MOLECULAR TECHNOLOGY FOR DEVELOPING DURABLE RESISTANCE TO THE SUGAR BEET ROOT MAGGOT (*TETANOPS MYOPAEFORMIS*)

Haiyan Li and Ann C. Smigocki
USDA-ARS Molecular Plant Pathology Laboratory, 10300 Baltimore Ave.,
Beltsville, MD 20705

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Abstract

Sugar beet root maggot (SBRM), *Tetanops myopaeformis* von Röder, is a major economic insect pest of sugar beet in North America. While several moderately resistant breeding lines have recently been registered, they do not offer complete control. A significant amount of knowledge about how plants protect themselves against insect invasion is being provided by advances being made in bioinformatics and functional genomics, however, complementary molecular studies on insect adaptive mechanisms used to overcome host resistance and develop tolerance to many insecticides are lacking. This study was initiated to establish a transcriptomic profile of SBRM genes and to identify physiologically valuable genes that can serve as targets for bio-insecticides and RNA interference mediated pest control. PCR-select suppressive subtractive hybridization (SSH) was used to produce an annotated SBRM EST dataset as a reference point for genes whose expression is modulated by interactions with resistant or susceptible sugar beet roots. This data will provide new insights into the molecular response elicited by SBRM in interactions with sugar beet roots and will advance the development of novel approaches for more effective SBRM control.

Introduction

Little information is available on how insects evolve adaptive mechanisms to overcome host resistance and develop tolerance to many insecticides used for their control. To gain a better understanding of insect biochemistry and molecular biology, this study was initiated to establish a transcriptomic profile of the sugar beet root maggot (SBRM, *Tetanops myopaeformis*) and to identify physiologically valuable genes that could serve as targets for development of pest control strategies. PCR-select suppressive subtractive hybridization (SSH) was used to generate cDNA libraries of SBRM genes that were specifically up- or down-regulated when the insect was feeding on resistant or susceptible sugar beet roots (Li et al., 2011). In this study, we report on the continued characterization of cloned SBRM genes that were selected for differential expression in interactions with roots of the sugar beet host plant. Functional annotation of the identified clones will provide knowledge on the possible molecular mechanisms that drive resistant and susceptible SBRM interactions with sugar beet roots. Information obtained from this study will be used for devising biotechnological approaches for managing pest adaptation and for pursuing sustained and environmentally sound insect control measures.

Materials and Methods

Plants and insects used for cDNA libraries

Sugar beet breeding lines F1016 with moderate resistance to SBRM and a susceptible line F1010 were used in this study (Campbell, 1990; Campbell et al., 2000). Three F1016 and F1010 plants (3 to 6-month old) were harvested and the roots washed in water. Plants were placed in a glass tray and the roots were infested with SBRM second- or early third-instars that were kept on **water**-moistened **nylon membranes** for 72 h prior to the start of the feeding experiment (Figure 1). Twenty larvae were frozen at 0, 1, 6, 24, 48 and 72 h after plant infestation and frozen at $^{-}80^{o}$C for preparation of RNA.

Figure 1. Sugar beet roots infested with second and early third instar root maggots.

RNA isolation

Total RNA was isolated using the Trizol Reagent (Invitrogen, Carlsbad, CA) and purified with RNeasy Spin Columns (Qiagen, MD). Genomic DNA was removed with RNase-Free DNase I (Qiagen). Poly $(A)^{+}$ RNA was prepared using a Poly(A) Purist Kit (Ambion Inc., Austin, TX).

cDNA library screening, sequencing and annotation

Suppressive subtractive hybridization (SSH) was carried out using the PCR-Select cDNA Subtraction Kit (Clontech Laboratories, Inc., Mountain View, CA) as previously reported (Li et al., 2011). Selected clones were sequenced and annotated using BLASTN to identify a unique set of ESTs. The ESTs were computed against the FlyBase (http://flybase.org/blast) and the NCBI non-redundant protein (Nr) database (http://blast.ncbi.nlm.nih.gov) using BLASTX algorithm with a cut-off E-value of 10^{-5} to indicate homology between *T. myopaeformis* sequences and insect sequences.

Gene ontology (GO) annotations were performed using Blast2GO v.2.4.2 (Conesa et al., 2005; Gotz et al., 2008). Peptide sequences were uploaded into the Blast2GO program, and BLASTP with a minimum E-value of 10^{-5} was performed by the program prior to mapping and

annotation into GO terms. The same settings were used to confirm significant ontology which was categorized as Molecular Function, Biological Process, or Cellular Component.

RT-PCR

mRNA expression patterns of selected SBRM clones were examined by RT-PCR. Pooled RNAs used for first strand cDNA was synthesis using SuperScript II (Superscript 1st Strand Synthesis System, Invitrogen). RT-PCR analysis was repeated three times and the transcript levels were determined by densitometry. Averaged results were analyzed using the student's *t*-test to calculate p-values.

Results and discussion

To understand the molecular mechanisms in the interaction of sugar beet and SBRM, SSH technology was initially utilized to establish a transcriptomic profile of sugar beet genes responsive to SBRM infestation (Puthoff and Smigocki, 2007). Present study utilizes similar approach to establish a transcriptomic profile of SBRM genes that can serve as targets for bio-insecticides and RNA interference mediated pest control.

Using the SSH enrichment technology, three subtracted cDNA libraries enriched in SBRM genes responsive to interactions with sugar beet roots that are resistant (F1016) or susceptible (F1010) to the root maggot were prepared (Li et al., 2011). Over 2000 clones were selected from these cDNA libraries and screened for genes that are up-regulated (forward subtracted) and down-regulated (reverse subtracted). More than half were randomly selected from each of the two subtracted libraries prepared from F1016 or F1010 intra-genotype subtractions and the other half of the clones came from inter-genotype treatment (F1016 subtracted with F1010 and vice versa). The library subtracted from the inter-genotype treatment generated fewer clones; however, such studies have been shown to be useful for identification of genes that are reciprocally regulated (Puthoff et al., 2003; Puthoff and Smigocki 2007). More than 760 SBRM ESTs were identified as being differentially expressed within the 72 hours of exposure to the resistant F1016 and/or susceptible F1010 roots. The expression pattern of six randomly selected clones was confirmed by RT-PCR analysis (Table 1).

SBRM sequences were compared with the FlyBase (http://flybase.org/blast) and the NCBI non-redundant protein (Nr) database (http://blast.ncbi.nlm.nih.gov) using the BLASTX program. Of the more than 760 assembled sequences, 342 (45%) matched at least one known protein with an e-value threshold of 1×10^{-5}, and the remaining clones showed no significant similarity to any known genes. Of the 342 known genes, 136 were determined to be unique clones. Distribution of e-value scores of the matched sequences was as low as 1×10^{-175}, but more than half of the sequences fell between 1×10^{-25} and 1×10^{-50}, strongly suggesting possible biological and biochemical functions for the cloned genes (Figure 2). The size of clones ranged from 200 to 1000 bp, with an average insert size of 480 bp. Furthermore, Blast2GO search shows that the species distribution of total BLAST hits of reads from the SBRM transcriptome is primarily driven by fruit fly *Drosophila melanogaster*, which has the most represented insect sequences in GenBank.

Table 1. RT-PCR analysis of six randomly selected SBRM clones whose expression is modulated by feeding on resistant (F1016 fed) and susceptible (F1010 fed) sugar beet roots compared to expression in unfed insects. * Student's *t*-test p-value ≤ 0.05. NC = no change.

The assembled sequences were then analyzed using Blast2GO software for a global visualization of the Blastx results. Gene ontology (GO) annotation provides descriptions of gene products in terms of their associated biological process, molecular function and cellular component (Berardini et al., 2004). GO functional interpretations for insects are primarily based on the *Drosophila* genome. Using Blast2GO, the assembled SBRM sequences revealed a dominance of metabolic and catalytic genes **that are involved in the initial interactions of the SBRM larvae with the sugar beet root.**

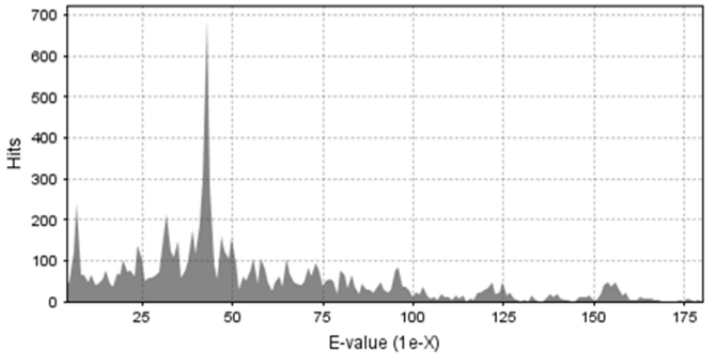

Figure 2. Distribution of e-value scores from BLASTx searches of the assembled SBRM cDNA sequences.

Since research on plant-insect interactions is currently limited by the lack of a genetically tractable herbivore that feeds from a well-studied model plant species, identification of candidate genes of *T. myopaeformis* depends primarily on genes from other insects where the immune/stress responses to pathogens or to insecticides have been described. Being the first EST data set for the SBRM, it establishes a complementary connection between the interaction of SBRM and its host and the basis for more in-depth molecular and genomic studies that will lead to development of strategies for better control of this insect pest of sugar beet.

ESTs	RT-PCR expression fold change *	
	F1016 fed	F1010 fed
SBRM EST1	1.2	5.4
SBRM EST2	3.3	-2.8
SBRM EST3	2.8	4.7
SBRM EST4	4.8	4.2
SBRM EST5	-3.2	1.5
SBRM EST6	NC	2.2

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