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Molecular insights into the compatible and incompatible interactions between sugar beet and the beet cyst nematode

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Abstract

Background: Sugar beet (*Beta vulgaris* subsp. *vulgaris*) is an economically important crop that provides nearly one third of the global sugar production. The beet cyst nematode (BCN), *Heterodera schachtii*, causes major yield losses in sugar beet and other crops worldwide. The most effective and economic approach to control this nematode is growing tolerant or resistant cultivars. To identify candidate genes involved in susceptibility and resistance, the transcriptome of sugar beet and BCN in compatible and incompatible interactions at two time points was studied using mRNA-seq.

Results: In the susceptible cultivar, most defense-related genes were induced at 4 dai while suppressed at 10 dai but in the resistant cultivar Nemakill, induction of genes involved in the plant defense response was observed at both time points. In the compatible interaction, alterations in phytohormone-related genes were detected. The effect of exogenous application of Methyl Jasmonate and ET-generator ethephon on susceptible plants was therefore investigated and the results revealed significant reduction in plant susceptibility. Genes putatively involved in the resistance of Nemakill were identified, such as genes involved in phenylpropanoid pathway and genes encoding CYSTM domain-containing proteins, F-box proteins, chitinase, galactono-1,4-lactone dehydrogenase and CASP-like protein. Also, the transcriptome of the BCN was analyzed in infected root samples and several novel potential nematode effector genes were found.

Conclusions: Our data provides detailed insights into the plant and nematode transcriptional changes occurring during compatible and incompatible interactions between sugar beet and BCN. Many important genes playing potential roles in susceptibility or resistance of sugar beet against BCN, as well as some BCN effectors with a potential role as *avr* proteins were identified. In addition, our findings indicate the effective role of jasmonate and ethylene in enhancing sugar beet defense response against BCN. This research provides new molecular insights into the plant-nematode interactions that can be used to design novel management strategies against BCN.

Keywords: Sugar beet, *Heterodera schachtii*, CYSTM domain-containing proteins, Ethylene, Jasmonate, Transcriptome

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Background

Sugar beet (*Beta vulgaris subsp. vulgaris*) is a biennial, outbreeding and diploid ($2n = 18$) plant from the family Amaranthaceae, that is cultivated in temperate and subtropical regions [1]. Sugar beet is one of the most important crops worldwide, grown in 58 countries [2], and providing about 30% of the total world sugar production. It is also important as a source for bioethanol and animal feed [1].

Many crops are damaged by different diseases and pests including plant-parasitic nematodes. The annual global crop losses caused by plant-parasitic nematodes have been estimated at 157 billion dollars [3]. The beet cyst nematode (BCN, *Heterodera schachtii* [4]) has been identified as a plant pathogen since 1859 in Germany [5] and is now widely distributed throughout most of the beet-growing areas in the world, causing considerable yield losses (up to 60%). Infected beet plants exhibit symptoms including stunting and reduced growth, wilted leaves, and abnormal root development, also known as “bearded roots” [6]. The host range of the BCN is very wide as it can infect more than 200 plant species, mainly plants of the families Amaranthaceae (many species of *Beta* and *Chenopodium*) and Brassicaceae (e.g. *Brassica oleracea*, *B. napus*, *B. rapa*, *Raphanus sativus* and *Arabidopsis* sp.) [7].

The second-stage juveniles (J2) of the BCN penetrates the host root and migrates intracellularly through the cortical cells towards the vascular cylinder to find a proper cell for feeding site induction. After selection of a single cell as initial syncytial cell (ISC), it secretes molecules through its stylet and starts feeding from the ISC. Then, partial cell wall dissolution and protoplast fusion of several hundred neighboring cells occurs, leading to the formation of a highly metabolically active and multinucleated syncytium. The syncytium is the only nutrition source throughout the nematode's life. After starting the feeding process, the J2 becomes sedentary and matures after three molts [8]. Adult males leave their syncytia in the roots to mate with females while female nematodes remain attached to the feeding site and, following fertilization, produce several hundred eggs inside their enlarged body. After the completion of egg development, females die and their body wall hardens to form a cyst, which protects the eggs until hatching [9]. Completing the life cycle depends on the successful induction and maintenance of the feeding structure. Nematode secretions from esophageal glands, amphids and cuticle cause cellular reprogramming events related to major changes in the plant gene expression profile. Several nematode effectors from root-knot and cyst nematodes, such as cellulases, pectinases, expansins, chorismate mutase and calreticulin, have been reported [10–15].

Phytohormones play important roles in the formation of nematode feeding sites and regulation of gene

expression in plant defense/susceptibility responses [16–20]. The role of salicylic acid (SA) in plant defense against plant-parasitic nematodes has been investigated in *Arabidopsis*. Wubben et al. [19] found that SA-deficient mutants of *Arabidopsis* exhibited increased susceptibility to BCN and SA-treated wild type plants showed decreased BCN infection. Kammerhofer et al. [17] suggested that SA does not play a major role early during *H. schachtii* infection, but can suppress syncytium and female development at later time points. Moreover, Kammerhofer et al. [17] showed that mainly jasmonic acid (JA) can trigger plant defense against BCN in *Arabidopsis*. Also in other plant species, a number of studies have shown that exogenous application of JA on roots or shoots of plants enhanced resistance to plant parasitic nematodes [21–23]. In *Arabidopsis*, Ethylene (ET) positively affects BCN attraction to the root [17] and a positive role for ET in syncytium formation has also been demonstrated [20, 24].

The transcriptional changes occurring during the compatible *Arabidopsis-H. schachtii* interaction have been studied using differential display and microarrays [25–27]. Puthoff et al. [26] used Affymetrix GeneChip microarrays to compare gene expression in whole roots of *Arabidopsis* infected by *H. schachtii* or *Heterodera glycines* (soybean cyst nematode, SCN) at 3 days after infection (dai) and identified 128 and 12 genes, respectively, with altered mRNA levels following the BCN or SCN infection. Szakasits et al. [27] reported that gene expression in syncytia induced by *H. schachtii* in *Arabidopsis* roots did not strongly differ when comparing two time points, 5 and 15 dai. They analyzed the expression of 21, 138 genes and identified 3893 and 3338 genes, respectively, with higher or lower expression levels in syncytia compared with control roots. Their results revealed that genes involved in degradation of cell walls (such as pectate lyases and expansins), chloroplast proteins (such as glyceraldehyde 3-phosphate dehydrogenase A, cytochrome B6-F complex iron-sulfur subunit) and chlorophyll a-b binding proteins were up-regulated. Among the strongly down-regulated genes, peroxidases and major intrinsic proteins (including aquaporins) were observed.

Although sugar beet is the main host of *H. schachtii*, there is limited information about the alterations of gene expression in this host plant, either in susceptible or resistant plants. Resistance to BCN has not been found in cultivated beets. However, some commercial resistant varieties (eg. Nemakill, Evasion, Nematop) have been generated through interspecific crosses between *B. vulgaris* and the wild relative species *Patellifolia procumbens* [28–31]. The resistant varieties contain a translocated fragment of *P. procumbens* chromosome 1 harboring the resistance gene *Hs1^{Pro-1}*. Although *Hs1^{Pro-1}* is the first cloned nematode resistance gene and encodes a 282-amino acid

protein with leucine-rich repeats (LRR) and a transmembrane domain, its role in the resistance of sugar beet against BCN is still in doubt [32]. The existence of a second nematode resistance gene named *Hs1-2* in the vicinity of *Hs1^{pro-1}* has been suggested although further characterization is lacking [33, 34].

Resistant sugar beets are invaded by J2 of *H. schachtii* and can establish a feeding site, but syncytia degenerate before nematode maturation, hence hindering nematodes to complete their lifecycle [32, 35]. Samuelian et al. [36] used the cDNA-AFLP technique to identify sugar beet genes induced upon infection with the BCN. They analyzed 8000 transcript-derived fragments (TDFs) from infected hairy root clones of susceptible and resistant sugar beet (carrying the resistance gene *Hs1^{pro-1}*). They found that TDF_6, *Beta vulgaris Ki1*, was differentially expressed in both materials but more strongly in resistant plants. Upon transgenic over-expression this gene was able to inhibit the development of BCN in susceptible hairy roots.

Considering the importance of sugar beet and *H. schachtii* as a major limiting factor of beet production, our research aimed at performing a transcriptome study of compatible and incompatible interactions of sugar beet with BCN at two time points. The results revealed expression changes in genes involved in defense response, hormone pathways, metabolism, nutrition, and transcription regulation. Also, our experiments showed that exogenous application of methyl jasmonate and ET-generator ethephon on the susceptible sugar beet plants causes induced defence against BCN. Next to that, we have identified putative candidate genes that are involved in the resistance of cultivar Nemakill and nematode effectors that are expressed during infection in either a susceptible or a resistant cultivar.

Results

Sequencing and mapping

In this study, mRNA of root tissues of uninfected and BCN-infected susceptible and resistant sugar beet plants was sequenced at two time points upon inoculation: 4 days after inoculation (dai, early stage) and 10 dai (late stage), and in 2 independent biological replicates leading to a total of 16 sequenced samples. In a compatible interaction, the nematodes are at the J2 stage at 4 dai, while at 10 dai they are at the late J3 stage. As susceptible cultivar we worked with line 7112*SB36 (Sugar Beet Seed Institute, Iran), while the resistant cultivar was Nemakill [29, 31, 37, 38].

A total of 442,691,707 raw reads were obtained from the transcriptome sequencing. The average number of trimmed reads per sample was 26,581,145. A total of 425,298,335 trimmed reads that were 73–74 bp in length were aligned against the reference genome sequence of sugar beet (Refbeet1.1, [1]) using STAR and 90–91% of the sequenced reads could be uniquely mapped across all samples. The total number of uniquely mapped reads was 385,810,355, representing an average per sample coverage of 7.33X of the sugar beet transcriptome. An overview of the sequencing data and mapping results is shown in Table 1. The sequencing data have been deposited at the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) under the accession number (GSE135555). The number of differentially expressed genes (DEGs) obtained by all executed pairwise comparisons is shown in Fig. 1, and is described in detail below.

Differentially expressed genes (DEGs) in the compatible sugar beet-BCN interaction

Early stage (4 dai)

One hundred two transcripts were significantly differentially expressed in the infected susceptible roots vs their

Table 1 Summary of the transcriptome data from sugar beet roots in the compatible and incompatible interaction with *Heterodera schachtii*

Sample	Number of trimmed reads	Number of uniquely mapped reads	Percentages of uniquely mapped reads
Uninfected susceptible roots at 4 dai (UnS-4 dai)	55,453,474	50,263,420	90.66
Infected susceptible roots at 4 dai (IS-4 dai)	54,891,104	49,904,470	90.71
Uninfected resistant roots at 4 dai (UnR-4 dai)	52,935,813	48,014,740	90.74
Infected resistant roots at 4 dai (IR-4 dai)	50,732,663	46,245,798	91.19
Uninfected susceptible roots at 10 dai (UnS-10 dai)	55,008,558	50,229,976	91.29
Infected susceptible roots at 10 dai (IS-10 dai)	49,755,520	45,227,314	90.88
Uninfected resistant roots at 10 dai (UnR-10 dai)	54,885,472	49,741,102	90.63
Infected resistant roots at 10 dai (IR-10 dai)	51,635,731	46,183,535	90.25
Total	425,298,335	385,810,355	
Transcriptome size	246,730,718 bp		
Average per sample coverage of the sugar beet transcriptome		7.33X	

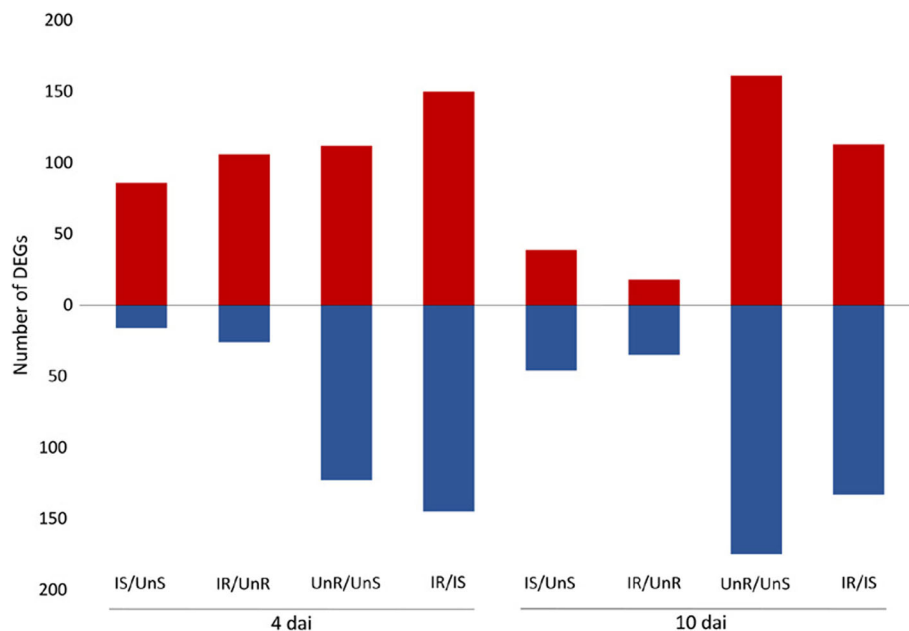


Fig. 1 Number of significant differentially expressed genes (DEGs) identified by all pairwise comparisons executed in this study, to analyze sugar beet in interaction with *Heterodera schachtii*. Red: up-regulated; blue: down-regulated genes. IS, infected susceptible roots; UnS, uninfected susceptible roots; IR, infected resistant roots; UnR, uninfected resistant roots; dai, days after inoculation

corresponding uninfected controls at 4 dai, of which 86 genes were up-regulated and 16 down-regulated, indicating a general pattern of induction of genes at this early stage of BCN infection (Additional file 1: Table S1). It should be noted that among these 102 DEGs, 34 genes were not functionally annotated in PLAZA 3.0. Gene set analysis revealed particular enrichment for GO terms, metabolic process, single-organism process, cellular process, response to stimulus, multi-organism process, catalytic activity, binding, oxidoreductase activity and transferase activity (Fig. 2).

Late stage (10 dai)

At the later time point (10 dai), 85 DEGs were identified, of which 39 genes were up-regulated and 46 genes were down-regulated (Additional file 1: Table S1). The GO terms, response to stimulus, metabolic process, single-organism process, cellular process, biological regulation, binding, catalytic activity, oxidoreductase activity and transferase activity, were the most enriched (Fig. 2).

General changes in the compatible interaction

Comparing the transcriptome of infected roots vs uninfected controls at both time points in the compatible interaction showed significant alterations in the expression of genes involved in metabolism, cell wall architecture, developmental process, transport, plant defense responses, transcription factors and hormone metabolism. Genes related to cell wall architecture such as pectinesterase and polygalacturonase (cell wall degradation), glycine-rich proteins (cell wall proteins) and glycosyltransferases

(cell wall synthesis) were significantly up-regulated at both time points (Additional file 2: Table S2). Also, expression of transporter genes including lipid transporters, ion transporters, ABC transporter, peptide and nitrate transporters was significantly changed at both time points (Additional file 2: Table S2). In addition, the expression of some genes involved in plant hormone metabolism, including genes related to auxin (e.g. indole acetic acid, IAA), gibberellic acid (GA), abscisic acid (ABA) and cytokinin (CK), as well as SA, JA and ET was altered (Additional file 2: Table S2). When focusing on genes involved in the plant defense response, we observed that the majority of these genes were induced at the early stage (4 dai), while suppressed at the later stage (10 dai) in the susceptible cultivar. For example, genes encoding NADPH-cytochrome P450 reductase and trans-cinnamate 4-monoxygenase - involved in the phenylpropanoid pathway - were up and down-regulated at 4 and 10 dai, respectively (Additional file 2: Table S2). The expression of four proteinase inhibitor genes belonging to the Kunitz family trypsin and protease inhibitor protein family was up-regulated at 4 dai. Genes encoding Defensin-like proteins, glutathione S-transferase genes (GST), two BRASSINOSTEROID INSENSITIVE 1-associated receptor (BAK1) genes and some peroxidase genes were induced at both stages. On the other hand, genes encoding chitinase and galacto-1,4-lactone dehydrogenase were down-regulated at 4 dai and two amorpha-4,11-diene-12 monoxygenase genes, several heat shock proteins (HSP), two DIBOAGlucoside dioxygenase BX6 genes - involved in

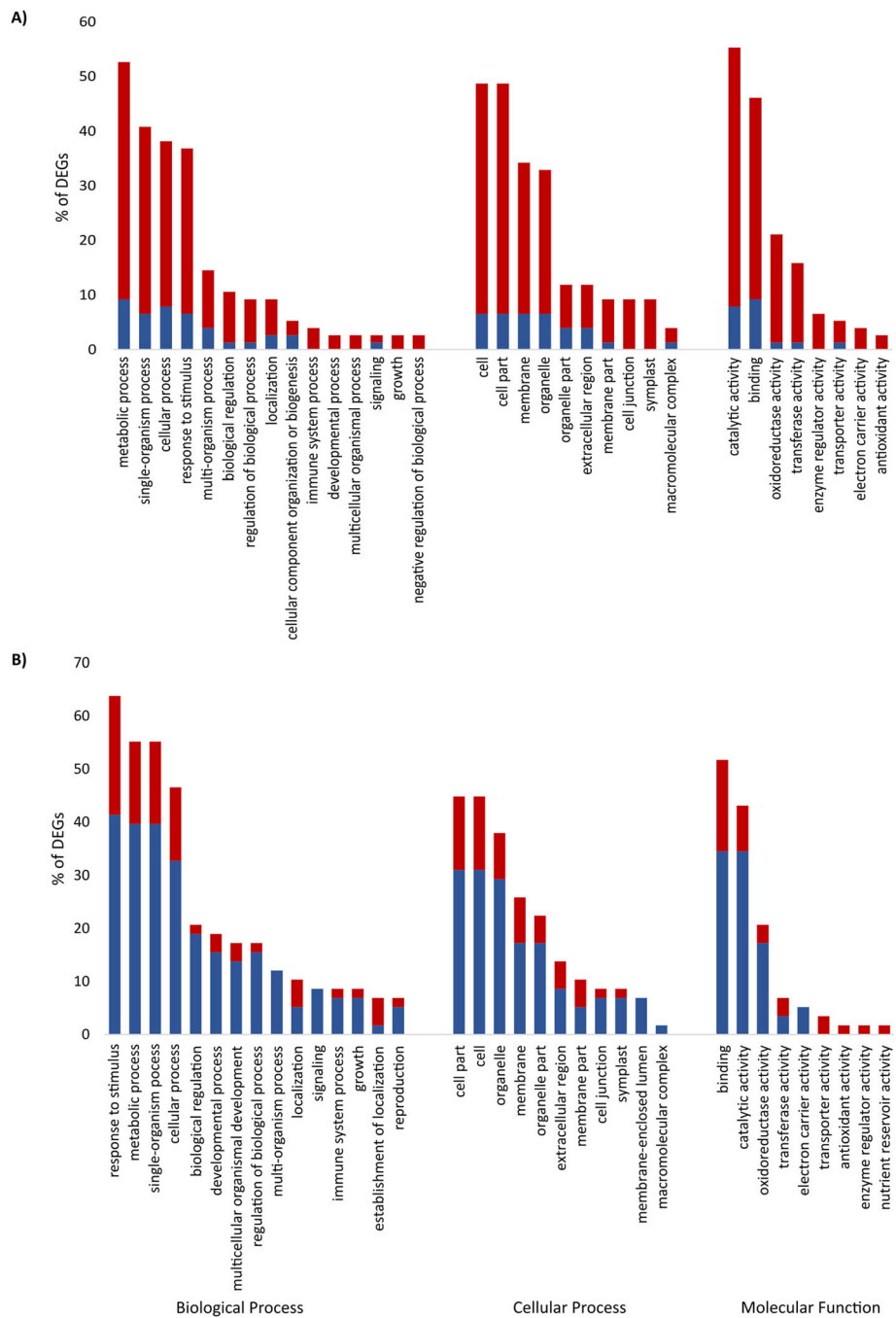


Fig. 2 Classification of significant DEGs obtained from the BCN-infected susceptible sugar beet roots compared to the uninfected susceptible sugar beet roots at **a** 4 days after inoculation (IS vs UnS-4 dai) and **b** 10 days after inoculation (IS vs UnS-10 dai) in three main Gene Ontology (GO) categories. Up-regulated and down-regulated GO terms are shown in red and blue bars, respectively

benzoxazinone synthesis - were suppressed at 10 dai. The analysis also revealed alteration in the expression of some transcription factors (TFs) in the susceptible sugar beet plants: a gene encoding a zinc finger TF was up and down-regulated at 4 and 10 dai, respectively. The expression of two genes encoding basic helix-loop-helix family (bHLH) TFs were up-regulated at 4 dai.

Two linoleate 9S-lipoxygenase genes, involved in JA biosynthesis genes were up-regulated at 4 dai. Three ET-responsive genes such as one APETALA2/ethylene-responsive transcription factor (AP2/ERF) gene were down-regulated in the infected roots at 10 dai (Additional file 2: Table S2).

Considering the alterations of some genes involved in plant defense hormones ET and JA in BCN infected roots and the lack of knowledge on the role of these hormones in sugar beet defense against BCN, the effect of external stimulation of these hormones on sugar beet immunity to BCN was investigated. To this aim, we treated the susceptible sugar beet with MeJA, or ET-generator Ethephon, 24 h prior to inoculation. The number of J2s and females at 4 and 21 dai, respectively, were

compared on treated and control plants. Upon exogenous application of Eth, the number of J2s per plant (4 dai) was lower in Eth-treated plants compared to controls. However, the results obtained at the later time point (21 dai), where females were counted, were inconsistent between experiments (Fig. 3). On the other hand, the results showed that MeJA-treated plants had significantly fewer J2s (at 4 dai) and females (at 21 dai) per plant than control plants in all experiments.

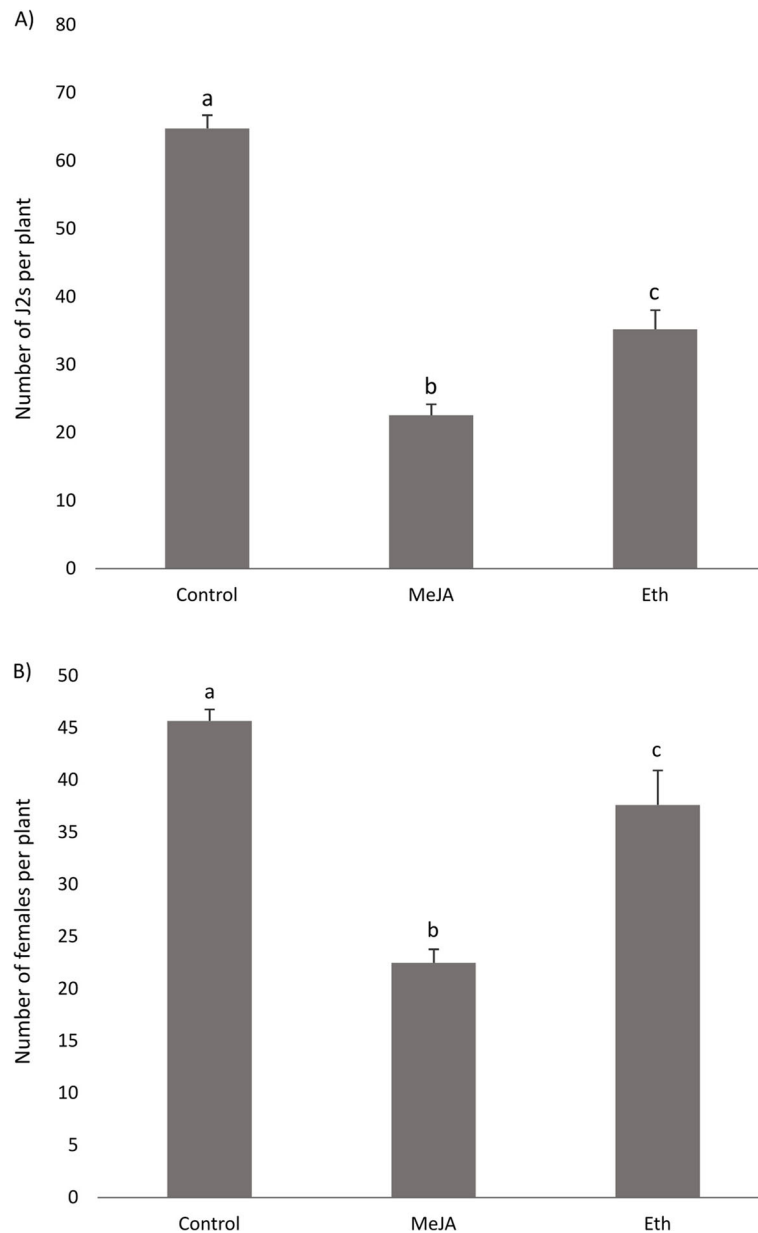


Fig. 3 Effects of exogenous application of methyl jasmonate (MeJA, 100 μ M) or ethephon (Eth, 500 μ M) on invasion and development of *Heterodera schachtii* in susceptible sugar beet plants (line 7112*SB36). **a** infection rate at 4 days after inoculation (invasion) and **b** number of females at 21 days after inoculation (development). Chemicals were applied 24 h before nematode inoculation. Bars represent means + SE of 10 plants. Different letters indicate statistically significant differences ($P < 0.05$). The experiment was independently repeated three times, with similar results, except for the female number at 21 dai upon Eth treatment, which was not significantly affected in one of the repeats

Differentially expressed genes (DEGs) in the incompatible sugar beet-BCN interaction in cultivar Nemakill

Early stage (4 dai)

Comparing the transcriptome of infected roots of the resistant cultivar Nemakill with uninfected roots at 4 dai, 132 DEGs were identified, of which 106 genes were up and 26 genes were down-regulated (Additional file 3: Table S3), again showing a general pattern of gene induction. Forty eight of these genes were not functionally annotated in PLAZA 3.0. Gene set analysis revealed particular enrichment for following GO terms, cellular process, single-organism process, metabolic process, response to stimulus, localization, multi-organism process, binding, catalytic activity, transporter activity, oxidoreductase activity and transferase activity (Fig. 4).

Late stage (10 dai)

At 10 dai, 53 transcripts were differentially expressed, of which 18 genes were induced and 35 genes were suppressed (Additional file 3: Table S3). The GO terms, response to stimulus, metabolic process, single-organism process, cellular process, biological regulation, catalytic activity, binding, transferase activity and enzyme regulator activity, were the most enriched (Fig. 4).

General changes in the incompatible interaction

When considering both time points, the results generally showed changes in the expression of genes related to metabolism, cell wall architecture, transport, plant defense responses, transcription factors and hormone metabolism. The expression of some genes involved in cell wall architecture, including genes involved in cell wall degradation (polygalacturonase, xylan 1,4-beta-xylosidase, cell wall-associated hydrolase and expansin) and cell wall proteins (arabinogalactan peptide, glycine-rich proteins and proline-rich proteins) were altered (Additional file 4: Table S4). Several genes encoding transporters - amino acid transporter, sugar transporter, lipid transporters, peptide transporters, aquaporins, ABC transporters and ion transporters - were induced at 4 dai (Additional file 4: Table S4). When focusing on plant defense response genes, proteinase inhibitor genes of the kunitz family trypsin and protease inhibitor proteins, defensin-like protein, lignin-forming anionic peroxidases, endochitinase, dirigent protein 23, BURP domain-containing proteins, PR1, PR6, CYSTM domain-containing proteins and a 'cell killing protein' showed up-regulation in the incompatible interaction. Also, two genes of the zinc finger TF family, and a gene encoding basic-leucine zipper (bZIP) TF were induced in the infected sugar beet roots (Additional file 4: Table S4). The expression of three genes involved in IAA biosynthesis were up-regulated at 4 dai. Genes related to ABA (2 at 4 dai and 1 gene at 10 dai) were induced in the infected

roots compared to the uninfected samples. Moreover, a JA-responsive gene was up-regulated at 4 dai while some JA-induced proteins were suppressed at 10 dai (Additional file 4: Table S4).

In addition, the expression of three genes containing a leucine-rich repeat (LRR) domain were up-regulated at 10 dai (Additional file 4: Table S4). The *HsI^{pro-1}*-gene was strongly expressed under both infected and uninfected conditions in the resistant cultivar.

Comparison of the sugar beet transcriptome between compatible and incompatible interactions

Firstly, it should be mentioned that, since the genome of sugar beet has been sequenced only recently [1], there is no well-annotated genome available. Secondly, the sugar beet genotypes which were used in this study have a different genetic background making it challenging to compare their transcriptional response to BCN infection. Therefore, the transcriptome of two sugar beet cultivars was first compared in uninfected root systems at both time points, to elucidate general differences related to this varying genetic background. The results indicated that 236 genes were significantly differentially expressed between uninfected roots of the two sugar beet cultivars (UnR vs UnS) at 4 dai. At 10 dai, the comparison of uninfected roots of the two cultivars (UnR vs UnS) led to the identification of 337 DEGs. Among them several genes, including examples encoding caffeoyl-CoA O-methyltransferase, dirigent proteins, chalcone synthases, tricetin 3',4',5'-O-trimethyltransferase, citrate synthase and endopeptidase inhibitors, showed higher expression in uninfected resistant plants compared to the susceptible plants at both time points (Additional file 5: Table S5). Using a similar comparison between infected plants of both cultivars (IR vs IS), 295 DEGs were found at 4 dai and 247 at 10 dai. Among these genes, a methyltransferase, phosphoinositide phosphatase SAC6, genes related to the phenylpropanoid pathway (such as caffeoyl-CoA O-methyltransferase and anthocyanin 3'-O-beta-glucosyltransferase), genes involved in signaling (including calcium-binding proteins, protein phosphatases 2C-type, protein phosphatase methylesterase and lactosylceramide 4-alpha-galactosyltransferase), F-box proteins, aquaporins, papain-like cysteine protease and some unknown genes that were highly induced in IR vs IS at 4, 10 or both time points (Additional file 5: Table S5).

To identify candidate genes related to the resistance, we compared the BCN-induced responses in both cultivars and looked for stronger or exclusive induction in resistant cultivar Nemakill. An aquaporin, bisphosphate carboxylase small chain, F-box protein, receptor-like protein kinase, trypsin inhibitor, galactono-1,4-lactone dehydrogenase, CASP-like protein, chitinase, and 16 unknown genes were highly induced in IR vs IS at 4 dai

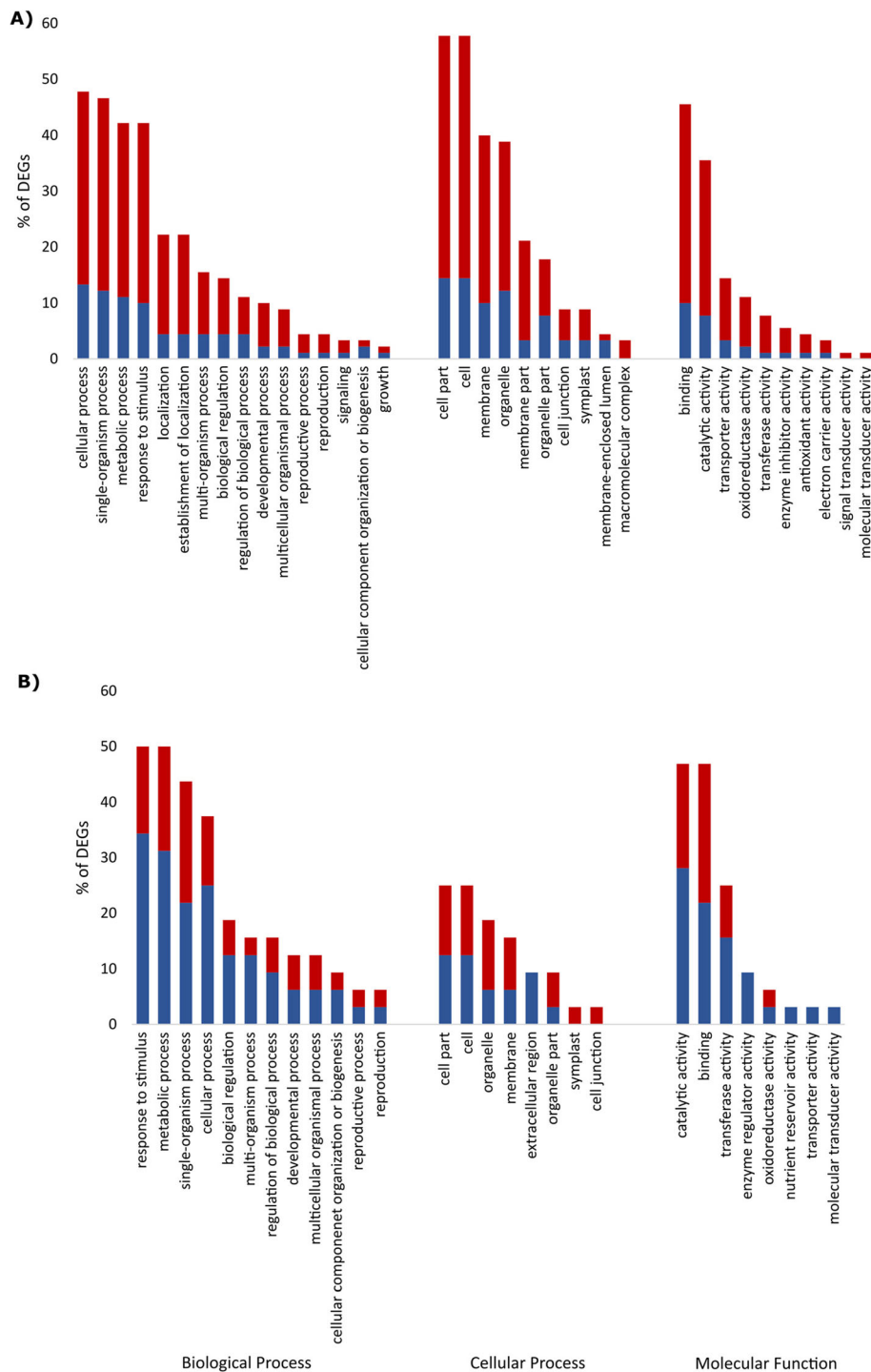


Fig. 4 Classification of significant DEGs obtained from the BCN-infected resistant sugar beet roots compared to uninfected resistant sugar beet roots at **a** 4 days after inoculation (IR vs UnR-4 dai) and **b** 10 days after inoculation (IR vs UnR-10 dai) in three main Gene Ontology (GO) categories. Up-regulated and down-regulated GO terms are shown in red and blue bars, respectively

($6.89 \geq \log_2FC \geq 2.0$, Additional file 6: Table S6). Three putative disease resistance genes, three HSPs, a nucleor-edoxin, ET-responsive transcription factor, multiprotein bridging factor 1, and one unknown gene were strongly

induced in IR vs IS at 10 dai ($6.02 \geq \log_2FC \geq 2.19$, Additional file 6: Table S6). CYSTM domain-containing proteins, 14-3-3 protein, an ABC transporter, UDP-glucose flavonoid 3-O-glucosyltransferase and some unknown

genes were also only induced in the infected resistant roots comparing to their controls. These genes could be responsible for the previously confirmed incompatible response of cultivar Nemakill [29, 31, 37, 38].

Validation of the transcriptome data

Quantitative real time-PCR (qRT-PCR) was performed to validate the mRNA-seq data. From the 10 initially selected genes, specific amplification was successful for six transcripts. The results of data analysis confirmed consistent expression patterns between the qRT-PCR and mRNA-seq data (Table 2).

Detection of *H. schachtii* transcripts in infected roots of sugar beets

Non-sugar beet mapped reads of the infected susceptible and resistant roots were further analyzed as explained in details in the Materials and Methods section. In total, 1707 transcripts of *H. schachtii* were identified from the infected samples. At early stage (4 dai), 342 and 469 genes were identified in the susceptible and in the resistant samples, respectively. At this time point, 211 nematode genes were common between both cultivars (Additional file 7: Table S7). At late stage (10 dai), 1321 and 901 nematode transcripts were identified in the susceptible and resistant samples, respectively. There were 323 common nematode genes between the cultivars (Additional file 7: Table S7).

Several nematode housekeeping genes including actin, tubulin, ubiquitin, ribosomal proteins, initiation and elongation factors and heat shock proteins were expressed in all infected sugar beet roots of both cultivars. Also, some genes involved in transport and lipid metabolism were identified. Among the transcripts, some genes encoding cell-wall degrading enzymes (cell wall-associated hydrolases and polygalacturonases) and peptidases (aspartic, metallo and serine) were detected (Additional file 7: Table S7).

Regarding to parasitism, genes encoding antioxidants such as thioredoxin-related transmembrane protein (in

all treatments), superoxide dismutase (IS-10 dai and IR-10 dai), glutathione S-transferase (IS-10 dai and IR-10 dai), and cytochrome C-peroxidases were detected. In addition, several known or putative effector genes were found, including transcripts encoding cathepsin (one transcript in IS-4 dai, two transcripts in IR-4 dai, three transcripts in IS-10 dai and IR-10 dai), ubiquitin extension protein (IS-4 dai, IR-4 dai, IS-10 dai), 14–3-3 protein (one transcript in IS-4 dai, IR-4 dai and IR-10 dai and two transcripts in IS-10 dai), calreticulin (two transcripts in IR-4 dai, IS-10 dai and IR-10 dai), C-type lectins (two transcripts in IS-10 dai and IR-10 dai), transthyretin-like protein (five transcripts in IS10, three transcripts in IR10), esophageal gland cell secretory proteins (six transcripts in IS-10 dai and three transcripts in IR-10 dai), and uncharacterized putative effector proteins (two transcripts in IR-4 dai and IR-10 dai, three transcripts in IS-10 dai). A gene encoding an autophagy-related protein and one encoding a cold-shock-like protein were only detected in the resistant roots at both time points, suggesting that the nematodes are under stress in the incompatible interaction. Also, a gene encoding Bax inhibitor 1 protein, a cell death suppressor, was only detected in nematode transcripts of the infected resistant roots at 10 dai.

Discussion

The global need for sugar, as one of the most important components of foods and drinks, is increasing rapidly. Sugar beet accounts for almost all sugar production in Europe and for over a quarter (30%) of the total world production. Among the factors that reduce the sugar beet yield, *H. schachtii* (BCN) is known as a major limiting factor, yet little is known about the interaction between sugar beet and BCN at the molecular level [36]. To increase our knowledge, transcriptome analyses were carried out using next generation sequencing (NGS) technology to investigate the compatible and incompatible sugar beet reaction to infection with *H. schachtii* and as a result in total about 443 million bp raw reads

Table 2 Expression profiles of six selected genes in the *Heterodera schachtii*-infected and uninfected sugar beet roots at 4 dai, as determined by qRT-PCR and mRNA-seq

Gene ID (RefBeet1.2)	Gene description	log ₂ fold change							
		UnR vs UnS		IR vs IS		IR vs UnR		IS vs UnS	
		qRT-PCR	mRNA-seq	qRT-PCR	mRNA-seq	qRT-PCR	mRNA-seq	qRT-PCR	mRNA-seq
BVRB_003160	Polymerase	0.57	0.06	3.11	3.14	1.15	0.91	-1.39	-0.65
BVRB_004820	Defense response	-1.57	-0.77	-1.60	-1.61	-0.31	-0.77	-0.28	0.05
BVRB_3g067160	Binding to nucleic acid	-1.51	-2.21	-0.87	-1.55	0.92	0.03	4.23	3.70
BVRB_3g070680	Unknown	-1.97	-0.83	3.68	2.16	1.12	1.03	1.54	0.41
BVRB_4g093270	Unknown	1.70	0.98	-4.04	-2.67	-0.73	-0.06	4.98	1.86
BVRB_9g225900	Unknown	-0.55	0.40	8.20	3.34	0.43	0.07	-8.38	-5.23

dai days after infection, UnR uninfected resistant roots, UnS uninfected susceptible roots, IR infected resistant roots, IS infected susceptible roots

were acquired. We identified a large number of genes related to cell wall architecture, metabolism, nutrition, signal transduction, stress, defense responses and phytohormones, for which the expression was significantly altered upon BCN infection. Genes that are only up-regulated in the incompatible interaction but rather unaffected or even suppressed in the compatible interaction could potentially be related to the resistance response of sugar beet against BCN.

For example, three genes encoding CYSTM domain-containing proteins were up-regulated in the resistant roots of sugar beet upon BCN infection. It has been suggested that CYSTM proteins are part of a cellular protective mechanism against stress in eukaryotes, including humans [39]. The *PCCI* gene in Arabidopsis, belonging to this group, encodes an 81-amino acid protein, with a cysteine-rich domain that is involved in development and defense response to stresses including pathogens [40]. So far, the role of these genes in plant-nematode interactions has not been reported.

A gene encoding chitinase, was down-regulated early in the BCN-infected susceptible plants, while significantly up-regulated in the infected resistant plants. Alteration of chitinase gene expression in different plants as a defense mechanism against nematodes has been reported [18, 41]. Guimaraes et al. [42] suggested a role of *AsCHI2* in the defence response of *Arachis stenosperma* to *Meloidogyne arenaria*.

In addition, a gene encoding SKIP23, an F-box protein containing a domain of unknown function (DUF295), was highly induced in the infected resistant plants compared to the susceptible plants at 4 dai. F-box proteins are components of SCF (Skp I, Cullin, and an F-box protein) ubiquitin-ligase (E3) complexes, which mediate ubiquitination and subsequent proteasomal degradation of target proteins. These proteins play diverse roles in different processes such as plant development [43–45], circadian clock regulation [46, 47], hormone perception and signaling [48–53], response to abiotic stresses [54–57] and plant-pathogen interactions [58–61]. The protein SKIP23 was found to interact with ASK1, a component of, for example, the strigolactone SCF receptor complex, [62, 63] and also with 14–3–3 proteins in Arabidopsis [64]. Interestingly, a nematode-derived transcript encoding a 14–3–3 protein was accumulating in the infected resistant roots compared to the controls. The role of the F-box protein SKIP23 in the resistance of sugar beet cultivar Nemakill seems likely although more investigations are needed to elucidate the exact role of this gene. On the other hand, a similar F-box protein with DUF295 domain (Ascorbic acid Mannose Pathway Regulator 1, AMR1) has been shown to be involved in modulating the expression of several genes in the ascorbate biosynthesis pathway in Arabidopsis [65].

Ascorbate is an important natural compound with high antioxidant activity. The main biosynthetic pathway of ascorbate in plants is the “Smirnoff-Wheeler” pathway [66], in which the last step is the oxidation of L-galactono-1,4-lactone to L-ascorbate by the enzyme *L-galactono-1,4-lactone dehydrogenase (GLDH)* in the mitochondria [67]. The here-observed suppression of the *GLDH* gene during the compatible interaction, but significant induction during an incompatible interaction at 4 dai confirms a potential role of ascorbate in resistance of sugar beet to *H. schachtii*. Similarly, [68] have shown a significant accumulation of ascorbic acid in roots of root knot nematode-resistant tomato cultivars upon nematode infection, but not in susceptible plants.

Suppression of some genes involved in the phenylpropanoid pathway was observed in the susceptible cultivar, while several phenylpropanoid related genes were induced in the resistant cultivar, such as dirigent proteins, chalcone synthases, caffeoyl-CoA O-methyltransferase, anthocyanin 3'-O-beta-glucosyltransferase and UDP-glucose flavonoid 3-O-glucosyltransferase. This indicates the importance of this pathway in resistance of sugar beet against BCN. Phenylpropanoids are a large class of secondary metabolites including SA, lignin, flavonoids, coumarins, lignans etc. [69]. Suppression of genes in this pathway has been previously observed in compatible plant-RKN interactions [18, 70], while induction was reported in compatible plant-CN or plant-migratory nematode interactions [18, 71].

Induction of a gene encoding a casparian strip membrane domain protein (CASP)-like protein, belonging to “uncharacterized protein family UPF0497” was detected in the infected resistant roots compared to the uninfected resistant plants and to the susceptible plants at 4 dai. CASPs mediate Casparian strip formation, composed of a lignin polymer and acting as para-cellular barrier for selective nutrient uptake and stress resistance, also against nematodes [72]. It also plays a role in the activation of hormone signaling pathways [73–76].

Among the genes involved in hormone pathways, genes related to JA biosynthesis and responses such as lipoxigenases and bHLH TFs were up-regulated in the susceptible roots at early stage while their expression was not induced at later stage. Similarly, Kammerhofer et al. [17] reported up-regulation of genes related to JA biosynthesis early upon nematode inoculation. This induction could be related to a damage response caused by intracellular nematode penetration, or could be related to a plant defense response to nematode presence. Our experiments showed that upon foliar treatment of the susceptible sugar beets with MeJA, infection rates of J2s (at 4 dai) and the number of females (at 21 dai) were significantly lower compared to untreated plants. These results reveal the positive role of JA in systemic defense of sugar beet against *H. schachtii*.

In our transcriptome data, genes belonging to the ET pathway were generally suppressed in the susceptible plants, while ET-responsive TF were induced in the infected resistant roots at 10 dai. To investigate the role of ET in the defense response of sugar beet against BCN, we applied Eth on shoots of susceptible sugar beet plants and our results showed lower J2s infection and female development rates on Eth-treated plants compared to controls, although in one experiment the effect on female development was not significant. In contrast to our results, a higher infection rate at 24 hai and no significant differences in number of penetrating BCN J2s and female counts in Eth-treated *Arabidopsis* roots has been reported [17]. Indeed, contradictory roles of ET in nematode attraction, feeding site formation and development, and plant defense have been reported [17, 20, 24, 77–83], probably because of its pleiotropic role in development and defense [84]. Taken together, it seems likely that ET is playing a role in defense of sugar beet against BCN but further investigations are needed to reveal the exact role of ET at different stages of nematode and feeding site development and plant defense.

Regarding to the presence of the *Hs1^{Pro-1}* gene in the resistant cultivar, the expression level of this gene was assessed and our analysis showed that the gene was highly expressed under both infected and non-infected conditions in the resistant cultivar. In addition, three other putative disease resistance genes, two genes with LRR and the other one with LRR and NBS-ARC domains, were significantly induced in the BCN-infected resistant roots compared to the uninfected resistant or infected susceptible roots at 10 dai. These data reveal a potential activation of an R-gene complex in the Nema-kill cultivar upon BCN infection.

Conclusions

This study is the first transcriptome analysis of sugar beet in compatible and incompatible interactions with *H. schachtii* and increases our knowledge of the molecular mechanisms underlying BCN resistance in sugar beet. A large number of DEGs, including many important genes playing potential roles in susceptibility or resistance of sugar beet against BCN were identified. In addition, several genes encoding nematode effectors were identified and some of them were only detected in the resistant roots, suggesting a potential role as *avirulence* (*avr*) protein that needs to be further elucidated. The effective role of application of jasmonate and ethylene in enhancing the basal defense response of sugar beet against BCN was showed. The results of this research extend our knowledge about plant-nematode interactions and can be used for breeding programs targeting BCN resistance in sugar beet.

Methods

Plant material and nematode infection

Seeds of a susceptible sugar beet line (7112*SB36, Sugar Beet Seed Institute (SBSI), Iran) and a resistant cultivar (Nemakill, Syngenta) were used in this study. Presence of the *Hs1^{Pro-1}* gene was confirmed in Nemakill by PCR amplification and Sanger sequencing (genbank accession number MT845291). The susceptible cultivar does not contain this gene. Sugar beet seeds were germinated on sterile soil for 3 days at 28 °C. The seedlings were transferred to SAP substrate (Sand Absorbent Polymer [85];) and were kept in a growth chamber at 25 ± 2 °C, with 16 h light/8 h darkness. A pure population of *H. schachtii* that originated from one cyst was multiplied on a susceptible sugar beet cultivar (Jolgeh, SBSI, Iran) in sterile potting soil under the same conditions. The J2s were harvested from cysts that were soaked in 3 mM ZnCl₂ to stimulate hatching. Fifteen-day-old roots of the sugar beet plants were inoculated with 300 fresh J2s of *H. schachtii*. Control plants were mock-inoculated with water. Whole root tissue of infected and control plants was collected at two time points, 4 (early stage) and 10 (late stage) dai, were then washed in water, immediately frozen in liquid nitrogen and stored at –80 °C until RNA extraction. For each time point, two independent biological replicates were collected and each replicate consisted of a pool of six individual plants.

RNA isolation, library preparation, and sequencing

Total RNA was extracted from whole roots of infected and uninfected plants at 4 and 10 dai with the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's protocol, with an additional sonication step for 30 s after addition of buffer RLT. The quantity and quality of each RNA sample was evaluated using a NanoDrop 2000c (Thermo Fisher Scientific, Belgium). For each sample, 2 µg of total RNA was used for library preparation using QuantSeq 3' mRNA-Seq Library Prep Kit (Lexogen) as following: an oligodT primer containing an Illumina-compatible linker sequence at its 5' end was hybridized to the RNA and reverse transcription was performed. After first strand synthesis, the RNA was removed and second strand synthesis was initiated by random priming and a DNA polymerase. The random primer also contained an Illumina-compatible linker sequence at its 5' end. The second strand synthesis was followed by a magnetic bead-based purification step. Amplification of libraries was performed, and addition of barcodes was executed during the PCR amplification step. In the final step, the double-stranded libraries were purified by magnetic beads to remove all reaction components. Quality of the libraries was confirmed using an Agilent Bioanalyzer 2100. After cluster generation, 16 libraries were sequenced on a NextSeq 500 Illumina sequencing

platform to produce single-end 76 bp reads. The samples were multiplexed to minimize lane effects. Library construction and sequencing were carried out by the NXTGNT sequencing center (Ghent University, Belgium).

mRNA-Seq data analysis

A summary of the data-analytical pipeline is shown in Fig. 5. For each sample, sequencing data quality was assessed by FastQC [86]. Trimmomatic [87], with a 5-base sliding window, was used to improve data quality: Bases with a phred score lower than 20 were trimmed, and reads shorter than 40 nt were removed. The trimmed reads from each sample were aligned to the *Beta vulgaris subsp. vulgaris* reference genome (Refbeet1.1, [1]) using the STAR software [88]. Reads that did not map on the sugar beet genome, were kept aside to identify nematode transcripts (see ‘Detection of nematode transcripts in infected root samples’). Transcriptome size was calculated as the sum of the lengths of all primary transcripts.

The number of trimmed reads mapped to each annotated gene per condition was counted using the summarizeOverlaps functions of the GenomicAlignments R package [89]. A gene was considered to be expressed if it

had a raw count value higher than 1 in both replicates of each condition. The reads were normalized and differentially expressed genes (DEGs) were identified using the R-package ‘DESeq2’ [90]. In this software, the counts are divided by sample-specific size factors determined by median ratio of gene counts relative to geometric mean per gene. Empirical null modelling was performed using the fdrtool package (version 1.2.15) using the Wald statistic from DESeq2 as input [91]. Adjusted *P*-values for each estimate of False Discovery Rates (FDRs) were calculated using the Benjamin-Hochberg algorithm. Transcripts were considered to be significant DEGs when the adjusted *P*-value was < 0.05 . For each time point, the expression level of each transcript in the infected samples was compared to the controls, moderated \log_2 -transformed fold-changes (FC) values were further used throughout this study.

Gene ontology analysis

Gene Ontology (GO) analysis was performed using PLAZA 3.0 (https://bioinformatics.psb.ugent.be/plaza/versions/plaza_v3_dicots/). PLAZA contains information about structural and functional annotation, gene families, protein domains, etc. in plants [92]. Using the

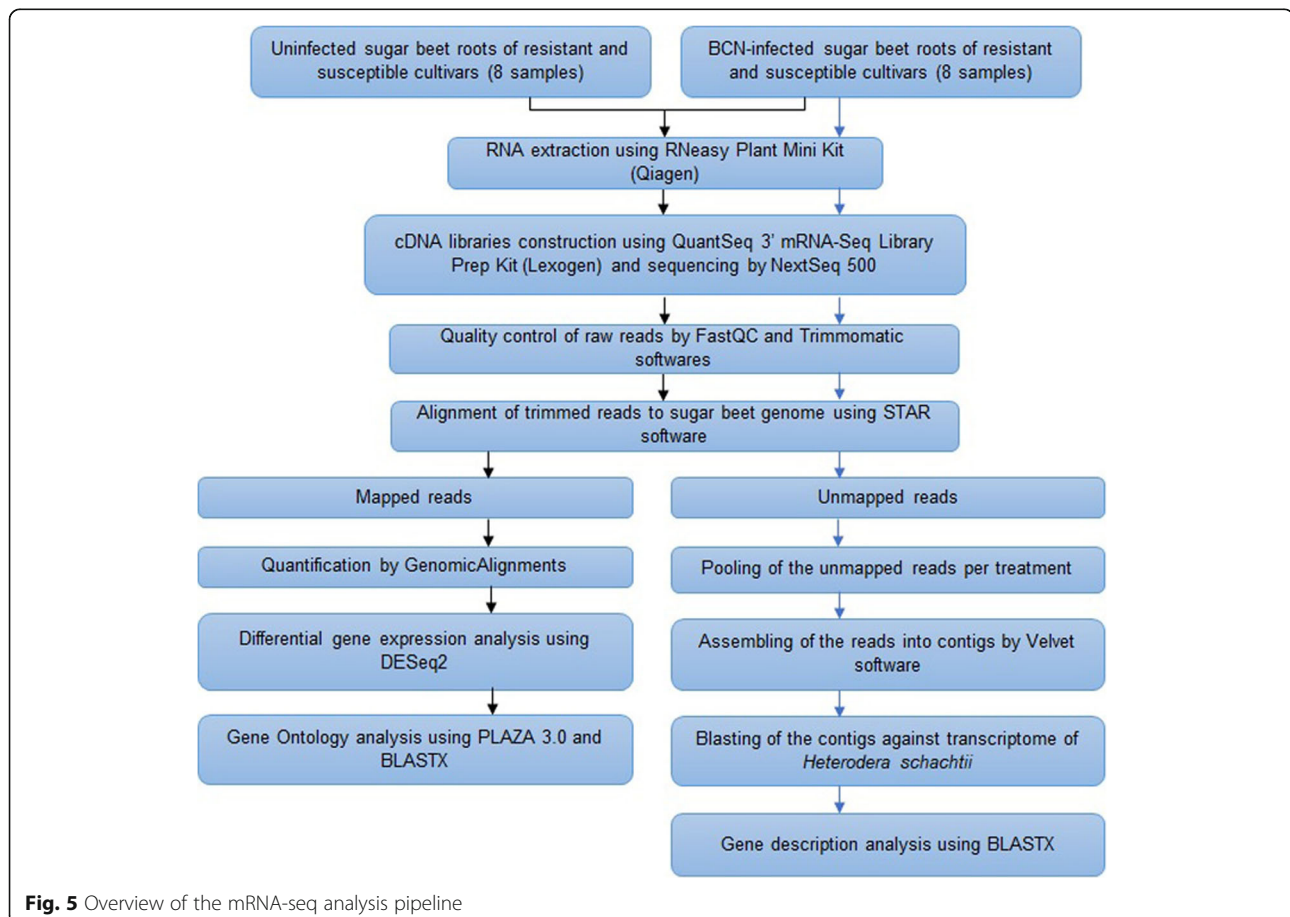


Fig. 5 Overview of the mRNA-seq analysis pipeline

PLAZA 3.0 tool, differentially expressed transcripts were assigned to GO categories and functionally annotated. Genes without annotation in PLAZA 3.0 were BLASTX-ed against the non-redundant protein sequences using NCBI online blast service (E-value <1e-5).

qRT-PCR validation

Based on the mRNA-seq results, 10 genes were selected for validation. Gene-specific primers were designed using Primer3 (<http://bioinfo.ut.ee/primer3-0.4.0/>) and ApE v2.0.49.10 softwares. Glyceraldehyde 3-phosphate dehydrogenase, *BvGAPDH*, was used as reference gene [93, 94]. For qRT-PCR, independent samples of the susceptible and resistant plants at 4 dai were harvested for RNA-extraction. All reactions were done with two biological and three technical replicates. A total of 2 µg of each RNA sample was treated with 2 U of DNaseI enzyme (ThermoFisher). The cDNA synthesis was performed using 200 U of Tetro Reverse Transcriptase enzyme and Oligo (dT)₁₈ primer (Tetro cDNA Synthesis kit, Biorad, Germany) according to the manufacturer's protocol. Quantitative real-time PCR was performed using SensiMix™ SYBR NO-ROX (Biorad, Germany) on a CFX connect real-time PCR machine (Biorad, USA) as following: 10 min of initial denaturation at 95 °C and 40 amplification cycles (25 s at 95 °C, 25 s at 58 °C and 20 s at 72 °C). After the last step, specificity was tested using a melting curve by gradually increasing the temperature to 95 °C. Data were analyzed using Rest 2009 [93]. The expression level of all genes was normalized using the internal control *BvGAPDH* and the relative expression level of target genes was calculated using $2^{-\Delta\Delta C_t}$ method [94]. All primers used in this study are listed in Additional file 8: Table S8.

Detection of nematode transcripts in infected root samples

The unmapped reads of replicates of each cultivar at each time point were pooled and assembled into contigs using the Velvet software (version 1.2.10 [95]). The contigs were blasted against the transcriptome of *H. schachtii* [96] and those contigs with a bit score higher than 50 were considered as transcripts of *H. schachtii*. BLASTX was performed against the non-redundant protein sequences using NCBI online blast service (E-value <1e-5).

Chemical treatments and statistical analyses

The susceptible sugar beet line (7112*SB36) was used. Seedlings were transferred to sterile soil in polyvinylchloride (PVC) tubes and further grown at the same conditions as described above. Solutions of methyl jasmonate (MeJA, 100 µM, Sigma-Aldrich), or ET-generator ethephon (Eth, 500 µM, Sigma-Aldrich) were prepared in distilled water containing 0.02% (v/v) Tween

20 as surfactants. Leaves of two-week-old sugar beet plants were sprayed with vaporizers until run off with a fine mist of either compound. For control plants, distilled water containing 0.02% (v/v) Tween 20 was applied. One day after chemical treatments, roots of sugar beet plants were inoculated with 300 fresh J2s, as described in previous steps. The infection level of the plants was evaluated at 4 and 21 dai by counting the number of J2s and females, respectively. To visualize the J2s, roots were stained with acid fuchsin at 4 dai [22]. Ten plants per treatment were included in each experiment and all experiments were independently repeated three times.

Statistical analyses were done using SPSS Statistics V22.0 software. After confirming normality and homoscedasticity of the data, one-way ANOVA and Duncan's multiple range test were applied to test for significant differences between the treatments ($P < 0.05$).

Supplementary information

Supplementary information accompanies this paper at <https://doi.org/10.1186/s12870-020-02706-8>.

Additional file 1 Table S1. Significant differentially expressed genes (DEGs) of sugar beet in the compatible sugar beet-beet cyst nematode (BCN) interaction.

Additional file 2 Table S2. Significant sugar beet DEGs related to cell wall architecture, metabolism, developmental process, transport, defense response, hormone metabolism and transcription factors in the compatible sugar beet- BCN interaction.

Additional file 3 Table S3. Significant sugar beet DEGs in the incompatible sugar beet- BCN interaction.

Additional file 4 Table S4. Significant differentially expressed sugar beet genes related to cell wall architecture, transport, defense response, hormone metabolism and transcription factors in the incompatible sugar beet- BCN interaction.

Additional file 5 Table S5. Significant DEGs in comparison of resistant and susceptible sugar beet cultivars in uninfected and BCN-infected conditions.

Additional file 6 Table S6. Sugar beet genes that show a differential response to BCN infection when comparing the resistant and susceptible cultivar. Statistical significance was evaluated in DESeq2 by evaluating significant interaction between infections status and sugar beet variety.

Additional file 7 Table S7. Transcripts derived from beet cyst nematode (BCN) genes detected during the compatible and incompatible interaction with sugar beet.

Additional file 8 Table S8. Primer sequences of sugar beet genes used for qRT-PCR expression analysis.

Abbreviations

BCN: Beet cyst nematode; dai: Days after inoculation; DEG: Differentially expressed gene; ET: Ethylene; Eth: Ethephon; IS: Infected susceptible roots; IR: Infected resistant roots; JA: Jasmonic acid; MeJA: Methyl jasmonate; qRT-PCR: Quantitative real time-PCR; TF: Transcription factor; UnR: Uninfected resistant roots; UnS: Uninfected susceptible roots

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Authors' contributions

RG performed the experiments and some of the data analysis and also wrote the manuscript. EP and TK conceived the project, designed the experiments and improved the manuscript. BV performed the bioinformatics analyses.

SBM prepared some materials and edited the manuscript. NS and RM designed the experiments and edited the manuscript. Next Generation Sequencing and Bioinformatic analysis was performed under the supervision of TDM. All authors have read and approved the manuscript.

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Availability of data and materials

The partial sequence of the *Hs1^{pro-1}* gene from sugar beet cultivar Nemakill has been deposited at the GenBank database with accession number MT845291. The mRNA datasets generated during the current study are available at the NCBI Gene Expression Omnibus (GEO) under the accession number GSE135555. The *Beta vulgaris subsp. vulgaris* reference genome and the transcriptome of *H. schachtii* were obtained from the <http://bvseq.boku.ac.at/Genome/Download/RefBeet-1.1/> and <https://www.ncbi.nlm.nih.gov/sra/?term=SRX381021>, respectively.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests. Tina Kyndt is member of the editorial board of BMC Plant Biology.

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