Project title:	Insecticide resistance in the Tomato leafminer
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Project leader:	Chris Bass, University of Exeter
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Location of project:	Exeter University
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The results and conclusions in this report are based on an investigation conducted over a one-year period. The conditions under which the experiments were carried out and the results have been reported in detail and with accuracy. However, because of the biological nature of the work it must be borne in mind that different circumstances and conditions could produce different results. Therefore, care must be taken with interpretation of the results, especially if they are used as the basis for commercial product recommendations.

AUTHENTICATION

We declare that this work was done under our supervision according to the procedures described herein and that the report represents a true and accurate record of the results obtained.

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CONTENTS

GROWER SUMMARY

Headline:

- Resistance to important IPM compatible chemicals has been confirmed in some UK populations.
- Molecular basis of this resistance has been determined.
- Molecular-based resistance diagnostics are under development.
- Ability to 'resist' novel mating disruptors is investigated in UK populations.

Background:

Tuta absoluta.

T. absoluta is a micro lepidopteran species belonging to the Gelechiidae family which consists of about 500 genera and 4700 species. It is endemic to South America, first described in Peru in 1917, and genetic studies reveal that its expansion throughout Europe, Africa and the Middle East came from a single introduction into Eastern Spain from central Chile in 2006. Its arrival in the UK is believed to be due to importation of infected produce from Spain in 2009. *T. absoluta* arrived in the UK armed with resistance to many traditional insecticides.

T. absoluta host plants include economically important crops in the family solanaceae and since the outbreak of *T. absoluta* in 2009 its economic impact has been felt by many UK tomato growers. Its life cycle lasts about one month and females have been recorded laying in excess of 200 eggs. Favourable temperatures for development are similar to those of tomato glasshouses in Europe and its host plant preference is greater for cultivated tomato varieties than that of wild types. Control of this pest after its insurgence required the development of an integrated pest management (IPM) strategy. This included biocontrol from the predatory hemipteran bug *Macrolophus pygmaeus* and applications of two pesticides with novel modes of action, Coragen and Conserve. A more recent addition in the fight to control *T. absoluta* is the introduction of Isonet-Ts mating disruptor.

Conserve.

The active compound in Conserve is spinosad, a bio-pesticide derived from the soil bacteria *Saccharopolyspora spinosa*. This insecticide targets the nicotinic acetylcholine receptor (nAChR), a nerve receptor involved in rapid neurotransmission. nAChR has long been recognised as a target for insecticidal action. Binding of spinosad to the nAChR causes loss

of function of the receptor, allowing the movement of chemical charge, depolarising the muscle membrane. This electrochemical transduction activates many responses in the cell including contraction of muscles. Intoxication by spinosad ultimately results in paralysis, feeding cessation and death.

High levels of spinosad resistance have been reported in field populations of South American and European *T. absoluta.* An amino acid substitution in the nAChR was identified in a resistance-selected strain, resulting in alteration of the structure of nAChR. The same mutation was observed in other resistant insects.

A second mechanism of resistance in *T. absoluta* is exon skipping. Exon skipping is the exclusion of discrete regions of RNA that are usually present in normal RNA transcripts, RNA transcripts which then go on to direct the assembly of the protein receptors. This 'missing' region of RNA results in the loss of a whole section of the protein. 100% of resistance-selected *T. absoluta* expressed receptor protein transcripts lacking the normally present exons. This resulted in the loss of the spinosad binding region of the nAChR.

Coragen.

Coragens active compounds are in the chemical class diamides which have excellent effectiveness at controlling lepidopteran pests. Diamides have a novel mode of action, targeting another nervous system receptor, the ryanodine receptor (RyR). This receptor is involved in calcium signalling which regulates many processes in insects, including muscle contraction. In spite of Coragens great lepidopteran profile, resistance has recently evolved in the field. 2010-2011 South Chinese populations of Diamond back moth *Plutella xylostella* developed target-site resistance resulting in 2000-fold resistance increase. A mutation resulting in an amino acid substitution was presumed to confer resistance in Thailand, Philippine and Chinese populations. Resistance to diamides has also been reported in *T. absoluta.* Italian populations showed 2414-fold resistance and in resistant Greek populations an amino acid alteration at the same site as the resistant *P. xylostella* was identified as being causal.

Isonet-T

Mating disruptors were introduced in 2016 and after great success in controlling *T. absoluta,* have immediately become an integral part of IPM. They work by inundating the glass house atmosphere with a synthetic sex pheromone identical to that released by females. This

confuses males as to the location of females, preventing reproduction, and has proved a highly effective control measure. While this introduction has relaxed the pressure on the chemical controls there are reports in the scientific literature of parthenogenesis (asexual reproduction) in *T. absoluta*. With *T. absoluta's* track record of overcoming control measures and reports of control failure at one U.K. site this year, it is important to asses the U.K. populations ability to reproduce parthenogenetically.

Summary

Objectives.

The main aims of this research project are to test for resistance in the field, characterise the molecular basis of resistance to Conserve and Coragen. Once the molecular basis for these pesticides has been identified, DNA based diagnostics will be developed with the aim of replacing the classical leaf dip assays.

As this is an applied study, the aims of this project must stay flexible and track changes as they occur in the grow houses around the U.K. - this includes changes in levels of resistance. Often the evolution of resistance occurs through a trade-off with pest fitness, so discontinuation of pesticide use may cause population level susceptibility to return, thus restoring the efficacy of the pesticide. This project also aims to monitor new mating disruption control products and to asses the ability for *T. absoluta* to reproduce asexually.

Results and discussion.

Overview.

Previous work on U.K. populations has described spinosad resistance and this report not only confirms this, but also describes alternative mechanisms for this resistance and a rapid method for its detection. This work also shows low-level field resistance to diamides and further laboratory selection of this population generated high levels of resistance. These findings have a serious implication, potentially rendering current IPM strategies used by many growers in the U.K. severely restricted. However evidence of *T. absoluta's* ability to reproduce in the absence of sexual reproduction was not detected.

Conserve.

Leaf dip assays showed high levels of resistance in U.K. strains. These populations were then tested for target site alterations. Amplified nAChR transcripts from resistant populations showed reduction in size compared to the susceptible strain. Disparity in size between the

resistant and susceptible amplified transcripts can be observed by a process called gel electrophoresis. This gives a distinct picture of the variation in transcript size and therefore resistance status.

The redundancy of spinosad was confirmed from the bioassay results, and analysis of the nAChR gene transcript clearly showed exon 4 had been skipped when compared to the full complement of exons in a susceptible population. The exclusion of exon 4 from this reading frame resulted in a 109 nucleotide deletion and a shift in the reading frame, producing a premature stop codon in exon 5 at position. RNA translation would produce a massively truncated protein and would structurally render the protein completely non-viable.

More likely however is that nonsense mediated decay (NMD) would digest RNA before energy was wasted, translating it to a non-functional protein. NMD is a surveillance pathway that prevents expression of genes with PSC translating into incomplete proteins.

This result however does offer the ability to create an RNA based diagnostic capable of identifying the size difference between full nAChR transcripts and those with exon 4 skipped, based on PCR and gel electrophoresis. This would drastically reduce the time taken to assess frequencies and locations of resistant phenotypes from ~2 months to 24-48 hours.

Coragen

Bioassays for Coragen resistance showed some moderate tolerance to diamides in field populations. The RyR transcripts were amplified, targeting the regions surrounding the binding site of diamides. Sequencing of these amplicons showed no obvious mutations when compared to the susceptible population in most cases. However, low level variation at one target site was indicated in a population with some tolerance. Selection of this population with increasingly higher doses of diamides caused a rise in the frequency of this mutation within the population until it was present in all individuals tested.

This mutation is present in resistant European populations and so is likely to have been imported with U.K. populations at low frequency. This means it is only a matter of time before it is selected for in the field.

Isonet-T

90 virgin females across three populations were tested for their ability to reproduce asexually. Although over 90% of these females laid eggs, none of the eggs developed into larvae. Results from these experiments did not support previously reported findings and

revealed no evidence to support the claim that *T. absoluta* can reproduce parthenogenetically.
Since this research has been conducted, much of the *T. absoluta* problems faced by growers has been alleviated by the introduction of a new control measure - the Isonet-T mating disruptor. However control failure has been reported in one grow house this season. Understanding this failure is vital to optimising the use of this control measure and maintaining its effectiveness.

Maintaining optimum IPM effectiveness requires a plethora of control measures each targeting a different aspect of the insect's biology. Over use of any particular control measure can select for fixation of resistance rendering the control measure useless. The loss of any control measure increases selection for resistance to remaining control measures. If Coragen and Conserve are used without prior knowledge of resistance in the population they could lose their efficiency and be lost altogether. Although the mating disruptor may seem to be a 'silver bullet', reliance solely on this will select for resistance. Coragen and Conserve need to be preserved to support the mating disruptor and maintain a diverse array of control measures.

Financial benefits

Insecticide resistance tests currently done by scientists are time consuming, expensive and slow to provide results. Molecular assays could reduce the time taken from 8-10 weeks to within 2-3 days and reduce the cost from £2000 to around £100 - £200.

The careful monitoring of control measure effectiveness will dictate their integration into glasshouse specific management strategies. This will result in the choice of the most effective control measure, as well as a relaxation of resistance selection on less effective measures preventing their redundancy. Reliance on only one control measure will result in resistance and resurgence of *T. absoluta* populations, causing losses of up to £50k per ha.

Action Points

Further research

- Test UK population's ability to overcome mating disruptors.
- Uncover potential mechanism if resistance is present
- Test diagnostic capability to asses UK resistance frequency.

- Advise growers on use of control method combinations based on resistance profile of populations.

SCIENCE SECTION

Introduction

The tomato leaf miner, *Tuta absoluta,* is an economically important pest species of tomatoes*,* which radiated from South America to Spain in 2006, and subsequently has spread throughout Europe, Africa and the Middle East (Desneux *et al*, 2011). Global tomato production exceeds 163 million tonnes and has an export value of nearly $14 billion (FAOSTAT 2013). In many tomato producing regions *T. absoluta* has reached epidemic status destroying whole crops and in some cases inflating market prices by 400% (Toesland, 2016), resulting in it being sensationally referred to as 'tomato Ebola' (The Guardian, 2016) and the 'tomatopocalypse' (Parker, 2016) The devastating effect of this pest is felt by commercialised growers and subsistence growers throughout its expanding range.

Food production and security is one of the grandest challenges facing the global community. Despite a growing global population, the number of undernourished people has declined over the last 10 years by 167 million. The overall number however, remains incredibly high at 793 million (FAO, 2016). Secure and stable food production is vital for three main reasons: (1) Nutritional health; malnutrition is the largest contributor to disease in the world causing the death of over 5 million children every year (FAO, 2014). (2) Economic security, in 2010 2.6 billion people were dependant on agriculture for their livelihoods and the 2011 global agriculture output was valued at $2.4 trillion (Alston & Pardey, 2014). (3) Social stability, with food prices being linked to civil unrest, evidence of which was observed during the 'food price spike' of 2008 (Bellemere,2015). The fact that approximately 50% of agricultural production is wasted each year (Lundquist *et al,* 2008) highlights real targets where farmers and scientists can collectively focus efforts to increase productivity. Research into mitigating losses should be further incentivised when considering limited land resources for agricultural expansion and a global population expected to hit 9 billion by 2050 requiring a 60% increase in global food production (FAO, 2014).

Life cycle

T. absoluta is a micro lepidopteran species belonging to the Gelechiidae family which consists of about 500 genera and 4700 species (Zhang, 2011). It is endemic to South America and first described in Peru in 1917 (Meyrick, 1917) with genetic studies revealing that it's expansion throughout Europe, Africa and the Middle East came from a single introduction into Eastern

Spain from central Chile in 2006 (Guillemaud *et al*., 2015). *T. absoluta is* a multivoline oligophagous herbivore of species in the Solanaceae family. It is holometabolous with 5 instar phases throughout larval development during which all herbivory is done. Larvae mine through the leaf cuticle and feed on the parenchyma creating characteristic galleries in the process (Fig 1). The development times of *T. absoluta* from egg to adult are both host plant (Table 1) and temperature dependant with life cycles ranging from 18.3 days to 115.4 days on tomato cultivars at 30°C and 10°C respectively (da Silva Krechemer, 2015)). Development time was faster on tomato plants when compared to potato (Pereyra & Sanchez, 2006)) and variation in development on different potatoes cultivars overlapped with that of a tomato cultivar (Megido *et al,* 2013).

Figure 1. Damage to tomato leaf caused by *T. absoluta.*

Table 1. Development times for life stages tomato cultivars, Bravo and Tex317 at 25C (Silva *et al*, 2015).

Life stage	Bravo (days)	Tex317 (days)
Egg	3.56	3.92
Instar I	2	2.4
Instar II	1.82	1.53
Instar III	1.27	2.33
Instar IV	3.82	3.6
Instar V	8.91	9.78
Male pupae	7.14	6.14

Female pupae	6.57	6
Total time to maturity	35.09	35.7

Mating

Reproduction is primarily sexual in *T. absoluta,* although it has also been reported to reproduce parthenogenetically (Caparros Megido, 2012). From the first day of eclosion into maturity, female *T. absoluta* attract males through the emission of a volatile pheromone (3E,8Z,11Z)-3,8,11tetradecatrien-l-yl acetate (Attygalle *et al*, 1996). Calling females unfold their antennae and remain immobile, while elevating their abdomen over the wings and exposing their ovipositor (Lee *et al*,2014), as well as their intersegmental glandular membrane positioned at the tip of their abdomen where pheromone is released. Longevity and fecundity was highest in polyandrous females. 67% of females mated more than once and oviposition peaked at 2-3 days with 72% being laid in the first 7 days. On average 74.2 eggs were produced per female with egg viability at 87.17 % (Lee *et al*,2014).

Resistance

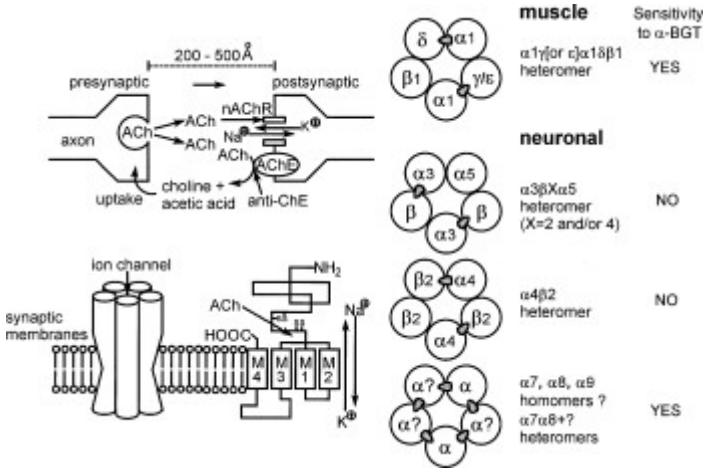
Since *T. absoluta'*s crop pest status was recognised in South America in the 1960's, it has been controlled with the use of chemical pesticides. Methamidophos (organophosphate), cartap (carbamate), avermectins and the pyrethroids deltamethrin and permethrin were initially used to control *T. absoluta* (Guedes & Picanco, 2012). With up to 36 pesticide applications in one growing season, selection was strong and resistance to these compounds quickly evolved. This has put pressure on the development and production of new pesticides with novel modes of action such as spynosyns and diamides.

Spynosyns

Spynosyns are secondary metabolites produced by the bacteria *Sachharopolyspora spinose.* Spinosad was introduced to the market in 1997 and is comprised of spinosyn A and D (Salgado, 2001). Spinosads targets the nicotinic acetylcholines receptor (nAChR), a receptor involved in rapid neurotransmission (Tomizawa & Casida, 2001), that has long been recognised as a target for insecticidal action (Gepner *et al*, 1978). The AChR is made up from 5 subunits (Fig 2) (Arias, 1997) arranged in a pentameric transmembrane complex arranged around a central channel (Tomizawa & Casida, 2001). Ten to twelve subunit genes have

been identified in a range of insects (Rinkevich & Scott, 2009) and further splicing of these genes increases subunit diversity with 18 unique transcripts from 1 subunit gene being identified in *Tribolium castaneum* (Rinkevich & Scott, 2009).

Figure 2. Structure of acetylcholine receptor showing function, ACh binding location and variation in subunit composition.



Specific binding of agonists allows the movement of ions down their electrochemical gradient depolarising the membrane (Arias, 1997). This electrochemical transduction activates many responses in the cell; contraction of muscles, neurotransmitter release, glucose use and transference of frequency encoded information for memory and learning processes. Loss of function of the receptor can be brought about by competitive antagonists which compete with ACh for binding sites. The binding site for spinosad is the nAChR α6 subunit.

High levels of spinosad resistance have been reported in field populations of *T. absoluta* (Campos *et al*, 2015). A G275E amino acid substitution in the α6 subunit was identified in resistant selected strains with resistant field populations being 67.5% homozygous for the mutation (Silva *et al*, 2016). The same mutation was observed in *F. occidentalis* with *a* 350000-fold increase in resistance to spinosad (Puinean *et al*, 2013). Another mechanism of α6 alteration in *T. absoluta* is exon skipping, the exclusion of discrete regions of RNA that are usually spliced together to form mature RNA. 100% of clones from resistance-selected lines expressed protein transcripts lacking the normally mutually exclusive exon 3a or 3b which result in the loss of an ACh binding loop in the α6 subunit. Instead of genetic changes resulting

in non-specific binding sites for spinosad, methylation may have played a role (Shukla *et al*, 2011). The methylation ratio of the selected strain were 30% compared to 0% and 0.5% in the unselected strains at a CpG 21bp downstream of exon 3a. This position matched sequences thought to be targets of splice binding proteins. Another co-factor however is the fact that transcripts encoding likely splice factor proteins were significantly downregulated (Berger *et al*, 2016).

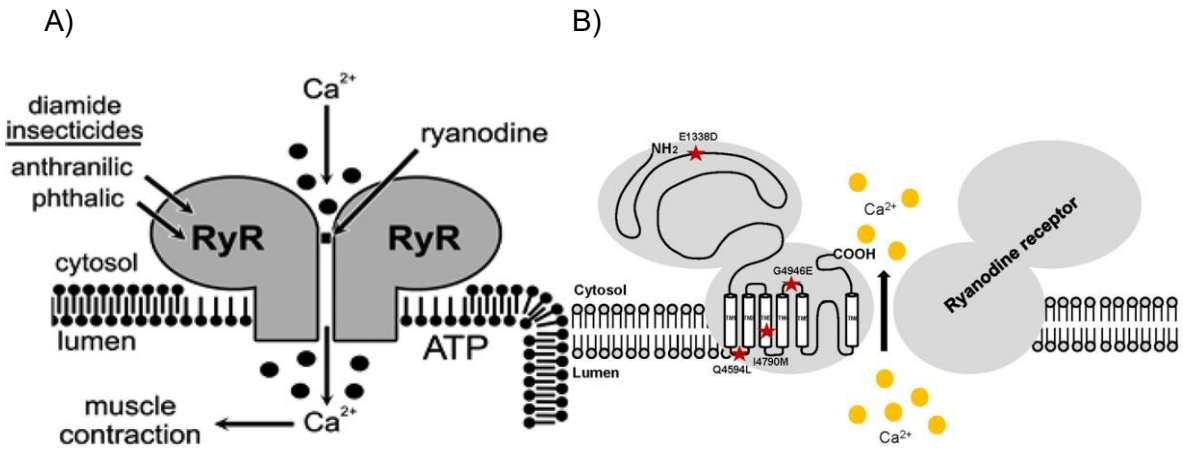
Diamides

Diamides were brought to the market in 1997 and have an excellent profile against several economically important pest species. By 2013 global turnover of this pesticide class was

$1.2 billion (Sparkes & Nauen, 2015). Diamides have a novel mode of action, targeting the ryanodine receptor (RyR) involved in Ca2+ signalling. Ca2+ regulates many processes in eukaryotic cells including gene regulation, membrane transport processes, hormone biosynthesis, muscle contraction, neurotransmitter release and apoptosis (Lummen, 2012).

RyRs are homotetrameric proteins that surround a large release channel that regulates Ca^{2+} from intra cellular stores in the endo/sarcoplasmic reticulum (Fig 3). They respond to surface membrane action potentials and/or change in concentration of a secondary messengers and are linked to muscle excitation-contraction coupling (Meissner, 1994). The RyR is named after a natural alkaloid ryanodine (Ry) which is a natural pesticide and was important in the RyR's characterisation. Ryanodine inhibits both mammalian and insect RyR by locking channels in a sub-conductance state after activation by plasma membrane voltage-gated Ca2+ channels. In contrast, insect specific diamides directly activate RyRs. Both mechanisms have effectively the same result, depleting internal Ca2+ stores interfering with muscle contraction and ultimately resulting in paralysis (Cordova *et al*, 2006). In spite of chlorantraniliproles great lepidopteran profile resistance has recently evolved in the field. In 2010-2011 populations of *Plutella xylostella* from Southern China were suggested to have developed target site resistance resulting in 2000-fold resistance increase (Wang & Wu, 2012). A mutation resulting in the amino acid substitution G4946E was subsequently shown to confer resistance in Thailand, Philippine and Chinese populations (Guo, *et al*, 2014, Trockza *et al*, 2012). Resistance to diamides has also been reported in *T. absoluta* (Roditakis *et al*, 2015). Italian populations showed 2,414 fold resistance and in resistant Greek populations the amino acid substitutions G4946V and I4790M were identified as potentially being causal. These substitutions were introduced into the RyR of *Drosophila* by CRISPR/Cas9 genome editing and confirmed that G4946V contributes to high chlorantraniliprole and flubendiamide resistance (Douris *et al*, 2017).

Figure 3. A) Ryanodine receptor showing ryanodine and diamide binding sites (Isaacs *et al*, 2012). B) Ryanodine receptor displaying positions of mutations in resistant strains of *P. xylostella.*



Control

Insecticide resistance is an inevitable evolutionary consequence of massive selection pressures applied by chemical agents targeting pest populations. Insect resistance management (IRM) strategies are used within a larger integrated pest management (IPM) system with the goal of protecting crops whilst preventing or slowing resistance from occurring. The current method of control for *T. absoluta* in the U.K. employs IPM and the combinatory approach has three basic components: (1) Monitoring pest populations in the field for changes in density; (Toesland, 2016) focusing on economic injury levels, and (3) integrating multiple control strategies. These practices can provide crop protection whilst reducing pesticide use slowing resistance (IRAC, 2007).

Monitoring is the first step in IRM, informing when pesticides should be applied based on numbers of pests and natural enemies preventing unnecessary applications. It should also occur after application to assess efficacy. The threshold value for a population density where control should be used can be defined as sufficient numbers of pest to cause economic yield losses of a greater value than the cost of the pesticide plus its application.

Once pest densities reach this threshold integrated approaches to control are implemented. This does include the use of pesticides but limits there use by incorporating biological control agents, better farming practices, crop rotation, use of resistant crop varieties, chemical attractants and deterrents. When the pesticides are applied, they should be preferentially selected on their specificity as broad spectrum pesticides may have off-target effects on

beneficial insects such as pollinators or biocontrols. Also, to minimise resistance, alternations of pesticides should be considered, the use of pesticides that have different modes of action, targeting different sites within the pest reducing overall selection pressure at each target site. Finally, it is important to destroy any crop residue where pests may persist minimising pest establishment in the next growing season. It is especially important where any remaining pests are resistant causing a founder effect proliferating new populations with population level resistance.

IRM and IPM are vital strategies in the control of *T. absoluta* in the U.K., especially as currently there are limited effective pesticide classes left with the two most used being spinosad and diamides, which are effective at supressing populations without harming off target beneficial insects. *T. absoluta* populations are monitored early in the growing season by delta traps in which a pheromone attractant lures males to sticky paper. Distributing these throughout the crop can give a picture of the numbers and spread of the pest throughout the crop. The next step in the IPM is the introduction of the biocontrol agent, *Macrolophus pygmaeus. M. pygmaeus* feeds on caterpillars through the leaf but is slow to establish a useful population size. Pest population growth must also be slowed down. This is done by application of spinosad through the irrigation, just after mines are visible about 45 weeks into the crop. If biocontrol is ineffective a second round of pesticide application is required. This time chlorantraniliprole is used, and having a different mode of action to spinosad should reduce the selection for resistance. At the end of the growing season it is important to eliminate any remaining reservoirs of the pest thereby delaying establishment in the next growing season. This is done with indoxacarb. Indoxacarb penetrates the leaf and kills the larvae. It has a different mode of action to spinosad and chlorantraniliprole but effects pollinators and biocontrol agents so can only be used at the end of the season.

This IPM approach has had much success in limiting the pest status of *T. absoluta* but in spite of the IRM strategies included in the IPM protocols, resistance to spinosad and chlorantraniliprole have been confirmed. IPM therefore has to be well informed through understanding the molecular mechanisms of insecticide resistance and their frequencies within populations. They also need to be flexible and adapt in accordance with pest evolution. This involves appropriate application of pesticides, the search for new classes of chemicals with new modes of action and the search for new and effective biocontrols compatible with new and existing chemistries.

Objectives

The main aims of this research project are to characterise the molecular basis of resistance in *Tuta absoluta* to contemporary pesticide classes used in the field, with specific emphasis on spinosad and chlorantraniliprole. Once the molecular basis for these pesticides has been identified DNA based diagnostics will be developed with the aim of replacing the classical leaf dip assays. Although leaf dip assays are the 'standard' test for resistance they can take months to complete. Samples received from the field need to be reared to suitable population size and assays taking 72 hours completed. DNA based diagnostics would be able to reduce this time to ~24 hours giving the grower 'real time' population level resistance data and so would be invaluable in informing IPM strategies and thus prevent the miss use of pesticides, slowing the evolution of resistance in the field and maintaining the pesticides in the arsenal of useful control agents.

As this is an applied study the aims of this project must stay flexible and track changes as they occur in the grow houses around the U.K., this includes changes in levels of resistance. Often the evolution of resistance occurs as a trade-off with fitness so discontinuation of pesticide use may cause population level susceptibility to return, returning efficacy of the pesticide. This project also aims to monitor new pest control products. This year the introduction of Isonet's mating disruptors has appeared to be a massive success. However personal communications with industry consultants has revealed that in some grow houses implementing this control measure resurgence of the pest has occurred. This could be due to one of two reasons; firstly, *T. absoluta* has the ability to reproduce parthenogenetically. The lack of necessity for sexual reproduction may select for obligate parthenogenesis maintaining populations at pest status. Secondly, selection for resistance to mating disruption. This could be due to reasons such as selection of variant phenotype in pheromone signature and detection or other behavioural strategies. Further aims of this study then are to select for parthenogenesis in *T. absoluta* populations as well as to confirm if sexually reproducing *T. absoluta* have the ability to resist this method of control and if so isolate the mechanism by which they do this.

To summarise, *T. absoluta* has become resistant to two of the major compounds used to control it the short term objectives are to:

1. Test for resistance in field populations

2. **Test for mutations which may confer target site resistance and functionally validate**

3. **Create DNA based diagnostics that can rapidly assess frequency and location of resistance**

4. **Test for metabolic resistance**

5. **Identify candidate detoxification genes**

The project's long-term aims are:

6. **Continue to monitor resistance frequencies and locations of U.K. populations**

7. **Test usefulness of synergists to overcome metabolic resistance**

8. **Evaluate potential for *T. absoluta* to overcome novel pest control strategies**

Materials and methods

Rearing T. absoluta.

T. absoluta larvae were collected from tomato grow houses in the U.K., R & L Holt and sons, Evesham (EVH strain) and Bezemer and sons, North Yorkshire (BS strain). A third strain was received from Rothamsted (TA1). They were housed in bug domes in controlled environment rooms under a 16:8 hour light dark cycle at 25°C and 55% relative humidity.

Larvae were placed on potted tomato plants (variety Money maker) with additional plants being added as required. Upon eclosion ~50 adults were collected using a bug vacuum (Backyard safari, U.K.) and transferred to a new bug dome with a fresh tomato plant.

Leaf-dip Bioassay

Leaf dip bioassays were conducted in accordance with IRAC 22 protocol. LC_{50} values were calculated using probit analysis (Microsoft excel).

RNA extraction

Larvae were snap frozen in liquid nitrogen to prevent any stress state gene expression response. RNA was then extracted using Bio Line isolate II RNA mini kit according to their protocol.

cDNA synthesis

cDNA was synthesised using the Maxima H Minus First Strand cDNA Synthesis Kit (ThermoScientific, USA) according to their protocol.

PCR

PCR was conducted using ThermoScientific DreamTaq Green PCR Master Mix (2X) protocol.

PCR purification

PCR products were purified with GeneJET PCR purification kit (Thermo Scientific) in accordance with their protocol. Purified DNA was then quantified using a spectrophotometry (NanoDrop, Thermo-Fisher).

Sequencing

All sequencing was completed by Eurofins Genomics with samples submitted in accordance with their guidelines.

Metabolic resistance

To test for metabolic resistance strains with different resistance phenotypes (Table 2) had their transcriptomes sequenced, allowing differentially expressed genes to be identified, specifically genes associated with xenobiotic metabolism. The expression of these genes can then be quantitatively validated using qPCR and their causality verified through their inhibition using synergists or through functional validation by transgenic expression.

Transcriptomes were sequenced using next generation Illumina sequencing from four populations. These transcripts were then *de novo* assembled using Trinity (Grabherr et al, 2011). Differentially expressed genes were identified using DEseq (Anders, 2010) and edgeR (Robinson *et al*, 2010). For each software package of each comparison there were in excess

of 100 000 differentially expressed genes. To isolate candidate genes from these lists the genes that didn't meet the following criteria were excluded: (1) Present in both DEseq and edgeR. (2) Adjusted p values

< 0.05. (3) Fold change was >±2. (4) Had a blast annotation. (5) Present in all three comparisons. These criteria returned a list that had 368 genes (Fig 9). From here a literature search was undertaken for the name of each gene and a new column assigned to the spreadsheet for the function of that gene. This column was then searched through with the search terms in column 1 of table 6. The genes who's expression were in a correlated order compared to the resistance level of the strains were then put forward to the final list of candidate genes to be quantitatively validated using qPCR.

Table 2. Populations used for transcriptome analysis.

Population	Location	Resistance (ppm)
SUS	Murcia, Spain	LC50 = 0.15
MUR	Murcia, Spain	LC50 = 1.6
SIC	Sicily,Italy	LC50 = 47.6
RES	SIC selected	LC30 = 250

Results

Target site resistance to spinosad

Leaf dip assays were conducted to generate LC_{50} values for the 2016 BS strain (Table 3). Spinosad concentrations ranged from 0 (control) to 2500ppm spanning the field dose rate of 200 ppm. In these bioassays the 2016 BS strain exhibited marked resistance, indeed it was so high (13.3% mortality at 500 ppm) that it was impossible to calculate an LC_{50} value.

The EVH 2016 strain was compared to the a strain collected at the same site a year earlier that had shown storng resistance to spinosad (Table 4, Fig 2). No spinosad had been applied in the field during this period. EVH 2016 were compared to EVH 2015 as well as a positive

control (TA1) to test if susceptibility had returned to the population. Mortality had increased slightly, from 30.43% to 41.67% in the 200 ppm treatment and 33.33 to 41.67 in the 500 ppm however, compared to TA1 the population still remained resistant.

These populations were then tested for target site resistance. Gel electrophoresis of amplified nAChR transcripts revealed variation in amplicon size between the susceptible TA1 strain and the two resistant BS and EVH strains (Fig 3). These were then sequenced and analysed. Comparison of the sequences sequence revealed 109 bp missing from the resistant BS and EVH strains (Fig 4). These missing base pairs coincided with exon 4 of the full transcript. The result of this was a frame shift in the exon 5 region and the introduction of a premature stop codon (PSC) 146 bp after the start of exon 5 (Fig 5). This was not present in TA1 which displayed a transcript with the full complement of 12 exons.

Target site resistance chlorantraniliprole

Bioassays for diamide resistance showed LC_{50} value at 33.063 ppm with the 95% confidence intervals ranging from 11.17 ppm to 97.9 ppm for BS (Table 5) and 9.5 ppm for EVH. These were both higher than the field application rate of 1.4 ppm The RyR transcripts were amplified with the regions surrounding the known binding site of chlorantraniliprole being targeted (Fig 6). Sequencing of the amplicons showed no missense mutations when compared to TA1. Low level heterozygosity was indicated in EVH strain at bp 1503 which would result in a G to T SNP translating to a G4946V substitution (Fig 7). However, selection of this population at 1 ppm appeared to show no increase in the frequency of this potential resistance mutation (Fig 8).

Metabolic resistance to chlorantraniliprole

Analysis of differentially expressed transcripts between strains was conducted using two software packages, DEseq and edgeR. From the 368 differentially expressed transcripts (Fig 9) six were selected (Table 7); Three cytochrome P450's, gelsolin, a short-chain dehydrogenase, and nose resistant protein. These were selected because they fitted the criteria described in the methods and all had increasing/decreasing fold changes in expression in the correct order corresponding to the resistance levels in the strains tested.
These values remain to be validated by qPCR.

Table 3. Leaf dip assay testing spinosad resistance of BS.

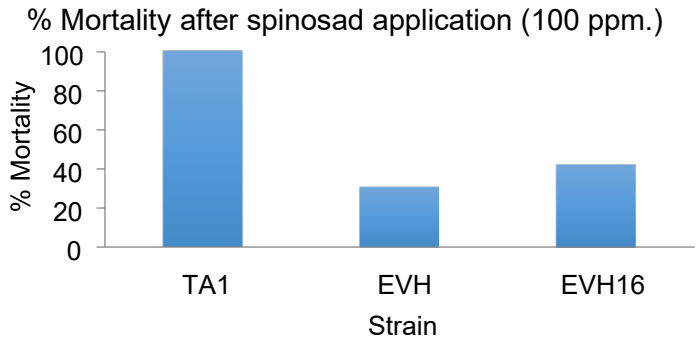
Dose (PPM)	Mortality of replicate (%) 1	2	3	4	5	6	Average mortality (%)
0	0	0	0	0	0	0	0.00
4	0	0	0	0	0	0	0.00
20	0	0	0	0	0	0	0.00
100	0	0	0	20	0	0	3.33
500	0	0	20	0	20	40	13.33
2500	20	20	0	0	20	0	10.00

Table 4. Spinosad discriminating dose leaf dip assay for TA1, EVH and EVH16.

Strain	Dose (PPM)	Mortality of replicate (%) 1	2	3	Average mortality (%)
TA1	0	28.6	25	50	34.53
TA1	100	100	100	100	100
TA1	500	100	100	100	100
EVH	0	25	0	0	8.33
EVH	100	28.6	12.7	50	30.43
EVH	500	12.5	37.5	50	33.33
EVH16	0	0	0	0	0
EVH16	100	50	50	25	41.67
EVH16	500	25	25	75	41.67

Figure 4. Percent mortality after spinosad application of TA1 EVH 2015 and EVH 2016 at a) 100 ppm and b) 500 ppm.

a)



b)

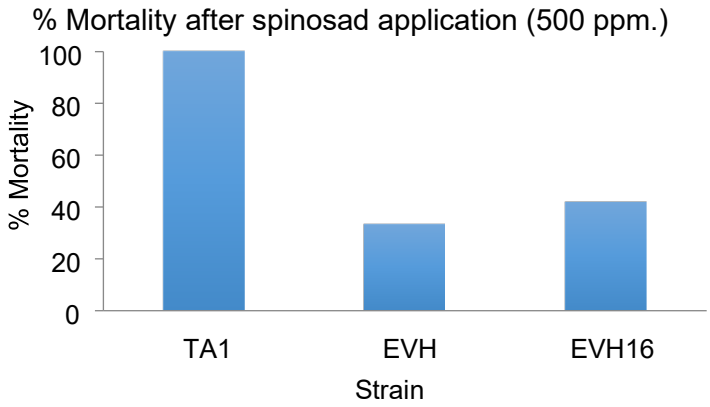


Figure 5. Gel picture showing the amplification of the nAChR receptor for TA1 BS and EVH. Disparity In size between the three bands indicates variation in nAChR transcript size between resistant and susceptible strains.

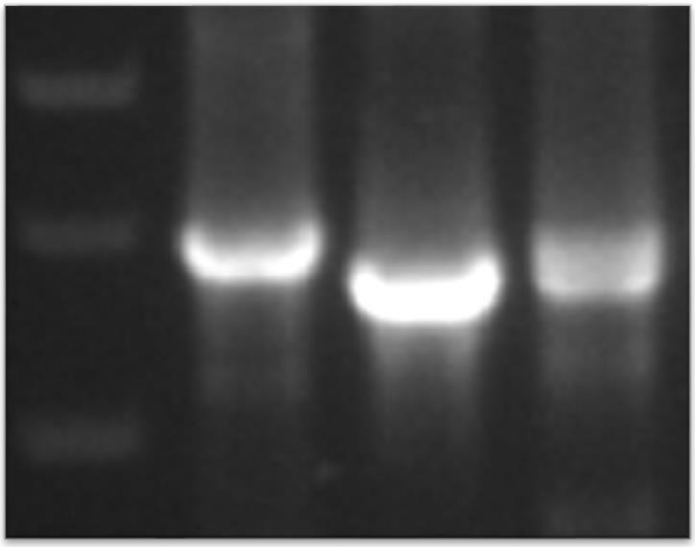


Figure 6. Eurofins Sequencing results showing excluded transcript region (exon4) of BS and EVH compared to TA1.

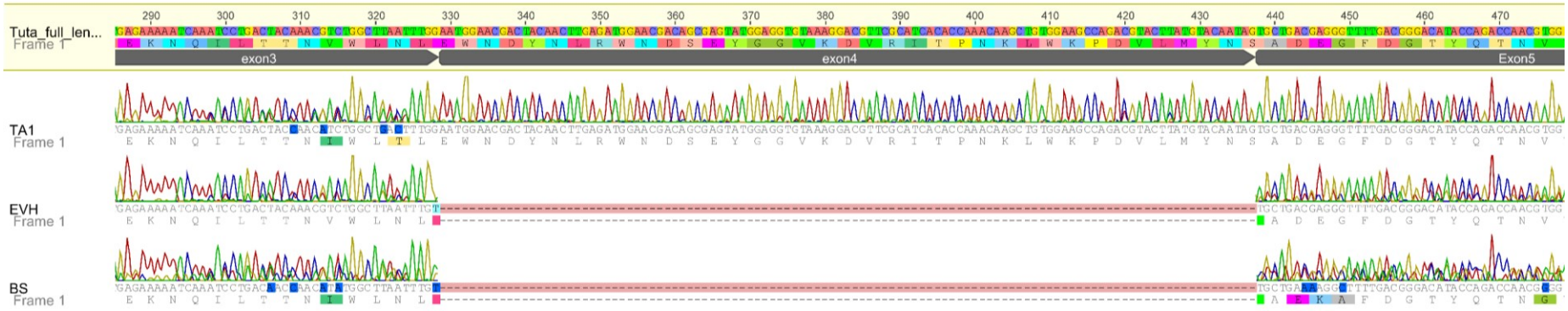
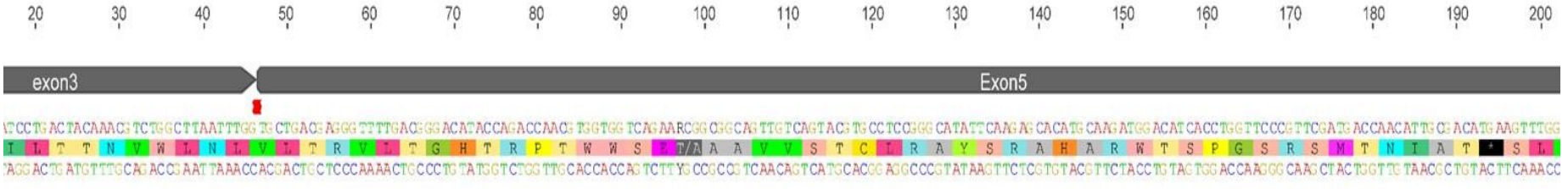


Figure 7. Position of premature stop codon (black rectangle) with the exclusion of exon 4.



RYR

Table 5. leaf dip assay with chlorantraniliprole on BS. Chlorantraniliprole concentrations ranged from 0 (control) to 4000 ppm. The LC_{50} value was calculated to be 33.063 ppm with the 95% confidence intervals ranging from 11.17 ppm to 97.9 ppm.

Concentration (ppm)	Mortality (%)
0	3.4
0.4	3.3
4	16.7
40	53.3
400	93.3
4000	96.6

Figure 8. Gel picture showing successful amplification of *T.absoluta* RyR cDNA.

Figure 9. Eurofins sequencing for EVH 2016 candidate target site substitution position.

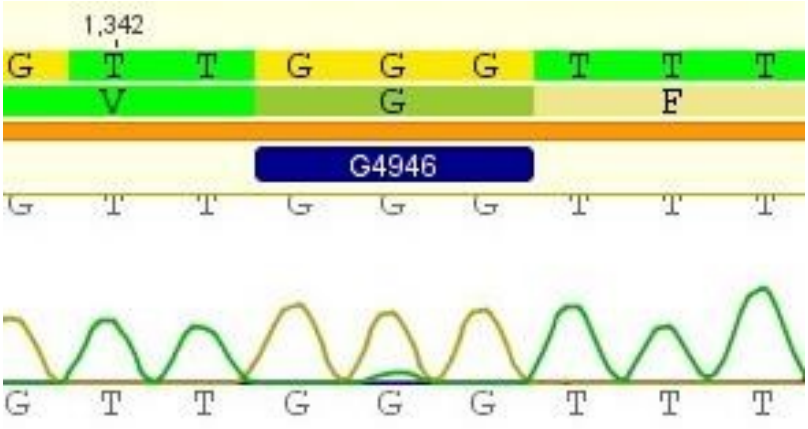


Figure 10. Sequencing results of RyR G4946 region of EVH 2016 strain selected at 1 ppm

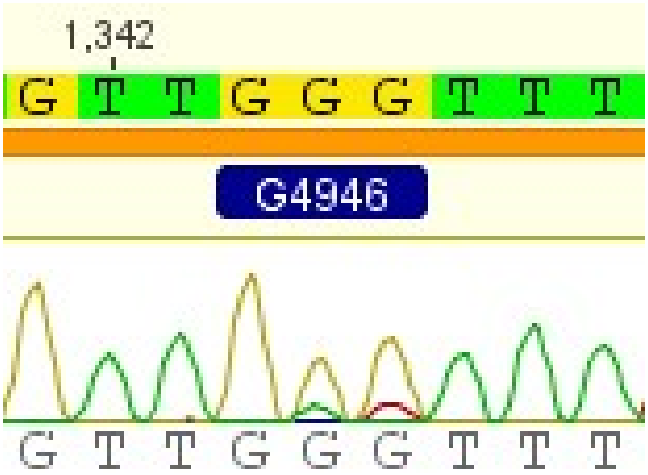


Figure 11. Number of differentially expressed genes in DEseq, edgeR and DEseq vs edgeR analysis.

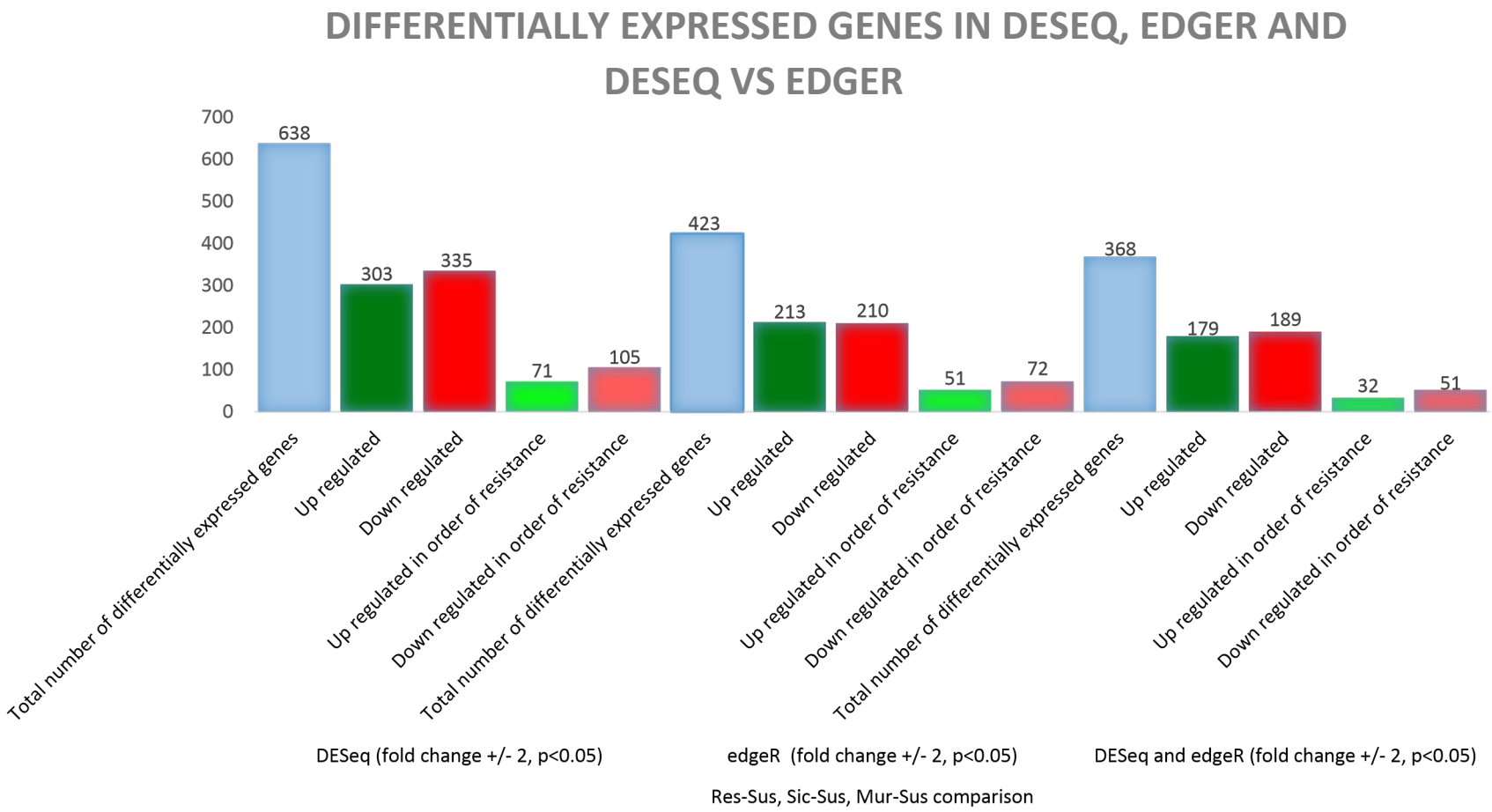


Table 6. Number of hits for search terms in genes fulfilling selection criteria (see methods).

	Res,Sic,Mur-Sus				
Search terms	Differentially regulated	Up regulated	Up regulated in order of resistance	Down regulated	Down regulated in order of
P450	34	33	6	3	1
Glutationine	2	1	0	1	0
GST	0	0	0	0	0
Transferase	17	6	0	11	2
Esterase	3	2	1	1	0
Chitin	6	2	1	4	1
Cuticular	1	1	0	0	0
ABC transporters	0	0	0	0	0
transporter	9	1	0	8	2
transport	22	5	0	17	8
UDP	4	4	0	0	0
Facilitator	1	0	0	1	1

Table 7. Final selection of candidate genes including fold changes.

			Deseq			edgeR		
Transcript ID	Name	Full transcript (Y/N)	Mur-Sus	Sis-Sus	Res-Sus	Mur-Sus	Sis-Sus	Res-Sus
TR28655	Gelsolin	Y	8.90	9.02	22.88	21.61	23.45	78.21
TR12902	cytochrome P450	N	3.68	3.93	5.11	3.70	3.99	5.31
TR21047	short-chain dehydrogenase	Y	3.47	3.48	3.68	3.56	3.64	3.91
TR24445	cytochrome P450	Y	6.18	5.49	8.21	6.47	5.73	8.85
TR20974	cytochrome P450	Y	6.86	6.17	6.88	7.49	6.65	7.69
TR80162	Nose resistant to fluoxetine protein 6 like (*Bombyx mori*)	N	0.17	0.00	0.00	0.12	0.00	0.00

Discussion

Insects have a remarkable ability to evolve pesticide resistance and have done so to most chemistries applied to them. This has resulted in the necessity of new chemistries and strategies aimed at preventing and slowing evolution of this resistance such as those employed in current IPM strategies. *T. absoluta* is no exception, having invaded Europe armed with resistance to pyrethroids, organophosphates and carbamides it has since developed spinosad and chlorantraniliprole resistance. Previous work on U.K. populations has described spinosad resistance and this report not only confirms this but also describes an alternative mechanism for this resistance with a rapid method for its detection. It also indicates the possibility of low level resistance to chlorantraniliprole and identifies potential candidate genes for this resistance. These findings have a serious implication, potentially rendering current IPM strategies used by many growers in the U.K. redundant.

Spinosad resistance

The redundancy of spinosad was confirmed from the bioassay results in both EVH and BS strains and analysis of the nAChR gene transcript clearly showed exon 4 had been skipped when compared to the full complement of exons in the susceptible TA1 strain. Full length cDNA from the susceptible TA1 strain had an open reading frame of 1530 bp. The exclusion of exon 4 from this reading frame resulted in a 110 bp deletion and a shift in the reading frame resulting in the introduction of a PSC in exon 5 at position 584. RNA translation would produce a massively truncated protein. It would include exon 3, part of the acetylcholine binding site but the translated polypeptide would be missing amino acid residues 110 to 146 and from residue 196 to 540, the total number of amino acids in the protein would be 160 and would structurally render the protein non-functional as important ligand binding loops present in these regions would be missing.

More likely, however, is that nonsense mediated decay (NMD) would hydrolyse the RNA before it was translated to a non-functional protein. NMD is a surveillance pathway that prevents expression of genes with PSC translating into incomplete polypeptides. Therefore the structure of the resistant nAChR pentameric transmembrane protein would lack the α6 subunit altogether and it would likely be replaced with another nAChR subunit gene present in the *T. absoluta* genome. It would be interesting to use qPCR compare the expression of the alternative subunit genes between susceptible and resistant to identify a candidate replacement of the α6 subunit and test how this in turn affected fitness.

This result however does offer the ability to create an RNA-based diagnostic capable of identifying the size difference between full nAChR transcripts and those with exon 4 skipping based on PCR and gel electrophoresis. This would drastically reduce the time taken to assess frequencies and locations of resistant phenotypes from ~2 months to 24-48 hours. Before this could be considered a robust diagnostic a national level assessment of resistance mechanisms must take place to ensure this is the common mechanism throughout the U.K. Also further research into the underlying cause of the exon skipping event may provide an even cheaper and quicker method. If a causal mutation in genomic DNA could be identified then samples could be collected by growers stored in ethanol and posted for immediate analysis.

Alternative splicing is a ubiquitous mechanism for explaining the expansive proteomic diversity present in organisms from the limited number of genes in eukaryotic genomes. It is believed to occur in over 60% of eukaryotic transcripts and miss/alternative splicing is vital to understanding many pathologies. Although the 'players' in the system have been reasonably well described predicting which *cis*- or *trans-acting* elements (sequences in DNA, or proteins that make up the spliceosome and bind to those sequences) or combinations of elements in this degenerate system interact and cause an alternative splicing event is not fully understood. Further investigation into this phenomenon in *T. absoluta* offer a window into a process that would not only directly aid the objectives of this research but also the wider biological community working on alternative splicing. The simultaneous extraction of gDNA and RNA of resistant and susceptible individuals would give insight into how genomic changes alter translation and transcription, including co-translational editing by splicing of the nAChR α6 gene.

Chlorantraniliprole resistance

Chlorantraniliprole resistance is likely in the U.K. based on reports of application failure coming from communications with growers and bioassay results supporting this. Bioassays for chlorantraniliprole resistance indicated low-level resistance (LC 50 at 33 ppm and 9.5 ppm for BS and EVH respectively) although with large 95% confidence limits caution must be taken when interpreting the results. The amplification and sequencing of the RyR showed no missense mutations indicating no target site resistance in the BS strain, however, when pooled individuals from EVH were sequenced a small second peak in the G4946 region was observed. This region has previously been associated with

chlorantraniliprole resistance. The peak, translating to a G4946V substitution, was present suggesting the possibility of a low frequency resistance mutation in the population. This region was previously associated with the resistant phenotype of the diamond back moth, *Plutlella xylostella* (Trockza *et al*, 2012). The population was then selected at 1 ppm and the region re-analysed. The result was the same and the mutation did not seem to have increased in frequency. Further selection is underway with healthy populations having survived a higher (5 ppm) selection.

Metabolic resistance is another potential route to resistance to this compound.

From the transcriptomic analysis of the Sus, Mur, Sic and Res strains 6 genes were selected as candidate resistance genes. Gelsolin was a tentative candidate, as it is involved in the assembly and dis-assembly of actin filaments which play an important role in muscle contraction. In a high calcium environment, an environment induced by chlorantraniliproles excitement of the RyR, it is activated and severs actin microfilaments. Nose resistant to fluoxetine protein 6-like is another tentative candidate, as it is involved in the uptake of molecules including xenobiotics from the intestine to surrounding tissues. Therefore its down regulation may prevent uptake of chlorantraniliprole. Short-chain dehydrogenase have previously been shown to be overexpressed in lepidopterans resistant to organophosphates and were believe to be pivotal in toxicogenomic response of *C. elegans* to the toxic compound PCB. However, perhaps the most likely candidates are the three cytochrome P450's. P450's are a large family of well-characterised detoxification enzymes. They are commonly upregulated in many organisms for xenobiotic metabolisms. Within this population, they remain to be tested for causal links to metabolic resistance. This can be done in vivo through either their expression in transgenic Drosophila lines or in vitro in insect cell lines. Alternatively further validation could be done by inhibiting their function with inhibitors such as piperonyl butoxide.

Knowledge and Technology Transfer

- The project has been strongly supported by the British Tomato Growers' Association Technical Committee since it was originally proposed.
- The student took the initiative to work with the tomato industry in the months leading up to the start of this project.
- The student visited leading commercial tomato growers during that time and established laboratory cultures of *Tuta absoluta* pests that were thought to be resistant to important insecticides. Results of this early testing provided information that influenced several growers' pest control strategies.
- In the first year of the project, the student joined the TGA's 'Tuta absoluta Think Tank' that has met at a commercial nursery and communicated by conference call and e-mail.
- The student has subsequently made visits to key tomato growers, shared results, and modified the research programme to take into account the two-way flow of information.

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Appendix 1

Dissertation plan

Proposed Table of Contents - (completion status/estimated completion date)

1. General Introduction

1.1 - Food security (Completed)

1.2 - Insect as pests (Completed)

1.3 - *Tuta absoluta* (Completed)

1.4 - Pest management strategies (Completed)

1.5 -Previous control methods for *T. absoluta* (Completed)

1.6 -Current Methods for control (Completed)

1.6.1 -Spinosyns (Completed)

1.6.2 – Diamides (Completed)

1.6.3 - Mating disruption (Completed)

1.7 - Resistance mechanisms (Completed)

1.7.1 -Target site (Completed)

1.7.2 – Metabolic (Completed)

1.8 - Current resistance to contemporary control (Completed)

1.9 - Objectives (Mostly completed but need to track changes in the field as they occur)

2. General materials and methods

2.1 *T. absoluta* populations (Completed)

2.2 RNA extraction (Needs expanding – completed by Dec 2017)

2.3 cDNA synthesis (Needs expanding – completed by Dec 2017)

2.4 PCR (Needs expanding – completed by Dec 2017)

2.5 qPCR (Needs expanding – completed by Dec 2017)

2.6 PCR purification (Needs expanding – completed by Dec 2017)

2.7 Sequencing (Needs expanding – completed by Dec 2017)

2.8 gDNA extraction (Needs expanding – completed by Dec 2017)

2.9 Transcriptome analysis (Needs expanding – completed by June 2018)

2.10 Selection experiments (Expected completion – June 2018)

2.11 Genome assembly (Expected completion – Dec 2018)

3. Resistance to Spinosad

3.1 Introduction (Completed)

3.2 Methods (Completed)

3.3 Spinosad Bioassays (Completed 2015 & 2016 strains. 2017 + strains to complete)

3.4 Amplification and sequencing of α6 subunit transcripts (cDNA) (Completed 2015 & 2016 strains.

3.5 2017 + strains to complete)

3.6 3.5 Amplification and sequencing of α6 gDNA intronic regions and identification of mutations linked to exon 4 skipping (Partially sequenced for TA1, BS & EVH pooled samples. Replication required for individuals for each strain. Dec 2017).

3.7 3.6 Development of DNA diagnostics (RNA Diagnostic developed. DNA diagnostic dependant on gDNA sequencing of individuals. June 2018)

3.8 3.7 Analysis of results (Dec 2018)

3.9 3.8 Discussion (Dec 2018)

4. Resistance to Chlorantraniliprole

4.1 Introduction (Completed)

4.2 Methods (Expand on transcriptome methods. Dec 2017)

4.3 Chlorantraniliprole Bioassays (Completed 2017 strains. Awaiting further field strains)

4.4 Amplification and sequencing of RyR (Completed 2017 strains. Awaiting further field/selected strains)

4.5 Illumina sequencing of chlorantraniliprole resistant and susceptible strains (Completed)

4.6 Transcriptome assembly and gene annotation (Completed)

4.7 Identification of differentially expressed genes (Completed)

4.8 qPCR validation of differentially expressed genes (Some genes qPCR validated, further genes may need testing. Dec 2017)

4.5 Discussion (June 2018)

5. Genome sequencing and assembly

5.1 Introduction (Dec 2018)

5.2 Methods (Dec 2018)

5.3 Inbreeding strains (completed)

5.4 DNA extraction (Oct 2017)

5.5 Sequencing (June 2018)

5.6 Assembly (Dec 2018)

5.7 Gene annotation and manual curation of specific gene families (Dec 2018 +)

5.9 Reassembly of RNAseq data generated in chapter 4 (Dec 2018 +)

5.10 Discussion (June 2019)

6. Functional validation of resistance genes and mutations

6.1 Introduction (Dec 2019)

6.2 Methods (Dec 2019)

6.3 Possible approaches:

- Expression of genes in Drosophila
- Genome editing in Drosophila and/or *T. absoluta* using CRISPR-CAS (June 2019)
- Recombinant enzyme expression
- Expression of insect receptors in Xenopus oocytes

6.4 Results (June 2019)

6.5 Discussion (Dec 2019)

7. Parthenogenesis evolution

7.1 Introduction (Dec 2018)

7.2 Mating disruption (Dec 2018)

7.3 Selection experiments

Comparison of frequency of parthenogenesis between resistant and susceptible populations (June 2018)

Select virgin lines for;

individuals with highest asexual fecundity (Start Jan 2018)

sex ratios shifted (Start Jan 2018)

7.4 Results

7.5 Discussion

8. General Discussion

7.1 Resistance to spinosad (Dec 2019)

7.2 Resistance to Chlorantraniliprole (Dec 2019)

7.3 Genome and Transcriptome analysis (Dec 2019)

7.4 Diagnostic development (Dec 2019)

7.5 Evolution of parthenogenesis (Dec 2019)

7.6 Future work (Dec 2019)

