RNAi CONTROL OF TOBACCO THRIPS: ILLUMINA TRANSCRIPTOMICS

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Abstract

The whole-body transcriptome for nymphs of the tobacco thrips was sequenced on an Illumina® Genome Analyzer IIx sequencer. Illumina sequences produced 6.6 gigabases of sequence data distributed across 66 million 101-base reads. Approximately 55% of these reads had a minimum quality score of Q20 across the entire length of the read and 85% of the bases among all reads had a minimum quality score of Q30. The reads were assembled with Trinity® software into 64,280 contiguous sequences (contigs). The contig lengths ranged from approximately 100 bps (base pairs) to greater than 2,000 bps. The assembled contigs were batched BLASTed, mapped and annotated with Blast2GO® software. The number of contigs with BLAST hits was 22,174. The vast majority of these were associated in the order of highest to lowest number of GO (Gene Ontology) assignments to binding, catalytic activity, transporter activity, structural molecular activity and molecular transducer activity. To illustrate the depth of contig information in our global putative functional analysis, messages are further characterized which are involved in hormonal regulation of development, steps in juvenile hormone (JH) biosynthesis, critical messages involved in JH degradation and ecdysteroid metabolism, known commercial insecticide targets, and enzymes involved in general in insecticide detoxification. There results provide many new leads for the development of both RNAi and artificial antibody approaches of control of this important insect pest of cotton and other crops where currently there is no commercial plant transgenic approach to tobacco thrips control. In addition, this transcriptome provides opportunities for the global evaluation of risk to insecticide resistance, the study of plant virus transmission and insect vectored-plant diseases in general, and the study of the basic physiology and ecology of tobacco thrips and the crops on which they feed.

Introduction

The tobacco thrips, *Frankliniella fusca*, is polyphagous and exploits a wide variety of both cultivated crops and weed species (Kahn et al., 2005). It is an economic pest of cotton and peanuts through feeding damage and is a major vector of phtopathogenic viruses and bacteria to other crops. The small size (~ 1 mm long) and long, fringed wings of *F. fusca* adults aid in their dispersal by trade and localized wind currents. *F. fusca* is traditionally distributed in North America east of the Rocky Mountains. The recent spread of this species, perhaps due to global trade, has been documented as far as Japan (Nakao et al., 2011). The tobacco thrips is polyphagous and exploits a variety of both cultivated crops and weed species (Kahn et al., 2005). *F. fusca* populations have the capacity to adapt to local conditions and plant hosts (Mound and Teulon, 1995) and have a 16 to 25 day generation time, are multivoltine and can reproduce parthenogenetically. *F. fusca* females lay eggs inside of plant tissue and have two feeding larval stages followed by two non-feeding stages, the pre-pupal and pupal instars and adult. The tobacco thrips has demonstrated pesticide resistance (Mound and Teulon, 1995).

Thrips also are the only known vectors of tospoviruses, the sole plant-infecting genus in the family *Bunyaviridae*. A single member of this genus, the tomato spotted wilt virus (TSWV) is responsible for approximately 1 billion dollars in damage worldwide (Prins and Goldbach, 1998). *F. fusca* can transmit TSWV and impatiens necrotic spot virus (Prins and Goldbach, 1998) as well as the bacterial agent responsible for center rot of onion (Gitaitis et al., 2003). The tobacco thrips is considered the primary vector of TSWV in the Southeastern United States, which presents an economic threat to tobacco, peanut, pepper and tomato in North Carolina (Groves et al., 2003). TSWV can only be acquired by immature *F. fusca* in its two larval forms and is only transmitted by *F. fusca* adults which remain infectious for life. TSWV is a propagative virus that depends on being actively transported across cellular membranes and circulated in the insect's hemocoel (Andret-Link and Fuchs, 2005). New research into potential

virus receptors for TSWV has identified receptor candidates located in the midgut (Bandla et al., 1998). Thrips species vary in their capacity to transmit viruses and may gain or lose the capacity to vector virus isolates over time (Wijkamp et al., 1995). Although thrips do not vector diseases in cotton, their damage to seedlings can produce significant yield losses and maturity delays in much of the cotton belt.

Thrips consistently present the largest economic threat to North Carolina and Virginia cotton production but is also a significant pest in the Midsouth and elsewhere (Bacheler, 2012; Akin et al., 2010). Direct feeding on seedling cotton can result in malformed leaves, stunted plants, delayed maturity and yield reduction. Thrips damage in cotton is magnified by cool and dry conditions early in the growing season which extends the window of cotton seedling vulnerability to this pest complex through slower plant growth (Bacheler, 2010). Additionally, tobacco thrips is the most abundant species present throughout most of the cotton belt (Reed, 2010). Presently, thrips are often expensive and difficult to control, requiring the use of both chloronicotinoid seed treatments and foliar sprays (Toews et al., 2012).

The prospect of developing transgenes to plant bugs, stink bugs and thrips may lie in the use of RNAi (RNA interference) to suppress or silence critical genes of the pest species (Gordon and Waterhouse, 2007). RNAi disrupts gene expression/protein synthesis in the cytoplasm of target organisms at the gene transcript (messenger RNA (mRNA)) level (Huvenne and Smagghe, 2010). The agent of RNAi is dsRNA (double stranded RNA) or siRNA (small interfering RNA). The mechanism by which this disruption occurs is the cleavage of introduced dsRNA into siRNAs (short-interfering RNAs) or the introduction of siRNA directly which are complementary in their nucleotide sequence to the targeted insect mRNA. These complementary siRNAs interact with the target mRNA and prevent the message from being translated into proteins that are essential to the physiology of the pest organism.

Several labs have reported using RNAi to cause varying levels of gene suppression in targeted insects fed or injected with dsRNA or siRNA. Walker and Allen (2010) suppressed expression of a salivary enzyme in the tarnished plant bug, *Lygus lineolaris* (Palisot de Beauvois) (Hemiptera: Miridae); Chen et al. (2010) targeted trehalose phosphate synthase in brown planthopper, *Nilaparvata lugens* (Hemiptera: Delphacidae); and, Rosa et al. (2010) decreased levels of actin mRNA in glassy-winged sharpshooter, *Homalodisca vitripennis* (Hemiptera: Cicadellidae). Working with Lepidoptera, Griebler et al. (2008) targeted JH (juvenile hormone) regulating neuropeptides in the fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae); Kumar et al. (2009) suppressed acetylcholinesterase in cotton bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae); and Tian et al. (2009) reduced levels of chitin synthase in beet armyworm, *S. exigua.* The efficacy of transforming plants to express dsRNAs for pest control has been demonstrated with two non-hemipterans. Baum et al. (2007) reported that corn, *Zea mays*, had been transformed with transgenes expressing dsRNA that reduced expression of a target gene in the western corn rootworm, *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae). Mao et al. (2011) reported reduced weights of *H. armigera* larvae fed leaves of cotton transformed to express dsRNA targeting a P450 that allows larvae to feed on gossypol, a phenolic cotton secondary metabolite with insect antifeedant activity.

A first step in the development of an RNAi-based insect control method is to identify potential genes of the pest species that can be targeted with dsRNA or siRNA. The objective of the work reported here was to use Illumina® sequencing technology to sequence for the first time the transcriptomes of the tobacco thrips and then use bioinformatics to analyze the resulting sequences to identify targets for RNAi. The work can also be used to develop a global approach for the evaluation and diagnosis of thrips resistance to chemical insecticides. Other uses for this technology are the global identification of RNA viruses in general, leads for better understanding plant disease transmission and mechanisms in thrips for the maintenance and transmission process, and the general study of the physiology and ecology of this important pest.

Materials and Methods

Insects

Tobacco thrips, *Frankliniella fusca*, larvae were obtained from colonies in the laboratory of G. G. Kennedy at North Carolina State University, Raleigh, NC and were originally collected from sites in North Carolina. Adults and nymphs were kept in 1-gallon plastic tubs covered with one layer of cheesecloth, fed on pole beans and maintained at 25 ± 1° C, 55-60% relative humidity, 14 hours light: 10 hours dark until needed for RNA extraction. Six hours before extraction, the beans were removed from the rearing containers. This starvation period allowed the insects to

clear their digestive system of plant material (as confirmed by observation and seeing no green in the gut as viewed across the insect cuticle).

Total RNA isolation

Whole live larval thrips (200 insects) were flash frozen with liquid nitrogen and then ground to a fine powder with a mortar and pestle. Lysis buffer (600µl) was added to this powder just after grinding. Total RNA was extracted using a RNAeasy Mini Kit (QIAGEN) from animal tissue following the manufacture's protocol.

cDNA library preparation and Illumina sequencing

A cDNA library was prepared from ≥ 5 µg total RNA following vendor recommendations (Illumina, Inc., San Diego, CA) for sequencing on the Illumina Genome Analyzer IIx sequencer. The cDNA was sequenced on three lanes of an Illumina eight-lane flow cell. Trinity software (20 August 2011 release; www.trinityrnaseq.sourceforge.net) was used to assemble the larval reads into contigs (contiguous nucleotide sequences), with a k-mer length of 25.

Bioinformatics

Blast2GO® software (Conesa et al., 2005) was used to align, map and annotate the contigs. Blast2GO analysis was concluded in Dec. 2011. For the alignment step, the contigs were translated to proteins in all six reading frames and compared to the GenBank nr (non-redundant) protein database using the BLASTx (Basic Local Alignment Search Tool) algorithm with E-value cut-off set at E-3 (10^{-3}). BLAST hits (thrips query contigs with database-sequence matches where E-value ≤ E-3) were mapped and annotated with GO (Gene Ontology) terms. These GO terms assigned the translated query sequences to categories of putative protein function (GO level 2 functional categories) on the basis of sequence and functional conservation among organisms represented in publicly-accessible protein/gene-product sequence databases (Gene Ontology Consortium; Ashburner et al., 2000).

Results and Discussion

Sequencing of reads & assembly of contigs

Illumina sequences produced 6.6 gigabases of sequence data distributed across 66 million 101-base reads. Approximately 55% of these reads have a minimum quality score of Q20 across the entire length of the read and 85% of the bases among all reads had a minimum quality score of Q30. The reads were assembled with Trinity software into 64,280 contiguous sequences (contigs). The contig lengths are shown in Fig. 1 and ranged from approximately 100 bps to greater than 2,000 bps.

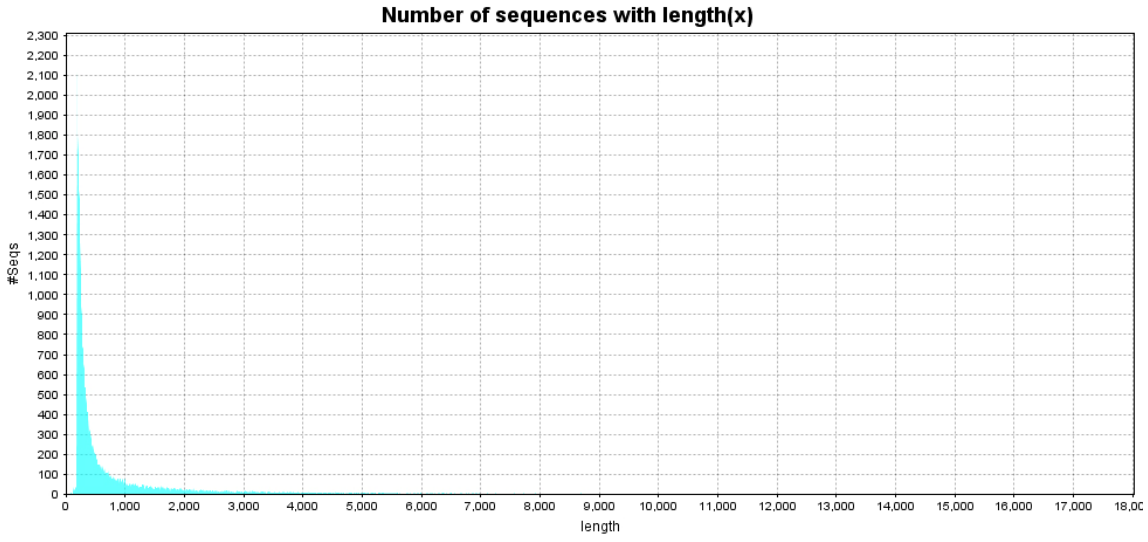

Fig. 1. Contig length of assembled reads from Illumina sequencing of the transcriptome from whole bodies of larval tobacco thrips.

Global analysis of contigs

The assembled contigs were batched BLASTed, mapped and annotated with Blast2GO software. Contigs with BLAST hits were 22,174. Fig. 2 shows the species distribution for these hits. As expected, the greatest number of

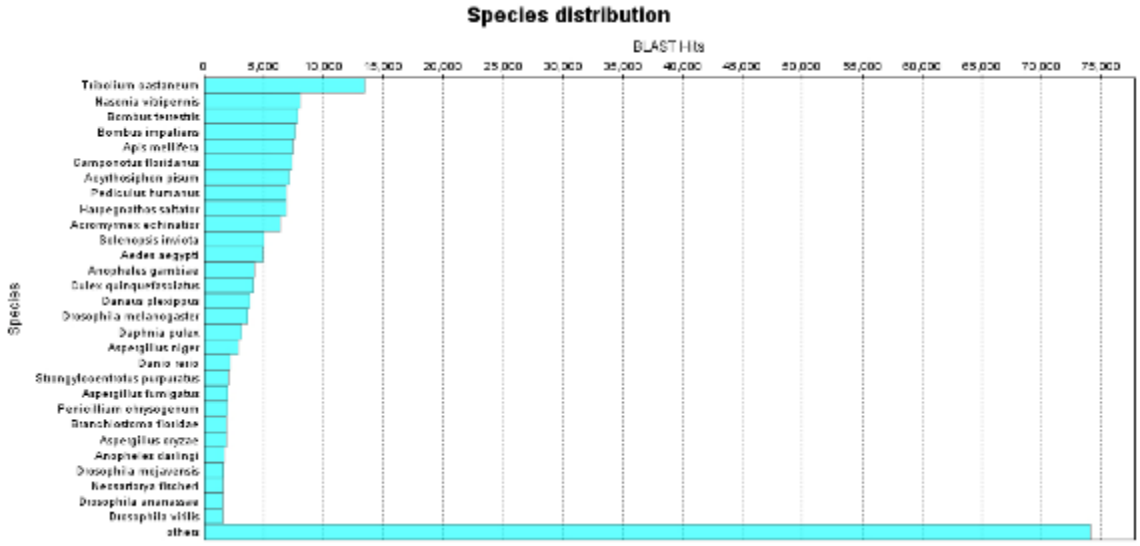

Fig. 2. Species distribution of blast hits for the contigs shown in Fig. 1 for whole body, larval tobacco thrips.

hits is associated in some cases with insects where the genome is available. The number of contigs annotated with Gene Ontology terms was 9,586. This classification is shown in Fig. 3. The vast majority of these were associated in the order of highest to lowest number of GO assignments to binding, catalytic activity, transporter activity, structural molecular activity and molecular transducer activity.

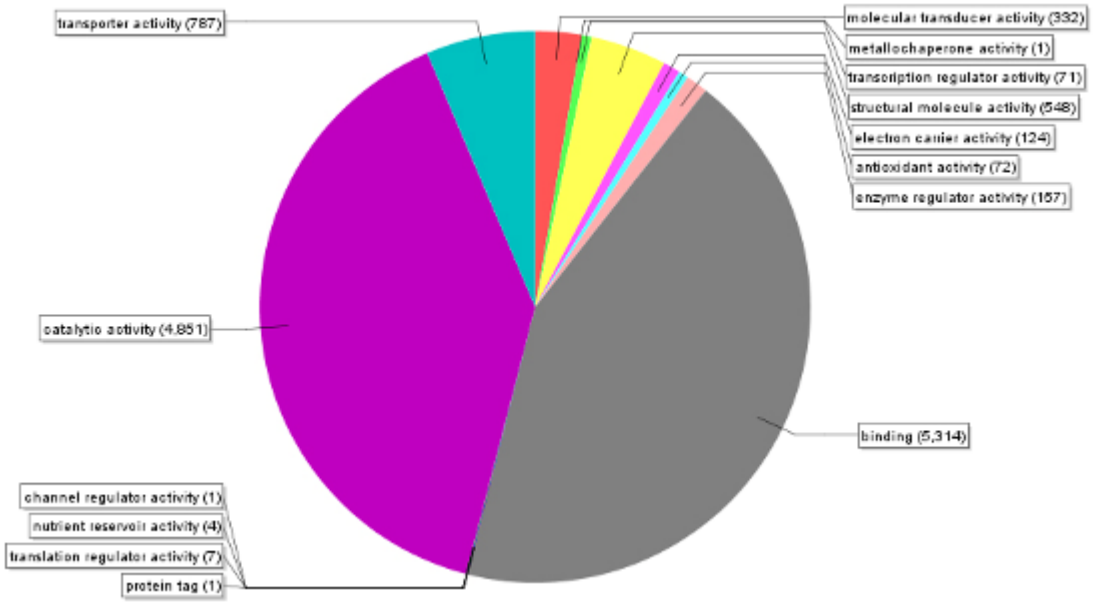

Fig. 3. Results of GO analysis for whole body larval tobacco thrips.

Annotation of contigs of specific functional interest

It is not possible in this paper to discuss the putative function of all (over 20,000) of the contigs obtained. However, to demonstrate some of the specific content, a few messages associated with insect growth and development and insecticide mode of action are briefly characterized. However, there is much more in our database for study.

Table 1 shows some examples of insect hormones that were found with significant matches in the available, public databases. Glycoprotein hormones have been found in a number of invertebrates including insects but the function of this hormone is poorly understood. The neuropeptide, allatostatin (Table 1), is produced in the insect head and

Table 1. Examples of putative hormones found in the whole body, larval transcriptome of the tobacco thrips.

Message	No. Bp	E-value
glycoprotein hormone	421	4.94E-50
allatostatin	1858	2.09E-15
eclosion hormone	1252	5.98E-17

although it may have several functions in insects, most research has been on its role in the regulation of juvenile hormone (JH) biosynthesis; JH is responsible for many aspects of insect development including metamorphosis, reproduction, diapause, migration, color, and much more. The eclosion hormone (Table 1) is another neuropeptide involved in the fundamental process in insects of molting. In general, the discovery of these relatively small neuropeptides which are typically in low abundance but especially so for a whole body transcriptome is a demonstration of the power of the use of Illumina and the deep sequencing of many short reads with this technology in the discovery of important regulatory elements associated with insect endocrinology. Table 2 shows some examples of putative hormone receptors.

Table 2. Examples of hormone receptors found in the whole body, larval transcriptome of the tobacco thrips.

Message	No. Bp	E-value
diuretic hormone receptor	714	5.37E-54
adipokinetic hormone receptor	1513	2.53E-129

JH biosynthesis in insects occurs in a small pair of endocrine organs, the corpora allata, behind the insect brain. Table 3 also demonstrates how deep sequencing was successful in obtaining at the least the putative partial sequence of some of the enzymes in this pathway. Farnesyl pyrophosphate synthetase for example is involved in the final stages of JH III biosynthesis prior to the JH III branch, and the JH acid methyl transferase is found in the JH branch and is responsible for the addition of a methyl group to farnesoic acid to produce methyl farnesoate, the next to last step before the synthesis of JH III by the addition of a C10,11 epoxide.

Table 3. Examples of putative proteins involved in juvenile hormone (JH) biosynthesis found in the whole body, larval transcriptome of the tobacco thrips.

Message	No. Bp	E-value
farnesyl pyrophosphate synthetase	246	2.06E-43
JH acid methyltransferase	997	7.01E-60

The regulation of JH titer in insects is unique compared to other animals since two processes are involved: biosynthesis as just discussed and dynamic changes in JH degradation. Table 4 shows two of the most important putative enzymes in this degradation process: (i) JH epoxide hydrolase which converts the epoxide of JH to a diol; and (ii) JH esterase, which removes the JH methyl ester, producing JH acid. Ecdysteroids are steroid hormones that

regulate insect molting, metamorphosis, diapause, reproduction and other developmental processes in insects. Table 4 highlights a putative P450 that might be involved in the synthesis of the active form of this hormone.

Table 4. Putative enzymes involved in juvenile hormone (JH) degradation and ecdysteroid synthesis found in the whole body, larval transcriptome of the tobacco thrips.

Message	No. Bp	E-value
JH epoxide hydrolase	2337	4.69E-170
JH esterase	2438	7.60E-144
ecdysone 20-monooxygenase	3478	0

Finally, Table 5 illustrates some of the potential targets for current commercial insecticides and putative xenobiotic enzymes which in general can be responsible for insecticide resistance. There were many additional contigs with putative identifications in these categories of function that were not shown.

Table 5. Putative insecticide receptors and xenobiotic enzymes found in the whole body, larval transcriptome of the tobacco thrips.

Message	No. Bp	E-value
acetylcholine receptor alpha-like	1451	0
aminopeptidase	3061	0
cadherin	230	3.86E-37
glutathione S-transferase	224	4.50E-25
cytochrome p450	1858	1.08E-129
carboxylesterase	1723	4.36E-67

Summary

In summary, we report here the first Illumina transcriptome to the tobacco thrips, *Frankliniella fusca*, a serious yield-reducing pest of seedling cotton in the Southeast and Midsouth regions of the US. Both the global analysis of these data and characterization of specific messages show that the transcriptome was successful in the identification of rare and small messages, even when the transcriptome was constructed for mRNA from a whole body homogenate. These results provide many new leads for the development of both RNAi and artificial antibody approaches of control of this important insect pest of cotton and other crops, where currently there is no commercial plant transgenic approach to thrips control. In addition, this transcriptome provides opportunities for the global evaluation of risk to insecticide resistance, the study of plant virus transmission, and the study of the basic physiology and ecology of this pest and the crops on which they feed.

Acknowledgements

We gratefully acknowledge financial support from Cotton Inc. and a NIH training grant in the Biotechnology Program at NC State University. We thank Dr. Jenn Schaff for sequencing services and Mr. Jonathan Keebler for assembly of the Illumia individual reads into contigs, in the NC State University Genome Sequencing Laboratory.

References

Akin, D.S., A.R. Monticello, GM. Lorenz III, G.E. Sudebaker, S.D. Stewart, et al. 2010. Evaluation of automatic insecticide applications following preventative insecticides for thrips: preliminary results from a regional project. *In*, Proceedings, 2010 Beltwide Cotton Conferences, National Cotton Council, Memphis, TN. pp. 1119-1121.

Andret-Link, P. and M. Fuchs. 2005. Transmission specificity of plant viruses by vectors. J. Plant Pathol. 87:153-165.

Ashburner, M., C.A. Ball, J.A. Blake, D. Botstein, H. Butler, J.M. Cherry, A.P. Davis, K. Dolinski, S.S. Dwight, J.T. Eppig, et al. 2000. Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat. Genet. 25:25-29.

Bacheler, J.S. 2010. Managing insects on cotton. 2010 Cotton Information. http://ipm.ncsu.edu/cotton/insectcorner/PDF/Chapter11.pdf

Bacheler, J.S. 2012. New products technologies and concerns: Cotton Insects. Consultants Conference. *In*, Proceedings, 2012 Beltwide Cotton Conferences, National Cotton Council, Memphis, TN (in press).

Bandla, M., L. Campbell, D. Ullman and J. Sherwood. 1998. Interaction of tomato spotted wilt tospovirus (TSWV) glycoproteins with a thrips midgut protein, a potential cellular receptor for TSWV. Phytopathol. 88:98-104.

Baum, J.A., T. Bogaert, W. Clinton, G.R. Heck, P. Feldmann, O. Ilagan, S. Johnson, G. Plaetinck, T. Munyikwa, M. Pleau, T. Vaughn and J. Roberts. 2007. Control of coleopteran insect pests through RNAi interference. Nature Biotech. 25:1322-1326.

Chen, J., D. Zhang, Q. Yao, J. Zhang, X. Dong, H. Tian, J. Chen and W. Zhang. 2010. Feeding-based RNA interference of a trehalose phosphate synthase gene in the brown planthopper, *Nilaparvata lugens*. Insect Mol. Biol. 19:777-786.

Conesa, A., S. Gotz, J.M. Garcia-Gomez, J. Terol, M. Talon and M. Robles. 2005. Blast2GO: a universal tool for annotation, visualization and analysis in functional genomics research. Bioinformatics 21:3674-3676.

Gitaitis, R., R. Walcott, M. Wells, J. Perez and F. Sanders. 2003. Transmission of Pantoea ananatis, causal agent of center rot of onion, by tobacco thrips, *Frankliniella fusca* RID B-5933-2011. Plant Dis. 87:675-678.

Gordon, K.H. and P. M. Waterhouse. 2007. RNAi for insect-proof plants. Nat. Biotechnol. 25:1231-1232.

Griebler, M., S.A. Westerlund, K.H. Hoffmann and M. Meyering-Vos. 2008. RNA interference with the allatoregulating neuropeptide genes from the fall armyworm *Spodoptera frugiperda* and its effects on the JH titer in the hemolymph. J. Insect Phys. 54:997-1007.

Groves, R., J. Walgenbach, J. Moyer and G. Kennedy. 2003. Seasonal dispersal patterns of *Frankliniella fusca* (Thysanoptera : Thripidae) and tomato spotted wilt virus occurrence in central and eastern North Carolina. J. Econ. Entomol. 96:1-11.

Huvenne, H. and G. Smagghe. 2010. Mechanisms of dsRNA uptake in insects and potential of RNAi for pest control: a review. J. Insect Phys. 56:227-235.

Kahn, N., T. Walgenbach and G. Kennedy. 2005. Summer weeds as hosts for *Frankliniella occidentalis* and *Frankliniella fusca* (Thysanoptera: Thripidae) and as reservoirs for tomato spotted wilt tospovirus in North Carolina. J. Econ. Entomol. 98:1810-1815.

Kumar, M., G.P. Gupta and M.V. Rajam. 2009. Silencing of acetylcholinesterase gene of *Helicoverpa armigera* by siRNA affects larval growth and its life cycle. J. Insect Phys. 55:273-278.

Mao, Y., X. Tao, X. Xue, L. Wang and X. Chen. 2011. Cotton plants expressing CYP6AE14 double-stranded RNA show enhanced resistance to bollworms. Transgenic Res. 20:665-673.

Mound, L. and D. Teulon. 1995. Thysanoptera as phytophagous opportunists. Thrips Biol. Manag. 276:3-19.

Nakao, S., C. Chikamori, S. Okajima, Y. Narai and T. Murai. 2011. A new record of the tobacco thrips *Frankliniella fusca* (Hinds) (Thysanoptera: Thripidae) from Japan. Appl. Entomol. Zool. 46:131-134.

Prins, M. and R. Goldbach. 1998. The emerging problem of tospovirus infection and nonconventional methods of control. Trends Microbiol. 6:31-35.

Reed, J., A. Catchot, S. Akin, G. Lorenz, G. Studebaker, A. Herbert, C. Daves, S. Raymond, J. Greene, M. Toews, R. Leonard, S. Stewart, D. Cook, J. Gore, D. Kerns, J. Bacheler, P. Roberts and S. Micinski. 2010. Regional thrips trial, 2009: thrips species composition. *In*, Proceedings, 2010 Beltwide Cotton Conferences. National Cotton Council, Memphis, TN. pp. 906-912.

Rosa, C., S.G. Kamita, H. Dequine, H. Wuriyanghan, J.A. Lindbo and B.W. Falk. 2010. RNAi effects on actin mRNAs in *Homalodisca vitripennis* cells. J. RNAi Gene Silencing 6:361-366.

Tian, H., H. Peng, Q. Yao, H. Chen, Q. Xie, B. Tang and W. Zhang. 2009. Developmental control of a lepidopteran pest *Spodoptera exigua* by ingestion of bacteria expressing dsRNA of a non-midgut gene. PLoS ONE 4:e6225.

Toews, M., P. Roberts, J. Herbert, S. Tubbs, G. Harris, R. Srinivasan, R. Smith, J. Greene, J. Bacheler, D. Reisig and A. Herbert. 2012. Thrips management in seedling cotton with starter fertilizer and a single foliar application. *In*, Proceedings, 2012 Beltwide Cotton Conferences, National Cotton Council, Memphis, TN (in press).

Walker, W. and M.L. Allen. 2010. Expression and RNA interference of salivary polygalacturonase genes in the tarnished plant bug, *Lygus lineolaris*. J. Insect Sci. 10:173 available online: insectscience.org/10.173.

Wijkamp, I., N. Almarza, R. Goldbach and D. Peters. 1995. Distinct levels of specificity in Thrips Transmission of Tospoviruses. Phytopathol. 85:1069-1074.