Aphid-induced transcriptional regulation in near-isogenic wheat

by

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DECLARATION

I, the undersigned, hereby declare that the dissertation submitted herewith for the degree *Magister Scientiae* to the University of Pretoria, contains my own independent work and has not been submitted for any degree at any other university.

Leon van Eck

April 2007

PREFACE

Most arable land devoted to a single crop is allocated for the production of wheat (*Triticum aestivum* L.). Indeed, wheat occupies one sixth of the world's total crop acreage. As the demand for wheat and other staple crops escalates with continued population growth, biotechnology is of obvious value in assisting the development of crops with more nutritional value, higher yield, and the ability to endure environmental stress and disease. An especially pressing problem in wheat cultivation is attack by phloem feeding insects. Due to its cryptic behaviour and biotypic diversity, the Russian wheat aphid (*Diuraphis noxia* Mordv.) has proven a particularly difficult pest to manage in many parts of the world. The development of cultivars with genetic aphid resistance must form part of an integrated pest management strategy. Therefore, it is vital to understand the aetiology of the symptoms caused by *D. noxia* and to identify the molecular mechanisms preventing resistant wheat cultivars from succumbing to attack.

Chapter 1 of this dissertation comprises a brief overview of our current knowledge of wheat resistance to the Russian wheat aphid, *Diuraphis noxia*. This chapter highlights the idiosyncrasies of the wheat genome, the biology and epidemiology of *D. noxia*, and places particular emphasis on the symptoms caused by *D. noxia* feeding in wheat and the genetics of wheat resistance to *D. noxia*. This plant-insect interaction is put into context by the discussion on general defence mechanism, including *R*-gene mediated resistance and the hypersensitive response.

The different genetically controlled mechanisms available to wheat in combating *D. noxia* attack are poorly characterized. Although it is known that resistant wheat can respond to aphid attack by being antibiotic, antixenotic or tolerant, the exact biochemical pathways involved in establishing these resistant wheat phenotypes are unclear. **Chapter 2** describes the

transcriptional profiling of the susceptible wheat line Tugela and three resistant near-isogenic lines developed from it, Tugela-*Dn1*, Tugela-*Dn2* and Tugela-*Dn5*, during *D. noxia*-induced stress. Various transcripts involved in or affected by the wheat responses to *D. noxia* are revealed by cDNA-AFLP and further characterized using qRT-PCR analysis; possible functions for some transcripts in *D. noxia* resistance are postulated.

Chapter 3 consists of a summary describing the significance of the study and detailing the different mechanisms and biochemical pathways that the wheat lines containing the three resistance genes *Dn1*, *Dn2* and *Dn5* employ in initiating and sustaining successful defence responses against aphid attack.

Novel sequences obtained from this study (see **Appendix**) were entered into the GenBank EST database (www.ncbi.nlm.nih.gov/) with the following accession numbers: EL563871; EL563872; EL563873; EL563874; EL563875; EL563876; EL563877; EL563878; EL563879; EL563880; EL563881; EL563882; EL563883; EL563884; EL563885; EL563886; EL563887.

The findings presented in this dissertation represent the outcomes of a sudy undertaken from March 2004 to October 2006 in the Department of Genetics, University of Pretoria, under the supervision of Prof. AM Botha-Oberholster and Prof NLV Lapitan. Chapter 2 is being prepared as a manuscript to be submitted for review to the journal *Plant Physiology*. The following manuscript and conference poster presentations were generated based on the results of this study:

Van Eck L, Lapitan NLV, Botha A-M (2007) Three near-isogenic lines of *Triticum aestivum* L. with distinct modes of resistance to *Diuraphis noxia* Mordv. exhibit dissimilar patterns of transcriptional regulation during aphid infestation. Plant Physiology (In preparation).

Van Eck L, Lapitan NLV, Botha-Oberholster A-M (2006) Expression profiling of near-isogenic lines of hexaploid wheat (*Triticum aestivum*) upon infestation with the Russian wheat aphid (*Diuraphis noxia*) using cDNA-AFLP and quantitative PCR analysis. South African Genetics Society 19th Congress: Bloemfontein, RSA, 2-4 April 2006. Programme and Abstracts, p. 43-44.

Van Eck L, Myburg AA, Lapitan NLV, Botha-Oberholster A-M (2006) Profiling Russian wheat aphid (*Diuraphis noxia*) induced transcripts in near-isogenic wheat (*Triticum aestivum* L.) lines using cDNA-AFLP and quantitative PCR analysis. 17th Biennial International Plant Resistance to Insects (IPRI) Workshop: Purdue University, West Lafayette, IN, USA, 9-12 April 2006.

Van Eck L, Myburg AA, Lapitan NLV, Botha A-M (2005) Expression profiling of near-isogenic lines of hexaploid wheat (*Triticum aestivum*) upon infestation with the Russian wheat aphid (*Diuraphis noxia* Mordvilko) using cDNA-AFLP analysis. Society for Experimental Biology Annual Main Meeting: Universitat Autonoma de Barcelona, Barcelona, Spain, 11-15 July 2005. Abstracts/Comparative Biochemistry and Physiology, Part A 141 S266.

Botha-Oberholster A-M, Matsioloko MT, Du Preez FB, Van Eck L, Walters RSG (2004) Russian wheat aphid-mediated elicitation of the wheat defense-transcriptome. 16th Biennial International Plant Resistance to Insects (IPRI) Workshop: Baton Rouge, LA, USA, 21-23 March 2004.

Botha-Oberholster A-M, Matsioloko MT, Du Preez, FB, Van Eck L, Walters RSG

(2004) Russian wheat aphid-mediated elicitation of the wheat defense-transcriptome.

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LIST OF ABBREVIATIONS

ABC	ATP-binding cassette
AFLP	amplified fragment length polymorphism
cDNA-AFLP	complementary DNA AFLP
CICR	calcium-induced calcium release
ET	ethylene
FBPase	fructose-1,6-*bis*phosphatase
HR	hypersensitive response
JA	jasmonic acid
NB-LRR	nucleotide-binding leucine-rich repeat
qRT-PCR	quantitative real-time PCR
PCR	polymerase chain reaction
PDI	protein disulfide isomerase
RAPD	random amplification of polymorphic DNA
RFLP	restriction fragment length polymorphism
ROS	reactive oxygen species
SA	salicylic acid
SAR	systemic acquired resistance
SSH	suppression subtractive hybridization
SV	slow-activating vacuolar
TDF	transcript-derived fragment
UDP-GTase	UDP-glucosyl transferase
VOC	volatile organic compound

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CHAPTER I

Literature review:

genetic resistance of hexaploid wheat (*Triticum aestivum* L.)

against the Russian wheat aphid (*Diuraphis noxia* Mordv.)

1.1 WHEAT: ITS ORIGINS AND GENOMIC ORGANIZATION

The genome of bread wheat (*Triticum aestivum* L.) is massive, containing 16.72 x 10^9 bp per haploid nucleus (Venter and Botha, 2000; Gupta et al., 2005). By comparison, rice (*Oryza sativa*), the model plant for cereal research, has a genome of about 450Mb (Ogihara et al., 2003) and *Arabidopsis thaliana* has a genome comprising a mere 140Mb (Lagudah et al., 2001). More than 70% of the gigantic *T. aestivum* genome is thought to consist of repetitive DNA (Feuillet et al., 2003) which separates 18 major and 30 minor gene-rich regions (Erayman et al., 2004).

It has been estimated that 70% of angiosperms were polyploid at some stage during their evolution (Masterson, 1994). This includes rice and *A. thaliana*, where extensive duplications and rearrangements can be found (Ogihara et al., 2003). *T. aestivum* is an allohexaploid, with its three distinct – yet closely related – sub-genomes designated A, B, and D (Lagudah et al., 2001; Gupta et al., 2005). These have been derived from three progenitor species. The A sub-genome of allopolyploid wheat shares a high degree of homology with the AA diploid *T. urartu*. This species hybridized about half a million years ago with the donor of the B sub-genome, an unidentified species most closely resembling *Aegilops speltoides*. The D sub-genome is similar to that found in *Ae. tauschii.* Subsequent hybridization between *Ae. tauschii* and the AABB allotetraploid is estimated to have occured 8 000-10 000 years ago, creating modern hexaploid wheat (Devos and Gale, 1997; Feldman and Levy, 2005). *T. aestivum* has proven to be the model system for studying the genomes of polyploid plants, especially since its progenitor species have been determined (Ogihara et al., 2003).

When cDNA clones are hybridized to aneuploid lines of hexaploid wheat, most genes are shown to be present on all three genomes (Devos and Gale, 1997). Retention of such a triplicate homeoallelic set makes gene redundancy quite common (Lagudah et al., 2001) and the compensating ability of chromosomes of different ancestral origin have made the classification of the 21 chromosomes of *T. aestivum* into 7 homeologous chromosomes for each discrete genome possible. Thus: $2n = 6x = 42$, AABBDD. Genomes with large chromosomes and where $x = 7$ are characteristic of the temperate grasses, as represented by such cereals as wheat, barley (*Hordeum vulgare*), oats (*Avena sativa*) and rye (*Secale cereale*). These form a monophyletic group (Figure 1.1) as confirmed by molecular phylogenetic analysis (Kellog, 2001).

Although wheat is the most extensively studied crop at the cytogenetic level (Gupta et al., 2005), at the genomic level rice has been the most thoroughly analyzed (Sasaki, 1998). Because of its small size, rice has been the best analogue for the ancestral grass genome (Devos and Gale, 1997). The genomes of the Triticeae tribe have been characterized by rearrangements relative to the rice genome. Rice chromosome 10 is inserted into the proximal region of the long arm of R5, forming the wheat group 1 homeologous chromosomes; R8 is inserted into R6 to form the homeologous group 7 chromosomes; and R7 into R4 to form the homeologous group 2 chromosomes (Devos and Gale, 1997).

The convergence of the maps of the three genomes of hexaploid wheat is not an isolated phenomenon, however. Comparative genetics using cross-mapping of RFLPs have resulted in evidence for extensive synteny across the grass family (Devos and Gale, 1997; Gale and Devos, 1998; Gupta et al., 2005). With the exception of some local sequence artefacts, such as tandem duplications, inversions, deletions and translocations, genes in the large Poaceae family of almost 10 000 species (Kellog, 2001) tend to be ordered colinearly on chromosomes despite the enormous disparity in DNA content between them (Ilic et al., 2003). Within the

Triticeae tribe wheat, *T. monococcum*, *Ae. tauschii* and barley have genomes that are highly colinear, with the presence of some small inversions (Devos and Gale, 1997). Although colinearity is preserved in the rearranged segments, a minimum of 7 translocations distinguishes rye and *Ae. umbellulata* exhibits at least 11 such events. These species-specific incidents might occur during or post-speciation. In contrast to the colinearity between wheat genomes, maize (*Zea mays*) is a palaeopolyploid, a tetraploid approaching the diploid state (Ilic et al., 2003), with such advanced differentiation that the homeologous chromosomes cannot really be discerned any longer (Devos and Gale, 1997).

Comparative wheat genomics can facilitate gene discovery through the use of micro-synteny between the model rice genome and that of wheat, as well as the sequence similarities between the gene products of wheat and their homologues in *Arabidopsis* (Lagudah et al., 2001). However, the high levels of gene duplication in wheat compared to rice have so far restricted the use of rice information in the positional cloning of Triticeae genes. Previous efforts at cross-mapping genes found in the *Adh1-Adh2* region of rice onto maize indicate that colinearity is maintained for housekeeping genes, but not for the more agronomically important disease resistance genes (Gale and Devos, 1998). Many multigene families, such as the resistance genes and storage proteins are in non-homoeologous loci even within the wheat tribe (Lagudah et al., 2001) and disease resistance genes may exhibit a faster rate of evolution, as observed from mapping some disease resistance gene homologues across rice, barley and foxtail millet (Gale and Devos, 1998). Comparative genomic approaches can therefore not be employed to successfully identify cereal resistance genes. A functional genomics approach to gene discovery would be a more suitable approach when dealing with rapidly radiating idiosyncratic gene families.
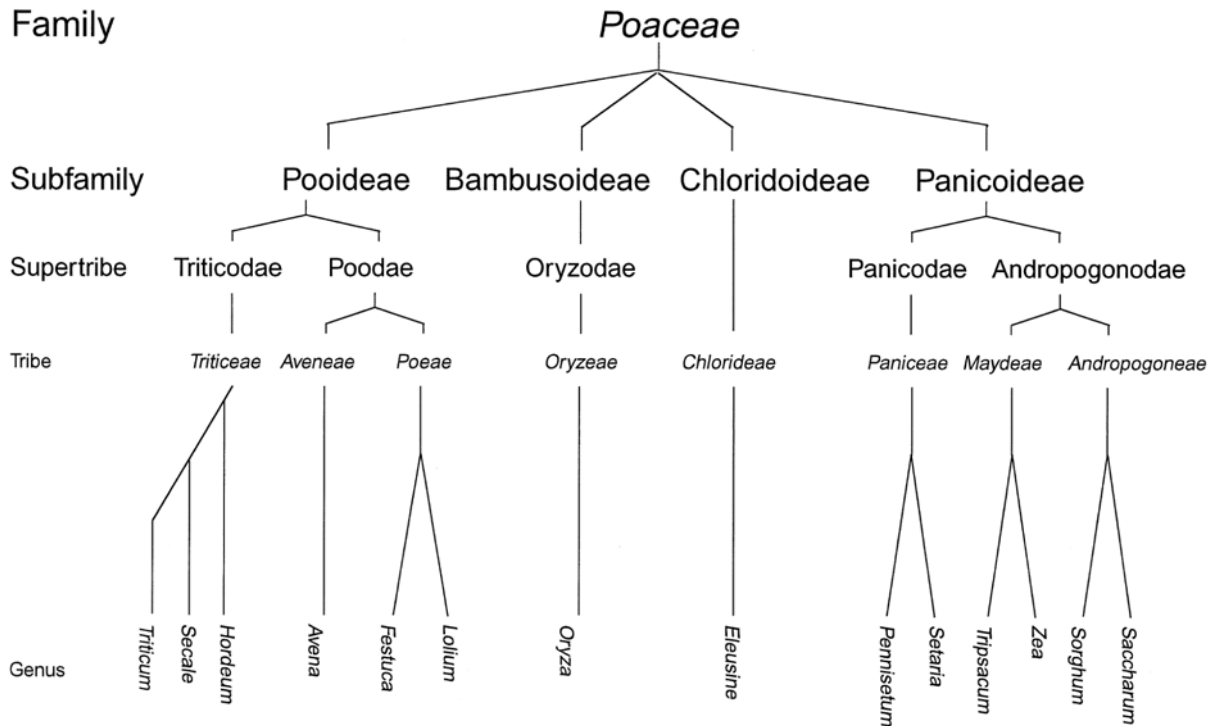
Figure 1.1

Taxonomic relationships within the grass family (taken from Devos & Gale, 1997). The Triticeae are a monophyletic group.

1.2 RUSSIAN WHEAT APHID RESISTANCE

1.2.1 Characterization of *D. noxia* damage

The Russian wheat aphid (*Diuraphis noxia,* Mordvilko) is a pest of wheat and barley causing devastation in many countries of the world (Smith et al., 1992). Economic losses result from reduced yield as well as increased production costs (Anderson et al., 2003). The greatest damage happens early in the South African and US growing seasons (Baker et al., 2003). The damage is assumed to occur because of a phytotoxin injected by the aphids during feeding,

which causes chloroplast and cellular membrane breakdown. As yet, no such toxin has been isolated or characterized. Feeding damage by *D. noxia* is typified by longitudinal white and yellow streaking (Figure 1.2) and stunted growth (Burd and Burton, 1992; Puterka et al., 1992) and chlorophyll deficiency due to infestation reduces yields by up to 50% (Burd and Burton, 1992). *D. noxia* damage disrupts osmoregulatory processes and drought-stress symptoms are observed in the leaves of infested plants (Burd and Burton, 1992). Cell bleaching has been shown to be related to induced water imbalances (Van der Westhuizen et al., 1998) and drought stress has been shown to accelerate the damage caused by *D. noxia* feeding (Liu et al., 2001). *D. noxia* typically aggregates on the new growth of the host (Burd and Burton, 1992), where it probes the vascular bundles of the leaves in search of the phloem (Ni and Quisenberry, 1997). The use of an alternating current electronic insect feeding monitor has shown that *D. noxia* probes more often, but for a shorter duration diurnally, whereas fewer probes of longer duration constitute nocturnal feeding behaviour (Ni and Quisenberry, 1997).

The damage inflicted by probing and feeding *D. noxia* prevents new leaves from unfurling properly and causes leaf rolling in the already spread out leaves (Figure 1.2). *D. noxia* feeding apparently reduces leaf turgor below the threshold required for elongation and cell wall extensibility, causing a reduction in leaf size and preventing the new leaves from unfolding correctly (Burd and Burton, 1992). Rolled leaves shelter the aphids from chemical and biological control methods (Smith et al., 1992). Feeding on the flag leaf results in the developing grain head becoming trapped and interferes with self-pollination and grain-filling (Van der Westhuizen et al., 1998).

Ultrastructural studies showed limited chloroplast breakdown of the leaf cells of resistant cultivars after feeding. *D. noxia*-infested barley shows more collapsed auto-

fluorescent cells typical of a hypersensitive cell death response (Van der Westhuizen et al., 1998).

Figure 1.2

Symptoms of *D. noxia* infestation on susceptible wheat, indicating leaf rolling (*left*) and chlorotic streaking (*right*).

1.2.2 *D. noxia* resistance in wheat

The management of *D. noxia* includes the use of systemic insecticides and biological control agents, such as *Diaeretiella rapae*, an endoparasitic wasp and the natural enemy of over 60 different aphid species worldwide (Baker et al., 2003). The efficacy of biological control can be enhanced by coupling it with resistant genotypes: since resistant cultivars exhibit less leaf-rolling, these provide predators and parasitoids with better access to developing aphid

colonies (Jyoti and Michaud, 2005). Genetic plant resistace to *D. noxia* is also considered a more desirable alternative to the use of expensive and dangerous pesticides (Puterka et al., 1992; Ni and Quisenberry, 1997).

Numerous sources of natural resistance to *D. noxia* have been identified in members of the Triticeae tribe and are used extensively in the breeding of resistant cultivars (Anderson et al., 2003; Lage et al., 2004; Ceoloni et al., 2005; Randolph et al., 2005). Wheat resistance to *D. noxia* is classified into three categories: antibiosis, antixenosis and tolerance (Painter, 1958). The genetic background in which a specific resistance gene is bred may play a role in the successful establishment of a resistant phenotype (Van der Westhuizen et al., 1998) and in some instances a resistant cultivar exhibits a combination of these categories of resistance (Smith et al., 1992).

Antibiosis indicates how fertile the aphids are on that host, and is measured through parameters such as fecundity (mean number of nymphs produced per female), nymphipositional period (mean number of days from production of first progeny to last progeny), mean maximum number of nymphs produced per day of the nymphipositional period, mean number of nymph production days, and mean adult longevity (Smith et al., 1992). Mean *D. noxia* fecundity is lower on resistant varieties, and nymphs are produced over a shorter period of time, with less daily nymphal production and lower mean maximum number of nymphs produced per day. Adult longevity is also shorter. Differences in antibiosis may also be detected by examining the rate of nymph production and any related lag time (Unger and Quisenberry, 1997).

Antixenosis indicates the preference of the aphid for the plant in terms of oviposition, shelter or food (Painter, 1958), and is measured by the parameters such as number of aphids found on plants of different genotypes offered in a choice test of short-term duration (Smith et al., 1992).

Measures of tolerance encompass the height and fresh weight of infested plants, as well as the dry weight, standardized against uninfested samples to yield a relative degree of reduction. Susceptible plants suffer greater weight and height reductions compared to tolerant varieties. Burd and Burton (1992) demonstrated that the duration of the aphid infestation might be more important to the host than the level of infestation. Infested plants cease growth, but have the ability to rapidly recover after the aphids are removed: carbon assimilation increases during recovery, with increased leaf and root growth.

Eleven *D. noxia* resistance *R* genes, known as *Dn* genes, have so far been found in wheat and its relatives: *Dn1-Dn9*, *Dnx* and *Dny* (Liu et al., 2005).

Dn1 is a single dominant gene conferring antibiosis and was obtained from the germplasm accession PI (plant introduction) 137739 (South African wheat accession SA1684) (Du Toit, 1987).

Dn2 is a single dominant gene found in PI 262660 (South African wheat accession SA2199) (Du Toit, 1987), and confers mostly tolerance with only a low level of antibiosis (Smith et al., 1992).

A plant introduction from the former Soviet Union, PI 372129 contained *Dn4*, a single dominant gene first identified in the United States in 1987. *Dn4* confers tolerance and has been mapped to the short arm of chromosome 1D (Ma et al., 1998; Liu et al., 2001). The resistance afforded by *Dn4* is utilized in 'Halt', released to growers by the Colorado Agricultural Experiment Station in 1994 and the resistant cultivar most often found in the US (Liu et al., 2001). The antixenotic single dominant *Dn7* gene was transferred from rye into a wheat background via a RS/1BL translocation and confers a higher level of resistance than *Dn4*, although linkage studies have shown that *Dn7* and *Dn4* may be orthologous (Anderson et al., 2003).

PI 294994 (South African accession SA463) is a winter wheat accession from Bulgaria exhibiting antixenosis and moderate antibiosis; its resistance is labelled *Dn5* (Marais and Du Toit, 1993; Zhang et al., 1998; Heyns et al., 2006). The mode of inheritance of *Dn5* has been variously described as either a single dominant gene; one dominant and one resistant gene; two dominant genes in coupling-phase linkage, allelic to the other *Dn* genes; or a totally separate entity (Zhang et al., 1998). This confusion could possibly stem from the heterogeneity in the original PI 294994 accession and the effects of different parents used in the crosses made for linkage analysis (Heyns et al., 2006). According to Liu et al. (2001) PI 294994 contains three resistance genes, collectively designated *Dn5+*. One of these is *Dn8*, located distally on chromosome 7DS (Liu et al., 2001) and therefore loosely linked to *Dn5* (Zhang et al., 1998). The other, *Dn9*, was positioned by Liu et al. (2001) on chromosome 1DL in PI 294994.

Several molecular markers have been developed to assist in identifying and localizing *Dn* genes. The use of RAPD markers has yielded some molecular markers linked to *Dn2* (Myburg et al., 1998) and *Dn5* (Venter and Botha, 2000). *Dn1*, *Dn2* and *Dn5* are located on chromosome 7D, but have been capriciously mapped to the long arm (Ma et al., 1998), or the short arm of chromosome 7D (Liu et al., 2001). Bulk segregant analysis found the microsatellite locus *Xgwm111* to be tightly linked to *Dn1*, *Dn2, Dn5* and *Dnx* (Liu et al., 2001). Aneuploid analysis pinpointed its location on chromosome 7DS. This has led to the hypothesis that these resistance genes could be allelic at the same locus, or different, but tightly linked on the short arm of chromosome 7D, proximal to the centromere. More recently, physical mapping again moved the location of *Dn5* to 7DL (Heyns et al., 2006). The same study reiterated that the original PI 294994 introduction is a heterogenous source of *Dn* genes and that any cultivar bred from it may contain a number of linked or allelic loci (Heyns et al., 2006).

One way of limiting time-consuming segregation analyses is to use near-isogenic lines (NILs), which have great advantages in developing new markers. By crossing the susceptible parent with the parent carrying the gene of interest and subsequently backcrossing the offspring to the susceptible parent, a line is produced that closely resembles the susceptible line, but contains the resistance gene of interest. The only difference between NILs is introduced by linkage drag, which enables researchers to identify polymorphisms in the region bracketing the introgressed gene. Suitable DNA markers in this window are present in the individuals with the introgressed gene when in coupling phase, but absent in those without it. With development of more closely linked molecular makers, breeders can lessen linkage drag from the progenitor species into the cultivar genome, resulting in little alteration of the developing cultivar (Botha and Venter, 2000).

The 'Tugela' cultivar ('Kavkaz'/'Jaral') is a high yielding hard red intermediate wheat with a medium growth period, released in South Africa in 1985 (Tolmay et al., 2006). The Tugela resistant NILs were created at the Small Grain Institute, Bethlehem, South Africa, by consecutive backcrosses using single-seed selection in each generation. The final backcross line was selfed twice to produce defined homozygous resistant lines. The following NILs are available: Tugela (*D. noxia* susceptible line), Tugela-*Dn1* (Tugela*4/PI 137739), Tugela-*Dn2* (Tugela*4/PI 262660), and Tugela-*Dn5* (Tugela*4/PI 294994) (Lacock et al., 2003; Tolmay et al., 2006).

1.2.3 Biotypes and aphid distribution

The Russian wheat aphid (Figure 1.3) was discovered in the United States in March 1986, near Lubbock, Texas (Burd and Burton, 1992; Jyoti and Michaud, 2005), having been introduced via the Texas Panhandle. It crossed the Canadian border into southern Alberta in

1988 (Jyoti and Michaud, 2005); by 1990 it was reported from all wheat-producing countries except Australia (Basky, 2003). *D. noxia* collected from around the world show biotypic differences (Smith et al., 1992; Smith et al., 2004). Biotypes are defined as populations within an insect species that are able to damage plant genotypes previously resistant to that insect (Puterka and Burton, 1990). The large amount of potential variation is of major concern when developing plant resistance, since it commonly occurs in aphids targeted for such a pest management strategy, such as the greenbug (*Schizaphis graminum*). The category of plant resistance – such as antibiosis, antixenosis and tolerance (Painter, 1958) – and the genetic diversity of the insect, as well as the prevalence of specific resistant cultivars and the number of resistance genes they harbour can all affect the occurrence of virulent biotypes (Smith et al., 2004).

The high degree of biotypic diversity found in worldwide *D. noxia* populations implies a geographical limit on the usefulness of any particular resistant plant germplasm. However, this could also suggest that cultivars susceptible to *D. noxia* in one geographic area might actually be resistant in another geographical region (Puterka et al., 1992). It is imperative to bear in mind that a single genetic source of resistance expressed in vast acres of monoculture for several growing seasons may pose a considerable directional selection for the emergence of virulent *D. noxia* populations (Jyoti and Michaud, 2005).

Countries where *D. noxia* has been established for many years may have a number of biotypes (Puterka et al., 1992) and single heavily infested sites might be potential sources of *D. noxia* expansion, such as the Carpathian Basin, being a model for *D. noxia* expansion throughout much of Eastern Europe. *D. noxia* is widely adaptable to differing altitudes and latitudes, but seems to prefer hot and dry areas similar to its place of origin in Central Asia (Starý et al., 2003). The pest is endemic to the former Soviet states, the Balkans and much of

the Middle East. Consequently, many sources of genetic resistance have been found in wheat accessions originating here (Harvey and Martin, 1990; Du Toit, 1992).

Percentage leaf chlorosis is one character used for detecting biotypic variation in *D. noxia* on cereals, although the extent of chlorosis is closely related to aphid numbers (Puterka et al., 1992). DNA fingerprinting techniques such as AFLP and RAPD PCR are also used to detect polymorphisms between US and South African populations of *D. noxia*. There are numerous reports of resistance-breaking strains of *D. noxia*: the wheat cultivar 'Amigo' was resistant to the South African aphid strain in 1985. By 1987, decreased resistance was reported in the USA (Basky, 2003). In the spring of 2003, an outbreak of *D. noxia* in southeastern Colorado was reported from fields planted with previously resistant cultivars (Haley et al., 2004; Jyoti and Michaud, 2005). This new virulent population, now termed US biotype 2, quickly gained ground and was soon reported in Nebraska and Texas (Smith et al., 2004). Only the *Dn7* gene has proved resistant to the new US biotype 2, since the *Dn4*-containing 'Halt' cultivar produced the susceptible symptoms of chlorotic streaking and leaf rolling when attacked by it (Collins et al., 2005).

The Hungarian *D. noxia* is more virulent than the South African aphid, the South African aphid being more susceptible to antibiosis. None of the South African resistant cultivars tested were resistant to Hungarian *D. noxia* populations. Because of its increased virulence, the Hungarian biotype is potentially more damaging. Although it occurs in Hungary, Germany and the Czech Republic, *D. noxia* is not recognized as a pest of wheat in these countries. Agricultural practice could play a part: aphid numbers might be negatively influenced by the increased relative humidity in Hungarian wheat fields, where wheat is sown at a density of 200-220 kg/ha. In South Africa and the United States, wheat is sown at a much lower density of 20-120 kg/ha (Basky, 2003).

Most *D. noxia* found are parthenogenic and viviparous, and less than 1% of those collected are oviparous (Unger and Quisenberry, 1997). Male sexual morphs have not been discovered in the United States (Puterka et al., 1992) or South Africa, but might be present at levels too low to be detected. Sexual reproduction could be the primary mechanism for generating aphid biotypic diversity. Different types of resistance will place aphid populations under different levels of adaptive pressure. Lack of high levels of reproductive antibiosis should negate or delay the development of *D. noxia* biotypes (Smith et al., 1992) and resistant plants showing tolerance should enable *D. noxia* to survive on plants that will support predator and parasite populations (Puterka et al., 1992). Utilizing gene pyramiding in plant breeding in order to furnish polygenic plant resistance will result in plants that cope better with the arrival of new virulent aphid biotypes.

Figure 1.3

The Russian wheat aphid (*Diuraphis noxia*) feeding on wheat leaves, indicating the cryptic behaviour exhibited by this aphid (*left*) and the morphology of alate (winged) and apterous (wingless) forms (*right*).

1.3 MECHANISMS OF PLANT DEFENCE

1.3.1 Plant-insect interactions

Plants have well-known structural defence mechanisms, such as thorns, silica and trichomes. Trichomes form a physical obstacle to *D. noxia* feeding: resistant Tugela-*Dn1* plants had 1.7 times greater trichome density than the susceptible Tugela line, which may prevent *D. noxia* from finding a suitable feeding site (Bahlman et al., 2003).

Direct chemical defence also plays a significant part in plant defence against insects. This huge array of chemical defences includes: proteinase inhibitor proteins that enhance plant resistance to insects by actively inhibiting their proteolytic digestive enzymes; polyphenol oxidases that decrease the nutritive value of the plant by cross-linking proteins or catalyzing the oxidation of phenolic secondary metabolites; and toxic compounds, such as alkaloids, terpenoids and phenolics that poison broad-spectrum herbivores, forcing specialists to invest their resources in detoxification mechanisms that in turn incur growth and development costs (Trumble et al., 1993; Kotanen and Rosenthal, 2000; Kessler and Baldwin, 2002).

Indirect plant defence mechanisms rely on the release of volatile organic compounds (VOCs) in order to signal the presence of herbivores to their predators and parasitoids. These insects feed on or deposit eggs into the larvae of the herbivorous insect (Dangl and Jones, 2001). Volatile organic compounds are mainly C_6-alcohols and –aldehydes, terpenes and molecules derived from shikimate (Kessler and Baldwin, 2002).

Chewing insect damage usually elicits a wounding response, mediated chiefly by ethylene (ET), as well as jasmonic acid (JA), a linolenic acid derivative. The primary wound signal is represented by systemins, the first described oligopeptides with phytohormonal function (Kessler and Baldwin, 2002). They are 18 amino-acid peptides cleaved from a 200-amino acid precursor called prosystemin, are active at femtomolar levels and are known to be transported in the phloem (Baker et al., 1997). Plasma membrane proteins initiate a complex wound cascade after binding systemins (Kessler and Baldwin, 2002), which includes the induction of proteinase inhibitors at remote wound sites (Baker et al., 1997). Oligogalacturonides are a local, intermediate step in signalling following systemin production that transiently elicits the production of reactive oxygen species. The wound cascade concludes with the activation of phospholipase A_2, which releases linolenic acid from the plasmamembrane, supplying the substrate for the initial step in the octadecanoid (C_{18}-fatty acid) biosynthetic pathway (Baker et al., 1997; Gachomo et al., 2003).

The plant response to aphid feeding via piercing-sucking mouthparts differs from that elicited by the more mechanical damage inflicted by other insects with chewing mouthparts, since they exhibit a more intimate and sophisticated mode of biotrophic parasitism. The biochemical origin of wheat resistance to *D. noxia* is still relatively unclear (Van der Westhuizen et al., 2002). Since the aphid probes mainly intercellularly before the stylet penetrates the phloem, several defence-related products have been shown to accumulate in the apoplast of resistant wheat cultivars, including pathogenesis-related proteins like chitinases and oxidative enzymes like peroxidases (Van der Westhuizen et al., 1998). Resistance is not constitutively expressed and is induced by *D. noxia* infestation. The level of the response varies in different resistant cultivars and the genetic background in which the *Dn* gene is bred plays a role in the effectiveness of the resistance response (Van der Westhuizen et al., 1998).

Aphid feeding induces not only wound-responsive JA-regulated genes as activated by a chewing insect, but also the expression of pathogen-responsive genes regulated by salicylic acid (SA) (Montesano et al., 2003). For example, plants attacked by aphids have higher mRNA levels of *PDF1.2* (encoding defensin) and *LOX2* (encoding lipoxygenase), both of which are wound inducible and involved in the JA signalling cascade. Green peach aphids (*Myzus persicae*) induce SA-dependent transcription of *PR-1* and *BGL2* in wild-type *Arabidopsis* plants, but not in *npr1* mutants, which are deficient in SA signalling (Kessler and Baldwin, 2002). SA- and JA-dependent genes have also been induced in plants attacked by phloem-feeding whiteflies (Kessler and Baldwin, 2002). The activation of both JA- and SA-induced genes during aphid feeding contradicts reports indicating that the two systems work antagonistically and that SA and other cyclooxidase inhibitors actually inhibit JA production and JA-elicited gene expression (Dangl and Jones, 2001). During tobacco mosaic virus (TMV) infection for example, SA production inhibits wound-inducible JA accumulation and secondary metabolite accumulation (Kessler and Baldwin, 2002).

The activation by aphids of disease resistance pathways usually engaged in pathogen defence indicate highly specific interactions between plants and phloem-feeding insects. This is based on recognition of aphid-derived agents by the plant, and these eliciting agents are then induce physiological or biochemical responses associated with the expression of resistance (Kogel et al., 1988). In *D. noxia*, salivary proteins are the most likely elicitors of resistance, since they come into sufficiently close contact with the plant during feeding and resistant wheat injected with *D. noxia* proteins showed induction of defence-related enzymes (Lapitan et al., 2005).

1.3.2 Disease resistance as a gene-for-gene relationship

The strategies plants use to defend themselves show similarities to animal innate and adaptive immunity. Plants not only have preformed defences that provide non-specific protection against a wide range of organisms, but also have active host-specific responses such as the hypersensitive response (HR) and systemic acquired resistance (SAR) (Jackson and Taylor, 1996).

Compatible reactions result in disease symptoms and incompatibility results when the resistance reaction severely restricts or prevents pathogen growth (Jackson and Taylor, 1996). Incompatibility is the result of a single interacting pair of genes in the host and pathogen (Staskawicz et al., 1995). This is therefore a very specific type of resistance and although this relationship has been best characterized in plant-pathogen reactions, it directly applies to *D. noxia* resistance in wheat, since the *Dn* genes condition resistance in an analogous way. The gene of plant origin in this interacting pair is termed the resistance (*R*) gene and the gene of pathogen origin is termed the avirulence (*Avr*) gene. This relationship of *R* genes in the host interacting in paired combinations with pathogen *Avr* genes to condition resistance is known as the gene-for-gene hypothesis (Flor, 1955). An *Avr* gene gives the pathogen an avirulent phenotype on a host plant carrying the corresponding *R* gene (Staskawicz et al., 1995).

The gene-for-gene interaction depends on the recognition by the *R* gene products of specific elicitor signal molecules produced directly or indirectly by the pathogen *Avr* genes, whereupon the *R* gene products are responsible for initiating directed plant defence responses (Staskawicz et al., 1995; Bent, 1996). A receptor-ligand model has been proposed for gene-for-gene relationships, whereby the R proteins must act as receptors that recognize *Avr* gene-dependent ligands as well as activate an intracellular signalling cascade (Bent, 1996; Hammond-Kosack and Jones, 1997). There is sufficient evidence to suggest that *D. noxia-*

derived elicitors are recognized in such a *Dn* gene product-mediated way in order to affect resistance (Van der Westhuizen et al., 1998; Botha et al., 2005; Lapitan et al., 2005). In the case of fungal or bacterial infection, the activated signalling cascades coordinate the initial plant defence responses to impair pathogen entry. Therefore, R proteins need to be constitutively expressed in healthy, unchallenged plants in order to detect attacks at any time. RNA gel blot analysis confirmed that the *RPM1 R* gene among others is constitutively expressed at low levels in healthy plants (Hammond-Kosack and Jones, 1997).

Certain *R* genes, like *Pto* of tomato, cannot independently condition a resistance response, yet are essential for *R* gene activity. This indicates that R proteins do not necessarily interact directly with Avr proteins, but might function in a protein complex that is the functional receptor (Jackson and Taylor, 1996). The occurrence of Pto-based resistance introduces the guard hypothesis. In this model system, AvrPto of *P. syringae* targets Pto, and another protein, Prf, guards Pto by activating defence upon detecting AvrPto-Pto complexes (Van der Biezen and Jones, 1998). Here, the R protein shows no solitary interaction with the Avr protein, but rather monitors whether another cellular protein is under attack from the pathogen effector protein (Dangl and Jones, 2001).

The concept that pathogens would retain dominant *Avr* genes that elicit a concerted resistance response from their host is counterintuitive. In fact, many Avr proteins have important functions as viral coat proteins, or the structural components of bacteria, like glucan, chitin and flagellin (Montesano et al., 2003). Any gene expressed by a pathogen could become an *Avr* gene if an *R* gene capable of recognizing its product and triggering a strong defence response evolved in the host plant (Bent, 1996). The re-establishment of virulence after an *R* gene has evolved in the resistant plant could take place via the deletion of *Avr* genes, frameshifts leading to truncated Avr proteins or ectopic expression from altered promoters. If vital pathogen components such as those involved in replication are targeted, the

effectiveness of the corresponding *R* gene may be extended (Jackson and Taylor, 1996). Compatibility and disease result from any alteration or loss to either the plant resistance *R* gene or pathogen avirulence *Avr* gene (Hammond-Kosack and Jones, 1997; Buell, 1998). Microbes expressing Avr proteins have higher fitness when infecting susceptible plants not carrying the cognate *R* gene (Staskawicz et al., 1995; Dangl and Jones, 2001) and incur fitness penalties when mutations convert avirulence to virulence (Jackson and Taylor, 1996; Hammond-Kosack and Jones, 1997).

The co-evolution of plants with their pests and pathogens has resulted in the development of a molecular arms race. As soon as virulent pests or pathogens evolve, with the ability to infect the host, the susceptible host undergoes selective pressure to recognize attack and mount a suitable response. The pathogen responds to this novel resistance with an alternative mechanism, thus restoring virulence. This cycle will continue as a dynamic ongoing evolutionary battle, generating a large diversity of disease resistance genes (Staskawicz et al., 1995; Jackson and Taylor, 1996). Depending on population structure, plant pathogens may exhibit a high mutation rate from avirulence to virulence that renders individual *R* genes obsolete. Natural selection favours virulent races and therefore plants must evolve novel R protein variants to either detect the modified Avr determinant or another pathogen component (Hammond-Kosack and Jones, 1997).

1.3.3 *R* gene evolution, organization and structure

It is now more than a decade since the first *R* genes were isolated. The functional *R* genes isolated so far encode resistance to bacterial, viral and fungal pathogens and even nematodes and insects (Dangl and Jones, 2001). *R* genes may have evolved from proteins involved in endogenous recognition and signalling systems utilised during growth and development. A

significant number of the mammalian, yeast, and insect proteins related to plant R proteins control endogenous signalling, development and cell-to-cell adhesion. Two plant proteins encoded by the *Arabidopsis erecta* and *clavata* genes determine floral organ shape and size and are similar to the rice R protein Xa21 (Jackson and Taylor, 1996; Hammond-Kosack and Jones, 1997). Molecular analysis of the TMV resistance gene suggests the presence of an array of related genes at the *N* locus in tobacco. An *N*-like cluster has been identified in tomato, which suggests that this complex locus arose in a progenitor species (Baker et al., 1997). This indicates either ancient specificity or convergent evolution driven by prevalent pathogen ligands (Hammond-Kosack and Jones, 1997).

R genes are often organized into linkage groups that contain separate resistance loci, each with a number of different allelic specificities and each conditioning resistance to a different pathogen. Thus, *R* genes are often members of large multigene families (Bent, 1996; Baker et al., 1997) arranged in large arrays forming complex loci that can be conserved in distantly related plant species (Jackson and Taylor, 1996). The tight linkage between *Dn1*, *Dn2*, *Dn5* and *Dnx* on chromosome 7D of wheat (Liu et al., 2001) might be an example of this.

Tight clustering of *R* genes probably arose due to the duplication of a segment carrying an ancestral gene through unequal crossing-over during meiotic mispairing. Genetic analysis of the highly unstable *Rp1* complex in maize using flanking DNA markers has shown that diversity arose through cross-overs as well as gene conversion (Hammond-Kosack and Jones, 1997). Novel specificities may evolve via frequent recombination and mispairing events such as duplications and deletions, followed by divergence (Bent, 1996). Gene dosage effects, nonallelic and epistatic interactions, together with the host background genotype can all influence the inheritance and phenotype of *R* genes. Unequal crossing over within clusters of similar sequences could accelerate the appearance of new *R* gene alleles. Plants with newly

developed specificities will have a selective advantage in the face of rapidly evolving pathogen populations (Bent, 1996; Jackson and Taylor, 1996; Baker et al., 1997).

Most plant disease resistance genes are of the NB-LRR kind, encoding proteins with nucleotide binding (NB) and leucine-rich repeat (LRR) domains (Bai et al., 2002; Lacock et al., 2003). Annotation of the *Arabidopsis* genome indicates the presence of roughly 150 genes that encode R proteins of the NB-LRR kind, more if the truncated proteins are included (Dangl and Jones, 2001; Bai et al., 2002). It is therefore probable that the wheat *Dn* genes encode such NB-LRR proteins (Botha et al., 2005), constitutively expressed at low levels in resistant plants in order to recognize aphid elicitors in a fast, elicitor-specific way. No function other than disease resistance has yet been assigned to the NB-LRR class of genes (Bai et al., 2002).

LRR domains are multiple serial repeats of a motif about 24 amino acids in length. LRRs contain leucines or other hydrophobic residues at regular intervals and regularly spaced prolines and asparagines. The short repeat lengths found in plant disease resistance genes are thought to adopt a structure resembling a *β*-helix with a linear structure (Hammond-Kosack and Jones, 1997). Functional specificity is thought to reside with the intervening exposed amino acids and not with the conserved hydrophobic residues of the LRRs, which are oriented internally to serve as scaffolding for the structure (Bent, 1996; Hammond-Kosack and Jones, 1997). The LRR is the receptor domain and mediates protein-protein interactions during recognition between the *R* and *Avr* gene products (Bent, 1996; Jackson and Taylor, 1996). Glycosylation patterns can also influence its binding capability (Hammond-Kosack and Jones, 1997). The LRR domain is under strong diversifying selection pressure, reflecting its role in the recognition of constantly evolving pathogen ligands. It has a size range of 350 to 700 amino acids for most genes, and only occasionally shows a conserved repeat structure (Bai et al., 2002).

NB domains are found in proteins with ATP- or GTP-binding activity, such as ATP synthase *β*-subunits, adenylate kinases and G proteins (Baker et al., 1997; Hammond-Kosack and Jones, 1997; Lacock et al., 2003). The common occurrence of NB domains in both plants and animals could be an indication of similar functioning (Lacock et al., 2003). Site-directed mutagenesis of key residues implicated in nucleotide binding abolishes the capacity of *R* genes with NB domains to induce HR upon infection (Baker et al., 1997). The NB domain is involved in recognition signalling (Bai et al., 2002), possibly by the activation of kinases or G proteins (Hammond-Kosack and Jones, 1997).

NB-LRR proteins are typically divided into two classes in plants, depending on whether they include a coiled-coil/leucine zipper (LZ) or Toll/interleukin-1 receptor (TIR) domain (Baker et al., 1997; Bai et al., 2002). LZ domains consist of heptad repeat sequences (consensus XXXYXXL, with Y being a hydrophobic residue) that can facilitate protein-protein interactions by promoting the formation of coiled-coil structures. They have well-defined roles in homo- and heterodimerization of especially eukaryotic transcription factors, but their exact function in *R* genes is uncertain (Bent, 1996). It is suggested that R proteins could be monomers until pathogen challenge, and then dimerize upon activation (Hammond-Kosack and Jones, 1997). The *Arabidopsis R* genes *RPM1* and *RPS2* both encode R proteins with LZ domains at their N-terminal, followed by the NB domain, an internal hydrophobic domain and 14 imperfect LRRs at the C-terminal (Jackson and Taylor, 1996; Hammond-Kosack and Jones, 1997; Buell, 1998).

The TIR motif is similar to the cytoplasmic signalling domains on the *Drosophila* Toll protein and the mammalian interleukin-1 receptor (IL1-R) (Lacock et al., 2003). The N-terminal of both the tobacco (*Nicotiana tabacum*) N protein exhibits a TIR domain (Hammond-Kosack and Jones, 1997). The TIR domain shows moderate homology to Toll/IL-1R cytoplasmic domains and may function in a similar manner, since one component of the

N-mediated defence response is the generation of reactive oxygen species (ROS) (Bent, 1996; Baker et al., 1997).

In *Arabidopsis*, 60% of the sequences are of the TIR-NB-LRR type and 40% of the LZ-NB-LRR type (Dangl and Jones, 2001). Although the TIR class of NB-LRRs forms the largest proportion of genes in the *Arabidopsis* genome, it is probably absent in cereals, as it was not detected in the assembled rice genome sequence or in other cereal EST databases (Bai et al., 2002). Most cereal NB-LRRs are similar to the dicot non-TIR, or coiled-coil class. The N-terminals of these genes often contain either a leucine zipper or coiled-coil sequence, which would facilitate protein-protein interactions (Lacock et al., 2003). It is reasonable to assume that if the *Dn* genes for *D. noxia* resistance encode NB-LRR proteins, they would be of this class. Cereal NB-LRR genes may be more homogeneous in domain architecture than similar dicot genes. Where only about 50 genes in *Arabidopsis* are non-TIR NB-LRRs, rice is estimated to have as many as 660 (Bai et al., 2002) to 1500 non-TIR NB-LRR sequences (Menezes and Jared, 2002). Some do not code for the coiled-coil domain at the N-terminal at all and fifty of the genes found in rice encode an NB without an adjoining LRR region. This group may be cereal or monocot-specific. By contrast, the tomato *Cf* genes encode extracellular LRRs, but no NB sequences (Staskawicz et al., 1995; Hammond-Kosack and Jones, 1997). Cf proteins may be associated with a membrane receptor complex that recruits a kinase domain for intracellular signalling upon direct or indirect recognition of the elicitor peptide (Staskawicz et al., 1995; Hammond-Kosack and Jones, 1997).

Other structural classes of R proteins exist entirely without NB-LRR domains and several require additional factors with NB-LRRs to function. Serine/threonine kinases and NB-LRRs may often function together in signal-transduction pathways (Dangl and Jones, 2001). The serine/threonine kinases are protein kinases that phosphorylate serine or threonine residues (Lacock et al., 2003) as part of a kinase/phosphatase signalling cascade (Jackson and

Taylor, 1996). The phosphorylation state of a protein is a common and important way of regulating its activity, as evidenced by the several conserved residues found among serine/threonine kinases (Bent, 1996). Pto of tomato is such a serine/threonine kinase and, as such has obvious signal transduction capacity, but lacks Avr recognition capacity (Buell, 1998). This is why Pto requires the product of the *Prf R* gene, which does exhibit a LZ-NB-LRR structure, in order to function (Buell, 1998) Yeast two-hybrid interaction has indicated that Pti1, a second serine/threonine protein kinase, acts as a substrate for phosphorylation by Pto and acts downstream of Pto in a protein kinase cascade leading to HR The characterization of *Pto* demonstrated the central role of kinase-mediated signal transduction in gene-for-gene plant disease resistance (Bent, 1996).

The rice gene *Xa21* combines the properties of the tomato *Pto* and *Prf* genes by possessing an extracellular LRR domain, a single transmembrane domain and an intracellular serine/threonine protein kinase domain. These types of proteins are also known as receptor-like kinases (RLKs), since the extracellular LRR domains are involved in signal perception and the kinase domains are involved in intracellular signal transduction via MAP kinases and the activation of transcription factors (Montesano et al., 2003).

R proteins are also classified according to their subcellular localization. The existence of cytoplasmic and transmembrane classes of R protein indicates that some are specialized to detect secreted ligands or surface components from the pathogen, and some are dedicated to recognize ligands that appear inside the cell (Dangl and Jones, 2001). For cytoplasmic R proteins, the LRR domain is regarded as the recognition domain by default, as the NB, LZ and TIR domains have an obvious signal transduction capacity (Figure 1.4) (Hammond-Kosack and Jones, 1997).

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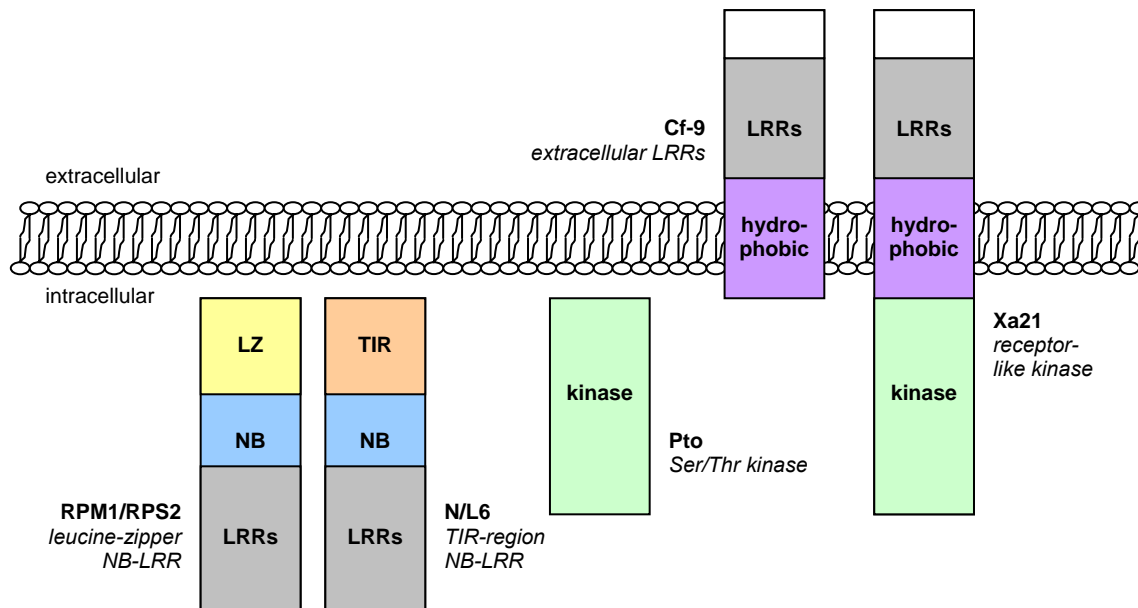
Figure 1.4

A comparison between different *R* gene products, indicating cellular localization and domains (Adapted from Hammond-Kosack & Jones, 1997).

1.3.4 The hypersensitive response and systemic acquired resistance

Although the hypersensitive response (HR) involves only a small fraction of cells of the total plant, it is an important survival mechanism (Staskawicz et al., 1995; Jackson and Taylor, 1996). This is a response to invasion by all classes of pathogens and phloem feeding insect attack and the most common feature associated with active host resistance.

After pest or pathogen recognition via matched *R* and *Avr* specificities, several changes in the cell are initiated (Figure 1.5), involving MAP kinase and phosphatase activation cascades, cellular decompartmentalization and altering of membrane potentials (Bent, 1996; Buell, 1998; Ono et al., 2001). Signalling cascades converge and induce the general responses of HR which are similarly elicited by a wide range of organisms (Hammond-Kosack and

Jones, 1997). This response includes an influx of Ca^{2+} and exchanges of H^+ for K^+ inside the cell, leading to alkalinization of the extracellular space and a decrease in intracellular pH (Dangl et al., 1996). This seems to be essential to the oxidative burst, the rapid generation of reactive oxygen species (ROS) such as O_2^-, H_2O_2, OH^- and NO, involved in pathogen elimination and downstream signalling (Staskawicz et al., 1995; Baker et al., 1997). ROS have several downstream signalling functions, and the effects of peroxide include an increase in salicylic acid (SA)-biosynthetic enzymes, transcription of *PR* genes and the potential generation of lipid peroxides via the action of the hydroxyl radical (Dangl et al., 1996). Increased activity of oxidative enzymes like peroxidases and other oxidative enzymes like polyphenol oxidases and lipoxygenases is also observed (Jackson and Taylor, 1996). Western blotting has indicated that peroxidase-related proteins increase quantitatively as *D. noxia* infestation of resistant wheat proceeds, not only at the site of feeding, but also spreading systemically. Peroxidases are known to be involved in an array of defence-related reactions which collectively contribute to *D. noxia* resistance (Van der Westhuizen et al., 1998). Potential roles for oxidative injury in defence against herbivorous insects are direct oxidative injury to the insect; indirect injury through oxidative damage to dietary compounds and signal transduction for eliciting plant defensive systems (Botha et al., 2005). Oxidative cross-linking and strengthening of the plant cell wall occurs during HR, with callose and suberin deposition and lignification (Ryals et al., 1996). When these oxidative events occur in resistant wheat, mesophyll cell-wall thickness increases by 12%, which could likely hinder aphid probing as they seek out the sieve elements of the phloem (Van der Westhuizen et al., 1998).

Other proteins are also expressed during HR, such as glutathione *S*-transferases, proteinase inhibitors, and various biosynthetic enzymes. These include enzymes involved in salicylic acid (SA) and ethylene (ET) biosynthesis and the production of low molecular weight antimicrobial metabolites called phytoalexins (Klessig et al., 2000).

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HR leads to cell death induced locally at the infection site, observed as small necrotic lesions (Dangl et al., 1996). It is thought to actively and directly limit pathogen development to those areas immediately surrounding the initially infected cells by providing a physical barrier to pathogen growth, as well as restricting the of nutrient supply (Menezes and Jared, 2002). The same presumably applies to aphid feeding, as cell death at the site of stylet penetration would also obstruct feeding. Cell death may facilitate the mass release of antimicrobial enzymes and metabolites into the extracellular matrix and the release of signals for disease resistance (Yu et al., 1998).

Cell death during HR is programmed. Death is not caused directly by the pest or pathogen, but rather the activation of pathways encoded by the plant genome upon recognition of elicitors (Mittler and Lam, 1995; Dangl et al., 1996). This requires active metabolism. Programmed cell death in plants is analogous to apoptosis in mammals, which has been much better characterized. A hallmark of apoptosis is the systematic condensation and degradation of nuclear DNA, which can be observed by agarose gel electrophoresis as DNA laddering. Chromatin cleavage is a key step in apoptosis, as inhibition of nuclear DNA degradation prevents apoptosis from occurring (Mittler and Lam, 1995). Similarly, the HR induced by cowpea against the cowpea rust fungus is accompanied by degradation of the host DNA into oligosomal fragments (Dickman et al., 2001) and *Arabidopsis* leaves treated with cryptogein, a fungal elicitor of HR, demonstrated nuclear condensation, DNA fragmentation, and production of apoptotic bodies (Dangl et al., 1996; Asai et al., 2000). There are clear differences between programmed plant cell death and apoptosis in animals, however. Programmed cell death in tobacco is accompanied by increased ion leakage, but animal apoptosis is not. This is expected, since the plant cell's contents cannot be engulfed by neighbouring cells (Mittler and Lam, 1995). Activation of the HR cell death pathway in

tobacco is correlated with the induction of several deoxyribonucleases which are not induced during senescence or necrosis.

It is thought that ROS are a key trigger for the induction of programmed cell death. ROS such as O_2^- and H_2O_2 form an integral part of HR and are both generated early after infection, although peroxide seems to be the key factor in cell death, since it is the most stable and can cross membranes (Dangl et al., 1996). The oxidative burst, SA production and *PR* gene expression all occur before HR cell death starts (Yu et al., 1998).
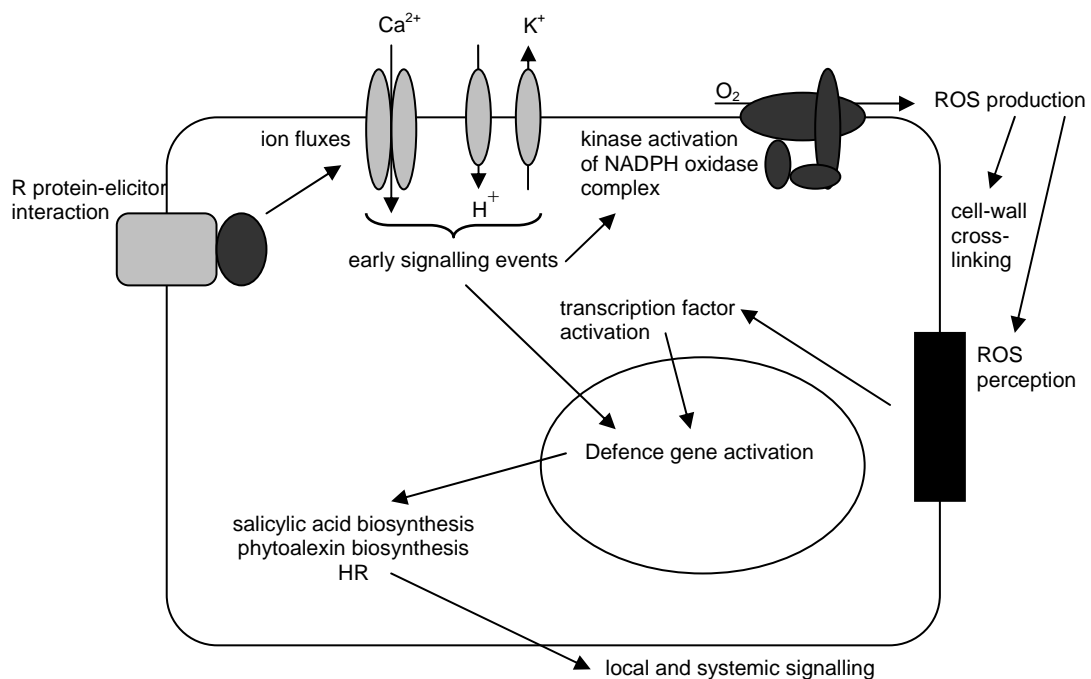
Figure 1.5 Cellular events immediately following pathogen recognition (adapted from Dangl et al., 1996).

Local HR is associated with the onset of systemic acquired resistance (SAR) in distal tissues. Several signalling molecules, including JA, ET and SA, as well as ROS like NO, are involved in the induction of HR (Montesano et al., 2003) and the subsequent signalling leading to SAR (Staskawicz et al., 1995). The SAR pathway is inducible by pest or pathogen exposure at

localized sites of HR (Ryals et al., 1996), and is fully induced within 2 days after the pest or pathogen is encountered (Delaney et al., 1995). SAR can be distinguished from other disease resistance responses by both the spectrum of protection and the associated changes in gene expression. The long-lasting resistance imparted by SAR acts non-specifically throughout the plant (Jackson and Taylor, 1996; Menezes and Jared, 2002) by reducing the severity of disease caused by all classes of pest and pathogens (Staskawicz et al., 1995).

The accumulation of SA is required for SAR signal transduction (Delaney et al., 1995). SA levels increase by several hundred-fold in tobacco after pathogen infection. Adding exogenous SA to soybean cell suspensions greatly facilitates HR programmed cell death triggered by pathogens (Asai et al., 2000). Exogenously applied SA also induces SAR and the expression of SAR-specific proteins such as the PR proteins, and normally susceptible tissues develop highly resistant responses (Jackson and Taylor, 1996).

The analysis of transgenic plants expressing the bacterial *nahG* gene encoding salicylate hydroxylase, an enzyme that catalyzes the conversion of SA to catechol, has shown that these plants do not accumulate free SA and are compromised in local and systemic resistance responses, therefore being incapable of mounting a SAR response to viral, fungal or bacterial pathogens (Delaney et al., 1995; Jackson and Taylor, 1996; Ryals et al., 1996). *In vivo* labelling of SA during TMV infection of tobacco has proved that there is a marked increase in SA at the inoculation site and in the distal parts (Buell, 1998). However, grafting experiments with tobacco plants indicated that SA is not the long-distance signal, since TMV inoculation of *nahG* expressing rootstocks resulted in very little SA accumulation in infected tissue compared to wild type plants (since SA is actively degraded), but transmission of the systemic signal out of those rootstocks was unaltered and distal grafted wild type tissue still displayed elevated levels of SAR gene expression and induced resistance similar to ungrafted wild type plants (Jackson and Taylor, 1996; Ryals et al., 1996; Buell, 1998). This demonstrates a direct

link between signalling involved in local lesion formation (HR) and SAR signal transduction (Jackson and Taylor, 1996).

The *NPR1/NIM1* gene of *Arabidopsis* has been cloned and is a key regulator of SAR responses. It is an inducer of downstream *PR* genes. Its homologues have been identified in maize and wheat, indicating the ubiquitous presence of this gene. NPR1 is a key regulator in transducing the SA-dependent signal leading to SAR in distal tissues and local HR and is required by certain R proteins. *NPR1* is therefore thought to function downstream of SA accumulation, probably as a *PR* gene transcription regulator (Delaney et al., 1995; Baker et al., 1997). *NPR1* is not required for the role SA plays during programmed cell death, as *npr1* mutants act like the wild type upon induction of programmed cell death (Asai et al., 2000).

Expression levels of NPR1 are doubled after SA treatment or inoculation with fungal or bacterial pathogens. The protein only becomes activated at SAR induction, because the constitutively expressed form does not cause *PR* gene expression. When activated, NPR1 causes a stronger, but not quicker, induction of *PR* genes (Delaney et al., 1995; Cao et al., 1998). NPR1 is normally inactive until the SA signalling pathway is induced, and can therefore be seen as a master regulator of *PR* genes, in charge of transmitting SA signals to downstream components in the signalling pathway (Cao et al., 1998; Asai et al., 2000).

The SAR signal transduction pathway appears to function as a modulator of other disease mechanisms: when SAR is activated, a normally compatible plant-pathogen interaction can be converted into an incompatible one (Ryals et al., 1996). The induction of a set of genes called *SAR* genes is tightly correlated with the onset of SAR in distant uninfected tissue. A protein is classified as a SAR protein when its presence or activity correlates tightly with maintenance of the resistance state. Many of these proteins have been shown to exhibit antimicrobial activity either *in vitro* or *in vivo* (Klessig et al., 2000). Many SAR proteins belong to the class of pathogenesis-related (PR) proteins (Table 1.1), which originally were

identified as novel proteins accumulating after TMV infection of tobacco leaves (Jackson and Taylor, 1996). In this case, the set of SAR markers consists of at least nine families comprising acidic forms of PR-1 (PR-1a, PR1-b and PR-1c), β-1,3-glucanase (PR-2a, PR-2b and PR-2c), class II chitinase (PR-3a and PR-3b), hevein-like protein (PR-4a and PR-4b), thaumatin-like protein (PR-5a and PR-5b), acidic and basic isoforms of class III chitinase, an extracellular β-1,3-glucanase and the basic isoform of PR-1. In *Arabidopsis* the *SAR* marker genes are *PR-1*, *PR-2* and *PR-5* (Buell, 1998). These genes have been cloned and characterized and are often used in evaluating the onset of SAR (Klessig et al., 2000). However, several secondary metabolites such as saponins, melanins, tannins, alkaloids and flavonoids are also synthesized during SAR (Menezes and Jared, 2002).

PR proteins induced in response to pathogen attack can actively alter symplastic transport by regulating plasmodesmata function or structure, which SAR defence responses may rely on. Maize PR proteins are localized at the plasmodesmata (Baker et al., 1997). The identity and relative expression levels of *SAR* genes vary between different plant species. A number of genes homologous to *SAR* genes from dicots have been identified in monocot species. Homologues of the PR-1 family have been characterized in maize and barley, and additional PR proteins have been identified in maize. Markers for chemically activated SAR have been described in wheat. These wheat chemically induced *WCI* genes encode a novel lipoxygenase, a cysteine protease, and three other proteins with unknown function. *SAR* genes are strongly expressed when resistance is maintained and are causally associated with resistance (Ryals et al., 1996).

Table 1.1

Pathogenesis-related protein types and their putative functions (adapted from

Gachomo et al., 2003).

Protein family	Putative identity	Target in pathogen defence
PR-1	Pathogenesis-related protein 1 precursor	Membrane
PR-2	β-1,3-glucanase	Cell wall glucan
PR-3	Endochitinase	Cell wall chitin
PR-4	Endochitinase	Cell wall chitin
PR-5	Osmotin	Membrane
PR-6	Proteinase inhibitor	Proteinase
PR-7	Proteinase	Not defined
PR-8	Endochitinase	Cell wall chitin
PR-9	Peroxidase	Cell wall reinforcement
PR-10	RNase	Pathogen RNA
PR-11	Endochitinase	Cell wall chitin

The β-1,3-glucanase PR protein is induced by *D. noxia* infestation of wheat. Inter- and intracellular isoforms are known. It is localized at the cell walls of the vascular bundles and selectively accumulates in the apoplast of resistant cultivars, as well as intracellularly in chloroplasts, where it is implicated in preventing damage to the photosynthesis system by *D. noxia* feeding (Van der Westhuizen et al., 2002). β-1,3-glucanase is reported to be involved in defence against fungal pathogens in several cereals, including oats, barley and wheat. β-1,3-glucanase is induced upon leaf rust (*Puccinia triticina*) infection in wheat and shows a synergistic action with chitinases. A function of the β-1,3-glucanase enzyme could be the regulation of the amount of β-1,3-glucans formed. This substrate accumulates at sites of attempted pathogen penetration. Its role in plant interaction with insects is still unclear. β-1,3-

glucanase and endochitinase are induced in susceptible cultivars as well as the resistant cultivars (Neu et al., 2003), although induction may occur too late or not strongly enough to realize resistance.

The expression of chitinases in uninfested wheat plants is low and constant. A dramatic increase occurs within 48 hours after *D. noxia* infestation in resistant cultivars. This increase is also seen in susceptible cultivars, but again seems to occur too late and to levels too low to facilitate any resistance response. Chitinase induction is also systemic, with enzyme levels being higher at the point of feeding. Although chitinase function has not been well defined in insect attack, it has a clear role in microbial attack. The chitinase isoforms produced during wounding and *D. noxia* infestation are known to differ (Van der Westhuizen et al., 2002). It is not known whether the probing *D. noxia* releases chitinous compounds, but the digestion of such molecules by chitinases will produce oligosaccharide fragments that may act as elicitors of the defence reaction. *N*-acetylchito-oligosaccharide elicitors are known for inducing cellular responses including phytoalexin synthesis, ROS production and expression of several defence-related genes (Ono et al., 2001). Increased chitinase function might also be a general part of the hypersensitive response (Van der Westhuizen et al., 1998). PR proteins can enhance resistance when introduced into transgenic crops (Botha et al., 1998; Van der Westhuizen et al., 2002).

1.4 FUNCTIONAL GENOMICS

The improvement of agricultural crops comprises breeding new cultivars through the combining of traits from parental lines or wild relatives (Botha and Venter, 2000). However, phenotype-based selection is time-consuming and often not very accurate (Myburg et al., 1998). Biotechnology has vastly improved not only the efficiency at which useful traits can be bred into cultivars through marker-assisted selection, but also our understanding of the molecular principles underlying agronomically desirable traits such as disease resistance. Such an understanding is critical for the advancement of 21st century agriculture.

The comprehensive investigation of specialized cellular metabolism has found some of its most powerful tools in functional genomics (Dixon, 2001). Analysis on a genomic or transcriptomic scale has been accelerated through the development of techniques such as the cDNA microarray (Schena et al., 1995), suppression subtractive hybridization (Diatchenko et al., 1996) and cDNA-AFLP (Bachem et al., 1996).

Valuable information on coordinated gene expression has been obtained from cDNA microarray analysis on complex cellular events, from xylogenesis in hybrid aspen (Hertzberg et al., 2001) to the *Arabidopsis* defence response against fungal pathogens (Schenk et al., 2000). cDNA microarrays allow the patterns of expression of thousands of genes to be investigated in a single hybridization (Yang et al., 1999).

Functional genomics approaches allow not only the discovery of novel genes, but place special emphasis on how the expression of those genes is integrated into the biochemical pathways and metabolic events under investigation. This has proved particularly useful in understanding cellular events during plant resistance responses.

Suppression subtractive hybridization (SSH) (Diatchenko et al., 1996) has been successfully used in constructing subtracted libraries of differentially regulated transcripts. This method has assisted in the identification of many cereal genes involved in interactions with pests and pathogens. In rice, for example, several transcripts of low abundance were isolated from JA-induced libraries. These included novel members of MAP kinase cascades and other rare transcripts not normally present in cDNA libraries (Xiong et al., 2001). The responses to phloem feeding insects have also been investigated using this method. SSH of rice induced by planthopper (*Nilaparvata lugens*) feeding yielded 27 planthopper-responsive genes involved in diverse cellular processes (Yuan et al., 2005). SSH has also been successfully used in identifying genes induced by *D. noxia* feeding in wheat. Various subtractive hybridizations between infested and uninfested wheat, or susceptible and resistant near-isogenic lines, resulted in the characterization of several novel sequences (Van Niekerk and Botha, 2003) as well as implicating several resistance gene analogs in the defence response (Lacock and Botha, 2003; Boyko et al., 2006).

cDNA microarrays can be combined with SSH, allowing for effective and high throughput analysis of the expression of the cDNA clones generated by SSH (Yang et al., 1999). The wheat response to *D. noxia* has been investigated in this way: a cDNA microarray including ESTs previously isolated from *D. noxia*-induced SSH libraries indicated significant differential regulation of transcripts involved in cellular processes like signal transduction and photosynthesis (Botha et al., 2006).

cDNA amplified fragment-length polymorphism (cDNA-AFLP) is a high throughput method of identifying differentially regulated genes (Bachem et al., 1996). Visualization of gene expression via rapid RNA fingerprinting is an attractive alternative to cDNA library screening and other traditional techniques. Microarray-based approaches to transcriptome analysis usually require prior development of large EST or cDNA clone collections (Schenk

et al., 2000; Hertzberg et al., 2001), but cDNA-AFLP does not require prior sequence information and allows for the identification of novel genes (Goossens et al., 2003). Using cDNA-AFLP, global transcriptomic changes in all samples can be concurrently compared (Bachem et al., 1996).

Because of these advantages, cDNA-AFLP has become a major tool in investigating plant responses to stressful stimuli. These have included differential regulation of transcripts involved in heat stress in cowpea (*Vigna unguiculata*) nodules (Simões-Araújo et al., 2002) and differential regulation of transcripts during the hypersensitive response in cassava (*Manihot escuenta*) (Kemp et al., 2005). cDNA-AFLP has also been employed in investigating gene expression during *Peronospora* infection of *Arabidopsis*, identifying differentially regulated genes from both fungal pathogen and host (Van der Biezen et al., 2000). Cereal research has similarly advanced using cDNA-AFLP analysis, particulary in t analyzing transcriptome-wide changes in wheat induced by *D. noxia* infestation (Matsioloko and Botha, 2003).

Modern biotechnology has established valuable techniques for the high throughput analysis of gene regulation during agriculturally important biological phenomena, such as the wheat resistance response against *D. noxia*. Current and future research in plant defence is set to vastly improve the pace and quality of crop production worldwide.

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CHAPTER 2

Wheat (*Triticum aestivum* L.)

transcriptional regulation during

Russian wheat aphid (*Diuraphis noxia* Mordv.) infestation

2.1 ABSTRACT

Russian wheat aphid (*Diuraphis noxia*, Mordv.) feeding on susceptible cultivars of hexaploid wheat (*Triticum aestivum*, L.) leads to leaf rolling, chlorosis and the eventual death of the plant. Although several resistance genes have been identified and are employed extensively in breeding agronomically useful cultivars, no *Dn* gene has ever been cloned or characterized. Plants expressing these *Dn* genes are afforded distinct modes of resistance to *D. noxia* infestation: the *Dn1* gene confers an antibiotic effect to lower aphid fecundity; *Dn2* confers tolerance to high aphid pressure; *Dn5* confers antixenosis, and aphids do not prefer such plants as hosts. Little is known about the components involved in establishing a successful defence response against *D. noxia* attack and how these differ between the different resistance categories. We consequently investigated the downstream components involved in or affected by the generation of these resistance mechanisms by comparing the responses of Tugela near-isogenic lines with different *Dn* genes to *D. noxia* infestation at a transcript level. cDNA-AFLP analysis yielded 121 differentially regulated transcript-derived fragments (TDFs) grouped into eight expression clusters. We cloned and sequenced 49 representative TDFs, which were further classified into five broad functional categories based on inferred similarity to database sequences. Transcripts involved in such diverse processes as stress, signal transduction, photosynthesis, metabolism and gene regulation were found to be differentially regulated during *D. noxia* feeding. Many TDFs demonstrated homology to proteins with unknown function and several novel transcripts with no similarity to previously published sequences were also discovered. Detailed expression analysis using qRT-PCR and RNA hybridization provided evidence that the time and intensity of induction of specific pathways is critical for the development of a particular mode of resistance. This includes: the generation

of kinase signalling cascades and the induction of several ancillary processes such as ubiquitination, leading to a sustained oxidative burst and the hypersensitive response during antibiosis; tolerance as a passive resistance mechanism countering aphid-induced symptoms through the repair or *de novo* synthesis of photosystem proteins; and the possible involvement of ethylene-mediated pathways in generating volatile organic compounds during antixenosis. This study is the first report on the involvement of KCO1, a vacuolar K^+ channel, in assisting cytosolic Ca^{2+}-influx and preventing leaf rolling, as well as the role of iron homeostasis as a gene regulatory mechanism for sustaining the oxidative burst during the antibiotic defence response.

2.2 INTRODUCTION

Aphids are the largest group of phloem-feeding insects and their enormous reproductive potential makes them the most devastating pests to crop production. They have a more intimate association with the plants they feed on than chewing insects do, and are known to elicit the expression of plant genes more commonly associated with bacterial and fungal pathogen attack (Moran and Thompson, 2001). The interaction between wheat (*Triticum aestivum* L.) and the Russian wheat aphid (*Diuraphis noxia* Mordvilko) has been of major interest to researchers in this field, particularly the identity and mechanics of the *Dn* resistance genes. Categories of resistance to *D. noxia* are defined as antibiosis, where the plant reduces the reproductive fitness of aphids feeding on it; tolerance, which is seen as a lack of plant height and biomass reduction because of feeding; or antixenosis, the non-selection of a cultivar as host (Painter, 1958; Smith et al., 1992; Unger and Quisenberry, 1997). The genetic background in which a specific *Dn* gene is bred may play a role in the successful establishment of a resistant phenotype (Van der Westhuizen et al., 1998) and in some instances a resistant cultivar exhibits a combination of these categories of resistance (Smith et al., 1992).

None of the ten designated *D. noxia* resistance genes (*Dn1-Dn9*, *Dnx* and *Dny*) have been cloned and results from mapping efforts have often been contradictory or inconclusive (Ma et al., 1998; Myburg et al., 1998; Liu et al., 2001; Heyns et al., 2006). *Dn1*, *Dn2* and *Dn5* are located on chromosome 7D, but whether they are allelic at the same locus or independent but tightly linked is not yet clear (Liu et al., 2001; Heyns et al., 2006). Some heterogeneity in the original PI 294994 accession *Dn5* was acquired from, may explain why conflicting results are obtained by different research groups (Zhang et al., 1998). It is speculated that the *Dn*

genes would function like classic pathogen resistance genes by encoding proteins that could recognize aphid-specific elicitors in a gene-for-gene manner and launch a defence response by initiating signalling cascades (Lacock et al., 2003; Botha et al., 2006). Upon *D. noxia* feeding, antibiotic cultivars like those containing *Dn1* initiate a hypersensitive response, such as seen with plant-pathogen interactions. This is associated with the production of reactive oxygen species like hydrogen peroxide (H_2O_2) and programmed cell death at the site of aphid probing. These reactive oxygen species induce the accumulation of salicylic acid, which in turn stimulates the expression of pathogenesis-related (PR) proteins, like chitinases (Botha et al., 1998), peroxidases (Van der Westhuizen et al., 1998) and β-1,3-glucanases (Van der Westhuizen et al., 2002). PR proteins accumulate in the apoplast of resistant plants within 24 hours of infestation (Botha et al., 1998). While the exact function of these proteins in aphid defence remains unclear, it has been suggested that chitinases might generate oligosaccharide elicitors from chitinous compounds released during aphid feeding (Van der Westhuizen et al., 1998).

Aphid feeding causes chlorosis in the leaves of susceptible wheat. *D. noxia* seems to interfere with the osmoregulation of leaf turgor during cell elongation (Burd and Burton, 1992), preventing the proper unfolding of new leaves. Leaf chlorophyll content is reduced by *D. noxia* infestation (Heng-Moss et al., 2003) and longitudinal chlorotic streaking is characteristic of infested susceptible cultivars. This results in decreased photosynthetic potential and the eventual collapse of the plant (Burd and Burton, 1992). Aphid damage has historically been ascribed to a phytotoxin injected during feeding, which is responsible for chloroplast disintegration (Fouché et al., 1984). Such a phytotoxin has never been described or isolated. Ultrastructural studies revealed limited chloroplast breakdown in the leaves of resistant cultivars after feeding (Van der Westhuizen et al., 1998), but since cell fluorescence data has shown that *D. noxia* feeding causes reduced photosynthetic capacity even in intact

chloroplasts (Haile et al., 1999), this chloroplast rupture mechanism seems unlikely. *D. noxia* feeding probably induces malfunctioning of the photosynthetic apparatus of the stacked region of the thylakoid membrane, but the exact site of interference has not been determined (Burd and Elliott, 1996; Heng-Moss et al., 2003). The chlorosis induced by *D. noxia* differs from normal chlorophyll degradation during leaf senescence (Ni et al., 2001). *D. noxia* feeding stimulates an increase in the activity of Mg-dechelatase, a catabolic enzyme that converts chlorophyllide *a* to pheophorbide *a* as the final step in the chlorophyllase pathway (Ni et al., 2001; Wang et al., 2004b).

Since the phenotypes afforded by different *Dn* genes vary – *Dn1* gives antibiosis, *Dn2* tolerance and *Dn5* a combination of antibiosis and antixenosis (Wang et al., 2004a) – the presence of these genes in near-isogenic lines must therefore activate transcription of defence-related genes differently. Total chlorophyll concentration assays indicate that *D. noxia* feeding causes a marked decrease in chlorophyll levels in Tugela, but that the reduction in the antibiotic near-isogenic line Tugela-*Dn1* is much less severe (Botha et al., 2006). Antibiotic Betta-*Dn1* plants are also unable to compensate for chlorophyll loss, which has been attributed to an increase in defence compound production. Tolerant Betta-*Dn2* plants have very stable chlorophyll content during *D. noxia* feeding, suggesting that they can compensate for chlorophyll loss in some way (Heng-Moss et al., 2003). The global transcriptional reprogramming induced by *D. noxia* feeding in wheat is poorly understood. Relatively little is known about the components involved in establishing a successful defence response against *D. noxia* attack. In this study we investigate the downstream components involved in or affected by the generation of active (antibiosis) or passive (tolerance) resistance mechanisms by comparing the responses of Tugela near-isogenic lines with different *Dn* genes to *D. noxia* infestation at the transcript level. We present evidence that the time and intensity of induction of specific pathways is critical for the development of resistance.

2.3 MATERIALS AND METHODS

2.3.1 Plant material and aphid treatments

Hexaploid wheat (*Triticum aestivum* L.) germplasm of the near-isogenic lines (NILs) Tugela, Tugela-*Dn1* (Tugela*4/SA1684), Tugela-*Dn2* (Tugela*4/SA2199) and Tugela-*Dn5* (Tugela*4/SA463) was obtained from the Small Grain Institute, Bethlehem, South Africa (Liu et al., 2001; Tolmay et al., 2006). The NILs are bred to closely resemble their susceptible Tugela parent via successive rounds of backcrossing, but contain the resistance genes of interest. Seeds were sown into 5 pots for each cultivar and thinned to 3 seedlings per pot after 5 days. Plants were grown for 14 days (2-3 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 25°C ± 2°C. Plants of each cultivar were infested with 5 adult, apterous *Diuraphis noxia* and incubated for 2 h, 6 h, 12 h, or 24 h. Control plants remained uninfested. All leaves except the first leaf were harvested into liquid N_2 and stored at -80°C prior to RNA isolation.

2.3.2 RNA isolation and cDNA synthesis

Frozen plant tissue was ground in liquid N_2 using a mortar and pestle. Total RNA was extracted using a guanidine thiocyanate buffer method (Chomczynski and Sacchi, 1987) and the RNeasy kit with on-column DNaseI digestion (Qiagen GmbH, Hilden, Germany). 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.

2.3.3 cDNA-AFLP analysis

50 ng of cDNA from each sample was used for cDNA-AFLP analysis (Bachem et al., 1996). cDNA-AFLP reactions were performed using the Expression Analysis kit (Li-Cor Biosciences, Lincoln, NB) according to the manufacturer's instructions for the generation of TaqI+0/MseI+0 pre-amplification PCR products, which were assayed for yield and quality by 1% agarose gel electrophoresis. Using the approximate yield as a guide, the pre-amplification products were diluted accordingly in sterile dH_2O and used as template for the final selective amplification. Selective amplifications were performed using primer combinations from the kit (Table 2.1).

Table 2.1

Primer combinations used in cDNA-AFLP analysis

Primer combination classification		Primer combination classification	
Combination	Two-digit primer code	Combination	Two-digit primer code
Msel-AC / Taql-AC	18	Msel-AC / Taql-GA	11
Msel-AG / Taql-CA	26	Msel-AG / Taql-GT	22
Msel-CT / Taql-CA	46	Msel-CT / Taql-CT	45
Msel-CT / Taql-GT	42	Msel-CT / Taql-TC	43
Msel-GA / Taql-AC	58	Msel-GA / Taql-CA	56
Msel-GT / Taql-AC	68	Msel-GT / Taql-AG	67
Msel-GT / Taql-CA	66	Msel-GT / Taql-GA	61
Msel-TG / Taql-AC	88	Msel-TG / Taql-CA	86

Each primer combination was assigned a two-digit primer code, the first digit and second digits corresponding to the MseI+2 and TaqI+2 primers used, respectively. The TaqI+2 primers were IRDye700-labeled, which fluoresces in the infrared register to aid visualization. cDNA-AFLP profiles were separated on Li-Cor IR^2 4200S automated DNA sequencers using an 8% (v/v) LongRanger acrylamide gel solution (Cambrex Corp., East Rutherford, NJ) as previously described (Myburg et al., 2001). cDNA-AFLP images were saved in 16-bit .TIFF format for image analysis.

2.3.4 TDF recovery 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). TDFs were eluted from polyacrylamide gel slices into 10 mM Tris-HCl using rapid freeze-thaw cycles and centrifugation at 13 000 rpm for 10 min. TDFs were re-amplified from the eluate by standard PCR with primers corresponding to the original primer combination used in selective amplification. These products were checked qualitatively by 2% agarose gel electrophoresis, before being ligated into the pGem-T Easy plasmid vector (Promega Corporation, Madison, WI). Competent DH5α *E. coli* were prepared (Inoue et al., 1990) and transformed with the vector. After blue/white colony screening, white putative transformants were positively identified by colony PCR (Güssow and Clackson, 1989). 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 (Altschul et al., 1997). Expectation values where $E = e^{-02}$ and lower were considered significant. Cloned TDFs were assigned clone identification numbers of format AmoLve-*xx.xxx*, denoting a two-digit primer code as listed

in Table 2.1 and the approximate band size in base pairs of the fragment as determined from cDNA-AFLP analysis.

2.3.5 *In silico* analysis and clustering of gene expression patterns

cDNA-AFLP band intensities of differentially expressed TDFs were scored using AFLP-Quantar*Pro* (ver. 1.0 Keygene B.V. Wageningen, The Netherlands). Lane finding, band finding and sizing were performed as described in the AFLP-Quantar*Pro* user manual, with band finding and scoring parameters previously described for Li-Cor gels (Myburg et al., 2001). Band intensities were normalized among lanes based on the total lane intensity to correct for inconsistencies in loading and other technical artefacts. This was automatically performed *in silico* by multiplying the band intensity by the normaliation factor obtained when the total mean lane intensity is divided by the mean intensity for a single lane. Cluster analysis was performed on the normalized band intensities using the Cluster program from EisenSoftware (Eisen et al., 1998). Groups of TDFs with similar expression patterns across cultivars and time intervals were thus identified. During hierarchical clustering, distances were calculated using the Spearman Rank correlation similarity metric with complete linkage clustering. Java TreeView (Saldanha, 2004) was used to produce the cluster diagram.

2.3.6 Quantitative RT-PCR confirmation of differential gene expression

Quantitative real-time PCR (qRT-PCR) was performed using the iScript One-Step RT-PCR Kit (Bio-Rad Laboratories, Hercules, CA) and analysed using the iCycler iQ Real-Time PCR Detection Instrument (Bio-Rad). After primer design (Table 2.2) from TDF sequence

information using Primer Designer 5 (ver. 5.03, Scientific and Educational Software, Cary, NC) purified salt-free primers were synthesized (Integrated DNA Technologies, Coralville, IA). Five ng of total RNA and 10 µM of each primer was used per reaction and all PCR reactions were carried out in triplicate. Relative quantification was done using the Tugela_0hpi (Tugela at 0 h post-infestation) sample as calibrator, and a serial dilution of the Tugela-*Dn1*_24hpi sample to generate the standard curve. The unregulated chloroplast 16S rRNA transcript was selected as endogenous control and used for normalization during relative quantification of target genes (Pfaffl, 2001).

Table 2.2

PCR primers used in qRT-PCR

Clone ID	PrimerA	PrimerB
AmoLve-11.250	5'-CAATAATGCCGCCATCCG-3'	5'-GAGACCCAAGGACAACAC-3'
AmoLve-11.270	5'-GTAAACAACTCGGAGAACAG-3'	5'-TTCATCGGTCTTGGAAGTAG-3'
AmoLve-11.450	5'-ATTTCTCCCAGGACCCATCTC-3'	5'-GACACCTTTGCCTTCAGGAAC-3'
AmoLve-22.210	5'-TACTGCCGAGAAGCACAAAAC-3'	5'-GCTACTGGATTCACCAAAAAG-3'
AmoLve-22.225	5'-ACCGTCACTTCAGAGACATC-3'	5'-GCTGGGAGGAAATCATTCAC-3'
AmoLve-22.275	5'-CACCAGGCTAACAACACCAAG-3'	5'-ACCGAGTAGAAGCAGCATCAG-3'
AmoLve-26.265	5'-CCTTGGTTGGTGACACATTC-3'	5'-GGTTCAGCCCATTTCTTTGC-3'
AmoLve-26.355	5'-GAGTAAAGACCAAATCCGAGAA-3'	5'-CAACCGTATGAGTAGCAAGTAA-3'
AmoLve-61.272	5'-GTATTCGCCTGATGGAAC-3'	5'-CTGCCTTCACTGGATAAC-3'
AmoLve-68.344	5'-CAGATGAAAGGAGCCAAAGC-3'	5'-AAGCCCAATGCCGATGATAG-3'
AmoLve-88.303	5'-AACTGGGACACGCCTACTGC-3'	5'-GCTTTCCGCTTGGGCTCTTC-3'
ATP synthase	5'-TTCCCCCAGGCAAGTTACC-3'	5'-TCATCAACCCGTCCGTAGC-3'

2.3.7 Confirmation of differential gene expression by RNA hybridization analysis

Fifty ng of PCR product amplified using the primer sets employed in qRT-PCR served as template for the synthesis of fluorescein-11-dUTP-labeled probes using the *Gene Images* Random Prime Labeling kit (Amersham Biosciences, Little Chalfont, UK) according to the manufacturer's instructions. Incorporation of the fluorescein label was monitored by comparing the fluorescence with a reference strip of serially diluted nucleotide mix containing the fluorescein-11-dUTP molecules. Two hundred ng of RNA from each sample was blotted onto Hybon-N+ nylon membrane (Amersham Biosciences) using the BioDot-SF device (Bio-Rad Laboratories) according to the recommendations in the user manual. The RNA was cross-linked to the membrane using the UVIlink CL508 ultraviolet crosslinker (UVItec Ltd., Cambridge, UK) set at 0.240 J for 3 min. Probe hybridization was performed in a Techne HB-1D hybridization chamber (Techne Inc., Burlington, NJ). Pre-hybridization of RNA was performed at 65°C in 0.125 ml.cm^{-2} hybridization buffer for 30 min, whereafter the denatured probe was added and allowed to hybridize overnight at 65°C. Probe detection was carried out with the *Gene Images* CDP-*Star* Detection kit (Amersham Biosciences) according to the instructions of the manufacturer. Blots were visualized by exposure to Hyperfilm ECL chemiluminescence film (Amersham Biosciences) overnight.

2.4 RESULTS

2.4.1 Aphid treatments

Whereas adult aphids fed on Tugela and Tugela-*Dn2* showed nymph production by 12 h, aphids fed on Tugela-*Dn1* and Tugela-*Dn5* only started to visibly multiply after 24 h. The necrotic spotting indicative of a hypersensitive programmed cell death response was observed on Tugela-*Dn1* plants directly at the sites of feeding. No changes in plant phenotype, whether hypersensitive lesions or chlorotic streaking, were observed in the other lines.

2.4.2 RNA and cDNA quality

Total RNA purified from all samples was of sufficient quantity (mean yield = 77640 ng; σ = 29532 ng) and quality, with no genomic DNA contamination (Figure 2.1). mRNA was isolated from all samples (mean yield = 566 ng; σ = 199 ng) and successfully used as template for cDNA synthesis (mean yield = 301 ng; σ = 150 ng) with a calculated efficiency of 96.8%.

2.4.3 cDNA-AFLP differential expression patterns

Pre-amplification for cDNA-AFLP analysis yielded fragments with sizes ranging from 50 bp to 1 000 bp as revealed by 1% agarose gel electrophoresis (Figure 2.1). Several differentially

expressed transcript-derived fragments (TDFs) were identified by cDNA-AFLP analysis (Figure 2.2). Approximately 1 500 fragments were generated from 16 different primer combinations (Table 2.1). TDFs that were selected for further analysis were polymorphic between near-isogenic lines or time points and exhibited visually discernable differential regulation of at least two-fold. Based on these criteria, 121 differentially regulated TDFs were selected to be quantitatively scored using the AFLP-QuantarPro software.
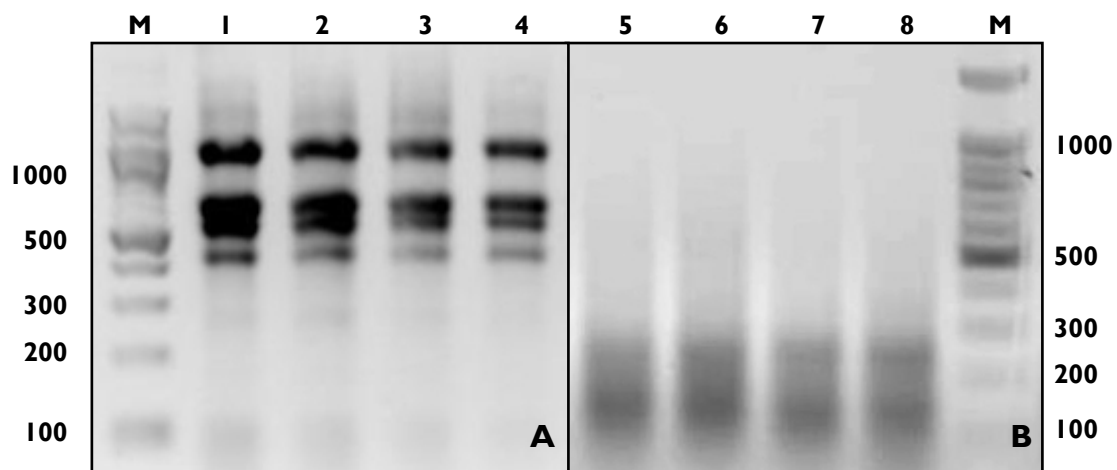
Figure 2.1

A. Typical quality of purified total RNA as revealed by 2% agarose gel electrophoresis. Band sizes are indicated to the left in number of base pairs. M: 100 bp DNA ladder (New England Biolabs, Inc. Beverly, MA); 1: Tugela-Dn2_6hpi; 2: Tugela-Dn5_6hpi; 3: Tugela_12hpi; 4: Tugela-Dn1_12hpi. **B.** Typical quality of pre-amplifications for cDNA-AFLP analysis as revealed by 1% agarose gel electrophoresis. Band sizes are indicated to the right in number of base pairs. 5: Tugela-Dn1_0hpi; 6: Tugela-Dn1_2hpi; 7: Tugela-Dn1_6hpi; 8: Tugela-Dn1_12hpi; M: 100 bp DNA ladder.
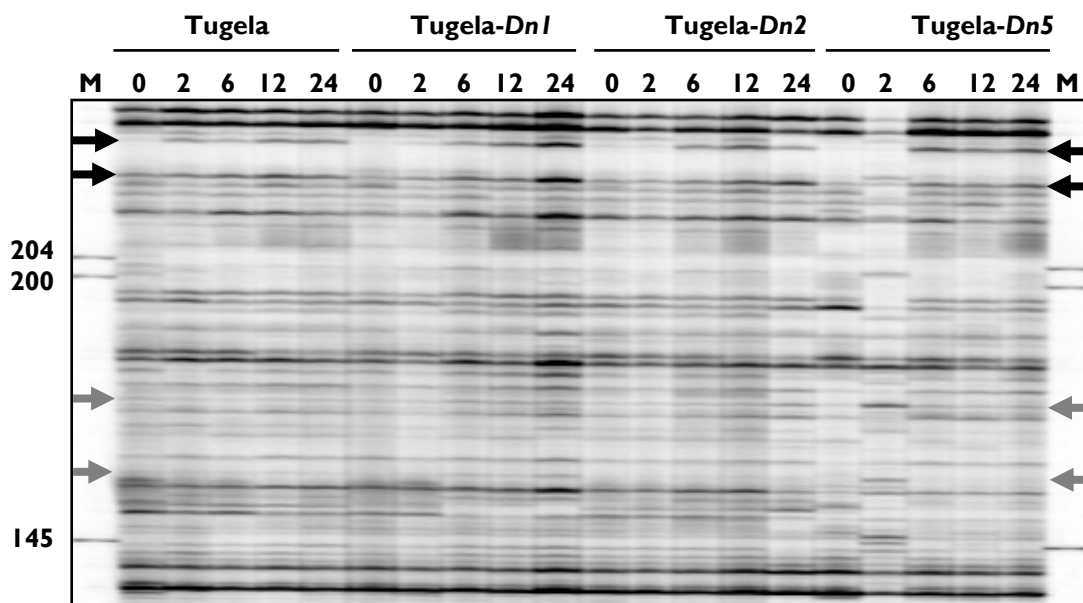
Figure 2.2

Section of a typical cDNA-AFLP expression profile obtained from the four near-isogenic wheat cultivars, infested for increasing periods of time. M: IRDye700-labeled molecular size standard. Sample numbers under cultivar names indicate number of hours post-infestation. Black arrows indicate TDFs differentially regulated between time points and grey arrows indicate TDFs differentially regulated between near-isogenic lines.

2.4.4 *In silico* image analysis and clustering of expression patterns

A total of 121 differentially regulated TDFs were scored and grouped into 8 clusters, labelled A to H (Figure 2.3). Distinct clusters were obtained with increases in expression over time as well as clusters of TDFs exhibiting decreases in expression over time. To better illustrate this within-cluster relationship among near-isogenic lines, the mean normalized band intensities for all TDFs within a cluster were plotted (Figure 2.3). Cluster A is characterized by TDFs showing increased expression in Tugela-*Dn1* and Tugela-*Dn5* at 2 h post-infestation. Cluster B is typified by a rise in the levels of TDFs in Tugela-*Dn5* at 2 h post-infestation and a sharp increase in the expression of TDFs in Tugela-*Dn2* at 24 h post-infestation. TDFs in cluster C show a decrease in expression at 2 h post-infestation in Tugela-*Dn5*. TDFs are most abundant during the final time points after infestation, peaking at 6 h post-infestation in Tugela-*Dn5*, 12 h post-infestation in Tugela and Tugela-*Dn2*, and at 24 h post-infestation in Tugela-*Dn1*. TDFs in cluster D exhibit a slight rise in expression as infestation proceeds, attaining the highest levels in Tugela-*Dn1* at 24 h post-infestation. TDFs in cluster E exhibit constitutively higher expression in Tugela-*Dn1*, peaking at 2 h post-infestation. Cluster F contains TDFs peaking at 2 h post-infestation, with a decline thereafter in all lines except Tugela-*Dn5*, which only peaks at 12 h post-infestation. TDFs in cluster G exhibit highest levels in Tugela and Tugela-*Dn2* at 12 h post-infestation, with slight peaks for Tugela-*Dn1* and Tugela-*Dn5* at 6 h post-infestation. Cluster H shows a general decrease in TDF expression over time in all lines except Tugela-*Dn2*, which shows a sharp rise in expression at 6 h post-infestation. Representatives from each of these eight clusters were chosen for qRT-PCR analysis.
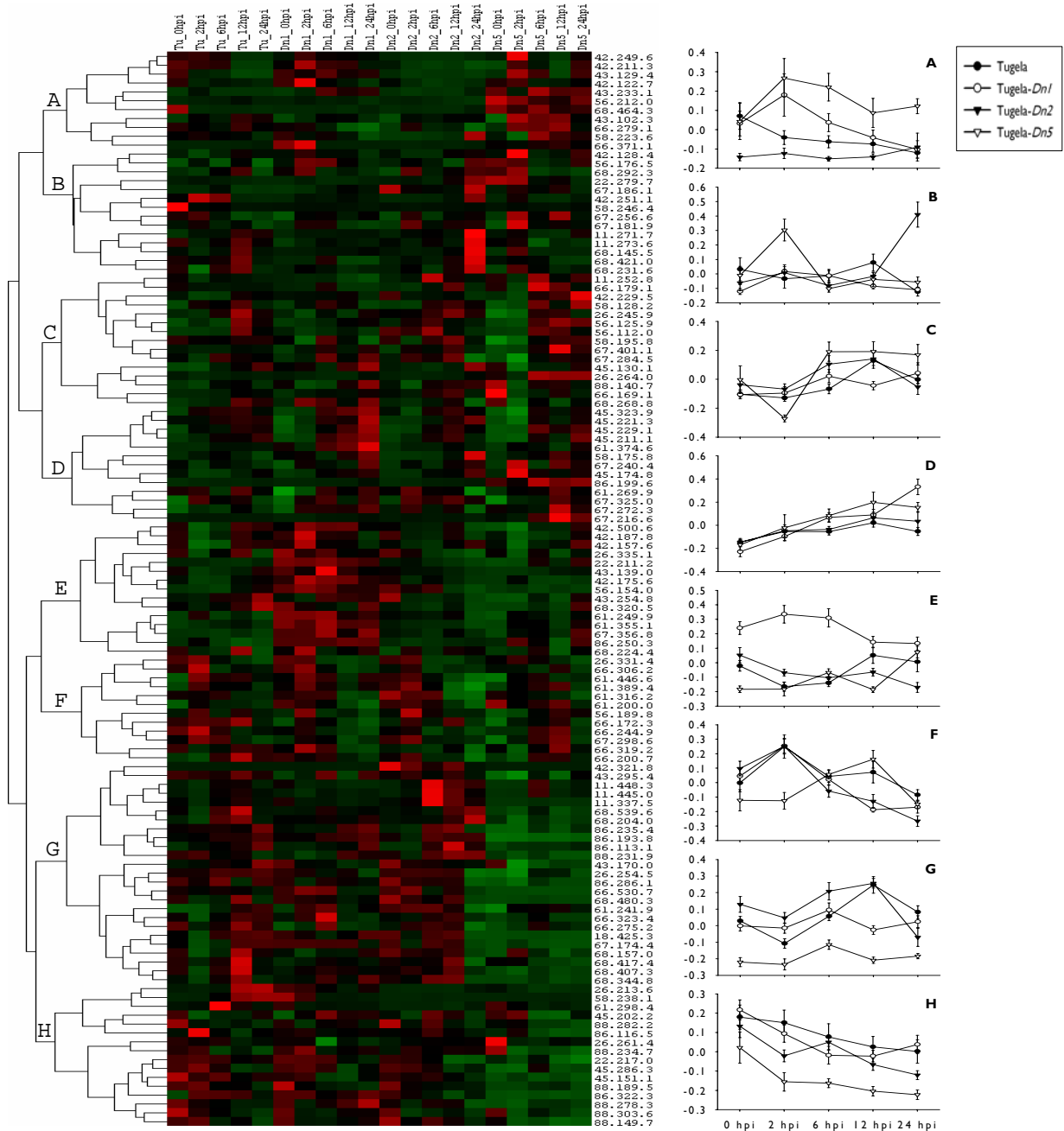
Figure 2.3

Hierarchical clustering of 121 TDFs differentially regulated during the wheat response to *Diuraphis noxia* feeding. Each column corresponds to a specific genotype at a specific time post-infestation, and each row represents the expression profile of a single TDF. Normalized expression is represented by a colour scale from red (highly up-regulated) to green (highly down-regulated). Black represents no differential regulation. TDF identification numbers are composed of the two-digit primer combination code as stipulated in Table 2.1, appended by the fragment size assigned *in silico* by the AFLP-Quantar*Pro* software. Mean normalized band intensity plots are given for each cDNA-AFLP expression cluster to the *right* of the hierarchical cluster analysis.
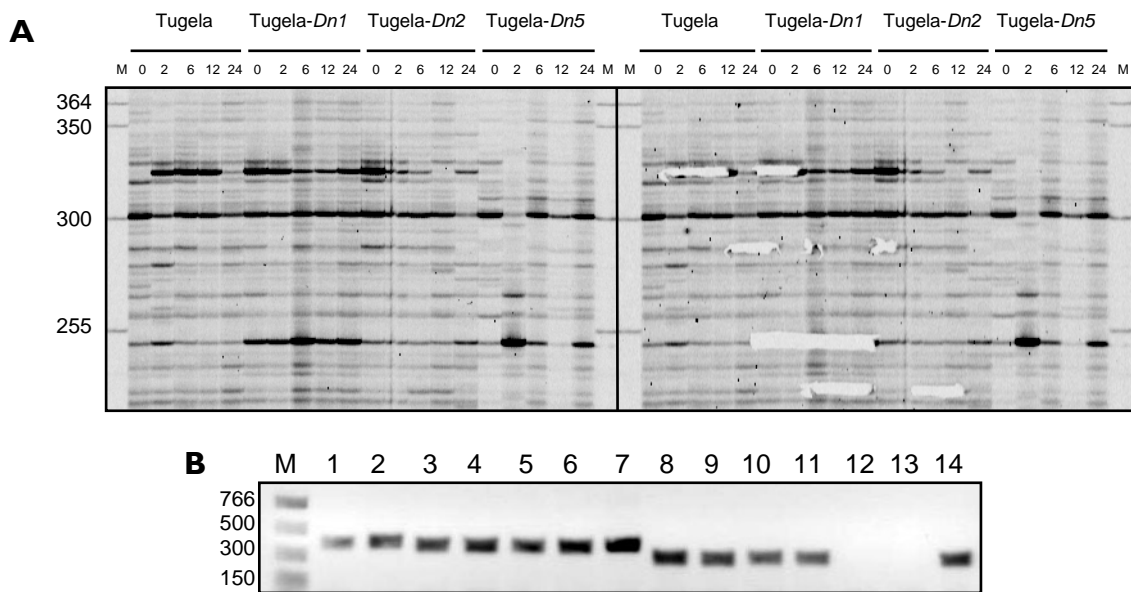
Figure 2.4

A. Odyssey gel images demonstrating the process of TDF targeting and excision. The partial polyacrylamide gel image at *left* illustrates the bands of interest, whereas the image at *right* shows the same gel after successful band excision. **B.** Example of verification of successful cloning of TDF via colony PCR from putative transformants. Band sizes are indicated to the left in number of base pairs. M: PCR marker (New England Biolabs, Inc. Beverly, MA); lanes 1-7: amplifications from separate colonies for AmoLve-45.323; lanes 8-14: amplifications for separate colonies for AmoLve-43.233. Note that lanes 12 and 13 did not amplify and therefore represent false positives.

2.4.5 TDF recovery and identification

Forty-nine differentially regulated TDFs representing all eight expression clusters were excised from the acrylamide gel, re-amplified and cloned (Figure 2.4). After the sequences were obtained, putative identities were assigned to the clones using BLASTx or BLASTn (Table 2.3). Based on the putative functions of the proteins inferred by similarity, the TDFs were classified into five broad functional categories (Figure 2.5). Of the total amount of

sequenced TDFs, 14% was involved in general gene regulation and metabolism, 25% in stress

and signal transduction and 12% in photosynthesis. The remainder of TDFs either exhibited

similarity to hypothetical proteins or proteins of unknown function, or were classified as

TDFs with no significant similarity to proteins in the non-redundant database. All novel

sequences were entered into the Genbank EST database as listed in the Appendix. TDFs

categorized as regulatory transcripts included a lingual lipase-like gene, TPA cysteine

protease, a putative transfactor, a methyl CpG-binding protein, ethylene-responsive RNA

helicase, C4-type zinc finger protein, ubiquitin and ubiquitin-protein ligase I (Table 2.3). The

stress and signal transduction category included such diverse transcripts as a mitochondrial

half-ABC transporter, a mechanosensitive ion channel protein, kinases (i.e. GHMP kinase and

serine/threonine protein kinase), inorganic pyrophosphatase, a stress related-like protein

interactor, isomerases (i.e. PDI-1 protein disulfide isomerase 1 and IDI2 isopentenyl-

diphosphate delta isomerase 2), a 66 kDa stress protein and KCO1 outward-rectifying

potassium channel (Table 2.3). Several TDFs grouped together functionally as components of

photosynthesis. This category clearly indicated the importance of the Rubisco small subunit

during the wheat response to *D. noxia*, with three of the TDFs obtained showing such

similarity. Other transcripts included a TMP 14 kDa thylakoid membrane phosphoprotein,

fructose-1,6-*bis*phosphatase and aconitate hydratase (Table 2.3).

Table 2.3

Analysis of TDF sequence similarity using BLASTx and BLASTn

Clone ID	Size (bp)	Homology	E value
Regulatory			
AmoLve-26.340	657	Lingual lipase-like	$4e^{-41}$
		[*Oryza sativa*[a]]	
AmoLve-45.211	215	TPA putative cysteine protease	$2e^{-24}$
		[*Hordeum vulgare* subsp. *vulgare*[a]]	
AmoLve-56.112	115	Putative transfactor	$1e^{-02}$
		[*Oryza sativa*[a]]	
AmoLve-66.371	377	Ubiquitin	$2e^{-23}$
		[*Triticum aestivum*[a]]	
AmoLve-68.344	390	Putative ubiquitin-protein ligase 1	$4e^{-05}$
		[*Oryza sativa*[a]]	
AmoLve-88.234	243	Ethylene-responsive RNA helicase	$4e^{-15}$
		[*Lycopersicon esculentum*[a]]	
AmoLve-88.250	252	Hypothetical C4-type zinc finger protein TraR-family	$7e^{-35}$
		[*Escherichia coli* O157:H7[a]]	
Stress and signal transduction			
AmoLve-11.250	257	Putative mitochondrial half-ABC transporter	$1e^{-14}$
		[*Oryza sativa*[a]]	
AmoLve-11.270	323	Putative mechanosensitive ion channel protein	$3e^{-14}$
		[*Oryza sativa*[a]]	
AmoLve-11.450	454	GHMP kinase-like protein	$2e^{-57}$
		[*Oryza sativa*[a]]	
AmoLve-22.210	248	Serine/threonine protein kinase domain	$9e^{-14}$
		[*Triticum aestivum*[a]]	
AmoLve-22.225	223	Inorganic pyrophosphatase	$2e^{-30}$
		[*Oryza sativa*[a]]	

AmoLve-26.245	249	Putative UDP-glucose glucosyltransferase 1	$8e^{-15}$
		[*Oryza sativa*[a]]	
AmoLve-26.250.1	257	Clathrin heavy chain	$3e^{-28}$
		[*Oryza sativa*[a]]	
AmoLve-26.265	263	Putative stress related-like protein interactor	$6e^{-35}$
		[*Oryza sativa*[a]]	
AmoLve-45.323	322	PDI-1 protein disulfide isomerase 1	$3e^{-149}$
		[*Triticum aestivum*[b]]	
AmoLve-61.272	338	Outward-rectifying potassium channel (KCO1) mRNA	$3e^{-16}$
		[*Hordeum vulgare* subsp. *vulgare*[b]]	
AmoLve-66.172	300	Putative 66 kDa stress protein	$1e^{-40}$
		[*Oryza sativa*[a]]	
AmoLve-88.300	166	IDI2 isopentenyl-diphosphate delta isomerase 2	$3e^{-16}$
		[*Hordeum vulgare* subsp. *vulgare*[a]]	
Photosynthesis			
AmoLve-11.445	448	TMP 14 kDa thylakoid membrane phosphoprotein	$2e^{-14}$
		[*Arabidopsis thaliana*[a]]	
AmoLve-66.306	199	Putative Rubisco small subunit	$2e^{-07}$
		[*Triticum turgidum* subsp. *durum*[a]]	
AmoLve-66.319	155	Putative Rubisco small subunit	$2e^{-07}$
		[*Triticum turgidum* subsp. *durum*[a]]	
AmoLve-66.323	394	Putative aconitate hydratase	$2e^{-44}$
		[*Oryza sativa*[a]]	
AmoLve-86.322	324	Putative Rubisco small subunit	$2e^{-07}$
		[*Triticum turgidum* subsp. *durum*[a]]	
AmoLve-88.303	305	Fructose-1,6-*bis*phosphatase	$8e^{-44}$
		[*Pisum sativum*[a]]	

Unknown

AmoLve-26.250	255	Expressed protein	$3e^{-33}$
		[*Oryza sativa*[a]]	
AmoLve-43.129	265	OSJNBb0085C12.17	$4e^{-14}$
		[*Oryza sativa*[a]]	
AmoLve-45.370	375	OSJNBa0023J03.10	$4e^{-30}$
		[*Oryza sativa*[a]]	
AmoLve-61.374	388	Hypothetical protein	$2e^{-09}$
		[*Oryza sativa*[a]]	
AmoLve-66.244.1	340	Unknown protein	$4e^{-04}$
		[*Oryza sativa*[a]]	
AmoLve-68.292	334	OSJNBb0016D16.15	$3e^{-05}$
		[*Oryza sativa*[a]]	
AmoLve-88.231	144	P0046G04.25	$4e^{-17}$
		[*Oryza sativa*[a]]	

No homology

AmoLve-11.340	345	No match[a,b]
AmoLve-22.270	281	No match[a,b]
AmoLve-22.275	322	No match[a,b]
AmoLve-26.220	215	No match[a,b]
AmoLve-26.270	257	No match[a,b]
AmoLve-26.355	336	No match[a,b]
AmoLve-42.151	194	No match[a,b]
AmoLve-42.321	324	No match[a,b]
AmoLve-43.139	180	No match[a,b]
AmoLve-45.229	235	No match[a,b]
AmoLve-66.169	170	No match[a,b]
AmoLve-67.181	217	No match[a,b]
AmoLve-67.216	218	No match[a,b]
AmoLve-68.268	292	No match[a,b]

AmoLve-68.407	410	No match[a,b]
AmoLve-86.113	114	No match[a,b]
AmoLve-88.189	219	No match[a,b]

[a]Similarity analysis of TDF sequence using BLASTx

[b]Similarity analysis of TDF sequence using BLASTn
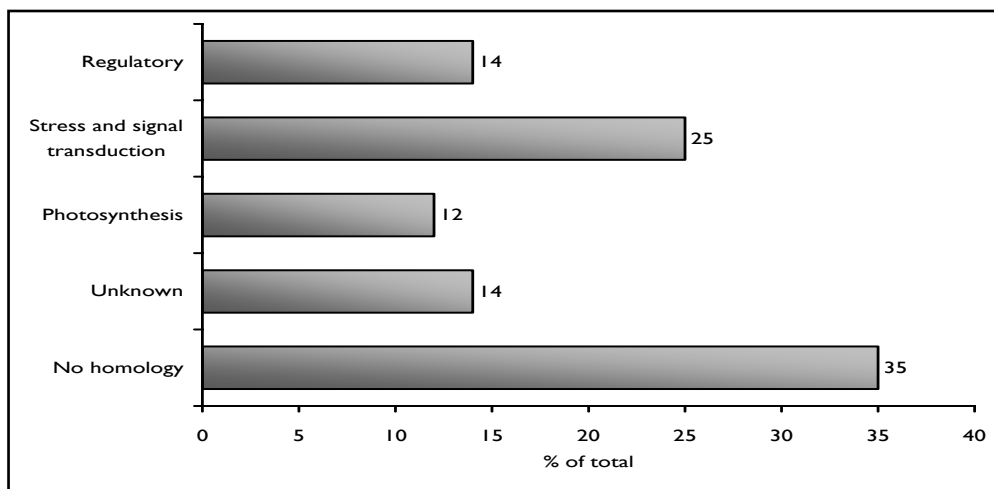
Figure 2.5

Classification of putative TDF identities based on BLASTx and BLASTn results. TDFs classed as unknown demonstrate significant homology to sequences for proteins with unknown function. TDFs with no homology had no significant similarity to any database sequences via BLASTx or BLASTn.

2.4.6 Confirmation of TDF expression patterns by qRT-PCR and RNA hybridization analysis

The results obtained using cDNA-AFLP analysis was verified using qRT-PCR on TDFs representative of the eight expression clusters and the five functional categories. Generally, the differential transcript regulation obtained with qRT-PCR corresponded well with the band intensity for each particular TDF obtained from the cDNA-AFLP procedure and supported the classification of the TDFs into their respective expression clusters. In addition to qRT-PCR, the expression of three different transcripts was successfully visualized by RNA hybridization

(Figure 2.6). In all three cases, band intensities indicated similar trends to those obtained with qRT-PCR. AmoLve-26.265, a member of cDNA-AFLP cluster C (Figure 2.3), was identified through database similarity as a putative stress related-like protein interactor from *Oryza sativa* (Table 2.3). qRT-PCR analysis indicated a decrease in transcript abundance in all lines from 6 h post-infestation onwards, with the lowest levels at 6 h and 12 h post-infestation in Tugela and Tugela-*Dn2*, and at 24 h post-infestation in Tugela-*Dn1*. RNA hybridization analysis also suggested a decrease in transcript abundance below detectable levels from 6 h post-infestation onwards in all resistant near-isogenic lines. The transcript was not detectable in the susceptible Tugela line at any of the five time intervals (Figure 2.6).

AmoLve-61.272, a member of cDNA-AFLP cluster D (Figure 2.3), showed significant similarity to a KCO1 outward-rectifying potassium channel from *Hordeum vulgare* (Table 2.3) and was induced by *D. noxia* feeding. qRT-PCR and RNA hybridization analyses agreed well, supporting an increase in AmoLve-61.272 transcript abundance. RNA hybridization indicated an increase to high levels from 2 h post-infestation onwards and qRT-PCR suggested about 2-fold induction by 6 h post-infestation in all lines, maximizing at almost 9-fold induction in Tugela, 6-fold induction in Tugela-*Dn1*, roughly 5-fold induction in Tugela-*Dn2* and more than 3-fold induction in Tugela-*Dn5* by 24 h post-infestation (Figure 2.6).

TDF AmoLve-22.225, a member of cDNA-AFLP cluster H (Figure 2.3), shares similarity to inorganic pyrophosphatase from *O. sativa* (Table 2.3). qRT-PCR data indicate a decrease in expression as aphid infestation progresses. The transcript was downregulated between 6 h and 12 h post-infestation in the resistant near-isogenic lines to levels 0.4-fold that of the calibrator (Pfaffl, 2001), with the susceptible Tugela line already demonstrating a decrease between 2 h and 6 h post-infestation to about 0.6-fold compared to the calibrator. Blotting analysis also indicated a drop in expression, with a decrease below detectable levels from 6 h post-infestation onwards (Figure 2.6).
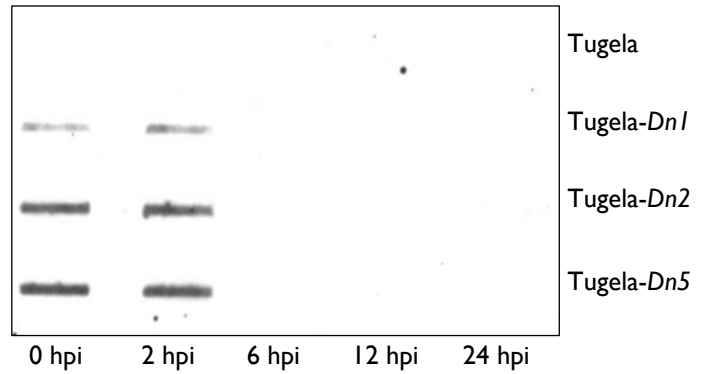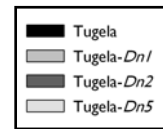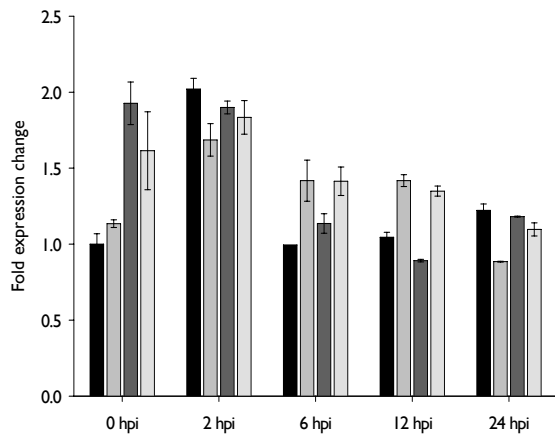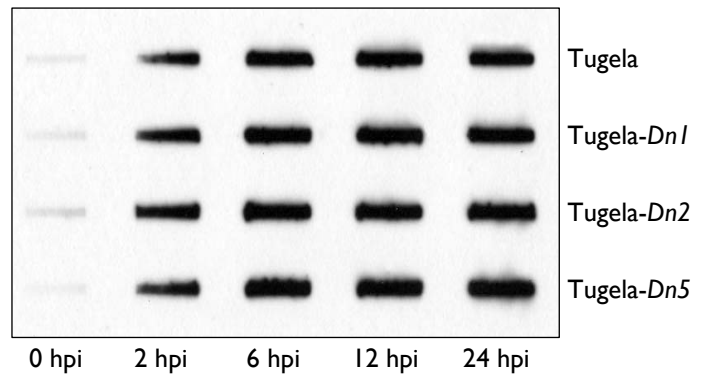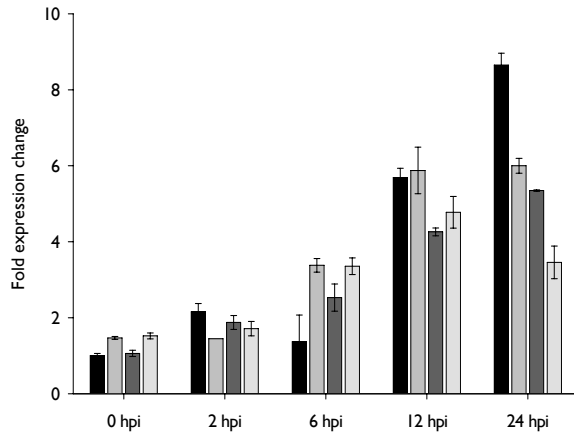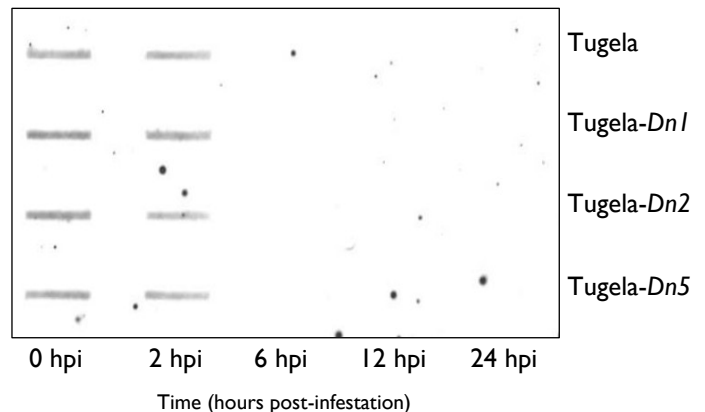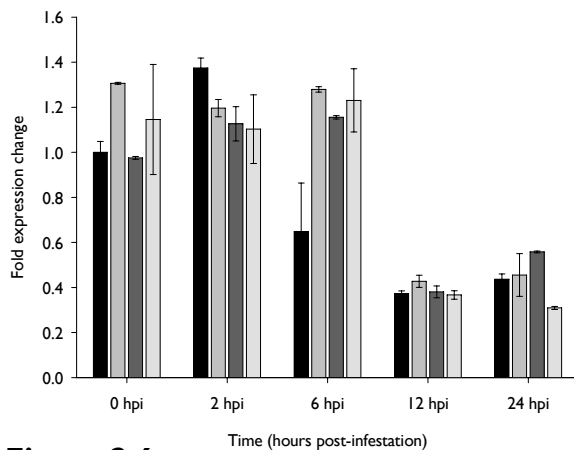
Figure 2.6

Comparison of expression profiles of wheat transcript-derived fragments (TDFs) during aphid infestation using qRT-PCR and RNA hybridization. In all cases, the *X*-axis represents time in h post-infestation. The *Y*-axis on bar charts indicates the fold expression change obtained by qRT-PCR.

Several TDFs could be classified into a broad functional group of transcripts involved in general gene regulation and metabolism (Figure 2.5). These included AmoLve-66.371, with similarity to ubiquitin, which formed part of cDNA-AFLP cluster A (Figure 2.3). The normalized cDNA-AFLP band intensity plot indicated a higher constitutive level of this TDF in Tugela-*Dn1*, with an increase in expression at 2 h post-infestation, whereafter transcript abundance decreases to levels comparable to the other lines (Figure 2.7). qRT-PCR data for AmoLve-68.344, the putative ubiquitin-protein ligase, also indicated induction of this transcript at 2 h post-infestation in Tugela-*Dn1* (Figure 2.7). This induction was 4-fold in Tugela-*Dn1* as compared to the uninfested susceptible Tugela line.

cDNA-AFLP profiling data grouped AmoLve-45.211 into cluster D (Figure 2.3). This TDF showed significant homology to a TPA putative cysteine protease from *H. vulgare* (Table 2.3). The normalized cDNA-AFLP band intensity plot indicated that the transcript increased in quantity over time, especially in Tugela-*Dn1* (Figure 2.7). Transcript abundance peaked at 12 h post-infestation in Tugela-*Dn2*, and by 24 h post-infestation band intensities for Tugela and Tugela-*Dn2* indicated much lower abundance compared to the other lines.

AmoLve-88.234 is an ethylene-responsive RNA helicase (Table 2.3) from cDNA-AFLP cluster H (Figure 2.3). qRT-PCR analysis of AmoLve-88.234 demonstrated it to be highly expressed in uninfested Tugela-*Dn5* compared to the other uninfested lines, with consequent peaks in expression for Tugela at 2 h post-infestation, Tugela-*Dn1* at 2 h post-infestation, and Tugela-*Dn2* at 6 h post-infestation (Figure 2.7).
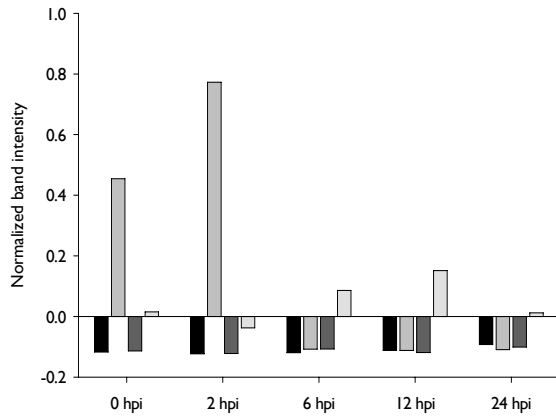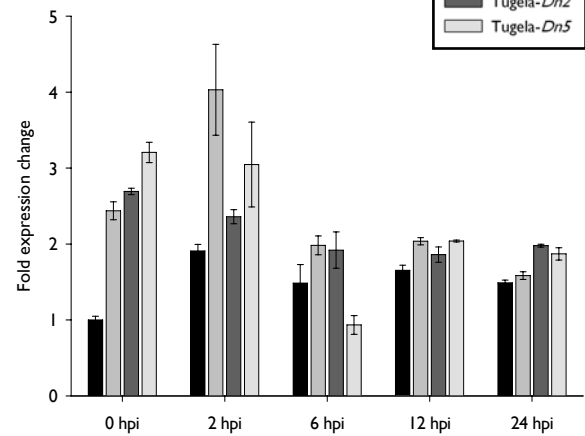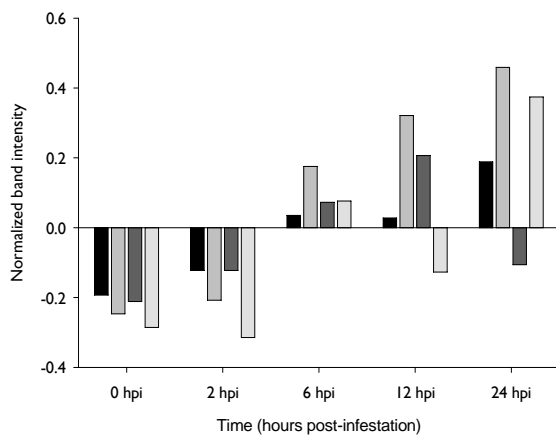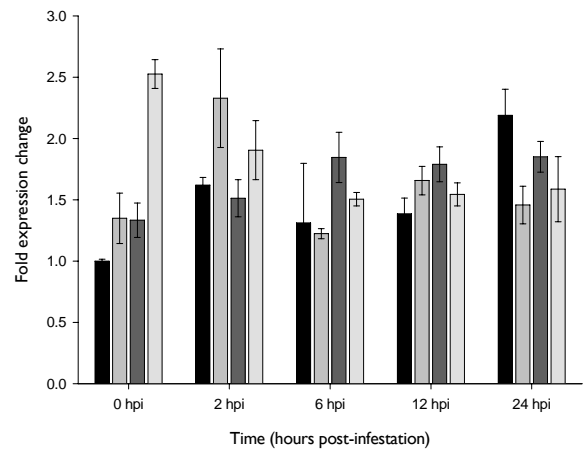
Figure 2.7

Expression profiles of differentially regulated wheat transcript-derived fragments (TDFs) involved in general regulatory mechanisms. In all cases, the *X*-axis represents time in h post-infestation. On cDNA-AFLP expression plots (*left*) the *Y*-axis indicates the normalized band intensity obtained by cDNA-AFLP using the AFLP-Quantar*Pro* software. The *Y*-axis on qRT-PCR expression plots (*right*) indicates the fold expression change obtained by qRT-PCR, where the expression level is calculated relative to the expression level of the Tugela_0hpi sample and is normalized to the expression of the chloroplast 16S rRNA transcript.

A number of TDFs could be functionally grouped together as transcripts involved in stress and signal transduction (Figure 2.5). AmoLve-11.250, a TDF from cluster C (Figure 2.3), has significant amino acid similarity to a putative mitochondrial half-ABC transporter from *O. sativa* (Table 2.3). Normalized cDNA-AFLP band intensity plots indicated that no regulation was observed in Tugela or Tugela-*Dn1*, but that Tugela-*Dn2* and Tugela-*Dn5* both exhibited a sharp increase at 6 h post-infestation, with a subsequent sharp decrease to previous levels by 12 h post-infestation (Figure 2.8).

TDF AmoLve-45.323 possesses very high similarity to the *T. aestivum* PDI-1 protein disulfide isomerase (Table 2.3) and is part of cDNA-AFLP cluster D (Figure 2.3). It exhibited a general increase in abundance according to normalized cDNA-AFLP band intensity plots (Figure 2.8). Levels in Tugela and Tugela-*Dn2* peaked at 12 h post-infestation. The TDF was more abundant in Tugela-*Dn1* than in the other lines from 6 h post-infestation onwards, peaking at 24 h post-infestation. Tugela, Tugela-*Dn2* and Tugela-*Dn5* had comparable amounts of this transcript at 24 h post-infestation, transcript abundance in Tugela-*Dn1* being higher at this time point.

AmoLve-22.210 has very high homology to a wheat serine/threonine protein kinase (Table 2.3). This TDF from cDNA-AFLP expression cluster E has a qRT-PCR expression pattern indicating responsiveness in Tugela, Tugela-*Dn1* and Tugela-*Dn5*, with transcript abundance in Tugela and Tugela-*Dn1* peaking at 5-fold that observed in the uninfested Tugela sample by 24 h post-infestation (Figure 2.8).

AmoLve-66.172 is a putative 66 kDa stress protein (Table 2.3) from cDNA-AFLP cluster F (Figure 2.3). The normalized cDNA-AFLP band intensity plot indicated higher transcript levels in the susceptible Tugela line at all time intervals when compared to the resistant near-isogenic lines. Induction appeared to be bimodal, with increased transcript

abundance in all lines at 2 h post-infestation, followed by a decrease at 6 h and subsequent increase at 12 h post-infestation (Figure 2.8).

AmoLve-11.450 is a TDF from cDNA-AFLP expression cluster G (Figure 2.3) exhibiting similarity to a rice GHMP kinase (Table 2.3). qRT-PCR analysis indicated strong induction of this transcript by *D. noxia* feeding. By 12 h post-infestation, transcript abundance was more than 20-fold in Tugela, Tugela-*Dn1* and Tugela-*Dn2*, and more than 8-fold in Tugela-*Dn5*, as compared to the uninfested control. By 24 h post-infestation, transcript abundance increased to more than 35-fold in Tugela and 20-fold in Tugela-*Dn1* and Tugela-*Dn2*, whereas transcript abundance in Tugela-*Dn5* leveled off at 8-fold that observed in the uninfested control (Figure 2.8).

Figure 2.8 (*overleaf*)

Expression profiles of differentially regulated wheat transcript-derived fragments (TDFs) involved in stress and signal transduction pathways. In all cases, the *X*-axis represents time in h post-infestation. On cDNA-AFLP expression plots (*left*) the *Y*-axis indicates the normalized band intensity obtained by cDNA-AFLP using the AFLP-Quantar*Pro* software. The *Y*-axis on qRT-PCR expression plots (*right*) indicates the fold expression change obtained by qRT-PCR, where the expression level is calculated relative to the expression level of the Tugela_0hpi sample and is normalized to the expression of the chloroplast 16S rRNA transcript.

AmoLve-45.323
(PDI-1 protein disulfide isomerase 1)
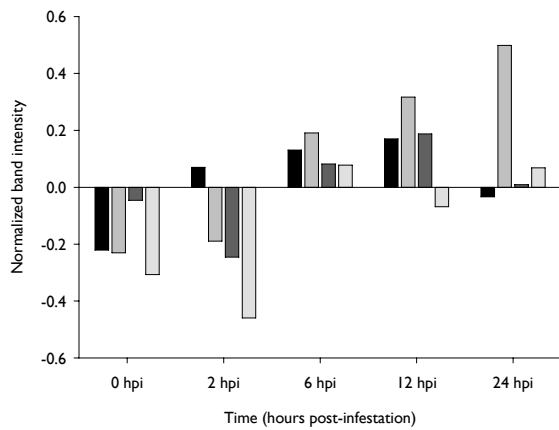
AmoLve-11.450
(GHMP kinase-like protein)
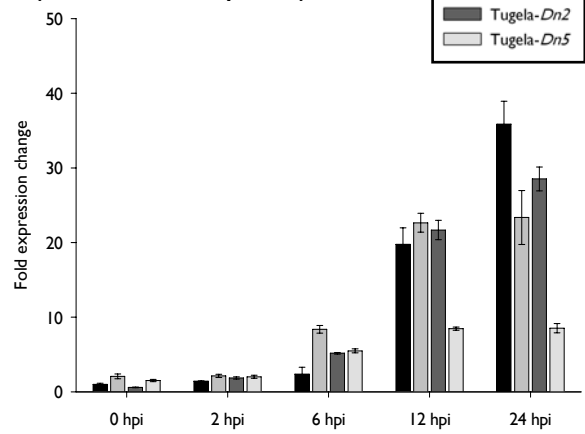
AmoLve-11.250
(mt half-ABC transporter)
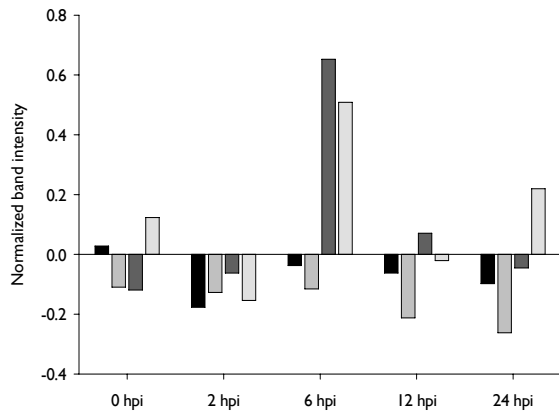
AmoLve-22.210
(serine/threonine protein kinase)
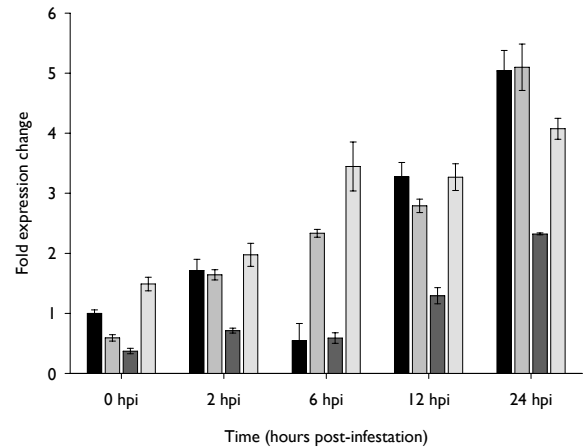
AmoLve-66.172
(66 kDa stress protein)
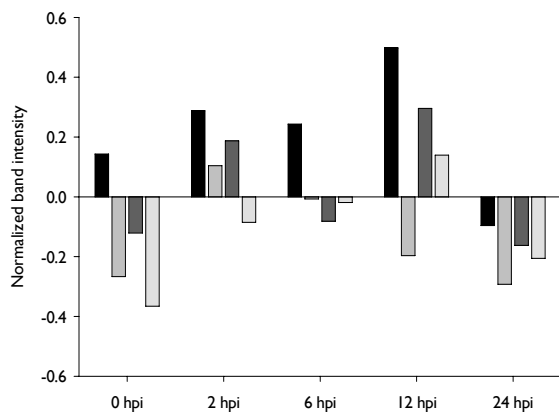
Transcripts involved in photosynthesis formed another distinct functional category (Figure 2.5). This category included a TDF from cDNA-AFLP cluster G (Figure 2.3): AmoLve-66.323, with similarity to a putative aconitase enzyme from *O. sativa* (Table 2.3), has a cDNA-AFLP expression pattern indicating a massive induction in Tugela-*Dn1* at 6 h post-infestation, before returning to baseline levels (Figure 2.9). Only slight increases in expression between 6 h and 12 h post-infestation were observed in the other near-isogenic lines.

The TDF AmoLve-11.445 from cDNA-AFLP cluster G (Figure 2.3) exhibits significant similarity to a 14 kDa thylakoid membrane phosphoprotein from *Arabidopsis thaliana* (Table 2.3). Its normalized cDNA-AFLP band intensity plot indicated a massive induction of this transcript in the tolerant Tugela-*Dn2* line triggered 6 h post-infestation. To a lesser extent, this was also observed in Tugela-*Dn5* (Figure 2.9).

qRT-PCR of AmoLve-88.303, a TDF from cDNA-AFLP cluster H (Figure 2.3) with high homology to fructose-1,6-*bis*phosphatase from *Pisum sativum* (Table 2.3), indicated a severe decrease in transcript abundance in all lines from 6 h post-infestation onwards, except in Tugela-*Dn2*, where at 6 h an increase in excess of 4-fold above the expression in Tugela at 0 h is observed (Figure 2.9).

qRT-PCR analysis was also performed on the b subunit of CF_1 ATP synthase in order to investigate its regulation during aphid feeding. This revealed induction of this transcript in all lines. The susceptible Tugela and tolerant Tugela-*Dn2* achieved higher levels of this transcript at 24 h post-infestation than observed in Tugela-*Dn1* and Tugela-*Dn5*.

AmoLve-66.323
(aconitate hydratase)
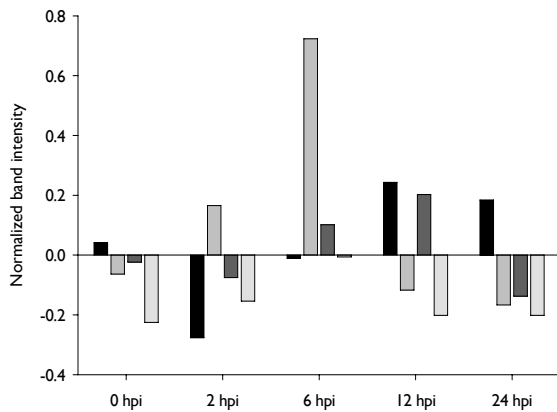
AmoLve-88.303
(fructose-1,6-*bis*phosphatase)
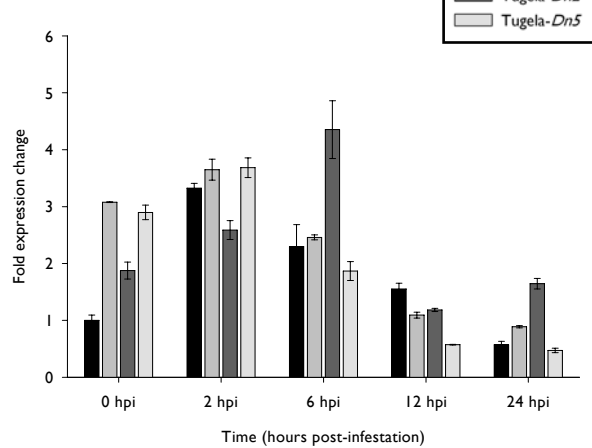
AmoLve-11.445
**(TMP14 thylakoid membrane phosphoprotein)**
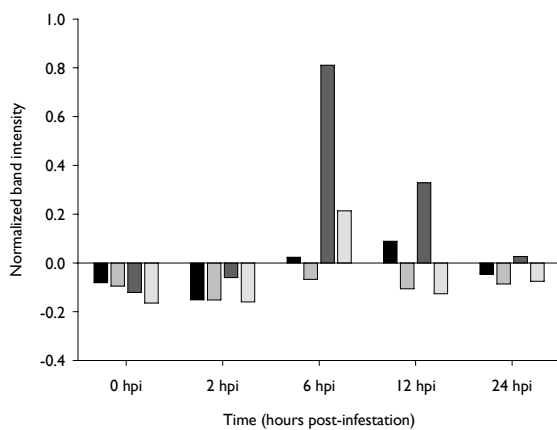
ATP synthase CF$_1$
(b subunit)
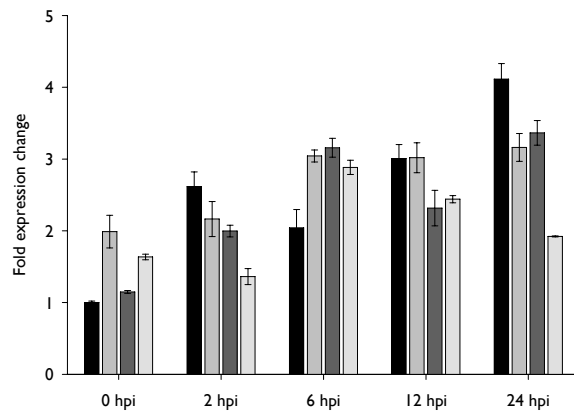
Figure 2.9

Expression profiles of differentially regulated wheat transcript-derived fragments (TDFs) involved in photosynthesis. In all cases, the *X*-axis represents time in h post-infestation. On cDNA-AFLP expression plots (*left*) the *Y*-axis indicates the normalized band intensity obtained by cDNA-AFLP using the AFLP-Quantar*Pro* software. The *Y*-axis on qRT-PCR expression plots (*right*) indicates the fold expression change obtained by qRT-PCR, where the expression level is calculated relative to the expression level of the Tugela_0hpi sample and is normalized to the expression of the chloroplast 16S rRNA transcript.

2.4.7 Amino acid sequence alignment of AmoLve-26.245 with known UDP-glucose glucosyl transferases

AmoLve-26.245 is a TDF from cDNA-AFLP cluster C (Figure 2.3). This TDF has a sequence similarity to a putative UDP-glucose glucosyltransferase 1 from *O. sativa* (Table 2.3). The normalized cDNA-AFLP band intensity plot for AmoLve-26.245 exhibited a marked increase in band intensity in all cultivars up to 12 h post-infestation, indicating induction of this gene by *D. noxia* feeding. At 2 h post-infestation, the resistant lines Tugela-*Dn1*, exhibiting antibiosis, and Tugela-*Dn5*, exhibiting antixenosis and moderate antibiosis, had lower levels than the susceptible Tugela and tolerant Tugela-*Dn2* lines (Figure 2.10.A). This discrepancy between the susceptible or tolerant lines and the antibiotic lines became even more striking by 12 h post-infestation, which indicated that negative regulation of the target molecule is more prominent in the lines that do not rely on a hypersensitive reponse.

In order to provide support for the hypothesis that AmoLve-26.245 encodes a UDP-glucose glucosyltransferase with specificity for salicylic acid as target for glycosylation, a ClustalW multiple protein sequence alignment was constructed of AmoLve-26.245 with eight plant UDP-glucose glucosyltransferases (Figure 2.10.B), including a UDP-glucose:salicylic acid gluosyltransferase (gi:7385017) from tobacco (Lee and Raskin, 1999). The alignment indicated that the translated AmoLve-26.245 sequence encompasses the last 51 residues at the C-terminal of the protein.

**AmoLve-26.245
(UDP-glucose glucosyltransferase 1)**

A

B

```
AmoLve-26.245                        1 MELMEGENGRVMKKKAQEWREKAVKAT-KPGGSSHDNFDALIRDVLAP---SRHG------
gi:51964394|Origin: O. sativa      436 AELMEGQKGKEMRRKAEEWREKAIRAA-KPGGSSHRNFEELVRHVLLA---KES-------
gi:50912923|Origin: O. sativa      436 AELMEGQKGKEMRRKAEEWREKAIRAA-KPGGSSHRNFEELVRHVLLA---KES-------
gi:50922975|Origin: O. sativa      444 REAMEGKKGRETRRRAQEWKEKAVRVT-LPGGPGDTNLDRVIHDVLISCKDKTSRVNGESV
gi:79349376|Origin: A. thaliana    436 RELMDGEKGKKMREKAVEWRRLAEKATKLPCGSSVINFETIVNKVLLG---KTPNT-----
gi:7385017 |Origin: N. tabacum     408 KIVMEEKKGKKIRENAKKWKELARKAV-DEGGSSDRNIEEFVSKLVTIASVES--------
gi:86439711|Origin: T. aestivum    422 REMMVGRKGMEAREKTLEWKRLAEDAT-KEGGSSCANLDRFVEDVLIK---GL--------
gi:50726639|Origin: O. sativa      419 REVMEGERSKEFMENANGWKEKARNAM-CEGGSSDKNIVEFIAKIGVDRKFK---------
gi:34911582|Origin: O. sativa      444 RVMGEGEEGEAMRKKVKELREKARSAV-KEGGSSYDDAGRLIDELMARRSSGTS-------
consensus                          452 relmegekgkemrekaqewrekArkat kpgGss rnfeelvrhvlla   kis
```

Figure 2.10

A. Normalized cDNA-AFLP band intensity plot for AmoLve-26.245, a transcript-derived fragment (TDF) with sequence similarity to UDP-glucose glucosyltransferase. **B.** Alignment of the predicted amino acid sequence for the cDNA clone AmoLve-26.245 with the amino acid sequences of several plant UDP-glucose glucosyl transferases, including several with specificity for salicylic acid as target for glycosylation. Conserved residues at a specific site are highlighted in black, whereas similar residues are highlighted in grey. Dashes indicate gaps to maximize alignment.

2.5 DISCUSSION

2.5.1 cDNA-AFLP as a valid method of assaying wheat gene regulation

In this study we identified several transcript-derived fragments (TDFs) differentially regulated during the wheat response to *Diuraphis noxia* by using cDNA-AFLP analysis. The band intensities were scored as a measure of transcript abundance, and representative TDFs from each expression cluster were excised, sequenced and assigned putative identities through database homology searches (Table 2.3). Transcripts were grouped into eight expression clusters (Figure 2.3), depending on their pattern of induction during the infestation period. Results also reflected differences between the susceptible Tugela line and the different resistant near-isogenic lines. TDFs representative of the different expression clusters were selected for qRT-PCR analysis to verify the cDNA-AFLP results.

cDNA-AFLP analysis has proved useful in investigating stress responses in plants in general (Simões-Araújo et al., 2002; Kemp et al., 2005), and the interactions between wheat and its pests and pathogens in particular (Matsioloko and Botha, 2003; Zhang et al., 2003). Several recent studies investigating the expression of wheat genes in response to *D. noxia* feeding have also relied on suppression subtractive hybridization (SSH) technology (Lacock and Botha, 2003; Van Niekerk and Botha, 2003; Botha et al., 2006; Boyko et al., 2006). Whereas the SSH performed by Botha et al. (2006) was performed by subtracting between the same susceptible Tugela and resistant Tugela-*Dn1* lines used

in this study, the SSH performed by Boyko et al. (2006) was performed using bulked $F_{2:3}$ plants from a cross between the susceptible 'Sando's Selection' cultivar and a resistant accession containing *Dnx*. *Dnx* was not included in the present study, but the fact that it affords a combination of antibiosis and tolerance (Boyko et al., 2006) makes the data from both studies analogous. The presence of transcripts such as the stem rust resistance protein DW986142 (serine/threonine protein kinase) and protein disulfide isomerases DW986151 and DW986152 in their SSH cDNA library validate the cDNA-AFLP clones AmoLve-22.210 (serine/threonine protein kinase) and AmoLve-45.323 (PDI-1, protein disulfide isomerase 1) obtained here.

Several parallels can be drawn between the results of these experiments. A number of wheat transcripts determined to be of importance during aphid infestation in the SSH-based studies (Botha et al., 2006; Boyko et al., 2006) were again discovered via cDNA-AFLP analysis. The significance of certain cellular processes (Figure 2.5) is also emphasized by being independently detected in all these studies, such as recognition, signalling and defence responses, the oxidative burst, cell maintenance, photosynthesis and energy production. Components of the ubiquitin/26 S proteasome pathway, such as ubiquitin-protein ligase (Botha et al., 2006) and ubiquitin fusion degradation protein (Boyko et al., 2006) discovered to be differentially regulated upon *D. noxia* exposure, support the differential expression of AmoLve-66.371 (ubiquitin) and AmoLve-68.344 (E3 ubiquitin-proteinligase 1). Components of the photosynthetic machinery, such as the photosystem I P700 apoprotein A1 and chloroplast ATP synthase (Botha et al., 2006), the photosystem I antenna and assembly proteins and photosystem II protein D1 (Boyko et al., 2006) are also implicated in the wheat response to *D. noxia* and serve as supporting

evidence for the participation of several chloroplast genes in countering the effects of aphid-elicited stress. These include AmoLve-11.445 (TMP14 thylakoid membrane phosphoprotein) and AmoLve-88.303 (fructose-1,6-*bis*phosphatase).

Jointly, expression pattern analysis and database homology information from differentially regulated TDFs provide a representation of the kinds of cellular processes and biochemical pathways sensitive to *D. noxia* elicitation. By examining global transcriptional changes occurring in wheat within a 24 h time period after *D. noxia* feeding in susceptible Tugela, antibiotic Tugela-*Dn1*, tolerant Tugela-*Dn2* and antixenotic Tugela-*Dn5* it is possible to associate these biochemical changes with each of the three distinct modes of resistance.

2.5.2 Defence signal transduction

Certain TDFs identified as being differentially regulated are well known to be associated with plant stress or the hypersensitive response. These include: AmoLve-26.265, with amino acid sequence homology to a putative stress related-like protein interactor from *Oryza sativa*; AmoLve-66.172, exhibiting significant similarity to a 66 kDa stress protein from *O. sativa*; and two TDFs similar to kinase transcripts. AmoLve-11.450, with similarity to a rice GHMP kinase-like protein has a qRT-PCR expression pattern indicating induction of this TDF by aphid feeding (Figure 2.8). AmoLve-22.210 has very high homology to a wheat serine/threonine protein kinase (Table 2.3). The qRT-PCR expression pattern of this TDF indicated responsiveness in Tugela, Tugela-*Dn1* and Tugela-*Dn5*, with transcript abundance in Tugela and Tugela-*Dn1* peaking at 5-fold that

observed in the uninfested Tugela sample by 24 h post-infestation. It is interesting to note that Tugela-*Dn1*, showing antibiosis, and Tugela-*Dn5*, with antixenosis and moderate antibiosis, manage to exceed the 2-fold threshold by 6 h post-infestation, whereas the susceptible Tugela line responds sharply at 12 h post-infestation. This delayed kinase response in Tugela might be too late to be efficacious.

Kinase signalling cascades are well described in the plant pathology literature (Hammond-Kosack and Jones, 1996; Dangl and Jones, 2001; Lam et al., 2001). These kinases activate downstream proteins through phosphorylation, propagating a recognition signal which eventually leads to defence responses, most often in the form of a hypersensitive response. The tomato *Pto* gene conferring resistance against *Pseudomonas syringae* pv. *tomato* carrying the *avrPto* gene is such a serine/threonine kinase (Hammond-Kosack and Jones, 1997). It interacts directly with the pathogen's avirulence protein as well as a tomato NBS-LRR protein encoded by the *Prf* gene (Buell, 1998). AmoLve-22.210 demonstrates sequence similarity with the barley stem rust resistance gene *Rpg1* (E value = $1e^{-09}$), as did the serine/threonine protein kinase discovered by Boyko et al. (2006). It has been suggested that the products of the *Dn* genes could be serine/threonine protein kinases (Boyko et al., 2006), or proteins of the NBS-LRR resistance class (Lacock et al., 2003; Botha et al., 2006). Since aphid elicitation of the wheat resistance response requires high specificity, the identity of the Dn proteins as NBS-LRR type proteins is likely (Swanepoel et al., 2003; Botha et al., 2005). It is therefore conceivable that the protein encoded by AmoLve-22.210 might interact directly or indirectly with the *Dn* gene product in order to generate the appropriate defence responses. This 'guard hypothesis' has been well-described in the tomato *Pto* model (Van

der Biezen and Jones, 1998; Dangl and Jones, 2001). Ligand binding in protein activation cascades often leads to positive feedback induction loops (Dangl et al., 1996). A suboptimal fit between the *Dn2* gene product and AmoLve-22.210 might explain why *Dn2* doesn't induce a strong hypersensitive response like *Dn1* (Van der Westhuizen et al., 1998), and why the abundance of AmoLve-22.210 is lower in Tugela-*Dn2*. The generation of antibiosis therefore relies on timely kinase signalling activation as observed in Tugela-*Dn1* and to a lesser extent in Tugela-*Dn5*. Delayed (Tugela) or reduced (Tugela-*Dn2*) induction is not effective in initiating this kind of defence. Further characterization of AmoLve-22.210 and its possible target is required before its involvement in signal generation during aphid attack can be fully understood.

2.5.3 The ubiquitin/26 S proteasome pathway

TDFs AmoLve-66.371 and AmoLve-68.344 demonstrate sequence homology to ubiquitin from *T. aestivum* and a putative E3 ubiquitin-protein ligase 1 from *O. sativa*, respectively (Table 2.3), implying the involvement of the ubiquitin/26 S proteasome pathway in the wheat response to *D. noxia* feeding. Ubiquitin-protein ligase was previously identified as part of a collection of transcripts from *D. noxia* induced SSH libraries (Botha et al., 2006). The ubiquitin/26 S proteasome pathway is engaged in diverse cellular processes, from regulating the cell cycle and embryogenesis, to pathogen resistance and senescence (Vierstra, 2003). During ubiquitination the 76 amino acid ubiquitin protein is covalently attached to a specific protein target in a reaction cascade, thereby tagging it for degradation (Vierstra, 2003). Poly-ubiquitinated proteins displaying chains of

concatenated ubiquitin molecules are recognized and degraded by the 26 S proteasome, a multi-subunit protease (Devoto et al., 2003; Vierstra, 2003). Ubiquitination is conducted via a complex assembly of enzymes. Of these, the E1 (activating) and E2 (conjugating) enzymes are responsible for activating a C-terminal cysteine residue of the ubiquitin in an ATP-dependent manner through a series of enzyme-conjugated intermediates (Vierstra, 2003). The E3 enzymes, or ubiquitin-protein ligases, are the specificity determinants that mediate the final transfer of ubiquitin from E2 to the ε-amino group on lysine residues of target proteins (Devoto et al., 2003). Almost 1 200 different E3 genes have been discovered in *A. thaliana*, each one with a specific protein as target. Four different types of plant E3 ubiquitin-protein ligase complexes are found, based on sequence motifs and supposed assembly during conjugation (Vierstra, 2003).

cDNA-AFLP band intensity plots for AmoLve-66.371, with similarity to ubiquitin, indicated a higher constitutive level of this TDF in Tugela-*Dn1*, with an increase in expression at 2 h post-infestation, whereafter transcript abundance decreases to levels compare to the other lines (Figure 2.7). qRT-PCR data for AmoLve-68.344, the putative ubiquitin-protein ligase, also indicated induction of this transcript at 2 h post-infestation in Tugela-*Dn1* (Figure 2.7). This induction was 4-fold in Tugela-*Dn1* as compared to the uninfested susceptible Tugela line. The expression levels in Tugela and Tugela-*Dn2* showed no noteworthy regulation. Ubiquitination has been implicated as a regulatory mechanism durng plant resistance to pathogen attack (Liu et al., 2002; Peart et al., 2002; Devoto et al., 2003). The ubiquitin-protein ligase complex probably targets proteins constituting negative regulators of the defence response for 26 S proteasome degradation. Cell death inhibitors or transcription factors preventing induction of defence

response genes are likely candidates for ubiquitination (Liu et al., 2002). It is possible that R proteins themselves are targets of E3 complexes whose association and breakdown are triggered by a compatible pathogen infection (Vierstra, 2003). The expression patterns obtained for both AmoLve-66.371 and AmoLve-68.344 suggest that directed protein degradation via the ubiquitin/26 S proteasome pathway plays a role in manifesting the antibiotic resistance response against *D. noxia* in Tugela-*Dn1*, but is not activated to such an extent during susceptibility or tolerance responses.

2.5.4 Ethylene-mediated response

TDF AmoLve-88.234 has strong homology to a putative ethylene-responsive RNA helicase from *Lycopersicon esculentum* (Table 2.3). RNA helicases are tissue specific proteins with highly conserved catalytic central domains and a range of N- and C-terminal extensions (Aubourg et al., 1999). They unwind RNA molecules during post-transcriptional mRNA maturation events such as RNA splicing and are involved in ribosome assembly and the initiation of translation (Aubourg et al., 1999; Zegzouti et al., 1999). The helicases are grouped into several protein families depending on the presence of certain defining conserved motifs. AmoLve-88.234 also showed significant homology to a DEAD box RNA helicase from *Zea mays* (E value = $5e^{-06}$) and probably belongs to this group. The eukaryotic initiation factor eIF-4a is the best characterized in the DEAD box RNA helicase family (Aubourg et al., 1999). qRT-PCR analysis reveals AmoLve-88.234 to have an unusual expression pattern, being highly expressed in uninfested Tugela-*Dn5* compared to the other uninfested lines, and with consequent peaks in

expression for Tugela at 2 h post-infestation, Tugela-*Dn1* at 2 h post-infestation, and Tugela-*Dn2* at 6 h post-infestation (Figure 2.7). *ER68*, an ethylene-responsive mRNA helicase-like gene isolated from tomato fruit also exhibited an intriguing, almost bimodal expression pattern during the ethylene-dependent ripening process (Zegzouti et al., 1999). *D. noxia* feeding is known to elicit ethylene-dependent signalling pathways, although the response is not as potent as that observed during wounding or herbivory by chewing insects (Miller et al., 1994; Botha et al., 1998; Kessler and Baldwin, 2002; Botha et al., 2005). AmoLve-88.234 seems to be involved in regulating the expression of transcripts involved in ethylene-mediated signalling. Since the basal level of this transcript in Tugela-*Dn5* appears to be higher, the antixenotic response of this line might be more dependent on ethylene-induced genes compared to the other lines, although noteworthy ethylene-regulated gene expression in the other lines cannot be excluded.

2.5.5 Potassium and calcium ion flux

TDF AmoLve-61.272 showed significant similarity to a KCO1 outward-rectifying potassium channel from *H. vulgare* and was quantified using qRT-PCR and RNA hybridization (Figure 2.6). An increase in AmoLve-61.272 transcript abundance was observed with both techniques. RNA hybridization indicated an increase to high levels from 2 h post-infestation onwards and qRT-PCR suggested about 2-fold induction by 6 h post-infestation in all lines, maximizing at almost 9-fold induction in Tugela, 6-fold induction in Tugela-*Dn1*, roughly 5-fold induction in Tugela-*Dn2* and more than 3-fold induction in Tugela-*Dn5* by 24 h post-infestation (Figure 2.6).

Potassium ions are involved in adjusting the membrane potential, as well as osmoregulation and turgor-driven movements (Van Volkenburgh, 1999). Regulation of redox homeostasis occurs in response to aphid attack (Kehr, 2006) and *D. noxia* feeding in wheat notoriously impedes osmoregulation of leaf turgor during cell elongation, causing symptoms such as leaf rolling (Burd and Burton, 1992). Upregulation of AmoLve-61.272 could prove an effective way of sustaining turgor and preventing leaf rolling.

KCO1 belongs to a class of two pore (2 P-domain) K^+ channels. Six members of the *kco* K^+ channel gene family (*AtKCO1-AtKCO6*) have been identified in *Arabidopsis* (Sinnige et al., 2005). The barley HvKCO1 and *Arabidopsis* AtKCO1 exhibit the two pore domains, four transmembrane helices and two C-terminal Ca^{2+}-binding EF-hand motifs which characterize this class (Czempinski et al., 1997; Sinnige et al., 2005). In addition, the N-terminal contains a 14-3-3 protein binding motif rich in lysine and arginine residues (Sinnige et al., 2005). Activation of KCO1 is strongly dependent on the presence of nanomolar concentrations of cytosolic Ca^{2+}, providing a link between calcium-mediated signalling processes and K^+ ion transport in higher plants (Czempinski et al., 1997). An increase in cytosolic Ca^{2+} initiates long-distance calcium-activated protein kinase signalling cascades (Kehr, 2006). Sustained cytoplasmic Ca^{2+} elevation forms a vital post-recognition molecular switch, transmitting primary recognition responses to multiple downstream effectors, including activation of the oxidative burst and the hypersensitive response (HR) (Grant et al., 2000).

The calcium concentration in the sieve elements is very low compared to the surrounding parenchymatous cells (Kehr, 2006). However, when the aphid stylet

punctures the phloem sieve elements during feeding, massive influx of Ca^{2+} into the lumen of the sieve element is initiated. Soluble proteins in the phloem rapidly coagulate, effectively blocking the flow of phloem sap by clogging the sieve plates (Tjallingii, 2006). Sieve elements are also sealed off by the production of callose in the cell wall outside the plasma membrane (Will and Van Bel, 2006). Aphids can overcome Ca^{2+}-induced plugging of the sieve plates upon stylet insertion and are thought to achieve this through Ca^{2+}-binding factors in the watery saliva or inhibition of calcium influx via the gel-like sheath saliva (Kehr, 2006; Tjallingii, 2006; Will and Van Bel, 2006). Studies using AtKCO1-GFP fusion proteins in *Arabidopsis* have established that AtKCO1 localizes to the vacuolar membrane; it is part of the slow-activating vacuolar (SV) channel (Sinnige et al., 2005). The SV channel conducts both K^+ and Ca^{2+} ions and the activity is strongly dependent on cytosolic Ca^{2+} concentrations. The SV channel therefore constitutes a positive feedback system involved in calcium-induced calcium release (CICR). Ca^{2+} entering the cytosol from the vacuole serves to further activate the SV channel (Sinnige et al., 2005). However, a local increase in Ca^{2+} concentration near the cytosolic side of the barley HvKCO1 channel was found to enable the binding of 14-3-3 proteins, which modulate the activity of the K^+ channel and reduce SV channel activity and Ca^{2+} release (Sinnige et al., 2005).

The rapid induction of AmoLve-61.272, putatively encoding the wheat TaKCO1 K^+ channel, would assist in sustaining elevated cytosolic Ca^{2+} levels. This could potentially induce sealing of sieve elements by protein coagulation and callose deposition, as well as initiate signalling events leading to downstream resistance responses such as the generation of ROS and the hypersensitive response. Expression

analysis using qRT-PCR and RNA hybridization did not highlight any compelling differences in AmoLve-61.272 abundance between the susceptible and resistant lines: all plants showed induction of AmoLve-61.272 to varying degrees, irrespective of genotype. In fact, qRT-PCR analysis indicated that the susceptible Tugela line accumulated the highest level of AmoLve-61.272 by 24 h post-infestation (Figure 2.6).

Since the mRNA for this study was extracted from whole leaves, the histological localization of AmoLve-61.272 into only sieve elements and companion cells, or also in other cell types such as parenchyma and epidermal cells could not be determined. It is conceivable that Tugela and its resistant near-isogenic lines all elevate Ca^{2+} levels in an SV channel-mediated way, but that the resistant genotypes are able to prevent sequestering of Ca^{2+} by aphid saliva and allow the transduction of downstream signals to seal off the phloem and initiate other resistance responses. Resistant plants might also mobilize more Ca^{2+} than the aphid saliva can bind in order to affect a successful coagulation response. This can be achieved in an SV channel-independent manner, since the apoplast also serves as a source of Ca^{2+} ions (Will and Van Bel, 2006). The discovery of differential regulation of a Ca^{2+}-regulated outward-rectifying K^+ channel during the wheat response to *D. noxia* attack introduces a novel avenue of investigation. The histological localization of this potassium channel and its accompanying effect on calcium influx in susceptible and resistant wheat lines needs to be fully characterized. This will facilitate our understanding of recognition events at the moment of stylet penetration.

2.5.6 The oxidative burst and iron homeostasis

The database similarity search for AmoLve-45.211 assigned it homology to a TPA cysteine proteinase precursor from *Hordeum vulgare*, which is known to be expressed during leaf senescence and wounding responses (Scharrenberg et al., 2003).

By the turn of the century, 60 cysteine proteases had been isolated from plants (Ueda et al., 2000). The cysteine protease from *H. vulgare* is part of the papain-like C1A subfamily and is translated as a preproenzyme containing a prepropeptide. Cysteine proteases synthesized as preproproteins which are processed autocatalytically or with the aid of a processing enzyme are usually glycosylated and typically secreted or transported to the vacuole and stored as nonactive precursors (Ueda et al., 2000; Krüger et al., 2002; Scharrenberg et al., 2003). The enzyme is activated once the 100-150 amino acid N-terminal precursor is cleaved off (Scharrenberg et al., 2003). The caspase family of cysteine proteases in animals are very target-specific, and caspase-mediated protein fragmentation eventually leads to cell dismantling. A cysteine protease shown to be a functional analog of animal caspases is activated during HR in tobacco (Chichkova et al., 2004). Cysteine protease mRNA accumulates under environmental stress such as wounding, or during programmed cell death stages (Ueda et al., 2000). Cysteine proteases are activated during HR and programmed cell death in order to mobilize amino acids or to break down crucial cellular proteins. They are therefore a regulatory mechanism, whether advancing cell death by activating inactive cell death promoters or degrading proteins inhibiting cell death (D'Silva et al., 1998). Cysteine proteases are also

thought to positively regulate *R* gene-dependent resistance by processing either the R or Avr protein, or other protein interactors (Krüger et al., 2002).

A *D. noxia*-induced increase in AmoLve-45.211 transcript abundance could be attributed to the hypersensitive response, which is especially prominent in the antibiotic Tugela-*Dn1* line. Supporting evidence for the participation of cysteine proteases in *R*-gene mediated resistance has been acquired from studies with tomato. *Rcr3* is also a papain class cysteine protease and is rapidly induced during successful Cf-2-mediated resistance to *Cladosporium fulvum* in tomato (Krüger et al., 2002). Active Rcr3 is secreted into the apoplastic intercellular fluid and has a pattern of expression similar to that of PR proteins. Not surprisingly, PR proteins are also secreted into the apoplast during *D. noxia* feeding in wheat, where primary recognition of the aphid elicitors occurs (Van der Westhuizen et al., 1998).

TDF AmoLve-26.245 exhibits significant sequence similarity to a putative UDP-glucose glucosyltransferase 1 from *Oryza sativa* (Table 2.3). UDP-glucose glucosyltransferases are a large group of enzymes that transfer glucose molecules from UDP-glucose to various specific substrates, including terpenes, anthocyanins and flavonoids. A glucosyltransferase was part of a set of jasmonic acid-inducible genes isolated by suppression subtractive hybridization in rice (Xiong et al., 2001). Several are regulated by salicylic acid (SA) and are known to be induced during leaf senescence (Scharrenberg et al., 2003). Curiously, SA also serves as a substrate for glycosylation (Yalpani et al., 1992), forming glucosylsalicylic acid. This reaction is catalyzed with high specificity by a UDP-glucose:salicylic acid glucosyltransferase enzyme. The enzyme in *Nicotiana tabacum* has been cloned and consists of 459 amino acids, with a molecular

mass of 48 kDa (Lee and Raskin, 1999). The only sugar source for this enzyme is UDP-glucose, although SA is not the only possible target molecule and other related phenolics can also act as acceptors (Lee and Raskin, 1999). Since glycosylation of SA eliminates its function, a UDP-glucose glucosyltransferase with SA as its target would constitute a negative regulator of SA-dependent pathways (Yalpani et al., 1992). UDP-glucose:salicylic acid glucosyltransferase expression is rapidly induced by incompatible pathogens and the application of exogenous SA in *N. tabacum* (Enyedi and Raskin, 1993; Lee and Raskin, 1999), with the localized accumulation of the glucosylsalicylic acid product only in leaves exhibiting a hypersensitive response. The pattern of enzyme induction in tobacco matches the transcriptional activation of *PR-1* genes, with a 6.7-fold induction 72 h after tobacco mosaic virus inoculation (Enyedi and Raskin, 1993). This is paralleled by the cDNA-AFLP data for AmoLve-26.245, since this TDF exhibits a marked increase in band intensity in all lines up to 12 h post-infestation, indicating induction of this gene by *D. noxia* feeding (Figure 2.10.A). At 2 h post-infestation, the resistant lines Tugela-*Dn1*, exhibiting antibiosis, and Tugela-*Dn5*, exhibiting antixenosis and moderate antibiosis, had lower levels than the susceptible Tugela and tolerant Tugela-*Dn2* lines. This discrepancy between the susceptible or tolerant lines and the antibiotic lines became even more striking by 12 h post-infestation, which indicates that negative regulation of the target molecule is more prominent in the lines that do not rely on a hypersensitive reponse. This expression pattern strongly suggests identity of AmoLve-26.245 as a UDP-glucose glucosyltransferase with SA as target molecule. Further support was obtained from a ClustalW multiple protein sequence alignment of AmoLve-26.245 with eight plant UDP-glucose glucosyltransferases (Figure 2.10.B),

including a UDP-glucose:salicylic acid gluosyltransferase (gi:7385017) from tobacco (Lee and Raskin, 1999). The alignment indicated that the cloned AmoLve-26.245 sequence encompasses the last 51 residues at the C-terminal of the protein. Since the average length of the full-length sequences is 482 residues, the TDF probably only constitutes 10% of the full length of the wheat transcript.

TDF AmoLve-66.323, with similarity to a putative aconitase enzyme from *O. sativa*, has a cDNA-AFLP expression pattern indicating a massive induction in Tugela-*Dn1* at 6 h post-infestation, before returning to baseline levels (Figure 2.9). Only slight increases in expression between 6 h and 12 h post-infestation were observed in the other near-isogenic lines. Aconitase is a citric acid cycle enzyme responsible for the isomerization of citrate to isocitrate via an enzyme-bound *cis*-aconitate intermediate. The mitochondrial isoform is a component of the citric acid cycle, whereas the cytosolic isoform is part of the glyoxylate cycle. The *Arabidopsis* genome has three nuclear encoded aconitase genes; the plant mitochondrial and cytosolic forms are encoded by the same genes and are probably localized to the mitochondria through inefficient importing (Navarre et al., 2000; Carrari et al., 2003; Moeder et al., 2006). Lowered levels of aconitase have been linked to higher chlorophyll levels and increased rates of CO_2 assimilation via sucrose synthesis (Carrari et al., 2003). The brief increase in transcript abundance in Tugela-*Dn1* after *D. noxia* feeding contrasts with work done by Navarre et al. (2000), who reported that *Nicotiana tabacum* aconitase was not induced by tobacco mosaic virus (TMV) infection. The Tugela-*Dn1* line is known to initiate a rapid oxidative burst upon *D. noxia* feeding, resulting in a hypersensitive cell death response at the feeding site (Van der Westhuizen et al., 1998). The aconitase enzyme is especially

sensitive to reactive oxygen species (ROS) such as H_2O_2 and inactivation by nitric oxide (NO) (Navarre et al., 2000). Aconitase enzymatic activity requires the presence of a 4Fe-4S iron-sulfur cluster formed with three cysteine residues in the active site (Navarre et al., 2000; Carrari et al., 2003; Moeder et al., 2006). This iron-sulfur cluster is lost during oxidative stress or in the presence of NO, and the aconitase becomes an mRNA-binding protein able to negatively regulate the expression of specific transcripts. While the binding of IRE (iron-responsive) elements has been demonstrated in animals, in tobacco and *Arabidopsis* aconitase specifically binds the 5' UTR of the chloroplastic CuZn superoxide dismutase 2 (*CSD2)* mRNA, which does not contain an IRE consensus sequence (Moeder et al., 2006). 5' binding prevents translation, whereas 3' binding actually improves transcript stability (Navarre et al., 2000). The CSD2 enzyme has a significant role in the antioxidant defence network, lowering intracellular ROS levels. Aconitase is required for efficient cell death induction in *N. benthamiana* triggered by the interaction of R/Avr proteins. Studies using aconitase knockouts in *Arabidopsis* and *N. benthamiana* silenced for aconitase using virus-induced gene silencing (VIGS) established that reduced levels of aconitase resulted in enhanced resistance to oxidative stress, higher levels of the *CSD2* transcript and poorly induced HR and cell death upon pathogen exposure (Moeder et al., 2006). Since Tugela-*Dn1* relies on sustained induction of ROS to establish the hypersensitive response, programmed cell death and antibiosis, negative regulation of superoxide dismutase would be necessary. Massive induction of aconitase in an enzymatically inactive but transcript-binding form might therefore constitute part of the regulatory mechanism ensuring persistence of the oxidative burst in this line.

BLASTx results indicate that AmoLve-11.250 has significant amino acid similarity to a putative mitochondrial half-ABC transporter from *O. sativa* (Table 2.3). Normalized cDNA-AFLP band intensity plots indicated that no regulation was observed in Tugela or Tugela-*Dn1*, but that Tugela-*Dn2* and Tugela-*Dn5* both exhibited a sharp increase at 6 h post-infestation, with a subsequent sharp decrease to previous levels by 12 h post-infestation (Figure 2.8). The putative rice mitochondrial half-ABC transporter has high amino acid sequence homology with the Sta1 (STARIK1) ABC transporter from *A. thaliana*, grouping it in subfamily B as a probable iron-sulfur cluster transporter. The ABC transporters constitute a very large protein family and although they have been assigned diverse functions in membrane transport (Kushnir et al., 2001; Campbell et al., 2003), the roles of most members are yet to be established (Grec et al., 2003). Since each ABC transporter is relatively target-specific, a huge variety of these proteins is required to transport the large number of secondary metabolites plants produce (Grec et al., 2003). All such transporters are characterized by a highly conserved ATP-binding cassette (ABC) domain (Kushnir et al., 2001) and hydrolyze ATP during active transmembrane transport of various molecules from small ions to proteins, often against a concentration gradient (Kushnir et al., 2001; Campbell et al., 2003). ABC transporters have been associated with various host-pathogen interactions (Xiong et al., 2001; Campbell et al., 2003). Several ABC transporters in the pleiotropic drug resistance (PDR) family can be induced by salicylic acid, methyl jasmonate and ethylene and are involved in exporting fungitoxic metabolites such as terpenoids and diterpenoids to the site of fungal infection (Campbell et al., 2003; Grec et al., 2003; Kobae et al., 2006; Stein et al., 2006). Such PDR-type ABC transporters have previously been implicated in *D. noxia* defence in

wheat, putatively functioning by secreting cytotoxins to actively damage aphid biology (Boyko et al., 2006).

Half-ABC transporters such as Sta1 in *Arabidopsis*, are structurally distinguished from the full ABC transporters by having only one transmembrane and one ATP-binding domain, expressed in a single polypeptide. The Atm1p of budding yeast mitochondria is such a half-transporter, with its ABC domain facing the mitochondrial matrix. It functions as an exporter in the maturation of cytosolic iron-sulfur proteins (Kushnir et al., 2001). Fe-S clusters are prosthetic groups whose chemical characteristics define the redox, catalytic, and regulatory properties of many proteins, including the aconitase encoded by TDF AmoLve-66.323. Sta1 in *Arabidopsis* is a functional ortholog of yeast Atm1p and has significant sequence homology to TDF AmoLve-11.250. The Sta1 polypeptide spans 728 amino acids. Plant cells, like yeast and mammals, possess mitochondrial iron-sulfur cluster biosynthesis machinery resembling that of bacteria (Kushnir et al., 2001). *Arabidopsis* plants with a mutation in the *STARIK* gene exhibited dwarfism and chlorosis, as well as upregulation of genes for Cu/Zn superoxide dismutases; since chlorosis is light-dependent, such mutants might be experiencing photo-oxidative damage (Kushnir et al., 2001). This indicates the possibility that upregulation of AmoLve-11.250 in Tugela-*Dn2* and Tugela-*Dn5* might assist in countering the chlorotic symptoms associated with *D. noxia* feeding on susceptible lines. AmoLve-11.250 was not regulated in Tugela-*Dn1* antibiotic wheat and *Arabidopsis sta1* mutants had no differences in aconitase activity compared to the wild-type (Kushnir et al., 2001). This might explain why only Tugela-*Dn1* has a successful hypersensitive response, since upregulation of the RNA-binding form of aconitase (without the iron-

sulfur cluster provided by the half-ABC transporter) would ensure downregulation of the Cu/Zn superoxide dismutase and a sustained oxidative burst. It is clear that the further study of iron homeostasis during *D. noxia* feeding in wheat will shed much needed light on exactly how resistant lines can generate defence responses, and how they discriminate between active antibiotic and passive tolerance responses.

2.5.7 Photosynthesis

TDF AmoLve-45.323 possesses very high similarity to the *T. aestivum* PDI-1 protein disulfide isomerase (Table 2.3). It exhibited a general increase in abundance according to normalized cDNA-AFLP band intensity plots. Levels in Tugela and Tugela-*Dn2* peaked at 12 h post-infestation. The TDF was more abundant in Tugela-*Dn1* than in the other lines from 6 h post-infestation onwards, peaking at 24 h post-infestation. Tugela, Tugela-*Dn2* and Tugela-*Dn5* had comparable amounts of this transcript at 24 h post-infestation, transcript abundance in Tugela-*Dn1* being higher at this time point (Figure 2.8).

PDI is part of a superfamily of multifunctional chaperone proteins involved in signal transduction pathways. It is characterized by two double-cysteine, redox-active sites and one or more copies of the highly conserved thioredoxin fold domain. PDI assists in protein folding by catalyzing the formation of disulfide bonds by oxidation of sulfhydryl groups of cysteine residues and is typically found in the endoplasmic reticulum (Kim and Mayfield, 1997). The PDI gene family in wheat consists of three members located on chromosomes 4BS, 4DS and 4AL (with a fourth on 1BS probably being non-functional) and its involvement in regulating the folding and deposition of

storage protein bodies in the wheat endosperm has been investigated (Johnson et al., 2001; Johnson and Bhave, 2004). However, these proteins have also been implicated in pathogen responses. A wheat protein disulfide isomerase was induced within 3 h after inoculation with the fungal pathogen *Mycosphaerella graminicola* (Ray et al., 2003). In the resistant wheat line Tadinia, this response peaked at 12 h post-inoculation, comparable to the response of TDF AmoLve-45.323 to *D. noxia* feeding in the susceptible Tugela and tolerant Tugela-*Dn2* lines. It is thought that the antioxidant properties of PDI could help limit potential cell damage by reactive oxygen species generated during plant-pathogen interactions (Ray et al., 2003). The increase in AmoLve-45.323 transcripts upon *D. noxia* infestation might constitute part of such a post-hypersensitive clearance mechanism following the oxidative state initiated by aphid feeding. This would particularly explain the elevated quantity of this TDF observed in Tugela-*Dn1*, the antibiotic line exhibiting a strong oxidative burst.

However, the situation is complicated by the fact that, besides their chaperone function, the PDI protein family associates with transcriptional complexes regulating genes responding to various stimuli. In this role, the protein disulfide isomerase RB60 from the green algae *Chlamydomonas reinhardtii* has been implicated in the maintenance of photosynthetic capacity by enabling the replacement of photo-damaged D1 protein, an integral part of the photosynthetic reaction centre of photosystem II (PSII). In a mechanism providing a direct association between the quantity of light absorbed and the rate of synthesis of the D1 protein, reducing equivalents generated by photosynthesis and donated by ferredoxin and ferredoxin-thioredoxin reductase to RB60 allows it to reversibly catalyze the reduction of chloroplast polyadenylate-binding protein (cPABP).

The reduced form of cPABP is then able to bind the 5' UTR of *psbA* mRNA, resulting in increased synthesis of the D1 protein (Kim and Mayfield, 1997). Since the aphid-derived compounds responsible for chlorosis are thought to act by causing photooxidative damage to the photosynthetic apparatus (Heng-Moss et al., 2003), and damage to PSI is thought to lead to over-reduction of PSII and irreversible photoinactivation (Botha et al., 2006), induction of PDI provides a persuasive mechanism for the increased turnover of components of PSII. Although it is clear that AmoLve-45.323 is involved in the wheat resistance response to *D. noxia*, it is uncertain whether it acts as a regulator of the oxidative burst, assists in maintaining photosynthetic capacity, or possibly both. AmoLve-45.323 is a candidate for further characterization: it would be interesting to identify the target of this PDI and to elucidate its specific role in wheat defence against *D. noxia*.

qRT-PCR of AmoLve-88.303, with high homology to fructose-1,6-*bis*phosphatase from *Pisum sativum* (Table 2.3), indicated a severe decrease in transcript abundance in all lines from 6 h post-infestation onwards, except in Tugela-*Dn2*, where at 6 h an increase in excess of 4-fold above the expression in Tugela at 0 h is observed (Figure 2.9). Fructose-1,6-*bis*phosphatase (FBPase) catalyzes the formation of fructose-6-phosphate and is considered the enzyme where the Calvin cycle and starch biosynthetic pathways intersect. The activity of this enzyme is regulated by light-dependent changes in redox potential through the ferredoxin-thioredoxin system. The enzyme is only weakly active in the oxidized state, but becomes highly activated when reduced by thioredoxin (Ruelland and Miginiac-Maslow, 1999).

The integrity of PSI and especially the PSI-D protein is essential for the docking and subsequent reduction of ferredoxin (Haldrup et al., 2003). The decline in chlorophyll *a* observed in wheat suffering from *D. noxia* infestation (Burd and Elliott, 1996; Ni et al., 2001; Wang et al., 2004) is indicative of damage to the reaction centre of PSI (Botha et al., 2006). Therefore, when the function of the PSI-D protein is compromised, the light-dependent regulation of FBPase via the ferredoxin-thioredoxin system is impaired (Ruelland and Miginiac-Maslow, 1999; Haldrup et al., 2003). A decrease in FBPase transcript levels as seen in the susceptible Tugela and resistant Tugela-*Dn1* and Tugela-*Dn5* plants after *D. noxia* feeding leads to a reduction in photosynthetic capacity, since transgenic potato plants with reduced levels of chloroplastic FBPase had increased levels of fructose-1,6-*bis*phosphate and its precursors, retarded growth rates due to reduction in photosynthetic activity and displayed reduced total leaf carbon content (Kossmann et al., 1994). The converse is also true: transgenic tobacco plants expressing a cyanobacterial chloroplastic fructose-1,6/sedoheptulose-1,7-*bis*phosphatase exhibited enhanced efficiency of CO_2 fixation and accumulation of carbohydrates, resulting in rapid growth (Miyagawa et al., 2001). The increased levels of FBPase were thought to reduce levels of ribulose-1,5-*bis*phosphate, thereby inducing ribulose-1,5-*bis*phosphate carboxylase/ oxygenase (Rubisco) and increasing the rate of photosynthesis (Miyagawa et al., 2001). It is likely that the rate of photosynthesis in Tugela-*Dn2* is also accelerated in this way, since Rubisco (AmoLve-86.322) formed part of the collection of differentially expressed TDFs in cluster H (Figure 2.3). Tugela-*Dn2* is a tolerant line, capable of maintaining the integrity of the photosynthetic machinery during high aphid pressure. Upregulation of

enzymes directly involved in photoassimilation suggests a method for coping with the detrimental effects of aphid-derived agents.

TDF AmoLve-22.225 shares similarity to inorganic pyrophosphatase from *O. sativa* (Table 2.3). qRT-PCR data indicated a decrease in expression as aphid infestation progresses. The transcript was downregulated between 6 h and 12 h post-infestation in the resistant near-isogenic lines to levels 0.4-fold that of the calibrator, with the susceptible Tugela line already demonstrating a decrease between 2 h and 6 h post-infestation to about 0.6-fold compared to the calibrator (Figure 2.6). Blotting analysis also indicated a drop in expression, with a decrease below detectable levels from 6 h post-infestation onwards (Figure 2.6).

Pyrophosphatase catalyzes the formation of inorganic phosphate from inorganic pyrophosphate. The function of the chloroplast CF_1 ATP synthase is dependent on the availability of inorganic phosphate for the phosphorylation of ADP and chloroplasts display a very high pyrophosphatase activity. Transgenic tobacco expressing a bacterial cytosolic inorganic pyrophosphatase showed a change in photoassimilate partitioning. With the increase in soluble sugars, a reduction of up to 85% in chlorophyll was also observed (Sonnewald, 1992). This experiment had effects comparable to those achieved from the silencing of FBPase in transgenic tobacco conducted by Kossmann et al. (1994). The decrease in transcript abundance of AmoLve-22.225 upon *D. noxia* feeding might constitute part of the stress response, as additional metabolic demands are made on the increasingly strained physiology of the plant. The resistant near-isogenic lines might be better enabled to sustain the required metabolic capacity as compared to the susceptible Tugela line, which succumbs to the pressure of aphid feeding. It would be interesting to

determine the cellular localization of AmoLve-22.225, whether cytosolic or within the chloroplast, and to investigate its effects on carbohydrate metabolism and chlorophyll content during *D. noxia* feeding.

The TDF AmoLve-11.445 has a normalized cDNA-AFLP band intensity plot indicating a massive induction of this transcript in the tolerant Tugela-*Dn2* line triggered 6 h post-infestation (Figure 2.9). To a lesser extent, this is also observed in Tugela-*Dn5*. AmoLve-11.445 has significant homology with the thylakoid membrane phosphoprotein TMP14 of *Arabidopsis thaliana* (Table 2.3). The plant photosystem I (PSI) is a pigment-protein complex located in the thylakoid membrane. It acts as an oxido-reductase which accepts electrons on the luminal side delivered from PSII through a series of electron carriers and transfers them to the outer side of the membrane to reduce the $NADP^+$ required for CO_2 assimilation. PSI is known to be composed of 12 core subunits: PSI-A to –L, PSI-N and PSI-O and 4 light-harvesting complex I (LHCI) antenna proteins. TMP14 has recently been shown to be a subunit of PSI, designated PSI-P (Khrouchtchova et al., 2005). Together with PSI-D, PSI-P (TMP14) is one of the first phosphorylated PSI subunits to be described, with 25% of total TMP14 in *Arabidopsis* being phosphorylated. Phosphoproteins are plentiful in the thylakoid, the LHCII and the PSII core proteins D1, D2 and CP43 being the most abundant. Phosphorylation of the LHCII proteins plays an important role in balancing the excitation energy between the two photosystems during state transitions. In state 1, the plastoquinone pool is oxidized and LHCII is not associated with PSI. In state 2, light leads to reduction of the plastoquinone pool, LHCII becomes phosphorylated and a fraction moves to PSI to complete the electron transport chain. The phosphorylation status of TMP14 does not

seem to be influenced by state transitions and probably represents constitutive post-translational modification affecting the electrostatic interactions among PSI subunits (Khrouchtchova et al., 2005).

Chlorosis due to *D. noxia* infestation is thought to originate from interference with electron transport (Burd and Elliott, 1996; Haile et al., 1999; Heng-Moss et al., 2003; Botha et al., 2006). Susceptible wheat shows decreased levels of chlorophyll *a* upon infestation by *D. noxia* (Burd and Elliott, 1996; Ni et al., 2001; Wang et al., 2004b) which indicates damage to PSI (Botha et al., 2006). If this is indeed the case, it has serious implications for susceptible wheat under aphid attack. PSI catalyzes the electron transport from plastocyanin to ferredoxin (Haldrup et al., 2003). This reduced ferredoxin pool is mostly employed in generating NADPH for CO_2 assimilation, but is also used in regulating the activity of, among others, CF_1-ATP synthase and several enzymes in the Calvin cycle (Ruelland and Miginiac-Maslow, 1999). Under-reduced ferredoxin directly diminishes the plant's ability to synthesize ATP and carbohydrates. qRT-PCR analysis of the CF_1-ATP synthase response to aphid feeding indicated an increased demand for ATP synthase transcripts as infestation progresses (Figure 2.6). Since damaged PSI can no longer act as electron acceptor from PSII via the cytochrome b_{6-f} complex, inefficient reoxidation of the reduced plastoquinone occurs, halting state transitions and resulting in over-reduction of PSII (Burd and Elliott, 1996). This leads to photoinactivation of PSII, the irreversible decline in functional PSII complexes, because the absorbed light energy exceeds the amount that can be employed in electron transport (Kornyeyev et al., 2006).

In experiments using the near-isogenic lines Betta-*Dn1* and Betta-*Dn2*, the antibiotic Betta-*Dn1* suffered chlorophyll losses when infested with *D. noxia*, whereas

the tolerant Betta-*Dn2* maintained chlorophyll levels under infestation (Heng-Moss et al., 2003). In similar studies the resistant Tugela near-isogenic lines managed to sustain levels of chlorophyll *a* and *b* (and therefore functional photosystems) during *D. noxia* feeding, whereas susceptible Tugela suffered a severe decline in chlorophyll (Wang et al., 2004; Botha et al., 2006). Acute induction of AmoLve-11.445, a putative component of PSI, is observed in Tugela-*Dn2* and Tugela-*Dn5*, indicating transcriptionally regulated photosynthetic compensation. Upregulation of PSI complexes would ensure the integrity of electron transport from PSII during state 2 as well as increased levels of NADPH and possibly increased CO_2 assimilation. In growth tolerance experiments, the tolerant PI 262660 line containing the *Dn2* gene maintained vigorous growth during aphid infestation when compared to the susceptible Arapahoe and antibiotic PI 137739 line (Haile et al., 1999). Increased photosynthetic capacity via upregulation of photosystem components provides a tantalizing mechanism for passive resistance against *D. noxia* feeding. Future research focusing on chloroplast gene expression analysis with particular emphasis on the thylakoid membrane proteins involved in electron transport will provide helpful information regarding the mechanics of aphid-induced chlorosis and plant tolerance.

2.5.8 Antibiosis, antixenosis and tolerance

We can conclude from this study that the wheat response to feeding by *D. noxia* is not an isolated event, affecting the expression of a few genes, but a concerted cellular reprogramming event. It involves many genes and many processes, encompassing a

repertoire of defence-related genes, housekeeping and regulatory genes and components of the photosynthetic apparatus. Further analysis of the many uncharacterized genes with unknown functions discovered in this study will facilitate our future understanding of the complex interaction between wheat and aphid.

In susceptible wheat, such as Tugela, symptoms caused by *D. noxia* feeding are biphasic, with an immediate early response resulting in leaf rolling and a delayed response resulting in chlorosis, energy depletion, and death. It is clear that several distinct mechanisms need to be in place to generate each mode of resistance.

A cultivar is deemed antibiotic when aphids feeding on it show decreased fecundity (Smith et al., 1992; Unger and Quisenberry, 1997). The plant rapidly deploys an active resistance mechanism geared towards obstructing aphid feeding and even injuring the aphid and impeding oviposition. The antibiotic near-isogenic line Tugela-*Dn1* very rapidly recognizes aphid stylet penetration via a *Dn1*-mediated recognition event, leading to a substantial influx of Ca^{2+} into the cytosol, assisted by the vacuolar SV channel. Kinase signalling cascades are induced within 2 h after feeding commences, leading to increased levels of SA and the oxidative burst. The significance of ubiquitination in the activation of HR is also now becoming clear. Levels of ROS are finely regulated by several systems involving iron homeostasis, such nitric oxide, RNA-binding genes such as aconitase 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, which leads to localized necrotic lesions and prevents aphid feeding altogether. *PR* gene expression is induced as HR gives way to the long-term protection of SAR, with enforced

cell walls proving more resistant to subsequent attack and the expression of a pool of arthropod-damaging pathogenesis-related enzymes (Botha et al., 1998; Van der Westhuizen et al., 1998).

Tolerant cultivars cope with high aphid pressure without incurring reductions in growth (Burd and Burton, 1992). Tolerant plants have passive resistance mechanisms in place, not focusing on eradicating the aphid, but rather on dealing with the drain placed on energy and nutrients and the damaging effects of aphid-derived molecules on chlorophyll levels. By about 6 h after aphid infestation, the tolerant near-isogenic line Tugela-*Dn2* induces photosynthetic compensation in a *Dn2*-mediated way. 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 (such as TMP14 of PSI) and enabling the rapid replacement of damaged components of PSII (by the upregulation of protein disulfide isomerases), as well as increasing levels of enzymes involved in photoassimilation (such as fructose-1,6-*bis*phosphatase and ATP synthase), Tugela-*Dn2* manages to retain active photosynthesis and prevents chlorosis from occurring. This comes at the cost of orthodox plant defence responses and Tugela-*Dn2* does not exhibit the frenetic signalling capacity and oxidative burst associated with a classic hypersensitive response.

Although the dubious resistance background of any line bred to contain the antixenotic *Dn5* gene (Heyns et al., 2006) impairs accurate assessment of its effects during aphid feeding, differential expression of genes in the Tugela-*Dn5* near-isogenic line seem to link antixenosis 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 (Chamberlain et al., 2000; Kessler and Baldwin, 2002). Since Tugela-*Dn5* also demonstrates moderate antibiosis, this line probably counters aphid attack in a similar way to Tugela-*Dn1*, albeit with slight idiosyncrasies and significantly more crosstalk between SA-mediated and ethylene and JA-mediated pathways.

These three resistant near-isogenic lines bred from the susceptible Tugela wheat line (Tolmay et al., 2006) constitute a superb opportunity to study the effects of single resistance genes on an identical genetic background. From the evidence presented here it is clear that initial aphid recognition in a *Dn* gene-specific manner coupled with the time and intensity of subsequent gene activation is critical in the eventual development of a resistant phenotype, whether an active antibiosis, or a passive photosynthetic compensatory tolerance.

2.6 ACKNOWLEDGEMENTS

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CHAPTER 3

Summary

3. SUMMARY

Aphid-induced transcriptional regulation in near-isogenic wheat

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This study represents the first comprehensive analysis of gene regulation underlying the distinct categories of resistance afforded to wheat (*Triticum aestivum*, L.) by different *Dn* genes. Russian wheat aphid (*Diuraphis noxia*, Mordv.) feeding on susceptible wheat cultivars causes leaf rolling, chlorosis and the eventual death of the plant. Plants expressing *Dn* genes are resistant to *D. noxia* infestation, but different *Dn* genes afford phenotypically distinct modes of resistance: the *Dn1* gene confers an antibiotic effect to lower aphid fecundity; *Dn2* confers tolerance to high aphid pressure; and *Dn5* confers antixenosis, and aphids do not prefer such plants as hosts.

Little is known about the components involved in establishing a successful defence response against *D. noxia* attack and how these differ between the distinct resistance categories. It is assumed that the *Dn* genes function as classic *R* genes in plant defence, being receptors for elicitors in aphid saliva. Upon recognition, defence response signalling is initiated, but the exact mechanics of subsequent cellular events in aphid resistance have only recently come under investigation. Evidence from cDNA microarray and subtractive

hybridization experiments indicated the involvement of kinase signalling cascades and photosynthetic proteins in the response against *D. noxia.* However, expression analysis describing how these processes differ between plants carrying different *Dn* genes and how these differences account for antibiosis, antixenosis or tolerance had not been conducted.

We consequently investigated the downstream components involved in or affected by the generation of these resistance mechanisms by comparing the responses in transcript regulation of Tugela near-isogenic lines with different *Dn* genes to *D. noxia* infestation. cDNA-AFLP analysis was selected as an appropriate functional genomics tool, since it is semi-quantitative, does not require prior sequence information and allows for the discovery of novel genes. cDNA-AFLP analysis yielded 121 differentially regulated transcript-derived fragments (TDFs) grouped into eight expression clusters. We cloned and sequenced 49 representative TDFs, which were further classified into five broad functional categories based on inferred similarity to database sequences. Transcripts involved in such diverse processes as stress, signal transduction, photosynthesis, metabolism and gene regulation were found to be differentially regulated during *D. noxia* feeding. Many TDFs demonstrated homology to proteins with unknown function and several novel transcripts with no similarity to previously published sequences were also discovered.

Detailed expression analysis using quantitative RT-PCR and RNA hybridization provided evidence that the time and intensity of induction of specific pathways is critical for the development of a particular mode of resistance. This includes: the generation of kinase signalling cascades and the induction of several ancillary processes such as ubiquitination, leading to a sustained oxidative burst and the hypersensitive response during antibiosis; tolerance as a passive resistance mechanism countering aphid-induced symptoms through the repair or *de novo* synthesis of photosystem proteins; and the possible involvement of

ethylene-mediated wounding pathways in generating volatile organic compounds during antixenosis.

This is the first report on the involvement of KCO1, a vacuolar K^+ channel, in assisting cytosolic Ca^{2+}-influx and preventing leaf rolling, as well as on the role of iron homeostasis as a gene regulatory mechanism for sustaining the oxidative burst during the antibiotic defence response. This study opens up several areas of investigation heretofore unexplored in cereal-aphid interaction research. Of particular interest is the induction of genes involved in photosynthetic compensation during *Dn2* tolerance responses, since these constitute a novel, passive resistance mechanism exclusive to aphid defence as opposed to the active resistance triggered in the presence of the *Dn1* gene in the form of a general hypersensitive response.

APPENDIX

dbEST entries

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LOCUS       EL563871                 345 bp    mRNA    linear   EST 12-FEB-2007
DEFINITION  CG2007_001 cDNA-AFLP of wheat response to Diuraphis noxia Triticum
            aestivum cDNA clone AmoLve-11.340 5', mRNA sequence.
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VERSION     EL563871.1  GI:125654740
KEYWORDS    EST.
SOURCE      Triticum aestivum (bread wheat)
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            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 345)
  AUTHORS   Van Eck,L., Lapitan,N.L.V. and Botha-Oberholster,A.M.
  TITLE     Aphid-induced transcriptional regulation in near-isogenic wheat
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
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            Tel: 27 12 420 3945
            Fax: 27 12 420 3947
            Email: ambothao@postino.up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
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            BACKWARD: SP6
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                     noxia"
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                     cDNA-AFLP of wheat leaf mRNA after induction of resistance
                     response to the Russian wheat aphid, Diuraphis noxia."
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      181 tacataggtg tccaaaaatt cgtctggctt ttgccttgta ctgctgctcc tgcattctgt
      241 ggatgctttg tattataaag ggagcggcat gacaaacagt ttattatgtc aacttgaagc
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ACCESSION   EL563872
VERSION     EL563872.1  GI:125654741
KEYWORDS    EST.
SOURCE      Triticum aestivum (bread wheat)
  ORGANISM  Triticum aestivum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 281)
  AUTHORS   Van Eck,L., Lapitan,N.L.V. and Botha-Oberholster,A.M.
  TITLE     Aphid-induced transcriptional regulation in near-isogenic wheat
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
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            Tel: 27 12 420 3945
            Fax: 27 12 420 3947
            Email: ambothao@postino.up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
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                     /clone_lib="cDNA-AFLP of wheat response to Diuraphis
                     noxia"
                     /note="Organ: leaf; Vector: pGem-T Easy; Site_1: EcoRI;
                     Site_2: EcoRI; Transcript-derived fragments generated via
                     cDNA-AFLP of wheat leaf mRNA after induction of resistance
                     response to the Russian wheat aphid, Diuraphis noxia."
ORIGIN
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      121 tgttcatagc acaagagaga agtctcgttt acatgtagat gtcaagtttg ctctatacgt
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            aestivum cDNA clone AmoLve-22.275 5', mRNA sequence.
ACCESSION   EL563873
VERSION     EL563873.1  GI:125654742
KEYWORDS    EST.
SOURCE      Triticum aestivum (bread wheat)
  ORGANISM  Triticum aestivum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 322)
  AUTHORS   Van Eck,L., Lapitan,N.L.V. and Botha-Oberholster,A.M.
  TITLE     Aphid-induced transcriptional regulation in near-isogenic wheat
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
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            Institute
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            Tel: 27 12 420 3945
            Fax: 27 12 420 3947
            Email: ambothao@postino.up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
            PCR PRimers
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                     response to the Russian wheat aphid, Diuraphis noxia."
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ACCESSION   EL563874
VERSION     EL563874.1  GI:125654743
KEYWORDS    EST.
SOURCE      Triticum aestivum (bread wheat)
  ORGANISM  Triticum aestivum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 215)
  AUTHORS   Van Eck,L., Lapitan,N.L.V. and Botha-Oberholster,A.M.
  TITLE     Aphid-induced transcriptional regulation in near-isogenic wheat
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
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            Tel: 27 12 420 3945
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            Email: ambothao@postino.up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
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                    response to the Russian wheat aphid, Diuraphis noxia."
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VERSION     EL563875.1  GI:125654744
KEYWORDS    EST.
SOURCE      Triticum aestivum (bread wheat)
  ORGANISM  Triticum aestivum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 257)
  AUTHORS   Van Eck,L., Lapitan,N.L.V. and Botha-Oberholster,A.M.
  TITLE     Aphid-induced transcriptional regulation in near-isogenic wheat
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
            Department of Genetics and Forestry and Agricultural Biotechnology
            Institute
            Faculty of Agriculture and Natural Science, University of Pretoria
            74 Lunnon Str. Hillcrest, Pretoria, Gauteng, ZA0002, South Africa
            Tel: 27 12 420 3945
            Fax: 27 12 420 3947
            Email: ambothao@postino.up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
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                     noxia"
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                     Site_2: EcoRI; Transcript-derived fragments generated via
                     cDNA-AFLP of wheat leaf mRNA after induction of resistance
                     response to the Russian wheat aphid, Diuraphis noxia."
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ACCESSION   EL563876
VERSION     EL563876.1  GI:125654745
KEYWORDS    EST.
SOURCE      Triticum aestivum (bread wheat)
  ORGANISM  Triticum aestivum
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            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 336)
  AUTHORS   Van Eck,L., Lapitan,N.L.V. and Botha-Oberholster,A.M.
  TITLE     Aphid-induced transcriptional regulation in near-isogenic wheat
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
            Department of Genetics and Forestry and Agricultural Biotechnology
            Institute
            Faculty of Agriculture and Natural Science, University of Pretoria
            74 Lunnon Str. Hillcrest, Pretoria, Gauteng, ZA0002, South Africa
            Tel: 27 12 420 3945
            Fax: 27 12 420 3947
            Email: ambothao@postino.up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
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                     response to the Russian wheat aphid, Diuraphis noxia."
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VERSION     EL563877.1  GI:125654746
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  ORGANISM  Triticum aestivum
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            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 194)
  AUTHORS   Van Eck,L., Lapitan,N.L.V. and Botha-Oberholster,A.M.
  TITLE     Aphid-induced transcriptional regulation in near-isogenic wheat
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
            Department of Genetics and Forestry and Agricultural Biotechnology
            Institute
            Faculty of Agriculture and Natural Science, University of Pretoria
            74 Lunnon Str. Hillcrest, Pretoria, Gauteng, ZA0002, South Africa
            Tel: 27 12 420 3945
            Fax: 27 12 420 3947
            Email: ambothao@postino.up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
            PCR PRimers
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                     response to the Russian wheat aphid, Diuraphis noxia."
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135

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VERSION     EL563878.1  GI:125654747
KEYWORDS    EST.
SOURCE      Triticum aestivum (bread wheat)
  ORGANISM  Triticum aestivum
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            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 324)
  AUTHORS   Van Eck,L., Lapitan,N.L.V. and Botha-Oberholster,A.M.
  TITLE     Aphid-induced transcriptional regulation in near-isogenic wheat
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
            Department of Genetics and Forestry and Agricultural Biotechnology
            Institute
            Faculty of Agriculture and Natural Science, University of Pretoria
            74 Lunnon Str. Hillcrest, Pretoria, Gauteng, ZA0002, South Africa
            Tel: 27 12 420 3945
            Fax: 27 12 420 3947
            Email: ambothao@postino.up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
            PCR PRimers
            FORWARD: T7
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                     /lab_host="Eschericia coli (DH5z)"
                     /clone_lib="cDNA-AFLP of wheat response to Diuraphis
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                     /note="Organ: leaf; Vector: pGem-T Easy; Site_1: EcoRI;
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                     response to the Russian wheat aphid, Diuraphis noxia."
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ACCESSION   EL563879
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KEYWORDS    EST.
SOURCE      Triticum aestivum (bread wheat)
  ORGANISM  Triticum aestivum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 180)
  AUTHORS   Van Eck,L., Lapitan,N.L.V. and Botha-Oberholster,A.M.
  TITLE     Aphid-induced transcriptional regulation in near-isogenic wheat
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
            Department of Genetics and Forestry and Agricultural Biotechnology
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            Tel: 27 12 420 3945
            Fax: 27 12 420 3947
            Email: ambothao@postino.up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
            PCR PRimers
            FORWARD: T7
            BACKWARD: SP6
            Insert Length: 180   Std Error: 0.00
            Seq primer: T7
            POLYA=No.
FEATURES             Location/Qualifiers
     source          1..180
                     /organism="Triticum aestivum"
                     /mol_type="mRNA"
                     /cultivar="Tugela"
                     /db_xref="taxon:4565"
                     /clone="AmoLve-43.139"
                     /dev_stage="2-3 leaf stage"
                     /lab_host="Eschericia coli (DH5z)"
                     /clone_lib="cDNA-AFLP of wheat response to Diuraphis
                     noxia"
                     /note="Organ: leaf; Vector: pGem-T Easy; Site_1: EcoRI;
                     Site_2: EcoRI; Transcript-derived fragments generated via
                     cDNA-AFLP of wheat leaf mRNA after induction of resistance
                     response to the Russian wheat aphid, Diuraphis noxia."
ORIGIN
        1 tcccggccgc catggcggcc gcgggcattc gattgatgga gtcctgagta actcaactca
       61 acttgaaatt actcgggcgg gacgggcgaa ttcacagttt tacagaagca aattgacagt
      121 gtcgtccacg gttttacagc tccacgaact caccacaaac tgatcggtac gcagtctaca
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LOCUS       EL563880                235 bp    mRNA    linear   EST 12-FEB-2007
DEFINITION  CG2007_010 cDNA-AFLP of wheat response to Diuraphis noxia Triticum
            aestivum cDNA clone AmoLve-45.229 5', mRNA sequence.
ACCESSION   EL563880
VERSION     EL563880.1  GI:125654749
KEYWORDS    EST.
SOURCE      Triticum aestivum (bread wheat)
  ORGANISM  Triticum aestivum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 235)
  AUTHORS   Van Eck,L., Lapitan,N.L.V. and Botha-Oberholster,A.M.
  TITLE     Aphid-induced transcriptional regulation in near-isogenic wheat
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
            Department of Genetics and Forestry and Agricultural Biotechnology
            Institute
            Faculty of Agriculture and Natural Science, University of Pretoria
            74 Lunnon Str. Hillcrest, Pretoria, Gauteng, ZA0002, South Africa
            Tel: 27 12 420 3945
            Fax: 27 12 420 3947
            Email: ambothao@postino.up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
            PCR PRimers
            FORWARD: T7
            BACKWARD: SP6
            Insert Length: 235   Std Error: 0.00
            Seq primer: T7
            POLYA=No.
FEATURES             Location/Qualifiers
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                     /organism="Triticum aestivum"
                     /mol_type="mRNA"
                     /cultivar="Tugela"
                     /db_xref="taxon:4565"
                     /clone="AmoLve-45.229"
                     /dev_stage="2-3 leaf stage"
                     /lab_host="Eschericia coli (DH5z)"
                     /clone_lib="cDNA-AFLP of wheat response to Diuraphis
                     noxia"
                     /note="Organ: leaf; Vector: pGem-T Easy; Site_1: EcoRI;
                     Site_2: EcoRI; Transcript-derived fragments generated via
                     cDNA-AFLP of wheat leaf mRNA after induction of resistance
                     response to the Russian wheat aphid, Diuraphis noxia."
ORIGIN
        1 gatgagtcct gagtaactaa actcaatgtt gattggacaa aaaacaaact aggagggtaa
       61 acaaggattc ttcccccttg aaaccatctt aggttcacaa gtatacttgg acagtttcct
      121 ctttgcaact actaaaacag acagacagag cggcagattc acagacagac gcaaataaac
      181 tcagaagact acattccttt ccaactcatg ataacaagtc ggtacgcagt ctaca
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138

```
LOCUS       EL563881                170 bp    mRNA    linear   EST 12-FEB-2007
DEFINITION  CG2007_011 cDNA-AFLP of wheat response to Diuraphis noxia Triticum
            aestivum cDNA clone AmoLve-66.169 5', mRNA sequence.
ACCESSION   EL563881
VERSION     EL563881.1  GI:125654750
KEYWORDS    EST.
SOURCE      Triticum aestivum (bread wheat)
  ORGANISM  Triticum aestivum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 170)
  AUTHORS   Van Eck,L., Lapitan,N.L.V. and Botha-Oberholster,A.M.
  TITLE     Aphid-induced transcriptional regulation in near-isogenic wheat
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
            Department of Genetics and Forestry and Agricultural Biotechnology
            Institute
            Faculty of Agriculture and Natural Science, University of Pretoria
            74 Lunnon Str. Hillcrest, Pretoria, Gauteng, ZA0002, South Africa
            Tel: 27 12 420 3945
            Fax: 27 12 420 3947
            Email: ambothao@postino.up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
            PCR PRimers
            FORWARD: T7
            BACKWARD: SP6
            Insert Length: 170   Std Error: 0.00
            Seq primer: T7
            POLYA=No.
FEATURES             Location/Qualifiers
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                     /organism="Triticum aestivum"
                     /mol_type="mRNA"
                     /cultivar="Tugela"
                     /db_xref="taxon:4565"
                     /clone="AmoLve-66.169"
                     /dev_stage="2-3 leaf stage"
                     /lab_host="Eschericia coli (DH5z)"
                     /clone_lib="cDNA-AFLP of wheat response to Diuraphis
                     noxia"
                     /note="Organ: leaf; Vector: pGem-T Easy; Site_1: EcoRI;
                     Site_2: EcoRI; Transcript-derived fragments generated via
                     cDNA-AFLP of wheat leaf mRNA after induction of resistance
                     response to the Russian wheat aphid, Diuraphis noxia."
ORIGIN
        1 gtagactgcg taccgacatc atggttcatc cgatgagatc atcgtggaac atgtgggagc
       61 caacatgggt atccagatcc cgctgttggt tattgaccga agagtcgtct cggtcatgtc
      121 tgcttgtctc ccgaacccgt agggtctaca cacttactca ggactcatca
//
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```
LOCUS       EL563882                217 bp    mRNA    linear   EST 12-FEB-2007
DEFINITION  CG2007_012 cDNA-AFLP of wheat response to Diuraphis noxia Triticum
            aestivum cDNA clone AmoLve-67.181 5', mRNA sequence.
ACCESSION   EL563882
VERSION     EL563882.1  GI:125654751
KEYWORDS    EST.
SOURCE      Triticum aestivum (bread wheat)
  ORGANISM  Triticum aestivum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 217)
  AUTHORS   Van Eck,L., Lapitan,N.L.V. and Botha-Oberholster,A.M.
  TITLE     Aphid-induced transcriptional regulation in near-isogenic wheat
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
            Department of Genetics and Forestry and Agricultural Biotechnology
            Institute
            Faculty of Agriculture and Natural Science, University of Pretoria
            74 Lunnon Str. Hillcrest, Pretoria, Gauteng, ZA0002, South Africa
            Tel: 27 12 420 3945
            Fax: 27 12 420 3947
            Email: ambothao@postino.up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
            PCR PRimers
            FORWARD: T7
            BACKWARD: SP6
            Insert Length: 217   Std Error: 0.00
            Seq primer: T7
            POLYA=No.
FEATURES             Location/Qualifiers
     source          1..217
                     /organism="Triticum aestivum"
                     /mol_type="mRNA"
                     /cultivar="Tugela"
                     /db_xref="taxon:4565"
                     /clone="AmoLve-67.181"
                     /dev_stage="2-3 leaf stage"
                     /lab_host="Eschericia coli (DH5z)"
                     /clone_lib="cDNA-AFLP of wheat response to Diuraphis
                     noxia"
                     /note="Organ: leaf; Vector: pGem-T Easy; Site_1: EcoRI;
                     Site_2: EcoRI; Transcript-derived fragments generated via
                     cDNA-AFLP of wheat leaf mRNA after induction of resistance
                     response to the Russian wheat aphid, Diuraphis noxia."
ORIGIN
        1 cccgtgaatt gtaatacgac tcactatagg gaaccggacc cctttgtgat ggggcaactc
       61 gcatcacaaa cattacgact ctcccaaaat gtcagaaatg ccagaaaaaa aagttggggg
      121 tcacttcctc tggactcgtc aatcgacaac tagaattcgc ggccgcctgc aggtcgacca
      181 atatgggaga gctgcccaaa ggagatggat gcagagg
//
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```
LOCUS       EL563883                218 bp    mRNA    linear   EST 12-FEB-2007
DEFINITION  CG2007_013 cDNA-AFLP of wheat response to Diuraphis noxia Triticum
            aestivum cDNA clone AmoLve-67.216 5', mRNA sequence.
ACCESSION   EL563883
VERSION     EL563883.1  GI:125654752
KEYWORDS    EST.
SOURCE      Triticum aestivum (bread wheat)
  ORGANISM  Triticum aestivum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 218)
  AUTHORS   Van Eck,L., Lapitan,N.L.V. and Botha-Oberholster,A.M.
  TITLE     Aphid-induced transcriptional regulation in near-isogenic wheat
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
            Department of Genetics and Forestry and Agricultural Biotechnology
            Institute
            Faculty of Agriculture and Natural Science, University of Pretoria
            74 Lunnon Str. Hillcrest, Pretoria, Gauteng, ZA0002, South Africa
            Tel: 27 12 420 3945
            Fax: 27 12 420 3947
            Email: ambothao@postino.up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
            PCR PRimers
            FORWARD: T7
            BACKWARD: SP6
            Insert Length: 218   Std Error: 0.00
            Seq primer: T7
            POLYA=No.
FEATURES             Location/Qualifiers
     source          1..218
                     /organism="Triticum aestivum"
                     /mol_type="mRNA"
                     /cultivar="Tugela"
                     /db_xref="taxon:4565"
                     /clone="AmoLve-67.216"
                     /dev_stage="2-3 leaf stage"
                     /lab_host="Eschericia coli (DH5z)"
                     /clone_lib="cDNA-AFLP of wheat response to Diuraphis
                     noxia"
                     /note="Organ: leaf; Vector: pGem-T Easy; Site_1: EcoRI;
                     Site_2: EcoRI; Transcript-derived fragments generated via
                     cDNA-AFLP of wheat leaf mRNA after induction of resistance
                     response to the Russian wheat aphid, Diuraphis noxia."
ORIGIN
        1 gatgagtcct gagtaagtct ggcttattct tttattccat ctctagccgc cccgtggctg
       61 ttgctgcagc ggctccaaac attgttcaca aacttacaac aaactgccat atcatctacc
      121 aggatcttgc ttgcaacttt tagattgtgt ggcgattgtc ggggtggtcc agctgtctaa
      181 gcagcagctt ccgcctgctc ttcggtacgc agtctaca
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```
LOCUS       EL563884                292 bp    mRNA    linear   EST 12-FEB-2007
DEFINITION  CG2007_014 cDNA-AFLP of wheat response to Diuraphis noxia Triticum
            aestivum cDNA clone AmoLve-68.268 5', mRNA sequence.
ACCESSION   EL563884
VERSION     EL563884.1  GI:125654753
KEYWORDS    EST.
SOURCE      Triticum aestivum (bread wheat)
  ORGANISM  Triticum aestivum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 292)
  AUTHORS   Van Eck,L., Lapitan,N.L.V. and Botha-Oberholster,A.M.
  TITLE     Aphid-induced transcriptional regulation in near-isogenic wheat
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
            Department of Genetics and Forestry and Agricultural Biotechnology
            Institute
            Faculty of Agriculture and Natural Science, University of Pretoria
            74 Lunnon Str. Hillcrest, Pretoria, Gauteng, ZA0002, South Africa
            Tel: 27 12 420 3945
            Fax: 27 12 420 3947
            Email: ambothao@postino.up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
            PCR PRimers
            FORWARD: T7
            BACKWARD: SP6
            Insert Length: 292   Std Error: 0.00
            Seq primer: T7
            POLYA=No.
FEATURES             Location/Qualifiers
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                     /mol_type="mRNA"
                     /cultivar="Tugela"
                     /db_xref="taxon:4565"
                     /clone="AmoLve-68.268"
                     /dev_stage="2-3 leaf stage"
                     /lab_host="Eschericia coli (DH5z)"
                     /clone_lib="cDNA-AFLP of wheat response to Diuraphis
                     noxia"
                     /note="Organ: leaf; Vector: pGem-T Easy; Site_1: EcoRI;
                     Site_2: EcoRI; Transcript-derived fragments generated via
                     cDNA-AFLP of wheat leaf mRNA after induction of resistance
                     response to the Russian wheat aphid, Diuraphis noxia."
ORIGIN
        1 ggctgcatgc ggccggggaa atcgattgag actgcgtacc gaagttttca ggatcagtca
       61 gaattctgat gataattctc aagaaaatgt ttacttgcac cccgatctag gtttcttcct
      121 cattctgatt tttgttttcg ttttatggta ttttcactat aaagaaaaac caatcctttc
      181 tatgcagaag gattggactc ttttttttggg gggagtactc atctcctttt ttggtgcgga
      241 atcgtcctgc gacgaaggaa tctccagcgg gccacttact caggactcat ca
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```
LOCUS       EL563885               410 bp    mRNA    linear   EST 12-FEB-2007
DEFINITION  CG2007_015 cDNA-AFLP of wheat response to Diuraphis noxia Triticum
            aestivum cDNA clone AmoLve-68.407 5', mRNA sequence.
ACCESSION   EL563885
VERSION     EL563885.1  GI:125654754
KEYWORDS    EST.
SOURCE      Triticum aestivum (bread wheat)
  ORGANISM  Triticum aestivum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 410)
  AUTHORS   Van Eck,L., Lapitan,N.L.V. and Botha-Oberholster,A.M.
  TITLE     Aphid-induced transcriptional regulation in near-isogenic wheat
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
            Department of Genetics and Forestry and Agricultural Biotechnology
            Institute
            Faculty of Agriculture and Natural Science, University of Pretoria
            74 Lunnon Str. Hillcrest, Pretoria, Gauteng, ZA0002, South Africa
            Tel: 27 12 420 3945
            Fax: 27 12 420 3947
            Email: ambothao@postino.up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
            PCR PRimers
            FORWARD: T7
            BACKWARD: SP6
            Insert Length: 410   Std Error: 0.00
            Seq primer: T7
            POLYA=No.
FEATURES             Location/Qualifiers
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                     /organism="Triticum aestivum"
                     /mol_type="mRNA"
                     /cultivar="Tugela"
                     /db_xref="taxon:4565"
                     /clone="AmoLve-68.407"
                     /dev_stage="2-3 leaf stage"
                     /lab_host="Eschericia coli (DH5z)"
                     /clone_lib="cDNA-AFLP of wheat response to Diuraphis
                     noxia"
                     /note="Organ: leaf; Vector: pGem-T Easy; Site_1: EcoRI;
                     Site_2: EcoRI; Transcript-derived fragments generated via
                     cDNA-AFLP of wheat leaf mRNA after induction of resistance
                     response to the Russian wheat aphid, Diuraphis noxia."
ORIGIN
        1 ccagtgcatt gtaatacgac tcactatagg gtaacccccc cccctactta cctagggatt
       61 ttccggccga ctgcgggccg aacaaatgtg aaatagctcc caaggcgagg ttatgtcttt
      121 cattgagtat ggactcatga atcctaaatg aattgcgcgg cccccggccg gagcagcttc
      181 cgggagaaat accgaaatcg gtggatgatt cccttgagca ttctatcggg acagcctaaa
      241 ttgcttgagc ctggtgtggg taatgagtga gcccagtgag aaaatgtgat tcgctcacaa
      301 tgtcccgctt ccagcgagg cagaaacgga taaggcggac aggcttgagg agaccgaacg
      361 agggaggggga agtaggattg aatgggggttg gggtgactgt tccgctttcc
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143

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LOCUS       EL563886                114 bp    mRNA    linear   EST 12-FEB-2007
DEFINITION  CG2007_016 cDNA-AFLP of wheat response to Diuraphis noxia Triticum
            aestivum cDNA clone AmoLve-86.113 5', mRNA sequence.
ACCESSION   EL563886
VERSION     EL563886.1  GI:125654755
KEYWORDS    EST.
SOURCE      Triticum aestivum (bread wheat)
  ORGANISM  Triticum aestivum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 114)
  AUTHORS   Van Eck,L., Lapitan,N.L.V. and Botha-Oberholster,A.M.
  TITLE     Aphid-induced transcriptional regulation in near-isogenic wheat
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
            Department of Genetics and Forestry and Agricultural Biotechnology
            Institute
            Faculty of Agriculture and Natural Science, University of Pretoria
            74 Lunnon Str. Hillcrest, Pretoria, Gauteng, ZA0002, South Africa
            Tel: 27 12 420 3945
            Fax: 27 12 420 3947
            Email: ambothao@postino.up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
            PCR PRimers
            FORWARD: T7
            BACKWARD: SP6
            Insert Length: 114   Std Error: 0.00
            Seq primer: T7
            POLYA=No.
FEATURES             Location/Qualifiers
     source          1..114
                     /organism="Triticum aestivum"
                     /mol_type="mRNA"
                     /cultivar="Tugela"
                     /db_xref="taxon:4565"
                     /clone="AmoLve-86.113"
                     /dev_stage="2-3 leaf stage"
                     /lab_host="Eschericia coli (DH5z)"
                     /clone_lib="cDNA-AFLP of wheat response to Diuraphis
                     noxia"
                     /note="Organ: leaf; Vector: pGem-T Easy; Site_1: EcoRI;
                     Site_2: EcoRI; Transcript-derived fragments generated via
                     cDNA-AFLP of wheat leaf mRNA after induction of resistance
                     response to the Russian wheat aphid, Diuraphis noxia."
ORIGIN
        1 tagactgcgt accgacaatg gaacatacca ccaaacaaaa tttactggta tatatatagt
       61 ggtatgagcc tggaattttg cattgcaaca ctacgccatt actcaggact catc
//
```

```
LOCUS       EL563887                 219 bp    mRNA    linear   EST 12-FEB-2007
DEFINITION  CG2007_017 cDNA-AFLP of wheat response to Diuraphis noxia Triticum
            aestivum cDNA clone AmoLve-88.189 5', mRNA sequence.
ACCESSION   EL563887
VERSION     EL563887.1  GI:125654756
KEYWORDS    EST.
SOURCE      Triticum aestivum (bread wheat)
  ORGANISM  Triticum aestivum
            Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
            Spermatophyta; Magnoliophyta; Liliopsida; Poales; Poaceae; BEP
            clade; Pooideae; Triticeae; Triticum.
REFERENCE   1  (bases 1 to 219)
  AUTHORS   Van Eck,L., Lapitan,N.L.V. and Botha-Oberholster,A.M.
  TITLE     Aphid-induced transcriptional regulation in near-isogenic wheat
  JOURNAL   Unpublished (2007)
COMMENT     Contact: Botha-Oberholster AM
            Department of Genetics and Forestry and Agricultural Biotechnology
            Institute
            Faculty of Agriculture and Natural Science, University of Pretoria
            74 Lunnon Str. Hillcrest, Pretoria, Gauteng, ZA0002, South Africa
            Tel: 27 12 420 3945
            Fax: 27 12 420 3947
            Email: ambothao@postino.up.ac.za
            Transcript-derived fragments generated via cDNA-AFLP of wheat leaf
            mRNA after induction of resistance response to the Russian wheat
            aphid, Diuraphis noxia.
            PCR PRimers
            FORWARD: T7
            BACKWARD: SP6
            Insert Length: 219   Std Error: 0.00
            Seq primer: T7
            POLYA=No.
FEATURES             Location/Qualifiers
     source          1..219
                     /organism="Triticum aestivum"
                     /mol_type="mRNA"
                     /cultivar="Tugela"
                     /db_xref="taxon:4565"
                     /clone="AmoLve-88.189"
                     /dev_stage="2-3 leaf stage"
                     /lab_host="Eschericia coli (DH5z)"
                     /clone_lib="cDNA-AFLP of wheat response to Diuraphis
                     noxia"
                     /note="Organ: leaf; Vector: pGem-T Easy; Site_1: EcoRI;
                     Site_2: EcoRI; Transcript-derived fragments generated via
                     cDNA-AFLP of wheat leaf mRNA after induction of resistance
                     response to the Russian wheat aphid, Diuraphis noxia."
ORIGIN
        1 accggccgtc atgcggccgc gggtaatcga tgatgagtcc tgataatgcc cttgtcccct
       61 accatcaggt taggtaggaa acactataca agcaactgta ccaaccacct cgctttttac
      121 atttgtaccc ttgtttacgt tgccatctgt cggtgacccc tttagcgtat aaaaggaggc
      181 ccatgcgcaa cgtagaaggg gttcggtacg cagtctaca
//
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