# Profiling *Diuraphis noxia* (Hemiptera: Aphididae) Transcript Expression of the Biotypes SA1 and SAM Feeding on Various *Triticum aestivum* Varieties

N. F. V. Burger,[1,2] E. Venter,[3] and A.-M. Botha[1,4]

[1]Genetics Department, Stellenbosch University, Private Bag X1, Matieland 7601, South Africa (nfvburger@sun.ac.za; ambo@su- n.ac.za), [2]Genetics Department, University of Pretoria, Hillcrest, Pretoria, South Africa, department of Botany and Plant Biotechnology, University of Johannesburg, Auckland Park, Johannesburg, South Africa (eduardv@uj.ac.za), and Corresponding author, e-mail: ambo@sun.ac.za

**Abstract**

The intimate relationship between an aphid and its host is mediated by the composition of the secreted saliva. In the present study, aphid heads were sampled and transcript profiling conducted after aphids were fed on their preference host and transferred to a variety of preference and nonpreference hosts. It was found that the virulent *Diuraphis noxia* (Kurdjumov) (Hemiptera: Aphididae) biotype SAM was able to selectively up-regulate more transcripts when confronted with feeding on a variety of hosts, than was the case with the less virulent D. *noxia* biotype SA1, suggesting increased genomic regulation when coping with a stressful environment. Collectively, the observed transcriptomic changes are supported by previous findings that host changes induce significant changes in the proteome of phytophagous hemipterans, unlike in many other entomopha- gous generalist species. The current data suggest that highly specialized hemipterans may be able to counter plant defenses with inducible salivary transcripts with resulting protein biosynthesis, as demonstrated here.

**Key words:** plant-insect interaction, Russian wheat aphid, *Diuraphis noxia*, cDNA-AFLP, salivary expression profile

Phloem-feeding aphids represent the largest group of insect pests (~4,700 species) owing to their enormous reproductive potential (Davis 2012). This is also true in the case of *Diuraphis noxia* (Kurdjumov) (Hemiptera: Aphididae), with reports of economic losses amounting to roughly US$800 million over a period of 10 yr in the United States alone (not accounting for other predominantly affected regions, i.e., Argentina and South Africa; Morrison and Peairs 1998). Aphids have evolved a more intimate association with their plant hosts than herbivorous insects and elicit the expression of plant genes more commonly associated with bacterial and fungal pathogen attack (Moran and Thompson 2001; Boyko et al. 2006; Botha et al. 2010, 2014a,b; Smith et al. 2010).

Resistant wheat varieties activate an arsenal of defensive pathways upon feeding by *Diuraphis noxia* defined by the presence of Dn *(Diuraphis noxia)* genes that confers resistance against *D. noxia* (Botha et al. 2014a). Aphids feeding on wheat containing these genes evoke resistant responses, phenotypically categorized by evaluating both host and aphid, manifesting as either antibiosis, where the plant reduces the reproductive fitness of aphids feeding on it; tolerance, which is seen as a lack of plant biomass reduction despite continuous feeding; or antixenosis, the nonpreference of a cultivar as host (Painter 1951, 1958; Smith et al. 1992; Unger and Quisenberry 1997). Resistant cultivars may exhibit combinations of these categories of resistance (Smith et al. 1992, Haile et al. 1999), and the genetic background in which a specific *Dn* gene is bred also plays a role in the successful establishment of a resistant phenotype (Van der Westhuizen et al. 1998a,b). The interaction between wheat *(Triticum aestivum* L.) and *D. noxia* has been of major interest to researchers in the field of biotic plant

resistance, particularly the identity and function of *D. noxia* effectors and wheat *Dn* resistance genes.

For susceptible wheat varieties, feeding of *D. noxia* causes chlorotic and longitudinal streaking in addition to rolling of the wheat leaf. Consequently, susceptible wheat leaves exhibit head trapping and reduced leaf chlorophyll content, leading to a lowered photosynthetic capacity (Burd and Burton 1992; Burd and Elliott 1996; Heng-Moss et al. 2003; Wang et al. 2004a,b; Botha et al. 2006). Under extreme levels of *D. noxia* infestation the biochemical stress, experienced by these plants, associated with feeding may lead to premature death (Fouche et al. 1984, Walters et al. 1980). Until the early 2000s, it was believed that the phenotypic symptoms experienced by *D. noxia*-infested wheat were the result of an injected phytotoxin presumed responsible for chloroplast disintegration and later due to viral infection (Von Wechmar and Rybicki 1981). However, this belief has been refuted, as no phytotoxic effector has ever been isolated or characterized, nor has the presence of plant viruses in *D. noxia* ever been confirmed (Gilchrist et al. 1984, Kovalev et al. 1991, Botha et al. 2010).

In a study to determine the composition of D. *noxia* effectors, purified extracts (i.e., proteins and carbohydrates respectively extracted from complete aphids) were injected into healthy susceptible plants to visualize phenotypic responses expressed in the host normally associated with D. *noxia* feeding (Lapitan et al. 2007a). The authors found that leaf rolling and the induction of pathogenesis related (PR) proteins associated with D. *noxia* feeding could only be induced after an injection with the protein extract, suggesting that the effector was proteinaceous in composition (Lapitan et al. 2007a). In a followup study, salivary glands from D. *noxia* were isolated, protein extracted, and purified on high performance liquid chromatography (HPLC; 100x). The purified protein extracts were then injected into susceptible and resistant wheat varieties, where after the host's responses were phenotypically and biochemically assessed. Observations confirmed that the effector was a protein compound ~58kDa in size and originated from the salivary glands (Van Zyl 2007, Van Zyl and Botha 2008). In *Myzus persicae* (Sulzer, green peach aphid) (Hemiptera:Aphididae), studies have also demonstrated that salivary components of *M. persicae* contains proteinaceous effectors (3-10 kDa in size) that induced defense responses in *Arabidopsis thaliana* (De Vos andJander 2009, Bos et al. 2010).

Multiple studies have focussed on elucidating the effectors present in aphid-plant interactions. A study performed by Bos et al. (2010) attempted to elucidate effector proteins from *M. persicae* through an elaborate study of thousands of expressed sequence tags (ESTs), which included both the in silico characterization of possible effectors and their subsequent over expression within *Nicotiana ben- thamiana*. Although they were able to identify a few elicitors that suppressed certain aphid targeted defense responses, they were unable to conclusively identify a specific effector responsible for the detection of *M. persicae*. A possible reason for this may include the fact that no single effector is responsible for the detection of an aphid feeding on a host, but rather that a combination of effectors (such as C002 and flg22; Felix et al. 1999; Mutti et al. 2006, 2008) may be used by a host plant to identify aphid feeding.

A recent study illustrated that salivary proteins from *Diuraphis* sp. are similar when aphids were fed on artificial diets (Cooper et al. 2011), and that there was little comparison when compared to two other aphid species. Several issues regarding the use of artificial diets to study salivary composition have been raised (Miles 1999), and studies have shown that aphids fed on a variety of artificial diets produce different enzymes at varying levels (Habibi et al. 2001). This limits the usefulness of artificial diets to characterize genes induced in aphids when confronted with a specific *R*-gene within the varied genetic background of most commercial crops. Another shortcoming was the use of mass spectrometry for the identification of proteins, as results obtained are generally biased to known proteins of which there are relatively few when considering aphid elicitors. Therefore, the objective of this study was first, to conduct cDNA-AFLP transcript profiling to visualize the extent of altered transcription induced in *D. noxia* when confronted with feeding on a variety of hosts containing different resistance *Dn* genes, and second, to survey selective transcripts that were induced or suppressed during feeding in response to hosts with varying genetic backgrounds or source of resistance.

## Materials and Methods

### Aphid Colonies

*Diuraphis noxia* biotypes used during the study included SA1 and SAM, which were reared in insect BugDorm Insect cages (MegaView Science Co. Ltd, Taiwan) at 20 6 2 $^{0}$C to prevent crosscontamination with continuous artificial fluorescent lighting as described in Botha et al. (2014a) (Supp. File 1 [online only]).

### Plant Material

The differential set used amounted to a total of 16 varieties and consisted of hexaploid wheat *(Triticum aestivum* L.) germplasm of the near-isogenic lines (NILs) Tugela, Tugela-Dn! (Tugela*4/SA1684), Tugela-Dn2 (Tugela*4/SA2199), and Tugela-Dn5 (Tugela*4/ SA463); Betta, Betta-Dn! (Betta*4/SA1684), Betta-Dn2 (Betta*4/ SA2199), and Betta-Dn9; Karee and Karee-Dn8 were obtained from the Small Grain Institute, Bethlehem, South Africa (Du Toit 1988, Marais and Du Toit 1993, Liu et al. 2001, Tolmay et al. 2006). While NILs Gamtoos-S and Gamtoos-R (Dn7), SA2199 (Dn2), and W861 (Dn7) came from the Stellenbosch Germplasm Collection (Marais et al. 1994, Anderson et al. 2003, Lapitan et al. 2007b). Near isogenic lines Yuma and Yumar (Dn4) (Saidi and Quick 1996, Haley et al. 2004) were kindly provided by Prof C.M. Smith (Department of Entomology, Kansas State University, Manhattan, Kansas). Seeds were sown into five pots for each cultivar and thinned to three seedlings per pot after 5 d. Plants were grown for 31 d (5-6 leaf stage) under greenhouse conditions in a 1:2:2:1 mixture of perlite (Chemserve, Olifantsfontein, South Africa), sifted bark compost, loam and sand at 20 6 2 $^0$C (Supp. File 1 [online only]).

### Aphid Virulence Screening

The virulence profile (VP) of each *D. noxia* biotype was determined using the disease phenotypes (i.e., leaf rolling, chlorotic streaking, and death scores) of the plants over a period of 6 wk. Plants were incubated in BugDorm Insect cages to prevent cross-contamination at 20 6 2 $^0$C with continuous artificial fluorescent lighting. Individual plants were infested with 10 apterous *D. noxia* adults (biotype SA1 or SAM) and incubated for 6 wk during which their scores were determined (Weiland et al. 2008, Botha et al. 2014a; Supp. File 1 [online only]).

### Experimental Lay-Out and Aphid Head Sampling

Aptera *D. noxia* adults were reared under normal greenhouse conditions at 20 6 2 $^0$C on their preference hosts (i.e., Tugela for *D. noxia* biotype SA1 and Tugela-Dn! for *D. noxia* biotype SAM), where after they were carefully removed (by use of a fine camel hair brush) and placed at 4 $^0$C for 1 h to decrease metabolic activity and induce starvation. After the 1-h cold treatment, the aphids (n = 960) were carefully placed on the wheat differential set to feed, where after aphids (n = 30, 3 replicates of 10) were collected for RNA extraction after feeding periods of 4 and 48 h, respectively. Aphid heads were dissected using a sterile hypodermic needle (precooled in liquid nitrogen) and transferred directly to liquid nitrogen and stored at −80$^0$C until RNA extraction could be performed. Controls for cDNA-AFLP analysis included aphids obtained directly from the stock population (i.e., did not undergo cold treatment) and designated as 0 h samples and fridge controls (aphids that had been collected from stock populations and placed at 4$^0$C for 1 h where after heads were dissected).

### Total RNA Extraction and cDNA Synthesis

Laboratory equipment and solutions used during RNA extractions were pretreated as described by Sambrook et al. (1989). Collected aphid heads were pooled and ground in liquid nitrogen with the use of a sterile micro pestle. RNA extraction of samples were performed using the Qiagen RNeasy kit (Plant tissue protocol; Qiagen, Limburg, Netherlands) as per manufacturer's instructions.

Double-stranded cDNA was synthesized from each sample making use of the Roche cDNA synthesis system (Roche Life Sciences, Indianapolis, USA) with 100ng total RNA as template. The manufacturer's guidelines were followed, except that half of the suggested volumes were used. Quantification of RNA and synthesized cDNA was achieved through use of the NanoDrop ND-1000 spectrophotometer v3.0.1 (Supp. File 1 [online only]).

### cDNA-AFLP Profiling and Transcript-Derived Fragment (TDF) Identification

cDNA-amplified fragment length polymorphisms (cDNA-AFLP) analysis was carried out as originally described by Bachem et al. (1996), using the AFLP Expression Analysis Kit (Li-Cor, Lincoln, USA) with some adjustments (Zaayman et al. 2009).

Selective PCR amplifications were carried out with 64 TaqI/ Msel (+2/+2) primer combinations using the 0h control samples of both D. *noxia* biotypes SA1 and SAM to screen for the most informative primer set (Ps) combinations, namely, MTG/TGT (Ps2), MTG/ TCA (Ps3), and MGA/TGA (Ps4).

Samples were then mixed with 5 pl loading dye and resolved on 8% denaturing polyacrylamide gels (8% Long Ranger gel solution [Lonza, Basel, Switzerland], 7.0 M urea and 0.8 x TBE consisting of 71.2mM Tris,

71.2mM boric acid and 1.6mM EDTA) on the Li- Cor DNA analyzer 4200S (at 1,500 V and 45 °C). Images generated on the Li-Cor DNA analyzer during electrophoresis were used to calculate band intensities of fragments judged to be differentially expressed, using Sequentix's GelQuest v3.2.1 program (available from http://www.sequentix.de/gelquest/download.php, accessed 17 January 2017; Supp. File 1 [online only]).

### Fragment Isolation, Cloning, and Identification of Differentially Expressed Transcripts

Selected fragments (n = 150) showing significant differential expression between the treatments (only selecting unambiguous bands, and based on band presence/absence and difference in intensity) were subsequently isolated, cloned, and sequenced for putative identification as was performed previously (Zaayman et al. 2009). Transformants were screened by colony PCR to confirm the presence of inserts (Giissow and Clackson 1989). Samples successfully cloned (confirmed by colony PCR) were sequenced by Inqaba biotech (Pretoria, South Africa; Supp. File 1 [online only]).

### Clustering and Data Analysis

The resulting band intensities were first normalized using the Cluster program (Eisen et al. 1998, available from http://rana.lbl. gov/EisenSoftware.htm), with mean-centering applying Spearman's rank correlation. A cluster image of the differentially expressed fragments that share similar expression patterns was generated with Java TreeView (Saldanha 2004, available from http://jtreeview.sour ceforge.net/) and statistical analyses were performed as previously reported (Zaayman et al. 2009; Supp. File 1 [online only]).

### Functional Annotation

Sequences obtained from Sanger sequencing were screened for vector contamination using the NCBI's VecScreen tool (available from http://www.ncbi.nlm.nih.gov/tools/vecscreen/, accessed 17 January 2017) after which contaminating vector and low-quality sequences were removed using the FinchTV v1.4 program (available from http:// www.geospiza.com/ftvdlinfo.html, accessed 17 January 2017). Sequences were assigned identity by making use of the BLASTx tool (Altschul et al. 1990) and then functionally annotated through use of the BLAST2GO application (Conesa et al. 2005, available from https://www.blast2go.com/, accessed 17 January 2017).

## Results

### Virulence Screening

Phenotypic assessment of the 16 cultivars over a period of 6 wk after infestation with D. ***noxia*** biotype SAM under greenhouse conditions, revealed a significant breakdown of resistance in plant genotypes that were previously reported as D. noxia-resistant cultivars (http:// www.arc.agric.za/arc-sgi/Pages/ARC-SGI-Homepage.aspx, accessed 17 January 2017; Table 1; Fig. 1). *Diuraphis noxia* biotype SA1 only expressed virulence against the susceptible wheat varieties as previously reported (Tolmay et al. 2006, Jankielsohn 2011). The antixe- notic cultivars containing the Dn5 and Dn7 gene expressed the highest level of resistance against *D. noxia* biotype SAM and had a virulence score (VS) of 9/10 after the 6-wk screening period, but was still classified susceptible according to its virulence profile (VP), confirming the results of a previous study performed by Botha et al. (2014a) All the other cultivars had expressed VSs of 10 and were classified as susceptible to feeding by *D. noxia* biotype SAM, including plants containing resistance genes (i.e., *Dnl*, *Dn2*, *Dn4*, *Dn8*, and Dn9).

### Profiling of Transcripts in Aphid Biotypes After Feeding

Visual assessment of the resulting profiles indicated significant changes in the expression profiles obtained from heads, both within (Supp. Fig. 1 [online only]) and between, D. *noxia* biotypes, suggesting major changes in the salivary glands constituents. Further analysis of cDNA-AFLP patterns among the *D. noxia* biotype x plant genotype combinations (Supp. Table 1 [online only]) using the three different enzyme-primer combinations yielded ~10,573 TDFs. Of these, 58.37% of the TDFs did not exhibit any significant change from the 0 h post infestation (hpi) control values (i.e., Tugela [0 hpi] for *D. noxia* biotype SA1 and Tugela-*Dnl* [0 hpi] for SAM) at $P < 0.05$ in expression across the treatments, that resulted in a total of 7,749 informative bins.

The average expression levels of all the TDFs were calculated and presented (Fig. 2). When a comparison was drawn between the relative expression (as measured in band intensities after normalization) of transcripts between *D. noxia* biotypes SA1 and SAM after feeding on the wheat differential set, it was apparent that


**Fig. 1.** Photos of selected cultivars after 3 wk ofvirulence screening with *Diuraphis noxia* biotype SAM illustrating variations in phenotypic symptoms associated with susceptibility. (**A.1** and **A.2**) *Triticum aestivum* cv. Betta *Dn9* leaves with slight chlorotic streaking; (**B.1** and **B.2**) *Triticum aestivum* cv. Karee *Dn8* leaves with moderate chlorotic streaking andsignsof leaf rolling; (**C.1** and **C.2**) *Triticum aestivum* cv. Karee with severe leaf rollingand chlorotic streaking.

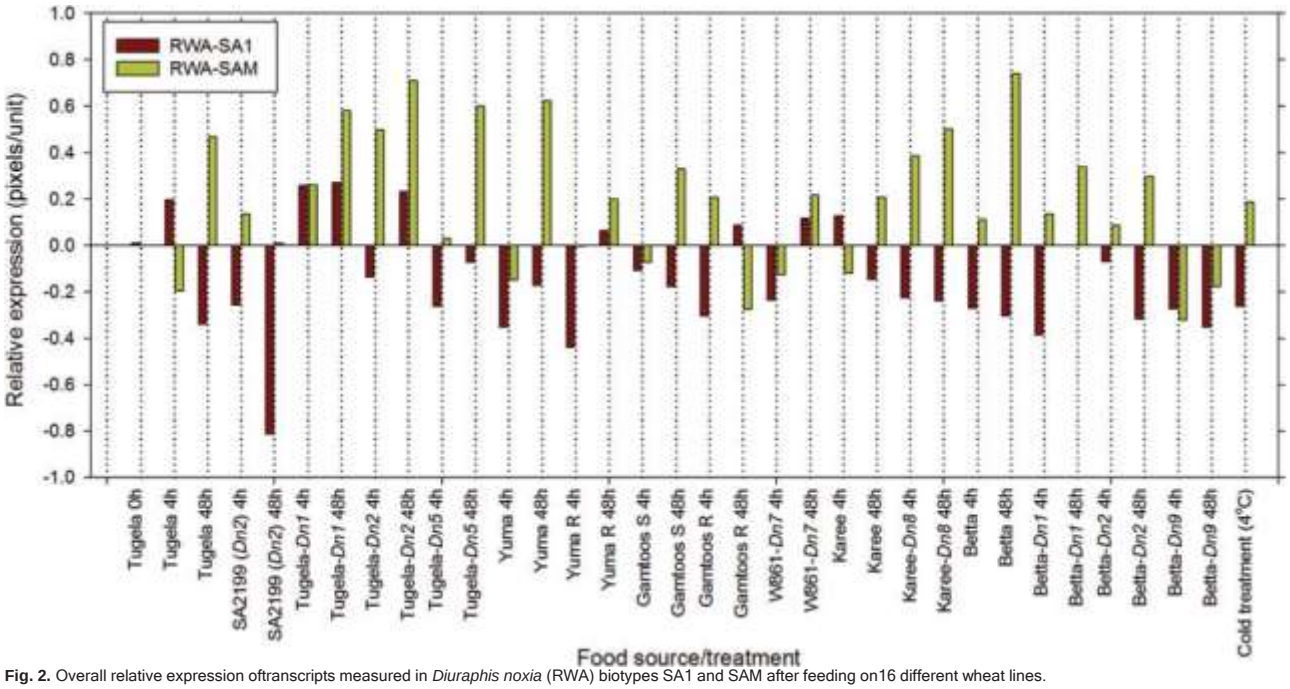


**Fig. 2.** Overall relative expression oftranscripts measured in *Diuraphis noxia* (RWA) biotypes SA1 and SAM after feeding on16 different wheat lines.

**Table 1.** Virulence profile (VP) of the two *Diuraphis noxia* biotypes (SA1 and SAM) based on chlorosis, streaking, leaf rolling (LR), and virulence scores (VS)

| Aphid biotype** | Host genotype | Host response to feeding | | | | | Host virulence profile** |
|---|---|---|---|---|---|---|---|
| | | Streaking** | Leaf rolling** | Chlorosis | Aphid fertility** | Virulence score** | |
| SA1 | Tugela | V | V | 3 | >50 | 9/10 | S |
| SAM | Tugela | V | V | 4 | >50 | 10 | S |
| SA1 | Tugela-Dnl[a] | □ | □ | 1 | <20 | 1/2 | R |
| SAM | Tugela-Dnl | V | V | 4 | >50 | 10 | S |
| SA1 | Tugela-Dn2[b] | □ | □ | 1 | >35 | 1 | R |
| SAM | Tugela-Dn2 | V | V | 3 | >50 | 10 | S |
| SA1 | Tugela-Dn5[c] | □ | □ | 1 | <20 | 1 | R |
| SAM | Tugela-Dn5 | V | V | 3 | >50 | 9/10 | S |
| SA1 | Betta | V | V | 3 | >50 | 9/10 | S |
| SAM | Betta | V | V | 4 | >50 | 10 | S |
| SA1 | Betta-Dnl[a] | □ | □ | 1 | <20 | 1/2 | R |
| SAM | Betta-Dn! | V | V | 4 | >50 | 10 | S |
| SA1 | Betta-***Dn2***[b] | □ | □ | 1 | >35 | 1 | R |
| SAM | Betta-***Dn2*** | V | V | 3 | >50 | 10 | S |
| SA1 | Betta-Dn9 | □ | □ | 1 | <20 | 1 | R |
| SAM | Betta-Dn9 | V | V | 3 | >50 | 9/10 | S |
| SA1 | Karee | V | V | 3 | >50 | 9/10 | S |
| SAM | Karee | V | V | 4 | >50 | 10 | S |
| SA1 | Karee-Dn8 | V | V | 3 | <20 | 1/2 | R |
| SAM | Karee-Dn8 | V | V | 4 | >50 | 10 | S |
| SA1 | Yuma | □ | □ | 1 | <20 | 1/2 | R |
| SAM | Yuma | V | V | 4 | >50 | 10 | S |
| SA1 | Yumar ***(Dn4)***[d] | □ | □ | 1 | <20 | 1/2 | R |
| SAM | Yumar ***(Dn4)***[d] | V | V | 4 | >50 | 10 | S |
| SA1 | Gamtoos | V | V | 3 | >50 | 9/10 | S |
| SAM | Gamtoos | V | V | 4 | >50 | 10 | S |
| SA1 | Gamtoos-R ***(Dn7)***[e] | □ | □ | 1 | <20 | 1/2 | R |
| SAM | Gamtoos-R ***(Dn7)***[e] | V | V | 4 | >50 | 9/10 | S |
| SA1 | W861 ***(Dn7)***[e] | □ | □ | 1 | <20 | 1/2 | R |
| SAM | W861 ***(Dn7)***[e] | V | V | 4 | >50 | 9/10 | S |
| SA1 | SA2199 ***(Dn2)*** | □ | □ | 1 | >35 | 1 | R |
| SAM | SA2199 ***(Dn2)*** | V | V | 3 | >50 | 9/10 | S |

For streaking and leaf rolling: V = visible, □ = none visible. For chlorosis: 1 = 20% or less chlorosis per leaf and 4 = 100% chlorosis (dead leaf/tissue). Aphid fertility is presented as number of aphids. Virulence score and Virulence profile is resistant (R) = 1-3; intermediate (I) = 4-6; and susceptible (S) = 7-10 (Weiland et al. 2008; $n = 30$).

** = significant at $P < 0.05$.

[a]Du Toit (1987) (PI 137739, 'Gandum I Fasai').

[b]Du Toit (1988) (PI 262660, 'Turtsikum').

[c]Marais and Du Toit (1993) (PI 294994, 'Strelinskaya Mestnaya').

[d]Saidi and Quick (1996).

[e]Marais et al. (1993).

transcripts were mostly up-regulated in the *D. noxia* biotype SAM, while they were mostly down-regulated in *D. noxia* biotype SA1. The only exceptions to this trend were similar levels in expression observed in both

biotypes after feeding on Tugela-*Dnl* 4hpi, and similar trends in expression after feeding on Tugela-*Dnl* 48 hpi, Tugela-*Dn2* 48 hpi, Yuma 4 hpi, Gamtoos-S 4 hpi, W861-Dn7 4 hpi and 48 hpi, and Betta-Dn9 4 hpi and 48 hpi. The only measured instances where transcripts were down-regulated in *D. noxia* biotype SAM samples, but not in *D. noxia* biotype SA1 samples, were after feeding on Tugela 4 hpi, Karee 4 hpi, and Gamtoos-R 48 hpi.

## Cluster Analysis

The 7,749 bins found to display significant differential expression were clustered into groups of transcripts that share similar expression patterns (Fig. 3). Cluster analysis of the TDFs revealed two major clusters and two arrays indicated as Clusters 1 and 2, and Arrays 1 and 2. The profiles obtained from *D. noxia* biotype SA1 all grouped together in cluster 1, while all those obtained from D. *noxia* biotype SAM grouped together in cluster 2 irrespective of the host cultivars and time points.

Cluster 1 formed subclusters (1A, 1Bi, and 1Bii) where groupings formed predominantly according to host genotype and not by the presence/absence of specific ***Dn*** genes within the host. Time of sampling did not

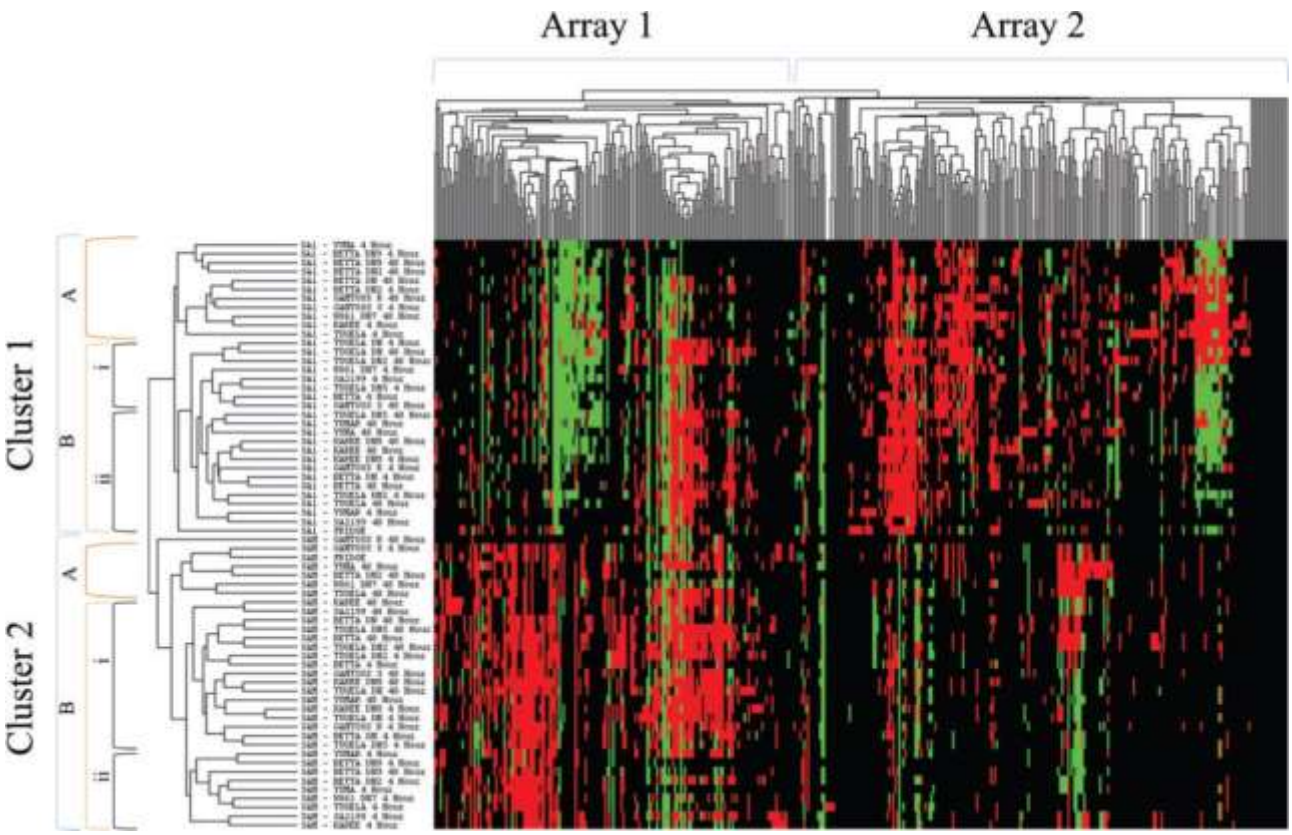


**Fig. 3.** Cluster image generated by Java TreeView (Saldanha 2004). Red bands show up-regulated transcripts, whereas green bands show down-regulated transcripts, relativeto 0 hpi feeding on preference hosts (controls).

follow any strict grouping, as 4 hpi and 48 hpi samples were spread out evenly between the clusters.

Cluster 2 also displayed subclusters (2A, 2Bi, and 2Bii) but groups formed according to the time point of sampling irrespective of the host genotype, or the presence/absence of *Dn* genes within the host. The only exception to this was for D. *noxia* samples Karee *Dn8* 48 hpi and Betta *Dn9* 48 hpi. *Diuraphis noxia* samples from all genotypes were spread across clusters 2A, 2Bi, and 2Bii, which also proved true for the different *Dn* genes. Interestingly, the *D. noxia* biotype SAM Gamtoos-R 48 hpi sample was the only sample that did not form part of a sub cluster and showed the least number of TDFs experiencing significant differential expression.

Hierarchical clustering of the samples also produced two arrays. In array 1, TDFs obtained from *D. noxia* biotype SA1 (cluster 1) were mostly down-regulated as a result of the host shifts while the TDFs from *D. noxia* SAM (cluster 2) showed a general up- regulation. In array 2, cluster 1 possessed a wider array of TDFs differentially expressed than when comparing them to cluster 2.

Most of the *D. noxia* biotype SA1 samples that were obtained 4 hpi from susceptible cultivars (i.e., no *Dn* gene present) did not group with their NILs that contained *Dn* genes, with the only exception being the *D. noxia* biotype SA1 sample obtained from Gamtoos-S 4 hpi. The D. *noxia* biotype SA1 samples that were collected at 48 hpi from susceptible cultivars grouped with NILs that contained a Dn gene, while the only exception was Gamtoos-S 48 hpi which grouped with Betta 4 hpi.

### Excision and Identification of Transcripts

Following the cDNA-AFLP analysis approach, we were able to excise and sequence characterize 41 differentially regulated TDFs (Supp. Table 2 [online only]). After the sequences were obtained, putative identities were assigned to the clones using BLAST2GO (Conesa et al. 2005). Only one of the sequences had no corresponding BLAST match and was therefore completely unknown (Supp. Fig. 2 [online only]), while 31.7% produced significant Blast hits without assigned gene ontology (GO) function and 65.8% showed similarity to well characterized sequences in NCBI (http://www. ncbi.nlm.nih.gov/Blast.cgi, accessed 17 January 2017) with matches to the GO database. Based on the putative functions of the transcripts inferred by similarity, the TDFs were assigned to broad functional GO categories (Supp. Fig. 3 [online only]). These included amino acid synthesis (12%), cellular homeostasis (23%), lipid and fatty acid metabolism (24%), and the largest group being energy metabolism associated (41%). The functional categories were further refined into groups with associated pathways and enzymatic groupings (Supp. Fig. 3B and C [online only]). These included the following: biotin metabolism, fatty acid metabolism, ascorbate and aldarate metabolism, biosynthesis of unsaturated fatty acids; pentose phosphate pathway, glutathione metabolism, sphingolipid metabolism, glycine, serine and threonine metabolism, and methane metabolism, with most transcripts falling into the amino sugar and nucleotide sugar metabolism groups. The TDFs of interest to this study included transcripts with suggested detoxification properties and involved in apoptosis (e.g., laccase 2, cullin-3, 6-phosphogluconate decarboxylating, sphingomyelin phosphodiesterase, midnolin) and transcripts involved in membrane function and transport (e.g., proton-associated sugar transporter a-like, monocarboxylate transporter, collagen alpha-1 partial, transmembrane protein mitochondrial isoform x2, acidic mammalian chitinase-like; Supp. Table 2 [online only]).

## Discussion

The intimate relationship between an aphid and its host is mediated by the secretion of abundant amounts of saliva during all the stages of feeding, including probing and ingestion (Will et al. 2013). Two types of saliva are secreted during feeding, the "sheath saliva"— once secreted, although initially in liquid form, hardens around the stylet to form a path in the plant cells and remains in the plant; and the second form that remains as a liquid to facilitate feeding—known as "watery saliva." Previous studies suggested four phases of salivary secretion: namely, intercellular sheath secretion; followed by the intracellular salivation into cells along the stylet path; then phloem salivation into sieve elements; and lastly, phloem feeding salivation i.e., feeding interspersed with sporadic periods of salivation (Martin et al. 1997, Tjallingii 2006).

The composition of aphid saliva and the specific functions of certain salivary proteins have become clear only recently, driven primarily by the increase in available genomic resources for aphids, particularly the sequenced genome of the pea aphid *Acyrthosiphon pisum* (Harris) (International Aphid Genome Consortium 2010). Salivary gland EST libraries for A. *pisum* and the green peach aphid *M. persicae* have been exploited to provide candidate salivary proteins (Mutti et al. 2006, Bos et al. 2010, Carolan et al. 2011). Subsequent characterisation of a number of these proteins in these and other aphid species have indicated their importance in facilitating the plant-aphid interaction (Habibi et al. 2001, Harmel et al. 2008, Mutti et al. 2008, Carolan et al. 2009, Cooper et al. 2011, Nicholson et al. 2012, Roa et al. 2013). However, most of these studies made use of artificial diets during harvesting of salivary proteins in vitro, posing the question to what extent the published data represents the actual in vivo and real-time salivary composition. As the aphid's salivary

glands (through means of excreted saliva), the stylet (through physical penetration) and the gut (through means of digestion) are the only components molecularly interacting with the host, they would represent as viable targets for studying aphid-plant interactions. The use of severed heads would exclude any regulatory changes experienced by the gut, as only the stylet and salivary glands would be present. Aphid stylets can be considered to be inert, as sheath saliva is excreted (Tjallingii 2006) to create a path to the feeding site, thus enveloping the stylet and separating it physically from its host. As aphids are believed to alter their salivary composition when faced with varied diets, be they artificial or natural (Miles 1999, Habibi et al. 2001), it can be expected that transcription rates within salivary glands would be substantial. Thus, it was deemed plausible that most, if not all, observed changes in expression obtained from whole heads would represent changes in salivary gland transcription.

From the cluster analysis, it was clear that *D. noxia* biotype SA1 (cluster 1 in Fig. 3) grouped predominantly according to host genotype, regardless of the presence of any *Dn* gene. This has some interesting implications for future breeding programmes wanting to introduce known Dn genes into different cultivars, as it appears that the genotypic background plays a significant role in the composition of the salivary transcriptome of less virulent aphids, i.e., those who cannot escape detection by the host, no matter what *Dn* gene it is faced with. This is in agreement with the combined findings of Heng-Moss et al. (2003) and Botha et al. (2006) where the introduction of the *Dnl* gene into two different cultivars resulted in a varied ability to protect the respective plants from aphid mediated chlorophyll loss.

The time points sampled were specifically chosen to coincide with the defensive responses of wheat when faced with a biotic stressor (Botha et al. 2006, 2010). The 4-h time point sampling corresponds with induction of the hypersensitive response (HR) in wheat, activated by effectors in a naive host (as reviewed in Martin et al. 2003). The 48-h time point was selected as this overlaps with the induction of systemic acquired resistance (including the expression of PR proteins) and production of secondary metabolites (Botha et al. 1998). The low virulence of *D. noxia* biotype SA1 may explain why 48 hpi samples obtained from susceptible cultivars clustered with NILs containing *Dn* genes, with the exception of samples obtained from the Gamtoos genotype. This implies that although D. *noxia* biotype SA1 is able to escape early detection in susceptible hosts, it will eventually experience a similar host environment as that of a *Dn* containing host (assuming that similarities in salivary expression is a measure for the defense response experienced by the aphid) as defense responses mediated by the host will be genotype specific (Van der Westhuizen et al. 1998a,b). Interestingly, 48 hpi samples from the host cultivars Tugela and Betta also grouped with 4 hpi samples fed on *Dn* gene containing NILs. This may illustrate part of the role that *Dn* genes plays within host defense through early detection of effector recognition, and thus the initiation of an earlier and more effective defense response.

For the laboratory-evolved *D. noxia* biotype SAM, the clustering of different samples were based on the time of sampling, with the exception of Gamtoos-R 48 hpi. The clustering of samples from the same time point from different genetic backgrounds, and with different *Dn* genes, illustrates that the only shared characteristic affecting the salivary transcriptome composition for biotype SAM, is that of generalized host defense responses launched (either HR or SAR) in response to any damage as is the case, for example, when faced with mechanical wounding (Van der Westhuizen et al. 1998a,b). Seemingly, biotype SAM escapes detection from resistant hosts, as it displays a comparable clustering configuration as D. *noxia* biotype SA1 4 hpi after feeding on susceptible cultivars, in that there is no association in the clustering of samples in regards to host genotype or by the type of *Dn* gene present. This again supports findings of the study conducted by Botha et al. (2014a) where the defense response (measured in enzyme activity) in Dn-containing hosts fed on by *D. noxia* biotype SAM was similar to that of susceptible NIL hosts that were fed on by biotype SA1.

The fact that *D. noxia* samples from both biotypes SA1 and SAM obtained from the Gamtoos genotype clustered differently than samples obtained from other genotypes can be explained by the mosaic nature of the Gamtoos genotype's parentage. The Gamtoos cultivar was established through a crossing of the Mexican spring wheat cultivar and the German winter wheat cultivar Kavkaz, that contains a rye translocation, and included rust resistant genes *Sr31* and *Lr26* (as reviewed in Lapitan et al. 2007b). The Gamtoos-R cul- tivar was then established through an additional rye chromosomal translocation from a Turkish cultivar (Marais et al. 1994). This varied geographical background may explain the altered salivary tran- scriptomes of D. ***noxia*** biotypes. The presence of the rust resistance genes alone would dramatically change the host environment as previous studies have hinted to the increase in *D. noxia* resistance by wheat cultivars in possession of pathogen resistance genes (Boyko et al. 2006, van Eck et al. 2010).

The naivety of the host being fed on, in regards to previous aphid infestation, also has a significant effect on the salivary transcriptome of the aphid. This can be concluded from *D. noxia* biotype SA1 samples Tugela 4 hpi and 48 hpi and *D. noxia* SAM sample Tugela *Dnl* 4 hpi and 48 hpi that showed significant altered regulation when moved from the stock colonies (i.e., Tugela for SA1 and Tugela *Dnl* for SAM). This provides molecular evidence that is in agreement with previous studies that have reported on the varied conditioning effects (mostly through aphid host choice and fecundity) present in plants that have undergone aphid infestation (Formusoh et al. 1992, Messina et al. 2002, Qureshi and Michaud 2005).

Most of the transcripts found in the present study were reported earlier in the salivary proteome of hemipterans (i.e., detoxifying and membrane associated defense transcripts, e.g., p-galactosidase, glucose dehydrogenases, chitinases; Nicholson et al. 2012, Roa et al. 2013), while transcripts not previously reported in

this group included midnolin and sphinomyelinase. The role of midnolin-like proteins in insects is unclear, although it has been reported in the salivary glands of the frog biting fly, *Corethrella appendiculata* (Ribeiro et al. 2014), but it is thought to be important in cellular signalling of mammalian adult tissues where it is suggested to regulate glucokinase enzyme activity in pancreatic beta cells in an attempt to regulate cellular homeostasis (Hofmeister-Brix et al. 2013).

Sphingolipids are nearly ubiquitous constituents of membranes in most living organisms (Merrill and Sweeley 1996) and located predominantly in the outer leaflet of the plasma membrane and modulate cell behaviour at both the level of cell-surface receptors and intracellular signal transduction. Known functions include mediation of interactions between cells and the extracellular matrix, cell-cell communication and diverse functions of the immune system. However, some are also found intercellularly which participate in signal transduction events such as, regulation of cell growth and differentiation, differentiated cell functions (such as responses to cytokines) and programmed cell death (apoptosis). Sphingolipids are released from cells by either secretion or shedding; membrane internalization, recycling, and degradation; and hydrolysis to release bioactive products that participate in cell signalling (Merrill and Jones 1990). The sphingomyelin phosphodiesterase (EC 3.1.4.12, neutral sphingomyelinase or SMase) is a hydrolase enzyme belonging to the DNasel superfamily of enzymes and is responsible for breaking sphingomyelin down into phosphocholine and ceramide. The activation of SMase has been suggested as a major route for the production of ceramide in response to cellular stresses (Hannun and Obeid 2002). Sphingomyelin phosphodiesterase has been found to be highly upregulated in *Anopheles gambiae* (Diptera:Culicidae) saliva undergoing desiccation stress (Wang et al. 2011), while transcripts coding for sphingolipid metabolism were upregulated in *Aphis gossypii* (Hemiptera:Aphididae) resistant to thiamethoxam (Pan et al. 2015). In *Schizaphis graminum* (Hemiptera:Aphididae) a sphingolipid (StAR-related protein) was found to be upregulated when comparing the overall proteome of a highly virulent biotype relative to that of other lower virulent biotypes (Pinheiro et al. 2014).

In the present study, aphid heads were harvested and transcript profiling conducted after aphids were fed on their preference host and transferred to a variety of preference and nonpreference hosts. It was found that the virulent *D. noxia* biotype SAM was able to selectively up-regulate more transcripts when confronted with feeding on a naive host, than was the case with the less virulent *D. noxia* biotype SA1, suggesting increased genomic regulation when coping with a stressful or changing environment (Fig. 2). Further supporting evidence for this notion came from an observation that unlike for the lesser virulent *D. noxia* biotype SA1, the more virulent *D. noxia* biotype SAM was seemingly able to avoid detection by its host (Botha et al. 2014a). Collectively the observed results support earlier observations that changes in the host induced significant changes in the proteome of phytophagous hemipterans, unlike in many other entomophagous generalist species [e.g., spined soldier bug *Podisus maculiventris* (Say) (Hemiptera:Pentatomidae); Habibi et al. 2001], thus motivating the use of similarities in salivary expression, between different biotypes feeding on different hosts, to elucidate similarities in host environment in regards to the effect of defense responses. The data generated here suggest that highly specialized hemipterans possesses the ability to regulate their salivary transcrip- tome in a real-time fashion to adapt to their feeding environment, thus countering plant compounds through inducible salivary transcripts with resulting protein biosynthesis.

## Acknowledgments

This work was supported by the Winter Cereal Trust, the National Research Foundation, the Technology and Human Resources for Industry Programme, and the Department of Trade and Industry. The research was funded through THRIP Grant: TP2010072900011; NRF Competitive Programme for Rated Researchers (CPRR) Grant: CPR20110615000019459; and NRF Incentive Funding for Rated Researchers Programme (IFR) Grant: IFR201004200013.

## References Cited

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. J. Mol. Biol. 215: 403-410.

Anderson, G. A., D. Papa, J. H. Peng, M. Tahir, and N.L.V. Lapitan. 2003. Genetic mapping of Dn7, a rye gene conferring resistance to the Russian wheat aphid in wheat. Theor. Appl. Genet. 107: 1297-1303.

Bachem, C.W.B., R. S. van der Hoeven, S. M. de Bruijn, D. Vreugdenhil, M. Zabeau, and R.G.F. Visser. 1996. Visualization of differential gene expression using a novel
method of RNA fingerprinting based on AFLP: analysis of gene expression during potato tuber development.

Plant J. 9: 745-753.
Bos, J.I.B., D. Prince, M. Pitino, M. E. Maffei, J. Win, and S. A. Hogenhout. 2010. A functional genomics approach identifies candidate effectors from the aphid species
*Myzus persicae* (green peach aphid). PLoS Genet. 6: e1001216. doi:10.1371/journal.pgen.1001216.
Botha, A.-M., M.A.C. Nagel, A. J. van der Westhuizen, and F. C. Botha. 1998. Chitinase isoenzymes in near-isogenic wheat lines challenged with Russian wheat aphid, exogenous ethylene, and mechanical wounding.
Bot. Bull. Acad. Sin. 39: 99-106.
Botha, A.-M., L. Lacock, C. van Niekerk, M. T. Matsioloko, F. B. Du Preez, S. Loots, E. Venter, K. J. Kunert, and C. A. Cullis. 2006. Is photosynthetic transcriptional regulation in *Triticum aestivum* L. cv. 'TugelaDN' a contributing factor for tolerance to *Diuraphis noxia* (Homoptera: Aphididae)? Plant Cell Rep. 25: 41-54.
Botha, A.-M., Z. H. Swanevelder, and N.L.V. Lapitan. 2010. Transcript profiling of wheat genes expressed during feeding by two different biotypes of *Diuraphis noxia.* Environ. Entomol. 39: 1206-1231.
Botha, A.-M., L. Van Eck, N.F.V. Burger, and Z. H. Swanevelder. 2014a. Hypervirulent *Diuraphis noxia* (Kurdjumov) biotype SAM avoids detection by its host *(Triticum aestivum* L.) (Poales: Poaceae) during feeding. Environ. Entomol. 43: 672-681.
Botha, A.-M., L. Van Eck, N.F.V. Burger, and Z. H. Swanevelder. 2014b. Near-isogenic lines of *Triticum aestivum* with distinct modes of resistance exhibit dissimilar transcriptional regulation during *Diuraphis noxia* feeding. Open Biol. 3:1116-1126. doi:10.1242/bio.201410280.
Boyko, E. V., C. M. Smith, V. K. Thara, J. M. Bruno, Y. Deng, S. R. Starkey, and D. L. Klaahsen. 2006. Molecular basis of plant gene expression during aphid invasion: wheat *Pto*- and Pti-like sequences are involved in interactions between wheat and Russian wheat aphid (Homoptera: Aphididae). J. Econ. Entomol. 99: 1430-1445.
Burd, J. D., and R. L. Burton. 1992. Characterization of plant damage caused by Russian wheat aphid (Homoptera: Aphididae). J. Econ. Entomol. 85: 2015-2022.
Burd, J. D., and N. C. Elliott. 1996. Changes in chlorophyll a fluorescence induction kinetics in cereals infested with Russian wheat aphid (Homoptera: Aphididae). J. Econ. Entomol. 89: 1332-1337.
Carolan, J. C., C.I.J. Fitzroy, P. D. Ashton, A. E. Douglas, and T. L. Wilkinson. 2009. The secreted salivary proteome of the pea aphid *Acyrthosiphon pisum* characterised by mass spectrometry. Proteomics 9: 2457-2467.
Carolan, J. C., D. Caragea, K. T. Reardon, N. S. Mutti, N. Dittmer, K. Pappan, F. Cui, M. Castaneto, J. Pulain, C. Dossat, et al. 2011. Predicted effector molecules in the salivary secretome of the pea aphid (*Acyrthosiphon pisum*): a dual transcriptomic/protemic approach. J. Proteome Res. 10: 1505-1518.
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.
Cooper, W. R., J. W. Dillwith, and G. J. Puterka. 2011. Comparisons of salivary proteins from five aphid (Hemiptera: Aphididae) species. Environ. Entomol. 40: 151-156.
Davis, G. K. 2012. Cyclical parthenogenesis and viviparity in aphids as evolutionary novelties. J. Exp. Zool. 318: 448-459.
De Vos, M., and G. Jander. 2009. *Myzus persicae* (green peach aphid) salivary components induce defence responses in *Arabidopsis thaliana*. Plant Cell Environ. 32: 1548-1560.
Du Toit, F. 1987. Resistance in wheat *(Triticum aestivum*) to *Diuraphis noxia* (Hemiptera: Aphididae). Cereal Res. Commun. 16: 175-179.
Du Toit, F. 1988. Another source of Russian wheat aphid (*Diuraphis noxia*) resistance in *Triticum aestivum*. Cereal Res. Commun. 16: 105-106.
Eisen, M. B., P. T. Spellman, P. O. Brown, and D. Botstein. 1998. Cluster analysis and display of genome-wide expression patterns. Proc. Nat. Acad. Sci. USA95: 14863-14868.
Felix, G., J. D. Duran, S. Volko, and T. Boller. 1999. Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. Plant J. 18: 265-276.
Formusoh, E. S., G. E. Wilde, and J. C. Reese. 1992. Reproduction and feeding behavior of greenbug biotype E (Homoptera: Aphididae) on wheat previously fed upon by aphids. J. Econ. Entomol. 85: 789-793.
Fouche, A., R. L. Verhoeven, P. H. Hewitt, M. C. Walters, C. F. Kriel, and J. C. De Jager. 1984. Russian aphid *(Diuraphis noxia*) feeding damage on wheat, related cereals and a *Bromus* grass species, pp. 22-33. *In* M. C. Walter (ed.), Progress in Russian wheat aphid (Diuraphis noxia Mordv.) research in the Republic of South Africa, Technical Communication No. 191, Department of Agriculture, Republic of South Africa.
Gilchrist, L. I., R. Rodriguez, and P. A. Burnett. 1984. The extent of Freestate streak and *Diuraphis noxia* in Mexico, pp. 157-163. *In* Barley yellow dwarf, a Proceeding of the Workshop, CIMMYT, Mexico.
GUssow, D., and T. Clackson. 1989. Direct clone characterization from plaques and colonies by the polymerase chain reaction. Nucleic Acids Res. 17: 4000.
Habibi, J., E. A. Backus, T. A. Coudron, and S. L. Brandt. 2001. Effect of different host substrates on hemipteran salivary protein profiles. Entomol. Exp. Appl. 98: 369-375.
Haile, F. J., L. G. Higley, X. Ni, and S. S. Quisenberry. 1999. Physiological and growth tolerance in wheat to Russian wheat aphid (Homoptera: Aphididae) injury. Environ. Entomol. 28: 787-794.
Haley, S. D., J. S. Quick, J. J. Johnson, F. B. Peairs, J. A. Stromberger, S. R. Clayshulte, B. L. Clifford,J. B. Rudolph, O. K. Chung, and B. W. Seabourn. 2004. Occurrence of a new Russian wheat aphid biotype in Colorado. Crop Sci. 44: 1589-1592.
Hannun, Y. A., and L. M. Obeid. 2002. The Ceramide-centric universe of lipid-mediated cell regulation: stress

encounters of the lipid kind. J. Biol. Chem. 277: 25847-25850.
Harmel, H., E. Letocart, A. Cherqui, P. Giordanengo, G. Mazzucchelli, F. Guillonneau, E. De Pauw, E. Haubruge, and F. Francis. 2008. Identification of aphid salivary proteins: a proteomic investigation of *Myzus persicae*. Insect Mol. Biol. 17: 165-174.
Heng-Moss, T. M., X. Ni, T. Macedo, J. P. Markwell, F. P. Baxendale, S. S. Quisenberry, and V. Tolmay. 2003.
Comparison of chlorophyll and carotenoid concentrations among Russian wheat aphid (Homoptera: Aphididae) -infested wheat isolines.J. Econ. Entomol. 96: 475-481.
Hofmeister-Brix, A., K. Kollmann, S. Langer, J. Schultz, S. Lenzen, and S. Baltrush. 2013. Identification of the ubiquitin-like domain of midnolin as a new glucokinase interaction partner. J. Biol. Chem. 288: 35824-35839.
International Aphid Genome Consortium. 2010. Genome sequence of the pea aphid *Acyrthosiphon pisum*. PLoS Biol. 8: e1000313. doi:10.1371/ journal.pbio.1000313.
Jankielsohn, A. 2011. Distribution and diversity of Russian wheat aphid (Hemiptera: Aphididae) biotypes in South Africa and Lesotho. J. Econ. Entomol. 104: 1736-1741.
Kovalev, O. V., T. J. Poprawski, A. V. Stekolshchikov, A. B. Vereshchagina, and S. A. Gandrabur. 1991. *Diuraphis* Aizenberg (Hom., Aphididae): key to apterous viviparous females, and review of Russian language literature on the natural history of *Diuraphis noxia* (Kurdjumov, 1913). J. Appl. Entomol. 112: 425-436.
Lapitan, N.L.V., Y.-C. Li, Y. Peng, andA.-M. Botha. 2007a. Fractionated extracts of Russian wheat aphid eliciting defence responses in wheat. J. Econ. Entomol. 100: 990-999.
Lapitan, N.L.V., J. Peng, and V. Sharma. 2007b. A high density map and PCR markers for Russian wheat aphid resistance gene *Dn7* on chromosome 1RS/ 1BL. Crop Sci. 47:811-820.
Liu, X. M., C. M. Smith, B. S. Gill, and V. Tolmay. 2001. Microsatellite markers linked to six Russian wheat aphid resistance genes in wheat. Theor. Appl. Genet. 102: 504-510.
Marais, G. F., and F. Du Toit. 1993. A monosomic analysis of Russian wheat aphid resistance in the common wheat PI 294994. Plant Breed. 111: 246-248.
Marais, G. F., M. Horn, and F. Du Toit. 1994. Intergeneric transfer (rye to wheat) of a gene(s) for Russian Wheat Aphid resistance. Plant Breed. 113: 265-271.
Martin, B., J. L. Collar, W. F. Tjallingii, and A. Fereres. 1997. Intracellular ingestion and salivation by aphids may cause the acquisition and inoculation of non-persistently transmitted plant viruses. J. Gen. Virol. 78: 2701-2705.
Martin, G. B., A. J. Bogdanove, and G. Sessa. 2003. Understanding the functions of plant disease resistance proteins. Ann. Rev. Plant Biol. 54: 23-61.
Merrill A. H., Jr, and D. D. Jones. 1990. An update of the enzymology and regulation of sphingomyelin metabolism. Biochim. Biophys. Acta 1044: 1-12.
Merrill A. H., Jr., and C. C. Sweeley. 1996. Chapter 12: Sphingolipids: metabolism and cell signalling, pp. 309-339. *In* D. E. Vance and J. E. Vance (eds.), Biochemistry of lipids, lipoproteins and membranes. Elsevier, Amsterdam, The Netherlands.
Messina, F. J., R. Taylor, and M. E. Karren. 2002. Divergent responses of two cereal aphids to previous infestation of their host plant. Entomol. Exp. Appl. 103: 43-50.
Miles, P. W. 1999. Aphid saliva. Biol. Rev. 74: 41-85.
Moran, P. J., and G. A. Thompson. 2001. Molecular responses to aphid feeding in Arabidopsis in relation to plant defence pathways. Plant Physiol. 125: 1074-1085.
Morrison, W. P., and F. B. Peairs. 1998. Response model concept and economic impact. Response model for an introduced pest - the Russian wheat aphid. Entomological Society of America, Lanham, MD.
Mutti, N. S., Y. Park, J. C. Reese, and G. R. Reeck. 2006. RNAi knockdown of a salivary transcript leading to lethality in the pea aphid, *Acyrthosiphon pisum*. J. Insect Sci. 6: 38.
Mutti, N. S., J. Louis, L. K. Pappan, K. Pappan, K. Begum, M.-S. Chun, Y. Park, N. Dittmer, J. Marshall, J. C. Reese, et al. 2008. A protein from the salivary glands of the pea aphid, *Acyrthosiphon pisum*, is essential in feeding on a host plant. Proc. Natl. Acad. Sci. USA 105: 9965-9969.
Myburg, A. A., D. L. Remington, D. M. O'Malley, R. R. Sederoff, and R. W. Whetten. 2001. High-throughput AFLP analysis using infrared dye labelled primers and an automated DNA sequencer. Biotechniques 30: 348-357.
Nicholson, S. J., S. D. Hartson, and G. J. Puterka. 2012. Proteomic analysis of secreted saliva from Russian Wheat Aphid (*Diuraphis noxia* Kurd.) biotypes that differ in virulence to wheat. J. Proteomics 75: 2252-2268.
Painter, R. H. 1951. Insect resistance in crop plants. Soil Sci. 72:481.
Painter, R. H. 1958. Resistance of plants to insects. Annu. Rev. Entomol. 3: 267-290.
Pan, Y., T. Peng, X. Gao, L. Zhang, C. Yang, J. Xi, X. Xin, R. Bi, and Q. Shang. 2015. Transcriptomic comparison of thiamethoxam-resistance adaptation in resistant and susceptible strains of *Aphis gossypii* Glover. Comp. Biochem. Physiol. Part D Genomics Proteomics 13: 10-15.
Pinheiro, P., M. S. Bereman, J. Burd, M. Pals, S. Armstrong, K. J. Howe, T. W. Thannhauser, M. J. MacCoss, S. M. Gray, and M. Cilia. 2014. Evidence of the biochemical basis of host virulence in the greenbug aphid, *Schizaphis graminum* (Homoptera: Aphididae). J. Prot. 13: 2094-2108.
Qureshi, J. A., and J. P. Michaud. 2005. Comparative biology of three cereal aphids on TAM 107 wheat. Environ. Entomol. 34: 27-36.
Ribeiro, J.M.C., A. C. Chagas, V. M. Pham, L. P. Lounibos, and E. Calvo. 2014. An insight into the sialome of the frog biting fly, *Corethrella appendi- culata*. Insect Biochem. Mol. Biol. 44: 23-32.
Roa, S.A.K., J. C. Carolan, and T. L. Wilkinson. 2013. Proteomic profiling of cereal aphid saliva reveals both ubiquitous and adaptive secreted proteins. PLoS ONE 8: e57413. doi: 10.1371/journal.pone.0057413.
Smith, C. M., D. J. Schotzko, R. S. Zemetra, and E. J. Souza. 1992. Categories of resistance in plant

introductions of wheat resistant to the Russian wheat aphid (Homoptera: Aphididae). J. Econ. Entomol. 85: 1480-1484.
Saidi, A., and J. S. Quick. 1996. Inheritance and allelic relationships among Russian wheat aphid resistance genes in winter wheat. Crop Sci. 36: 256-258.
Saldanha, A. J. 2004. Java treeview - extensible visualization of microarray data. Bioinformatics 20: 3246-3248.
Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbour Laboratory, New York, NY.
Smith, C. M., X. M. Liu, L. J. Wang, X. Liu, M. S. Chen, S. Starkey, and J. Bai. 2010. Aphid feeding activates expression of a transcriptome of oxylipin-based defence signals in wheat involved in resistance to herbivory. J. Chem. Ecol. 36: 260-276.
Tjallingii, W. F. 2006. Salivary secretions by aphids interacting with proteins of phloem wound responses. J. Exp. Bot. 57: 739-745.
Tolmay, V. L., F. Du Toit, and C. M. Smith. 2006. Registration of seven Russian wheat aphid resistant near isogenic lines developed in South Africa. Crop Sci. 46: 478.
Unger, L. M., and S. S. Quisenberry. 1997. Categorization of six wheat plant introduction lines for resistance to the Russian wheat aphid (Homoptera: Aphididae). J. Econ. Entomol. 90: 1408-1413.
Van der Westhuizen, A. J., X.-M. Qian, and A.-M. Botha. 1998a. p-1,3-gluca- nases in wheat and resistance to the Russian wheat aphid. Physiol. Plant. 103:125-131.
Van der Westhuizen, A. J., X.-M. Qian, and A.-M. Botha. 1998b. Differential induction of apoplastic peroxidase and chitinase activities in susceptible and resistant wheat cultivars by Russian wheat aphid infestation. Plant Cell Rep. 18: 132-137.
Van Eck, L., T. Schultz, J. E. Leach, S. R. Scofield, F. B. Peairs, A.-M. Botha, and N.L.V. Lapitan. 2010. Virus-induced gene silencing of WRKY53 and an inducible phenylalanine ammonia-lyase in wheat reduces aphid resistance. Plant Biotechnol. J. 8: 1023-1032.
Van Zyl, R. A. 2007. Elucidation of possible virulence factors present in Russian wheat aphid (Diuraphis noxia) biotypes' saliva. M.Sc. thesis. University of Pretoria, Genetics Department, South Africa.
Van Zyl, R. A., and A.-M. Botha. 2008. Eliciting proteins from *Diuraphis noxia* biotypes differ in size and composition, pp. 10-13. *In* IPRI-18th Biennial Workshop, 10-13 February 2008, Ft. Collins, CO.
Von Wechmar, M., and E. P. Rybicki. 1981. Aphid transmission of 3 viruses causes Freestate Streak disease. S. Afr. J. Sci. 77: 488^92.
Walters, M. C., F. Penn, F. Du Toit, T. C. Botha, K. Aalbersberg, P. H. Hewitt, and S. W. Broodryk. 1980. The Russian wheat aphid. Farming S. Afr. Leaf. Ser. Wheat G3: 1-6.
Wang, T., S. S. Quisenberry, X. Ni, and V. Tolmay. 2004a. Enzymatic chlorophyll degradation in wheat near-isogenic lines elicited by cereal aphid (Homoptera: Aphididae) feeding. J. Econ. Entomol. 97: 661-667.
Wang, T., S. S. Quisenberry, X. Ni, and V. Tolmay. 2004b. Aphid (Hemiptera: Aphididae) resistance in wheat near-isogenic lines. J. Econ. Entomol. 97: 646-653.
Wang, M. H., O. Marinotti, A. Vardo-Zalik, R. Boparai, and G. Y. Yan. 2011. Genome-wide transcriptional analysis of genes associated with acute desiccation stress in *Anophelesgambiae.* PLoS ONE 6: e26011.
Weiland, A. A., F. B. Peairs, T. L. Randolph, J. B. Rudolph, S. D. Haley, and G. J. Puterka. 2008. Biotypic diversity in Colorado Russian wheat aphid (Hemiptera: Aphididae) populations. J. Econ. Entomol. 101: 569-574.
Will, T., J. C. Carolan, and T. L. Wilkinson. 2013. Breaching the sieve element: the role of saliva as the molecular interface between aphids and the phloem. *In* G. A. Thompson and A.J.E. van Bel (eds.), Phloem: molecular cell biology, systemic communication, biotic interactions. Wiley-Blackwell, Ames, IA.
Zaayman, D., N.L.V. Lapitan, and A.-M. Botha. 2009. Dissimilar molecular defence responses are elicited in *Triticum aestivum* L. after infestation by different *Diuraphis noxia* (Kurdjumov) biotypes. Physiol. Plant. 135: 209-222.