GLOBAL ANALYSIS OF THE TOBACCO BUDWORM-COTTON MICROBIOME Anirudh Dhammi Loganathan Ponnusamy Madhavi Kakuman R. Michael Roe NC State University Raleigh, NC

Abstract

Insecticide resistance in insects has been attributed to many factors, namely target site modification, penetration, sequestration, metabolism, excretion and behavior. But the role of the bacteria microbiome present in insects associated with cotton is barely explored. Difficulty in studying the relationship between resistance and microbes is mostly because of the past reliance on a culture-based approach which does not provide a complete picture of total microbial diversity in a sample. In this study, Bt (*Bacillus thuringiensis*) susceptible and resistant strains of the tobacco budworm (TBW) on cotton were investigated by denaturing gradient gel electrophoresis (DGGE) for microbial diversity. The initial report of DGGE analysis has shown that susceptible and resistant strains of TBW have no difference in microbial diversity when fed on conventional cotton plants. However, the resistant strains had 3 fold more microbial diversity when fed on Bt cotton (Bollgard I and WideStrike) as compared to conventional cotton, suggesting an effect of transgenic versus conventional cotton on the insect bacterial community. These preliminary findings at the least argue for a needed future better understanding of the cotton and associated insect microbiome which should include high through put DNA sequencing techniques for a more in depth understanding of cotton bacteria communities.

Introduction

Microorganisms contributed a great deal towards the survival of any higher organism and play important roles in the establishment and success of insects. The insect gut for example is a highly nutrient rich ecological niche in which various groups of microbes survive and multiply (Rajagopal 2009). Absence of these microbes can affect significantly insect growth and development. For example, germ free crickets have a lower enzymatic hydrolytic activity as compared to its conventional counterpart, and these bacteria help in utilizing a wider range of food substrates (Domingo et al. 1998). Hemipterans like aphids survive exclusively on plant sap which is deficient in essential nutrients; the aphid is dependent on its associated gut symboints to use this food source (Rajagopal 2009). The stink bug without gut symbionts shows developmental delays, reduced ability to reproductive and die prematurely (Gross 2006). Axenic locusts are susceptible to infection by the entomopathogenic fungi, *Beauvaria* and *Metarhizium* (Rajagopal 2009). Similarly, axenic silkworms, *Bombyx mori*, larvae were found to be more susceptible to *Gluconobacter*, compared to flies with its normal gut micro flora (Ryu et. al. 2008). *Hamiltonella defensa* protects the aphid from attack by the parasitoid, *Aphididus ervi* (Oliver et al. 2005). Similarly, infection by the facultative symbiont, *Serratia symbiotica*, helps the aphid to tolerate higher temperatures (Russel and Moran 2006).

Symboints not only play an important role in host nutrition, development and immune responses but may also be important in insect resistance to pesticides. Insect resistance to pesticides is a worldwide problem. Mechanisms of insect resistance to pesticides include increased metabolism, target site modification, changes in behavior, reduced penetration, and increased excretion and sequestration (van Kretschmar et al. 2013). However, in the bean bug, *Riptortus pedestris*, the acquisition from soil of specific bacteria from the genus *Burkholderia* was found to induce resistance to the insecticide, fenitrothion (Kikuchi et al. 2012). Even in the diamond back moth, *Plutella xylostella*, where resistance to different insecticides have been studied and many mutations have been identified related to resistance, the microbiome is different between resistant and susceptible strains for the insecticides, chlorpyrifos and fipronil (Xia et al. 2013). In another study, microbe-free mosquito larvae were more susceptible to *Bacillus thuringiensis* (Bt) toxin compared to larvae with their natural flora. Also, a higher density of *Wolbachia* in *Culex pipens* has been seen in strains resistant to organophosphates (Berticat et al. 2002).

The evolution of insect resistance to transgenic plant technologies threatens their long-term effectiveness (Oppert et al 1997) and is expected at some time in the future, at least as some level. The addition of stacked protein toxins

with different sites of action and the use of an RNAi approach which can provide additional targets and an alternative nucleic acid chemistry is being used to help manage possible future resistance. Research on the importance of the plant and insect microbiome is lacking and greatly needed. For example, Bt protoxins need an alkaline pH for their activation (Wilson and Benoit 1993), and *Enterobacter faecalis*, found in higher pHs can acidify their environment though metabolite production (Manero and Blanch 1999). If this bacteria would invade the insect midgut, this could be the perfect scenario for protecting the insect from Bt toxicity (Broderick et al 2003). The work reported here is to provide preliminary data on the cotton-insect microbiome. In our work, we examined the microbial diversity of Bt resistant and susceptible strains of the tobacco budworm feeding on conventional versus Bt cotton using denaturing gradient gel electrophoresis (DGGE).

Materials and Methods

Tobacco Budworm

Bt susceptible (YDK strain) tobacco budworm neonates were fed for 24 h on 7 week old conventional cotton plants and compared with BT resistant (YHD2 strain) TBW neonates fed on 7 week old conventional cotton, Bollgard I, and WideStrike plants. Larvae were then collected and surface sterilized by 0.5% bleach followed by washing thoroughly with distilled water (5 times). The plants were grown in the green house at the same time under identical environmental conditions.

DNA Extraction

DNA was extracted from TBWs by a method described previously (Ponnusamy et al. 2014). Four TBW larvae were transferred to a 1.5 ml eppendorf tube and 160 μ l of lysis buffer 1 was added along with 20 μ l lysozymes and 20 μ l proteinase K to the samples. Samples were then homogenized and incubated at 37 °C for 1h. Subsequently, 200 μ l of pre-warm lysis buffer 2 was added with further incubation at 56°C for 1 h. DNA was recovered through phenol/chloroform extraction and ethanol precipitation, and the resulting DNA pellet was resuspended in 100 μ l ultrapure water. Subsequently, crude DNA was purified with the WIZARD DNA Cleanup System (Promega, Madison, WI, USA).

DNA Amplification

Purified DNA was used as template to amplify the V3 region of the 16SrRNA gene (specific to bacteria) with the universal primers <u>F357-GC</u> (5'-GC-clamp+ CCTACGGGAGGCAGCAG-3') and <u>518R</u> (5'-ATTACCGCGGCTGCTGG-3'). The GC-clamp was added at the 5' end of the forward primer to prevent a complete denaturation of the double-stranded fragments. The touchdown PCR was performed as described by Ponnusamy et al. (2010).

Denaturing Gradient Gel Electrophoresis

DGGE was performed using the DCode System (Bio-Rad) as described by Muyzer el al. (1993). Samples (15 μ l) were loaded onto 8 % polyacrylamide/bis (37.5:1) gels with denaturing gradients from 45–55 % [where 100 % is 7 M urea and 40 % (v/v) deionized formamide] in 1× TAE electrophoresis buffer. Electrophoresis was performed at 50 V at a temperature of 60°C for 18 h. The gel was stained with SYBR green I in 0.5X TAE buffer and digitally photographed with a ChemiDoc-It^{TS2} Imager (UVP, LLC Upland, CA).

Results and Discussion

We investigated the species composition of the bacterial community (species richness and evenness) in susceptible (YDK) and resistant (YDH2) TBW neonates by DGGE analysis of the 16S rRNA gene products amplified by PCR. Each band represents at least one unique phylotype, so the richness of the bacterial species in a sample was reflected in the number of DGGE-DNA bands; the intensity of a band reflected the relative abundance of the bacterial species in a sample.

Resistant (YDH2) and susceptible (YDK) strains have at least one prominent and four weak bands in all treatments (Figs. 1, 2 and 3). Additional bands are present in the resistance strain when fed on transgenic cotton (Bollgard I and WideStrike). The resistant strain showed a higher bacterial diversity when fed on Bollgard I. It consist of six prominent and eight weak bands which is about 3 fold higher than the bacterial diversity when fed on conventional cotton (Fig. 1).



Figure 1. DGGE profile of bacterial communities from susceptible (YDK) and resistant (YDH2) strains of neonates of the tobacco budworm fed on conventional cotton and the latter on Bollgard I cotton.

The resistant YDH2 strain also had a higher number of bacterial species when fed on WideStrike as compared to conventional cotton (Fig. 2). The resistance strain on transgenic plant had five (1 prominent and 4 weak bands) bacterial species which were also present when they are fed on conventional cotton. But it had an additional nine bacterial species (5 prominent and 4 weak bands) as compared to its feeding on conventional cotton (Fig. 2).



Figure 2. DGGE profile of bacterial communities from susceptible (YDK) and resistant (YDH2) strains of neonates of the tobacco budworm fed on conventional cotton and the latter on WideStrike cotton. Each lane represents a sample and each band represents a single bacterial species.

The resistant (YDH2) strain of the tobacco budworm as neonates on Bollgard I and WideStrike had essentially the same microbial diversity, which is peculiar since these are two different commercial products from different companies. There were minimal differences in bacteria abundance but essentially no difference in the species detected using DGGE (Fig. 3). This will be discussed further later.



Figure 3. DGGE side by side profile of bacterial communities from resistant (YDH2) strains of neonates of the tobacco budworm fed on Bollgard I and WideStrike cotton. Each lane represents a sample and each band represents a single bacterial species.

Results and Discussion

Preliminary analysis of the microbial diversity of resistant (YDH2) and susceptible (YDK) strains of the tobacco budworm by DGGE shows that their bacterial communities are similar when they are fed on conventional cotton. However, the resistant strain on transgenic cotton (Bollgard I and WideStrike) has a 3 fold higher bacterial diversity as compared to conventional cotton. The bacteria diversity and relative abundance of each species in the YDH2 neonates was also essentially the same on Bollgard I and WideStrike. These results suggest that the transgenic plants are affecting the microbiome of the larva different from the conventional plant. The presence of Bt toxin in the plant, the methods of genetic engineering and/or plant variety used for genetic engineering might be responsible for this difference in microbial diversity between insects feeding on transgenic cotton versus conventional cotton. The results are unexpected, however, since Bollgard I and WideStrike are products produced by different companies and further work is needed to validate these findings.

The results reported should be considered preliminary but argues that the cotton-insect microbiome ecosystem cannot be ignored in terms of the long term future sustainability of cotton production. At least the following areas of work should be considered in the future:

- Impact of Bt cotton on the plant and soil microbiome.
- Impact of the microbiome on the pest microbiome and physiology.
- Insect microbiome must be considered as a potential mechanism of insect resistance.
- Characterization of cotton microbiomics needs to move into more advanced molecular approaches using high throughput DNA sequencing.

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