Deciphering defense strategies that are elucidated in wheat containing different *Dn* resistance genes

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ABSTRACT

Russian wheat aphid (*Diuraphis noxia*, RWA, Homoptera: Aphididae) is a major pest problem in many wheat growing areas in the world. In the U.S. alone, the economic impact of RWA has been estimated at approximately $900M from 1987-1993. However, this estimate may be conservative, since several new biotypes were recently reported, presenting a difficult challenge for breeders. A lack of understanding of the interaction between the RWA and its host plant is a limitation in developing effective strategies for controlling the aphid. Wheat lines containing different resistance genes to the RWA exhibit different resistance or tolerance responses. We investigated these responses at transcriptome level in near-isogenic wheat lines (NILs) containing the *Dn1*, *Dn2* and *Dn5* resistance genes, respectively. Affymetrix gene technology (*i.e.* Affymetrix GeneChip® Wheat Genome Array) and cDNA-amplified fragment length polymorphism (cDNA-AFLP) transcript profiling were utilized. Following these approaches, we have identified genes and pathways associated with different resistance phenotypes afforded by the *Dn* genes. Detailed expression analyses using qRT-PCR and Northern blots provided further supporting evidence that regulation of specific pathways is critical for the development of a specific mode of resistance.

INTRODUCTION

Russian wheat aphid (*Diuraphis noxia* Kurdjumov, RWA) is a pest of wheat (*Triticum aestivum*) that occurs worldwide in wheat producing countries (with the exception of Australia) and causes great economic losses in South Africa[1] and the USA[2]. Symptoms associated with RWA feeding on the susceptible plants include: leaf rolling, the development of chlorotic streaking, a reduction of normal growth, which leads to a decrease in yield and even death, in the case of extreme infestation[3].

It is believed that the insects inject a phytotoxin into their hosts' phloem as part of their pierce-and-suck feeding process, and that this compound is responsible for the symptoms observed in the plants[4]. Its recognition by the resistant wheat plant elicits a defence response against RWA pathogenesis[5, 6]. It is believed that this recognition mechanism is a result of a "gene-for-gene" interaction between components in the host plant and those in the invading agent [2, 7].

Cells either defend themselves via cell wall thickening by up to 12%[6] or by experiencing programmed cell death. The latter is observed as necrotic lesions[8]. Subsequent to these activities, the systemic acquired resistance (SAR) commences through cascades mediated by salicylic acid and jasmonic acid, that lead to the expression of *pathogeneisis related* (*PR*)-genes, such as chitinase[5], β-1,3-glucanases[6] and peroxidases[5], and genes necessary for chloroplast maintenance, such as ATP synthase[7].

RWA resistance categories are defined as antibiosis (*i.e.* where the plant impedes the reproductive fitness of feeding aphids); tolerance (*i.e.* visible in a lack of plant height reduction because of feeding); or antixenosis (*i.e.* the non-selection of a cultivar as host)[9] [10]. The genetic background in which a specific *Dn* gene is bred may play a role in the successful establishment of a resistant phenotype[5, 6] and in some instances a resistant cultivar exhibits a combination of these categories of resistance[10]. Thus, understanding how this defence response is triggered provides the potential to screen wheat or other cereal populations for other potential genes with resistance against RWAs. Also, the very specific interaction between the RWA and wheat, make this an ideal model for studying insect-host interactions with the aim of applying the knowledge obtained on other phloem feeding insects' defence mechanisms.

MATERIALS AND METHODS

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Plant material Hexaploid wheat (*Triticum aestivum* L.) germplasm of the near-isogenic lines (NILs) Tugela, Tugela-*Dn1* (Tugela*4/SA1684; antibiotic), Tugela-*Dn2* (Tugela*4/SA2199; tolerant) and Tugela-*Dn5* (Tugela*4/SA463; antixenotic and low antibiosis) was obtained from the Small Grain Institute, Bethlehem, South Africa[11]. Plants were grown for 14 days (2-3 leaf stage) under greenhouse conditions at 25°C ± 2°C. Plants of each cultivar were infested with 5 adult, apterous *Diuraphis noxia*[12]and incubated for 2 h, 6 h, 12 h, or 24 h and 48 h. Control plants were not infested. All leaves except the first leaf were harvested into liquid N_2 and stored at -80°C prior to RNA isolation.

RNA preparation Total RNA was extracted using a guanidine thiocyanate buffer method[13] and the Qiagen RNeasy Plant Mini Kit with RNase Free/DNase (Qiagen, Valencia, CA) following the manufacturer's instructions. For cDNA-AFLP analysis mRNA isolation was performed using the Qiagen Oligotex mRNA kit. cDNA synthesis was performed using the cDNA Synthesis System (Roche Diagnostics GmbH, Mannheim, Germany) and the Qiagen MinElute Reaction Cleanup kit. Fifty ng of cDNA from each sample was used for cDNA-AFLP analysis[14]. cDNA-AFLP reactions were performed using the Expression Analysis kit (Li-Cor Biosciences, Lincoln, NB) for the generation of TaqI+2/MseI+2 pre-amplification PCR products, which were assayed for yield and quality by agarose gel electrophoresis.

Recovery of transcript derived fragments and identification Transcript derived fragments (TDFs) of interest were identified from gel images and excised from the polyacrylamide gel using the Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, USA). TDFs were eluted from polyacrylamide gel slices and re-amplified from the eluate by standard PCR with primers corresponding to the original primer combination used in selective amplification. Products were ligated into the pGem-T Easy plasmid vector (Promega Corporation, Madison, WI). Competent DH5α *E. coli* were prepared and transformed with the vector. After blue/white colony screening, white putative transformants were positively identified by colony PCR[15]. The sequences of cloned TDFs were obtained *via* dideoxy-dye terminator sequencing (Macrogen*USA*, Rockville, MD). Putative identities were assigned to TDFs by BLASTx and BLASTn homology searches in GenBank[16]. Expectation values where E = $e^{-05}$and lower were considered significant.

Hybridization to GeneChip® Wheat Genome Array (Affymetrix, USA) Integrity and quantity of the RNA was tested using Bio-Rad Experion RNA StdSen Chips (Bio-Rad, Hercules, CA). The RNA samples were sent to the Centre for Proteomic & Genomic Research (CPGR, Cape Town, South Africa), where additional quality control was performed. These facilities then performed RNA labelling, processing, and data gathering according to Affymetrix protocols.

Microarray data quality control and analysis A total of 12 samples were hybridized to arrays. Different quality control checks were performed including inspection of hybridized images, boxplots and histograms of $\log_2$(PM) values, examination of hybridization and PolyA controls. Data analysis was carried out using Bioconductor in R[17]. Data preprocessing and summarization were performed using Robust Multichip Average (RMA)[18], Affymetrix Microarray Suite5 (MAS5.0)[19], GeneChip Robust Multichip Average (GCRMA)[20], Variance Stabilisation (VSN)[21] and Probe level models (PLM)[22]. Only expression data significant to all normalization methods were included in further analyses. Statistical tests of differential expression were conducted using the moderated *t* test through the limma (Linear Models for Microarrays) package in Bioconductor. The Benjamini-Hochberg multiple testing adjustment was applied in order to control the comparison-wise false discovery rate[23].

Genes corresponding to probe sets with an absolute value of $\log_2$ fold change [$\log_2$(FC)] >1 and adjusted *P*-values of less than or equal to 0.05 were considered differentially expressed. The target sequences corresponding to genes identified as differentially expressed were obtained from Affymetrix. Target sequences were then searched against the KEGG[24] (http://www.genome.jp/kegg/), BRENDA[25] (http://www.brenda-enzymes.info/) and Gene Ontology (GO) databases (http://www.geneontology.org/) using BLASTx via the program PLAN[26]. Annotation was obtained for the top significant hit (using an e-value cutoff of $1e^{-10}$) for each target sequence.

RESULTS

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Several genes known to be involved in plant defense (*i.e.* reactive oxygen species, SAR associated and lignification) and stress were obtained from cDNA-AFLP and Affymetrix GeneChip® Wheat Genome Array analysis of the NILs after RWA feeding. The obtained significantly regulated genes also includes genes involved in cell signalling, wounding and ethylene signalling, transcription factors, proteins associated with energy production and carbon flux, as well as genes involved in the maintenance of photosystems PSI and PSII (Figures 1 and 2).

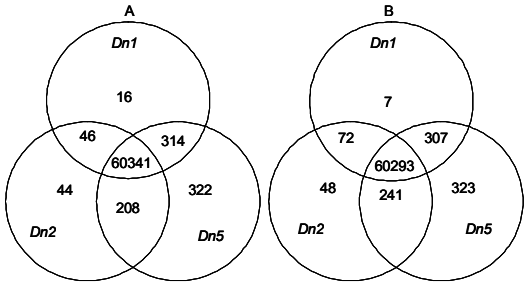

Figure 1. Genes significantly up (A) and down regulated (B) in NILs after RWA infestation. Genes represented were significantly up and down regulated after normalization ($\log_2$ fold change, $P < 0.001$).

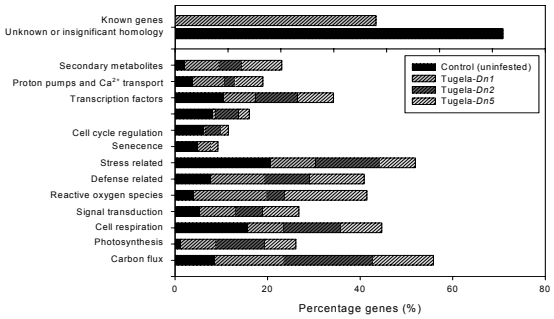

Figure 2. Representation of significantly regulated known genes according to functional categories in the cereal host during infestation by RWA. Genes represented were significantly up and down regulated after normalization ($\log_2$ fold change, $P < 0.001$)

DISCUSSION

The responses of RWA feeding on susceptible wheat are well documented[1,2,3-10]. RWA feeding causes immediate early responses generally associated with stress, such as loss of turgor pressure and leaf rolling[2,4,7,8]. This is followed by delayed responses such as chlorosis, and associated energy depletion and death as end result[2,3,4,7,8]. It is thus evident that several distinct mechanisms need to be in place to generate each mode of resistance, whether antibiosis, antixenosis or tolerance.

Antibiotic cultivars rapidly activate resistance mechanisms geared towards obstructing aphid feeding, even injuring the aphid and impeding oviposition[9, 10]. An antibiotic cultivar recognises the aphid stylet penetration, and immediate responses follow. These include: the activation signalling cascades and a substantial influx of Ca^{2+} into the cytosol. Results indicated that signalling cascades are induced within 2 h after feeding commences in Tugela-*Dn1*, leading to increased levels of SA and the oxidative burst. Levels of ROS are finely regulated by several systems involving iron homeostasis, RNA-binding genes and ABC transporters assisting in the movement of iron-sulfur clusters. Deposition of callose and sealing off of sieve elements interferes with aphid feeding. The production of ROS such as H_2O_2 elicits programmed cell death. PCD is visible as localized necrotic lesions on the leaves and is directed at the prevention of a "feeding" attack. *PR* gene expression is induced to provide long-term protection through SAR, and with enforced cell walls the cultivar are more resistant to subsequent attack.

Tolerant cultivars cope with high aphid pressure without incurring reductions in growth[9,10,27]. Tolerant plants use passive resistance mechanisms and strategies are directed at coping with the drain placed on energy and nutrients; and the damaging effects of aphid-derived molecules on chlorophyll levels, rather than deterring the insect. Photosynthetic compensation is deployed after 6 h to cope with the aphid associated stress. Photosynthetic compensation necessitates the upregulation of components of the photosystems. Aphid feeding interferes with the electron transport chain from PSII to PSI, leading to photobleaching of chlorophyll. By upregulating components of the electron transport chain and the rapid replacement of damaged components of PSII, as well as increasing levels of enzymes involved in photoassimilation, Tugela-*Dn2* manages to retain active photosynthesis and prevents chlorosis from occurring. Tolerant cultivars don't employ oxidative bursts associated with hypersensitive responses.

Antixenosis is associated with the expression of volatile organic compounds (VOCs). Expression of an ethylene-dependent RNA helicase was particularly prominent in Tugela-*Dn5*. This indicates that ethylene-mediated pathways might predominate in this line and that antixenosis constitutes a modification of the wounding response[28]. Significantly more crosstalk between SA-mediated and ethylene and JA-mediated pathways occurs. In addition to the specific applied mechanisms, it is also evident that the time and intensity of subsequent gene activation is critical in the development of specific resistant phenotypes, whether an active antibiosis, or a passive photosynthetic compensatory tolerance.

ACKNOWLEDGEMENT The authors express their sincere gratitude to the ARC-Small Grain Institute, Private Bag X29, Bethlehem, South Africa, 9700, for providing the seed stocks used in this study, and Winter Cereal Trust, the National Research Foundation and THRIP-DTI of South Africa for financial assistance, and the University of Pretoria for provision of infrastructure.

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