ASSOCIATIONS BETWEEN *WOLBACHIA*, MAIZE AND *DIABROTICA VIRGIFERA VIRGIFERA*

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The undersigned, appointed by the dean of the Graduate School, have examined the dissertation entitled

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A candidate for the degree of doctor of philosophy

And hereby certify that in their opinion it is worthy of acceptance.

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LIST OF ABBREVIATIONS

Abbreviation	Full Name
ABA	abscisic acid
ARS	Agricultural Research Service
Avr9	*avirulence9*
AZM	assembled *Zea mays*
BAC	bacterial artificial chromosome
BHC	β-hexachlorocyclohexane
bm1	*brown mid-rib1*
bm2	*brown mid-rib2*
bm3	*brown mid-rib3*
bx1	*benzoxazinless1*
bx2	*benzoxazinless2*
bx7	*benzoxazinless7*
bz1	*bronze1*
bz2	*bronze2*
Bt	*Bacillus thuringiensis*
C6	cycle 6
CAD	cinnamyl alcohol dehydrogenase
Cf-9	*Cladosporium fulvum9*
CI	cytoplasmic incompatibility
CRW3	corn rootworm 3
DDT	dichloro-diphenyl-trichloroethane
DIBOA	2,4-dihydroxy-1,4-benzoxazin-3-one
DIMBOA	2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one

DNA ..... deoxyribonucleic acid

dsDNA ..... double stranded deoxyribonucleic acid

ERS ..... Economic Research Service

EST ..... expressed sequence tag

F1 ..... first filial generation

FAC ..... fatty acid amino acid conjugate

GST ..... glutathione S-transferase

HPR ..... host plant resistance

IAA ..... indole acetic acid

IGL ..... β-hexachlorocyclohexindole-3-glyceral phosphate lyase

IGP ..... indole-3-glycerol phosphate

JA ..... jasmonic acid

LOX ..... lipoxygenase

MBOA 6-methoxy-2-benzoxazolinone

MCR ..... Mexican corn rootworm

mRNA ..... messenger ribonucleic acid

NASS ..... National Agricultural Statistics Service

NCARL ..... North Central Agricultural Research Laboratory

NCR ..... Northern corn rootworm

orp1 ..... *orange pericarp1*

orp2 ..... *orange pericarp2*

PAL ..... phenylalanine ammonium lyase

PCR ..... polymerase chain reaction

PD ..... peptide deformylase

PI ..... plant introduction

PR..... pathogenesis related

qRT-PCR..... quantitative reverse transcriptase polymerase chain reaction

RISC..... RNA induced silencing complex

RNA ..... ribonucleaic acid

RNAi ..... ribonucleic acid interference

ROS..... reactive oxygen species

RT-PCR..... reverse transcriptase polymerase chain reaction

S1 ..... first self

SA ..... salicylic acid

SAS ..... statistical analysis system

TIGR ..... The Institute for Genome Research

US ..... United States

USDA..... United States Department of Agriculture

UV..... ultraviolet

V3..... three true leaf stage

vp5..... *viviparous5*

vp8..... *viviparous8*

WCR ..... Western corn rootworm

ASSOCIATIONS BETWEEN *WOLBACHIA*, MAIZE AND *DIABROTICA VIRGIFERA VIRGIFERA*

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ABSTRACT

Diabrotica virgifera virgifera which is infected with *Wolbachia*, is the most significant and widespread pest to maize in North America and Europe. *Wolbachia* are obligate intracellular bacteria which seem to be limited to ecdyzoan hosts. Many *Wolbachia* hosts induce or vector serious human diseases resulting in the loss of millions of lives annually. The majority of differentially expressed genes identified in a microarray experiment performed to locate endogenous sources of *D. virgifera virgifera* resistance in maize, *Zea mays* L., are normally involved in microbial defense rather than wounding or insect attack. A second microarray experiment to determine whether *Wolbachia* were influencing the response of maize to *D. virgifera virgifera* attack indicated the presence of *Wolbachia* in the insect can down regulate genes in all plant defense classes. This may contribute to the success of *D. virgifera virgifera* as a maize pest. To further test this idea, assays on *D. virgifera virgifera* larval competitiveness and fertility were performed. Results of the assay suggest *D. virgifera virgifera* and *Wolbachia* share a commensal association.

Chapter 1. Introduction

Diabrotica virgifera virgifera

The western corn rootworm (WCR) *D. virgifera virgifera* LeConte is the most serious pest of maize, *Z. mays* L., in North America and parts of Europe (Chandler 1998, Hummel et al. 2005). In the US as of 1986, yield loss and management expenses cost producers over 1 billion dollars annually (Metcalf 1986). More metric hectares of farmland are treated with insecticides or transgenics to control corn rootworms than any other insect pest in the US (Suguiama and Carlson 1985, USDA-ERS 2006). Due to recent changes in its biology and distribution, the economic consequences of WCR are growing.

The genus *Diabrotica* arose from the neo-tropical regions of Mesoamerica (Webster 1895, Smith 1966). It belongs to the subtribe Galerucinae (Smith 1966), which contains primarily leaf beetles with root-feeding larvae (Branson and Krysan 1981). In North America, *Diabrotica* are divided into the *fucata* and *virgifera* groups (Wilcox 1972). The *virgifera* species are univoltine (Gillette 1912) and oligophagous (Smith 1966, Branson and Ortman 1970) and as a result, they are more specialized than the *fucata* group (Branson and Krysan1981). *D. virgifera,* and maize evolved in the tropical regions of Mesoamerica (Smith 1966, Galinat 1977, Branson and Kryson 1981). There is evidence that egg dormancy of the *virgifera* species evolved in response to the wet-dry seasons of the tropics (Krysan et al. 1977).

The WCR was first described by LeConte in 1868 after he had collected specimens near Fort Wallace, Kansas (Smith 1966). It was first recognized as a pest of maize in Colorado in 1909 (Gillette 1912). In less than 50 years WCR had become a significant pest in the US beginning in Nebraska, then Kansas and eventually spreading from South Dakota to Missouri (Tate and Bare 1946, Ball 1957). Today WCR can be found from Mexico to Canada and from Colorado to the Atlantic. It is also a major pest in parts of Europe (Hummel et al. 2005, Miller et al. 2005).

WCR are able to migrate long distances in search of food and oviposition sites (Coats et al. 1987, Hein et al. 1988). In general, adult beetles will move from field to field in large numbers in search of silking and pollinating maize (Hill and Mayo 1974) though factors such as population density, food quality and weather can affect movement and induce migration (Rankin and Rankin 1980).

The WCR overwinters in the egg stage in the ground near the base of maize plants (Ball 1957). The life cycle of WCR includes an egg stage, three larval instars, a pupal stage, and an adult stage. The first adults to emerge are males, though male emergence continues well after female emergence begins (Bergman 1986, Tollefson 1988, Quiring and Timmons 1990). Male WCR are not sexually mature upon emergence from the soil. Newly emerged females possess undeveloped ovaries with no oocytes (Short and Hill 1972) that develop upon mating (Sherwood and Levine 1993) which usually occurs within the first 24 hours following emergence (Quiring and Timmons 1990). Most females mate only once (Hill and Mayo 1975) however males are able to mate multiple times

(Branson et al. 1977, Quiring and Timmons 1990). Egg laying begins in early to mid-summer and continues until the killing frost (Ball 1957). Oviposition occurs randomly throughout the field (Onstad et al. 2001) with eighty percent of the eggs laid in the top 15.2 cm of the soil (Ball 1957). Branson and Johnson (1973) estimate the average lifetime fecundity of a female WCR is more than 1000 eggs.

Neonate larvae are responsible for initial host location. Larvae can move as far as 40 to 102 cm from their hatch location (Nowatzki et al. 2002, Suttle et al. 1967, Short and Leudtke 1970). Properties of the soil such as moisture (MacDonald and Ellis 1990), density (Strnad and Bergman 1987) and macropores (Gustin and Schumacher 1989) can influence larval movement. Although larvae prefer maize, they can survive to adulthood on several grass species (Wilson and Hibbard 2004, Oyediran et al. 2004). Neonate larvae locate their hosts through olfactory cues (Bjostad and Hibbard 1992). They are highly attracted to carbon dioxide (Hibbard and Bjostad 1988) discharged in the soil by root respiration. Once a host is located, larvae will feed on the root tip and burrow inside (Clark et al. 2006). Initial damage is concentrated at the root tip, followed by the region of cell differentiation at day three after infestation with damage observed throughout the root system after three days post infestation (Hibbard et al. 2003, Clark et al. 2006). If not properly controlled, WCR larvae can disrupt plant water relationships (Hou et al. 1997), increase susceptibility to lodging (Sutter et al. 1990, Spike and Tollefson 1991) and reduce grain yield (Godfrey et al. 1993, Gray and Steffey 1998, Urias-Lopez and Meinke 2001). Yield loss can be as great as half of the total crop (Godfrey et al. 1993).

Adult WCR will feed on nearly every part of a maize plant, but prefer the silk and pollen. Late in the summer they have been found on other grasses as well as, alfalfa (*Medicago sativa* L.), oat (*Avena sativa* L.), and soybean (*G. max* L.) (Rondon and Gray 2003). Nutrition plays an important role in WCR survivorship. When food supply is limited, the duration of larval development is increased and the percentage of larvae reaching adulthood is reduced (Weiss et al. 1985). The surviving adults are smaller with shorter life spans and reduced fecundity (Branson and Sutter 1982).

WCR has developed resistance to chemical and cultural control strategies more rapidly than many other insect pests. At one time over half the total US maize acreage was treated annually with insecticide to control corn rootworm (Metcalf 1986). Determining economically relevant insecticide thresholds for WCR control is difficult, as there is no correlation between adult and larval location and/or densities (O'Neal et al. 2001). For this reason many fields may be unnecessarily treated because producers use insecticides on a prophylactic basis (Foster et al. 1986). Soil insecticide at the time of planting is the most widely used chemical control measure (Pruess et al 1974, Mayo and Peters 1978).

Widespread organochlorine insecticide (DDT, BHC, aldrin, heptachlor) use led to high levels of insecticide resistance in WCR in Nebraska (Metcalf 1986, Ball and Weekman 1962). Terbufos, an organophosphate, was registered in 1974; by 1980, it had failed to control populations of WCR due to enhanced soil degradation (Feslot et al. 1982). Surveys have identified rootworm populations with resistance to carbaryl and methyl-parathion which were used for adult

control (Wright et al. 1996, Meinke et al. 1998, Miota et al. 1998, Scharf et al. 1998, Siegfried et al. 2004).

Cultural control through crop rotation, tillage, delayed planting, manure application or altered planting density has been used for almost a century in control of WCR (Chiang 1970, Krysan et al. 1977, Mayo 1980, Musick et al. 1980, Branson and Krysan 1981, Johnson and Turpin 1985, Spike and Tollefson 1991, Roth et al. 1995, Allee and Davis 1996, Gray et al. 1998). Cultural control methods are popular among producers due to their cost effectiveness. Crop rotation is a prevalent cultural tactic used to control WCR (Krysan et al. 1977, Branson and Krysan 1981; Gray et al. 1998). This strategy relies on the limited host preference of WCR by alternating soybean, a non-host, with maize, the preferred host limiting oviposition in the soybean fields and reducing damage to corn planted after soybean. In the 1980's increased WCR root damage was observed in corn plantings following soybean in Illinois (Levine and Oloumi-Sadeghi 1996). O'Neal et al. (2002) tested acceptance of soybean foliage using several populations of WCR collected from cornfields in areas with or without increased damage to corn roots following soybean rotation. They observed WCR feeding on soybean foliage in both choice and no choice assays with no difference in soybean leaf damage by WCR from regions where increased damage to corn following soybean was observed compared to those from regions without damage to corn after soybeans. Hibbard et al. (2002) observed no difference in attraction to soybean volatiles between WCR that oviposit in soybean fields vs. cornfields. Additional experiments by O'Neal et al. (2002) indicate that corn phenology

influences WCR feeding on corn vs. soybean and this difference could help to explain WCR oviposition in soybean fields.

Growers can reduce root damage by employing a no-till system (Johnson and Turpin 1985, Gray and Tollefson 1987). Under no-till conditions, maize plants have comparatively small root systems that result in a reduced food supply which, in turn, results in reduced survivorship of larvae (Roth et al. 1995). Delayed planting is another option available for growers. In this case reduced survivorship is observed resulting from lack of food for early hatching larvae (Roth et al. 1995). Mayo (1980) demonstrated that delaying planting until early June reduced rootworm damage. Manure applications to maize fields can reduce WCR larval populations and adult emergence (Chiang 1970). Allee and Davis (1996) showed that maize plots treated with manure had increased plant height, yields and root regrowth along with decreased lodging and early feeding injuries. Moderate plant density of 65,000 plants per hectare enhanced root size and the regrowth potential for certain hybrids (Spike and Tollefson 1989).

Host plant resistance (HPR) research targeting rootworm began in the 1930's (Bigger et al. 1938). In addition to the obvious reduction in input cost, HPR provides greater pest specificity and reduces effects on non-target organisms and beneficial insects compared to chemical control. Branson and Kryson (1981) identified both tolerance and antibiosis as mechanisms of rootworm HPR. In maize tolerance to rootworm is associated with larger root systems (Riedell and Evenson 1993). While tolerance minimizes yield loss, it does not aid in the

control of rootworm populations and may be effective only under low levels of larval infestation (Ortman et al. 1974, Riedell and Evenson 1993).

Several breeding programs are developing lines with native WCR resistance (Hibbard et al. 1999, Moeser and Vidal 2004). Hibbard et al. (1999) identified ten F1 hybrids with the greatest WCR resistance from a diallel study. Seven of these lines along with B37 and Mo47 were used to form the initial generation of the CRW3 population which has undergone multiple generations of selection for reduced root damage from WCR (Hibbard et al. 2007).

It is estimated that over half of the total maize acreage in the US is treated annually with insecticide in order to control corn rootworm (Levine and Oloumi-Sadeghi 1991). *Bt* is a naturally-occurring soil microorganism that has been used for many years for biological control (Schneph 1998). Many strains of *Bt* produce insecticidal proteins referred to as *Bt* proteins or *Bt* toxins which disrupt insect midgut membranes. Over 100 insecticidal genes encoding these proteins having been identified (Moellenbeck et al. 2001). In 2003, the first maize transgenic for corn rootworms control was approved (EPA 2003). Today more than half the total US maize acreage is planted with a Bt variety and rootworm targeted *Bt* represents half of the *Bt* maize (USDA-ERS 2006).

Given WCR ability to adapt to chemical and cultural control methods, development of insect resistance to single gene transgenics is a concern. Insect resistance to *Bt* was first reported for Indian meal moth (*Plodia interpunctella* Hübner) raised in grain bins containing *Bt*-treated maize (McGaughey, 1985). Subsequently, Bt resistant diamondback moths *(Plutella xylostella* L.) were

reported in the field in the US, and resistance had also developed in Asia (Liu and Tabashnik, 1997). Nearly a dozen species have developed resistance to *Bt* in the laboratory: European corn borer (*Ostrinia nubilalis* Hübner), tobacco budworm (*Heliothis virescens* Fabricius), pink bollworm (*Pectinophora gossypiella* Saud.), mosquito *(Culex quinquefasciatus* Say), almond moth (*Caudracautella spp*.), cottonwood leaf beetle (*Chrysomela scripta* Fabricius), beet armyworm (*Spodoptera exigua* Hübner), Egyptian cotton leafworm *(Spodoptera littoralis* Boisd.), tiger moth (*Trichoplusia ni* Hübner), Colorado potato beetle (*Leptinotarsa decemlineata* Say) and the yellow fever mosquito (*Aedes aegypti* Liston), (Tabashnik 1994, Gould et al. 1997, Wirth et al. 1997, Whalon and McGaughey 1998, Frutos et al. 1999, Huang et al. 1999, Liu et al. 1999).

Wolbachia

Wolbachia are gram negative, obligate intracellular alpha-Proteobacteria that share a monophyletic relationship with *Rickettsia* and *Ehrlichia*, which are also obligate endosymbionts (Anderson and Karr, 2001). *Wolbachia* seem to be limited to ecdyzoan hosts. They are cyptoplasmically inherited and tend to be concentrated in reproductive tissues of insects (Werren et al 1997). In arthropods, *Wolbachia* induce feminization, male killing, parthenogenesis and cytoplasmic incompatibility (reviewed in Werren 1997). These conditions affect host evolution, causing sexual selection (Jiggins et al. 2000), sex determination (Rigaud et al. 1997) or speciation (O'Neill and Karr 1990, Shoemaker et al. 1999, Bordenstein et al. 2001). *Wolbachia*-induced cytoplasmic incompatibility (CI) leads to the autonomous spread of infection by inhibiting the reproductive success

of uninfected females in matings to infected males thereby increasing the frequency of infected individuals. Insect mitochondrial mutation rates can be over 10 times greater in *Wolbachia*-infected insects versus non-infected counterparts during *Wolbachia* sweeps (vanOpijnen et al. 2005).

It is estimated that *Wolbachia* can be found in between 20% (Werren and Windsor 2000) and 75% of all insect species (Jeyaprakash and Hoy 2000) suggesting they may be the most prevalent symbiont in the world. Within the genus *Diabrotica, Wolbachia* have been identified in several species including WCR (Clark et al. 2001). *Wolbachia* have also been identified in populations of the northern corn rootworm, (NCR) (Roehrdanz et al. 2003). The presence of *Wolbachia* serves as a reproductive barrier between closely related WCR and Mexican corn rootworm (MCR) and populations of NCR (Giordano et al. 1997, Roehrdanz and Levine 2002, 2006). *Wolbachia* inhabit most filarial nematodes (Zimmer 2001) including those that cause river blindness and elephantiasis in humans. They also infect mosquitos which vector malaria, Dengue fever, and West Nile virus. In addition, *Wolbachia* infect several economically important insect species such as honey bees, *Apis mellifera*, (Jeyaprakash et al. 2003) and silk worms, *Bombyx mori*, (Puttaraju and Madhu. 2002) as well as those that cause significant economic loss such as WCR, fire ant, *Solenopsis invicta*, (Shoemaker et al. 2000), and cockroaches, *Blattella nipponica* and *Suprella longipalpa* (Vaishampayan et al. 2007).

Genome sequences of the *Wolbachia wMel* and *Wolbachia wBm* genomes are available (Wu et al. 2004, Foster et al. 2005, Salzberg et al. 2005). The

Wolbachia genome contains ten times more mobile-DNA elements than any other obligate intercellular bacteria. The genome consists of 1271 genes with 123 mobile-DNA elements, 81 transposases and 14 reverse transcriptases (Bordenstein and Reznikoff 2005). Recently lateral transfer of *Wolbachia* DNA ranging in size from 1000 bp to nearly intact genomes has been documented in several insect species (Arca et al. 2005, Kondo et al. 2005, Hotopp et al 2007).

Bacteriophages are widespread viruses infecting bacteria. *Wolbachia* are commonly infected with the bacteriophage WO. The WO bacteriophage, is one of the few to infect an intracellular host (Storey et al. 1989), as it is more common for selection pressure to eliminate parasitic DNA from endosymbionts (Anderson and Anderson 1999). Nearly 90% of all *Wolbachia* strains carry WO (Bordenstein and Wernegreen 2004) suggesting WO performs a service for its host that has yet to be identified.

Maize

Maize is a determinate annual plant, which produces separate male (tassels) and female (ears) inflorescences (Walden 1978). It is a cross-pollinating species with varied morphology and geographical range (Walden 1978). One of the most variable aspects of maize morphology is its endosperm (Walden 1978). All parts of the maize plant, excluding the roots, can be used for commercial purposes to produce anything from fuel to food, cosmetics and even plastic.

Total U.S. maize production in the last 50 years has increased several times although there has been a consistent reduction in cultivated acreage (USDA-ARS 2005, USDA-NASS 2007). On a global scale, the U.S. accounts for

nearly a third of the total world maize production (USDA-NASS 2005, USDA-NASS 2007). Aside from being an economically important species, maize lends itself well to genetic studies. The maize genome is large (2300 Mbp) and highly repetitive (Wei et al. 2007). The available gene assemblies include the Assembled *Zea mays* (AZM) sequences derived from methyl filtration and high-Cot libraries (Whitelaw et al. 2003; Chan et al. 2006) as well as the TIGR maize unigene set (Quackenbush et al. 2000) assembled from EST sequence. A robust physical map based on genetically anchored BAC (Wei et al. 2007) can be queried at the Arizona Genomics Institute website (www.genome.arizona.edu). Since there is a high level of collinearity between maize and other cereal species, it is often possible to utilize genetic resources from other grass species to understand maize biology. Gramene (Jaiswal et al. 2006) a cereal genomics database can be used to harness colinearity information. Numerous genetic maps are also available at MaizeGDB (www.maizegdb.org). A large collection of mutants are available for *in vivo* analysis through the Maize Genetics Cooperation Stock Center (maizecoop.cropsci.uiuc.edu). The University of Arizona has created a long-oligo-array in which contains nearly 60,000 probes representing most of the maize genome (www.maizearray.org). A draft sequence of the maize genome is available at http://www.maizesequence.org/index.html. These resources and others make genetic dissection of quantitative traits such as insect resistance feasible.

Plant Defense Against Insects

Plants employ three basic types of defense against insects and microbes: increased cell wall defenses, increased production of phytoalexins and production of pathogenesis-related proteins (PR-proteins) (reviewed by VanLoon et al. 2006). Cell wall defenses can involve increased production of structural components such as actin and glycoproteins. Other cell wall defenses are involved in programmed cell death, as dead cells are able to encapsulate an invading pathogen and prevent the establishment of infection. Phytoalexin production includes components involved in terpenoid, steroid, flavonoid and lignin synthesis. Pathogenesis related proteins are divided into 17 classes and are induced by pathogens or pests.

In plants, insect-mediated damage results in different physiological, biochemical and molecular response than mechanical damage (Reymond et al. 2000, Zhu-Salzman et al. 2004, Mohan et al. 2006). Plants are able to recognize and respond to insect attack by releasing volatiles which attract natural enemies (Pare and Tumlinson 1997 and 1999, Mattiacci et al. 1995, Schmelz et al. 2003) or by producing proteins or metabolites that hinder the biology of the pest (Chen et al. 2005, Mohan et al. 2006). Although most plants release the same basic classes of compounds, the blends and concentrations of the volatiles of plants vary in response to environmental factors that impact the plant physiology (Pare and Tumlison 1999). Maize remains a preferred system for understanding the nature of volatile mediated plant-insect interactions (Hoballah and Turlings 2005) and the investigation of the activation of secondary metabolites (Frey et al. 2000),

as a wealth of previous work links secondary metabolites and volatile emission to maize defense.

Damage to plant cells either through mechanical wounding or insect herbivory often results in the release of linolenic acid, which is converted to jasmonic acid (JA) through the octadecanoid pathway. JA is a primary intracellular defense signal, which mediates early defense signals as well as local and systemic defense genes (Ryan 2000). Although mechanical wounding and insect herbivory induce many of the same genes, it should be noted that insect feeding often results in lower levels of protein expression thought to be advantageous to the insect (Reymond et al. 2000, Felton 2005). Insect-derived elictors such as volicitin from beet armyworm increase JA and sesquiterpene volatile production (Schmelz et al. 2003).

In maize, fatty acid amino acid conjugates (FAC) from insect saliva induce plant defense. FAC's cause the release of terpenoids that can either aid in the attraction of natural enemies or act as a repellent to the offending insect (Turlings et al. 1995, Mattiacci et al. 1995, Alborn et al. 1997). Plant volatiles are often released within the first few hours following a wounding or feeding event (Korth and Dixon 1997) while secondary metabolites and proteins are often produced following more extended feeding periods (Korth and Dixon 1997, Moran et al 2002, Zhu-Salzman et al. 2004). Zhu-Salzman et al. (2004) found that many defense related genes had reached significant transcriptional levels 24 hours after insect feeding began in sorghum.

Frey et al. (2000) identified indole-3-glycerol phosphate lyase (IGL) as an enzyme activated by volicitin, which catalyzes the production of free indole. Indole is an intermediate product in DIMBOA and tryptophan synthesis. In the DIMBOA pathway, indole-3-glycerol phosphate (IGP) formation is catalyzed by the *bx1* gene product in maize and eventually is synthesized into DIMBOA through action of the products of the *bx2 – bx7* maize genes. Jabeen et al. (2006) demonstrated that β-glucosidase, which metabolises a DIMBOA precursor, is up-regulated in wounded tissue. DIMBOA/DIBOA production is higher in seedlings (Jabeen et al. 2006) whereas IGL is an inducible product that is activated later in maize development in response to insect herbivory (Frey et al. 2000). IGL is induced in several other plants and is an attractant to natural enemies (Frey et al. 2000).

In addition to the release of volatiles, insect herbivory results in the increased expression of pathogenesis related (PR) proteins. PR proteins are induced when challenged by pathogens, pests or other stress-related factors (Antoniw and White 1980). The expression of PR proteins is variable and dependent on the source of stress and the host plant genotype (McKenzie et al. 2002). PR proteins have been shown to directly inhibit insect growth and reproduction (Ryan 1990). In plants, PR proteins are grouped into 17 classes which include β -1,3-glucanases, chitinases, thaumatin –like proteins, protease inhibitors, proteinases, peroxidases and ribonuclease-like proteins (vanLoon 1999). β-1,3-glucanases and chitinases degrade glucan and chitin in the insect midgut (Moeller and Tiffin 2005). Cysteine proteases damage the insect

peritrophic matrix (Mohan et al. 2006). Protease inhibitors and proteinases alter and inactivate the digestive processes of insects and interfere with insect growth and development (Ryan 1990). Thaumatin-like proteins inhibit insect digestive enzymes (Kim et al. 2005). Peroxidases stimulate salicylic acid (SA) and ethylene production and induce expression of other related PR proteins (Wu et al. 1997). Ribonuclease-like proteins are induced shortly after insect or pathogen attack (Lo et al. 1999) and are speculated to function in selective degradation of mRNA species following attack (Walter et al. 1996).

During recent years, microarrays have been used to identify plant genes that respond specifically to insect feeding (Moran and Thompson 2001, Moran et al. 2002, Reymond et al. 2002, Zhu-Salzman 2004, Voelckel et al 2004, Chen et al. 2005). Zhu-Salzman et al. (2004) used cDNA microarrays and Northern blots in an effort to identify sorghum genes unique to greenbug feeding with JA and SA independent induction.

Experimental Objectives

To date no studies addressing global insect defense response in roots have been published. Given the importance of WCR as a pest of maize, we will perform experiments to address three objectives aimed at understanding the maize root transcriptome response to WCR feeding.

Objective 1. Evaluate the maize seminal root gene expression profile in plants from the sixth cycle of the CRW3 population 24 hours after infestation with neonate WCR larvae, 5 hours after mechanical wounding, and with no treatment (control) using a maize long-oligo-array.

Objective 2. Determine the effect of *Wolbachia* on maize seminal root gene expression by comparing RNA from maize plants following feeding by neonate larvae with *Wolbachia*, neonate larvae without *Wolbachia*, and no treatment using a maize long-oligo-array.

Objective 3. Determine whether the effect of *Wolbachia* on WCR size and fecundity.

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Chapter 2. A Genomic Survey of Maize Identifies Several New Insect Defense Targets

Abstract

Increasing demand for ethanol is motivating growers to increase maize production reducing production of other crops. WCR is the most significant and widespread pest to maize in North America and is becoming so in parts of Europe. It has surmounted chemical and cultural control strategies. Currently, selection for native resistance to WCR is driven by phenotypic expression. The goal of this study was to identify maize genes unique to WCR feeding with the hope of locating genes specific for insect defense. A maize microarray experiment was performed to identify WCR-responsive genes and mutants of candidate genes were assayed to confirm their involvement in WCR feeding response. Surprisingly the number of commonly employed insect defense genes which were differentially expressed was low. Interestingly, genes involved in bacterial defense, chromatin remodeling, and gene silencing were among those identified as WCR-specific. Nine genes currently not associated with insect defense in plants were differentially expressed in response to WCR feeding. The results suggest a bacterium capable of altering gene expression may contribute to the WCR feeding-specific changes in maize seminal root gene expression.

Introduction

Increasing demand for ethanol is motivating growers to increase maize production reducing production of other crops like soybean, *G. max* L., wheat, *Triticum aestivum* L., and rice, *Oryza sativa* L. (USDA-NASS 2007). WCR is a significant pest to maize in North America as well as parts of Europe (Chandler 1998, Hummel et al. 2005). It infests over 60% of the maize acreage in the United States. Over half of the total maize acreage has traditionally been treated with insecticide for rootworm control (Levine and Oloumi-Sadeghi 1991). *Bt* varieties specific for rootworm control are becoming increasingly used (USDA-ERS 2007). If not properly controlled, rootworm related yield loss can be as great as half of the total crop (Godfrey et al. 1993).

There are several rootworm species which threaten maize but WCR is more common throughout the majority of the Corn Belt. It inhabits the largest range of all the economically significant rootworm species and has demonstrated the ability to overcome diverse control tactics. Larval WCR feed below ground on maize root tissue while the adults feed on above ground tissue. Although WCR larvae can survive on a few other species of grasses (Wilson and Hibbard 2004, Oyediran et al. 2004), they are usually considered functionally monophagous on maize. In addition, WCR populations can supplant sympatric rootworm species (Woodson 1994).

WCR has developed resistance to organochlorine and methyl-parathion insecticides (Ball and Weekman 1962, Metcalf 1986, Wright et al. 1996, Meinke

et al. 1998, Miota et al. 1998, Scharf et al. 1998, Siegfried et al. 2004). As with insecticides, WCR has also circumvented crop rotation as a strategy for control (Levine and Oloumi-Sadeghi, 1996). While chemical and cultural practices are still used to control WCR, acreage planted with *Bt* transgenic varieties is increasing each year (USDA-ERS 2007). At present, 50% of maize acreage in the US is planted with *Bt* varieties; half of which is intended for control of maize rootworms (USDA-ERS 2007). Given the ability of WCR to develop resistance, deployment of single gene transgenics for durable resistance is a concern. For this reason, quantitative HPR is a preferable tactic for WCR control.

Currently, selection for HPR to WCR is driven by phenotypic expression. There are few traits known to confer resistance to WCR. Increased DIMBOA (Assabgui et al. 1995) and phytosterol (Moeser and Vidal 2004) content have been correlated with resistant germplasm. Increased β-caryophyllene emission by maize roots has been shown to result in significantly increased infection of WCR by *Heterorhabitis megidis*, an entomopathogenic nematode (Rasmann et al. 2005) however subsequent studies indicated that most US germplasm does not produce this compound due to reduced transcription of the underlying gene (Kollner et al., 2008). Identification of specific genes and associated favorable alleles that conferred insect resistance in maize would facilitate marker-assisted-selection in breeding for WCR HPR.

When stressed by either pathogens or wounding, plants activate three basic classes of defenses which include bolstering of cell wall defenses, production of phytoalexins and production of PR-proteins. Cell wall defenses

include strengthening of cell walls, senescence and lignification. Phytoalexins are chemicals produced by the plant following insect or microbial attack. They include volatiles, SA- and JA- related products and can be grouped into categories of phenolics, terpenoids and glycosteroids.

Under normal circumstances, plants recognize and respond to insect attack through a variety of physiological, biochemical and molecular responses which include the release of volatiles and the production of proteins or metabolites that hinder the biology of the offending insect. Insect attack of maize normally results in the upregulation of LOX, proteinase inhibitors, hydroperoxide lyase, PAL, methyl salicylate, methyl jasmonate, and a variety of volatile organic compounds (Cosgrove et al. 2000). These products can directly affect the insect by inducing the production of PR-proteins. Many of these products defend the plant indirectly as well by catalyzing the production of compounds that attract natural enemies or signal neighboring plants of impending attack (Pare and Tumlinson 1997 and 1999, Mattiacci et al. 1995, Schmelz et al. 2003). PR-proteins include chitinases, peroxidases, protease inhibitors, β-1-3-glucanases and other molecules that inhibit microbes or insects (van Loon 1999). They are non-detectable in healthy tissue and increase following microbial or insect attack.

During recent years, microarrays have been used to identify genes specific for plant response to insect feeding (Moran et al. 2002, Zhu-Salzman et al. 2004, Voelckel et al. 2004). Giovannai et al. (2006) observed no change in known defense response and wounding genes on Hessian fly (*Mayetiola destructor*) feeding. Reactive oxygen species indicative of oxidative stress also did not differ

between Hessian fly infested and uninfested roots (Giovanni et al. 2006). Zhu-Salzman et al. (2004) investigated the transcriptional response of sorghum (*Sorghum bicolor*) to greenbug aphids (*Schizaphis graminum*). They showed greenbug feeding did not alter or only slightly induced expression of several endogenous defense mechanisms including methyl jasmonate, involved in defense signaling, LOX, involved in JA biosynthesis, and dhurrin, a precursor of hydrogen cyanide. Both of these studies identified unexpected patterns of plant gene expression following insect feeding. These results suggest genome-wide analyses of gene expression are beneficial in understanding HPR to insects. For this reason we conducted a DNA microarray study to identify WCR-specific differentially expressed genes in maize seminal roots. Several maize mutants were tested to verify the contribution of individual genes identified in the microarray experiment towards WCR defense.

Materials and Methods

Insects. Non-diapausing, WCR eggs were obtained from the North Central Agricultural Research Laboratory (NCARL) in Brookings, SD. In the lab, eggs were incubated in the dark at room temperature. Soil was moistened as necessary to maintain optimal incubation conditions. Once hatching had commenced, only vigorously moving larvae were selected. Fifty larvae were selected with a small camel hair paintbrush and placed into a standard Petri dish. Viability of the selected larvae was verified by visualization with a dissection microscope. Any injured larvae were replaced with healthy larvae. Larvae were placed at the base of the maize plants by rinsing the Petri dish with a small amount of water.

Plant Tissue. For the microarray experiment, the CRW3 (S1) C6 line (Reg. No. GP-553, PI 644060) which had been selectively bred for WCR resistance was chosen (Hibbard et al. 2007). Maize seedlings were grown in a growth chamber. Lighting conditions were set at a 14 hour photoperiod and 10 hour scotoperiod. Both incandescent and florescent lights were used and a minimum light level of 650-700 microeinsteins was maintained. Temperatures were set at 28° C for the photoperiod and 22° C for the scotoperiod with humidity at 60% and 80% respectively. In order to mimic field conditions, maize seed was planted in soil containing 1% nitrogen, 0.5% potassium and 0.5% phosphorus fertilizer. Plants were grown in 360 ml plastic drinking cups which were perforated for drainage at the bottom of the cup. Plants were grown to the V3 stage where they were subjected to their respective treatments.

For the mechanical wounding treatment, a 1 inch putty knife was inserted, the depth of the cup, into the soil parallel to the edge of the cup and half way between the stalk and cup edge. Plants were allowed to rest for 5 hours before collection of tissue. For the WCR feeding treatment, 50 neonate larvae were placed at the base of the plant. Seminal root tissue was collected 24 hours post-infestation. Collection times for both the mechanical wounding and WCR feeding treatment were determined based on the literature which show that most mechanical wounding transcripts are up-regulated in ~ 5 hours to levels comparable to expression change levels measured 24 hours after insect feeding (Korth and Dixon 1997, Tamayo et al. 2000, Shen et al. 2000, Reymond et al. 2000). The control treatment was collected from uninfested, untreated maize plants at the V3 stage of development.

One hundred-fifty plants per treatment were pooled into a replicate. Three seminal root tips were collected per plant and pooled. Tissue from all three treatments was collected in the dark with the use of a green light. In 30 seconds or less, soil was dislodged from the roots, the roots were rinsed in room temperature water and then one centimeter of tissue from a seminal root tip was excised with a scalpel and placed into liquid nitrogen.

Microarray. RNA extraction, amplification, labeling and hybridization were performed using the products and protocols recommended by the Maize Oligonucleotide Array Project (www.maizearray.org/ maize_protocols.shtml). Residual sugars were removed from the RNA using the Qiagen RNAeasy Mini elute kit (Qiagen Cataloge # 74204). The amount of RNA recovered was

determined using a NanoDrop Fluorospectometer (NanoDrop Technologies, Wilmington, DE). Following hybridization, the slides were washed, dried and immediately scanned. A GenePix 4000B Axon scanner (Molecular Devices Corporation, Sunnyvale, California) was used. Slides were prescanned and a probe intensity curve with a count ratio of 1.0 +/- 0.1 was obtained before a final image was acquired. GenePix Pro version 6.0 software (Molecular Devices Corporation, Sunnyvale, California) was employed for slide normalization and spot-calling.

Data Analysis. Data were transformed by log2 and then normalized. Fixed effects were designated as: pin, dye, pin x dye, treatment, and treatment x dye. Random effects were designated as array and array x dye. SAS (SAS Institute, Cary, NC) was used to specify the denominator degrees of freedom and the covariance structure. SAS Proc Univariate was used to obtain the corrected sum of squares which reduced noise in the data for normalization. Once data had been normalized, they were subject to three separate analyses. The first analysis grouped the data by treatment type and the least squared means was used to compare the treatment types. The F-statistic was obtained for pairwise differences between all three treatments. This allowed us to observe changes in gene expression and determine the significance of relative expression changes for the three collective treatments. The second analysis consisted of a second pairwise comparison using the Wilcoxon signed-rank test which is a non-parametric test similar to a Student's t-test. The third analysis which was used for obtaining the expression profile subjected each probe I.D. value to a Kruskal-

Wallis test, which is a non-parametric procedure similar to an F statistic. The Wilcoxon signed-rank test and the Kruskal-Wallis test were performed in order to tighten the significance parameters of the results. These tests also allowed for more precise comparisons between two treatments. Only probes which received a p-value of less than 0.05 for both rank tests were included in our analysis.

Quantitative RT-PCR. Fifty differentially expressed probes were selected from the subset of one hundred thirty-five differentially expressed probes based on microarray analysis for verification by qRT-PCR (Table 2.1). Sequence specific primers were designed using PrimerQuest from IDT SciTools http://www.idtdna.com/Scitools/Applications/PrimerQuest/Default.aspx/ (Table 2.1). iScript One-Step RT-PCR Kit With SYBR Green (Biorad Cataloge # 170-8892) was used to quantify expression levels. Half reactions were performed with 30 ng of total RNA per sample. A standard curve was included on each plate along with a no template control. Standard curve concentrations were set at 100 ng, 50 ng, 25 ng, 12 ng, 6 ng and 3 ng of total RNA. RNA from the non-damaged control treatment with a primer selecting for a non-differentially expressed probe were used. An ABI7000 real-time PCR system (Applied Biosystems, Foster City, CA) was employed for mRNA quantification and verification of the microarray analyses. The standard curve was set with a regression value of of 0.99 or 1.0 for each plate and the values for the housekeeping probe were used to normalize the values for all of the probes on the plate. Cycles were programmed according to manufacturer's specifications in the iScript One-Step RT-PCR Kit with SYBR Green kit.

Root Tissue Damage Assay. The mutants tested included *brown midrib1* (*bm1), brown midrib2 (bm2), brown midrib3 (bm3), viviparous5 (vp5), viviparous8 (vp8), bronze1 (bz1), bronze2 (bz2),* and the *orange pericarp1/orange pericarp2 (orp1*/*orp2)* double mutant. All of the mutants are homozygous recessive. With the exception of *orp1/orp2* all result from a single gene mutation. Seeds of *bm1* (stock number 515D), *bm2* (stock number 119F), *bm3* (stock number 408B), *vp5* (stock number 103DA), and *vp8* (stock number 119B) were obtained from the Maize Genetics Cooperation Stock Center (http://maizecoop.cropsci.uiuc.edu) and subsequently increased and genotyped in the field or greenhouse in Columbia, MO. Seeds of *bz1, bz2*, and the *orp1/orp2* double mutant were obtained from Ed Coe. Mutant and wild-type sibling plants were grown and infested with WCR under similar parameters as for the microarray experiment. WCR fed for 48 hours prior to data collection to allow more damage to occur. Root tips were collected as for the microarray experiment. Four seminal root samples from a single plant were collected and pooled into one 1.5 ml eppendorf tube containing distilled water. For each sample, areas of insect feeding were counted and photographed. Images and counts were performed on a Leica-Eppendorf Microinjection Microscope (Leica Corporation, Wetzlar, Germany) at the University of Missouri Molecular Cytology Core under Bright Field light. Images were acquired at 20X exposure in QCapture Pro software. Damage areas were determined from digital images using AlphaEase software

(AlphaInnotech, San Leandro, California, USA). Data from the damage counts and areas were subjected to a variance stabilizing transformation; outliers greater than two standard deviations from the sample mean were removed from the analysis. The transformed and normalized data were then subjected to the Student's t-test; p-values less than 0.05 were considered statistically significant.

Results

Microarray. The maize long-oligo-array, printed by the University of Arizona, was used to determine maize seminal root gene expression patterns in wounded, WCR damaged, or control (untreated) roots. The array contains 70 mer oligonucleotides designed against nearly 60,000 maize unigenes on 2 slides. The statistical analysis identified relatively few significant probes specific for WCR feeding. Less than 500 probes on the microarray exhibited significant differential expression by both the Wilcoxon-Signed-Rank test and the Kruskal-Wallis test (Table 2.2). Individually each of the three statistical analysis methods also identified less than 500 significantly different probes indicating the low number of significant probes is not due to the statistical testing method.

Only 19 probes which encoded plant defense products were up-regulated in response to WCR feeding (Table 2.3). Of the defense probes exhibiting significant differential expression, several were down-regulated in response to rootworm feeding, and several that were up-regulated correspond to genes normally associated with microbial defense (Table 2.2). Other WCR-specific probes belong to the metabolism, defense, transcription factor, DNA repair and replication, hormone, signal transduction, architecture, chromatin remodeling and gene silencing ontology categories (Table 2.2).

Root Damage Assays. Maize mutants were compared against their wild-type siblings to identify differences in neonate WCR damage as measured by number of damaged sites per root and average damaged area per feeding site. Pair-wise comparisons via the Student's t-test identified significant differences in

one or both of the measures of WCR damage for *vp5*, *vp8*, *bm2*, *bz1*, *bz2*, and *orp1/orp2*. A significantly larger number of damaged sites per root were observed in comparison to wild-type in all of these mutants (Table 2.3). In addition, a significant reduction in the damage area per site was observed for the *bm2* mutant compared to wild-type (Table 2.3).

Discussion

The microarray expression profile indicated several genes, which had not previously been associated with insect defense, were up-regulated in response to WCR feeding. Surprisingly the number of known insect defense genes with differential expression in this study was low. Instead many up-regulated probes are directed at microbial defense. The probe encoding a xylanase inhibitor displayed increased differential expression in response to rootworm feeding (Table 2.3). Xylanase inhibitors protect the plant by preventing pathogens from compromising the cell wall and establishing infection. Plants typically produce xylanase inhibitors only in response to pathogen-derived factors (Beliën et al. 2006). Expression of maize peptide deformylase (PD) was also significantly increased in response to WCR feeding (Table 2.3). PD is involved in protein synthesis and degredation in eubacteria (Bouzaidi-Tiali et al. 2007). In bacteria, it is required for survival because it removes the N-terminal formyl group of nascent polypeptides (Meinnel et al. 1994, Mazel et al. 1994). In Arabidopsis, PDs are required for photosynthesis (Serero et al. 2001). The Arabidopsis PDs are most closely related to bacterial peptide deformylase, perhaps because of the bacterial origin of plant organelles. Moon et al. (2008) show that rice PD is required for chloroplast development and also affects mitochondrial development.

Avr9/Cf-9, a plant PR-gene up-regulated in response to WCR feeding (Table 1) is typically associated with resistance to the fungal pathogen, *Cladosporium fulvum,* in tomato (*Lycopersicon esculentum*). The tomato *Cf-9* gene encodes a highly glycosylated type I membrane protein characteristic of

receptor-like proteins (Van der Hoorn et al. 2005), which confers resistance to the fungus expressing the corresponding avirulence gene (*Avr9*). The protein encoded by *Cf-9* functions as part of a receptor complex that recognizes the *Avr* elicitor and initiates defense responses (Martin et al. 2003). One possible interpretation of the increased expression of this gene is that a gene-for-gene microbial resistance is elicited by WCR feeding on maize roots. The WCR-specific up-regulation in maize of the xylanase inhibitor, PD, and *Avr9/Cf-9* genes indicate that factor(s) other than insect elicitors may be contributing to the insect defense response in maize seminal roots.

A maize chitinase was significantly upregulated in response to WCR feeding as well (Table 2.3). As with the xylanase inhibitor and peptide deformylase, chitinase has not previously been associated with plant defense against insect feeding. Chitinases are often up-regulated in plants during pathogenesis of bacteria or fungi (reviewed in Heil and Bostock 2002). This up-regulation of maize chitinase by WCR could be further indicative of the involvement of a bacteria in the maize response to WCR feeding. An alternative explanation for WCR-specific up-regulation of the maize chitinase is that the chitinase has a direct role in protecting the plant against insect damage. Chitinases are used in biological control against insects. They can be derived from bacteria and fungi and are topically applied to the plant. Chitinases function as insecticides by permeablizing the insect cuticle which facilitates establishment of infection by other fungal and bacterial pathogens (Herrera-Estrella and Chet 1999). To date there is a single account of a endogenous plant chitinase

exhibiting a negative effect on insect biology (Lawrence and Novak 2006). The insect midgut is lined with chitin, proteoglycans and glycoproteins (Gullan and Cranston 2004). *In vivo* destruction of insect midgut peritrophic membranes by recombinant bacterial chitinase was demonstrated in *Spodoptera* (Regev et al. 1996). Under the alternative scenario for insect-specific induction of maize chitinase expression leads to permeabilization of the WCR cuticle or mid-gut membrane and is indicative of a novel plant defense against insect feeding.

Of the defense genes implicated in the microarray experiment, only a few characterized maize mutants are available for *in vivo* testing. Mutants selected for analysis were associated with flavonoid, carotenoid, tryptophan and lignin synthesis (Table 2.4). Though some of the mutants tested do not coincide with specific genes implicated on the microarray, they are located on corresponding biosynthetic pathways and contribute to the same end products.

The *bm1*, *bm2* and *bm3* encode gene products associated with reduction in lignin content (reviewed in Guillaumie et al. 2007). Lignin is a structural component of cell walls; during defense, it has been shown to accumulate around areas of attack and create a physical barrier against infection (Bonello and Blodgett 2003). *bm1* mutants have a 10-20% reduction in lignin content (Barriere et al. 2004) and an almost complete reduction (90-97%) in cinnamyl alcohol dehydrogenase (CAD) activity in roots (Halpin et al. 1998). The maize CAD gene is orthologous to a zinc-containing long-chain alcohol dehydrogenases in rice and eucalyptus (Goffner et al. 1992, Hawkins and Boudet 1994, Tobais and Chow 2005). The role of this CAD in lignin biosynthesis remains unresolved

(Guillaume et al. 2007). Guillaume et al. (2007) surveyed expression of a variety of phenylpropanoid and regulatory genes in the *brown-midrib* mutants. Their data suggests that *bm1* encodes a regulator of CAD. No significant difference between *bm1* mutant and wild-type was observed for either number of damaged sites or extent of insect damage. The maize *bm3* gene encodes caffeic O-methyl transferase (Vignols et al. 1995). Barriere et al. (2004) demonstrated *bm3* mutants display a 25-40% reduction in lignin content. As with the *bm1* mutant, no significant difference between mutant and wild-type was observed for either measure of insect damage for the *bm3* mutant. Together the lack of significant insect feeding associated changes for the *bm1* and *bm3* mutants suggests that lignin may not offer protection in maize seminal roots against WCR.

bm2 mutants in maize have a 15-25% reduction in lignin content (Barriere et al. 2004). Based on expression of genes involved in regulation and biosynthesis of phenylpropanoids, Guillaume et al. (2007) suggest *bm2* encodes a regulator affecting the RNA induced silencing complex (RISC) resulting in a lignin profile with lower ferulic acid ether levels and lower β-O-4-linked G units. The RISC in plants enables siRNA-mediated endonucleolytic cleavage of complementary RNA resulting in gene silencing (Scholthof 2007). In plants one function of RNAi mediated gene silencing is as a defense mechanism against invading RNA from microbial or viral pathogens (Scholthof 2007). Insect induction of RNAi silencing has not been observed in plants. A significant increase in the number of feeding sites was observed in *bm2* mutant vs. wild-type along with a reduction in the average damage area per feeding site (Table 2.4). Significan expression

differences were found in a number of genes involved in silencing on WCR feeding (Table 2.2. The significant differences observed in the *bm2* mutants, along with the differencing in silencing genes after WCR feeding further supports the possible involvement of a bacterial-mediated change associated with the maize-WCR interaction.

The expression data show that indole-3-glycerol phosphate lyase is up-regulated in response to WCR feeding (Table 2.4). The gene product indole-3-glycerol phosphate lyase is the first committed step in 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) synthesis and is part of the tryptophan biosynthetic pathway. Products of the tryptophan pathway include hydroxamic acids such DIMBOA which has been shown to have deleterious effects on WCR among other organisms (Assabgui et al. 1995). To further explore the effects of WCR on tryptophan biosynthesis, the *orp1/orp2* mutant was tested. The *orp1/orp2* mutant does not express the β subunit of tryptophan synthase and thus causes an accumulation of indole, anthranilic acid and indole-3-acetic acid (IAA) (Wright et al 1992). IAA is a ubiquitous plant auxin that is involved in many processes including plant defense. Recent work has shown that pathogenic microbes can interfere with auxin signaling to circumvent plant defenses and facilitate colonization (Navarro et al 2006, Spaepen et al. 2007). Our data show that the absence of tryptophan B synthase in the plant renders it more susceptible to insect damage as evident by the increase in number of feeding sites compared to its wild-type (Table 2.4).

Many ABA and carotenoid products are associated with plant defense against insects, especially in green tissue. The *vp5* gene encodes phytoene desaturase (Hable et al. 1998). It has reduced abscisic acid (ABA) and carotenoid levels. The *vp8* gene encodes a putative *altered meristems program1*-like peptidase (Suzuki et al. 2008) which affects ABA content but not carotenoid levels. One function of ABA is to protect the growing regions of the root against ROS. No difference was observed between mutant and wild-type for either *vp5* or *vp8* indicating these genes are not involved in maize response to WCR. In plants, microbial pathogenesis and in some cases insect damage can lead to oxidative bursts. Alternative oxydase, superoxide dismutase, or catalase up-regulation are markers for increased ROS. Our data indicates an insect-specific oxidative burst is unlikely. Lack of an oxidative burst would obviate the need for the antioxidant properties of ABA or carotenoids. Giovanni et al. (2006) also documented absence of the expected oxidative burst following Hessian fly feeding.

Flavonoids are a class of secondary metabolites in the plant that perform a myriad of functions including insect defense (McMullen et al. 1998), UV protection (Stapleton and Walbot 1994), protection against oxidative damage (reviewed in Ferrer et al. 2008), and phytoalexin induction (Iwashina 2003, Ferrer et al. 2008). *bz1* encodes the product flavonol 3-O-glucosyltransferase which catalyzes the conversion of anthocyanidin to anthocyanin (Klein and Nelson 1983, Dooner et al. 1991). Expression of this gene was up-regulated in response to WCR feeding in the microarray experiment (Table 2.3). *bz1* not only glycosylates anthocyanidins but flavonoids as well (Styles and Ceska 1977).

Maize produces the hydroxamic acid DIMBOA as well as its metabolite MBOA. Increased levels of DIMBOA have been shown to have deleterious effects on WCR larvae (Assabgui et al. 1995) while MBOA is used by WCR for locating maize root tissue (Bjostad and Hibbard 1992). We observed significantly more WCR damage events in *bz1* mutants vs. wild-types (Table 2.4) suggesting that a downstream product of *bz1* has WCR insecticidal properties. *bz2* encodes the product glutathione S-transferase (GST) which marks cyaniding-3-glucoside for vacuolar localization (Marrs and Walbot 1997). A significant increase in the number of WCR damage sites was observed in *bz2* mutants vs. wild-type (Table 2.4) indicating flavonoids have WCR insecticidal properties. Flavonol 3-sulfotransferase another enzyme involved in flavonoid biosynthesis was also up-regulated on WCR feeding in the microarray experiment (Table 2.3).

We observed increased expression of mobile DNA elements on the array following WCR feeding (Table 2.5). The activation of mobile DNA, gene silencing, and chromatin remodeling factors coupled with the up-regulation of anti-microbial plant defenses supports a hypothesis that a microbe may be involved in the response of maize to WCR attack. *Diabrotica* beetles have been shown to harbor three basic types of microbes; enterobacteria (Schalk et al. 1987), spiroplasma (Carle et al. 1997) and *Wolbachia* (Clark et al. 2001). Of these *Wolbachia* has been shown to cause chromatin remodeling leading to cytoplasmic incompatibility in *Drosophila* (Harris and Braig 2003). Genome sequencing of two strains of *Wolbachia* revealed a Type IV secretion system which likely functions in *Wolbachia*-host interactions (Wu et al. 2004, Foster et al. 2005).

Wolbachia are also capable of causing programmed cell death in host tissue (Pannebakker et al. 2007).

Other reports have been made of insects eliciting plant responses similar to those observed in our data set (McKenzie et al. 2002, Zhu-Salzman et al. 2004, Giovanini et al. 2006). Similarities include the lack of typical insect defense gene expression and oxidative bursts. The possibility of microbial-mediated alterations in host plant response indicates that the gene expression changes in maize-WCR interactions warrant closer examination.

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Table 2.1. Primers used for quantitative RT-PCR for verification of microarray results.

Probe I.D.	Forward Primer	Reverse Primer
MZ00020387	TGCCAACCTCCATCACTGGATCAT	TTAGCTGCCGAGGATGAACGAGAA
MZ00055963	TCCTAGCACCATGGATCCGCTTA	ACAGAAGCCTCCATGGCTAAGCTC
MZ00050488	TAGCGCTGCGCCTACCCAT	GTATTTGACTACCTCGAGTGGCGA
MZ00048528	TCCGGAGGCAGTTCTTCTTGGATT	GCAGTCGAAGGAGCACCATATTCA
MZ00047623	GCAGTCAGGGAACAATGCACAAGA	TCGGCTTGAAGCAGGAATGAATGC
MZ00047366	AGCTGTCCAGCGAGTACTATGGC	ACACGATGCTCGAGCTGTTGTCCA
MZ00046648	ATGGAGGATGACGAGTACGAGGAT	AGGCGTCGTCCTGCGTGATATCTT
MZ00046362	TCTTCCACGACTACTTCAACTGCC	AGGTGAGAGATGACCCAGGCGA
MZ00044942	ATACTGTAAGGGTGGTGATTGCCC	ACCCAACCACAAGTTGGCGTAGTA
MZ00044446	TGCAGGCGGCAAGGTGATAATAGT	TGACGTTCCTCCATTCCTGCTCAT
MZ00043484	GCTGTGGAAGGCATCAACCAGATT	CGATTCCGCCGTTGTTGATGATGA
MZ00041585	CCAGACGTAAGGAAGAAGAGAGACCT	ACAATTGCAAACCAGCACTCTCCC
MZ00041513	TGGTTCACGTACTGTGTGTTGTGC	AGCGTCACCTCTCTCTCTCTCTCTTT
MZ00041190	AATGGAAGGGTTGTGTCCTAGCCA	TAGTAAATTAAACGGCCGGGACGG
MZ00040567	AGCCTACTATGATGCCGAGGACTT	GCCCTGCTCATAGCAGCATGAAA

Probe I.D.	Forward Primer	Reverse Primer
MZ00037575	CATGGAGACCAAGCCTACCAACAT	GGCGCTTCTTGGCGCATAATAGTT
MZ00036611	TGAGTTGGATTGGAGTGGATTGAG	TCCCATCCAAACGCAGAATCTCCT
MZ00035793	CCTACGGCGAGAACCTCTTCTG	TCCGCCACATGATCTGGGTGTAGT
MZ00034239	ACCTAACGGGTATTTCGAGGCCAA	AGTAGACCTAATCTGCACTTGGGA
MZ00033251	CGCTGTGCGCTGCTTGATTAAGTT	TAGAGGTACTCCCTGCGCCT
MZ00032776	AGCACAGAATAGATGGAAGCGCAC	AGGTTTGCACTTGAGACAGGATGC
MZ00032371	AAGGAGTATGCCGACCTCAACCT	TGCCAAATCAAGAACCTCCCACTC
MZ00031952	CTGCCGCATGGTGATGACCAAATA	CATTCGGCAAGATTGAAGACCGTG
MZ00029811	AGCGTGGACCTTGACGACATTGAT	CCAGGAACGAATGGAAGCATAGCA
MZ00028668	GCTCACATTGTATGAGGTGGGCTA	ACCGCATAATTTGCGCCAATCCAA
MZ00027922	GCTCCACGCGGTTGTCTTTGAAAT	TTTGCCCGAGGATTTGGATGATCG
MZ00025730	GCTCCACGCGGTTGTCTTTGAAAT	TTTGCCCGAGGATTTGGATGATCG
MZ00024490	TGCTGGTGCAGTGACATTTGTGGT	AGTGACCTTATGGTCTCCATTCCC
MZ00024264	AGCCTCGTCGTTAGGACGTAAGGTAA	AGGAGGAGCATGAGGACGAGGA
MZ00023783	GGCGATGCCACACATGAACACAAA	CGCTATAGTTCACAAGAACGAACATGACG
MZ00023339	TCGGATACGCCTACGTCAACTTCA	AATGTTAGCAGATCCACTCCTGCG
MZ00023097	TCCTCGACAAGATGAAGAGGTCCA	ATATACAGCGCGAAAGCAACGAGC
MZ00022237	TGATAATCGCGCAATGGCCCTGT	GCAGTGGTCTAAGATGAGACGAGCTAGA
MZ00020916	TCAAAGTCGGAAGCGTCGAGCAAT	TGGCAACGTTGACCCTTCTATGGT

Probe I.D.	Forward Primer	Reverse Primer
MZ00019475	TATTCTGGGATTTGCCGCCCTTTG	AGCCGCTGCCTTTCGTTTCTTCTA
MZ00018539	TACGGTGTTAGAGCTGTACAGGGA	ACAGACCCATAATCAACCGCGTCT
MZ00018054	AGGGTGGCCCATCTCAAATACCAT	TTTGGCATAAATGGCGGGATCGGT
MZ00016670	ACAGCAGTGCTAGTGTTGGAGCAA	TTCCTGCGAATGTATGCCCAAAGC
MZ00014433	ATACCTCTACTACGATCTGCTGCCCA	TAACACAGCCAGCATAGCTGCAAG
MZ00010475	ATAGGCCAATACCCTCTGAACACTGC	TCAATGGCTGTTAGTGAGTATCCC
MZ00007502	TGCTAGATCCAGTAGATAGCCGAC	AGCGAGGAACCTAGAATCCCAAAC
MZ00005958	TGGTAGCGCAATGGTGAGACAGTT	CAACGTGCTTGCTAACACAGCCTA
MZ00005428	AAACGGAAATGATCACGGTGGCAG	TCGAGAAGCAGTAGTCGTGGAGC
MZ00004372	TATTCTGCCATGCATGCGGCTTTC	AAGTACGTGTTCTGGGACGAGT
MZ00003206	ACTGAACATGGCGGAGGTCCTA	TAGGAGGTACAATGTAGGCGACGA
MZ00002841	AAGCTGAAGCACAGGAACCTCGTC	TTGATGATCTTGTACCTCTGCGCC
MZ00002740	TTACCCATCGGTCTCGGTTGTCTT	GGTGAACACCAGTTCGCTTGGAAA
MZ00001736	TCATCGTACGAGGCCGTGGG	AAATCTCAGCATCCCGCCTCGTCT
MZ00001590	CTCCAGCTCGTCAACGTCCC	ACCAAGCGGATCAGATTTCAGGCA
MZ00045128	CTGCTCCTAGTCAAGAGCATTTAGT	AGATCCATTAGGGCACTGCTTC

Table 2.2. Maize microarray probes with significant changes in gene expression in maize between the control, mechanical wounding and WCR feeding, and their annotation based on the ver. 1.8 Arizona Maize long-oligonucleotide array. The F statistic was obtained for pairwise differences between all three treatments. Probes possessing a p-value of less than 0.05 are included below. Values in the control, wounding and WCR feeding columns represent the normalized and transformed treatment averages for relative gene expression. Negative values indicate gene down-regulation of relative expression while positive values represent up-regulation of relative expression.

Probe I.D.	**Annotation**	**Relative Expression**		
Signal transduction		**Control**	**Wounding**	**WCR Feeding**
MZ00050267	Putative myotubularin	0.3513	0.1621	-0.1690
MZ00034442	Serine peptidase leucine-rich repeat family protein	-0.2083	0.0709	0.1813
MZ00035972	Response regulator 7	0.5192	0.0727	-0.1448
MZ00014351	Acid phosphatase	0.5635	-0.0273	-0.2257
MZ00050267	Putative myotubularin	0.3513	0.1621	-0.1690
MZ00029811	Abscisic acid-inducible protein kinase	-0.8682	0.1787	-0.0027
Metabolism				
MZ00001506	Putative cytochrome P450	-0.0062	-0.0983	0.1265
MZ00002097	Vacuolar sorting receptor-like protein	0.0022	-0.0670	0.0372
MZ00049977	Putative GDSL-like lipase/hydrolase	-0.0417	-0.0256	0.1501
MZ00005238	Anionic peroxidase precursor	-1.0601	-0.0656	0.4213
MZ00009690	2-oxoacid dehydrogenase family protein	0.1170	-0.0646	0.1648
MZ00016200	Diphosphonucleotide phosphatase 2	0.0994	-0.0475	0.1645
MZ00021068	Phosphatidylcholine acyltransferase-like	0.0081	-0.0710	0.1891
MZ00024137	DTDP-glucose 4,6-dehydratase	-0.0032	-0.0682	0.0891
MZ00025723	Metallo-beta-lactamase-like	0.0369	-0.0398	0.0803
MZ00019270	Putative chloroplast nucleoid DNA-binding protein cnd41	0.0703	-0.0563	0.0950
MZ00001736	UDP-glucose:anthocyanin 3'-O-glucosyltransferase	-0.0220	-0.0617	0.0825
MZ00007822	Putative synaptic glycoprotein	0.0275	-0.0375	0.1023
MZ00016555	Thylakoid lumen protein	0.0239	-0.0607	0.1289
MZ00040543	Phosphogluconate dehydrogenase	-0.1787	0.4158	-0.4136
MZ00043364	Putative aminotransferase	0.1930	-0.0596	0.1189
MZ00048452	Hexose carrier protein	0.1628	0.0527	-0.1543
MZ00001766	Putative carbamoyl phosphate synthetase	0.3145	-0.0135	0.2508
MZ00016627	Rubredoxin family protein	0.0657	-0.0707	0.0279
MZ00031669	Putative polygalacturonase	0.0812	0.0409	-0.1480
MZ00023938	Phosphoenolpyruvate carboxylase	0.1172	0.0896	-0.2818

Probe I.D.	Annotation	Relative Expression		
Metabolism		**Control**	**Wounding**	**WCR Feeding**
MZ00029033	Coat/nuclear inclusion protein	0.0478	0.1112	-0.0475
MZ00035604	Putative 12-oxophytodienoate reductase	0.0212	0.0878	-0.1563
MZ00038546	Putative carboxypeptidase D	0.0179	0.0235	-0.0748
MZ00042848	Putative 12-oxophytodienoate reductase	1.0880	0.0056	-0.6255
MZ00010692	Membrane protein	0.7207	0.0236	-0.4236
MZ00035719	Methylmalonyl-CoA mutase	-0.0059	0.1823	-0.2493
Hormone				
MZ00037575	ABA- and ripening-inducible-like protein	-0.1494	0.3143	-0.3978
MZ00027642	Progesterone 5-beta-reductase, putative	0.8081	0.0562	-0.3582
MZ00041650	Ferritin 1 precursor	-0.1049	0.2954	-0.4012
MZ00041531	Oxysterol-binding protein	0.3140	-0.0228	-0.2468
MZ00041531	Oxysterol-binding protein	0.3140	-0.0228	-0.2468
Chromatin remodleing				
MZ00036611	Probable chromosome partitioning protein ParB	-0.2382	0.0292	0.1709
MZ00025730	HMGI/Y protein	-0.0195	-0.0770	0.0399
MZ00006107	Nucleosome assembly	0.8344	-0.0789	-0.4113
MZ00011180	Histone H4	0.2853	-0.0238	-0.3671
MZ00004507	Histone H3 type 1	0.3744	-0.0255	-0.3035
MZ00035004	Histone H2A	0.7241	0.0837	-0.4620
MZ00013517	Histone H2B	0.8288	0.0230	-0.0722
MZ00037646	Histone H2A.2.1	0.4184	0.0256	-0.3655
MZ00034239	Centromere protein-like	0.4189	-0.0476	-0.0817
MZ00037809	Histone H2B-like protein	0.8876	0.0604	-0.6818
MZ00036595	Similar to germinal histone H4 gene	0.5275	-0.0806	-0.6871
MZ00037830	Putative anti-silencing factor 1-like protein	0.3278	-0.0101	-0.0287
MZ00031797	GCN5-related N-acetyltransferase-like	0.3513	0.1874	-0.1708
Gene Silencing				
MZ00032371	TRNA (Guanine-N(7)-)-methyltransferase-like protein	-0.0736	0.0292	0.1284
MZ00009729	Putative N2,N2-dimethylguanosine tRNA methyltransferase	0.0813	-0.3428	-0.2340
MZ00055287	Probable O-GlcNAc transferase	0.2007	-0.0137	-0.2369
MZ00007307	ATP-dependent RNA helicase ste13	0.2915	-0.0403	-0.0238
MZ00044090	Methyltransferase	0.3716	-0.0348	-0.2344
MZ00036493	Putative RNA helicase	0.2639	0.0259	-0.1454
MZ00044446	Putative o-methyltransferase ZRP4	0.5939	0.0002	-0.0368
MZ00003995	Methyltransferase	0.8712	0.2242	-0.2750
Defense				
MZ00003206	5-azacytidine resistance protein-like	0.1985	-0.0065	0.3191

Probe I.D.	Annotation	Relative Expression		
Defense		**Control**	**Wounding**	**WCR Feeding**
MZ00005954	Leucine-rich repeat-like protein	-0.0438	0.1345	-0.0570
MZ00019475	Putative hydroxycinnamoyl transferase	-0.2028	0.1892	0.2373
MZ00023339	Avr9/Cf-9	0.0043	-0.0086	0.0308
MZ00024264	Cinnamoyl CoA reductase - maize	-0.0314	0.1297	0.3162
MZ00033251	Bax inhibitor-1	0.0340	-0.0626	0.1586
MZ00040567	Rust resistance protein RP1-D homolog	0.6177	-0.1332	0.4479
MZ00005958	Indole-3-glycerol phosphate lyase	-0.0274	-0.0039	0.7744
MZ00047366	Putative peroxidase	0.4460	-0.2207	-0.3858
MZ00032776	Putative disease resistance response protein	0.5589	0.0163	-0.3515
MZ00035793	Putative pathogenesis-related protein PR-1	0.4533	-0.0011	-0.0838
MZ00024490	Putative defensin	1.1257	0.0197	-0.4858
MZ00047188	Lipoxygenase	0.2840	-0.0885	0.0020
MZ00041443	Protein kinase C inhibitor -	0.3328	-0.0063	-0.1607
MZ00036599	chitinase	-0.1155	0.3086	1.2250
MZ00005428	4-hydroxycinnamic acid-CoA ligase	-0.5828	0.1075	0.1458
MZ00041513	Wound-induced protease inhibitor	-0.4879	0.0443	0.5652
MZ00019475	Putative hydroxycinnamoyl transferase	-0.2028	0.1892	0.2373
MZ00043484	Cysteine protease component of protease-inhibitor complex	0.2304	-0.1108	-0.0082
MZ00018539	Chorismate synthase 2	0.4547	-0.1590	0.2928
MZ00041190	Hydroxyproline-rich glycoprotein precursor	-0.4787	0.3774	0.5365
MZ00023097	Putative hydroxyproline-rich glycoprotein	-0.4304	0.0831	0.1421
MZ00012545	Glycine-rich protein	-0.5212	-0.0038	0.2246
MZ00014433	Putative xylanase inhibitor protein	-0.5237	0.0304	0.8004
MZ00007822	Putative glycoprotein	0.0275	-0.0375	0.1023
MZ00050488	Putative peptide deformylase	-0.0235	-0.0706	0.0637
MZ00001736	UDP-glucose:anthocyanin 3'-O-glucosyltransferase	-0.0220	-0.0617	0.0825
MZ00047623	Putative flavonol 3-sulfotransferase	0.0071	-0.0957	0.0803
Transcription				
MZ00010954	Putative GTP-binding protein	0.2058	0.0249	-0.1104
MZ00013482	Putative bZIP protein	0.0629	-0.0474	0.0596
MZ00024812	Probable ribophorin I	0.2813	-0.0927	0.1503
MZ00027518	Zinc finger	0.0537	0.2332	-0.1278
MZ00022093	F-box family protein-like	0.0828	0.0924	-0.1374
MZ00032738	Putative heterogeneous nuclear ribonucleoprotein A1	0.4705	-0.1194	-0.0810
MZ00022932	Putative ZF-HD homeobox protein	0.1685	0.0206	-0.1249

Probe I.D.	Annotation	Relative Expression		
Transcription		**Control**	**Wounding**	**WCR Feeding**
MZ00015814	F-box protein-like	0.1693	-0.0231	-0.1765
MZ00023783	Poly(A)-binding protein	0.6491	-0.0764	-0.1630
MZ00054910	Leucine zipper protein-like	-0.0267	-0.0449	0.0640
MZ00009390	Putative PHD-finger domain containing protein	0.1406	-0.0195	0.2606
MZ00011586	Zinc finger protein	-0.1132	0.1070	0.0040
MZ00020006	Small nuclear RNA activating complex polypeptide	-0.0220	-0.0654	0.0509
MZ00024488	KH domain-containing protein / zinc finger (CCCH type) family protein	-0.3510	0.0201	0.0886
MZ00044429	Putative Myb-like DNA-binding protein	-0.5464	0.1008	0.0274
MZ00026489	Putative RNA and export factor binding protein	-0.5016	0.0717	0.1922
MZ00006269	Putative DNA dependent trascription factor	-0.1372	0.0707	0.1908
MZ00017097	Putative GLE1L protein mRNA export factor	-0.1629	0.0011	0.3048
MZ00019270	Putative chloroplast nucleoid DNA-binding protein cnd41	0.0703	-0.0563	0.0950
Replication factors				
MZ00040646	MFP1 attachment factor 1	0.2252	-0.0496	0.0254
MZ00016191	Replication protein A 70b	0.6826	0.3512	-0.1278
MZ00030265	Putative DNA polymerase I	0.1068	-0.0840	0.1500
MZ00018901	DNA-directed DNA polymerase	0.1903	0.2324	-0.0970
Translation				
MZ00003737	Acidic ribosomal protein P0-	-0.0544	-0.0633	0.0580
MZ00009418	Putative eukaryotic translation initiation factor 4G	0.3128	0.2921	-0.0256
MZ00020836	Putative translation elongation factor EF-1 alpha	-0.0394	-0.0871	0.0155
MZ00042829	60S ribosomal protein L17	0.0959	0.2678	-0.3262
MZ00005507	Putative peptidyl-tRNA hydrolase	0.1751	-0.0218	-0.0542
MZ00042970	Putative protein translation factor Sui1	0.3935	-0.0176	-0.0241
MZ00026410	Dcp1-like decapping family putative	0.1369	-0.0834	0.0077
MZ00025730	Putative mitochondrial carrier protein	-0.0195	-0.0770	0.0399
MZ00047623	Putative mitochondrial carrier protein	0.0071	-0.0957	0.0803
MZ00056118	Protein synthesis initiation factor 4G	-0.6736	0.2922	0.0659
MZ00054549	Probable peptide synthetase protein	-0.0277	0.2027	0.2821

Probe I.D.	Annotation	Relative Expression		
Architecture		**Control**	**Wounding**	**WCR Feeding**
MZ00033251	Bax inhibitor-1	0.0340	-0.0626	0.1586
MZ00009004	GTP-binding protein-like; root hair defective 3 protein-like	-0.0022	-0.0634	0.0912
MZ00016627	Rubredoxin family protein	0.0657	-0.0707	0.0279
MZ00055963	BKRF1 encodes EBNA-1 protein-like antigen	0.2342	-0.0642	-0.0495
MZ00016839	Putative UBP1 interacting protein 1a	0.3515	-0.0523	-0.2557
MZ00031265	MDR-like ABC transporter -	0.0884	-0.2731	-0.5483
MZ00036517	Gigantea homologue	0.3029	-0.0742	-0.1207
MZ00014313	Non-symbiotic hemoglobin	-0.0941	0.5484	1.3025
MZ00044079	BRICK1	0.1713	0.0436	-0.3348
MZ00046081	Putative arp3	-0.1021	-0.1548	0.2578
MZ00014924	Nuclear movement protein-like	0.3016	-0.0794	-0.0101
MZ00039578	Actin	1.0002	-0.0709	-0.4419
MZ00038953	Actin	0.6493	-0.2310	-0.1994
MZ00039579	Actin	0.9001	-0.0099	-0.3089
MZ00039578	Actin	1.0002	-0.0709	-0.4419
MZ00039111	Actin	0.7686	-0.2921	-0.2480
MZ00041466	Actin	0.7936	-0.1379	-0.1581
MZ00039583	Actin	0.9386	-0.0806	-0.1415
MZ00039831	Actin 65	0.7615	-0.0233	-0.4282
MZ00039518	Putative actin depolymerizing factor	0.8883	-0.0359	-0.6154
MZ00043090	Beta-expansin 2	0.2288	0.1606	-0.4067
MZ00037557	Putative elongation factor 1 beta	-0.4791	0.5385	-0.5795
MZ00044866	Beta-expansin 2	0.2779	0.1625	-0.3551
MZ00036502	Profilin 5	0.5534	-0.0156	-0.2786

Table 2.3. Maize microarray probes with significant up-regulation in response to WCR feeding. The values in the control, wounding and WCR feeding columns represent the normalized and transformed treatment averages for relative gene expression. Negative values indicate gene down-regulation of relative expression while positive values represent up-regulation of relative expression. Annotation is for the 1.8 version microarray

Probe I.D. #	Annotation	Control	Wounding	WCR Feeding	Function
MZ00002740	Putative serine protease	-0.0160	-0.0423	0.0557	Interfere with insect digestion
MZ00046362	Putative PrMC3	-0.0016	-0.2214	0.0506	Hypersensitive response to pathogens
MZ00003206	5-azacytidine resistance protein	0.1985	-0.0065	0.3191	Anti-silencing factor
MZ00019475	Hydroxycinnamoyl transferase	-0.2028	0.1892	0.2373	Lignin Biosynthesis
MZ00023339	Avr9/Cf-9	0.0043	-0.0087	0.0308	Anti-virulence factor
MZ00024264	Cinnamoyl CoA reductase	-0.0314	0.1297	0.3162	Lignin Biosynthesis
MZ00033251	Bax inhibitor-1	0.0340	-0.0627	0.1586	Cell Regeneration
MZ00005958	Indole-3-glycerol phosphate lyase	-0.0274	-0.0040	0.7744	DIMBOA Synthesis
MZ00036599	Chitinase	-0.1156	0.3086	1.2250	Antifungal
MZ00005428	4-hydroxycinnamic acid-CoA ligase	-0.5829	0.1075	0.1458	Lignin Biosynthesis
MZ00041513	Wound-induced protease inhibitor	-0.4880	0.0443	0.5652	Interfere with insect digestion
MZ00041190	Hydroxyproline-rich glycoprotein precursor	-0.4787	0.3774	0.5365	Defense signaling and cell wall defense
MZ00023097	Putative hydroxyproline-rich glycoprotein	-0.4305	0.0831	0.1421	Defense signaling and cell wall defense
MZ00014433	Putative xylanase inhibitor protein	-0.5237	0.0304	0.8004	Anti-fungal
MZ00007822	Putative glycoprotein	0.0275	-0.0375	0.1023	Defense signaling and cell wall defense
MZ00050488	Putative peptide deformylase	-0.0235	-0.0706	0.0637	Antibacterial
MZ00001736	UDP glucose: anthocyanin 3'-O-glucosyltransferase	-0.0220	-0.0617	0.0825	Flavonoid Synthesis
MZ00047623	Putative flavonol 3-sulfotransferase	0.0071	-0.0957	0.0803	Flavonoid synthesis

Table 2.4. WCR feeding number of damaged sites and average damaged area per site for maize mutant and wild-types. Raw data from the damage counts and damage area were subjected to a variance stabilizing transformation; outliers greater than two standard deviations from the sample mean were removed from the analysis. The transformed and normalized data were then subjected to the Student's t-test; p-values less than 0.05 were considered statistically significant.

	Damage Counts		**Damage Area**	
Mutant I.D.	**Sample Average**	**p-value**	**Sample Average**	**p-value**
bm1	0.5950	0.73	5.3064	0.10
Bm1	0.6390		5.0483	
bm2	0.5083	0.05	4.3972	**0.01**
Bm2	0.1590		5.3414	
bm3	0.6588	0.73	4.6741	0.30
Bm3	0.6148		4.8361	
bz1	1.2896	**0.002**	5.6586	0.36
Bz1	0.2760		4.5480	
bz2	0.7942	**0.02**	5.7147	0.34
Bz2	0.2760		4.5480	
vp5	0.5083	**0.04**	4.6827	0.17
Vp5	0.1945		5.0242	
vp8	1.1743	**0.02**	5.7923	0.29
Vp8	0.4643		5.5756	
orp1/orp2	1.0349	**0.02**	5.3301	0.34
Orp1/Orp2	0.4330		5.1695	

Table 2.5. Viral and DNA transfer related genes exhibiting significant up-regulation in maize when exposed to WCR when compared with mechanical wounding and a non-damaged control. Annotation is from the 1.8 version of the microarray.

Probe I.D. version 1.8	Annotation
MZ00037829	VirF-interacting protein FIP1
MZ00037826	Prpol viral RNA-dependent RNA polymerase
MZ00019108	PR34 Rat cytomegalovirus
MZ00029759	PR34 Rat cytomegalovirus
MZ00045128	Reverse transcriptase
MZ00045001	Putative transposase
MZ00016044	Pit2 protein
MZ00034535	Putative pol protein
MZ00034536	Putative gag-pol precursor
MZ00056154	NA viral protein
MZ00045543	Putative gag-pol polyprotein

Chapter 3. *Wolbachia* Mediates the Silencing of Maize Defense via Its Insect Host

Abstract

Wolbachia are obligate intracellular bacteria limited to ecdyzoan hosts. Many infected hosts induce or vector serious human diseases resulting in the loss of millions of lives annually. *Wolbachia* also infect several insect species of economic importance. A previous microarray experiment performed to identify endogenous maize genes associated with WCR-feeding in maize produced a surprising paucity of insected related defense genes instead identifying genes normally involved in microbial defense. The results suggested that *Wolbachia* infection in WCR may contribute to the expression profile; thus a second microarray experiment was performed to determine whether *Wolbachia* influence the response of maize to WCR attack. Our results show that the presence of *Wolbachia* in the insect leads to down-regulation of multiple genes in all basic plant defense classes perhaps contributing to the success WCR as a maize pest.

Introduction

There are several *Diabrotica* species which threaten maize but only WCR has achieved widespread success as a crop pest. Although WCR can survive on a few select species of grasses (Wilson and Hibbard 2004), maize is its primary host (Metcalf 1986). Larval WCR feed below ground on maize root tissue while the adults feed on above ground tissue (Metcalf 1986).

During recent years, microarrays have been used to identify genes specific for plant defense against insect attack (Moran et al. 2002, Zhu-Salzman et al. 2004). Results of these studies indicate that plants coordinate defense gene expression through various biochemical pathways and may be dependent on individual modes of attack. Our previous microarray experiment performed to identify endogenous sources of WCR resistance in maize produced a surprising paucity of defense related probes, of which, the majority are normally involved in microbial defense (Chapter 2). In addition we observed down-regulation of many of the insect defense-associated probes in WCR treated roots relative to both the control and wounding treatments (Chapter 2). Other differentially expressed probes included those coding for chromatin remodeling, gene silencing and mobile DNA related factors. Results of WCR feeding experiments on maize mutants in pathways associated with insect and/or microbial defense also support the potential role of a microbe in maize-WCR feeding interactions (Chapter 2).

The activation of mobile DNA elements and gene silencing factors is normally associated with the plant's response to microbial attack. Insects, including WCR, play hosts to countless form of microbes. *Diabrotica* beetles

have been shown to harbor three basic types of microbes; *Enterobacteria* (Schalk et al. 1987), *Spiroplasma* (Carle et al. 1997) and *Wolbachia* (Clark et al. 2001). *Enterobacteria*, as the name implies, are found in the digestive tract of the insect. They are acquired as the insect ingests materials from its environment. *Enterobacteria* are thought to aid in digestive processes (Schalk et al. 1987). *Spiroplasma* can be found in rootworm heamolymph (Carle et al. 1997). Like enterobacteria, *Spiroplasmu* spp. are acquired from the environment, but their function in rootworms is unknown. *Wolbachia* are intracellular bacteria and can be found throughout the insect body though concentrations are highest in reproductive tissues (Werren 1997). Unlike *Spiroplasma* and *Enterobacteria*, *Wolbachia* are acquired through cytoplasmic inheritance.

Wolbachia are gram negative, obligate intracellular alpha-Proteobacteria that are related to *Rickettsia* and *Ehrlichia* (Anderson and Karr 2001). They can be found in up to 75% of all insect species (Jeyaprakash and Hoy 2000). *Wolbachia* infect the filarial nematodes that cause River Blindness and Elephantiasis. They also infect the mosquitos that vector malaria, Dengue fever, and West Nile Virus. Furthermore, *Wolbachia* infect several economically important insect species such honey bees and silk worms as well as cockroaches, fire ants, and WCR which cause significant annual losses. *Wolbachia* have a mutualistic relationship with filarial nematodes and aid in nematode metabolism (Bandi et al. 1999). In arthropods, *Wolbachia* infection induces feminization, male killing, parthenogenesis and CI (reviewed Werren 1997). In many insects including WCR, *Wolbachia* induce CI between infected males and non-infected

females. *Wolbachia* is unable to replicate outside the host employing CI as a drive mechanism for the autonomous spread of infection.

Recent work has demonstrated that *Wolbachia* are able to mediate genetic response on a tertiary level (Kramer et al. 2005, Bazzocchi et al. 2007, Morchón et al. 2007). Recent reports of host plant response to other *Wolbachia*-infected insects such as Hessian fly and greenbug aphid indicate they fail to elicit typical defense responses within host plants (McKenzie et al. 2002, Zhu-Salzman et al. 2004, Giovanini et al. 2006). These findings led us to hypothesize that the presence of *Wolbachia* in WCR may be responsible for the unusual plant gene expression pattern we observed in a previous microarray comparing feeding by the WCR with mechanical wounding (Chapter 2).

To determine whether *Wolbachia* within WCR influence the response of maize to WCR attack, a maize microarray experiment was performed comparing feeding of *Wolbachia* infected and non-infected WCR on maize seminal roots at the V3 stage.

Materials and Methods

Insect Culture. All *Wolbachia*-infected WCR used in these experiments were obtained from the North Central Agricultural Research Laboratory (NCARL) in Brookings, SD. A second *Wolbachia*-free colony maintained at the University of Missouri and derived from the colony at NCARL was the source of the *Wolbachia*-minus WCR. Both colonies of WCR are comprised of the non-diapausing WCR strain. *Wolbachia* infection status of the WCR was verified via PCR with *Wolbachia* specific primers coding for a 16s ribosomal RNA fragment (Werren et al. 1995). Infection status was verified prior to each experiment. Once hatching had commenced, only vigorously moving larvae were selected. Larvae were selected with a small camel hair paintbrush and placed into a standard Petri dish. Viability of the selected larvae was verified by visualization with a dissection microscope. Any injured larvae were replaced with healthy larvae. Larvae were placed at the base of the maize plants by rinsing the Petri dish with a small amount of water.

Plant Tissue Preparation. For the microarray experiment, the CRW3 (S1)C6 line (Reg. No. GP-553, PI 644060) which had been selectively bred for WCR resistance was chosen (Hibbard et al. 2007). Maize plants were grown in a growth chamber. Conditions were set at a 14 hour photoperiod and 10 hour scotoperiod. Both incandescent and florescent lights were used and a minimum light level of 650-700 microeinsteins was maintained. Temperatures were set at 28° C for the photoperiod and 22° C for the scotoperiod with humidity at 60% and

80% respectively. In order to mimic field conditions, maize seed was planted in soil containing 1% nitrogen, 0.5% potassium and 0.5% phosphorus fertilizer. Plants were grown in 360 ml plastic drinking cups which were perforated for drainage at the bottom of the cup. Plants were grown to the V3 stage where they were subjected to their respective treatments.

For the WCR feeding treatments, 50 neonate larvae were placed at the base of the plant. Maize seminal root tissue was collected 24 hours post-infestation. The control treatment was collected once V3 stage had been attained. Seventy-five plants per treatment were pooled into a replicate. Three seminal root tips were collected per plant and pooled. Tissue from all three treatments was collected in the dark with the use of a green light. Soil was dislodged from the roots, the roots were rinsed in room temperature water and then one centimeter of tissue from a seminal root tip was excised with a scalpel and placed into liquid nitrogen.

Microarray. RNA extraction, amplification, labeling and hybridization were performed using the products and according to the protocols of the Maize Oligonucleotide Array Project (www.maizearray.org/ maize_protocols.shtml). Residual sugars were removed from the RNA using the Qiagen RNAeasy Mini elute kit (Qiagen Cataloge # 74204). The amount of RNA recovered was determined using a NanoDrop Fluorospectometer (NanoDrop Technologies, Wilmington, DE). Following hybridization, the slides were washed, dried and immediately scanned. A GenePix 4000B Axon scanner (Molecular Devices Corporation, Sunnyvale, California) was used. Slides were prescanned and a

probe intensity curve with a count ratio of 1.0 +/- 0.1 was obtained before a final image was acquired. GenePix Pro version 6.0 software (Molecular Devices Corporation, Sunnyvale, California) was employed for slide normalization and spot-calling.

Microarray Experimental Design and Data Anaylsis. Data was transformed by log2 and then normalized. Fixed effects were designated as: pin, dye, pin x dye, treatment, and treatment x dye. Random effects were designated as array and array x dye. SAS (SAS Institute, Cary, NC) was used to specify the denominator degrees of freedom and the covariance structure. SAS Proc Univariate was used to obtain the corrected sum of squares which reduced noise in the data for normalization. Once data had been normalized, they were subject to 3 separate analyses. The first analysis grouped the data by treatment type and the least squared means was used to compare the treatment types. The F statistic was obtained for pairwise differences between all three treatments. This allowed us to observe changes in gene expression and determine the significance of relative expression changes for the three collective treatments. The second analysis consisted of a second pairwise comparison using the Wilcoxon signed-rank test which is a non-parametric test similar to a Student's t-test. The third analysis which was used for obtaining the expression profile subjected each probe I.D. value to a Kruskal-Wallis test, which is a non-parametric procedure similar to an F statistic. The Wilcoxon signed-rank test and the Kruskal-Wallis test were performed in order to tighten the significance parameters of the results. These tests also allowed for more precise comparisons between two treatments. Only

probes which received a p-value of less than 0.05 for both rank tests were included in our analysis.

Quantitative RT-PCR. Fifty differentially expressed probes were selected from the subset of one hundred sixty five differentially expressed probes based on microarray analysis for verification by qRT-PCR (Table 3.1). Sequence specific primers were designed using PrimerQuest from IDT SciTools http://www.idtdna.com/Scitools/Applications/PrimerQuest/Default.aspx/ (Table 3.1). iScript One-Step RT-PCR Kit With SYBR Green (Biorad Cataloge # 170-8892) was used. Half reactions were performed with 30 ng of total RNA per sample. A standard curve was included on each plate along with a no template control. Standard curve concentrations were set at 100 ng, 50 ng, 25 ng, 12 ng, 6 ng and 3 ng of total RNA. RNA from the non-damaged control treatment with a primer selecting for a non-differentially expressed probe were used. An ABI7000 real-time PCR system (Applied Biosciences, Foster City, CA) was employed for mRNA quantification and verification of the microarray analyses. The standard curve was set with a regression value of of 0.99 or 1.0 for each plate and the values for the housekeeping probe were used to normalize the values for all othe probes on the plate. Cycles were programmed according to manufacturer's specifications in the iScript One-Step RT-PCR Kit with SYBR Green kit.

Results and Discussion

Analysis of microarray expression data shows a genome-wide down-regulation of maize defense genes; over 30% of the total probes on the microarray exhibit statistically significant differential expression. For this paper, we limited our analysis to the 2,000 probes with the smallest p-value derived from the F statistic obtained for pairwise differences for all three treatments. Aside from several defense related genes which were identified in our analysis, many of the other differentially expressed probes included those coding for metabolic factors in lignin and phytoalexin biosynthesis and signal transduction (Table 3.2). Other differentially expressed probes were grouped into categories of DNA repair and replication, gene silencing, chromatin remodeling, and mobile DNA related factors (Table 3.1). A visual representation of the expression data for significantly affected defense-related genes in maize illustrates that feeding by *Wolbachia*-infected WCR results in down-regulation of all categories of plant defense to levels below that of the non-feeding control (Figure 3.1).

Plants activate three basic classes of defenses during pathogen attack: cell wall defenses, production of phytoalexins, and the production of PR proteins. Cell wall defenses prevent infection, deter feeding, trap microbes or make tissue less palatable to herbivoires. Phytoalexins include most toxic chemical produced following insect or microbial attack. These chemicals include volatiles as well as SA and JA related products. PR-proteins are unique from phytoalexins in that they are usually encoded by a single gene and are independent of pathways though they are often accompanied by increased production of phenylalanine

ammonia lyase (PAL), and peroxidases. PR-proteins are non-detectable in healthy tissue and exhibit increased levels following microbial or insect attack.

Insect attack of maize normally results in the up-regulation of lipoxygenase (LOX), proteinase inhibitors, hydroperoxide lyase, PAL, methyl salicylate, methyl jasmonate, and a variety of volatile organic compounds (Farag et al. 2005). These products can affect the insect by inducing the production of PR proteins that hinder the biology of the insect. Many of these products also defend the plant indirectly through increase production of compounds that attract natural enemies (Cosgrove et al. 2000).

Previous research has shown that the genes we found to be down-regulated by the presence of *Wolbachia* during WCR feeding on maize roots have a direct effect on the ability of maize to repel, defend and tolerate attack. The inability of a plant to express certain genes can make it susceptible to attack from other factors which are not normally a threat. Down-regulated probes included genes for cell wall structure and defense, phytoalexins and members 16 of 17 classes of PR proteins (Table 3.2, Figure 3.1). Affected cell wall factors included lignins, actin and glycoproteins (Figure 3.1C). Affected phytoalexins include phytosteroids, flavonoids and hydroxamic acids (Figure 3.1B). Differentially expressed PR proteins included PR-1 proteins, glucanase, chitinases (all 3 classes), thaumatin-like proteins, ribonucleases, peroxidases, protease and proteinase inhibitors, defensins, thionins, lipid transfer proteins, oxalate oxidases and glycoproteins (Table 3.2, Fig 3.1A). Notably, several genes within each category were suppressed. As a rule, defense genes were down-regulated in

maize when attacked by *Wolbachia*-infected WCR and were up-regulated following attack of non-infected WCR suggesting WCR-vectored *Wolbachia* dampen the host defense response to WCR feeding in maize seminal roots.

Lignin is a structural component of cell walls; during defense, lignin has been shown to accumulate around areas of attack and create a physical barrier against infection (Kawasaki et al. 2006). Cinnamoyl-CoA reductase and CAD catalyze the first steps in lignin synthesis; genes for both products are down-regulated in the presence of *Wolbachia*-infected WCR (Table 3.2). Since neonate WCR feed by burrowing inside the root, down-regulation of lignin associated products may indicate that the maize root is remaining palatable and/or digestible to the insect. Decreased amounts of lignin may make it easier for the larval WCR to burrow inside the root tissue. This data helps to explain the lack of significant differences in feeding damage of *bm1* and *bm3* maize seedling roots compared to their wild-type siblings (Chapter 2). Down regulation of cinnamoyl CoA reductase and CAD in wild-type plants would render them phenocopies of the *bm1* and *bm3* mutants.

Genes encoding maize glycoproteins are also down-regulated when *Wolbachia* are present in feeding WCR (Table 3.2). Glycoproteins are located in the cell wall and function by impeding infection of pathogens and eliciting the production of PR proteins. Decreased production of glycoproteins can make maize vulnerable to pathogens by weakening cell wall defenses (Garcia-Muniz et al. 1998). Actin, a structural component of cell walls, protects the plant from invading pathogens. Studies indicate that the inhibition of actin results in cell

wall permeablization through which both pathogenic and non-pathogenic microbes may pass (Kobayashi et al. 1997). Several actin-specific probes on the microarray are down-regulated in the presence of *Wolbachia*-infected WCR (Table 3.2). Decreased expression of structural components could signify that either the maize tissue is being rendered more digestible for the insect or the cell walls are being weakened to facilitate *Wolbachia* infection.

Another unanticipated effect of *Wolbachia* on maize was the down-regulation of senescence related proteins in comparison with the control treatment (Table 3.2). A recent report showed that *Wolbachia* from filarial parasites inhibited apoptosis of human neutrophil cells (Bazzocchi et al. 2007). Programmed cell death is a defense mechanism for countless organisms which hinders invading microbes. The down-regulation of senescence related proteins could indicate the attenuation of this possible defense mechanism of maize by *Wolbachia*.

Several phytoalexin-related genes were down-regulated in maize following feeding by W*olbachia* infected WCR (Fig 3.1B). Prior reports have examined the role of specific phytoalexins in plant defense. The data show that PAL is down-regulated in the presence of *Wolbachia* (Table 3.2); PAL marks the first committed step in flavonoid synthesis from which several defense products are derived. Flavonoids are often precursors to products which have repellant or deterrent effects on insects. Flavonoid related products such as dihydroflavonol 4-reductase have been shown to confer increased resistance to bacterial pathogens (Hayashi et al. 2005).

Glutathione S-transferase (GST), in the plant, is involved in the detoxification of xenobiotics and the degredation of peroxidases. Several GST encoding genes on the microarray are down-regulated (Table 3.2) which may suggest a problem in the plant with detoxification of toxins from the insect or bacteria. Glucosyltransferases are involved in the production of hydroxamic acids. Maize produces the hydroxamic acid DIMBOA as well as its metabolite MBOA. Increased levels of DIMBOA have been shown to have deleterious effects on WCR larvae (Assabgui et al. 1996) while MBOA is used by WCR for locating maize root tissue (Bjostad and Hibbard 1992). Probes which code for products on the hydroxamic acid pathway are down-regulated when *Wolbachia* are present (Table 3.2). This dynamic may support initial larval host location but reduce subsequent establishment by other larvae reducing competition for resources and increase the chances for larvae to reach adulthood. Decreased flavonoid production may be beneficial to *Wolbachia* by allowing them to persevere either in the insect or maize tissue. Decreased expression of flavonoids may render the maize plant more attractive to WCR.

Wolbachia also induce the down-regulation of a shikimate kinase gene involved in the synthesis of aromatic compounds (Figure 3.1B, Table 3.2) which can both deter insects from feeding and attract natural enemies (Pare and Tumlinson 1999). *Wolbachia*-infected WCR feeding resulted in the down-regulation of lipoxygenase (LOX) encoding probes (Table 3.2). Down-regulation or silencing of LOX results in decreased production of oxylipins and protease inhibitors as well as increased insect attack and colonization by insects which are

not normally associated with the plant (Kessler et al. 2004). The results of the microarray display a surprising lack of expression of genes encoding LOX metabolites such as jasmonic acid and hexanal which have been shown to facilitate volatile production in maize. Other probes coding for the production of volatiles such as indole, ethylene, beta-caryophyllene and sequiterpenes were not differentally expressed from the non-feeding control in maize attacked by *Wolbachia*-infected WCR. This suggests that the reduced expression of LOX results in the reduced expression of LOX-related metabolites. Furthermore, this may indicate that root tissue does not produce the same products as green leaf tissue when attacked by an insect.

Sixteen of seventeen classes of PR proteins are down-regulated in maize in the presence of *Wolbachia*-infected WCR (Table 3.2). PR proteins function as a defense mechanism. Plants with decreased expression of thaumatin-like proteins have been shown to be more susceptible to fungal attack (Polidoros et al. 2001); while increased levels of some thaumatin-like proteins confer resistance against several classes of fungi (Malehorn et al. 1994). Cysteine proteinases in maize are responsible for gut proteolysis in WCR (Brown et al. 2004). Decreased expression of aspartic protease renders the plant susceptible to water molds (Guevara et al. 2005). In the case of smut (*Ustilago maydis*), decreased expression of PR-1 proteins can render maize more susceptible (Basse 2005). Decreased expression of lipid transfer proteins results in increased microbial infestation of maize (Roy-Barman et al. 2006). Chitinases have been shown to inhibit *A. flavus* and *Fusarium* colonization in maize (Moore et al. 2004).

Defensins have also been shown to have antimicrobial properties in maize (Balandín et al. 2005). It is noteworthy that the majority of PR proteins effects have been demonstrated for microbial and not insect attack.

Many studies document the effect of *Wolbachia* surface proteins on the immune response of mammalian hosts (Kramer et al. 2005, Bazzocchi et al. 2007, Morchón et al. 2007, Porksakorn et al. 2007). Several studies have shown that surface proteins of *Ehrlichia* and Rickettsial bacteria silence mammalian immune response in order to establish infection and facilitate symbiosis (Zhang et al. 2004, Lin and Rikihisa 2004, Borjesson et al. 2006, Niu et al. 2007). Similar to the data in this study (Table 3.2), silenced mammalian systems included membrane trafficking, apoptosis and cytokines. Mammalian membrane trafficking and apoptosis can be compared with plant cell wall defense. The glycoproteins and actin down-regulated in maize roots by *Wolbachia* positive WCR feeding function in plant cell membrane trafficking and can be involved in programmed cell death. Cytokines are involved in initial immune and inflammatory response in mammalian systems. The repression of cytokines in mammals can be compared with the silencing of phytoalexins in plants, most notably, reactive oxygen species. Cytokines also induce the expression of other immunological factors as do many phytoalexins which are precursors to plant defense products.

An interesting feature of the data set is the altered expression of virus associated probes (Tables 3.2 and 3.3). Many commercially produced microarrays have non-specific probes printed throughout the slide to serve as controls for dye hybridization. Under normal circumstances, these probes do not

exhibit differential expression. The data set for the first microarray experiment showed that several viral and foreign DNA introgression factors were up-regulated in response to WCR feeding (Table 2.5). The data from the second microarray show that many of the same viral and foreign DNA introgression probes are up-regulated in maize when attacked by *Wolbachia*-infected WCR. Maize which had been exposed to *Wolbachia*-infected WCR showed increased expression of viral receptor and introgression factors as well as herpesvirus homologues (Table 3.3). Blasts of these sequences showed no homology to maize, WCR or *Wolbachia;* however, *Wolbachia* are commonly infected with bacteriophages.

Bacteriophages are widespread viruses infecting solely bacteria. Although phages are commonly lethal to the infected bacteria, beneficial associations have been observed when the phage codes for necessary products of the host (Miao and Miller 1999) or aids in the transfer of genetic material (Miller 2001). The *Wolbachia* phage, WO, is one of the few phages to infect an intracellular host (Storey et al. 1989) as it is more common for selection pressure to eliminate parasitic DNA from endosymbionts (Andersson and Andersson 1999). Perhaps the WO phage in *Wolbachia* performs a service for its host that has yet to be identified.

The structure of WO is an icosahedral capsid with a tail (Sanogo et al. 2005). Sequencing data supports the presence of tail structural genes, including the putative short contractile tail sheath protein gene (Masui et al. 2000, Fujii et al. 2004), suggesting that phage WO is a tailed bacteriophage capable of infecting

and injecting DNA during transduction. WO can spread between *Wolbachia* strains, and even to other species of bacteria though lateral transfer of WO it is thought to be relatively uncommon (Gavotte et al. 2007). The structure and biology of WO is quite similar to herpesviruses which are able to establish lifelong infections and can vector DNA into a host. Like WO, herpesviruses are large dsDNA viruses that can persist in a latent state within the host without ever causing disease. Currently, herpesviruses are used to vector and achieve long term, stable transgene expression for the treatment of several diseases (Burton et al. 2002). Like WO, herpesviruses can vector and transduce DNA from other sources. WO is involved in *Wolbachia* DNA transduction (Gavotte et al. 2007). Perhaps the up-regulation of foreign DNA introgression probes is an indication that WO may be involved in lateral transfer of genetic material into maize.

Lateral transfer of *Wolbachia* DNA has occurred in many insects (Hotopp et al. 2007). An interesting feature of *Wolbachia* is its genome contains 20% mobile DNA genes which is ten times more than any other obligate intercellular bacteria (Bordenstein and Reznikoff 2005). *Wolbachia* and the maize mitochondrial genome share a common ancestor in *Reclimonas americanus*, as well as significant sequence homology (Clifton et al. 2004). This homology may facilitate movement of *Wolbachia* into maize and perhaps the transfer of *Wolbachia* DNA into maize.

Our data illustrates that *Wolbachia* subvert plant defenses (Figure 3.1) indicating that WCR and *Wolbachia* may share a mutual association, where *Wolbachia* render maize susceptible to WCR, thus augmenting the

competitiveness of WCR and ultimately aiding in the perpetuation of *Wolbachia* infection within the insect population. It is tempting to speculate that the down-regulation of anti-microbial defense genes, as well as, the plant compounds and processes deleterious to the insect host of *Wolbachia* we observed may be another form of genetic drive, facilitating survival of the pathogen and therefore, survival of its host. *Wolbachia* are maternally inherited and cannot replicate outside of a host. They have been observed to cause genetic drive in insects, perpetuating themselves by cytoplasmic incompatibility and male killing among other mechanisms. The data suggest that *Wolbachia* and WCR may share a mutual association which is contrary to conventional view of a parasitic association in arthropods.

Our data demonstrate the unprecedented effect an intracellular organism can have at a tertiary level. Other reports have been made of insects eliciting plant responses similar to pathogens; however, these studies did not test the possibility of *Wolbachia* or other microbes as the causal agent (McKenzie et al. 2002, Zhu-Salzman et al. 2004, Giovanini et al. 2006) even though the insects in these studies harbor *Wolbachia* infections.

Our results support a new paradigm concerning plant-insect interactions involving insects that harbor *Wolbachia* where the insect-vectored bacteria cause large, significant changes in plant gene expression apart from changes associated with feeding by the insect vector itself. The *Wolbachia*-mediated changes we observed in gene expression suggest a revision in strategies for selection of insect resistant plants. The expression profile also warrants a re-evaluation of insect

resistant management programs which are mandated for transgenic crops. The data also suggest that the bacteriophage WO may be involved in the maize-WCR-*Wolbachia* complex. Whether WO is functioning to mediate the maize defense response or to facilitate lateral transfer of genetic material is unclear and warrants further investigation.

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Table 3.1. Primers used for quantitative RT-PCR expression verification of microarray results.

Probe I.D.	Forward Primer	Reverse Primer
MZ00020387	TGCCAACCTCCATCACTGGATCAT	TTAGCTGCCGAGGATGAACGAGAA
MZ00004041	CACTGCCCATCTGCTGAACAGAGAAA	TGTTCGAAGTTGGTTACAGGGCGT
MZ00004486	TCATCTGCAACTATTACCCGCCGA	ACACGCGCGTATGATTGATGCT
MZ00012518	CTTGCTCAACAACCTCACCACACA	AAGTGCTCGGCCGTCAACTACTAA
MZ00013897	ACCCTCGCGAAACCAAGAGTTTAC	GCTGCAAACGGAAAGTACAAGCAC
MZ00018372	TCAACCAGCAGCTTGTCAACTACC	ACTTGAAGCCTTGGCTCCTGGAAT
MZ00018389	AGGGAAGCTCCAATCCGAGTTCAA	TTCGGATACATCAGTCAGCTCGCA
MZ00020156	TTATCCACCGGCACCATACATCCA	AAGATTGGAGGCTAGGCATCACCA
MZ00020474	CAGTCACTCATCTAATGCAGCCGA	GCCAATGAAACCGCCACTGAAGTT
MZ00024006	GATGTGGTCACCTGGGTTTGCTTT	GGCACTCTCCGAAGCTTGAATGTA
MZ00025038	GAGTTATATGGATGTGATCCCACACG	CGAGAACGAATGTAGTATTTCGTGAGAC
MZ00027915	TCGTCAGGATGGTGGAGGCATA	TTGAAGCAACCAAACAGAGGAGCC
MZ00028247	GTCAAGTTGCCCGTCTTCTGATTC	CAAATCCGCTTTCGAAATCACATACGC
MZ00029688	TTCCTGATGGACCCTTGGTGGAAT	AACACCAGCTGCTTCTTCTGTTGC
MZ00030882	AATCTTGCAGCTCTCCCTCGAAGA	AATCACCGAGCTGTTAAGGACCGA
MZ00033520	TGGCAGGAGGTGAAGTAGCTAAGA	GCTGCAGATGATGTTGAACGGCTT
MZ00034469	CGCCAGTTGTGCACCTTCATTCAA	TTAAGTCTCCTTCCGCGCCTCTTT
MZ00036196	GTAGTTGCCATGTGTCAGCCAGTT	CATCTGTGTAGCACAAAGAGGCAG
MZ00036538	CAAGAGGAGCAGCTTTGGCTTCAA	AGTACACGTAGCAGCGTGCCTATT
MZ00036720	CGTCACCAAGTTCACCTCGTCCTAA	GTATGCCAGAATTATATATTGCACTGCTC
MZ00042168	GCAACAGCAACTACCAAGTCACCT	CGTGCACGTGTAATGCACGCTTAT
MZ00043996	CGTAGCTGGGTGAGCTACTAATGT	ACAACACATTGCCGGAGACACAAG
MZ00044023	TGTTTGCTAGCCCTGAGCTGAAGA	GTTTGCTGGCACCATTTCCCACTT

Probe I.D.	Forward Primer	Reverse Primer
MZ00002346	AATACCCGTCCCGCATTCACATCT	AAAGGTTGCTTTGTGGCACCTTCG
MZ00013100	CAAAGTTGTGAATGGTCTCGAATGC	AACTGCGAACTGTCAGACACACTC
MZ00013519	CTAAACATCACACACTGGGCACAACG	AAATCAGGCGCAACTGCAGGGTCAT
MZ00013547	CTGGAGACATGCAGGTGCAACATT	AGCCCTGGAATCAGAACCAGTGAA
MZ00018230	TAACACATCCAGGCAAGCACAGC	AAGAGAAAGGGAGCGCCAACGAAT
MZ00024529	CCCTACTAGACTGTCATGCATACTACA	TGGCTACAAATCGCCTGCATGGTT
MZ00025631	TGCATAGGAGTAACTGCCAGGGTT	CAGGCACATGCAACCAAACCAAAC
MZ00039022	GCATTTGCGGCTGTTGCTGTCTA	ACCAATACGGAACAGTGACACCAC
MZ00041692	TTGGACTGCACGACTACCATACGA	TTCCGGAACGTGAGACGCACATC
MZ00045168	ATGGGTTGAAGCAAGCATCTCGGA	TCAGAAATACAACAGCGCTCCCAC
MZ00000005	CATTGGCCTGCCAGCTTCAAGATT	GTGGGACTAATGTCCCTACAACA
MZ00014415	TCAAAGGGACGAGGTGGAGTACAA	CTCTGGACAGACAAAGGTGCAGTT
MZ00016076	AGACCACAGCTTCAAGTGGGACAA	AAGTACCGAACCCTGCTGAATCCT
MZ00024090	TCCGGATCATTCTGCCATGGAAAC	ATCAGATGGTATCGTCGCCAAGGA
MZ00039775	GAACAAGGACAAGGACGCCTACAT	ATAAACCGGGTGGATGTGGATGGA
MZ00042841	TCGAAGCCGTTCTTAGTTGGTGGA	AGCATTACCAGCGAGCTCCAGAA
MZ00044339	GACGCTGAATAAAGTCTTGTGTGT	CTTGTCTCCTCTATTTGTGCAACAGCC
MZ00029711	TGGAGAGCTTTCACGCTACCATGT	AGTAAGCGATGACCCAAGCTCCAA
MZ00029746	GATCTCATTTGGTGCATCGGAGAC	TTGTGCCTAGTGTGGCAGGACATT
MZ00033520	TGGCAGGAGGTGAAGTAGCTAAGA	GCTGCAGATGATGTTGAACGGCTT
MZ00035455	CGTGCTTGAGTGCATGTTTGTCGT	TCAACAAGATGGCATCCTACCCGA
MZ00036538	AAGAGGGTGATCCTGTGCGACAA	TGCACGCATGACATGTCCGAGTTA
MZ00041242	AAACATGTGCGGTGTTGCTACGTG	CACAACGGAGTTCCCGTTCTGTAT
MZ00043035	GACGCATGTCAGCTATTGTCTCGT	AGGGTTGTACCATAGGATCCGACA

Table 3.2. A cataloge of genes in maize exhibiting significant differential expression between the non-feeding control, feeding by *Wolbachia* infected WCR and feeding by non-*Wolbachia* infected WCR. Annotation is for the 1.12 version of the microarray. The F statistic was obtained for pairwise differences between all three treatments. Probes possessing a p-value of less than 0.05 are included below. Values in the control, + *Wolbachia* and - *Wolbachia* columns represent the normalized and transformed treatment averages for relative gene expression. Negative values indicate gene down-regulation of relative expression while positive values represent up-regulation of relative expression.

Probe I.D.	Control	+*Wolbachia*	-*Wolbachia*	Annotation
PR-1				
MZ00004486	0.28629	-0.70834	0.42204	pathogenesis related protein-1
MZ00042168	0.27636	-2.00827	1.73191	pathogenesis related protein-1
MZ00040855	-0.19455	-1.22957	1.42412	putative pathogenesis related protein-1
PR-3 and 4 chitinases				
MZ00013897	0.60544	-1.26679	0.66135	chitinase
MZ00025038	-0.13849	-0.49747	0.63596	pathogenesis-related protein 4 - barley
MZ00041276	0.13243	-1.00619	0.87376	chitinase
MZ00043035	0.29500	-1.26578	0.97078	chitinase
MZ00013319	0.50169	-0.82190	0.82692	chitinase
MZ00021210	0.20462	-0.01359	0.96283	chitinase
MZ00042332	-0.29349	-0.39356	0.68705	chitinase
MZ00033428	0.34643	-1.42077	1.07434	putative chitinase
PR-5 thaumatin-like proteins				
MZ00000977	-0.14194	-0.37386	0.51580	putative antifungal thaumatin-like protein
MZ00013547	-0.03908	-1.00381	1.04289	thaumatin-like protein
MZ00040435	-0.39901	1.14637	-0.74736	putative thaumatin-like protein
MZ00013285	-0.05678	-0.39325	0.45003	thaumatin-like protein
MZ00040435	-0.39901	1.14637	-0.74736	putative thaumatin-like protein
PR-6 proteinase inhibitors/ proteinases				
MZ00044339	0.26643	-0.53451	0.26808	cysteine proteinase inhibitor Scb-like protein
MZ00035455	-0.13164	-0.87217	1.00381	cysteine proteinase CP1
MZ00038348	0.43674	-0.92740	0.49066	cathepsin B-like cysteine proteinase
MZ00002045	-0.09321	-0.34906	0.44227	putative aspartic

Probe I.D.	Control	+*Wolbachia*	-*Wolbachia*	proteinase nepenthesin I **Annotation**
PR-6 proteinase inhibitors/ proteinases				
MZ00025326	-0.07839	-0.46348	0.54187	aspartic proteinase
MZ00036333	0.55414	-1.96349	1.40936	putative Sip1 protein encoded trypsin-like serine proteinases
PR-8 class III chitinases				
MZ00018372	-0.09187	-0.50369	0.59556	class III chitinase RCB4
MZ00013315	0.07022	-0.73177	0.66155	putative class III chitinase
MZ00037339	0.03418	-0.64691	0.61273	chitinase
PR-9 peroxidases				
MZ00012518	-0.29561	-0.78291	1.07852	putative peroxidase prx15 precursor
MZ00021144	-0.10106	-0.48489	0.58595	putative peroxidase 1 precursor
MZ00036720	-0.23328	-1.90109	2.13437	peroxidase
MZ00027710	1.86540	-0.26923	4.29454	putative peroxidase
MZ00027194	2.55130	0.07245	3.96909	putative peroxidase
MZ00004650	0.04505	-1.01169	0.65323	putative peroxidase
MZ00026737	0.51847	-1.08072	1.92925	peroxidase
MZ00027284	1.64292	-0.06051	2.91244	probable peroxidase
MZ00022313	-0.52294	-0.80129	0.52717	putative peroxidase
MZ00022582	0.86242	0.56453	1.92982	peroxidase 1 precursor-like protein
MZ00022862	-0.20443	0.01207	2.05420	class III peroxidase
MZ00016005	0.31038	-0.88950	1.58071	peroxidase
MZ00028399	0.46141	0.50925	3.08265	putative peroxidase
MZ00000932	0.53854	-0.63169	1.77146	putative peroxidase 1 precursor
MZ00056406	0.00373	-2.03948	2.03575	putative peroxidase
MZ00044248	-0.51997	-1.02688	1.54686	putative peroxidase
MZ00042699	0.03449	-1.92519	1.89071	putative peroxidase
MZ00033093	-0.01601	-1.55862	1.57463	putative peroxidase P7X
MZ00004650	0.11849	-0.87619	0.75770	putative peroxidase
PR-10 ribonucleases				
MZ00027915	-0.05187	-1.12610	1.17797	pathogenesis-related protein 10
MZ00028247	0.49832	-2.62245	2.12414	putative aleurone ribonuclease
MZ00019596	0.17618	0.19077	-0.36694	endoribonuclease L-PSP family protein-like
PR-12 defensins				
MZ00043949	0.08557	-1.05327	0.96771	defensin 1 precursor
PR-13 thionins				
MZ00016209	0.00094	0.64830	0.00000	Thionin

Probe I.D.	Control	+*Wolbachia*	-*Wolbachia*	Annotation
PR-14 lipid transfer proteins				
MZ00003835	0.33676	-1.67161	1.33486	putative lipid transfer protein
MZ00041611	-0.30180	-1.10390	1.40570	nonspecific lipid-transfer protein precursor
MZ00028450	4.97296	3.61081	6.88517	putative lipid transfer protein
Glycoproteins				
MZ00016315	0.07735	0.25262	-0.32998	hydroxyproline-rich glycoprotein DZ-HRGP
MZ00057018	-0.04165	0.44977	-0.40812	hydroxyproline-rich glycoprotein-like
Protease inhibitors				
MZ00044388	0.02651	-1.56254	1.53603	putative protease inhibitor
MZ00039775	0.10281	-0.48354	0.38073	xylanase inhibitor protein I
MZ00036538	-0.41536	-2.10232	2.51768	subtilisin/chymotrypsin inhibitor
MZ00041242	-0.02865	-0.86149	0.89014	cysteine protease
MZ00014433	-0.05364	-0.44424	0.49788	putative xylanase inhibitor
MZ00025431	-0.03244	-0.73077	0.76321	Bowman-Birk type trypsin inhibitor
MZ00011113	0.01716	0.15841	0.69393	OTU-like cysteine protease-like
MZ00057049	0.73904	-2.06462	1.32559	putative protease inhibitor
MZ00043179	-0.11591	-1.84501	1.96092	subtilisin/chymotrypsin inhibitor
MZ00037253	-0.05185	-1.77025	1.82210	subtilisin/chymotrypsin inhibitor
MZ00036636	-0.22862	-0.34046	0.56908	probable trypsin inhibitor
MZ00010105	-0.14471	-0.23952	0.38424	putative protease inhibitor
MZ00005891	-0.21905	-0.75460	0.97366	subtilisin inhibitor I
Lignin pathway				
MZ00020156	-0.59686	-1.15200	1.74885	putative cinnamyl alcohol dehydrogenase
MZ00005428	-0.81427	0.01629	0.79798	4-hydroxycinnamic acid-CoA ligase
MZ00044023	-0.03263	-0.69551	0.72814	cinnamoyl-CoA reductase
MZ00000781	1.46096	-0.07101	3.57397	putative caffeoyl-CoA O-methyltransferase 1
MZ00013937	2.05848	0.62999	3.29555	glutathione transferase
MZ00004041	-0.04038	-0.67719	0.71757	phenylalanine ammonia-lyase

Probe I.D.	Control	+*Wolbachia*	-*Wolbachia*	Annotation
Lignin pathway				
MZ00014292	1.32518	1.17489	3.59778	phenylalanine ammonia-lyase {Zea mays;}
MZ00012815	0.74538	-0.08293	1.92010	putative cinnamoyl-CoA reductase
MZ00004477	0.88302	-0.96477	1.92636	putative N-hydroxycinnamoyl/benzoyltransferase
MZ00025478	0.92187	0.18151	2.48570	cinnamyl-alcohol dehydrogenase (EC 1.1.1.195) - maize
MZ00021835	1.74355	-0.30253	2.27295	N-hydroxycinnamoyl/benzoyltransferase-like protein
MZ00017952	0.32885	0.07790	0.75354	putative Caffeoyl-CoA O-methyltransferase
MZ00014812	1.51136	0.12105	2.95847	putative cinnamyl alcohol dehydrogenase
MZ00025513	-0.55650	-0.46633	1.02284	cinnamic acid 4-hydroxylase
Flavonoids				
MZ00028764	0.07526	-0.46305	0.38778	dihydroflavonol4-reductase
MZ00013937	2.05848	0.62999	3.29555	glutathione transferase
MZ00012636	-0.24572	-0.39785	0.95595	glutathione S-transferase GST 29
MZ00026160	0.79660	-0.60977	1.92247	glutathione S-transferase GST 30
MZ00030444	1.49225	0.76489	1.95387	putative glutathione S-transferase
MZ00026581	-0.31322	-0.30955	0.62276	putative flavonol synthase
MZ00041712	-0.38460	-0.43680	0.82140	glutathione S-transferase GST 8
DIMBOA pathway				
MZ00015236	3.08190	2.10015	5.06733	UDP-glucosyltransferase BX9
MZ00001483	-0.15451	-0.71529	0.23164	putative glucosyltransferase
MZ00012679	-0.27730	-0.95516	1.04017	Probable hydroquinone glucosyltransferase
MZ00017936	1.22209	-0.86231	1.66262	putative glucosyltransferase
MZ00012710	0.90450	0.32156	1.82677	putative glucosyltransferase
MZ00056539	0.12180	-1.05076	0.92897	putative

Probe I.D.	Control	+*Wolbachia*	-*Wolbachia*	glucosyltransferase **Annotation**
LOX				
MZ00000005	-0.39050	-0.67223	1.06273	lipoxygenase
Programmed cell death				
MZ00036242	3.00384	1.54876	-4.55260	putative senescence-associated protein
MZ00036264	2.63298	1.33570	-3.96869	putative senescence-associated protein
MZ00036305	2.24268	1.96810	-4.21078	putative senescence-associated protein
MZ00036698	2.61540	2.27706	-4.89246	putative senescence-associated protein
MZ00037482	2.33638	0.42914	-2.76551	putative senescence-associated protein
MZ00037746	2.42116	2.38823	-4.80940	putative senescence-associated protein
Array I.D.	control	WT	no wol.	annotation
MZ00045590	2.65780	1.61750	-4.27530	putative senescence-associated protein
Miscellaneous defense genes				
MZ00043996	-0.16248	-0.94616	1.10864	bax inhibitor-1
MZ00016498	-0.11902	-1.75728	1.87630	disease resistance response protein-like
MZ00020474	-0.00204	0.34557	-0.34353	putative Arp2/3 complex 41kD subunit
MZ00015593	0.18531	-0.77224	0.58693	ZmNAS1
MZ00032776	0.02366	-0.30412	0.28045	putative disease resistance response protein
MZ00050488	0.00119	0.11420	-0.11538	putative peptide deformylase
MZ00014229	-0.96184	-1.32023	2.28207	pathogen-related protein
MZ00001975	-0.34109	-0.57538	0.91647	putative disease resistance protein
MZ00033060	-0.02876	-0.78830	0.81706	pir7b bacterial defense related esterase
MZ00022294	0.19851	-1.21384	1.01533	putative esterase
MZ00016683	-0.17156	-1.03108	1.20264	pathogenesis-related protein-like protein
Silencing and chromatin remodeling				
MZ00018389	-0.07870	0.27010	-0.19140	putative Helicase SKI2W
MZ00024006	0.19211	0.46966	-0.66178	putative RNA helicase
MZ00033520	-0.04707	0.47604	-0.42897	centromere protein-like
MZ00013519	-0.02593	-0.86143	0.88736	histone H2B.3
MZ00018230	0.11285	0.32883	-0.44168	putative cleavage and polyadenylation specific factor
MZ00024529	0.33793	0.44535	-0.78328	probable DNA (cytosine-

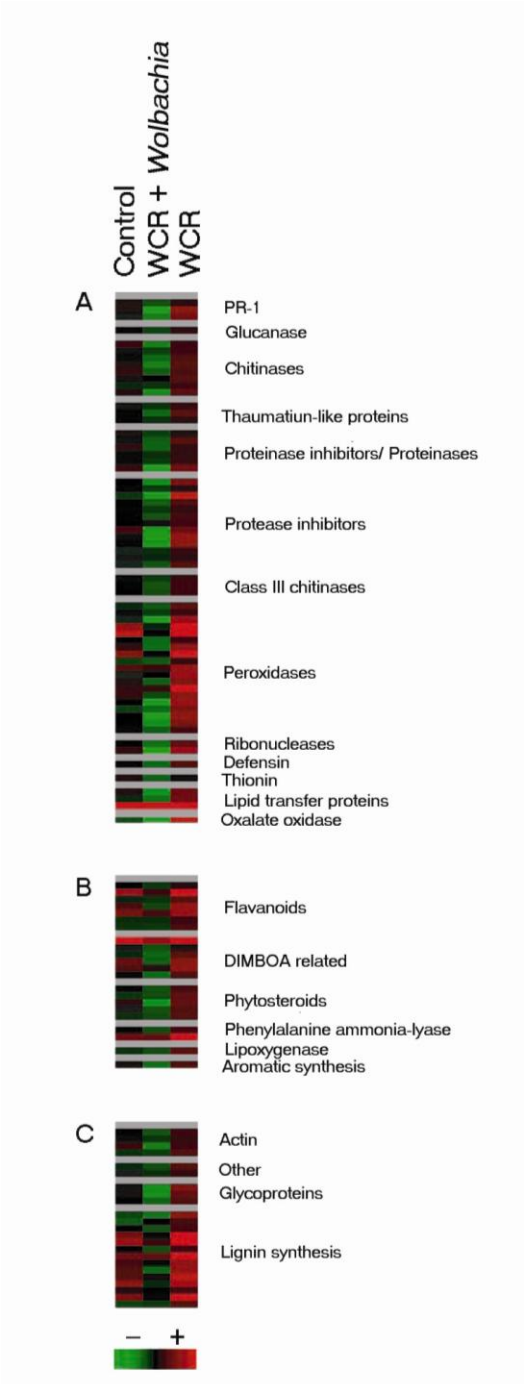
Probe I.D.	Control	+*Wolbachia*	-*Wolbachia*	5-)-methyltransferase **Annotation**
Silencing and chromatin remodeling				
MZ00025631	-0.15463	0.55266	-0.39803	putative ethylene-responsive RNA helicase
MZ00039022	-0.05608	0.26446	-0.20838	putative DEAD box-like RNA helicase
MZ00041692	-0.05051	0.52240	-0.47189	putative chromosome region maintenance protein
MZ00013642	-0.13699	0.66332	-0.52633	putative histone H2A protein
MZ00014415	-0.10369	0.45744	-0.35375	putative minichromosome maintenance deficient protein 5
MZ00014892	-0.05734	0.45207	-0.39473	putative pre-rRNA processing protein RRP5
MZ00016076	-0.24234	0.51238	-0.27003	putative Hec1 protein
MZ00042841	3.11877	0.64525	-3.76402	histone H2A.
MZ00029746	0.20065	0.44770	-0.64834	putative RNA helicase
MZ00033520	-0.04707	0.47604	-0.42897	centromere protein-like
MZ00001590	-0.27426	-0.09744	0.37169	AGO1
MZ00037830	0.05365	-0.19259	0.13894	putative anti-silencing factor 1-like protein
MZ00025730	0.00159	0.06215	-0.06374	HMGI/Y protein
MZ00016670	-0.00047	0.11744	-0.11697	parathymosin-like
MZ00022237	0.03514	0.07599	-0.11113	RNA helicase SDE3
MZ00036196	0.26023	0.61076	-0.87100	transcriptional co-repressor -like
MZ00025345	-0.10230	0.54396	-0.44166	transcriptional co-repressor -like
MZ00029711	-0.05665	0.37706	-0.32042	putative isoleucyl-tRNA synthetase
MZ00043264	1.40018	0.56696	-1.96714	putative RNA helicase
MZ00051062	0.25475	0.23600	-0.49074	putative DNA2-NAM7 helicase family protein
MZ00025021	0.15481	0.14925	-0.30406	putative RNA helicase, DRH1
MZ00017656	0.08780	0.00068	0.00000	
DNA repair and replication				
MZ00029688	0.02323	0.28285	-0.33180	putative DNA repair protein rhp16
MZ00030882	0.00298	0.59475	-0.59773	origin recognition complex subunit 3
MZ00034469	0.93638	1.58544	-2.52182	DNA-directed RNA polymerase
MZ00024090	0.14648	0.38845	-0.53493	putative DNA topoisomerase II

MZ00029517	0.04728	0.25175	-0.29902	HhH-GPD base excision DNA repair protein-related-like
Probe I.D.	**Control**	***+Wolbachia***	***-Wolbachia***	**Annotation**
DNA repair and replication				
MZ00029688	0.02323	0.28285	-0.33180	putative DNA repair protein rhp16
MZ00017604	0.42485	-0.28844	1.86343	MutT/nudix-like
MZ00027375	-0.48779	-0.32286	0.17464	5' repair endonuclease
MZ00036510	-0.07093	-1.34111	1.41205	exonuclease-like
MZ00046736	-0.07973	-0.13800	0.21773	putative DNA-damage-inducible protein P
MZ00041683	0.27323	0.19735	-0.47058	putative DNA damage binding protein 1
Mobile DNA related				
MZ00030586	0.04098	0.21214	-0.25312	putative copia-type pol polyprotein
MZ00039365	0.06624	0.17540	-0.24164	putative retrotransposon RIRE1 poly protein
MZ00002346	-0.26730	0.37999	-0.11268	putative DNA repair and recombination protein
MZ00013100	-0.11163	0.39963	-0.28801	putative MURBZC
MZ00035108	-0.01737	0.21153	-0.19417	putative gag/pol polyprotein
MZ00045168	0.00030	0.14325	-0.14356	reverse transcriptase
MZ00054755	0.01701	0.22216	-0.23918	putative PSTVd (potato spindle tuber viroid) RNA-biding protein
MZ00044385	-0.19427	-0.37640	0.57067	putative Tam3-like transposon protein
MZ00045479	-0.34905	-0.53677	0.88582	pol protein homolog

Table 3.3. Viral and DNA transfer related genes exhibiting significant up-regulation in maize when exposed to *Wolbachia* infected WCR. Annotation is for the 1.12 version of the microarray. The F statistic was obtained for pairwise differences between all three treatments. Probes possessing a p-value of less than 0.05 are included below. Values in the control, + *Wolbachia* and - *Wolbachia* columns represent the normalized and transformed treatment averages for relative gene expression. Negative values indicate gene down-regulation of relative expression while positive values represent up-regulation of relative expression.

	Relative Expression Value			
Probe I.D.	**Control**	**WCR + *Wolbachia***	**WCR - *Wolbachia***	**Annotation**
MZ00030586	0.04098	0.21214	-0.25312	Putative copia-type pol polyprotein
MZ00002346	-0.26730	0.37999	-0.11268	Putative DNA repair and recombination protein
MZ00035108	-0.01737	0.21153	-0.19417	Putative gag/pol polyprotein
MZ00045168	0.00030	0.14325	-0.14356	Reverse transcriptase
MZ00054755	0.01701	0.22216	-0.23918	Putative potato spindle tuber viroid
MZ00030586	0.040983	0.21214	-0.25312	Putative copia-type pol polyprotein
MZ00045001	-0.02441	0.129708	-0.1053	Putative transposase
MZ00045128	0.002603	0.131725	-0.13433	Reverse transcriptase
MZ00016044	-0.08003	0.21588	-0.13585	Pit2 protein - phosphate transporter and virus receptor activities
MZ00029759	0.007231	0.032301	-0.03953	PR34 Rat cytomegalovirus
MZ00037829	0.394966	0.401055	-0.79602	VirF-interacting protein FIP1
MZ00045128	0.002603	0.131725	-0.13433	Reverse transcriptase

Figure 3.1. Colormetric output of mean expression value of maize response to WCR feeding from TIGR Multiple Array Viewer. Sequence of probes corresponds to Table 3.1. (A) PR proteins, (B) Phytoalexins, (C) Cell wall associated factors.



Chapter 4. *Diabrotica virgifera virgifera* and *Wolbachia* Share a Commenal Association

Abstract

There are several rootworm species which threaten maize but WCR inhabits the largest range, has the biggest populations and has overcome both chemical and cultural control measures. WCR are infected with *Wolbachia* an obligate intracellular bacteria which causes reproductive isolation of WCR from NCR and MCR. *Wolbachia* have a parasitic relationship with many insects and a mutualistic relationship with filarial nematodes. Our microarray results indicate that WCR-vectored *Wolbachia* induce a global down regulation of maize defense gene expression which would aid in WCR infestation of maize. Global *Wolbachia*-induced reduction in defense gene expression along with the inability of *Wolbachia* to reproduce outside of its host suggest that *Wolbachia* and WCR may share a mutual association. To ask if *Wolbachia* are beneficial for WCR in other ways, assays on WCR host location and fertility were performed comparing larvae with and without *Wolbachia*. The data indicate that WCR and *Wolbachia* share at least a commensal association.

Introduction

WCR cause significant economic loss in maize in the US (Metcalf 1986). Although WCR can survive on a few other species of grasses (Wilson and Hibbard 2004, Oyediran et al. 2004), maize is its preferred and primary host (Oyediran et al. 2004). Larval WCR feed below ground on maize root tissue while the adults feed on above ground tissue (Metcalf 1986). WCR inhabits the largest range of all the economically significant rootworm species and due to its close association with maize its populations are more abundant and often supplant sympatric rootworm species (Metcalf 1986). It is unique among *Diabrotica* species in that it has repeatedly surmounted chemical and cultural control measures in far fewer generations (Ball and Weekman 1962, Feslot et al. 1982, Siegfried and Mullin 1989).

Wolbachia are gram negative, obligate intracellular alpha-Proteobacteria (Anderson and Karr 2001). They can be found in up to 75% of all insect species (Jeyaprakash and Hoy 2000). *Wolbachia* have been identified within populations of NCR (*Diabrotica barberi*) however, infection is sporadic and *Wolbachia* strains remain varied between populations (Roehrdanz et al 2007). *Wolbachia* infection of WCR causes a reproductive barrier between Mexican maize rootworm (MCR) and WCR. Once *Wolbachia* is removed from infected rootworms, viable hybrids can be obtained (Giordano et al. 1997).

Wolbachia is somewhat enigmatic in that it has been described as commensal, mutualistic, and parasitic depending on the organism it infects and the trait being studied. Bipartite associations can be classified as commensal,

mutualistic, amensal, or parasitic. A commensal association occurs when one organism benefits and the other derives neither benefit nor harm. In mutualistic associations both organisms benefit while in a parasitic association, one organism benefits to the detriment of the other. In amensal associations, one organism is harmed and the other is unaffected.

Wolbachia are classified as a parasite in many insects because an increase in fitness is observed following removal (Fry et al. 2004, Negri et al 2006, Fytrou et al. 2006). Negative effects on insect fitness due to *Wolbachia* infection have been observed on size (Hoffman and Turelli 1988), survival (Fleury et al. 2000, Fry et al. 2004), larval competitiveness (Huigens et al. 2004), fecundity (Hoffman and Turelli 1988, Fleury et al. 2000), male fertility (Snook et al. 2000) and immunity (Fytrou et al. 2006). *Wolbachia* are considered mutualists in association with filarial nematodes where they aid in metabolism of nematode hosts (Bandi et al. 1999). Recent experiments indicate that *Wolbachia* shares mutual relationships with *Exorista sorbillans*, *Drosophila simulans* and *Aedes albopictus* (Dobson et al. 2002, Puttaraju and Prakash 2005, Weeks et al. 2007). In arthropods, *Wolbachia* infection induces feminization, male killing, parthenogenesis and CI affecting insect evolution (reviewed by Werren 1997).

A microarray experiment was performed on maize roots fed on by WCR with or without *Wolbachia* to identify genes differentially expressed by the presence of *Wolbachia* in WCR. The experiment revealed that *Wolbachia* in WCR causes a global reduction in plant defense gene expression (Chapter 3). This reduction may be beneficial as it may facilitate WCR feeding by altering the

defense response of maize and may help explain the success of WCR as a pest of maize.

Based on the microarray results (Chapter 3) and the CI observed in WCR, we hypothesize that *Wolbachia* is not parasitic to WCR and the association between the two organisms may extend to more direct measures of fitness. To test our hypothesis a host location assay was conducted to determine whether or not *Wolbachia* infection affects the timing and ability of WCR larvae to locate a host plant. In addition, a hatch assay was conducted in which the larvae from an isolated sample of eggs were counted over time to identify the effect of *Wolbachia* on fertility. The effects of *Wolbachia* on fecundity and size were not tested as insects from *Wolbachia* infected and non-infected populations are housed in separate facilities to maintain quarantine status. Any effects that may have been observed between populations could not have been separated from environmental factors.

Materials and Methods

Insects. *Wolbachia*-free, non-diapausing WCR were obtained from a colony maintained at the University of Missouri. The *Wolbachia*-free colony was developed by feeding adults with an artificial diet of 0.1% tetracycline for the first generation, 0.2% tetracycline for the second generation and 0.3% tetracycline for the third generation. Insects were verified to be *Wolbachia*-free via PCR with *Wolbachia* specific primers coding for a 16S ribosomal RNA fragment (Werren et al. 1995). Selection was removed after verification. Infection status was verified prior to each experiment. Eggs from non-diapausing, WCR with *Wolbachia* were obtained from the North Central Agricultural Research Laboratory (NCARL) in Brookings, SD.

Hatch Assay. WCR with or without *Wolbachia* were allowed to oviposit in sterile oviposition plates for three days. Eggs were washed from the oviposition medium using a fine sieve under running water and examined under a microscope. One hundred viable eggs were placed in a standard Petri dish lined with Whatman filter paper that was kept damp with sterile water. The Petri dishes were sealed with parafilm and allowed to incubate at 25° C. Hatch counts were taken every 24 hours. Data was collected until seven days had passed without hatch. Eclosed larvae were counted and removed from the sample. Ten biological replications of 100 eggs each were performed for both *Wolbachia*-positive and *Wolbachia*-free WCR. Data was plotted to visualize a hatch curve. Statistical analysis was performed using a Student's t-test in Microsoft Excel.

Host Location Assay. Mo17 maize seed that had been imbibed for 24 hours was allowed to germinate for four days at 25° C. The germinated seed was placed at the center of a standard size Petri dish that has been lined with moistened Whatman filter paper. Sterile water was added to each dish to maintain adequate moisture levels. Both *Wolbachia*-positive and *Wolbachia*-free populations were tested at the same time. Because larvae usually emerge from the oviposition medium prior to collection, samples can be biased towards individuals with increased fitness. Therefore, neonate WCR larvae were collected from eggs that had been washed free of oviposition medium and incubated as in the hatch assay. Ten neonate larvae were collected with a camel hair brush and placed within the outer first centimeter of a dish containing a germinated maize seed. Any injured larvae were removed and replaced and the dish was then sealed with parafilm. Larvae were timed to quantify how long it took each population to locate the host plant. Counts of larvae that had located a host were taken every five minutes for an hour. Ten replications were performed for each of the two WCR populations. The data were analyzed using a Student's t-test.

Results and Discussion

To determine if *Wolbachia* infection affects WCR larval competitiveness, a host location assay was conducted and to identify the effect of *Wolbachia* on fertility, a hatch assay was conducted in which the eclosed larvae from an isolated sample of eggs were counted over time. If the association between WCR and *Wolbachia* is mutual, we expect to see a reduction in the time needed to locate a host in the *Wolbachia*-positive vs. *Wolbachia*-free population or a larger proportion of *Wolbachia*-infected WCR larvae locate a host compared to the *Wolbachia*-free population. If the association is amensal or parasitic, we expect to see an increase in the time it takes the Wolbachia-positive larvae to locate a host compared to the Wolbachia-free larvae or a reduction in the number of *Wolbachia*-infected WCR that locate a host. Finally, if the interaction is commensal, we should see no difference between *Wolbachia*-infected and non-infected WCR in their ability to locate a host plant or their rate of locating a host plant.

In the case of the hatch assay if a mutual association is present, we expect more larvae will hatch in the WCR with *Wolbachia* over the WCR which are not infected with *Wolbachia*. If the interaction is amensal or parasitic, the number of larvae hatching from *Wolbachia*-infected WCR should be lower than non-infected WCR. If the interaction is commensal, there should be no difference in larval hatch between *Wolbachia*-infected and non-infected WCR.

No significant difference in average hatch time (P=0.78) between larvae with or without *Wolbachia* infection was observed. The *Wolbachia*-free colony

exhibited an 89% total hatch percentage while the *Wolbachia*-infected, colony had an 88% total hatch percentage (p= 0.61). Both colonies have similar hatch distributions across time (Figure 4.1). A t-test comparing the total number of larvae from the *Wolbachia*-positive and *Wolbachia*-free populations that were able to locate their host (a germinated maize seed) was not significantly different (P= 0.54). A second t-test comparing the average length of time for host location to occur between the two colonies yielded a p-value of 0.75 further supporting the similarity between *Wolbachia*-positive and *Wolbachia*-free WCR. The distributions of number of larvae locating the host vs. time for the *Wolbachia*-positive and *Wolbachia*-free populations were similar (Figure 4.2).

The data show that *Wolbachia* infection in WCR does not affect either fertility or host location as indicated by the lack of statistically significant differences when comparing the populations. This data together with the facts that *Wolbachia* require WCR for replication, and that *Wolbachia* suppresses the maize defense response against WCR show that *Wolbachia* benefit through association with WCR and that WCR are not hindered by the relationship. The data indicate that WCR and *Wolbachia* share, at the least, a commensal association.

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Figure 4.1. Number of larvae hatching per day for two populations of WCR. Blue line indicates hatching data for *Wolbachia*-negative WCR. Pink line indicates hatching data for *Wolbachia*-positive WCR.

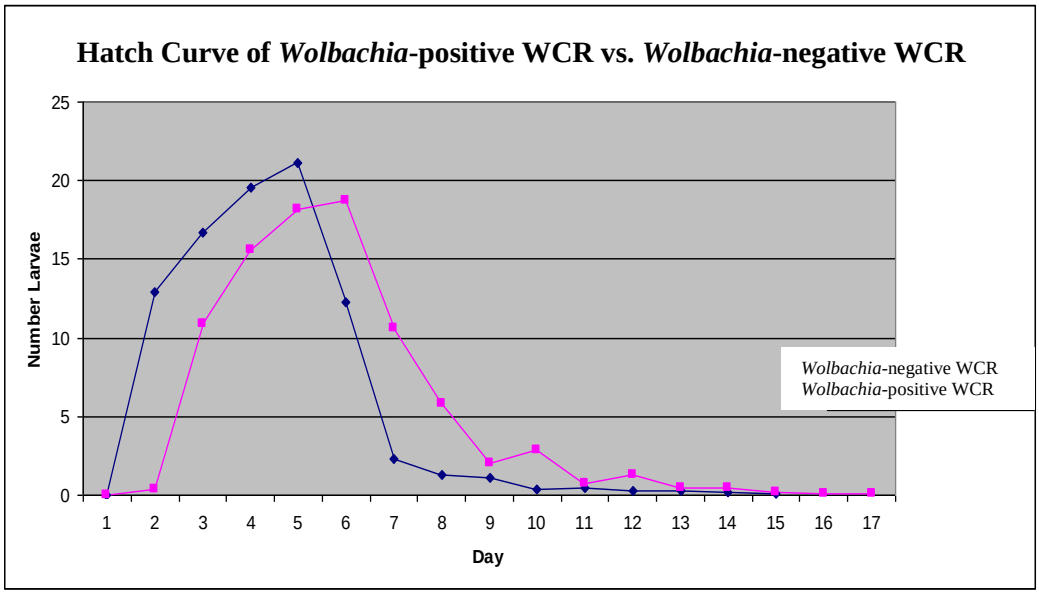
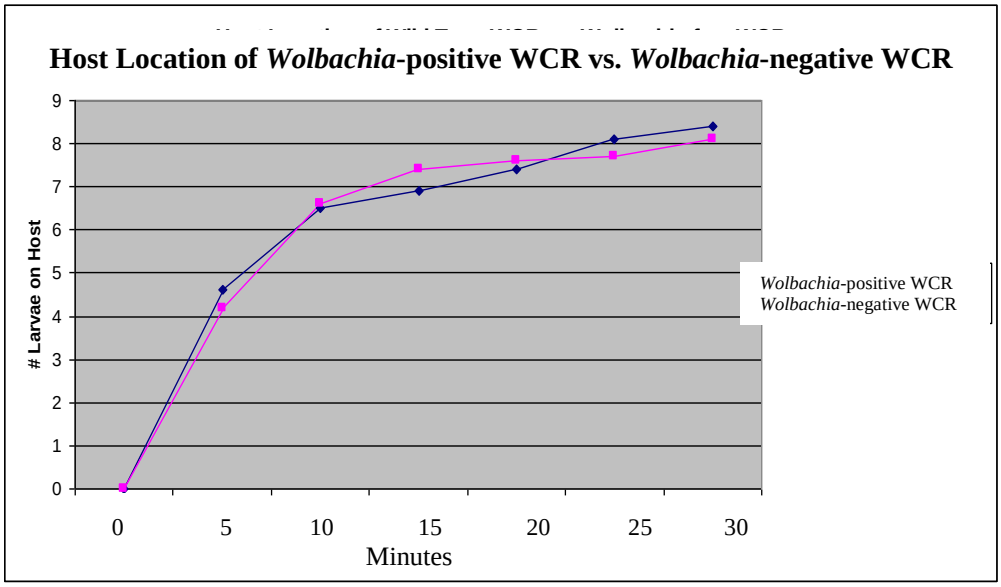


Figure 4.2. Results of WCR host location assay on Mo17 maize germinated seeds. Blue line indicates number of *Wolbachia*-positive WCR larvae finding maize host tissue every five minutes. Pink line displays the number of *Wolbachia*-negative WCR larvae finding maize tissue every five minutes.



Chapter 5. Conclusions and Future Directions

Insect-specific changes in maize seminal root gene expression patterns following feeding by neonate WCR larvae carrying *Wolbachia* identified a transcriptional response consistent with bacterial challenge to the maize root system (Chapter 2). These results led us to investigate the possibility that *Wolbachia*, a gram negative endosymbiont of WCR, was responsible for the transcriptional response we observed. The gene expression profile of maize seminal roots fed on by WCR with vs. without *Wolbachia* infection indicates that WCR-vectored *Wolbachia* affect gene expression in maize (Chapter 3). Among the changes we observed, HPR gene expression was down regulated by the presence of Wolbachia in WCR. This change could increase susceptibility of the host plant to insect damage benefiting the WCR. Down-regulation of HPR genes together with the inability of Wolbachia to replication outside its insect host suggest that the *Wolbachia*–WCR relationship is not parasitic. Additional data from host location and hatch assays identified no significant difference in total hatch, average hatch time, average time for host location or number of individuals finding the host (Chapter 4). This data together with the gene expression data and the obligate reproductive status indicate that WCR and *Wolbachia* share a commensal, perhaps even a mutual association.

Our results present a new paradigm in WCR-maize interactions where WCR-vectored *Wolbachia* mediates global changes in plant transcription, down-regulating many of the plant defense genes. This paradigm points to a number of

interesting areas and questions for future investigation. Our experiments demonstrated that neonate WCR larvae infected with *Wolbachia* can down-regulate host defense genes in maize seminal roots in V3 stage seedlings (Chapter 3). Understanding the mechanism by which *Wolbachia* mediate these broad changes in gene expression is the next step towards applying our results in WCR management. Among the areas to be tested to identify the mechanism underlying the altered transcription we observed, the role of the WO phage, if any, and the presence/extent of lateral gene transfer between *Wolbachia* and maize should be examined. Experiments testing the possibility of *Wolbachia*-mediated changes in root gene expression in older plants in a field setting are needed to determine the impact of our findings in a resistance management setting. *Wolbachia* may be present in as many as 70% of insect species. Given this fact, investigation to determine whether insect-vectored *Wolbachia* are capable of mediating transcriptional changes in other host plants should be undertaken. Our data suggest that *Wolbachia* share a commensal and possibly a mutatual relationship with WCR. A more detailed understanding of the effect of *Wolbachi*a infection on WCR gene expression would also be beneficial towards development of a resistance management plan.

VITA

Kelli L. Barr was born in Maquoketa, Iowa on February 16th, 1977 and raised in Santa Rosa, California. She married Jason Barr on May 20, 2000. She attended Iowa State University where she earned a Bachelor of Science in English Literature and a second Bachelor of Science in Classical Studies in May 2002. While earning her degrees, she worked in the laboratory of Dr. W. Allen Miller which researchered Barley Yellow Dwarf Virus and other leutoviruses like hepatitis C.

Upon matriculation, she began a graduate career at the University of Missouri in Classical Studies. After a year in the program, she realized that the sciences were more suited to her personal and professional goals so she joined the laboratory of Dr. Thomas Clark in the Entomology department. There she worked towards her Ph.D. until Dr. Clark left the University of Missouri for a corporate career. Kelli then joined the laboratory of Dr. Georgia Davis where most of her Ph.D. work was performed.

While completing her degree, Kelli had two children, a son and a daughter. Upon completion of her Ph.D. she accepted a postdoctoral position in the lab of Dr. Scott Michael at Florida Gulf Coast University. There she researched in virology with an emphasis on insect vectored viruses.