Transcriptome analysis of sugar beet root maggot (Tetanops myopaeformis) genes modulated by the Beta vulgaris host

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Abstract Sugar beet root maggot (SBRM, Tetanops myopaeformis von Röder) is a major but poorly understood insect pest of sugar beet (Beta vulgaris L.). The molecular mechanisms underlying plant defense responses are well documented, however, little information is available about complementary mechanisms for insect adaptive responses to overcome host resistance. To date, no studies have been published on SBRM gene expression profiling. Suppressive subtractive hybridization (SSH) generated more than 300 SBRM ESTs differentially expressed in the interaction of the pest with a moderately resistant (F1016) and a susceptible (F1010) sugar beet line. Blast2GO v. 3.2 search indicated that over 40% of the differentially expressed genes had known functions, primarily driven by fruit fly D. melanogaster genes. Expression patterns of 18 selected EST clones were confirmed by RT-PCR analysis. Gene Ontology (GO) analysis predicted a dominance of metabolic and catalytic genes involved in the interaction of SBRM with its host. SBRM genes functioning during development, regulation, cellular process, signaling and under stress conditions were annotated. SBRM genes that were common or unique in response to resistant or susceptible interactions with the host were identified and their possible roles in insect responses to the host are discussed.

Key words Beta vulgaris; resistant; sugar beet root maggot; suppressive subtractive hybridization; susceptible; transcriptome

Introduction

Sugar beet root maggot (SBRM, Tetanops myopaeformis) is the larvae of a small 2-winged fly that infests the roots of sugar beet (Beta vulgaris L.). It is the most destructive insect pest of sugar beet, affecting more than half of all North American sugar beet acreage. Infestations begin in late spring when the newly hatched larvae start feeding on roots of sugar beet seedlings. As the larvae develop, they continue to feed on roots throughout the growing season, inflicting significant crop damage and resulting in yield losses ranging from 10% to 100% (Campbell et al., 1998; Dregseth et al., 2003). To date, only 3 moderately resistant sugar beet lines have been released with at best 40% reduction of the SBRM damage (Campbell et al., 2000, 2011). Pesticides continue to be the primary control measure, but a critical need exists for an environmentally sound alternative that does not rely solely on pesticides. Thus, identifying insect molecular responses to their host will yield important new knowledge of plant–insect interactions and will facilitate the development of biotechnologically based insect pest-control practices.

Recent advances in bioinformatics and functional genomics is providing significant knowledge about how plants protect themselves against insect invasions (Zhu-Salzman et al., 2005). However, little information is available on how insects evolve adaptive mechanisms to overcome host resistance and develop tolerance to many insecticides used for their control (Dawkar et al., 2013; Ragland et al., 2015; Roy et al., 2016). So far the vast majority of research has focused on insect
immune responses to infectious microbes, primarily in model insects such as the flies (Drosophila spp., Irving et al., 2001; Boutros et al., 2002; Kaneko & Silverman, 2005; Cherry & Silverman, 2006; Lemaitre & Hoffmann, 2007; Sackton et al., 2007), mosquitoes (Anopheles gambiae, Aedes aegypti, Oduol et al., 2000; Christophides et al., 2002; Dimopoulos, 2003; Waterhouse et al., 2007), bees (Apis mellifera, Evans et al., 2006), aphids (Acrithosiphon pisum, Altincicek et al., 2008a; Gerardo et al., 2010), and beetles (Tribolium castaneum, Zou et al., 2007; Altincicek et al., 2008b). A study of nematode (Heteroder a glycines) gene expression in resistant and susceptible interactions with Glycine max roots focused on molecular mechanisms utilized by the nematode for invasion of the host plant (Klink et al., 2009). Recently, a few root-knot nematode (Meloidogyne incognita) genes involved in interactions with resistant and susceptible Alfalfa cultivars were identified; however, some potentially interesting or highly expressed M. incognita genes were not classified due to limited accessibility of current genome annotation of M. incognita (Postnikova et al., 2015).

Molecular responses in sugar beet host plants to SBRM feeding have been documented (Puthoff & Smigocki, 2007). Over 150 sugar beet root genes were identified in a moderately resistant F1016 or a susceptible F1010 sugar beet line (Puthoff & Smigocki, 2007). Several of these genes were characterized as to their functions in resistance mechanisms (Smigocki et al., 2008, 2009; Savić & Smigocki, 2012; Smigocki et al., 2013). In a reciprocal study, a PCR-select suppressive subtractive hybridization (SSH) was used to generate SBRM cDNA libraries enriched for larval genes modulated by the interaction of the pest with a moderately resistant or a susceptible sugar beet host (Li et al., 2011). In insects, SSH revealed transcriptional changes accompanying the interaction of an insect with an invading microbial pathogen in mosquito (Anopheles gambiae, Oduol et al., 2000), drone fly (Eristalis tenax, Altincicek & Vilcinskas, 2007a), greater wax moth (Galleria mellonella, Seitz et al., 2003), tobacco hornworm (Manduca sexta, Zhu et al., 2003), tsetse fly (Glossina morsitans morsitans, Hao et al., 2001), kissing bug (Rhodnius prolixus, Ursic-Bedoya & Lowenberger, 2007) and red flour beetle (Tribolium castaneum, Altincicek et al., 2008b).

In this study, we identified SBRM genes that are responsive to interactions with sugar beet hosts. Functional annotation of these genes may disclose molecular mechanisms driving resistant and susceptible SBRM interactions with sugar beet plants. Information obtained from this study may be useful for devising biotechnological approaches for managing pest adaptation and for pursuing sustained and environmentally sound insect control.

Materials and methods

Insects, plants, and insect infestations

SBRM larvae were collected from fields near St. Thomas, ND, and stored in field-collected soil at room temperature prior to the feeding experiments.

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). Plants were grown in the growth chamber at 25 °C during the day and 18–20 °C at night with a 16-h photoperiod.

For insect infestations, roots of 2-month old growth chamber grown plants were washed to remove the soil. Three plants of each genotype, F1016 or F1010, were placed on water-moistened nylon membranes (Roche, Mannheim, Germany) in a glass tray. One hundred second- or early third-instar SBRM, unfed for 72 h, were placed on the roots and allowed to feed for 6, 24, 48, and 72 h. Twenty maggots were collected at each time point, including the 0 h (unfed) and frozen at –80 °C for RNA isolation. Feeding experiments were repeated 3 times.

RNA isolation and suppressive subtractive hybridization

For RNA isolation, SBRM tissues were divided into 3 groups: SBRM unfed (A), fed on F1016 (B), and fed on F1010 (C). Groups B and C included pooled larvae from the F1016 or F1010 6, 24, 48, and 72 h time points, respectively. Each pooled group included all the larvae collected from all 3 feeding experiments.

Total RNA was isolated from each group with Trizol Reagent (Invitrogen, Carlsbad, CA, USA) and purified with RNeasy Spin Columns (Qiagen, MD, USA). 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, USA). SSH was carried out using the PCR-Select cDNA Subtraction Kit (Clontech Laboratories, Inc., Mountain View, CA, USA) according to the manufacturer’s specifications. Three complete subtractions were conducted: (1) F1016 fed versus unfed (B–A), (2) F1010 fed versus unfed (C–A), and (3) F1016 fed versus F1010 fed (B–C). For subtraction procedure (1) or (2), cDNA synthesized from the treated pool (B or C) was used as the tester, and that of the unfed control (A) was used as the driver for the forward subtraction. The reverse subtraction was performed with the control (A) as the tester and the treated tissues (B or C) as the driver. For
subtraction (3), each F1016 or F1010 fed pool (B or C, respectively) was used as the tester or driver in the forward or reverse subtractions. The resulting subtractive libraries were cloned in pCR2.1 TOPO vector (Invitrogen) and transformed into TOP10 E. coli cells (Invitrogen). Clones were plated on Luria Broth (LB) agar media containing 50 μg Kanamycin sulfate/mL (Kan-50), complemented with 40 mg/mL X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Recombinant white colonies were randomly picked into 96-well plates containing LB agar with Kan-50 and grown overnight.

Differential expression of subtracted cDNA clones were confirmed as directed by the PCR-Select Differential Screening Kit (Clontech). In brief, colony PCR was performed on over 3000 randomly picked colonies to amplify inserts with nested primers 1R and 2R provided in the screening kit. Two microtrols of resulting PCR products larger than 200 bp were identically spotted onto 4 sheets of positively charged nylon membranes (Roche) and UV cross-linked before blot hybridization. Probes generated from forward- and reverse- subtracted cDNAs were synthesized using the DIG-High Prime DNA labeling and Detection Starter Kit II (Roche). Probes were quantified as directed in the labeling kit in order to ensure equal amounts of probes were applied in all hybridizations. Prehybridizations and hybridization were performed at 42 °C using DIG Easy Hyb Granules (Roche) provided in the PCR-Select Differential Screening Kit. Probes were detected by using the DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche). Signals were visualized on Lumi-film chemiluminescent detection film (Roche). Signal intensity was quantified using an AlphaImager HP (Alpha Innotech, San Leandro, CA, USA). Gene expression fold-change was the ratio of hybridization signals between forward- and reverse- subtracted cDNA probes. The clones that had a ratio of at least 2 fold change were identified as differentially expressed and selected for sequencing.

Transcriptome analysis

Sequencing of differentially expressed clones was carried out at the Functional BioScience Inc., Madison, WI, USA. Raw sequences were stripped of contaminating vector and adaptor sequences and subjected to batch BLASTX search against the NCBI Nonredundant (Nr) database using Blast2GO v. 3.2 program (www.blast2go.com/blast2go-pro), the most up-to-date Blast2GO server. Using the same program, batch BLASTX was also conducted against the fruit fly (Drosophila melanogaster, taxa: 7227) database in order to identify related insect genes. Both BLASTX searches were computed using a cut-off expectation value (E-value) of 1.0E-5. The identical or highly similar fragments were removed by using CD-HIT program (http://weizhongli-lab.org/cd-hit/) to reduce the redundancy from combining the assemblies. A representative individual (nonredundant) clone was chosen from highly similar fragments for further analysis. The nonredundant EST sequences, excluding mitochondrial and ribosomal sequences, were submitted to dbEST (batch-sub@ncbi.nlm.nih.gov) to obtain GenBank accession numbers.

Gene ontology (GO) annotations were performed with Blast2GO program filtered by the database of flies (Diptera, taxa: 7147) to determine each putative protein’s role in biological process, molecular function and cellular component. In addition, the sequences were annotated according to Kyoto Encyclopedia of Genes and Genomes (KEGG) orthology (KO) and Enzyme Codes (EC) with default parameters. Each sequence was assigned with a unique EC number and was mapped to specific biochemical pathways according to the corresponding EC distribution in the KEGG database. Pathways of the annotated KO terms were identified using the KO Based Annotation System server 2.0, or KOBAS (http://kobas.cbi.pku.edu.cn) (Xie et al., 2011), which incorporates knowledge across 1327 species from 5 pathway databases of KEGG, PID (Schaefer et al., 2009), BioCyc (Karp et al., 2005), Reactome (Croft et al., 2011), and Panther (Thomas et al., 2003). In this analysis, the entire D. melanogaster gene set was used for background distribution.

RT-PCR

To confirm SSH data, RT-PCR analysis was carried out with 18 randomly selected SBRM clones using the same RNAs used for SSH. First-strand cDNA was synthesized using SuperScript II (Superscript 1st Strand Synthesis System, Invitrogen). Negative controls consisting of cDNA synthesis reactions without reverse transcriptase were also conducted to rule out genomic DNA as a source of template for PCR reactions. In order to ensure equal amounts of cDNA template were used in each PCR reaction, cDNA was quantified spectrophotometrically and confirmed visually with ethidium bromide staining. Amplification was conducted using gene-specific primers (Table S1) under the following conditions: initial incubation at 94 °C for 30 s; followed by 94 °C for 10 s; annealing temperature specific for each gene (48–80 °C) for 30 s; and 72 °C for 1 min. In order to obtain quantitative differences in samples, aliquots of PCR reactions were removed from tubes after 15, 20, 25, and 30 cycles to avoid being overcycled. RT-PCR analysis was repeated 3 times and gene expression was quantified by
Table 1 Overview of the SBRM EST libraries generated by SSH.

| Subtracted libraries | Differentially expressed ESTs§ | NCBI database†† | D. melanogaster database†† | Forward subtraction‡‡ | Reverse subtraction‡‡ |
|----------------------|-------------------------------|----------------|--------------------------|----------------------|----------------------|
| F1016 fed vs. unfed (B–A)† | 147                           | 68             | 59                       | 50                   | 18                   |
| F1010 fed vs. unfed (C–A)‡ | 71                             | 52             | 50                       | 47                   | 5                    |
| F1016 fed vs. F1010 fed (B–C)§ | 158                           | 56             | 53                       | 42                   | 14                   |
| Total                | 376                           | 176            | 162                      | 139                  | 37                   |

§ The number of ESTs that had a ratio of at least 2 fold change between the hybridization signals from forward- and reverse-subtracted cDNA probes.
†† The number of ESTs that had significant blast hits (e-value $< 10^{-5}$) against the NCBI nonredundant database or the fruit fly D. melanogaster database.
‡‡ The number of ESTs that had significant blast hits (e-value $< 10^{-5}$) against the NCBI nonredundant database.

**Results**

**Generation and assembly of SBRM ESTs**

PCR-select SSH generated 3 cDNA libraries that were enriched for SBRM genes differentially regulated by contact of the pest with a moderately resistant (F1016) and a susceptible (F1010) sugar beet line (Table 1). The first 2 subtracted libraries were made between F1016- or F1010-fed versus unfed larvae. These libraries should be enriched for SBRM genes that are upregulated (forward subtraction) and downregulated (reverse subtraction) by interactions with F1016 or F1010 plants. The third subtraction was made between F1016-fed versus F1010-fed larvae to identify any genes that are only induced in the F1016-fed larvae and/or genes with relatively higher expression levels in response to feeding on F1016 versus F1010 plants (forward subtraction) and vice versa (reverse subtraction). It added an additional comparison of gene expression between feeding on resistant and susceptible sugar beet genotype. These sequence data from the B–A, C–A, and B–C libraries have been submitted to the dbEST database (batch-sub@ncbi.nlm.nih.gov) under library accession numbers of LIBEST_028737, LIBEST_028738 and LIBEST_028739, respectively.

SSH analysis demonstrated 376 SBRM clones differentially expressed within 72 h of exposure to the moderately resistant and susceptible sugar beet plants (Table 1). BlastX search indicated that 176 (46.8%) or 162 (43.0%) of these differentially expressed clones had significant (e-value $< 10^{-5}$) blast hits with the NCBI Nr or the fruit fly D. melanogaster database, respectively.

Sequence mapping by Blast2GO indicated that the distribution of e-value scores of the matched sequences was as low as $10^{-175}$, but more than half of the sequences fell between $10^{-25}$ and $10^{-50}$ (Fig. 1A). The size of clones ranged from 200 to 1000 bp, with an average insert size of 480 bp (Fig. 1B). Furthermore, Blast search showed that the species distribution of the SBRM transcriptome was primarily driven by D. melanogaster, which has the most represented insect sequences in GenBank (Fig. 1C). These data indicated that the subtracted libraries contained good quality sequences that captured ample sequence homology to provide identification of a substantial fraction of the RNA messages produced during SBRM and sugar beet interaction.

**SBRM genes regulated by F1016 plant interaction**

The subtraction from the interaction of SBRM fed on F1016 roots versus unfed (B–A) revealed 147 differentially expressed SBRM ESTs within 72 h of interaction with the moderately resistant sugar beet line (Table 1). Among the 147 ESTs, 68 and 59 had similarities (e-value $< 10^{-5}$) with the NCBI Nr and D. melanogaster database, respectively. Of the 68 known genes, 50 were upregulated and 18 were downregulated upon interaction with the moderately resistant F1016 plants (Table S2A).
Fig. 1 Statistical analysis of BLASTX search of the assembled SBRM EST sequences against the NCBI nonredundant protein database using Blast2GO v. 3.2 program (www.blast2go.com/blast2go-pro) with default parameters. (A) Distribution of e-values. (B) Sequence length statistics. (C) Species distribution.

**SBRM genes regulated by F1010 plant interaction**

The subtraction of SBRM transcripts induced by feeding on F1010 tissues versus transcripts from unfed larvae (C–A) identified 71 differentially expressed SBRM ESTs after contact with the susceptible sugar beet line. Of those, 52 and 50 had known functions based on the NCBI and *D. melanogaster* database, respectively (Table 1). Among the 52 known genes, 47 were upregulated and 5 were downregulated by interaction with the F1010 plants (Table S2B).

**Comparison of SBRM genes regulated by F1016 and F1010**

In order to understand the genetic responses of SBRM to its host’s resistance, it is critical to identify SBRM genes that are common or unique in response to the resistant or susceptible genotype by comparing the first 2 libraries (Table 1, B–A and C–A). Interestingly, except for mitochondrial, ribosomal and hypothetical genes, only 1 characterized gene encoding transferrin was found to be regulated by both F1016 (JZ922760, Table S2A) and F1010 (JZ922885, Table S2B). Two pairs of clones (JZ922795 and JZ922892; JZ922789 and JZ922904) each shared over 99% sequence similarities but had no known functions. Other clones did not reveal exact sequence matches; however, common putative functions were identified. For example, 2 clones that only had 46.6% sequence similarity were found to encode glutactin or glutactin-like proteins, JZ922749 (Table S2A) and JZ922871 (Table S2B), and have the same match to the *D. melanogaster* gene encoding esterase Q.

The third subtracted library (B–C) expected to identify any genes that are only induced in the F1016-fed larvae and/or genes with relatively higher expression levels
in response to feeding on F1016 versus F1010 plants (forward subtraction) and vice versa (reverse subtraction) generated 158 differentially expressed ESTs. Of those 158 ESTs, 56 and 53 had known functions identified in the NCBI and fruit fly databases, respectively (Table 1). Among these 56 known genes 42 were from the forward and 14 from the reverse subtraction (Table S2C). There were 13 genes in this subtraction that were not identified from the first 2 libraries, including 7 identified from the forward subtraction, such as the genes encoding nascent polypeptide-associated complex subunit alpha (JZ922912), ras GTPase-activating-binding 1 (JZ922927), serine threonine-kinase 24 (JZ922928), and signal transducer and activator of transcription B (JZ922929). Six additional genes were found in the reverse subtraction and included genes encoding major royal jelly 1-like (JZ922935), exportin-1 (JZ922937), and glucose transporter 1 (JZ922938). A cathepsin L1 gene (JZ922867, Table S2B) induced by exposure to F1010 (C–A library) was found to have higher expression regulated by F1016 versus F1010 feeding (JZ922924, Table S2C). Although other clones did not share exact sequence matches, common putative functions were noted, for example, the genes encoding carboxylesterase and soluble NSF attachment proteins were also found in the F1016-fed versus unfed library (B–A).

Functional annotation of SBRM genes

The SBRM genes up- or downregulated by the interaction with F1016 or F1010 plants were subjected to GO analysis based on the *Drosophila* genome (Fig. 2). GO annotation describes gene products in terms of their associated biological process, molecular function and cellular component (Berardini et al., 2004). Blast2GO revealed that cellular and metabolic processes were the predominant biological terms for both F1016- and F1010-regulated SBRM genes within the biological process category (Fig. 2A). This was expected because the maggots used in this study were at the second or early-third instar stage during which many metabolic processes such as the cytoskeleton organization, cell cycle and generation and use of energy are involved. Other common processes represented by GO terms “localization,” “developmental process,” “biological regulation” were shared by genes regulated by both lines. A small number of genes, mostly regulated by F1016 plants, were grouped into the categories of “response to stimulus” and “signaling.” These genes, for example, encoded the defense responsive protein peptidoglycan recognition SB1 (JZ922754) and the signaling protein 14-3-3 zeta (JZ922732), were specifically upregulated by feeding on F1016 (Table S2A). Genes classified into “cellular component biogenesis and orga-n...
Table 2  Metabolic pathways involved and the number of clones identified in each SBRM subtraction library using the indicated databases.

| No. | Pathways                              | Database | Background genes | B–A | C–A | B–C |
|-----|---------------------------------------|----------|------------------|-----|-----|-----|
| 1   | Metabolism of proteins                | Reactome | 209              | 9   | 14  | 5   |
| 2   | Oxidative phosphorylation             | KEGG     | 138              | 4   | 4   | 2   |
| 3   | Nicotinic acetylcholine receptor signaling | Panther | 38               | 3   | 1   | 0   |
| 4   | Developmental Biology                 | Reactome | 98               | 1   | 1   | 1   |
| 5   | Apoptosis signaling pathway           | Panther  | 21               | 1   | 1   | 0   |
| 6   | FGF signaling pathway                 | Panther  | 38               | 1   | 0   | 0   |
| 7   | Ubiquitin mediated proteolysis        | KEGG     | 98               | 1   | 0   | 0   |
| 8   | Phagosome                             | KEGG     | 72               | 1   | 0   | 0   |
| 9   | Biosynthesis of unsaturated fatty acids | KEGG  | 16               | 1   | 0   | 0   |
| 10  | Catecholamine biosynthesis            | BioCyc   | 12               | 1   | 0   | 0   |
| 11  | Regulation of autophagy               | KEGG     | 14               | 1   | 0   | 0   |
| 12  | Glycolysis/gluconeogenesis            | KEGG     | 50               | 0   | 1   | 0   |
| 13  | Insect hormone biosynthesis           | KEGG     | 14               | 0   | 1   | 0   |
| 14  | Metabolism of RNA                    | Reactome | 52               | 0   | 1   | 0   |
| 15  | Drug metabolism—other enzymes         | KEGG     | 9                | 0   | 1   | 0   |
| 16  | RNA transport                         | KEGG     | 127              | 0   | 0   | 2   |
| 17  | Signal transduction                   | Rectome  | 302              | 0   | 0   | 1   |
| 18  | Lysosome                              | KEGG     | 87               | 0   | 0   | 1   |

1Numbers of genes present for the particular pathway in D. melanogaster database.

1B–A, C–A, and B–C represent the 3 subtracted libraries (see Table 1).

In terms of molecular function (Fig. 2B), most of the SBRM genes induced by F1016 and F1010 were classified into categories “binding,” “catalytic activity,” and “transporter activity.” Equal distribution of F1016- or F1010-regulated genes into categories “cell,” “organelle,” and “macromolecular complex” was observed (Fig. 2C).

**KEGG pathway assignment**

By using the pathway identification tool KOBAS, 61 SBRM genes were classified into 18 pathways (Tables 2 and S3). Most of the proteins mapped to KEGG pathways were metabolism proteins such as elongation factors and ribosomal proteins. These included 9, 14, and 5 genes identified in F1016 fed versus unfed (B–A), F1010 fed versus unfed (C–A) and F1016 fed versus F1010 fed (B–C) subtraction, respectively. These genes represented 13.4% (28/209) of the D. melanogaster genes for this pathway. Other pathways such as the oxidative phosphorylation, nicotinic acetylcholine receptor signaling, developmental biology, and apoptosis signaling pathway (No. 2–5 in Table 2) were associated with the SBRM interaction with both F1016 and F1010 plants. We found 6 pathways mapped only to the SBRM genes identified in the B–A subtraction. These included the FGF signaling pathway, ubiquitin mediated proteolysis, phagosome, biosynthesis of unsaturated fatty acids, catecholamine biosynthesis and the pathway of autophagy regulation (No. 6–11). These pathways involved the genes that encode 14-3-3 zeta (JZ922732), ubiquitin activating enzyme 1 (JZ922770), cytoplasmic dynein 1 intermediate chain isoform X1 (JZ922746), fatty acid desaturase (JZ922773), peptidylglycine alpha-hydroxylating monoxygenase (JZ922778), and gamma-aminobutyric acid receptor-associated protein (JZ922774). There were 4 pathways (No. 12–15) associated with the SBRM interaction with F1010 plants (C–A subtraction). Three pathways were found to be solely related to the third subtraction (No. 16–18), which involved genes that encode exportin-1 (JZ922937), translation elongation factor 1 alpha (JZ922943) and ras GTPase-activating -binding 1 (JZ922927) that were identified in the reverse subtraction, and cathepsin L1 (JZ922924) identified in the forward subtraction.

**RT-PCR confirmation of SSH data**

To confirm the SSH data, RT-PCR analysis was carried out with 18 differentially expressed genes chosen...
Table 3  Confirmation of the transcriptomic data (SSH) by RT-PCR.

| GenBank accession # | Description                                         | Libraries† | SSH‡ | RT-PCR‡ |
|---------------------|------------------------------------------------------|------------|------|---------|
| JZ922732            | 14-3-3 zeta                                          | B–A        | 3.61 | 4.13    |
| JZ922763            | Venom carboxylesterase-6-like                       | B–A        | 2.42 | 1.98    |
| JZ922762            | Ubiquitin carboxyl-terminal hydrolase 7             | B–A        | 5.72 | 4.85    |
| JZ922747            | Dopamine N-acetyltransferase-like                    | B–A        | 6.15 | 2.93    |
| JZ922758            | Na/K-transporting ATPase subunit alpha               | B–A        | 5.32 | 3.61    |
| JZ922749            | Glutactin                                            | B–A        | 4.51 | 3.90    |
| JZ922775            | Heat shock cognate protein 70–4                     | B–A        | -3.51| -2.89   |
| JZ922770            | Ubiquitin activating enzyme 1                        | B–A        | -4.41| -3.39   |
| JZ922778            | Peptidylglycine-alpha-hydroxylating monoxygenase     | B–A        | -6.81| 3.79    |
| JZ922885            | Transferrin                                          | C–A        | 6.91 | 5.43    |
| NS                  | Cytochrome c oxidase subunit III                     | C–A        | -4.86| -2.84   |
| JZ922869            | Elongation factor 1 alpha48D                        | C–A        | 4.30 | 4.79    |
| JZ922867            | Cathepsin L1                                         | C–A        | 5.71 | 4.32    |
| JZ922862            | Myosin heavy chain, isoform U                        | C–A        | 5.21 | 3.11    |
| JZ922887            | Uninitiated, partial                                 | C–A        | 2.56 | 1.59    |
| JZ922941            | Larval hexamerin Hex-L                               | B–C        | -2.11| 1.79    |
| NS                  | Ribosomal L10                                        | B–C        | 3.23 | 5.25    |
| JZ922936            | Fat body 2                                           | B–C        | -3.21| -3.39   |

†The 3 subtracted libraries (see Table 1).
‡The SSH and RT-PCR data represent the relative gene expressions regulated by sugar beet lines in each subtracted library. SSH, gene expression fold-change is the ratio of hybridization signals between forward- and reverse-subtracted cDNA probes. RT-PCR, a relative gene expression is the ratio of transcripts between the fed and unfed SBRM larvae (B–A and C–A subtractions) and between the F1016-fed and F1010-fed larvae (B–C subtraction). The correlation (P value) between SSH and RT-PCR data was analyzed by Student’s t test. In B–A and C–A subtractions, the plus and minus values represent the up- and downregulations, respectively. In B–C subtraction, the plus or minus values represent the genes with relatively higher expression levels induced by F1016 than by F1010, or vice versa.
NS = sequence not submitted.

randomly to represent both forward and reverse subtractions (Table 3). Of the 18 genes tested, 16 showed total agreement or slight differences between SSH and RT-PCR analyses (P < 0.1). The peptidylglycine-hydroxylating monoxygenase gene (JZ922778) that was downregulated by F1016 appeared to be upregulated by RT-PCR analysis. Another gene encoding larval hexamerin hex-L (JZ922941), identified by SSH as having more than 2 fold higher transcript levels when induced by F1010 versus F1016 feeding, was shown by RT-PCR with slightly higher expression regulated by F1016 exposure.

Discussion

Just as research on plant–pathogen interactions has benefited greatly from pairs of sequenced genomes, for example, Arabidopsis and Pseudomonas syringae, there will be synergistic effects in studying not only plant responses to insect herbivory, but also insect responses to plant defense. Whereas several insect genomes have been sequenced, there is currently no plant–insect model system available to conduct genetic and genomic studies using both partners in the interaction. Lacking are complementary molecular studies on insect adaptive mechanisms used to overcome host resistance and develop tolerance to insecticides. 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 & Smigocki, 2007). Using a similar approach, this study revealed over 300 root maggot genes that were differentially expressed in response to interaction with a moderately resistant or a susceptible sugar beet line. The SBRM EST database generated in this study will be coupled with our knowledge of resistance responses in sugar beet plants to serve as a reference point for molecular studies of nonmodel organisms.
Proteins involved in transcription and protein biosynthesis

We identified a large group of SBRM genes involved in protein synthesis, processing, and trafficking in all 3 subtracted libraries (Table S2 and Fig. 2). These include genes encoding ribosomal and mitochondrial proteins, elongation factors, translation initiation factor, sulfotransferase, and acetyltransferase. Ribosomal proteins play various roles, in addition to protein biosynthesis, termed extra-ribosomal functions, which include transcription, signal recognition, apoptosis, and nuclear transport protein synthesis (Lindstrom, 2009). The induced expression of proteins involved in the cellular transcription and translation machinery is commonly found in response to immune challenge in insects (Irving et al., 2001; Zhu et al., 2003; Johansson et al., 2005; Altincicek & Vilcinskas, 2007b; Ursic-Bedoya & Lowenberger, 2007). Appearance of these proteins in the SBRM subtracted libraries may reflect an increased demand by the insect fat body for producing various inducible proteins as a response to the host.

Catalytic proteins

A significant portion of sugar beet-induced SBRM genes encoded binding and catalytic proteins predominantly involved in cellular and metabolic processes. These included catalytic proteins with hydrolase (esterase/carboxylesterase), oxidoreductase (cytochrome b), and transferase (flavin adenine dinucleotide [FAD] synthetase) activities (Fig. 2 and Table S2). Insect metabolic and catalytic genes were demonstrated to function in insecticide resistance when they were identified in the midgut transcriptome profile of tobacco budworm, Heliothis virescens, exposed to biological and chemical pesticides (Zhu et al., 2011). In another report, their up-regulation led to a substantial increase of metabolic detoxification in acephate-resistant tarnished plant bug, Lygus lineolaris (Zhu et al., 2012). Similar response was also observed in a Bt-resistant strain of sugarcane borer, Diatraea saccharalis (Guo et al., 2012). The dominance of metabolic and catalytic genes found in the SBRM response to the sugar beet host suggested similar molecular mechanisms to those reported in analysis of other insects’ responses to insecticides.

Defense/resistance response proteins

Some of the identified SBRM genes played an important role in resistance or tolerance in other insects. Genes associated with defense responses were only found in the subtracted library of SBRM fed on the moderately resistant sugar beet line (B–A library, Table S2A), suggesting they play a role in the insect’s antiresistance during the insect–host interaction thus warranting further investigation. One of the genes shares high homology with the gene that encodes peptidoglycan recognition protein SB1 (PGRP-SB1, JZ922754). PGRPs activate intercellular immune signaling pathways in D. melanogaster upon interaction with PAMPs (pathogen associated molecular patterns) (Medzhitov & Janeway, 2002). PGRP-SB1 is 1 of the 13 PGRPs identified in the Drosophila genome whose expression was highly induced after infection, far more than for any other PGRP and to an extent similar to that of effectors such as antimicrobial peptide genes (Zaidan-Remy et al., 2011). This suggests that SBRM-PGRP-SB1 may be involved in SBRM-sugar beet interactions and may play a role in recognition of antiresistance peptide genes as well as in regulation of other immune responsive genes that have often been reported in other insects and vertebrates (Irving et al., 2001; Altincicek et al., 2008a).

Similarly, an SBRM gene encoding carboxylesterase-6 protein (JZ922763) was identified. Carboxylesterase proteins display active catalytic triad, which contains the active center essential for esterase activity. Besides their functions in insecticide resistance (Guerrero et al., 1999; Guerrero, 2000; Li et al., 2007; Zhu et al., 2011, 2012), their involvement in insect immune responses to bacterial (Shiotsuki & Kato, 1999) and fungal infection or mechanical wounding (Serebrov et al., 2001) have been reported. It was proposed that these immune inducible carboxylesterases function in degrading toxic substances generated during microbial infection. The SBRM carboxylesterase gene identified in this study was upregulated by the interaction with the moderately resistant F1016 genotype, which was in agreement with other reports where its upregulation was described more generally as part of the resistance/stress response.

Other identified proteins that were only associated with the SBRM-F1016 interaction are potentially involved in signal inductions. For example, the upregulated 14-3-3 zeta gene (JZ922732) is known to participate in fibroblast growth factors (FGFs) signal pathway and in regulatory processes by interacting with diverse target proteins in a sequence-specific and phosphorylation-dependent manner (Muslin et al., 1996; Bridges & Moorhead, 2005). The Ras GTPase-activating-binding gene (JZ922927) that was also induced by F1016 is required as a binding protein of GTPase activating proteins (GAPs) that negatively regulate Ras signaling pathway, which plays a role in cellular immune responses in Drosophila (Pazman et al., 2000; Lemaitre & Hoffmann, 2007). Ras-induced production of
reactive oxygen species (ROS) has been demonstrated as one of the early events important in plant–insect interactions (Maffei et al., 2007). In this study, upregulated GTPase-activating-binding gene may positively regulate Ras signaling pathway, which may contribute to the responses of SBRM to sugar beet.

**Common genes/proteins in resistant and susceptible interactions**

A few common genes/proteins involved in interactions with both F1016 and F1010 genotypes were demonstrated to be important in immunity and regulation in other insects. For example, 1 gene that encodes cathepsin L1 was found to be regulated by both F1016 and F1010 (Tables 3, S2B, and S2C). Cathepsin L1 is a lysosomal cysteine protease (CP) that belongs to peptidase C1A subfamily with a cathepsin propeptide inhibitor domain (I29). The I29 domain acts as an intramolecular inhibitor and is necessary for proper folding of the newly synthesized enzyme and its stabilization in denaturing pH conditions (Marchler-Bauer et al., 2011). In addition to its inhibitory role, cysteine proteinases (CPs) have different functions in various organisms (Matsumoto et al., 1995). Interestingly, parasitic CPs act extracellularly to help invade tissues and cells, to hatch or to evade the host immune system, which suggests that SBRM cysteine proteinase may play a role in evading its host system. Another gene upregulated by both F1016 and F1010 encodes a transferrin protein (Tables 3, S2A, and S2B). Insect transferrins have been postulated to have roles in immune response to bacterial infections since their expression levels are upregulated following exposure to pathogens (Yoshiga et al., 1999; Ampasala et al., 2004; Harizanova et al., 2005). Similarly, an SBRM gene that shares homology to the esterase Q gene of *D. melanogaster* (*D. melanogaster* ID: 7296447) was identified as upregulated by both F1016 and F1010 (Tables S2A and S2B). Esterase Q belongs to the glutactin class that is 1 of the 8 subfamilies of insect carboxylesterases (EC 3.1.1.1). Interestingly, the glutactin gene was identified recently in *Drosophila* as one of the hemocyte enriched genes involved in defense against nematode infection (Wang, 2012). Downregulation of the glutactin gene reduced production of glutactin in hemocytes thus weakening the protection provided by the extracellular matrix to impede entry (Wang, 2012).

**Conclusion**

This is a first report on the *T. myopaeformis* larval transcriptome data set of over 300 ESTs that are common or unique in response to resistant and susceptible interactions with the sugar beet host. This study definitively establishes a complementary connection between the SBRM larvae and its host. Moreover, it identifies SBRM genes that are critical in physiology, development, regulation and cellular processes, as well as genes responsive to environmental conditions. Since research on plant–insect interactions is currently limited by the lack of a genetically tractable herbivore that feeds on a well-studied model plant species, identifying candidate *T. myopaeformis* genes relied on the recognition of important genes in other insects that display immune/stress responses to pathogens or to insecticides. A closer examination of the respective genes is needed to decipher whether they are the same gene or members of a gene family that respond differently to various environmental stimuli (Wang & Constabel, 2004). The analysis of this data set is continually evolving and some of the conclusions may have to be revised as more advanced bioinformatic tools become available.

**Acknowledgments**

We thank Dr. Larry Campbell (USDA, ARS, Fargo, North Dakota, USA) for providing sugar beet seeds and sugar beet root maggot larvae. This work was supported in part by a Cooperative Agreement between the U.S. Department of Agriculture and the Beet Sugar Development Foundation.

**Disclosure**

The authors declare no conflict of interest. Mention and/or use of a commercial or proprietary product to the exclusion of others does not constitute endorsement by the USDA.

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article at the publisher’s web-site:

**Table S1** RT-PCR primers used in this study.

**Table S2A** SBRM genes modulated by moderately resistant sugar beet line F1016 (B–A).

**Table S2B** SBRM genes modulated by susceptible sugar beet line F1010 (C–A).

**Table S2C** SBRM genes regulated by F1016 versus F1010 (B–C).

**Table S3** Metabolic pathways involved in each subtracted cDNA library.