CHAPTER 1

INTRODUCTION

Diuraphis noxia Kurdjumov (Russian wheat aphid, RWA) is considered a major agricultural pest to the wheat and barley industry, with losses in the USA exceeding US$1 billion attributed to this pest. The introduction of various resistant wheat cultivars in the latter part of the 1980s significantly reduced the impact this pest had on the industry. However, the recent development of new RWA biotypes in the USA and South Africa nullify the resistance of many existing wheat cultivars. Again the RWA became a serious threat to wheat production in these regions. The new RWA biotypes, however, present an opportunity to investigate biotype development and the mechanisms aphids utilize to overcome cultivar resistance. Wheat transcriptome studies, where different cultivars are infested by one or more RWA biotypes, could explain the mechanisms of the different modes of plant resistance against the RWA. Furthermore, these studies could also highlight which resistant pressures influences aphid biotype development.

Literature is reviewed in Chapter 2. Here the literature on the members and the bacterium-aphid and aphid-plant interactions is reviewed. The chapter begins with an introduction to the aphid, *D. noxia*, which includes details like its origin, taxonomy, morphology, biology, preferred hosts, economic impact, symptoms and control. This is followed by a brief introduction on the symbiotic relationship between bacterial endosymbionts and their role in aphid success. The plant host, *Triticum aestivum*, is reviewed before the biotic interaction, *i.e.* aphid-plant interaction, is introduced. The chapter concludes with the aphid-plant interaction. Here the physical interaction between the aphid and plant, including plant defence evading mechanisms with special focus on the aphid salivary enzymes, are discussed.

Studies on plant-pest interactions usually deal with the specific pest or with the host plant (resistance). In the last couple of years, molecular techniques have increasingly driven the research in these fields and more studies now deal with both organisms. However, few plant-aphid investigations include the insects' endosymbiont. Endo- or symbiont-aphid interaction investigations highlighted the inter-dependencies of both on each other and the major influences that the endosymbiont(s) have on aphid biology and fitness as pest. Aphid success as plantsap feeders are directly contributed to an endosymbiont-aphid symbiosis. *Diuraphis noxia* have the bacterium *Buchnera aphidicola* as the endosymbiont. *Buchnera aphidicola* contains the biosynthetic pathways needed to produce leucine and tryptophan, two essential amino acids that are usually in low concentrations within the phloem. Aphids do not have the ability to produce these essential amino acids and are therefore dependent on their endosymbiont for amino acid production. The bacterium has moved rate limiting enzymes to multi-copy plasmids, thereby facilitating higher expression of

these essential amino acids. However, in the case of RWA-*B. aphidicola* relationship, the RWA seems less dependent on its endosymbiont since lower plasmid copy numbers and the presence of pseudogenes, have been reported for the endosymbiont of this aphid. Furthermore, *D. noxia* was shown to up-regulate leucine and tryptophan levels in phloem of susceptible wheat cultivars. Together, these finding suggests degradation in the aphid-endosymbiont relationship regarding essential amino acid production. The most cost effective regulation of RWA infestations are the employment of resistant wheat cultivars.

However, *D. noxia* is unable to up-regulate leucine and tryptophan in resistant wheat cultivars, therefore one could argue that the aphid was under severe selection pressure regarding essential amino acid production since the 1980s. This led to the first hypothesis of this study: There are no nucleotide differences in *B. aphidicola*'s leucine and selected tryptophan biosynthetic genes from the different RWA biotypes found in South Africa and the USA. Furthermore, the second hypothesis states the leucine plasmid copy numbers were the same for *B. aphidicola* of all the RWA biotypes. However, other aphid symbionts could also affect host fitness. In the first part of Chapter 3 the presence of other symbionts in the RWA biotypes was investigated using denaturing gradient gel electrophoresis (DGGE) of the bacterial 16S rRNA gene. With the confirmation of the RWA's monosymbiotic status, the chapter continues to investigate the first hypothesis, using *B. aphidicola* accessions from ten different RWA biotypes. Except for a single CCC-insert upstream of *leuA* gene in four of the ten RWA biotypes, no other differences were observed. Could this CCC-insert play a role in the regulation of the *leuA-leuB* operon? This led to a third hypothesis: The CCC-insert, found upstream of the *leuA* gene in *B. aphidicola* of some RWA biotypes, has a functional effect. This hypothesis was investigated in Chapter 4 by comparing accessions with and without the CCC-insert regarding the 5' UTR leader sequences, gene expression levels, *Rho*-independent terminator sites and predicted promoters. The chapter also investigated this region within other aphids as a possible regulatory mechanism within the family.

In the second part of the study (Chapter 5) the influences that statistical mechanisms have on the identification of differentially regulated genes within the RWA-host interaction, were investigated. The hypothesis of this chapter states that, though different background correction and normalization methods for Affymetrix datasets depend on different assumptions, they would eventually identify the same subset genes/probe sets as differentially regulated, especially under increased stringencies. In this chapter the influences of 5 different normalization and background correction methods, under three different confidence levels, with/without false discovery rate (FDR)

and family-wise type I error rate (FWER) correction, were investigated for two different experiments. The genes identified after these analyses as differentially regulated, were subsequently compared to identify how often a gene/probe set was deemed differentially regulated by all 5 smethods. The chapter's analyses were done using scripts written in the statistical program R.

The thesis's major results and conclusions are briefly discussed in the last chapter (Chapter 6). All the supplementary data for the different chapters are given in the appendices.

CHAPTER 2

APHID-PLANT-ENDOSYMBIONT INTERACTION: THE RUSSIAN WHEAT APHID, ITS HOSTS AND ENDOSYMBIONT, *BUCHNERA APHIDICOLA*

The symbiotic relationship between the bacterial endosymbiont *Buchnera aphidicola* and aphids enables highly specialised phloem feeding on host plants, thereby enabling aphids to feed almost undetected (Srivastava 1987; Douglas 1998; Moran *et al.* 2002; Voelckel *et al.* 2004). Plants try to counter this exploitation by employing constitutive and induced defences (Walling 2000). However, the influence that this aphid-plant interaction has on the bacterium, and the influence that the aphid-bacterium interaction has on overcoming plant resistance, have not been investigate very often (Walling 2000). This chapter aims to present an overview on the members, *i.e.* the Russian wheat aphid (*Diuraphis noxia* Kurdjumov), the endosymbiont (*Buchnera aphidicola* Munson *et al.*) and wheat (*Triticum aestivum* L.), and the biotic interactions between them.

Diuraphis noxia (Aphididae: Macrosiphini)

Diuraphis noxia (Russian wheat aphid, RWA) resides within the tribe Macrosiphini of the subfamily Aphidinae (Heie 1992). It forms part of the phytophagous suborder Sternorrhyncha and can therefore be either in the Homoptera (together with the Auchenorrhyncha) or the Hemiptera (the Homoptera and Heteroptera) (Miles 1999).

Origin

The RWA is palaearctic in origin, *i.e.* central Asia to the Middle East. However, various introductions have resulted in a worldwide distribution to all arid and semi-arid cereal producing regions, with the only exception being Australasia (Hewitt *et al.* 1984; Du Toit 1986; 1987; Zemetra *et al.* 1990; Souza *et al.* 1991; Gonzalez *et al.* 1992; Blackman & Eastop 2000; Stray 2000; Stary & Lukasova 2002; Baker *et al.* 2003; Haley *et al.* 2004). In the Republic of South Africa, it was first detected in 1978 with major yield losses resulting in subsequent years (Du Toit & Walters 1984; Du Toit 1987).

Description

Diuraphis noxia is a small, pale yellow-green or grey-green, spindle-shaped apterae (1.4-2.3 mm) that is often covered with a white powdery wax. Under adverse conditions it occurs as an alatae (1.5-2.0 mm) with pale-green abdomen (Hewitt *et al.* 1984; Walters *et al.* 1984; Gonzalez *et al.* 1992; Blackman & Eastop 2000). Overcrowding or a decline in host quality due to seasonal or

host morphological changes, trigger an ontogenetic switch from apterous (wingless) to alatae (winged) aphids that enables relocation and distribution (Walters *et al.* 1984; Dixon 1998). Characteristically to the RWA is the short antennae, a 'forked tail', *i.e.* a projection above the caudal, and an apparently absent siphunculi (Walters *et al.* 1984).

Biology

Females are viviparous and parthenogenetic, *i.e.* eggs commence development directly after ovulation. Nymphs also have the ability to produce embryos themselves, allowing for fast population expansions. Females can produce up to 4 nymphs per day that mature after approximately two weeks (Walters *et al.* 1984; Dixon 1998). Temperature affect the RWA, with higher temperatures having a negative effect on its the lifecycle (Girma *et al.* 1990).

Genetics

Very little is known about the genetics of the RWA. Aphids possesses holocentric chromosomes, *i.e.* chromosome with centromeric activity along the whole axis (Bizzaro *et al.* 2000 & references there in), that allow for karyotype rearrangements. This is thought to support the high occurrence of chromosomal polymorphism found in many aphid species (Manicardi *et al.* 2002).

Biotypes

Different RWA biotypes are distinguished from each other based on their ability to overcome host resistance, their fecundity and the amount of damage they cause to plants in a differential (Puterka *et al.* 1992; Jyoti & Michaud 2005; Burd *et al.* 2006; Jyoti *et al.* 2006; Weiland *et al.* 2008). *Diuraphis noxia* has a high worldwide biotypic diversity which is geographically limited (Puterka *et al.* 1992). Resistant lines is therefore only effective to the specific regions for which they've been bred, *e.g.* a Hungarian biotype was shown to be more virulent to South African resistant lines than the local biotype (Basky 2003). Similarly, Hungarian RWA populations were shown to be more virulent to resistant lines with *Dn1, Dn2, Dn4* and *Dn5* resistance genes (Basky 2003; Smith *et al.* 2004), while Russian, Syrian (Puterka *et al.* 1992), Chilean, Czech Republic and Ethiopian (Smith *et al.* 2004) populations showed resistance to *Dn4*. Czech populations showed resistance to *Dnx* and the Ethiopian population to *Dny* containing lines (Smith *et al.* 2004).

A second biotype has recently been added to the single biotype initially present in South African fields (Du Toit 1989; Tolmay *et al.* 2007), with a third biotype in the laboratory (*Figure 2.1*) (Van Zyl 2007). Today, eight biotypes are known to occur in the USA (Haley *et al.* 2004; Jyoti & Michaud 2005; Burd *et al.* 2006; Weiland *et al.* 2008). Molecular analysis of the USA biotypes showed little nuclear and mitochondrial variation (Lapitan *et al.* 2007b; Shufran *et al.* 2007), therefore suggesting adaptation or diversification of the existing USA biotype(s) rather than reintroductions. However, the biotype designation is assigned based on plant phenotypic responses to aphid feeding and no genetic, taxonomic or other differences can be presumed (Smith *et al.* 2005).
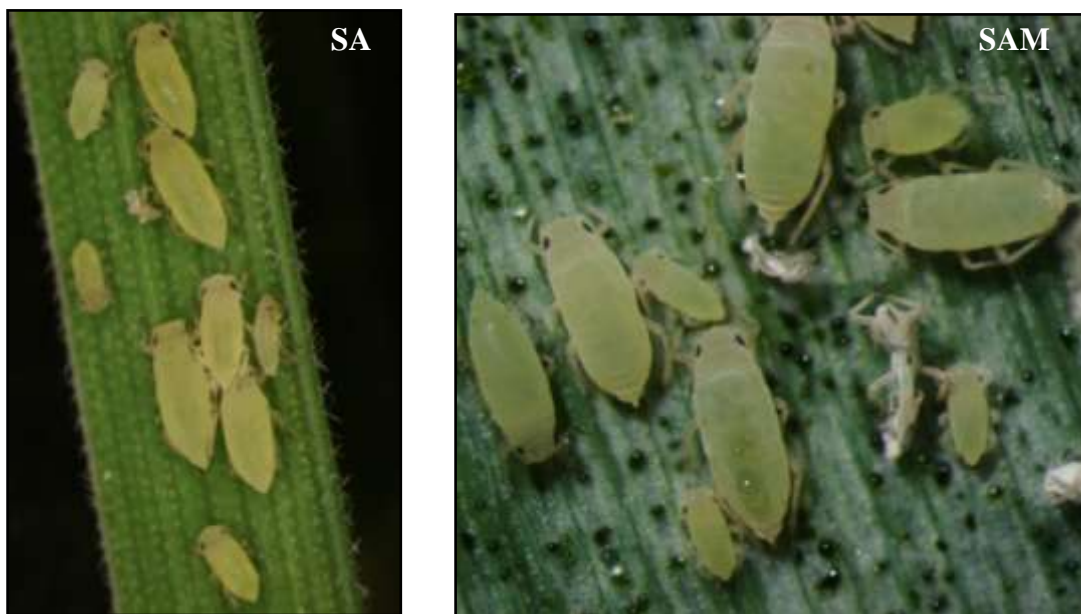

Figure 2.1 The South African (SA) biotype on the susceptible cultivar Scheepers, and the SA mutant (SAM) on the resistant wheat cultivar TugelaDN. No morphological differences are apparent. Photos taken under different magnifications.

Hosts

Host plants of the RWA include mainly barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*), but they have been found on other cereals and grasses. These include *H. murinum*, *T. cylindricum, Elymus arenarius* and *H. pusillum* (Butts & Pakendorf 1984b; Hewitt *et al.* 1984; alternative hosts reviewed in Kindler & Springer 1989; Kindler *et al.* 1992; Belefant-Miller *et al.* 1994; Blackman & Eastop 2000; Stray 2000). Post-harvest survival of RWA has been contributed to cultivation practices and volunteer plants (Hewitt *et al.* 1984; Kriel *et al.* 1986; Stray 2001). *Bromus*

species, *E. trachycaulum* and *E. agrotricum* may also play a role in the seasonal cycle of this aphid (Fouche *et al.* 1984; Hewitt *et al.* 1984; Kindler *et al.* 1992).

Symptoms

Several symptoms are characteristic of RWA infestation. These symptoms in susceptible cultivars include severe longitudinal streaking (*Figure 2.2*) or spotting that is white, yellow or purple in colour (chlorosis), and tightly inward curling leaf edges (leaf rolling) (Du Toit 1986). Growth can be retarded and heavily infested plants have a flattened appearance (Elsidaig & Zwer 1993). Flag leaf infestation leads to white bended ears and in severe cases susceptible cultivars die. Aphids are mainly found on the newest growth and axils of leaves. Infestations occur in a patchy distribution under field conditions and a 20 % infestation of the crop can escalate to 80 % in just 2 weeks if left unchecked. Resistant cultivars are identified by necrotic spots on leaves with no leaf rolling. Seed number, thousand-kernel mass, mass of ear per plant and total seed mass per plant for both susceptible and resistant lines is also reduced. Early infested plants usually have fewer productive tillers (up to 50 % less) and in many cases are dwarfed and of uneven height.
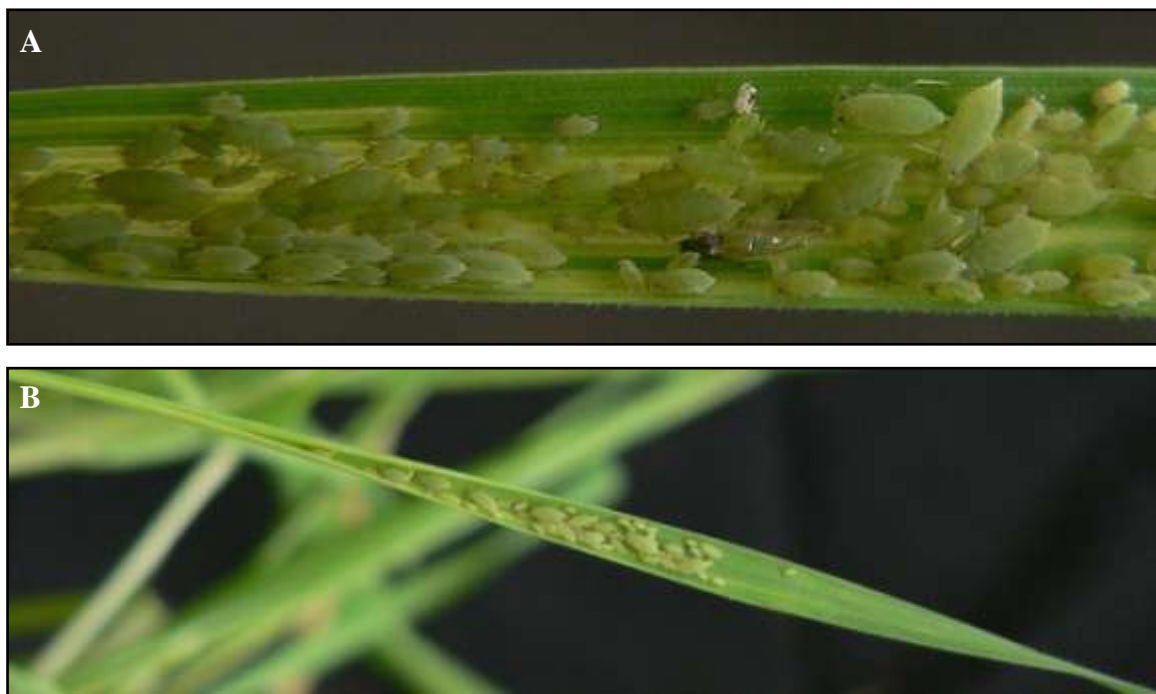

Figure 2.2 (A) Heavily infested wheat plants showing longitudinal streaking and (B) tightly inward curling of the leaf edges (leaf rolling).

Infested flag leaves can result in contorted grain heads that interfere with pollination and head extension (Butts & Pakendorf 1984c; Walters *et al.* 1984; Du Toit 1986; Kriel *et al.* 1986; Du Toit 1989; Quick *et al.* 1991; Smith *et al.* 1991; Dong & Quick 1995). Various screening keys have been developed to help quantify cultivar symptoms and resistance (Butts & Pakendorf 1984b; Du Toit 1987; Quick *et al.* 1991; Burd & Burton 1992). The longitudinal streaking/spotting and chlorosis observed during RWA feeding (Fouche *et al.* 1984; Hewitt *et al.* 1984; Kriel *et al.* 1984; Kriel *et al.* 1986) are sometimes mistakenly identified as virus transmission (Blackman & Eastop 2000; Kazemi *et al.* 2001).

Economic losses

Major yield losses over the years have been credited to the RWA. In the USA it is estimated to have caused losses exceeding US$ 1 billion (Quick *et al.* 1991; Gonzalez *et al.* 1992; Porter *et al.* 1998). Earlier research suggested yield losses of almost 35 % for plants (at growth stage 6) when infested with 9.1 aphids per plant, this converts to a reduction of 1 t/ha in yield (Du Toit & Walters 1984). Later reports calculated that RWA infested or damaged plants have yield reductions of 60-80 % for the 1987/88 season (Archer & Bynum 1992). Initial research suggested that major yield losses occurred when RWA feeding took place before growth stage 30 (stem elongation) (Tottman *et al.* 1979), but later studies suggest that wheat compensate for infestations stopped before this growth stage (Du Toit 1986; Kriel *et al.* 1986).

Control

Expensive systemic insecticides have proven relatively successful in controlling the RWA (Botha 1984; Walters *et al.* 1984; Du Toit 1989; Zemetra *et al.* 1990). Soil systemic insecticides were successful against the RWA but have a negative impact on yield (Du Toit 1984). Similarly, systemic insecticide treated seed controlled the RWA but were deemed economically unfeasible (Butts & Pakendorf 1984a). Leaf rolling as a result of RWA feeding, forms a protective enclosure for the aphids, making it difficult to reach them with contact insecticides and biological agents (Gonzalez *et al.* 1992; Bergeson & Messina 1998) — even though various natural enemies have been identify that may act as possible biological controls (Aalbersberg *et al.* 1984; Archer & Bynum 1992; Gonzalez *et al.* 1992; Reed *et al.* 1992; Clark & Messina 1998; Stray 2000; Bosque-Perez *et al.* 2002; Nowierski & Fitzgerald 2002; Prinsloo *et al.* 2002; Baker *et al.* 2003; Brewer *et al.* 2005).

The only viable options that remain are culture practices, *i.e.* delayed plantings, the eradication of volunteer wheat (Du Toit 1989), and resistant cultivars for the control of this pest. In recent years, the release of resistant cultivars in conjunction with biological controls, have reduced the effects of this pest on wheat fields (Bosque-Perez *et al.* 2002; Jyoti & Michaud 2005). Resistant cultivars, with normal root and shoot development, produced higher grain yields (Zwer *et al.* 1994). However, this scenario is changing with the new RWA biotypes discovered in the USA and South Africa (Haley *et al.* 2004; Jyoti & Michaud 2005; Tolmay *et al.* 2007) and with more virulent populations found across the globe (Puterka *et al.* 1992; Basky 2003; Smith *et al.* 2004).

Aphids and their endosymbiont, *Buchnera aphidicola*

Aphids primarily target the phloem sap of plants for all their nutritional needs (Srivastava 1987). Phloem sap, studied *via* stylet seepage (Bornman & Botha 1973), can be regarded as a excellent food source. It is high in carbohydrates, with nitrogen predominantly occurring as free amino acids. Generally, phloem is also toxin and feeding deterrent free, as these are usually localised within the vacuole and apoplast (Douglas 2006). Though high in sugars, phloem is low in nitrogen (~20 mol %), especially essential amino acids (Lai *et al.* 1996; Dixon 1998; Sandstrom & Moran 1999; Douglas 2006). This presents two problems to aphids: firstly, the ratio of non-essential to essential amino acids in phloem (1:4-1:20) is much lower than the 1:1 ratio found in animal proteins. Secondly, the highly concentrated sugars need to be regulated to prevent disturbing the osmotic pressure within the insect. This is done by the excretion of honeydew or by drinking from xylem (Douglas 2006; Will & van Bel 2006).

Aphids cannot produce essential amino acids themselves. This suggests that aphid growth is related to amino acid composition, rather than sucrose:amino acid ratio (Karley *et al.* 2002). However, essential amino acid concentrations within phloem, compared to that present in the aphid, cannot explain the high growth rates observed (Douglas 2006). It is a symbiotic relationship with an endosymbiont, *B. aphidicola*, that allows aphids to exploit phloem as food source (Douglas 1998).

Buchnera aphidicola's role in aphid nutrition

The primary endosymbiont of aphids, *B. aphidicola*, compensates for the deficiency in the diet by synthesising and recycling the necessary essential amino acids (Munson *et al.* 1991; Munson & Baumann 1993; Dixon 1998; Douglas 1998; Wilkinson 1998; Febvay *et al.* 1999; Sandstrom &

Moran 1999; Birkle *et al.* 2002; Moran *et al.* 2005). *Buchnera aphidicola* is transovarially and maternally inherited between generations, where it's maintained in specialised, aphid produced cells, called mycetocytes (or bacteriocytes) (Lai *et al.* 1994; Lai *et al.* 1996; Rouhbakhsh *et al.* 1996; Dixon 1998; Douglas 1998; Gil *et al.* 2004). Genome analysis highlighted the dependency of the endosymbiont on the host. Many genes from key pathways are absent in *B. aphidicola*, implying that the host provide many important substrates (Shigenobu *et al.* 2000; Tamas *et al.* 2002; Van Ham *et al.* 2003; Zientz *et al.* 2004). This supports the obligatory relationship known to exist between these organisms (Douglas 1998; Wernegreen & Moran 2000).

Genomics and transcriptomics of *B. aphidicola* demonstrated that most of the essential amino acid biosynthetic pathways, though not always complete, were retained and functional (Shigenobu *et al.* 2000; Tamas *et al.* 2002; Nakabachi *et al.* 2005). The type of essential amino acid biosynthetic pathways retained seems to be dependent on the aphid's diet (Tamas *et al.* 2002; Zientz *et al.* 2004). Depending on the need for a specific essential amino acid, *B. aphidicola* has duplicated genes or moved rate limiting enzymes, or even whole pathways, to single or multiple copy plasmids (Baumann *et al.* 1999). These rate limiting pathway enzymes are encoded on vertically, long-term transmission plasmids (Lai *et al.* 1996; Silva *et al.* 1998; Thao *et al.* 1998; Baumann *et al.* 1999; Soler *et al.* 2000; Wernegreen & Moran 2001).

Anthranilate synthase (AS), the first enzyme and limiting factor in tryptophan biosynthesis, is encoded as two subunits on the plasmid *TrpEG* (Lai *et al.* 1996). In most members of the Aphididae and even distantly related families like Pemphigidae, AS occurs as highly conserved repetitive units, inter-dispersed with less conserved intergenic regions on plasmids (Baumann *et al.* 1995; Rouhbakhsh *et al.* 1996; Van Ham *et al.* 1999). Tryptophan production is mainly controlled by feedback inhibition of the product, tryptophan (Lai *et al.* 1994; Lai *et al.* 1996). The overproduction of AS is an attempt to compensate for the inhibitory effect of tryptophan feedback inhibition (Lai *et al.* 1994). The rest of the genes in the pathway are located within the *B. aphidicola* genome (Baumann *et al.* 1998). The whole leucine gene pathway is also located on a plasmid, with gene organisation conserved in the Aphididae (Van Ham *et al.* 1997; Silva *et al.* 1998; Soler *et al.* 2000).

Buchnera aphidicola-RWA interaction

Conversely, RWA seems less dependent on its endosymbiont for essential amino acids (Telang *et al.* 1999; Porter & Webster 2000; Sandstrom *et al.* 2000; Ni *et al.* 2001). This hypothesis is supported by various observations. Firstly, the tryptophan plasmid of *B. aphidicola* occurs in lower copy numbers than in other aphids that feed on similar hosts (Lai *et al.* 1996; Thao *et al.* 1998). Secondly, this plasmid also contains seven pseudogenes, with amino acid measurements indicating a reduction in tryptophan availability (Lai *et al.* 1994; Lai *et al.* 1996; Wernegreen & Moran 2000). Retention of a single working *trpEG* gene copy by *B. aphidicola* is thought to be enough to allow sufficient tryptophan production when the RWA feeds on a less suitable host of preference. Thirdly, only a single copy of the leucine plasmid is retained (Thao *et al.* 1998; Baumann *et al.* 1999). This is in contrast to the 24 copies present in *B. aphidicola* of *Schizaphis graminum*, an aphid feeding on similar hosts (Thao *et al.* 1998). These reductions in copy numbers of genes involved in essential amino acid production is thought to have occurred in response to the ability of the RWA to alter the phloem sap composition of susceptible wheat cultivars (Telang *et al.* 1999; Porter & Webster 2000; Sandstrom *et al.* 2000; Ni *et al.* 2001).

Initially, like *S. graminum,* phloem sap of RWA susceptible cultivars are low in tryptophan and leucine (Thao *et al.* 1998). However, RWA feeding increases the essential amino acid component, especially leucine and tryptophan, in phloem of susceptible cultivars (Telang *et al.* 1999). The elevated levels of essential amino acids found in susceptible accessions infested by RWA could be the result of the elevated expression of amino acid transporter(s) (Van Niekerk 2003). Amino acid transporters, for specific amino acids, are known to be expressed under different environmental conditions (for review see Fischer *et al.* 1998; Delrot *et al.* 2000; Ortiz-Lopez *et al.* 2000). This observation suggests that a RWA diet of susceptible cultivars contain sufficient tryptophan and leucine, thereby removing the evolutionary pressure on the endosymbiont to retain all its gene copies (Lai *et al.* 1996; Telang *et al.* 1999). However, RWA cannot up-regulate these essential amino acids in resistant cultivars (Telang *et al.* 1999). This suggests that the selective pressure of lower leucine and tryptophan levels are back on *B. aphidicola* when the RWA is forced to feed on resistance wheat cultivars.

Triticum aestivum L. em. Thell. is believed to have originated ~10 000 years ago from the hybridisation of *T. tauschii* (Coss.) Schmal and *T. turgidum* L. This hybridisation resulted in today's allopolyploid bread wheat ($2n = 6x = 42$, AABBDD), where *T. tauschii* is considered to be the DD genome donor ($2n = 14$) and the allotetraploid *T. turgidum*, the AABB genome donor ($2n = 28$) (Gill *et al.* 1991 & references; Feldman & Levy 2005). *Triticum urartu* (AA) and an *Aegilops* species of section Sitopsis (BB) are considered the progenitor species for *T. turgidum* (Feldman & Levy 2005). This allopolyploidy of wheat, caused an evolutionary bottleneck with a narrow genetic base as shown by limited polymorphism found in its cultivars (Gill *et al.* 1991; Kubalakova *et al.* 2002).

Resistant wheat cultivars

Plant resistance to RWA have been broadly categorized into antixenosis, antibiosis and tolerance (Painter 1958; Kogan & Ortman 1978; Du Toit 1987). Antixenosis is the non-preference of a plant to provide an insect with shelter, food or oviposition, *i.e.* the inability to sustain and serve as host plant (Painter 1936; 1958; Kogan & Ortman 1978; Du Toit 1987; Bahlmann 2002). Antixenosis is attributed to multiple genes located on chromosomes 2B, 6A and 7D (Castro *et al.* 2001). Antibiosis of a resistant accession is the ability to adversely affect the insect's biology and is measured using aphid fecundity (Painter 1958; Bahlmann 2002). It is regarded as the most common form of wheat resistance (Du Toit 1987; 1989) and causes a decrease in aphid longevity and fecundity, a delay in development and increased restlessness (Kindler *et al.* 1992; Smith *et al.* 1992). Antibiosis to RWA is attributed to the involvement of different and independent genes on different chromosomes (Castro *et al.* 1999). Genes responsible for antibiosis and antixenosis resistance were shown to be different (Castro *et al.* 1999). Tolerant plants have the ability to survive infestations classified as fatal or severe injury causing to a susceptible cultivar (Painter 1958). Research suggests that adjustments to photosynthesis may be the underlining process used by tolerant cultivars to overcome infestations (Haile *et al.* 1999; Botha *et al.* 2006). Tolerance is also thought to be under multi-gene control with genes located on chromosomes 1A, 1D and 6D (Castro *et al.* 2001).

Initial, hexaploid wheat germplasm showed little resistance against the RWA (Du Toit 1987). Therefore, RWA resistance breeding programmes utilized related species as sources for resistance genes. These included *T. monococcum*, *T. timopheevii*, *T. ventricosum*, *T. tauschii*, *A. squarrosa*, *T. dicoccoides* and *Secale cereale* (rye) (Butts & Pakendorf 1984b; Du Toit 1987; Nkongolo *et al.*

1991a; Potgieter *et al.* 1991; Marais *et al.* 1994). Breeding endeavours helped in the identification of various RWA resistance genes (*Table 2.1*). Most of these resistance genes formed part of mapping studies (Ma *et al.* 1998; Myburg *et al.* 1998; Fritz *et al.* 1999; Venter & Botha 2000; Liu *et al.* 2001; Miller *et al.* 2001; Liu *et al.* 2002).

Table 2.1 *Triticum aestivum* RWA-resistant genes, their origins and mode of resistance.

Gene	Accession	General information
Dn1	PI 137739 /SA 1684	Hard white spring wheat, Iran, single dominant gene, antibiosis and antixenosis. One major gene on chromosome 7D (another, unidentified resistant gene on chromosome 7B)[1,3,5,6]
Dn2	PI 262660 /SA 2199	Hard white winter wheat, Bulgaria, single dominant gene, antibiosis and antixenosis[1,3,5]
Dn3	*T. tauschii* /SQ24	Recessive gene[2]
Dn4	PI 372129 /CORWA1	Single, dominant, nuclear gene, on chromosome 1DS, tolerance most significant resistance method, with least RWA damage to plants. Lower levels of chlorosis and fewer days to death than PI 137739 (*Dn1*) and PI 262660 (*Dn2*). High resistance with low RWA damage[3,8,10]
Dn5	PI 294994 /SA 463	Hard red winter wheat, Bulgaria, chromosome 7D. Possibly linked with *Dn1*. Resistance and inheritance unclear[3,4,5,8,11]
Dn6	PI 243781	Dominant gene[8,9]
Dn7	Rye	Unknown[7]
Dn8	PI 294994	Unknown[12]
Dn9	PI 294994	Unknown[12]
Dnx	PI 220127	Single, dominant gene[12,14]
Dny	'Stanton'	Unknown[13]

[1]Du Toit (1987; 1989), [2]Nkongolo *et al.* (1991a), [3]Nkongolo *et al.* (1991b), [4]Elsidaig & Zwer (1993), [5]Marais & Du Toit (1993), [6]Schroeder-Teeter *et al.* (1993), [7]Marais *et al.* (1994), [8]Dong & Quick (1995), [9]Saidi & Quick (1996), [10]Dong *et al.* (1997), [11]Zhang *et al.* (1998), [12]Liu *et al.* (2001), [13]Smith *et al.* (2004), [14]Boyko *et al.* (2006).

Plant resistance and defence mechanisms

Plants are at any moment bombarded with various biotic and abiotic stimuli. In the case of biotic interactions, plants employ an integrated defence strategy where stimuli may activate various signal transduction pathways that interlink (or cross talk) with each other to ensure the right defence response at the right time (Genoud & Metraux 1999; Pieterse & Van Loon 1999). This ensures a flexible defence with better energy and resource management (Baldwin & Preston 1999; Walling 2000).

Aphids, as phloem-feeders, produce smaller injuries during their long-lasting feeding interactions with plants, unlike chewing and cell-content feeders. Defence signals activated by aphid feeding are thought to be similar to those activated by bacteria, viral or fungal pathogens (*Figure 2.3*) (Walling 2000). The interactions between aphid/pathogen and plant could be compatible (slow reaction with hypersensitive response (HR) usually absent) or incompatible (fast reaction usually accompanied by HR) (Walling 2000). Incompatible interactions could again be divided into non-host resistance and host resistance (Odjakova & Hadjiivanova 2001). Non-host resistance, present in all plants, is the resistance of a species against a particular pathogen, *e.g.* general resistance for the majority of potential pathogens (Heath 2000b; Odjakova & Hadjiivanova 2001). Non-host resistance can include both preformed/constitutive and induced defences. Constitutive plant defences are various structural and chemical traits that physically limit/prevent insects accessing tissue, and/or deters colonization (antixenosis), growth, development, reproduction or survival (antibiosis) (Walling 2000; Peeters 2002). These include leaf morphological features, like waxy cuticula, cell walls (CW), silica depositions in CWs, callose, peptides, suberin and surface features, like trichomes and waxes, and stored allelochemicals (Hammond-Kosack & Jones 1996; Heath 2000b; Walling 2000; Dangl & Jones 2001; Peeters 2002; Walling 2008). Generally, sessile phloem feeders prefer thin leaf lamina and cuticles (Peeters 2002). In grasses, cutin and silica in epidermal cell walls and cuticles, are known to provide additional barriers to insects (Brett & Waldron 1990).

Plants have an active approach for non-host induced resistance. Elicitors, usually originating from the plant CW or the specific pathogen are recognized and activate constitutive and inducible defence responses *via* a complex signalling network, encompassing Ca^{2+} and H^+-ion fluxes, reactive oxygen species (ROS) intermediates, ethylene, salicylic acid (SA), jasmonic acid (JA), nitric oxide (NO) and membrane depolarisations. Inducible defence responses include
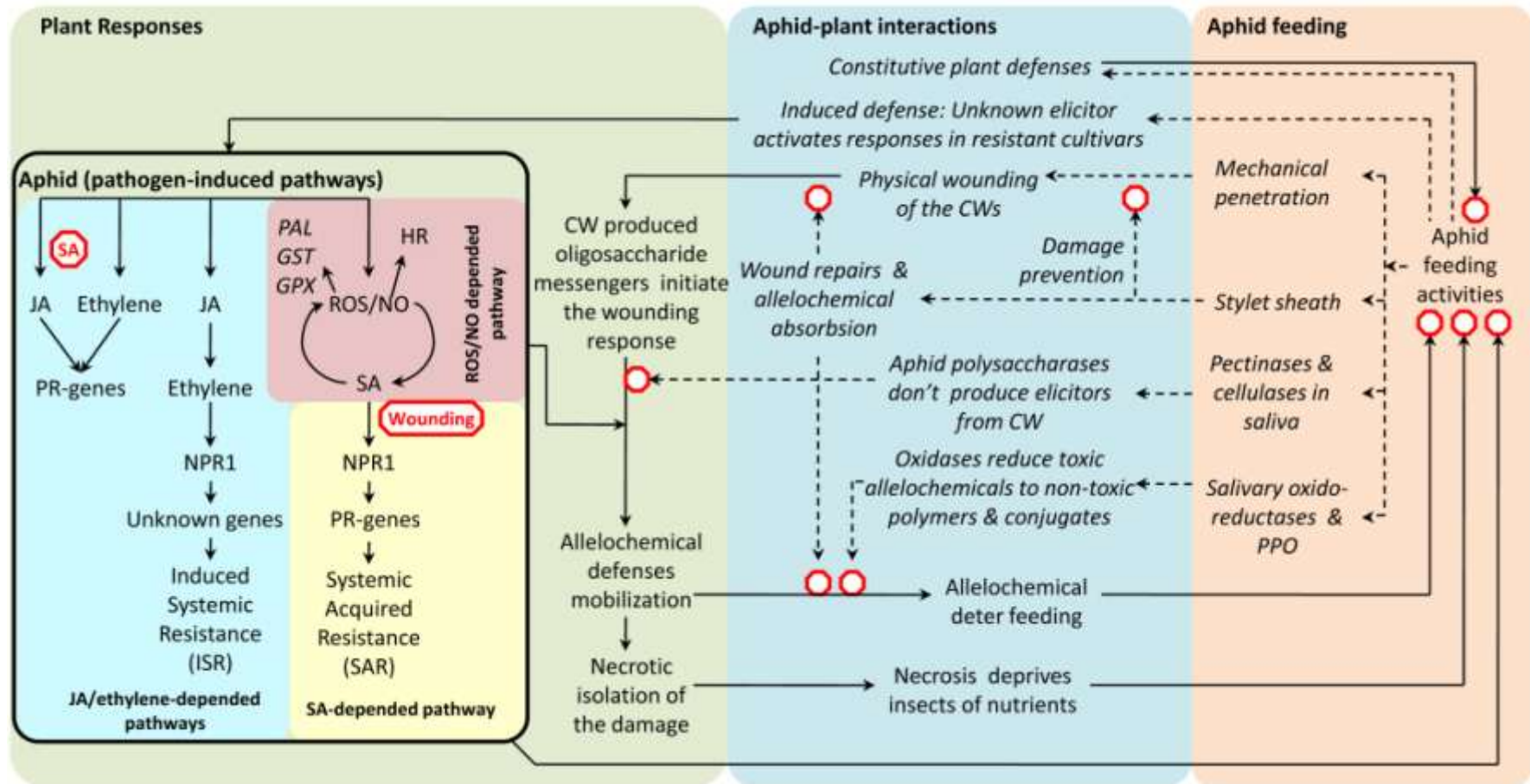
Figure 2.3 The interaction between aphids (italics and dashed lines) and the defence responses of plants (normal case and unbroken lines). Aphids activate similar signal transduction pathways as pathogens, which utilize the salicylic acid (SA), reactive oxygen species (ROS) and jasmonic acid (JA)/ethylene dependent pathways to activate gene expression in plants. Arrows indicate the interaction and red hexagons the inhibitions. Abbreviations: CW, cell wall; HR, hypersensitive response; NO, nitric oxide; PPO, polyphenol oxidase; PR-genes, pathogen related genes (Adapted from Miles 1999; Walling 2000).

the expression of pathogen-related proteins like protein kinases (PK), elements of the mitogen-activated protein kinases (MAPK) pathway, ROS, enzymes of the phytoalexin biosynthesis pathway and ultimately, programmed cell death (PCD) (Mittler *et al.* 1999; Heath 2000b; Walling 2000; Odjakova & Hadjiivanova 2001). These "non-specific" elicitors also plays a role in non-host HR generally associated with pathogen containment, elimination and the activation of defence genes (Heath 2000b). These collective pressures from various biotic and abiotic stresses have produced non-specific, broad-spectrum defences in response — an idea supported by the discovery of a cysteine protease with anti-fungal and feeding deterrent reactive sites (Joshi *et al.* 1999). The durability of non-host resistance, *i.e.* pathogens rarely alter host species range, makes it an ideal target for commercial breeding endeavours (Heath 2000b).

Host resistance (race/cultivar resistance), in contrast, is limited to certain expressed genotypes within an otherwise susceptible species and usually pathogen-genotype-specific or parasite-specific (Hammond-Kosack & Jones 1996; Heath 2000b; Odjakova & Hadjiivanova 2001). Host resistance is where a pathogen avirulence (*avr*) protein interacts/binds specifically with plant *R* protein(s), resulting in the recognition of the invader and the launch of the appropriate defence pathways (Hammond-Kosack & Jones 1996; Odjakova & Hadjiivanova 2001). Recognition by *R* genes can either be through this "gene-for-gene" ("receptor-ligand") interaction (van der Biezen & Jones 1998; Dangl & McDowell 2006), or through the recognition of *avr* mediated plant changes that promotes pathogen virulence ("guard hypothesis") (Dangl & Jones 2001; Chisholm *et al.* 2006). Both non-host and host resistance responses are proposed in aphid-plant interactions. A host resistant response, with a gene-for-gene interaction, is proposed for aphid-specific responses in resistant plants (Kaloshian 2004; Botha *et al.* 2006; Smith & Boyko 2007), while a non-host resistant response, activated as part of the general stress response after aphid tissue damage, is proposed for both susceptible and resistant plants (Van der Westhuizen *et al.* 1998b; Smith & Boyko 2007).

The origin and detection of aphid elicitors

Aphid probing can damage various tissues, many of which may produce defence responses as eliciting agents. Substances originating from the aphid's endosymbiont(s) are also possible elicitor candidates (Walling 2000; Smith & Boyko 2007 and references). Microbial elicitors (*Figure 2.4*), for example, can be peptides, proteins, lipids and oligosaccharides to name but a few (Montesano *et al.* 2003). Furthermore, saliva, as a foreign substance, may introduce elicitors into the plant. Digested cuticle products are well characterized in fungal recognition and defence response elicitors

in plants (Lequeu *et al.* 2003; Chassot & Metraux 2005). Exposure of a variety of plants and/or cell cultures to cutin monomers led to the effective protection of susceptible individuals, medium alkalinisation, ethylene production, activation of defence related genes and even H_2O_2 production (Chassot & Metraux 2005 & references therein). The complexity of the cuticle, consisting of surface wax, cutin and pectin (Brett & Waldron 1990), have many potential elicitors
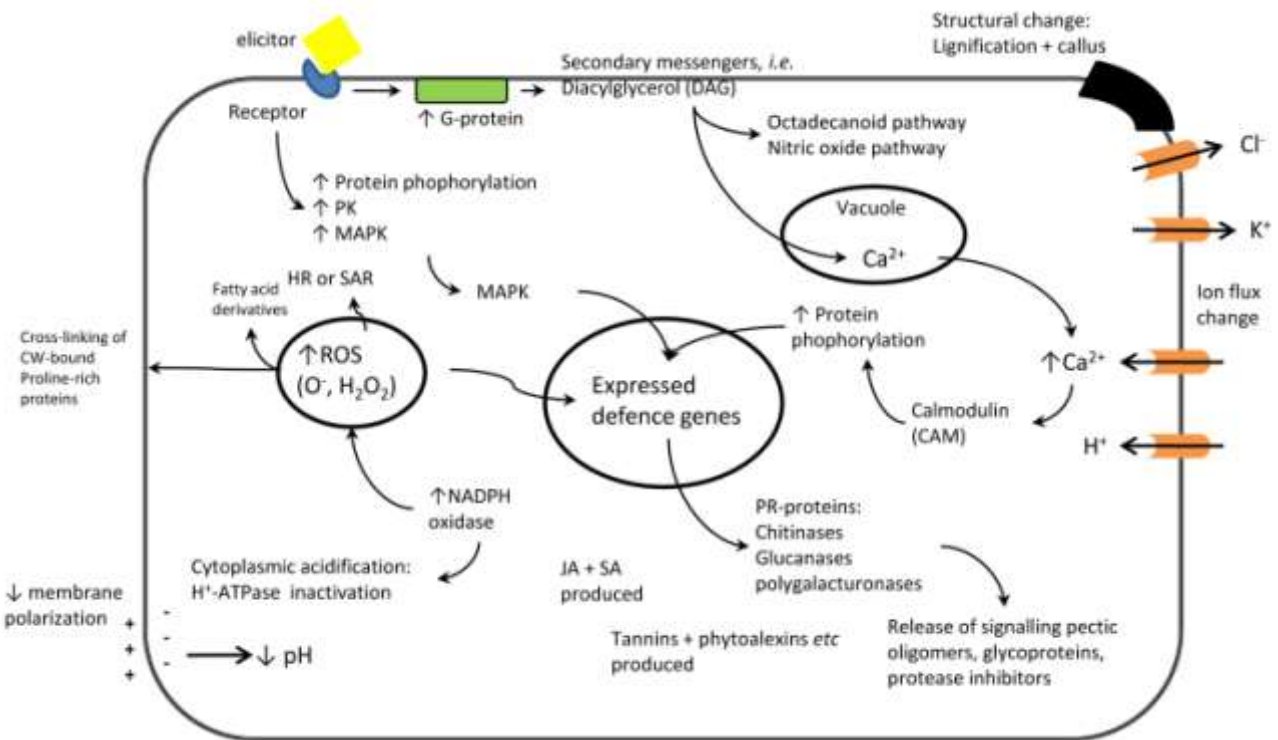

Figure 2.4 Biotic elicitation in plant-aphid interaction is thought to be similar to that of microbial pathogens. In microbial-pathogen interaction an elicitor binds to plasma membrane/CW receptor and activates protein phosphorylation, protein kinases (PK) and mitogen-activated PK (MAPK), as well as G-proteins (intracellular proteins that interact/function with receptors to regulate various enzymes and ion channels). Ion transporters are activated resulting in changes of ion fluxes (calcium and hydrogen ion influx increases). Calcium ions bind to calmodulin (CAM, non-enzymatic intracellular Ca^{2+}-binding proteins) which then binds to other proteins for regulation, resulting in the expression of defence genes. Secondary messengers are also activated, resulting in calcium release and the activation of various pathways. The cytoplasm acidifies as the result of NADPH oxidase activation, decrease in membrane polarization and the inactivation of H^+-ATPase. ROS activation and PR-protein expression can cause HR cell death at infection site or systemic acquired resistance (SAR) (compiled from Radman *et al.* 2003).

and may be the first step in RWA recognition. During a pathogen attack the CW degrading products, polysaccharides, diffuse into neighbouring cells triggering a defence response (Brett & Waldron 1990). Furthermore, phytoalexins, *i.e.* non-specific toxins produced by higher plants in response to pathogen attack, can also be induced from abiotic elicitors (*e.g.* from mechanical wounding), resulting in necrosis of host tissues (Brett & Waldron 1990). Proteinase inhibitors can also be produced in response to mechanical wounding (Brett & Waldron 1990).

Successful defence depends on the rapid recognition of a foreign invader which then triggers the appropriate response. The apoplast, with its interaction with the environment, is thought to play a key role in stress and pathogen detection (Foyer & Noctor 2005). Apoplastic transport moves various substances through the CW matrix (*i.e.* the apoplast) and across the plasma membrane located between neighbouring or widely separated cells (Brett & Waldron 1990). Many pathogenesis-related (PR) proteins accumulate in the apoplast and is therefore generally considered as the site where many eliciting signals of the defence responses originate and where defence products accumulates (Bowles 1990). Elicitors from fungal pathogens, can for example be detected by receptors in the plasma lemma (Nürnberger 1999) or CW (Heath 2000b), while bacterial and viral elicitors are perceived intracellular (Nürnberger 1999).

Plant resistance genes

In aphid-plant interactions, only a single plant resistance (*R*) gene has been cloned, *.e.g. Mi*, from the NBS-LRR (nucleotide binding site and leucine rich repeat) class, isolated from *Lycopersicon peruvianum* that confers resistance to the potato aphid, *Macrosiphum euphorbiae* (Kaloshian *et al.* 1995; Kanazin *et al.* 1996; Rossi *et al.* 1998; Smith & Boyko 2007). Other *R*-gene candidates in aphid-plant interactions include serine/theronine kinase, LRR-glycoproteins and leucine zipper (LZ)-NBS-LRR (reviewed by Smith & Boyko 2007). The various *Dn*-genes, characterized by the strength and type(s) of resistance they convey to cultivars, origin and mode of inheritance (*Table 2.1*), have yet to be successfully isolated. The current hypothesis is that the initial recognition and signalling of a co-ordinated defence response of resistant cultivars to the RWA, is due to specific plant *R*-genes, probably from the NBS-LRR protein class (Botha *et al.* 2006; Smith & Boyko 2007).

Resistance proteins are generally considered as mediators in recognising elicitors and activating downstream signalling responses, like the HR (Heath 2000a; Shirasu & Schulze-Lefert 2000; Moffett *et al.* 2002). Plant *R*-genes, based on amino acid homologies and characteristic

domain organisation, can be divided into five classes (Hammond-Kosack & Jones 1997; Young 2000; Dangl & Jones 2001). Intracellular or cytoplasmic serine or threonine PK forms the first distinct class (Hammond-Kosack & Jones 1997; Dangl & Jones 2001; Cannon & Young 2003), with sequence homology suggesting involvement in the signal transduction pathway, *e.g. Pto* of *Solanum lycopersicum* (syn. *Lycopersicon esculentum*) (Martin *et al.* 1993; Dangl & Jones 2001; Mysore *et al.* 2003). The second class contains a trans-membrane region connecting an extracellular leucine rich repeat (LRR) motif to a short cytoplasmic region, *e.g. Cf-X* genes of tomato (Jones *et al.* 1994; Cai *et al.* 1997; Thomas *et al.* 1997; Durrant *et al.* 2000; Dangl & Jones 2001; Cannon & Young 2003). The *Arabidopsis RPW8* is an example of the third class that consist of a trans-membrane or signal peptide and a coiled-coil (CC) domain (Dangl & Jones 2001; Xiao *et al.* 2001). The fourth class contains a large trans-membrane receptor, a large extracellular LRR domain and a cytoplasmic protein kinase domain, *e.g. Xa21* in *Oryza sativa* (Song *et al.* 1995; Pan *et al.* 2000a; Dangl & Jones 2001). The largest *R*-gene class contains both a nucleotide binding site (NBS) and a leucine-rich repeat (LRR) domain (Lagudah *et al.* 1997; Michelmore 2000; Dangl & Jones 2001; Halterman *et al.* 2001; Cannon & Young 2003).

NBS-LRR proteins are structurally characterized by a conserved nucleotide binding site (NBS), a variable N-terminal region and the varying number of LRRs at the carboxyl-terminal, (Hammond-Kosack & Jones 1997; Dangl & Jones 2001). The NBS region, a.k.a. nucleotide binding apoptosis *R*-gene & *CED-4* like domain (NB-ARC) or nucleotide binding domain (NBD), is further subdivided into small, highly conserved amino acid motifs (Bourne *et al.* 1991; Traut 1994; Cannon *et al.* 2002; Cannon & Young 2003). The NBS domain contains sequence motifs suggesting ATP binding or ATPases activity (Traut 1994; Pan *et al.* 2000b; Dangl & Jones 2001; Deslandes *et al.* 2002). LRR motifs provide the structural framework necessary in signalling transduction pathways for protein-protein, peptide-ligand and protein-carbohydrate interactions (Kobe & Deisenhofer 1994; 1995; Dangl & Jones 2001; Kobe & Kajava 2001). LRRs were also influential in determining the specificity of pathogen-specific gene-for-gene interactions, including the downstream signalling events (Ellis *et al.* 1999; Fluhr 2001; Kobe & Kajava 2001; Deslandes *et al.* 2002).

Redox signalling and plant defence

Redox signalling plays an important part in regulating defence gene expression (Baier & Dietz 2005 and references). In this signalling system, MAPKs play an important role and are again regulated by redox changes through phosphatase activities, with MAPK cascades resembling redox

signalling transduction pathways found in animals (Baier & Dietz 2005). Under normal conditions in the cytosol, antioxidants and antioxidant enzymes will maintain a reducing state, thus preventing ROS signals and the triggering of redox signalling pathways (Baier & Dietz 2005). ROS signalling of defences are determined by antioxidants, while the redox status of antioxidants, like ascorbate (AA), determines general plant defences to wounding and biotic stresses (Foyer & Noctor 2005). Indeed, changes in redox balance could induce defence-related genes, including PR-proteins. This can be seen when decreasing AA (or low levels of AA) leads to increase of PR-gene expression, this is similar for changes in glutathione (GSH), while dehydroascorbate (DHA) and oxidised glutathione (GSSH) accumulates during stress (Foyer & Noctor 2005; Noctor 2006). This 'redox sensing' by plants occurs when stresses change or adjust the redox state, thus leading to plant adjustments through other signalling systems like PK, phyto-hormones, protein phosphatases, ROS and calcium (Noctor 2006).

Transduction and defence pathways

Aphid feeding activates similar defence transduction pathways as bacteria, viruses or fungal pathogens, which include the JA/ethylene- and salicylic acid (SA)-dependent signalling pathways (*Figure 2.3*) (Walling 2000; Kaloshian 2004). These pathways act in a complex way to regulate each other and thus defence responses (Kunkel & Brooks 2002). The various signalling cascades results in local and systemic accumulation of defence RNAs and proteins, which includes enzymes capable of hydrolysing pathogen CWs, modifying plant CWs, synthesising secondary metabolites, producing signals for defence-signal modulation, protein turnover, *etc*. (Walling 2000). However, the aphid-plant interaction doesn't usually involve the wounding response. During the wounding response, breakdown products of plant tissues themselves act as elicitors to produce enzymatic cell breakdown (Wheeler 2001). Endogenous phenolics now leaking through broken membranes, are oxidized by oxidative enzymes and the production of these phenolics are stimulated in adjacent cells, thereby initiating repair processes like lignifications (Lamikanra 2002).

The HR is characterized by the rapid and localised death of plant cells, initiated by the plant (host) to prevent, restrict or confine the pathogen's growth and/or spreading (Heath 2000a; b; Lam *et al.* 2001; Odjakova & Hadjiivanova 2001). Typically, HR is recognised by one too many brown, dead cells at the infection site, usually as a result of ROS. Surrounding cells, not directly in contact or that is not physically invaded, may also die in HR, with sufficient numbers giving rise to necrotic lesions (Heath 2000a; Lam *et al.* 2001; Odjakova & Hadjiivanova 2001). Furthermore, cells

adjacent to these necrotic lesions become totally resistant to subsequent infections, *i.e.* show localised acquired resistance (Odjakova & Hadjiivanova 2001). HRs may or may not include cell death, but also includes the expression of *R*-genes (Heath 2000a; Lam *et al.* 2001). Host resistance that induces HR is generally controlled by gene-for-gene pathogen/parasite-specific *R*-genes, *i.e.* a specific *avr*-gene matching a specific *R*-gene (usually for fungal pathogens) or one *R*-gene capable of recognising multiple *avr*-genes (bacterial pathogens) (Heath 2000a).

Programmed cell death (PCD) is the activation of genetic programs inductive of cellular suicide and forms part of the HR, developmental programs, senescence, differentiation, development, seed germination and can even be induced by abiotic stresses (Beers & McDowell 2001; Lam *et al.* 2001). The systemic acquired resistance (SAR) pathway is activated after the HR response. The onset of SAR follows the accumulation of SA and is based on the expression of specific PR-genes that allows plants to maintain resistance against a pathogen, and includes PR-proteins like β-1,3-glucanase, chitinase, *etc*. (SAR reviewed by Ryals *et al.* 1996; Pieterse *et al.* 2001). SAR can be seen as a state of heightened defence and helps plants to protect themselves against a wide array of subsequent pathogen attacks (Kunkel & Brooks 2002; La Camera *et al.* 2004 reviewed microbial-plant interactions). Jasmonic acid (JA) or methyl JA (MeJA), known as jasmonates (JAs), are usually associated with chewing insects or the wounding responses (McConn *et al.* 1997; Titarenko *et al.* 1997; Halitschke & Baldwin 2005). However, JAs also plays a role in other cellular processes, like seed germination, development, senescence and leaf abscission (Seo *et al.* 2001). JA is produced by the octadecanoid pathway and forms part of the oxylipin signalling pathway (Halitschke & Baldwin 2005). Cross-talk, overlap or interaction of these various mechanisms exist (Beers & McDowell 2001), *e.g.* during HR the NO and ROS pathways interacts (Zaninotto *et al.* 2006) and SA, JA and ethylene pathways interact and control each other (Pieterse *et al.* 2001; Kunkel & Brooks 2002). Thus, though various expression studies have been done on insect-plant interactions (Fidantsef *et al.* 1999; Moran & Thompson 2001; Lacock *et al.* 2003; Mochida *et al.* 2003; Ogihara *et al.* 2003; Van Niekerk 2003) and on wheat-RWA interaction (Lacock *et al.* 2003; Van Niekerk 2003), the resistance mechanism involved in RWA host interaction is still unclear.

Aphid-plant interaction

Aphid probing and feeding

Aphids use a variety of chemical and physical stimuli in recognising their host of preference (Dixon 1987). A surface scan with the tip of the proboscis allows the detection of the vein contour, the preferred feeding site (Dixon 1998). This is confirmed with a drop of saliva, dissolving the cuticle which is then sensed by chemoreceptors on the labium tip (Srivastava 1987). The saliva also forms a flange on the surface through which the aphid will start probing (Miles 1968). Mandibular and maxillary stylets are arranged to form a needle-like structure with two deep grooves: one for pumping saliva out and the other for ingesting. This is used to probe into the plant while a continuous proteinaceous stylet sheath is secreted (Miles 1968; Dixon 1998; Brennan *et al.* 2001). The stylet sheath is thought to consist of mainly proteins, lipoproteins and phospholipids (Miles 1965; 1967; 1968). Sheath material, originating from the lateral lobe of the salivary gland, starts gelling immediately after secretion - probably due to hydrogen bonding and enzymatic oxidation of sulphydryl groups (Miles 1967; Dixon 1998). The product is a relatively impermeable, though flexible, salivary stylet sheath that provides some rigidity and directional control for the flexible stylet apex (Dixon 1998; Miles 1999).

Probing is the forward-backwards movement of the stylet, with a drop of saliva secreted before the next forward thrust. This movement drives the stylet through the gelling sheath material (Miles 1968; 1999), while brief stops allow sampling to determine the stylet position (Dixon 1998). These different actions produce two distinct EPG (electrical penetration graphs) wave patterns: one associated with salivation, while the other is associated with both salivation and feeding/testing (Dixon 1998). EPG, with transmission electron microscopy, showed that stylet paths follow an intercellular route, *i.e.* through the middle lamella, intercellular air spaces, secondary cell wall or amid the cell wall and plasmalemma, before going intracellular when entering the phloem (Dixon 1998; Miles 1999). However, when entering phloem cells, only the tip of the stylet penetrates and withdraw before sheath material is secreted (Miles 1968). RWA probing also follows an intercellular route, though the stylet tracks may have a branched appearance due to redirection (Fouche *et al.* 1984), that causes massive damage to the surrounding cells/tissue (Botha & Matsiliza 2004; Saheed *et al.* 2007b).

Following sieve element penetration, the viscous sheath forming saliva change into watery saliva (Miles 1965; Dixon 1998). Small volumes of watery saliva are also discharged intermittently

with sheath forming saliva when puncturing parenchyma cells and during intracellular probing. This is ingested with the surrounding substrate, thereby enabling the aphid to "taste" the current tissue being probed (Martin *et al.* 1997; Miles 1999). The functions of the watery saliva is unknown but could range from lubrication and food digestion, to food maintenance (reviewed by Miles 1999). Salivation is thought to take place when the aphid is not feeding or when the stylet is inserted/removed, but is continuous when parenchyma cells are used as food (Miles 1968; 1999). Aphids feeding on resistant plants usually don't continue with ingestion, but keep salivating or keep returning back to salivation (Will *et al.* 2007).

Pressure in the phloem elements is sufficient to drive phloem sap up the food canal of the stylets. Intake is controlled by a piston valve, though aphids do have the ability to suck/pump phloem sap. The phloem sap composition is sampled by gustatory epipharyngeal sensillae located in the pharynx (Dixon 1998). The alimentary tract is reviewed by Dixon (1987).

Evading the host defence

Aphids use various strategies to go unnoticed by plants while probing and feeding. These strategies range from preventing the release and/or synthesis of toxic compounds, to influencing redox poise, manipulating the wounding response, neutralising ROS and preventing induced defence pathways. Some of these approaches can also result in morphological changes in the host – mostly beneficial to the aphid. These strategies used in plant-aphid interactions can be either general or species specific.

Generally, aphid feeding may affect the intact cell layers around the stylet sheaths. These can demonstrate chloroplast degeneration, nucleus enlargement, the loss of starch and an increase in permeability. Free amino acids in aphid saliva were shown to cause increases in permeability and respiration, reduction in photosynthesis and monocot growth and even toxic reactions in plants (Miles 1968 and references). Sheath caused vascular blockages are thought to be responsible for localised pigmentation that can occur above blocked phloem vessels (as auxins and photosynthates accumulate) and wilting or decrease in transpiration in the case of xylem elements (Miles 1968 and references within). Aphid feeding can also cause hypertrophy in plant cells, *i.e.* the formation of leaf rolls (or pseudogalls). These pseudogalls are usually associated with cell growth on the opposite side of the aphid's feeding site and are thought to be as the result of increased auxin activity (Miles 1999).

Aphids need to neutralise any wounding or defence response while probing to enable a successful and long term feeding site. The probing process punctures numerous cells along the way, sometimes intentionally to determine the stylet position (Dixon 1998; Will & van Bel 2006). A small amount of watery saliva is released into the cell when a membrane is punctured (Miles 1968; Martin *et al.* 1997; Miles 1999; Saheed *et al.* 2007a). Salivary enzymes are thought to play a major role in suppressing host defence responses. The watery saliva also diffuse into the cells surrounding the advancing stylet track - no sheath material is produced until the stylet tip is removed (Miles 1999).

The stylet sheath prevents calcium inflow from the apoplast into the cell, thereby preventing a full launch of the wounding response (Yoo *et al.* 2002; Will & van Bel 2006; Will *et al.* 2007). Furthermore, no cell content, *i.e.* cytoplasm or ruptured vacuoles, can leak out into the intercellular spaces, thereby preventing the release/production of compounds that might trigger the defence responses in adjacent tissue (Miles 1999). Wound induced HRs are further suppressed when potentially toxic compounds (*e.g.* phenolics), that may promote necrosis, are absorbed and immobilised within the sheath material (Miles 1999). Sealing the wound with sheath saliva also prevents phloem sap losses and a drop in phloem turgor pressure. This is important since the loss of turgor pressure in the sieve elements (SE) may also induce the wounding response (Will *et al.* 2007/ and references). The small stylet volume and phloem sap flow regulation during feeding prevents a drop in SE turgor pressure (Dixon 1987; Will & van Bel 2006). Turgor pressure is also maintained during stylet penetration of the SE, thereby preventing the activation of stretch-activated calcium channels (Will & van Bel 2006).

Enucleated phloem SE was long thought to function as transporters of nutrients from source to sink tissues. However, this system's role in transport and signalling is far more complex. Molecules involved in plant defence (antioxidants, protease inhibitors, *etc.*), signalling (small RNAs, PK, SA, JA, *etc.*), and various macromolecules (mRNA and proteins), are all translocated *via* this system (Imlau *et al.* 1999; Ruiz-Medrano *et al.* 1999; Yoo *et al.* 2002; Lough & Lucas 2006; Gaupels *et al.* 2008; Kehr & Buhtz 2008; Le Hir *et al.* 2008). There is even indications of controlled transport of macromolecules to specific destinations in the plant (Aoki *et al.* 2005). Furthermore, proteins involved in RNA-binding, mRNA translation, macromolecule trafficking, *etc.* were found in phloem (Lin *et al.* 2008), thus suggesting protein synthesis and turnover – something not thought possible in SEs lacking nuclei and ribosomes (Yoo *et al.* 2002). The complexity of the system further increases when one considers that all the loading and unloading of phloem is done *via* the companion cells (Brett & Waldron 1990; Balachandran *et al.* 1997; Yoo *et al.* 2002; reviewed by Will & van Bel

2006; Le Hir *et al.* 2008; Braun & Slewinski 2009). Therefore, undetected breaching of this system poses numerous problems to aphids. Any breach will result in the rapid sealing of the SEs' sieve plates (as part of the wounding response). This is achieved by P-proteins (Dixon 1998; Miles 1999), or in the case of monocots like the Poaceae, by proteinaceous inclusions (bodies) of the SE plastids (Eleftheriou 1990; Miles 1999; Dinant *et al.* 2003; Will & van Bel 2006). In wheat the plastid envelope ruptures in heavily damaged sieve tubes, releasing the proteinaceous inclusions which seal the sieve-plate pores, which is followed by callose deposition (Bornman & Botha 1973; Brett & Waldron 1990; Eleftheriou 1990). Proteinaceous inclusion blocking is a fast reaction to wounding, while callose plugging of the sieve plates is thought to be a slower process (Will & van Bel 2006).

It has been suggested that the watery saliva prevents these blockages of the sieve plates when the stylet first break through the phloem CW (Dixon 1998). Calcium binding domains on salivary proteins suggest that they interact with Ca^{2+} on a molecular level, thereby preventing the calcium-initiated SE blocking (Will *et al.* 2007). The initial watery saliva injection may inhibit protein coagulation/redox-dependent precipitation, thus preventing blockages (Miles 1999). Aphid watery saliva can also unplug blocked SE (Will *et al.* 2007). However, stylectomy showed that phloem flow eventually stop, usually faster in a resistant cultivar (Miles 1999).

Salivary enzymes: Neutralising potential signals and suppressing defences

Aphid saliva is thought to play a role in overcoming toxic phenolics, ROS, wounding and defence responses. Enzyme composition usually differs between the proteinaceous and watery saliva, though the same enzymes might be in both (Miles 1967; 1968; Miles & Peng 1989; Urbanska *et al.* 1998; Miles 1999; Cherqui & Tjallingii 2000). Hydrolases, involved in degrading CW polysaccharides and oxidoreductase, in disrupting the plant's redox balance, are usually part of aphid salivary enzymes (Miles 1999). Pectinases and other polysaccharide depolymerising enzymes could actually counter wound-induced HR by pre-empting plant pectinases, *i.e.* by converting pectin or pectin-derived signals/elicitors to non-functional messengers that do not induce defence responses. An increase in salivation while probing, without HR, supports this theory (Miles 1999). Aphid pre-conditioning of hosts suggests that watery saliva have a greater effect on the surrounding phloem (Miles 1999). These systemic effects could possibly be attributed to watery saliva movement though the vascular system (Schotzko & Smith 1991; Rafi *et al.* 1996; Miles 1999). Oxidases are likely candidates since they move faster than reducing systems can counter their action through sensitive tissue (Miles 1999).

Salivary enzyme composition have led to the "redox-hypothesis" (Miles & Oertli 1993). This hypothesis suggest that the cellular redox homoeostasis, *i.e.* the soluble redox couples like NADPH, AA and GSH, are regulated by the aphid's salivary enzymes (Miles & Oertli 1993; Foyer & Noctor 2009). Plant phenolics are usually maintained in a reduced state by antioxidants in the cell (*Figure 2.5*). During aphid probing, plant cells oxidise and mobilise phenolic compounds in response to wounding (Miles & Oertli 1993). Plants use polyphenol oxidases (PPO or catechol oxidase) in the plastids to catalyse the oxidation of phenols to quinines (He *et al.* 2009). Phenolic monomers, and their oxidised quinones, are either toxic or may act as feeding deterrents to aphids (Miles & Oertli 1993; Grayer *et al.* 1994). Quinones redox cycling also play a role in defence: reducing equivalents are redirected to superoxide (and hydrogen peroxide) production, which may form either directly or indirectly part of the HR response (Cape *et al.* 2006 & references within). The final oxidised products, *i.e.* polymers and protein-phenol conjugates, are non-toxic cell sealants (Miles & Oertli 1993). Effective sealing and defence response in plant cells demand a controlled rate of phenolic oxidation (*Figure 2.6*) (Miles & Oertli 1993). Aphid salivary enzymes alter the redox state of cells (or SE), thereby interfering with coagulation and oxidation reactions of the phenolic compounds and proteins. This leads to faster oxidation, unordered sieve plate sealing and a reduction in the defence response (Miles & Oertli 1993).
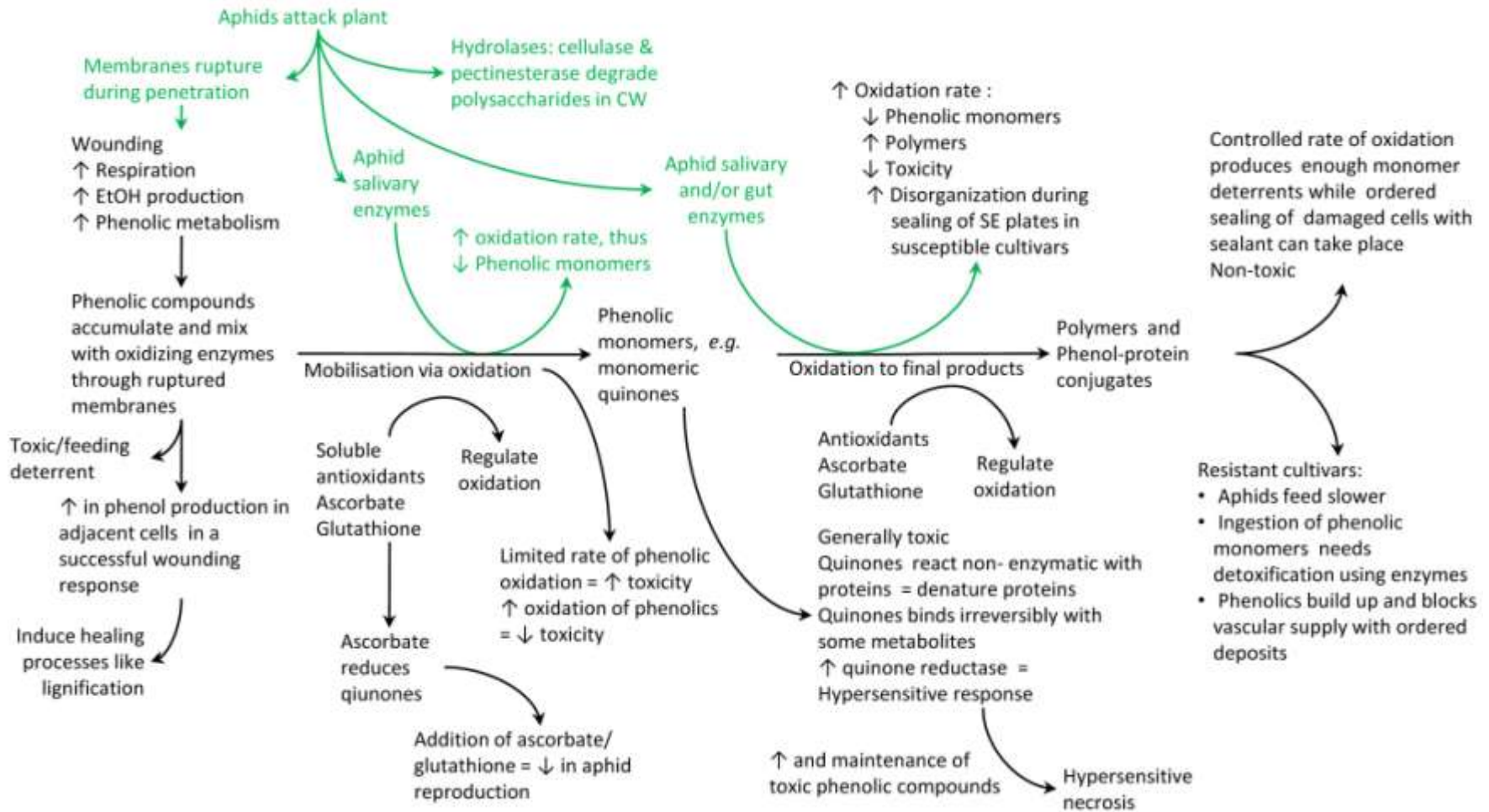
Figure 2.5 An overview of aphid feeding (green) and the responses of the host. Antioxidants in the plant regulate the redox condition of phenolic compounds in the cells by keeping them in a reduced form under normal cellular conditions, while regulating oxidation rates when wounded. Aphid salivary enzymes (green) alter the redox poise of the cell, thereby enhancing the oxidation of toxic phenolics/quinones into non-toxic phenol-protein conjugates and polymers. Refer to *Figures 2.6 & 2.7*. Compiled from Miles & Oertli 1993; Jarabak *et al.* 1997 and Ni *et al.* 2000.
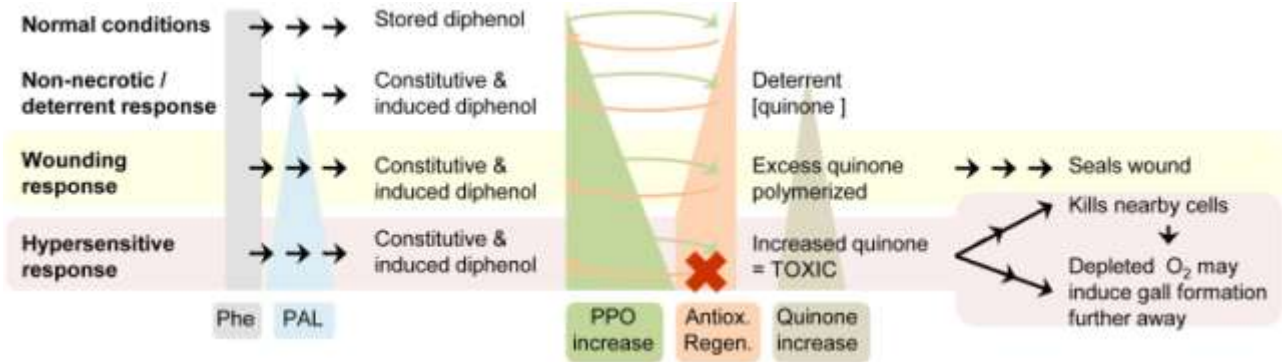
Figure 2.6 Plant defence responses during unsuccessful aphid infestation. Increases in expression or substrate of phenylalanine ammonia lyase (PAL) and polyphenol oxidase (PPO) lead to increases in quinones. The antioxidant regeneration system (Antiox Regen) regulates the conversion from diphenols to quinone, thereby keeping the supply of toxic compounds steady. When antioxidants are used faster than they can be replenished (X), quinones reach the HR level. Phe, phenylalanine. Also see *Figure 2.7*. Compiled from Miles & Oertli 1993 and Miles 1999.

Salivary enzymes of the RWA

Macerated RWA extracts showed no detectable polysaccharidase, phospholipase A, lipase or protease activity, but low levels of aminopeptidase (possibly from lysosomes) were observed (Fouche *et al.* 1984). In another study, total RWA extracts, confirmed with aphid head extracts, showed no amylase, pectinase or peroxidase (PX) activities, though cellulase, pectinesterase (PE), ascorbate oxidase (AO), superoxide dismutase (SOD), catechol oxidase (polyphenol oxidase, PPO) and catalase (CAT) activities were present (*Figure 2.5*) (Ni *et al.* 2000). The hydrolases (cellulase and PE) are involved with CW degradation while the oxidoreductase (AO, CAT, PPO and SOD) disrupts the plant's redox balance (Ni *et al.* 2000). Interestingly, CAT was only found in the RWA while PX was only present in *Rhopalosiphum padi* (Ni *et al.* 2000). The authors suggested that this difference in salivary composition could probably explain the different symptoms observed in susceptible hosts' in responses to these two aphids. CAT are probably more active as it needs no donor like PX when converting H_2O_2 to water and oxygen (Ni *et al.* 2000).

The possible effects of the RWA salivary enzymes on the plant are summarised in *Figure 2.7*. However, little is known about the role of aphid salivary AO. Recent studies on plant AO could possibly shed some light on the role of this enzyme in aphid-plant interactions.
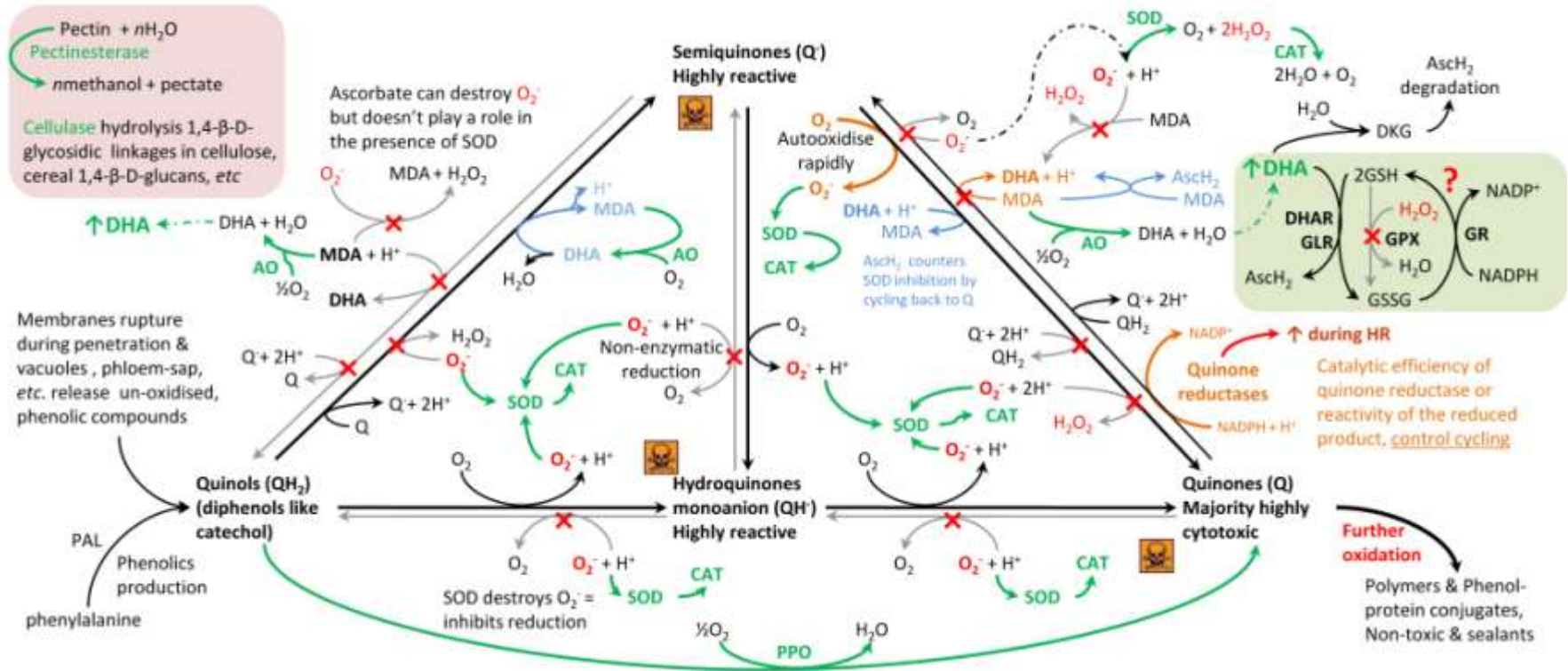
Figure 2.7 Known RWA salivary enzymes and their role in upsetting cellular and apoplastic redox poise in the host. In healthy tissue phenolics are kept in a reduced state by antioxidants and the continual reductase renewal. During wounding, membranes rupture and phenolics are released/produced. Quinone redox-cycling start (orange) and produce O_2^- and H_2O_2 resulting in ROS and HR. Quinone reductase transcription is up-regulated during this HR response, producing more ROS species. Toxic semi- and hydroquinones are also being recycled, thereby controlling the oxidation of quinones to non-toxic substances. Ingestion of un-oxidised phenolics/quinones, in the absence of the renewable antioxidants, autoxidise with proteins in the gut to form toxic substances. Salivary oxidoreductases shift the redox poise of the apoplast/cytosol and may even detoxify phloem sap *en route* to the gut. AscH$_2$, ascorbate; AO, ascorbate oxidase; APX, ascorbate peroxidase; CAT, catalase; DHA, dehydroascorbate; DHAR, DHA reductase; DKG, 2,3-diketo-l-gulonic acid; GR, glutathione reductase; GSH, reduced glutathione; GSSH, oxidised glutathione; GPX, glutathione peroxidase MDA, monodehydroascorbate; PPO, polyphenol oxidase (catechol oxidase); SOD, superoxide dismutase. (Miles & Oertli 1993; Jarabak *et al.* 1997; Miyake & Kurata 1998; Roginsky *et al.* 1999; Ni *et al.* 2000; Cape *et al.* 2006; Foyer & Noctor 2009); IUBMB Enzyme Nomenclature EC 1.6.5.5; Pfam PF01095; www.brenda-enzymes.org (EC3.1.1.11 – pectinesterase).

Ascorbate oxidase in plants is localised to the apoplast/CW (Pignocchi *et al.* 2003; Fotopoulos *et al.* 2006; Fotopoulos *et al.* 2008). The enzyme catalyses the oxidation of AA (AscH$_2$) to monodehydroascorbate (MDA) that disproportionate to AA and dehydroascorbate (DHA) (Fotopoulos *et al.* 2006; Fotopoulos *et al.* 2008; Foyer & Noctor 2009). Studies in plants showed that an increase in AO leads to a lower apoplastic AA (AA$_a$) *in vivo*, but has no effect on normal AA recycling gene expression (Pignocchi *et al.* 2003; Yamamoto *et al.* 2005; Fotopoulos *et al.* 2006). However, DHA levels increased though the overall AA, *i.e.* AscH$_2$, DHA, MDA, remained the same (Pignocchi *et al.* 2003). This suggests that the transport system could not cope with the high rate of AA oxidation, even though it tried to address the redox poise in the apoplast by increasing the total AA levels (Pignocchi *et al.* 2003). Plants with over-expressed AO subjected to oxidative stress, like an infection or environmental stress, also appear more sensitive to these stresses, with higher chloroplast loss and higher *in situ* H$_2$O$_2$ (Yamamoto *et al.* 2005; Fotopoulos *et al.* 2006; Fotopoulos *et al.* 2008). This suggests that an increased AO expression in plants reduce their capacity to up-regulate defences against ROS. Furthermore, Ca^{2+} channel expression decrease under higher AO expression levels, which could have major influences on signalling, elicitors, ROS-induced regulation, *etc.* (Fotopoulos *et al.* 2006). Over-expression of AO in tobacco plants led to partial stomatal closure, increase in water content, decline in peroxide-scavenging enzyme activities and an increase in leaf ABA levels (*Figure 2.6*) (Fotopoulos *et al.* 2008). The authors also suggested that AA$_a$ perceived environmental cues and then use DHA to regulate stomatal dynamics (Fotopoulos *et al.* 2008). Though all these results are based on over-expressed apoplastic plant AO, it does suggest a possible role for salivary AO in the host. The salivary AO could possibly suppress the wounding response initiated by Ca^{2+} movement/signalling, reduce the plants ability to launch defence responses and, depending on its location, could possibly play a role in diet enrichment by increasing AA. The increase observed in apoplastic H$_2$O$_2$ under higher AO levels is probably being addressed by RWA salivary CAT.

RWA-wheat interaction

Constitutive plant defences in resistant individuals cause the RWA to engage for longer periods in pre-penetration behaviour (Kindler *et al.* 1992; Webster *et al.* 1993). Epicuticular waxes are known to play a role in plant defences (Powell *et al.* 1999; Peeters 2002), however, cereal leaf waxes have little effect towards RWA resistance (Ni *et al.* 1998; Bahlmann 2002; Bahlmann *et al.* 2003). Furthermore, the average frequencies of leaf probing and the duration thereof, were similar in

both susceptible and resistant cultivars (Kindler *et al.* 1992). Conflicting results exist for trichomes and their influences on aphid feeding (Bahlmann 2002; Peeters 2002; Bahlmann *et al.* 2003). Trichome length, but not density, was shown to be directly linked to RWA resistance in some cultivars (Ni & Quisenberry 1997). Trichomes are usually located on leaf veins, possibly hindering aphids in finding their favourite feeding sites (Ni & Quisenberry 1997; Ni *et al.* 1998; Bahlmann 2002). However, the RWA was shown to have no preferential penetration sites or -patterns on wheat leaves but rather follows a randomised approach (Fouche *et al.* 1984).

The RWA prefers to feed from the thin walled phloem sieve tubes of the leaf basis and new growth, where the availability of photosynthetic assimilates are greater (Kriel *et al.* 1986; Botha & Matsiliza 2004). Aphids cease their feeding on resistant cultivars or non-host plants soon after the phloem element is penetrated, responses attributed to lectins binding to the chitin that surrounds the food canal and foregut of the aphid (Dixon 1998). However, other products, as part of the host defence response upon the recognition of a gene-for-gene interaction, might also deter aphid feeding (Kaloshian 2004; Botha *et al.* 2006; Smith & Boyko 2007). The average duration of salivation in feeding RWA was shown to be the same on both resistant and susceptible wheat cultivars, but less frequent in susceptible lines (Kindler *et al.* 1992). On non-hosts, the RWA salivates more and ingests less, taking up to four times longer to locate the phloem (Girma *et al.* 1992). RWAs also spend significantly longer time feeding on phloem of susceptible accessions than on resistant lines. Together with longer periods in the pre-penetration stage, these suggest that RWAs turned to non-phloem feeding for survival on resistant cultivars (Kindler *et al.* 1992). This hypothesis is also supported by a study of RWA feeding on barley (Webster *et al.* 1993).

There is a decrease in population size and an increase in alate female numbers when wheat becomes unsuitable for RWA feeding at the ear stage (see *Biology*) (Kriel *et al.* 1984; Walters *et al.* 1984). Aphid numbers peak at wheat growth stage 5, when photosynthetic rates are extremely high and products are directed towards the flag leaf and developing ears (Kriel *et al.* 1984). RWA "preconditioning" of its host over time, makes it easier to feed on the same plant for subsequent generations (Schotzko & Smith 1991; Rafi *et al.* 1996), thus two nymphs feeding on the same leaf results in faster development than if the two were to settle on different leaves (Rafi *et al.* 1996; Qureshi & Michaud 2005)

Feeding by the RWA causes leaf curling and the thickening of the epidermal cells in all lignin containing tissues (see *Symptoms*). No such reactions are induced by the same method of feeding as used by other aphids like *R. padi* and *S. graminum* (Fouche *et al*. 1984). Lignification after RWA feeding, as part of the HR, starts at the middle lamella and makes the CW impenetrable (Brett &

Waldron 1990; Mitchell *et al.* 1994; Mitchell *et al.* 1999). Initially, at these sites, it was hypothesized that a phytotoxin is injected that prevents the unfurling of the leaf (Smith *et al.* 1991). This phytotoxin was thought to be involved in the breakdown of the chloroplast (Fouche *et al.* 1984), with chlorophyll reductions of up to 85 % recorded (Kruger & Hewitt 1984), and thus the yellow streaking of the leaves. However, an ultra-structural study on the effects of RWA feeding on a susceptible barley cultivar, suggested that both xylem and phloem (parenchyma, thick-walled sieve tubes and companion cells) were extensively damaged during probing, thus leading to the typical RWA symptoms (yellow streaks, leaf rolling, *etc.*) (Saheed *et al.* 2007b). Similarly, severe RWA damage occurred to longitudinal vein phloem, including to most of the leaf phloem vascular system, of a susceptible wheat cultivar (Botha & Matsiliza 2004). Massive damage that can be observed as wound-related callose, is thought to re-route assimilates (with little or no transverse or longitudinal movement of assimilates) to form sinks at the aphid feeding sites (Botha & Matsiliza 2004). Furthermore, the RWA taps xylem to obtain water and in the process injects large amounts of watery saliva into the vessels. This saliva is electron-dense, smooth to amorphous, and lines the metaxylem thereby sealing the pit membranes between elements and those that connect the xylem vessels and xylem parenchyma (Saheed *et al.* 2007b). Callose further blocks the plasmodesmata of the phloem parenchyma elements and sieve tube-companion cells. A lot of the cells showed different degrees of plasmolysis which can be a result of the probing behaviour of the RWA that puncture cells. This results in oxidative stress with the occurrence of necrosis and chlorosis (Saheed *et al.* 2007b). The damage and sealing of the vascular system would interfere with apoplasmic and symplasmic transfer of water and nutrients, thus the white/yellow streaks and leaf rolling observations in RWA susceptible cultivars (Saheed *et al.* 2007b). These results are in contrast with an earlier study, conducted on *S. graminum* and the RWA, using isotope labelling (Burd 2002). Feeding of both aphids cause similar responses in the plant (Sandstrom *et al.* 2000). However, unlike greenbug, the RWA does not affect the rate of phloem loading and also has no or little influence on phloem translocation that occurs at the feeding site (Burd 2002). This suggests that the RWA feeding does not influence phloem function around the feeding site.

RWA feeding reduces chlorophyll fluorescence and photosynthetic rates of susceptible wheat and barley cultivars (Burd & Elliott 1996; Haile *et al.* 1999). A reduction in chlorophyll *a* and *b* content in susceptible cultivars, but not in resistant lines (Kruger & Hewitt 1984; Burd & Elliott 1996), or electron transport inhibition of the photochemical reaction centre (Miller *et al.* 1994; Burd & Elliott 1996), could explain these observations. Leaves treated with *D. noxia* extracts (2 h) showed that the chloroplasts initially lose their ordered arrangement next to the CW and are

Table 2.2 Morphological and physiological changes in cereals over time in response to RWA infestation.

Time interval (hours)	Susceptible cultivars infested	Resistant cultivars infested
2	Chloroplasts loosely ordered arrangement next to cell wall. Chloroplasts distributed throughout the cell (*in vitro*)[1]	
4	Chloroplast membranes disintegrate and the contents spread throughout the cell (*in vitro*)[1]	
5	Chloroplast homogenise and are no longer visible (*in vitro*)[1]	
6	Ethylene production begins (1800 nL h^{-1} g^{-1} fresh mass, control 400)[2]	Ethylene production begins (600 nL h^{-1} g^{-1} fresh mass (control 800))[2]
12	Ethylene production[2]	Ethylene production[2]
18	Ethylene production peak[2]	Ethylene production peak[2]
24 (1 d)		Chitinase activity is induced in TugelaDN, MolopoDN & BettaDN[4]
48 (2 d)	No increased inter- and intracellular β-1,3-glucanase activity observed in NILs: Tugela, Betta, Molopo[6]	Increased inter- and intracellular β-1,3-glucanaseactivity observed in *Dn1*-resistant cultivars: TugelaDN, BettaDN, MolopoDN, with 7 isoforms of the enzyme up-regulated intercellular which was background specific[6] Chitinase activity further increases for Tugela, Molopo & Betta[4]
72 (3 d)	Plasma lemma situated further from cell wall, convoluted appearance (*in vivo*)[1]	Ethylene production lower[2] Peroxidase activity starts to increase (TugelaDN,

	Ethylene production lowers[2]	MolopoDN & BettaDN)[4]
	Peroxidase activity starts to increase (Tugela, Molopo & Betta) [4]	Chitinase activity keeps increasing[4]
96 (4 d)	More prominent: plasmalemma situated further from cell wall, convoluted appearance. Some chloroplast membranes showed similar changes. Few grana were slightly swollen (*in vivo*) [1]	Ethylene patterns are the same till day 14[2] Peroxidase activity almost double that of the susceptible lines (TugelaDN, MolopoDN & BettaDN) [4]
	Reduction in chlorophyll a and b and total chlorophyll content[5]	Chitinase activity keeps increasing[4]
	Ethylene patterns are the same till day 14[2]	
	Peroxidase activity increases (Tugela, Molopo & Betta) [4]	
120 (5 d)	Wheat (average RWA host): Grana obviously swollen, frets showed swelling, chloroplast shape normal (*in vivo*) [1]	Peroxidase activity increases (TugelaDN, MolopoDN & BettaDN) [4]
	Chloroplast regarded as intermediately susceptible	Chitinase activity keeps increasing[4]
	Barley (good RWA hosts):	
	Convoluted cell membranes, swollen grana and fret disrupted, many osmiophilic globuli, many chloroplast disrupted completely	
	Chloroplast regarded as highly susceptible[1]	
	Oats (poor RWA hosts):	
	No changes in ultra-structure. Chloroplast regarded as resistant (*in vivo*) [1]	
	Peroxidase activity increases (Tugela, Molopo & Betta) [4]	
144 (6 d)	Chloroplast situated further from plasma lemma and cell wall. Grana-fret system swollen[1]	Peroxidase activity increasing (TugelaDN, MolopoDN & BettaDN) [4]
	Peroxidase activity increases for Molopo & Betta, levelling off for Tugela [4]	Chitinase activity peak for MolopoDN[4] Proteins (PR?) in IWF detected in resistant Dn1 lines

	Chitinases activity lowest for Molopo & Betta, peak for Tugela [4]	(upper 8cm used) (*in vivo*) [1]
	Induction of a 53 kD protein in Stephens and reduction of 47, 48 and 49 kD proteins [6]	
168 (7 d)	Chloroplast situated further from plasma lemma and cell wall, Grana-fret system swollen. Many osmiophillic globuli present (*in vivo*) [1] Strong burst of chitinase [2] Peroxidase activity increases for Molopo & Betta, decreasing for Tugela [4] Chitinases increasing for Molopo & Betta, decreasing for Tugela [4]	Peroxidase activity increasing (TugelaDN, MolopoDN & BettaDN) [4] Chitinase activity decreasing for TugelaDN & MolopoDN, increasing for BettaDN [4]
192 (8 d)	Double chloroplast membranes and plasma lemma extremely convoluted, Grana-fret system disrupted totally, many osmiophillic globuli present (*in vivo*) [1] Peroxidase activity increases for Molopo & Betta, decreasing for Tugela [4] Chitinases increasing for Molopo & Betta, decreasing for Tugela [4]	Peroxidase activity increasing (TugelaDN, MolopoDN & BettaDN) [4] Chitinase activity decreasing for TugelaDN & MolopoDN, increasing for Betta*Dn* [4]
216 (9 d)	No internal chloroplast definition remains (*in vivo*) [1] Peroxidase activity increases for Molopo & Betta, decreasing for Tugela [4] Chitinases increasing for Molopo & Betta, decreasing for Tugela [4]	Peroxidase activity increasing (TugelaDN, MolopoDN & BettaDN) [4] Chitinase activity decreasing for TugelaDN & MolopoDN, increasing for BettaDN [4]
240 (10 d)	Chloroplast membranes disintegrate, "osmiophillic globuli" present	Induction of chitinase [2] Peroxidase activity increasing for TugelaDN, peaking for

		MolopoDN & BettaDN[4]
	Purple (cyanidine containing cells) and white (bleached cells) streaking occurs on leaves—NO organelles left in cells (*in vivo*)[1]	Chitinase activity decreasing for TugelaDN & MolopoDN, peaking for BettaDN[4]
	Peroxidase activity increases for Molopo & Betta, decreasing for Tugela[4]	
	Chitinases increasing for Molopo & Betta, decreasing for Tugela[4]	
264 (11 d)	Reductions of 47, 48 and 49 kD proteins[6]	Induction of a 53 kD protein and reduction of a 47, 48 and 49 kD proteins[6]
288 (12 d)	Peroxidase activity increase for Molopo & Betta, decreasing for Tugela[4]	Peroxidase activity increasing for TugelaDN, decreasing for MolopoDN & BettaDN[4]
	Chitinases increasing for Molopo & Betta, decreasing for Tugela[4]	Chitinase activity increasing for TugelaDN, decreasing for MolopoDN & BettaDN[4]
336 (14 d)	Peroxidase activity still increase for Molopo & Betta, decreasing for Tugela[4]	Peroxidase activity increasing for TugelaDN, decreasing for MolopoDN & BettaDN[4]
	Chitinases peaking for Molopo & Betta, decreasing for Tugela[4]	Chitinase activity peaking for TugelaDN, decreasing for MolopoDN & BettaDN[4]

Compiled from: Fouche *et al.* (1984)[1], Nagel (1995)[2], Botha *et al.* (1998)[3], Van der Westhuizen and Pretorius (1996)[4], Burd and Elliott (1996)[5]; Van der Westhuizen *et al.*1998a[6]

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distributed through the cell content. After 4 hours (h) the chloroplast membranes started disintegrating and the contents spread throughout the cell, with no visible chloroplast left after 5 h (*Table 2.2*) (Fouche *et al.* 1984). RWA feeding seems to target the electron transport of photosystem II (PSII), leading to a decrease in the integrity of the thylakoid membrane system, which results in changes in the photosynthetic ability (Kruger & Hewitt 1984; Burd & Elliott 1996; Haile *et al.* 1999). However, Miller *et al.* (1994) observed no differences in PSII effectiveness of resistant and susceptible barley cultivars, but a decrease in the recovery of the quinone after illumination in susceptible cultivars. This suggests antioxidant involvement. Indeed, CAT (together with other oxidoreductases) in RWA saliva is thought to be responsible for the necrotic streaking observed in susceptible cultivars and the loss of chloroplast integrity (Ni *et al.* 2000). CAT, unlike PX, needs no donor when dealing with H_2O_2 and together with other oxidoreductases, they change the redox state of the cell, thus affecting the chloroplast electron transport chain (Ni *et al.* 2000). Rubisco subunits (LSU and SSU) also decrease in infected accessions (with the SSU more susceptible to RWA infestation) (Botha & van der Westhuizen 1992; Rafi *et al.* 1996).

RWA infestations also result in a proline increase in susceptible wheat lines faster than it does in resistant lines. However, during later stages, proline concentrations in resistant lines rapidly exceed and are maintained at higher levels than those of the susceptible lines (Botha & van der Westhuizen 1992). Moisture content observations of plants after RWA infection give conflicting results (Kruger & Hewitt 1984; Botha & van der Westhuizen 1992). Various other enzymes are also induced in resistant cultivars during RWA feeding (*Table 2.2 & 2.3*). RWA infestations also increase the accumulation of intercellular β-1,3-glucanase in resistant wheat cultivars (*Dn1* resistant cultivars), though cultivar dependent, but not in susceptible cultivars (Van der Westhuizen *et al.* 1998a; Mohase & van der Westhuizen 2002). Glucanases hydrolyse glucosidic linkages found in glucans, like callose (β-1,3,-glucan), which forms part of CW (Van der Westhuizen *et al.* 1998a). These CW glycoproteins can also activate plant defence responses. Indeed, glycoproteins in resistant wheat cultivars were shown to elicit defence responses after RWA infestation (Mohase & van der Westhuizen 2002). This suggest that RWA saliva could either release eliciting CW components in resistant cultivars (*via* salivary hydrolases) or could contain eliciting compounds that may activate defence responses, thereby resulting in increased PX, β-1,3-glucanase and PR-proteins, like chitinase, expression (Van der Westhuizen *et al.* 1998a; b; Mohase & van der Westhuizen 2002).

Studies on the apoplast of RWA infested cultivars showed many defence related genes up-regulated in the apoplasts of these cultivars after infestation (*Table 2.3*). The genetic background of cultivars may play a significant role in the expression of the same enzymes, as was shown for

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peroxidase and chitinase expression in the apoplast of the near isogenic lines (NIL) BettaDN and TugelaDN (Van der Westhuizen *et al.* 1998b). Intercellular washing fluid studies of TugelaDN revealed increases in chitinase activity in the symplastic tissue (Nagel 1995; Botha *et al.* 1998). Nagel (1995) showed these increases in the apoplast to be a result of endochitinase induction in TugelaDN. Eight chitinase isoforms were present in the Tugela and TugelaDN lines, with two extra chitinases induced after RWA infestation. A four-fold up-regulation of a third chitinase was also present in the resistant TugelaDN (Nagel 1995). Furthermore, ethylene induced a similar response to RWA feeding in the susceptible Tugela. Wounding had no effect on the chitinase activity in both lines. This indicated that ethylene and RWA infestations are similar in their effects on chitinase expression, but that wounding could not account for the observed inductions (Nagel 1995; Botha *et al.* 1998). Nagel (1995) further speculated that a chitosan-like substance could be responsible for the elevated chitinase expression observed after RWA infestation, with ethylene and salicylic acid (SA) acting as secondary elicitors. Both SA and ethylene are induced by chitosan (Nagel 1995). An elicitor-induced study, using phenylalanine ammonia lyase- (PAL), peroxidase-, chitinase- and glucanase-activities as measure, demonstrated that chitin and chitosan (and derivatives thereof) do elicit a non-selective defence response in 'Tugela' and 'TugelaDN' (Van der Westhuizen & Oberholster 1996). Lapitan *et al* (Lapitan *et al.* 2007a) confirmed the latter results when they showed that RWA specific responses were only obtained from the protein portion of aphid extracts, but not from chitin. This supports a gene-for-gene interaction with a protein elicitor (Lapitan *et al.* 2007a).

Table 2.3 Proteins present in the apoplast and vascular system with special focus on wheat.

Protein sizes	Enzyme and description
100 kD, 70 kD	Endoproteases (serine-like proteases)[1]
28-33, 22-24, 18.5-19.5 and 15.5-17 kD	Possible PR-proteins. From 3 cultivars with *Dn1* gene[2]
Unknown	Pathogenesis-related (PR) proteins[3]
36, 34, 27, 22 kD (apoplast) 61, 54, 49.5, 35,1 kD (vascular)	Chitinases (hydrolyse chitin) 36, 34 & 22 kD chitinases were induced in a resistant cultivar after RWA infestation[1,4,5,6]
Unknown	ß-1,3-glucanases[1]
100 kD, 70 kD, 63 kD, 55 kD, 40 kD fragments	No gluconase-6-phospate activity, caseinase activity or proteases activity[1]
Unknown	Peroxidase[6]

[1]Pinedo *et al.* (1993), [2]Van der Westhuizen & Pretorius (1996), [3]Bowles (1990), [4]Nagel (1995), [5]Botha *et al.* (1998), [6]Van der Westhuisen *et al.* (1998b).

Conclusion

The development of additional RWA biotypes have open new research areas in RWA-wheat research, especially on how new biotypes may develop in reaction to plant defence strategies. Additionally, RWA biotypes also allow us to investigate the RWA-endosymbiont interaction and the role of the endosymbiont(s) in aphid biotype development. The current theory on RWA-*B. aphidicola* interactions suggests that the RWA is increasingly less dependent on its bacterial endosymbiont, especially regarding essential amino acid production. Furthermore, increases in phloem leucine and tryptophan levels of susceptible wheat cultivars during RWA feeding, implies a highly specialized aphid-plant interaction with a lesser dependency on the endosymbiont. Since resistance wheat cultivars have lower RWA induced leucine and tryptophan levels, the RWA-*B. aphidicola* theory may suggests a change in the aphid that allows biotypes to induce higher levels of the required essential amino acids in resistant wheat cultivars as well. However, it was the aphid-bacterium symbiosis that first allowed phloem feeding. Therefore, if the aphid reverts back to utilizing *B. aphidicola*, which still has the means to produce the required essential amino acids, the RWA may feed on resistant cultivars. This hypothesis suggests that continuous selective pressures of resistant wheat cultivars could have selected for RWA biotypes with more beneficial essential amino acid producing *B. aphidicola* or

an advantages change in endosymbiont composition — in both cases sufficient levels of essential amino acids are produced to maintain a RWA population, and thus overcome host resistance. Furthermore, the various molecular studies on plant responses to RWA infestation have not yet given a definitive answer. Questions still remain on the details of the plant's responses to RWA infestation and how (if at all) different modes of resistance, *i.e.* antibiosis, tolerance and antixenosis, differ in their reaction to infestation. Currently, high-throughput array analyses are used in answering these questions. In order to obtain the differentially regulated genes with these methods, various statistical analyses are done on the datasets. The influences of different statistical normalization and background corrections methods on the dataset may have a large effect on the genes eventually deemed differentially regulated. This hypothesis is tested in the second part of this study.

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