SUGAR BEET ROOT MAGGOT (*TETANOPS MYOPAEFORMIS*) GENES MODULATED BY RESISTANT AND SUSCEPTIBLE INTERACTIONS WITH *BETA VULGARIS*

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Abstract

Suppressive subtractive hybridization (SSH) is a powerful tool for global analysis of gene expression and has been used in our laboratory to identify sugar beet root genes responsive to feeding by the sugar beet root maggot (SBRM, *Tetanops myopaeformis*). We are currently focusing our studies on the identification of SBRM genes whose expression is modulated by interactions with resistant or susceptible sugar beet germplasm. PCR-select SSH was used to generate cDNA libraries enriched for SBRM genes after contact of the pest with a moderately resistant F1016 and a susceptible F1010 germplasm. SBRM larvae were starved for 72 h and then fed F1016 or F1010 roots. At 1, 6, 24, 48 and 72 h after infestation, 20 larvae were collected for each time point for further analysis. Three complete subtractions were conducted using pooled tissues from the five time points: SBRM fed on F1016 vs. unfed, SBRM on F1010 vs. unfed, and SBRM on F1016 vs. on F1010. Screening of differentially expressed SBRM genes is ongoing. Genes identified as being important in resistant or susceptible pest-plant interactions will be selected for further analyses. New insights into the molecular response elicited by SBRM in interactions with sugar beet roots will advance the development of novel approaches for more effective SBRM control.

Introduction

Sugar beet root maggot (SBRM, *Tetanops myopaeformis*) is one of the most devastating insect pests of sugar beet that can reduce crop yields by as much as 100% (Cooke, 1993). While several moderately resistant breeding lines have recently been registered, they do not offer complete control and at best reduce the SBRM damage ratings by approximately 40% (Campbell et al., 2000 and 2011). Pesticides continue to be the primary control measure and alternative approaches are needed that do not rely on synthetic chemical pesticides and are environmentally sound. Investigating the molecular

responses elicited by the SBRM resistant and susceptible plants or by the SBRM pest in these interactions will provide new knowledge useful for developing alternative pest control approaches.

Suppressive subtractive hybridization is an efficient and systematic method for global analysis of gene expression where almost all genes are screened simultaneously, thereby providing a more complete picture of the response of an organism to an environmental challenge. This method has been used in a wide range of gene identification studies (Gepstein et al., 2003; Gu et al., 2004; Shim et al., 2004; Puthoff and Smigocki 2007; Rodriguez-Cabrera et al., 2008). Using this method our laboratory cloned more than 150 sugar beet root genes incited by SBRM in a moderately resistant F1016 or a susceptible F1010 sugar beet line (Puthoff and Smigocki 2007). Several of these genes are being characterized as to their functions in resistance mechanisms (Smigocki et al., 2008 and 2009). However, our knowledge of SBRM response to plant defense is limited. In this study, we report on the use of SSH to identify SBRM genes whose expression is modulated by the interaction of the pest with resistant or susceptible sugar beet breeding lines.

Materials and Methods

Insects, plants and insect infestations

SBRM larvae collected from fields near St. Thomas, ND (Pembina County) were obtained from Dr. Larry Campbell (USDA, ARS, Fargo, North Dakota) and stored in field-collected soil at room temperature prior to the feeding experiments.

Sugar beet breeding lines susceptible to SBRM, F1010, and moderately resistant, F1016, were used in this study (Campbell, 1990; Campbell et al., 2000). Seeds were germinated in the growth chamber at 25°C during the day and 18 to 20°C at night with a 16-h photoperiod. Seedlings were transferred to the greenhouse and cultivated under similar growth conditions.

For insect infestations, 220 larvae of second- or early third-instar maggots were starved for 72 h in a Petri dish on water-moistened nylon membrane. Three plants of each F1016 and F1010 line (3 to 6-month old) were washed to remove the soil and placed in a glass tray. After starvation, larvae were non-fed or fed with the roots of each sugar beet line for 1, 6, 24, 48 and 72 h. Twenty larvae were frozen at each time point, including a zero time point, and stored at - 80°C.

RNA isolation

Total RNA was isolated with Trizol Reagent (Invitrogen, Carlsbad, CA) and purified with RNeasy Spin Columns (Qiagen, MD). Genomic DNA was removed with RNase-Free DNase I (Qiagen). Quantity and quality of the total RNA was assessed using an ND-8000 Spectrophotometer (NanoDrop Technologies Inc., DE) and electrophoresis on denaturing agarose/formaldehyde gels. Poly(A)Purist Kit (Ambion Inc., Austin, TX) was used to purify mRNA.

SSH procedure and differential screening

SSH was carried out using the PCR-Select cDNA Subtraction Kit (Clontech Laboratories, Inc., Mountain View, CA). Three complete subtractions were conducted using pooled tissues from the five time points: SBRM fed on F1016 vs. unfed, SBRM on F1010 vs. unfed, and SBRM on F1016 vs. on F1010 (Table 1). The resulting subtractive libraries were cloned in pCR2.1 TOPO vector (Invitrogen) and transformed into TOP10 E. coli cells (Invitrogen). Clones were plated on LB media containing 50 µg/ml kanamycin (kan), complemented with 40 mg/ml X-gal. Recombinant white colonies were randomly picked into 96-well plates containing LB kan and grown overnight.

Clones with inserts from the subtractive cDNA library were verified by cDNA dot blots as directed in the “PCR-Select Differential Screening Kit” (Clontech). Four probes with both subtracted and non-subtracted cDNA were synthesized using a PCR DIG Probe Synthesis Kit (Roche, Mannheim, Germany). Hybridizations were performed at 42°C using DIG Easy Hyb Granules (Roche) supplemented with Blocking Solution supplied in the PCR-Select Differential Screening Kit. Detection of DIG probes was carried out as directed using CSPD Ready-to-Use (DIG-High Prime DNA Labeling and Detection Starter Kit II; Roche) and visualized on Lumi-film chemiluminescent detection film (Roche). Signal intensities were quantified using an AlphaImager HP (Alpha Innotech, San Leandro, CA). Clones identified as differentially expressed were selected for DNA sequencing.

Table 1. Sugar beet root maggot (SBRM) tissues used in SSH

SBRM treatment	Hours of treatment	Group of samples pooled	Subtractions
Unfed	0	A	1) B-A forward B-A reverse 2) C-A forward C-A reverse 3) B-C forward B-C reverse
F1016 fed	1	B	
	6		
	24		
	48		
	72		
F1010 fed	1	C	
	6		
	24		
	48		
	72		

1) Forward subtraction - cDNA from F1016 fed SBRM as tester and unfed as driver.
Reverse subtraction - cDNA from unfed SBRM as tester and F1016 fed as driver.
2) Forward subtraction - cDNA from F1010 fed SBRM as tester and unfed as driver.
Reverse subtraction - cDNA from unfed SBRM as tester and F1010 fed as driver.
3) Forward subtraction - cDNA from F1016 fed SBRM as tester and F1010 fed as driver.
Reverse subtraction - cDNA from F1010 fed SBRM as tester and F1016 fed as driver.

Results and discussion

The purpose of this study was to identify SBRM genes that are modulated by the interaction of the pest with resistant or susceptible sugar beet breeding lines. This newly gained knowledge of SBRM responses will provide the basis for developing effective, sustainable and environmentally sound SBRM control measures. The subtracted cDNAs used for preparation of the SBRM cDNA libraries are shown in Fig. 1 (a). Three complete libraries were made for both forward- (up-regulated genes) and reverse- (down-regulated genes) subtractions as indicated in Table 1. These subtractions should identify five major classes of SBRM genes 1) up-regulated or 2) down-regulated genes responsive to moderately resistant sugar beet (F1016) interaction (See 1 in Table 1), 3) up-regulated or 4) down-regulated genes responsive to susceptible sugar beet (F1010) interaction (see 2 in Table 1), and 5) genes reciprocally regulated by both resistant and susceptible sugar beet varieties (see 3 in Table 1).

To date, we have identified 768 forward-subtracted SBRM cDNA clones from the F1016 fed minus unfed interaction (1 in Table 1). Nearly 50% of the clones contained fragments ranging in size from 150 to 1000 bp (Fig. 1b). Only those clones larger than 200 bp were spotted on four identical nylon membranes and subjected to hybridization

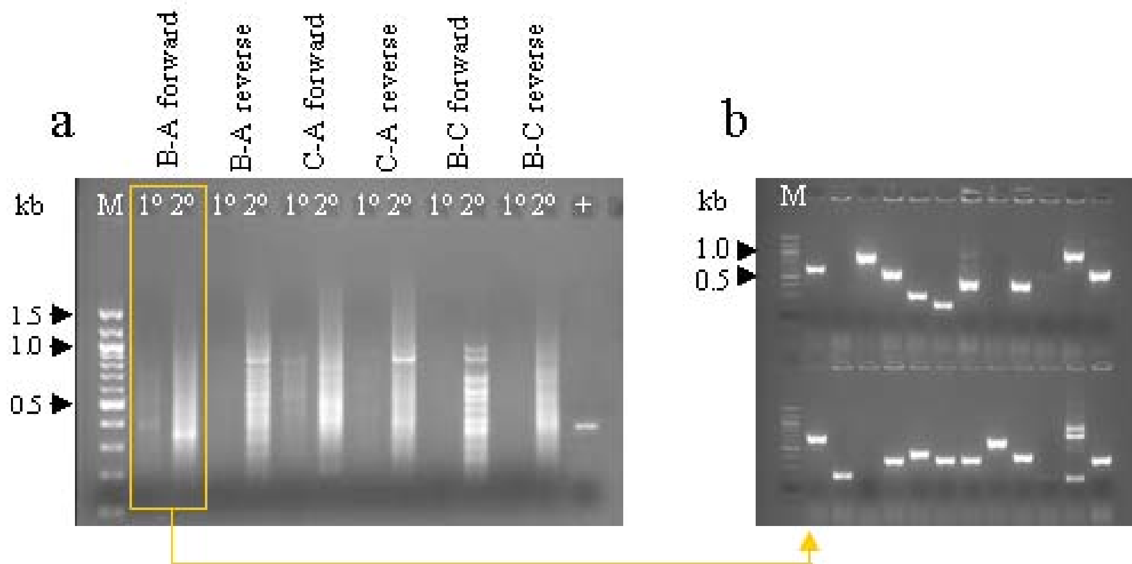

Figure 1. SBRM subtracted cDNA library construction. (a) First and second PCR of subtracted cDNA from A, B and C (see Table 1). Positive control template (+) is a subtracted mixture of Hae III-digested φX174 DNA provided with the PCR-select cDNA subtraction kit. (b) A sample of cDNA clones from the B-A forward subtraction (boxed in yellow in “a”). M, 100-bp DNA ladder.

with distinct probes (Fig. 2). We chose clones that had a ratio of at least 5 for the hybridization signal obtained with the forward-subtracted cDNA probe compared to the reverse-subtracted probe. Non-subtracted probes were also applied in differential screening to confirm or disprove ambiguous results obtained by using subtracted probes

which in general yielded more sensitive results than non-subtracted probes since rare sequences were retained. Thus, using non-subtracted probes would aid with the identification of clones that correspond to low-abundance transcripts. This screen helped identify 128 SBRM cDNA clones that had the most pronounced differential expression pattern following interaction with the moderately resistant F1016 sugar beet breeding line. These clones will be sequenced and functionally annotated. The most interesting clones will be selected for further analysis to confirm their expression in SBRM as it relates to their interaction with either the resistant or susceptible breeding line.

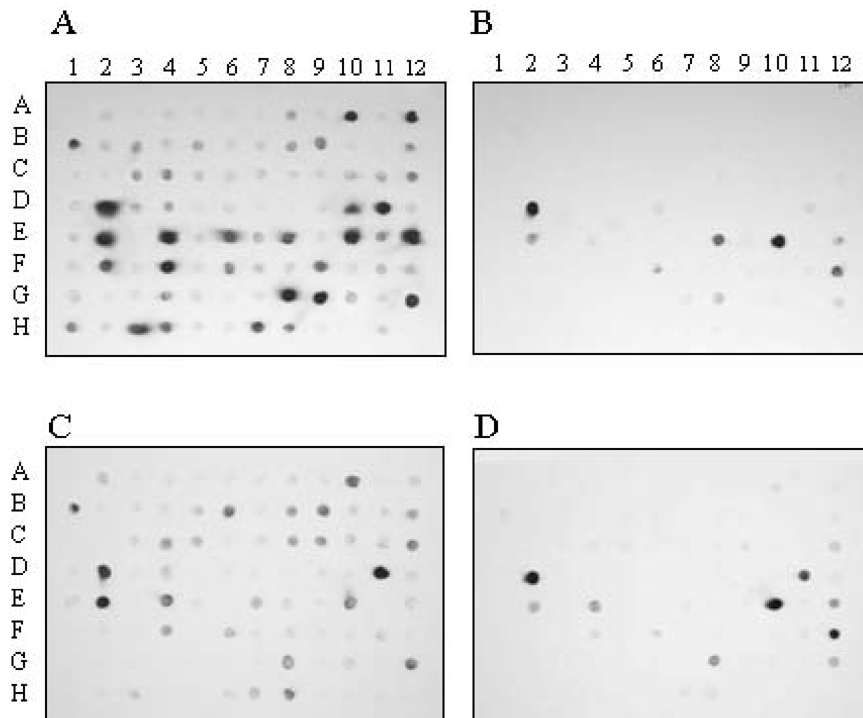

Figure 2. Differential screening of a subtracted sugar beet root maggot cDNA library. PCR-selected subtraction was performed using cDNA from F1016 fed SBRM as tester and unfed as driver. cDNA dot blots probed with (A) labeled forward-subtracted cDNA, (B) labeled reverse-subtracted cDNA, (C) labeled non-subtracted F1016 fed cDNA, (D) labeled non-subtracted non-fed cDNA.

Conclusion

In insects, SSH has been used successfully to study host-pathogen interactions in *Anopheles gambiae* (Oduol et al., 2000), *Glossina morsitans morsitans* (Hao et al., 2001) and *Manduca sexta* (Zhu et al., 2003). The results above show us that this technology can be applied to SBRM to identify differentially expressed genes regulated by the interaction of the pest with sugar beet roots. Further characterization of the selected gene clones should give us a better understanding of molecular mechanisms driving the SBRM infestation of sugar beet roots. The newly gained insights about insect-host interactions will be useful for developing more effective pest control strategies.

Acknowledgements

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