AN EFFICIENT CDNA-AFLP-BASED STRATEGY FOR THE IDENTIFICATION OF PATHOGENICITY FACTORS FROM THE STEM NEMATODE DITYLENCHUS DIPSACI

IDENTIFIKACE FAKTORŮ PATHOGENICITY FYTOPARASITICKÝCH NEMATOD DITYLENCHUS DIPSACI POMOCÍ CDNA-AFLP STRATEGIE

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ABSTRACT

Ditylenchus dipsaci, the stem nematode, is a migratory endoparasite of over 500 species of angiosperms. Biological responses and developmental processes are precisely controlled at the level of gene expression. Most pathogenicity factors are not expressed constitutively, but in highly coordinated way. A relatively simple method for profiling differential gene expression is cDNA-amplified fragment length polymorphism (cDNA-AFLP). We aimed to use the cDNA-AFLP technique to sample the *D. dipsaci* transcriptome and discover genes specifically up-regulated during host infection. A random sample of six TDFs representing gene transcripts more abundant or specifically PCR amplified from infested plant tissues cDNAs, but not from non-infested plant material and nematode suspension cDNAs were cloned and sequenced after PCR re-amplification. Each sequence was compared by the BLASTX algorithm to sequences within the GenBank database. Besides similarities with *Caenorhabditis elegans* proteins, one TDF was significant similar to chitinase from *Heterodera glycine*.

Keywords: stem nematode**,** *Ditylenchus dipsaci*, differential gene expression, TDF – transcript-derived fragment, cDNA-AFLP, bioinformatic analyses, pathogenicity factors

ABSTRAKT

Ditylenchus dipsaci je volně žijící osní fytoparasitický nematod, který se vyskytuje v mnoha biologických rasách. Identifikace a funkční analýza faktorů pathogenicity produkovaných fytoparasitickými nematody je nezbytným předpokladem efektivního šlechtění a transgenoze rostlin. Jednotlivé vývojové procesy v průběhu ontogenese jsou na molekulární úrovni určeny změnami v genové expresi. Jedním z efektivních přístupů ke studiu koordinovaných transkriptomů patogen-rostlina je strategie RNA „fingerprintingu“ označovaná jako cDNA-AFLP. V naší práci jsme použili tuto strategii pro charakterizaci transkriptomu *D. dipsaci* v průběhu infekčního procesu. Náhodně bylo vybráno šest diferenciálně exprimovaných fragmentů, které byly následně klonovány a sekvenovány. Získané nukleotidové sekvence byly porovnány se sekvencemi v databázi GenBank pomocí modulu

BLASTX. Kromě podobností s proteinovými sekvencemi *Caenorhabditis elegans*, jedna sekvence vykazuje vysokou míru podobnosti s chitinasou *Heterodera glycine*. Na základě těchto zjištění, v dalších krocích bychom chtěli připravit kompletní sekvenci cDNA genu pro chitinasu.

Klíčové slova: osní nematody, *Ditylenchus dipsaci*, diferenciální genová exprese, faktory pathogeicity, cDNA-AFLP profilování, bioinformatická analýza

INTRODUCTION

Ditylenchus dipsaci (Kühn) Filipjev, the stem nematode, is a migratory endoparasite of over 500 species of angiosperms (Fortuner, 1982). *D. dipsaci* is prevalent in wide range of climatic conditions, where moisture regimes enable nematode infection, multiplication and dispersal (Smith et al., 1992). The main method of control of *D. dipsaci* is crop rotation, but the presence of morphologically indistinguishable host races with different host preferences makes rotation difficult (Wendt et al., 1993).

Biological responses and developmental processes are precisely controlled at the level of gene expression. Information on the temporal and spatial regulation of gene expression often sheds light on the potential function of a particular gene. Most pathogenicity factors are not expressed constitutively, but in highly coordinated way. Powerful techniques, which are capable to reveal the differential gene expression patterns of a large number of genes reliably, would enable us to identify many pathogenicity-related genes. A relatively simple method for profiling differential gene expression is cDNA-amplified fragment length polymorphism (cDNA-AFLP) (Bachem et al., 1996; Money et al., 1996). This method involves restriction digestion of cDNAs with a combination of two enzymes, one recognising a 6 bp and the other a 4 bp sequence. Adaptors are ligated to the digested material and subsets of cDNA populations are selectively PCR amplified for comparison on polyacrylamide gels. Following electrophoretic fractionation, fragments of specifically up-regulated cDNAs can be excised from the dried polyacrylamide gel, reamplified by PCR, cloned and sequenced. Unlike differential display techniques that use of small random primers (Liang and Pardee, 1992), relatively high annealing temperature can be used, hence, cDNA-AFLP is more stringent and reproducible. In addition, cDNA-AFLP does not require any pre-existing sequence information, which makes it an excellent tool to identify novel genes.

In this investigation, we aimed to use the cDNA-AFLP technique to sample the *D. dipsaci* transcriptome and discover genes specifically up-regulated during host infection. Identification of genes up-regulated in these life cycle stages will form a platform for future functional analysis to determine their role in *D. dipsaci* infection processes and yield insights into molecular communication between parasite and host during their interaction.

MATERIALS AND METHODS

Nematode isolates and plant host: The nematode isolates used are listed in Table 1. Some populations were derived directly from the field and others had been in culture for a number years. As a plant host was used chicory (*Cichorium inthybus* cv. Jupiter), which was maintained in green-house conditions. Plant tissues were artificially infested with 20 individuals of the stem nematode *D. dipsaci*, during 3–4 weeks typical symptoms were formed.

Table 1 Sources, geographic origin and host of nematode isolates used in this study.

Species	Host	Source	Origin
	Cichorium inthybus	O. Douda, VURV Prague	Czech Rep.
Ditylenchus dipsaci	*Cichorium inthybus*	O. Douda, VURV Prague	Czech Rep.
	Cichorium inthybus	G. Urek, AI Ljubljana	Slovenia

RNA isolation and cDNA synthesis: Total RNA was prepared from nematode individuals, infected and non-infected chicory leaves using the RNeasy plant mini kit (Qiagen), following the protocol supplied by the manufacturer. Integrity and yield of the RNA was tested by agarose gel electrophoresis. PolyA$^+$ RNA was purified by using the mRNA purification kit (Promega) following the manufacturer's instructions. Synthesis of cDNA was performed with SuperScript™ II RNase H$^-$ Reverse Transcriptase (Invitrogen) starting with 1.2 μg polyA$^+$ RNA and T_{18} oligonucleotide primer.

Simplified cDNA-AFLP procedure: Besides modifications in electrophoretic fractionation, cDNA-AFLP was performed as described (Bachem et al., 1996). Double-stranded cDNA was digested by restriction endonucleases EcoR I and Mse I and ligated to EcoR I and Mse I adapters by using AFLP® analysis system for microorganisms (Life Technologies). Pre-amplification was performed in 20 cycles (94°C for 30 s; 56°C for 60 s and 72°C for 60 s), using primers (indicated as „E" and „M") corresponding to the EcoR I and Mse I adapters without extension (AFLP® microorganism primer kit). After 50 times dilution of the PCR products, the template was amplified again with one selective base extension at the 3´-end of the primers E and M using a standard AFLP touchdown selective amplification program. The PCR products were not denatured and were directly loaded (10 μl) in each lane of the gel. The gel was run at 150 V, 40 W for 5 h until the loading dye had just run off the bottom of the gel. As fragment size references on non-denatured 8% polyacrylamide gel (only 15 cm in length), a MassRuler 100 bp and 1 kbp DNA ladders (Fermetas, Lithuania) were used.

Ultrasensitive silver staining was accomplished following the protocol published by Caetano-Anollés and Greshoff (1994). After electrophoresis, the gel was placed on a platform

shaker in 0.5 liter of 7.5% acetic acid for 30 min to fix the DNA to the gel. This solution was saved after fixing to use as a stop solution during development. The gel was then rinsed 3 times for 2 min with 0.5 liter of double-distillated water. The gel was then placed in 0.5 l of staining solution containing 0.75 g of silver nitrate, 280 µl of 37% formaldehyde allowed to incubate with shaking for 30 min. The gel was rinsed 15 s in 0.5 liter of double-distilled water and then immediately placed in 0.5 liter of chilled (10°C) developer solution (15 g sodium carbonate, 280 µl 37% formaldehyde, 200 µg sodium thiosulfate). Just as the bands started to appear, the gel was transferred to a fresh 0.5 liter of developer solution (chilled). When the staining had reached the desired level, 0.5 liter of the saved 7.5% acetic acid solution was added to stop the reaction. After 2 min the gel was removed and rinsed two times with 0.5 liter of distilled tap water. Differentially expressed bands were immediately cut out with a razor blade from the gel and incubated in 50 μl of TE (10mM Tris pH 8.0, 1 mM EDTA pH 8.0) overnight at room temperature and then at 65°C for 2 h. AFLP fragments were recovered by PCR under the same conditions as used for the selective amplification.

Cloning, sequencing and bioinformatic characterization: The PCR products re-amplified by primer pair E-0/M-0 were directly cloned to pTZ57R/T vector using 3´-A overhangs generated by Taq polymerase (InsT/Aclone™PCR Product Cloning Kit, Fermentas, Lithuania) following the protocol provided by the supplier and transformed into *E. coli* DH5α. Clones were checked for the DNA fragment insert by PCR, and plasmid preparation were made with the Perfectprep Plasmid Mini Kit (Eppendorf, Germany). Fragments were sequenced using an automatic sequencing system (ABI Prism 377, Perkin Elmer, USA). Alignment and comparison of the DNA sequences from the TDFs was conducted through the National Centre for Biotechnology Information (NCBI) GenBank using the Basic Local Alignment Search Tool (BLAST) algorithm (Altschul et al., 1997). The BLASTX option were used to search GenBank databases for similar protein sequences.

RESULTS AND DISCUSSION

The identification of TDFs with a pathogenicity factor like expression pattern: Assuming that the kinetics of expression revealed by cDNA-AFLP indeed reflect the mRNA abundances in the mRNA populations, twenty-four primer combinations (E-O + M-A/T/G/C, M-O + E-A/T/G/C and E-A/T/G/C + M-A/T/G/C) were used to generate a large number of mRNA expression profiles. A random sample of six TDFs representing gene transcripts more abundant or specifically PCR amplified from infested plant tissues cDNAs, but not from non-infested plant material and nematode suspension cDNAs were cloned and sequenced after PCR re-amplification. None of the cDNA-AFLP fragments selected as specifically or more highly PCR amplified in infested plant tissues were polymorphic between the three different *D. dipsaci* isolates.

Fig. 1 Basic principle of cDNA-AFLP strategy used in this study. This RNA „fingerprinting" approach allows to sample the coordinated transcriptomes and discover genes specifically up-regulated during host infection. Identification of genes up-regulated in these life cycle stages will form a platform for future functional analysis to determine their role in D. dipsaci *infection processes and yield insights into molecular communication between parasite and host during their interaction. The arrows point to differentially expressed transcript-derived sequences (TDFs) from infested plant tissues.*

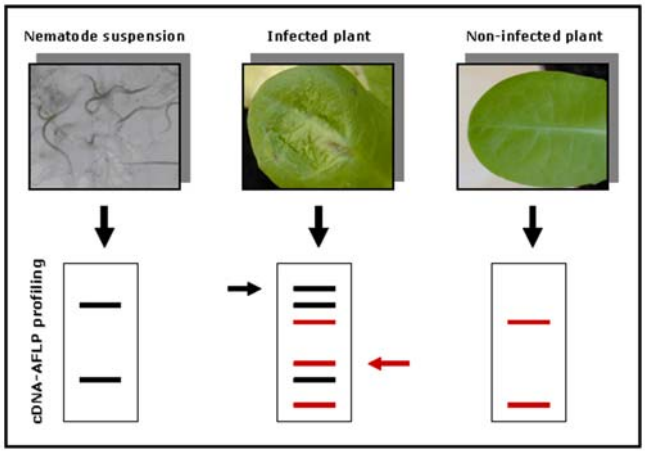
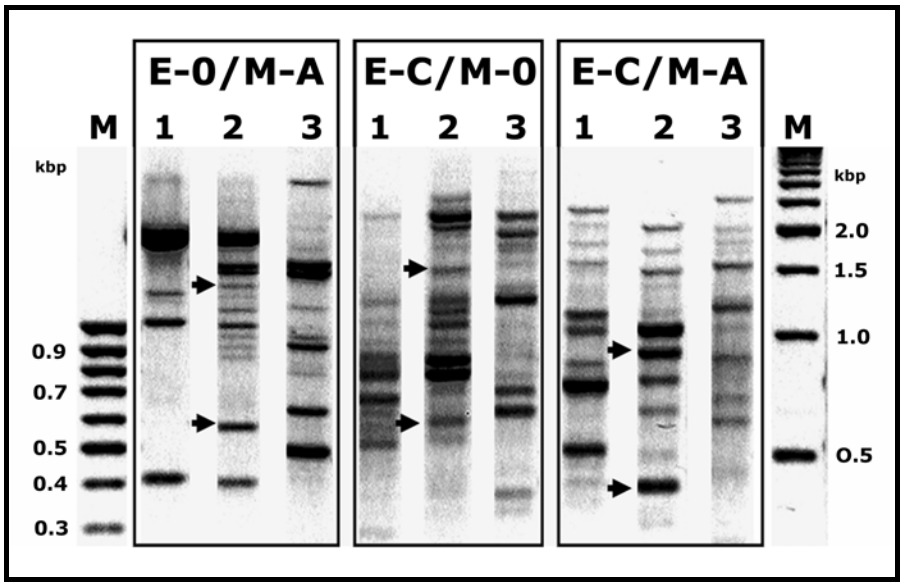


Fig. 2 An example of cDNA-AFLP expression pattern from nematode individuals in dormancy (1), infested plant tissues (2) and non-infested plant tissues (3). The arrows point to differentially expressed transcript-derived sequences (TDFs) from infested plant tissues that were excised from gel, re-amplified and sequenced. Primer combinations are shown at the top of cDNA-AFLP gel. The position of molecular size markers (M) are indicated at the left and the right.



Sequences from differentially expressed cDNA-AFLP products: Each sequence was compared by the BLASTX algorithm to sequences within the GenBank, DDJB and EMBL databases. Closest database similarities for each TDF sequence are shown in Table 2. Two TDFs were similar to *Caenorhabditis elegans* proteins, in the concrete to aconitate hydratase and glycerol-3-phosphate dehydrogenase. One TDF was significant similar to chitinase from *Heterodera glycine*. The other TDFs were matched to putative ABC transporter periplasmic binding protein from *Erwinia carotovora* subsp. atroseptica and hypothetical mitochondrial protein 29.1 from *Chondrus crispus*. Last TDF did not show significant similarity to any sequences residing in the GenBank database.

Table 2 Similarities at the protein level between TDFs from the stem nematode D. dipsaci *and sequences in databases.*

TDF Name	Database match – BLASTX	
	Closest similarity	**E-value**
DIT1	Q23500, Probable cytoplasmic aconitate hydratase [*Caenorhabditis elegans*] (Aconitase)	2e-42
DIT2	YP050925, putative ABC transporter periplasmic binding protein [*Erwinia carotovora* subsp. atroseptica SCRI1043]	5e-24
DIT3	None	
DIT5	AAN14978, chitinase [*Heterodera glycines*]	7e-06
D5F/Xho7	P34517, probable glycerol-3-phosphate dehydrogenase [NAD+], cytoplasmic	4e-27
D5F/Xho8	S59084, hypothetical protein 29.1 - red alga (*Chondrus crispus*) mitochondrion	2e-10

We report the use of cDNA-AFLP for identification of transcript-derived fragments (TDFs) potentially up-regulated in *D. dipsaci* during host infection process relative to their expression in dormancy. The application of cDNA-AFLP for gene discovery has been used previously for plant-parasitic nematodes. Qin et al. (2000) used this technique to identify putative pathogenicity factors from the potato cyst nematode *Globodera rostochiensis*.

This investigation has demonstrated the effectiveness of transcriptome-wide gene discovery tools such as cDNA-AFLP for simultaneously isolating many genes that are specifically up-regulated in certain developmental stages of plant-parasitic nematodes. Studying the functions of proteins encoded by these transcripts is the next major step towards determining their roles during infection of the host plant, potentially revealing novel targets for control of the stem nematode *D. dipsaci*.

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