THE EFFECT OF CYST NEMATODE (GLOBODERA ROSTOCHIENSIS) ISOLATE DDH1 ON GENE EXPRESSION IN SYSTEMIC LEAVES OF POTATO PLANT

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INTRODUCTION

The potato cyst nematode (Globodera spp.) is a major pathogen of potato plants (Solanum tuberosum), which contributes to the localized dedifferentiation of a restricted number of host cells into a specific feeding site called the syncytium (Gurr et al., 1991). The genus of cyst nematode (Globodera) has been reported in Egypt. Globodera rostochiensis was first found in 2017 on potato plants, in three governorates of Egypt; Alexandria, El-Behera and Sohag (Ibrahim et al., 2017). The parasitic root cyst nematode spends a great portion of their life cycles embedded in the roots of a host plant and thus exposed to a various of plant defence responses, consequently, decrease for both quality and crop yield in potato leaves included induction of pathogenesis related genes (G. rostochiensis) with similarity 77%. The constructed phylogenetic tree based on the DNA nucleotide sequence showed close relationship with a Japanese G. rostochiensis (AB207271). The functional transcription of the infected and non-infected potato plants was analyzed using Differential Display- PCR technique and about 57 up regulated and 22 down regulated genes. Seven genes (2 downregulated and 5 upregulated) were chosen at random and sequenced. The nucleotide sequences observed that the two down regulated genes were; Chitinase domain containing protein 1 and N-like putative resistance protein. Whereas, the five up regulated genes were; Glucan endo 1,3 beta glucosidase A precursor, STS 14 protein gene, Phytalexin deficient 4-1 protein gene, PEN1 gene and Linoleate 13S lipoxigenase 2-1, chloroplastic like, respectively. We can conclude that the regulation changes occurred for some pathogen related genes in systemically acquired immunity of infected potato plant compared with control were the main observation. Moreover, the cyst nematode could be able to suppress some of plant defense system genes and inducing another in the same time.

MATERIAL AND METHODS

Cyst nematode isolation and propagation

Approximately, 1.5 kg of sandy clay soil samples infected with cyst nematodes were collected from the soil layers at 20-40 cm depth and then placed in a plastic bags were labelled with date and soil type. These samples were collected from El-Beheira Governorate (El-Nubahia). Cyst nematodes were extracted from the soil using a pair of sieves (250 and 840 mesh size) (Shepherd 1985) and then stored in eppendorf tubes at room temperature. These cyst nematodes were propagated on the roots potato plants (Spunta cultivar under greenhouse conditions 27° C; 20-22 L: D photoperiod). Subsequently, the propagated cysts were isolated after 10 to 12 weeks' post inoculated the potato plant with the examined nematode according to (Cotton et al., 2014).
Identification of cyst nematode using ITS gene by specific PCR, sequencing and phylogenetic analysis

Total genomic DNA was extracted from the cyst nematode samples using Genomic DNA extraction kit (nItRoBiotechnology, USA). The genomic DNA was subjected to PCR amplification using the universal primers (ITS1: 5'-TCCGAGTGGTAAACCCGCTG-3' and ITS4: 5'-TTCCTCCGGCTTATTGATATG-3') (White et al., 1990). The PCR mixture reaction including: 12.5 µL PCR master mix (Applied biotechnology, Egypt), 1 µL of each ITS-primers 10 pmol/µL, and up to volume 25 µL from 4 mM Q water. PCR reactions were carried out in a thermal cycler (Gene Amp 9700, USA) with an initial denaturation step for 5 min at 94°C, followed with 35 cycles each for 1 min at 94°C, annealing temperature at 55°C for 1 min, elongation at 72°C for 1 min and final elongation at 72°C for 5 min, then the samples were held at 4°C. The product of PCR amplification was loaded into 2% agarose gel, stained with ethidium bromide and separated by electrophoresis as described by (Shaikh aldein et al., 2018). The PCR fragment was purified using the PCR clean up column kit (Maxim biotech INC, USA) and the purified PCR product was subjected to DNA sequencing by Sigma (Macrogen com, Korea). The obtained DNA nucleotide sequence was submitted to National Center for Biotechnology Information (NCBI) and the sequence alignment was compared with other cyst nematode ITS genes listed in the GenBank database. Phylogenetic tree was carried out using MEGA 4.0 program (Tamura et al., 2007).

Potato growth and nematode infection assay

Healthy potato tubers of Spunta cultivar were selected, surface sterilized with 75% ethanol for 5 min, followed by 10 min in 2.5% sodium hypochlorite and finally, the tubers were rinsed three times with sterile water. The sterile tubers were cultivated singly in 25 cm plastic pots in (3:1 mixture of autoclaved loam and sand) without fertilizers. Potato plants were cultivated at greenhouse under conditions (27°C, 20-22 L: D photoperiod) were inoculated with cyst nematode. The RT-PCR results revealed that, the infected potato plants which collected on intervals after the inoculation: 12h, 24h, 48 hr, 7d, 14d, 21d, 28d, 35d, 42d and 49 days post infection (dpi). Where, there was common band was observed after 14 days post infection with G. rostochiensis. Moreover, dependent on the result of DNA nucleotide sequence, the phylogenetic tree was generated as shown in (Fig. 1B). The constructed phylogenetic tree of Globodera rostochiensis was divided into two main clusters; one of them includes the Egyptian isolate of G. rostochiensis combined with Japanese isolate (AB202711), Additionally, the similarity between the Egyptian and the Japanese isolates was 77%.

Table 1 The arbitrary pathogen related protein primers and RAPD primers used in DD-PCR.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Type</th>
<th>Sequence 5 → 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endoglucanase (PR1)</td>
<td>Forward</td>
<td>TTC TTC CCT CGA AAG CTC AA</td>
</tr>
<tr>
<td>Chitinase (PR9)</td>
<td>Forward</td>
<td>CGG TGG TAC TCC TCC TGG ACC C</td>
</tr>
<tr>
<td>Chitin Binding Protein (PR8)</td>
<td>Reverse</td>
<td>CGG CCAC CGT GGT GCG CTG A</td>
</tr>
<tr>
<td>Peroxidase (PPO)(PR5)</td>
<td>Reverse</td>
<td>AGC ATG TTT CTG GAA TCA GCC TG</td>
</tr>
<tr>
<td>Phenylalanine Ammonia Lyase (PAL)</td>
<td>Forward</td>
<td>CATGCTCTTTGATGAGGCCGTA</td>
</tr>
<tr>
<td>RAPD 5</td>
<td>Forward</td>
<td>CAGGGGACAGA</td>
</tr>
<tr>
<td>RAPD 10</td>
<td>Forward</td>
<td>GAGGGGCAAC</td>
</tr>
<tr>
<td>RAPD 7</td>
<td>Forward</td>
<td>ACCCGCGGAG</td>
</tr>
</tbody>
</table>

Identification of cyst nematode using ITS gene, sequencing and phylogenetic analysis

The PCR product of ITS regions for Cyst nematode-DDH1 isolate showed two different amplicons 238 bp and 370 bp as shown in (Fig. 1A). The DNA nucleotide sequence of obtained the 238 bp band was aligned with the other published Globodera rostochiensis genes available in GenBank database (http://www.ncbi.nlm.nih.gov). The results confirm that the obtained sequence belong cyst nematode Globodera rostochiensis. Moreover, on the result of DNA nucleotide sequence, the phylogenetic tree was generated as shown in (Fig. 1B). The constructed phylogenetic tree of Globodera rostochiensis was divided into two main clusters; one of them includes the Egyptian isolate of G. rostochiensis combined with Japanese isolate (AB202711). Additionally, the similarity between the Egyptian and the Japanese isolates was 77%.

Figure 1 Amplified PCR products from cyst nematode G. rostochiensis generated by the ITS primers, where M: 1.5 kb DNA marker, lane C (negative control) and lane G.R (Globodera rostochiensis) PCR amplicons were 238 bp and 370 bp. B) Phylogenetic tree of Globodera rostochiensis Egyptian isolate with the other sequence published of Globodera rostochiensis alignment into GenBank.

Up and down regulation genes using DD-PCR analysis demonstrated a role of defense against pathogens

The DD-PCR results revealed that, the leaves of infected potato plants which collected on intervals after the inoculation; 12h, 24h, 48 hr, 7d, 14d, 21d, 28d, 35d, 42d and 49 days post infection (dpi). The general observation revealed that several common bands between control and infected potato plants. On the other hands, the presence of some bands in the infected plants those were absent in the control and vice versa. Results generated by PR3 primer showed only one up regulated band with molecular size 611 bp (12hr after infection), this band was down regulated after 24hr and 48hr post infection when compared with the control (Fig. 2 a). While, differential expression was observed in infected plants with primer PR3f and the results showed unique band with molecular size 590 bp after 35 days as shown in (Fig. 2 b) furthermore, a band of 900 bp in control plants has been disappeared completely (down regulated) just the infection occurred. Whereas, there was common band was observed after 14 days post infection with molecular size 1500 bp as a result of early infection with cyst nematode. The gene expression by DD-PCR using PPO primer represented 322 bp band with high intensity in the infected sample 28 days post infection as illustrated in (Fig. 2 c). DD-PCR profile using PPO primer represented similar bands in the same lengths when compared with control, while, these bands are differed in their
intensity during intervals of infection. Accordingly, genes induction of infected potato plants was accomplished as illustrated in (Fig. 2 d). High gene expression was noticed with infected plants after 48hr post infection with molecular sizes; 205 and 320 bp. As shown in (Fig. 2 e) using PAL primer, results revealed that a number of high intensive common bands between control and infected plants after 12hr, 24hr and 48hr post infection (290 and 300 bp), these two bands inverted into low intensive in early infection after 7 days post infection and subsequently, gradually disappeared in the late infection from 21 days post infection till became down regulated at 49 days post infection. The use of PR4 primer in DD-PCR was illustrated in (Fig. 2 f) where, there was induced band appeared in infected plants 48hr post infection with size 280 bp comparing with control. Consequently, the PR1 primer, the DD-PCR products revealed different genes in infected potato plants after 28 days post infection by G. rostochiensis with molecular size 690 bp compared with control plants (Fig 2 g). The induction as a result of infection, causes many alteration in gene expression regulation for both up or down with RAPD5 primer (Fig. 2 h), in contrast, only one band was observed with the control plants (295 bp, up regulated). In case of RAPD 7 primer, high gene expression was observed (up regulation) a 7 bands with molecular sizes ranged from 200-700 bp after 12, 24 and 48 hrs post infection compared with control (Fig. 2 i).

The DD-PCR amplification products of infected potatoes with nematode using RAPD10 primer (Fig 2 j), many different bands were up and down regulated genes that means, the pathogen (G. rostochiensis) induced the potato plant genes, therefore the changes in gene expression has been occurred and appeared in different intensity of numerous bands with assorted DNA with molecular sizes 390 bp and 400 bp after 12 hr to 7 days post infection respectively. Moreover, the negative control samples showed up regulated band with molecular size 700 bp, in addition up regulated band with molecular size 610 bp compared with control at 21 days after infection.

Figure 2 Up and down regulated genes of the DD-PCR using various arbitrary primers PR3r (a), PR3f (b), PP0r (c), PR0r (d), PALf (e), PR4r (f), PR1f (g), RAPD5 (h) RAPD7 (i) and RAPD10 (j) referring to the 7 selected bands for sequencing (arrows). M: 1.5 Kbp DNA marker, C; untreated potato plants (control), 12h; infected with G. rostochiensis after 12 hours, 24h; infected with G. rostochiensis after 24 hours, 48h; infected with G. rostochiensis after 48 hours, 7d; infected with G. rostochiensis after 7 days, 14d; infected with G. rostochiensis after 14 days, 21d; infected with G. rostochiensis after 21 days, 28d; infected with G. rostochiensis after 28 days, 35d; infected with G. rostochiensis after 35 days, 42d; infected with G. rostochiensis after 42 days and 49d; infected with G. rostochiensis after 49 days.

Sequence and phyllogentic analysis of potato pathogen related genes

The randomly selected DD-PCR bands with molecular sizes; 690, 590, 611, 280, 322, 295 and 700 bp as shown in table (2) up and down regulated genes were excised from the gel, purified and sequenced. The nucleotide sequences of these bands were aligned with the other deposited potato pathogen related genes available in the GenBank database and the results revealed that the band of 690 bp was "Glucanendo 1,3 beta glucosidase precursor. Whereas, the band of 590 bp is Linoleate 13S lipoxigenase 2-1 but the band of 611 bp was Phytoalexin deficient 4-1 protein gene. The band of molecular size 280 bp was PEN1 gene. Whenever, the amplicon of 322 bp is STS 14 protein gene but the band of 295 bp was identified as Chitinase domain containing protein 1* and finally, the 700 bp band was identified as N-like putative resistance protein (Table 2).

The DNA nucleotide sequences of the DD-PCR selected bands was used in construction of phylogenetic tree in compared with the other related genes listed on data base Fig. (3). The collective results indicated that, the Glucanendo 1,3 beta glucosidase A was induced as consequence of cyst infection and function an active role in hydrolysing β-1,3-glucan (a major structural component of pathogen cell walls), in addition it has significant role in the defence response against cyst nematode as a plant pathogenesis-related protein. In addition, the STS 14 protein gene is considered one of plant defense gene against plant pathogen and it is closely related to STS14-like proteins expressed in Solanum tuberosum. The Phytoalexin deficient 4-1 protein gene is identified as signal pathogenesis related proteins (R-gene signaling) which regulated SA mediated resistance response (S. tuberosum). The PEN1 gene its function is mediated secretory pathway in the pathogene trigger immunity against pathogen attacks. But, the Linoleate 13S lipoxigenase working as oixido-reductase activity and required for the wound-induced synthesis of jasmonic acid (JA) in leaves. Whenever, the Chitinase domain containing protein 1 had carbohydrate binding sites and was conserved very far back to organisms such as Caenorhabditis elegans. The PEN1 gene and Linoleate 13S lipoxigenase 2-1 are pathogen related genes. The N-like putative resistance protein is disease resistance protein and protects the plant against pathogens that contain an appropriate avirulence protein via a direct or indirect interaction with this avirulence protein. That triggers a defense system which restricts the pathogen growth where, response to the presence of a foreign body or the occurrence of an injury, which result in restriction of damage to the pathogen attacked or prevention or recovery from the infection caused by the attack.

Figure 3 Phylogenetic tree of the 7 (up and down) sequenced and aligned genes with the other published defensin genes and their accession numbers based on the nucleotide sequence alignment into GenBank. The scale for the branch length was given in substitutions per site.

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DISCUSSION

Globodera rostochiensis consider one of the plant parasitic cyst nematodes; was found in three governormates of Egypt in 2017 as previously mentioned by (Ibrahim et al., 2017). It is living in the soil and attacks the roots of plants. Moreover, it is obtained its nutrition from the cytoplasm of living plant cells causes terrible crop losses especially, potato plants which consider one of great economic crop and most essential crop in Egypt. Therefore, this investigation aimed to detect and identify the systemic induced genes of infected potato plants with cyst nematode via plant-nematode interactions. The cyst nematode samples were collected, isolated, propagated and identified and the well characterized nematode was used in studying the nematode-plant interaction on the molecular level.

In the current study, the ITS PCR showed two different amplified by the PR1 primer and amplified by the PR3 primer called PEN1 gene (Up regulated gene) in leaves; 590 bp. Similar result was obtained by PCR using G. rostochiensis specific primer and succeeded to identify G. rostochiensis ribosomal DNA sequence by Fleming et al., 1998 while, they found that, the PCR product of DNA was 238 bp. At the same investigation, the PCR result amplification product of internal standard template DNA was 748 bp according to their use of potato cyst nematode universal primer and it contradicted our result which was 370 bp. According to Fleming et al., 1998 they used an artificial internal control template to evaluate the quantity of target nematode DNA in a sample and thus to determine the viability and pest potential of the population. This could allow the population levels of cyst nematodes to be estimation over with species composition.

Infected potato plants with G. rostochiensis could differentially induce/suppressed a number of genes. DD-PCR results reflected the systemic response of plant cell according to G. rostochiensis infection. Subsequently, demonstration of some effective genes in plant defense against pathogens is very important. The sequence analysis identified a number of genes that putatively encode proteins that function in signal transduction during secondary pathway, control of gene expression and protect plant against pathogens. In this study, some genes were showed high similarity with the other pathogen related genes, these genes were differentially expressed in plant leaves and most of them were up regulated (induced by nematode infection). Glucanendo 1,3 beta glucosidase A amplified by the PR1 primer and it was induced in the infected potato plants after 28 days post infection with G. rostochiensis at late infection. Conversely, some studies reported significant increasing of β-1,3-endoglucanase to a maximum at 5 days post cyst nematodes infection during the gene expression and converted into down regulated by 9 and 14 days post infection wherever, the highest levels of β-1,3-endoglucanase expression during the plant–nematode interaction, enhance parasitism of cyst nematodes infection in the early stages (3–5 days post infection) (Hamamouch et al., 2012). Furthermore, (Hamamouch et al., 2011) reported increased of the expression level in Arabidopsis roots at 9 days after infection with the cyst nematode H. schachtii, using PR1 which considered one of the systemic acquired resistance markers. While, Beta endoglucanase is one of the important effector genes for plant infection with cyst nematode and up-regulated during the water uptake phase (Wang et al., 1999). In addition, (Goellner et al., 2000) found increasing in the expression of this gene before hatching of Globodera tabacum eggs. Also (Duceppe et al., 2017) mentioned that, the most essential polysaccharide degrading enzymes gene for plant colonization and prepare the environment for cyst nematode hatching of G. rostochiensis was beta-endoglucanases and it was up-regulated.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Name of sequence aligned genes</th>
<th>Length (bp)</th>
<th>Function of genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endoglucona se (PR₁)</td>
<td>Glucan endo 1,3 beta glucosidase A precursor. (Up regulated gene)</td>
<td>690</td>
<td>Has a vital role against pathogen attacks where, acting as a plant pathogenesis related protein, and induced by pathogen infection and it may hydrolysing a major structural component of pathogen cell walls which called β-1,3-glucon (Hamamouch et al., 2011).</td>
</tr>
<tr>
<td>Polynephol oxidase (PPO)</td>
<td>STS 14 protein gene (Up regulated gene)</td>
<td>322</td>
<td>Coding for a highly expressed mRNA in Solanum tuberosum explains similarity to pathogenesis related PR1 proteins of tobacco, Arabidopsis, barley, maize and tomato. The main function is protection against pathogens (Kombrink et al., 1988).</td>
</tr>
<tr>
<td>Chitinase (PR₂) Reverse</td>
<td>Phytoalexin deficient 4-1 protein gene (PAD4) (Up regulated gene)</td>
<td>611</td>
<td>One of the disease resistance signaling components (Signal pathogenesis related proteins), considering it essential regulator of salicylic acid mediated resistance responses (R-gene signaling) during SA dependent pathways where, regulates accumulation of the phenolic defense molecule (SA) in innate immunity of potato plants (Tamura et al., 2007 and Van Eldik et al., 1996).</td>
</tr>
<tr>
<td>Chitin Binding Protein (PR₃)</td>
<td>PEN1 gene (Up regulated gene)</td>
<td>280</td>
<td>One of important components of PTI (pathogen trigger immunity) against plant microbes in plants where, mediated secretory pathway, so the main function is consider one of defense signaling genes in potato leaves (Rasouli et al., 2015).</td>
</tr>
<tr>
<td>Chitinase (PR₄) Forward</td>
<td>Linolectase 13S lipoxigenase 2-1, chloroplastic like (Up regulated gene)</td>
<td>590</td>
<td>Has oxido-reductase activity, using linolenic acid as substrate in plants and may be involved in a number of diverse aspects of plant physiology including growth and development, pest resistance, and senescence or responses to wounding. Also, it required for the wound-induced synthesis of jasmonic acid (JA) in leaves.</td>
</tr>
<tr>
<td>RAPD 5</td>
<td>Chitinase domain containing protein 1 (CHDI1) (Down regulated gene)</td>
<td>295</td>
<td>This protein was recognized to have carbohydrate binding sites, which could be involved in carbohydrate catabolysis and was conserved very far back to organisms such as Caenorhabditis elegans.</td>
</tr>
<tr>
<td>RAPD 10</td>
<td>N-like putative resistance protein (Down regulated gene)</td>
<td>700</td>
<td>Is putative disease resistance protein where, considered it structural domains associated with resistance gene function in resistance response of potato against pathogens.</td>
</tr>
</tbody>
</table>
mediated membrane fusion events (Pratelli et al., 2004) and it proposed to have a role in the trafficking of secretory vesicles to the plasma membrane that contain cargo required for penetration resistance against plant microbes. Finally, Chitinase domain containing protein 1 gene was conserved very far back to organisms such as C. elegans. We assume that plant may produce chitinases to degrade the cell wall of the cyst.

CONCLUSION

This investigation considers the first report of characterizing cyst nematode (Globodera rostochiensis) on molecular level in Egypt. Where it showed the impact of this nematode on some plants and reduce their yield. Also, it showed the defense genes of the plant in response to cyst nematode infection. The results demonstrate the ability of RNAi and soil nematodes. This study strengthens the idea that nematodes can be used as a biological control agent against soil-borne pests.

REFERENCES


