Sugar beet (*Beta vulgaris* L.) genes regulated by sugar beet root maggot (*Tetanops myopaeformis*) infestation

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Introduction

Damage caused by the feeding of the sugar beet root maggot (SBRM, *Tetanops myopaeformis*) is quite devastating and can include the complete severing of young seedling roots and deformations of mature roots, and often leads to invasion by secondary pathogens. SBRM is the major insect pest on sugar beets (*Beta vulgaris* L.) in the U.S. and Canada and can cause major yield losses (10 – 100%) (Cooke, 1993). While moderately resistant sugarbeet lines are available, they do not offer complete control (Campbell et al., 2000). The identification of genes regulated by SBRM feeding in both susceptible and moderately resistant lines will prove useful for the development of future control methods including engineering new sugar beet varieties.

The Suppressive Subtractive Hybridization (SSH) method has been used in a wide range of gene identification studies (Gepstein et al., 2003; Gu et al., 2004; Shim et al., 2004). Use of SSH to identify genes regulated by SBRM larval feeding has several advantages over other methods including small amounts of starting material and rapid turn around time. In this study, we report on the use of SSH to identify sugar beet genes regulated by SBRM feeding in both moderately resistant (F1016) and susceptible (F1010) varieties.

Materials and Methods

Plant material and infestations

Two beet genotypes were used in this study: F1010, a susceptible line, and F1016, a moderately resistant line (Campbell, 1990; Campbell et al., 2000). Seeds were soaked

in water overnight, planted in soil and grown for 2.5 weeks in a growth chamber at 25°C.

An *in vitro* SBRM bioassay was used to collect SBRM-infested tissues (Smigocki et al., 2005a and b). Briefly, seedlings were removed from the soil with gentle washing, placed on water/agar (0.8%) plates and infested with 5 second-instar SBRM per plant. Larvae were collected from commercial sugar beet fields near St. Thomas, ND (Pembina County) and stored at 4°C (graciously provided by Dr. Mark Boetel, North Dakota State University). Root and hypocotyl tissues either uninfested or infested with SBRM for 24 and 48 h were harvested into liquid nitrogen, stored at -80°C and used for RNA extraction.

RNA extraction

RNA from root and hypocotyl tissue was extracted using RNeasy columns (Qiagen, Valencia, CA) and quantified spectrophotometrically. RNA quality was checked via denaturing gel analysis for the SSH procedure. Messenger RNA was isolated from each sample using Dyna Beads (Dynal, Oslo, Norway).

SSH procedure

Eight RNA samples were pooled to conduct three complete subtractions (both Forward and Reverse) according to Table 1. F1010 infested was compared to F1010 uninfested; F1016 infested was compared to F1016 uninfested and F1010 was compared directly to F1016. Forward subtractions identify genes up-regulated in the treated (infested) samples while Reverse subtractions identify genes up-regulated in control (uninfested) samples. SSH was carried out using the PCR-Select cDNA Subtraction Kit from BD Biosciences (San Jose, CA).

Table 1. Tissue samples used in Suppressive Subtractive Hybridization (SSH)

Sugar beet variety	Sample number	Treatment	Hours of treatment	Samples pooled for within variety subtractions	Samples pooled for between variety subtraction
F1010 susceptible	1	uninfested	24	F1010 uninfested	F1010 complete
	2	uninfested	48		
	3	SBRM	24	F1010 infested	
	4	SBRM	48		
F1016 moderately resistant	5	uninfested	24	F1016 uninfested	F1016 complete
	6	uninfested	48		
	7	SBRM	24	F1016 infested	
	8	SBRM	48		

First strand cDNA was synthesized using 2.0 μg of mRNA, primed with a modified oligo d(T) primer followed by conversion to double-stranded cDNA (dscDNA). Each dscDNA sample was digested with restriction enzyme Rsa I. These samples are referred to as the "driver" cDNA (Figure 1). Aliquots of the digested dscDNA were ligated to each of two adaptors in order to create two adaptor ligated tester cDNA samples (ALTC1 & ALTC2) (Figure 1).

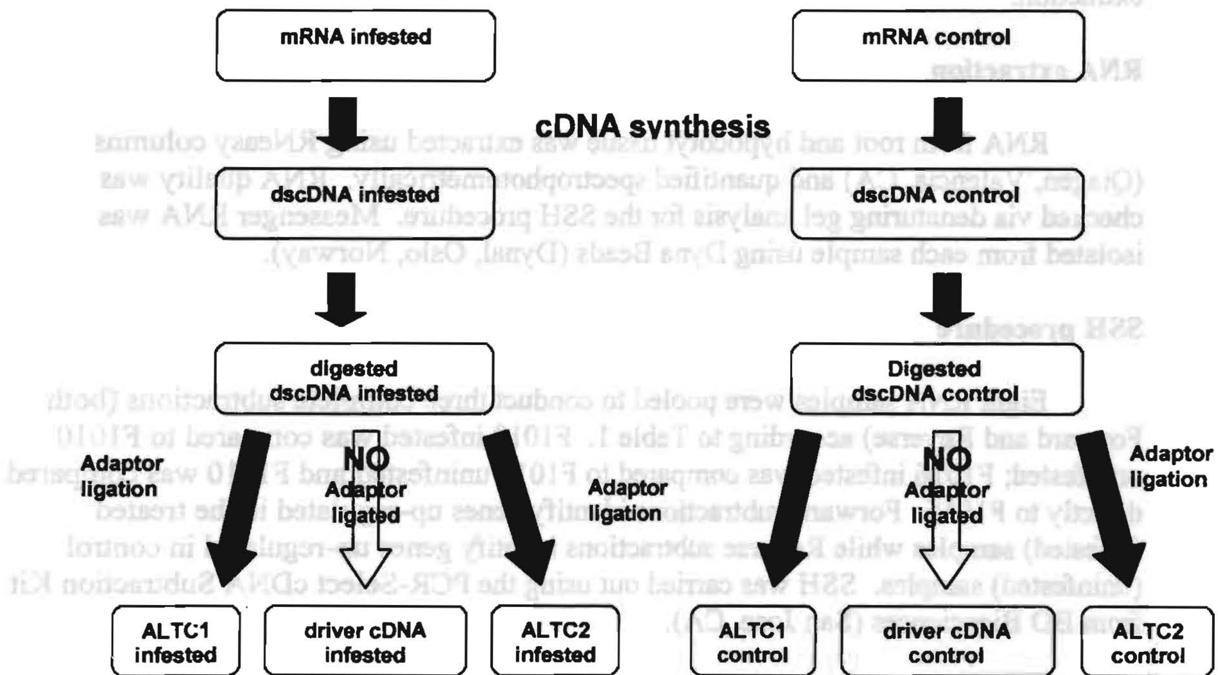

Figure1. Schematic drawing for the generation of "driver" and Adaptor Ligated "tester" cDNA (ALTC).

Each ALTC was hybridized with the driver from the opposite treatment (i.e. treated ALTC 1 or 2 with control driver cDNA and vice versa) resulting in four different primary hybridizations for each complete SSH procedure as indicated in Figure 2. Following seven hours of primary hybridization, the two hybridization reactions were combined, additional driver cDNA was added and the reactions hybridized over-night (Figure 2). The resulting pool of cDNA fragments was subjected to two rounds of suppressive PCR with primers specific to the ligated adaptors. A fraction of the resulting pool of cDNA fragments were cloned into pCR2.1 TOPO (Invitrogen, Carlsbad, CA), transformed into TOP10 cells (Invitrogen, Carlsbad, CA) and plated on LB medium supplemented with 50 ug/ml kanamycin sulfate.

Differential Screening

Recombinant colonies were picked and grown in 96-well plates containing LB medium supplemented with 50 ug/ml kanamycin sulfate. Inserted cDNA fragments were amplified using Taq DNA Polymerase (Fisher, Pittsburgh, PA). The resulting PCR products were denatured, spotted onto nylon membranes and neutralized. Following UV-cross-linking of DNA, membranes were hybridized with probes synthesized from the remaining subtracted pool of cDNA fragments.

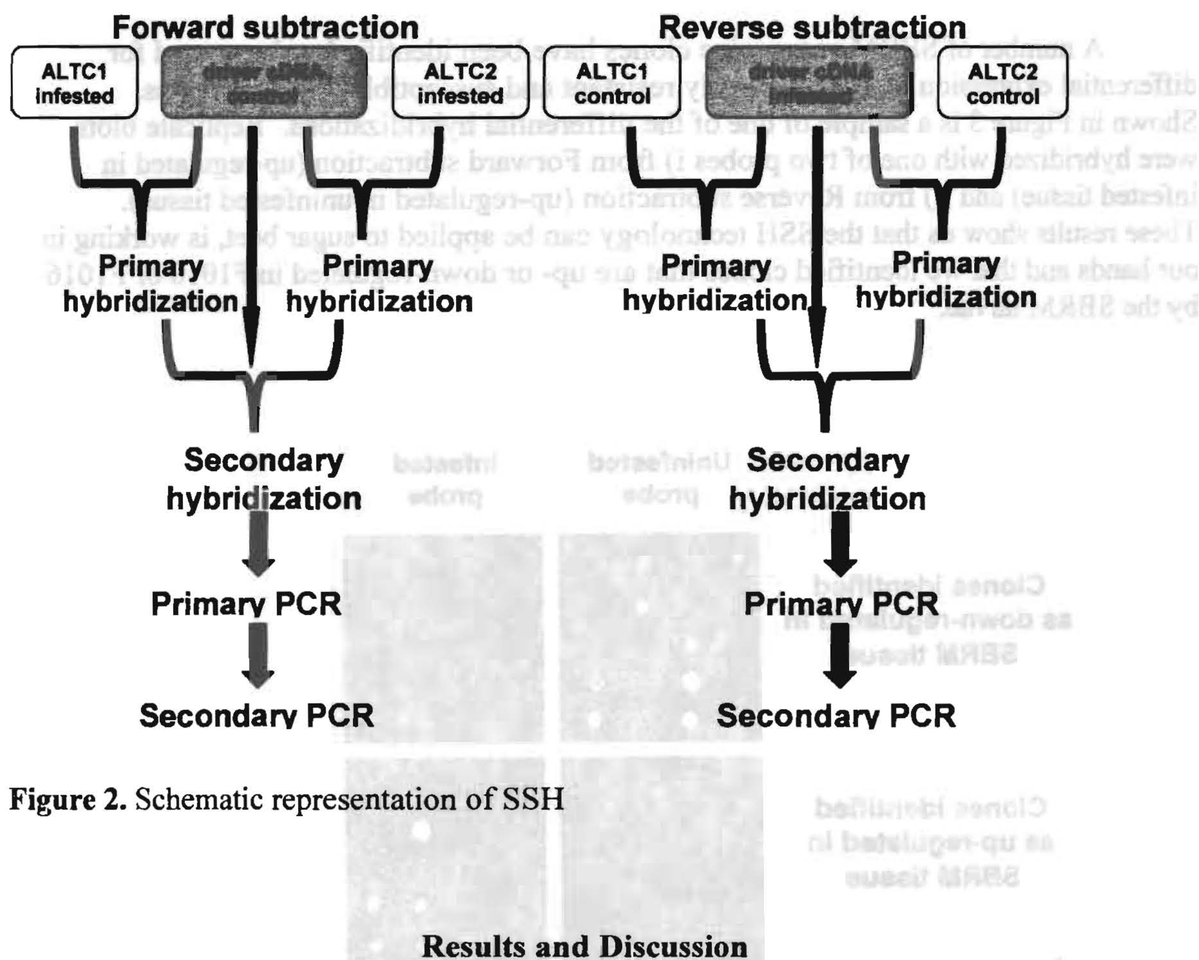

Figure 2. Schematic representation of SSH

Results and Discussion

The identification of genes regulated by SBRM feeding in both moderately resistant and susceptible varieties is anticipated to provide a knowledge base for the future control of this devastating insect pest. Our strategy was to identify SBRM-regulated genes in both F1010 (susceptible) and F1016 (moderately resistant) sugar beet genotypes. Eight RNA samples were combined according to Table 1 and used respectively for three complete suppressive subtractive hybridizations (SSH): i) F1010 uninfested vs. F1010 infested; ii) F1016 uninfested vs. F1016 infested; and iii) F1010 complete vs. F1016 complete. One advantage of using this system is that there is no clean-up or removal of dscDNA after hybridization resulting in the need for less starting material. Another advantage is that this method exponentially amplifies only

differentially expressed genes in the primary PCR. The secondary PCR, which uses nested primers, further enriches differentially expressed genes while at the same time reduces background. The three subtractions we performed should identify five major classes of SBRM-regulated sugar beet genes: 1) up-regulated in the susceptible variety, 2) down-regulated in the susceptible variety, 3) up-regulated in the moderately resistant variety, 4) down-regulated in the moderately resistant variety, and 5) genes reciprocally regulated between the two varieties. This last class of genes has been found in other plant/pathogen interactions when comparing resistant and susceptible interactions (Puthoff *et al.*, 2003).

A number of SBRM-responsive clones have been identified and screened for differential expression in the moderately resistant and susceptible sugar beet lines. Shown in Figure 3 is a sample of one of the differential hybridizations. Replicate blots were hybridized with one of two probes i) from Forward subtraction (up-regulated in infested tissue) and ii) from Reverse subtraction (up-regulated in uninfested tissue). These results show us that the SSH technology can be applied to sugar beet, is working in our hands and that we identified clones that are up- or down-regulated in F1010 or F1016 by the SBRM larvae.

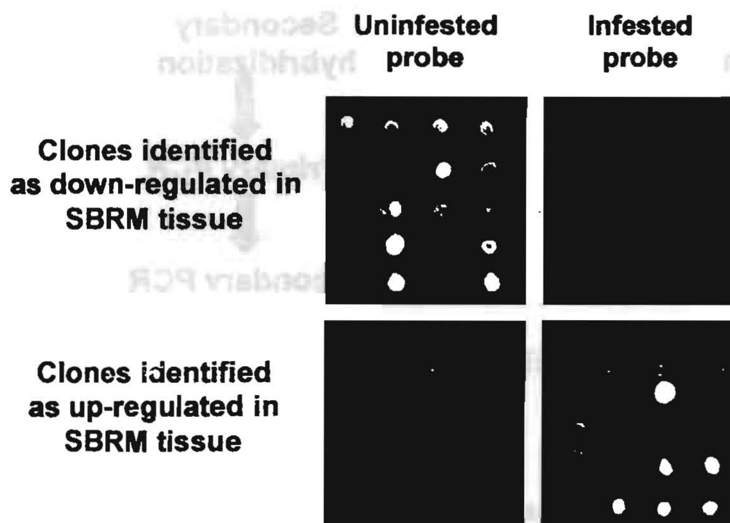

Figure 3. The identification of clones which are up- or down-regulated by SBRM feeding in F1016. Each row shows two replicate blots hybridized to one of two different probes (uninfested or infested).

To date, over 1000 cDNA fragments differentially expressed in response to SBRM feeding have been isolated. Further characterization will include, confirmation of differential expression, sequencing, full length cDNA cloning and expression profiling following various plant stresses. Clones shown to be regulated by SBRM feeding will be

sequenced and functionally annotated. Genes suspected to play a role in defense or susceptibility will be expressed in transformed sugar beet hairy root cultures and subsequently in transgenic plants and screened for increased or decreased resistance to SBRM and other sugar beet and taproot pests. Candidate genes identified from any or all of the subtractions will lead to a better understanding of the mechanisms of infestation, resistance and susceptibility.

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