Role of WRKY Transcription Factors in Tolerance of Winter Wheat *Triticum aestivum* to Aphid *Sitobion avenae* Infestation under Different Nitrogen Regimes

Rana Mohammed Alshegaihi

A Thesis Submitted for the Degree of Doctor of Philosophy

School of Natural and Environmental Science

Newcastle University

September 2018

Declaration

This thesis is submitted to Newcastle University for the degree requirements of Doctor of Philosophy in Biology. The research detailed within was performed during the period of 2014-2018, and was conducted in Newcastle University laboratories under the supervision of Prof Angharad Gatehouse and Dr Martin Edwards.

I hereby declare that this thesis is my own work and effort and that it has not been submitted anywhere for any award. Where other sources of information have been used, they have been acknowledged.

Rana Alshegaihi

Rana

بسم الله الرحمن الرحيم

In the name of God, the Most Gracious, the Most Merciful

To my parents, Noura and Mohammed Alshegaihi for their endless love, support and encouragement.

Abstract

The English grain aphid (*Sitobin avenae*) is one of the most damaging pests of wheat (*Triticum aestivum*), causing damage both by the abstraction of nutrients and, more importantly, as a vector of viral diseases. Changing agricultural conditions are leading to insect pests becoming a more serious threat to sustainable crop production and therefore understanding the molecular basis of endogenous tolerance to aphid infestation will help mitigate shortfalls in global crop yield. Limiting nitrogen input in wheat increases aphid tolerance, but reduces yield. The present study investigated the response of two commercial winter wheats (*Triticum aestivum*, Var. Cordiale and Grafton) to biotic (*Sitobion avenae*) and abiotic (nitrogen) stress. Different growth measurement parameters, including plant height, leaf area, chlorophyll content, NO_3^- ion accumulation and relative water content (RWC) were positively correlated with nitrogen level (Chapter 2). Both wheat genotypes exhibited significantly ($p < 0.01$) greater levels of resistance to aphids at low levels of nitrogen input (2.25 mM) than at intermediate (5.25 mM) or high (7.5 mM) nitrogen, with aphid fecundity reduced from 58 and 61 to 19 and 32 nymphs per adult for Cordiale and Grafton, respectively (Chapter 2). The role of changes in expression of genes for WRKY transcription factors in the stress response was studied in Var. Cordiale over time by RT-qPCR. *TaWRKY3* showed large changes in gene expression under aphid and nitrogen stress, suggesting a novel role for this TF in stress (Chapter 3). At 7.5 mM nitrogen maximum expression of *TaWRKY3* occurred 6 h after exposure to aphids, returning to basal by 9 h. At 5.25 mM nitrogen, expression occurred earlier and at higher levels than at 7.5 mM nitrogen, but again returned to basal at 9 h. The lowest nitrogen supply resulted in the same rapid onset of gene expression but the magnitude of the response (4-fold) was higher than with high nitrogen. In addition, the response was maintained for a longer period. To investigate the role of WRKY3 in the stress response TILLING lines with mutations to the *WRKY3* gene were grown under dual stress. As in the control plants, aphid fecundity on most mutant lines was greater at 7.5 mM than at 2.25 mM nitrogen. However, the mutant 1996 was more resistant than the WT at 7.5 mM nitrogen, and showed no difference between high and low nitrogen, suggesting that WRKY3 may play a role in the link between nitrogen stress and aphid tolerance (Chapter 4). In control plants, concentrations of jasmonic acid (JA) isomers increased as a result of aphid infestation, whereas the concentration of salicylic acid (SA) fell and there was little change in abscisic acid concentration. In the mutant lines, the SA concentration was initially lower than in control plants but increased in 1ine 1996, the concentration of SA was relatively high in line 1171, and the concentration of JA isomers was initially higher than in control plants, increased at 3 h, then decreased (Chapter 4). Protein-DNA interaction assays showed binding of the Wild Type WRKY3 protein to W-box elements (*TaPR1-23* flanking sequence, *PcPR1-1* promoter and synthetic) and that the mutation in TILLING line 1996 disrupts binding (Chapter 5). Regulation of *PR1* gene expression is important for activation of plant defence responses. The present work suggests that TaWRKY3 may regulate this response through binding to W-box elements in *PR1* genes. The data suggest that low nitrogen conditions may prime the defence of wheat against insect attack via a regulatory network of WRKY transcriptions factors. These results provide new knowledge and insight to help inform the effort to produce crops able to be grown under reduced nutrient input.

Acknowledgements

Above all, I am thankful to God the Almighty who grants me this unique opportunity to learn and to proceed successfully. This thesis is in its current form due to the assistance and guidance of many people.

First and foremost, I am incredibly grateful to **Prof. Angharad Gatehouse** for her helpful suggestions, her valuable guidance and without whose input and knowledge none of this would have come to pass. I will also forever grateful to **Dr Martin Edwards**, who thought me everything I needed to know and for huge support and patience.

My sincere gratitude goes to **Dr Jack Lee**, for his listening, offering me advice and supporting me through this entire process. Thank to **Ms Gillian Davison** for her guidance in using some of the equipment in the lab.

The team of the molecular lab in Devonshire Building have given power to my personal and professional experience at Newcastle. Further thanks must go to my best friends, **Baida Alshukri** and **Dr. Iman Nouioui** for her always support and to **Aishah Alwahaibi, Abdulrahman Alhudhaibi, Aishah Alatwi, Saddam Baba**, **Alistair Poll**, **Uma Kupsamy** and **Alanoud Alfagham** for excellent help and encouragement. Thanks to all group members.

This thesis wouldn't be possible without the love, support and blessings of my parents **Noura** and **Mohammed**, my sisters **Nouf**, **Reem**, **Nada** and **Salha**, and my brother **Abdullah**; I dedicate this achievement to them. I cannot thank you enough for all the support and love you have given me. And finally my greatest thanks go to my brother **Fahad**, no words can truly express the level of gratitude and appreciation I have for you. Thank you for continued support and love, especially during the worst moments.

This thesis is funded by King Abudulaziz University Saudi Arabia for Scientific Researcher.

Rana

Table of Contents

List of Figures

List of Tables

List of Abbreviations

ABA	Abscicis acid
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
FAO	Food and Agriculture Organization of the UN
JA	Jasmonic acid
MAPK	Mitogen activated protein kinases
PR	Pathogens related protein Pathogen-Associated
PAMP	Molecular Pattern
RT-qPCR	Real time quantitative polymerase reaction
RWC	Relative water content
SA	Salicylic acid
TFs	Transcription factors
TW	Turgid weight
M	Milligrams
mg	Moles/ litre
min	Minutes
ml	Millilitre
µl	Microliter
N	Nitrogen
EDTA	Ethylenediaminetetraacetic acid
ETI	Effector-triggered Immunity
HR	Hypersensitive Response
WT	Wild type
TaWRKY	Triticum aestivum WRKY transcription factor
mTaWRKY	Mutant Triticum aestivum WRKY transcription factors
AtWRKY	Arabidopsis thaliana WRKY transcription factor
ng	nomograms
FAO	Food and Agriculture Organisation
HAMPs	Herbivore associated molecular patterns
ETI	Effector-triggered immunity
HR	Hypersensitivity response

Chapter 1. General Introduction

1.1 Challenges to improving crop tolerance

Cereal crops are a major staple of people's diets across the world, and the continued cultivation of such crops is a major factor in a country's food security. The definition of food security by the Food and Agriculture Organization (FAO) is "having sufficient access and availability to healthy food that is nutritious and helps people to stay fit and active" (Fig.1-1) (Rahimifard *et al.*, 2018).

Although standards of living have risen in many countries with the increase of globalisation, there are still significant threats to food security. As the world's population is set to reach nine billion by 2050, an increase of 2.3 billion from current levels, demand for food will inevitably increase, by as much as 70%, which will mean that yields from agriculture will have to rise by 50% to meet the higher demand (FAO, 2015b; Adenle *et al.*, 2018). Another factor to take into consideration is the amount of available land for crop production, which has been decreasing, meaning that production on existing agricultural land will have to intensify. An important aspect of this is efficient irrigation, which has raised productivity on a quarter of the world's agricultural spaces (Rufin *et al.*, 2018). If there is better access to water, intensification of production is easier to achieve because fallow spells are shorter and the land can be cultivated more often (Gaur *et al.*, 2008; Domènech, 2015). A further threat is that of climate change, which entails an unknown capacity to endanger the production of food across the world (Schleussner *et al.*, 2018). Existing research has noted the effect that climate change has already had on crop yields; the most spectacular of such effects have been droughts and heat waves (Moore and Lobell, 2015; Lesk *et al.*, 2016). Climate change does not just affect crop productivity; it also adversely impacts the metabolism and physiology of plant life, the richness of soil and the microbial constitution of plants; it may alter the primary metabolic defences of plants and their ability to combat stresses, both biotic and abiotic (Noctor and Mhamdi, 2017).

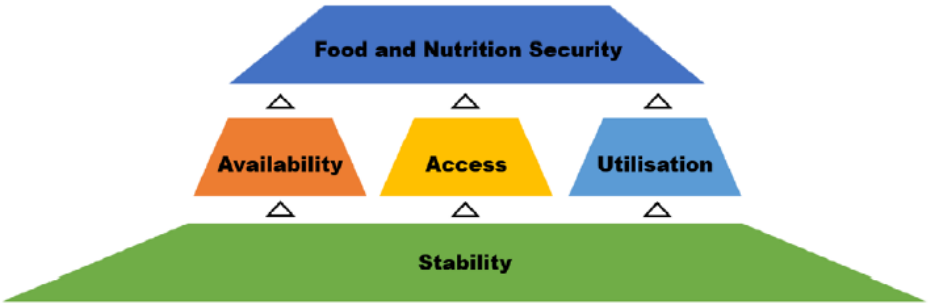


Figure 1-1. The UN Food and Agricultural Organisation FAO (2017c) has drawn up a list of what it considers to be the four pillars of global food and nutrition security. These are as follows: 1) availability: placing focus on production of sufficient quantities of food; 2) access: concerns over access to food in low-income families; 3) utilisation: feeding practices; and 4) stability: how likely it is that food will be available (Rahimifard *et al.*, 2018).

In practice, crops sustain several different stresses, whether biotic or abiotic. The factors involved in environmental or abiotic stresses include salinity, drought, high temperatures, stress from oxidation and a deficiency of nutrients. These can lead to a reduction in yield of > 50% (Wang *et al.*, 2003). Biotic or biological stresses can result from pests, pathogens and insects that are herbivorous, causing losses of between 10 and 20 per cent (Ferry *et al.*, 2004). Working to improve the way that plants counter stress and still yield a good harvest is a key feature of food security worldwide, and involves the understanding of the molecular processes involved, as regards both genetic manipulation and traditional agriculture (Takeda and Matsuoka, 2008). Genetic improvement through breeding is one especially favoured technique for making crops more resistant to stress. Genetic engineering (GE), or genetic modification (GM), has expanded the possibilities for crops to produce greater yields in challenging environments and has been shown to be an asset to world food security (Ferry and Gatehouse, 2009). Understanding regulatory molecular networks that help plants cope with various levels of stress – and the different interactions between stresses – will provide targets and routes that might be exploited.

Most agriculture across the world sustains damage from pests and pesticide chemicals are often applied to combat them. As resistance to agrochemicals increases among pests and pathogens, it is essential that we develop new ways of controlling them, so that future losses can be minimised. An alternative approach is to enhance endogenous host-plant resistance, but the effectiveness of this is not certain, because of the lack of information about which

genes confer resistance. To make control systems more effective, it is better to ascertain which genes have a controlling influence on the yield and concentrate on which molecular procedures are prominent in the interactions between plants, insects and pathogens. In order to do so, we must gain a deeper understanding of plant-insect interactions and in respect of this study, wheat-aphid interactions.

1.2 Wheat as a crop

Over thousands of years, wheat has been one of the most prolific crops in the world (Shiferaw *et al.,* 2013). Over 730 million tonnes are produced each year and the amount increases year on year. It is the third most prolific crop, behind rice and maize, and it amounts to an average of 20% of people's daily protein and calories (Shiferaw *et al.*, 2013). However, to meet future demand and to heighten world food security, the annual harvest needs to rise by 70% (FAO, 2015b).

The type of wheat that is produced most is bread wheat (*Triticum aestivum*), which accounts for 95% of wheat production and is able to flourish in numerous different climates. The genome size is around 16,000 Mb in hexaploid species (6x) which have 21 pairs of chromosomes (2n = 42), comprising three different ancestral genomes (termed A, B, and D) with seven chromosome pairs in each genome forming seven homoeologous groups (AABBDD) (Fig.1-2) (Petersen *et al.*, 2006). This happened because of polyploid hybridisation (Uauy *et al.*, 2009).

Wheat production is crucial in meeting world food needs, and agriculture must be sustainable on an ongoing basis in order to guarantee food security. Different strains of wheat and rice must be developed that generate increased yield, quality and stability, and have greater capacity to combat biotic and abiotic stress. To do this there has to be progress in genetic science that must amount to nothing less than a paradigm shift. The genome sequence of wheat can be used as a reference in developing and breeding new varieties of wheat, and this genetic knowledge can also be adapted for other major crops like rice, sorghum, and maize (International Wheat Genome Sequencing, 2014).

Wheat is important to Great Britain. It yields more tonnage per hectare than any other cereal crop, constituting 67% of the UK's production of cereals (DEFRA, 2015). There are numerous ways to raise the 'agricultural footprint' to help increase the crop yield, and this

will be a big part of future food strategy. As part of this question, we also need to look at the availability of nitrogen.

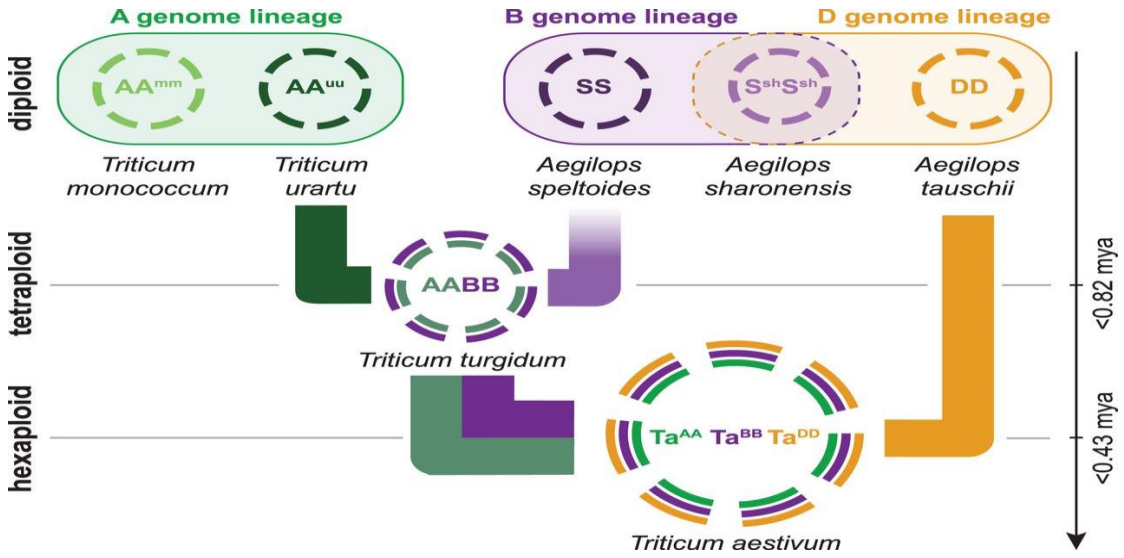


Figure 1-2. Schematic diagram of the polyploidisation within different wheat genomes.
The circles enclose the names and nomenclature for the genomes and provide a schematic representation of the chromosomal complement for each species to produce allohexaploid *Triticum aestivum* (2n=6x=42, AABBDD), which includes bread wheat (International Wheat Genome Sequencing, 2014).

1.3 Effect of stress on crop production

1.3.1 Effects of nitrogen stress in plants

Particularly costly methods employed by farmers to achieve target yields for their crops are fertilisers made from chemicals. In 2018, the amount of fertiliser being deployed on arable land was around 200 million tons globally (FAO, 2019). One of plants' most vital nutrients is nitrogen (N). As the population of the world swells possibly by two to three billion by 2050, the need for suitable agricultural land and nitrogen-based fertilisers will increase markedly (Zhang *et al.*, 2015). Increased numbers of plants will also mean more pathogens and insects, so careful stewardship of nitrogen stocks will be essential to service a rising population while also looking after the environment and people's health levels.

Nitrogen shortages negatively affect plant sustainability or even survival, so to enable good yields of crops, large quantities of nitrogen fertiliser are needed (Frink *et al.*, 1999). One of the major disadvantages from the over-use of nitrogen fertiliser has been resultant environmental impacts, such as nitrate contamination in groundwater (Socolow, 1999). It is therefore very important to ascertain plant tolerance systems and how they respond to low inputs of nitrogen fertiliser; when this is understood, it may be possible to reduce levels of nitrogen fertiliser. At present, plants have responded adaptively to low N levels by restricting growth, reducing photosynthesis, moving N to new organs and accumulating anthocyanins in large quantities (Ono *et al.*, 1996; Ding *et al.*, 2005; Diaz *et al.*, 2006). This is the basis of the Defence vs Growth hypothesis, whereby the growth-differentiation balance (GDB) structure combines theories of life history and phenotypic theories, which come together to create an overarching system that elucidates and predicts plant defence procedures and all the competing interactions in terms of ecology and evolution. The principle is that the plant has a finite amount of energy available to it, which can be distributed either towards growth or towards ensuring it is well prepared in the case of attack by pests or pathogens. The whole GDB idea of how plants defend themselves is based on a balance between the mechanisms of growth and differentiation. Treatments which increase the expression of defence related genes have been shown to down-regulate those associated with growth. The way in which these physiological trade-offs interact with competition, abiotic stress and herbivory illustrates how plants have evolved their defence mechanisms (Herms and Mattson, 1992).

Both plants and herbivorous insects respond to the process of nitrogen fertilisation, and the physiological and molecular bases of these responses remain elusive (Gao *et al.*, 2018). How the grain aphid's (*Sitobion avenae*) fecundity and associated regulatory signalling routes affect the way it feeds on commercial wheat cultivars at varying levels of nitrogen availability has been explored in this study. The level of tolerance of wheat to aphids is an important ongoing economic problem.

1.3.2 Impact of aphids on crop production

Economically, aphids (Hemiptera) are among the most important agricultural pests due to their particular feeding habits and high capacity for reproduction (Guerrieri and Digilio, 2008). Because of parthenogenesis and vivipary, aphids can multiply very quickly (Agarwala *et al.*, 2012), and they migrate and disperse over huge distances (Ali *et al.*, 2018). It is estimated that across the world hundreds of millions of dollars are lost annually due to aphid damage (Morrison and Peairs, 1998; Blackman and Eastop, 2000). In our quest to develop innovative approaches to pest control, it is important that we have an in-depth understanding of the impacts of aphids impact on their hosts. Such knowledge should enable us to exploit the insect resistance mechanisms that already exist within plants, thus facilitating successful breeding strategies.

Sitobion avenae (Fabricius) (Fig. 1-3), commonly known as the English grain aphid, constitutes one of the most serious pests of wheat in the UK. Crop losses can run between 20% and 80%, through the aphids feeding on phloem sap and the transmission of plant viruses such as barley yellow dwarf virus (BYDV) (Fereres *et al.*, 1988; Blackman and Eastop, 2000; Zhang *et al.*, 2018).

Figure 1-3. The English grain aphid (*Sitobion avenae*). Source: Influentialpoints.com (2018).

The feeding mode of aphids is highly specialised, and not like most other insect pests: they consume plant phloem by inserting a stylet in the cells. From here their stylets need to puncture the epidermis, the mesophyll and the vascular tissue, penetrate the phloem in a vascular bundle and select a suitable sieve element. The instant that the aphid comes into contact with the plant tissue, it secretes saliva containing effectors into its host to manipulate host cell processes and promote infestation. This carries on during the process of probing and feeding. Several authors have highlighted similarities between the roles of aphid effectors and the ways in which plant pathogens infect plants (Moreno *et al.*, 2011; Jaouannet *et al.*, 2014). To achieve this, effectors or saliva proteins are placed within the host, where they carry out a number of different tasks. As the effectors start to work, the plant's resistance may be activated, but several effector proteins have been shown to raise or lower virulence, while the activation or restraining of the defences of a plant may result from the effectors that the aphids' saliva holds (Fig. 1-4).

The way in which plants defend themselves against aphids is modified by viruses that the aphids transmit (Nalam *et al.*, 2019). Several distinct elements play their part in how effective aphids are as plant virus vectors, including: (i) the way in which a number of aphid types are polyphagous, such as *Myzus persicae*, which enables them to feed on numerous plant hosts, which is a major factor in spreading viruses amongst a wide range of species of plants; (ii) the capacity to reproduce parthenogenetically, thereby allowing masses of offspring to be produced very quickly; (iii) the existence of a stylet with a point like a needle, that can puncture the walls of plant cells and thus impregnate the host cell with viruses. The aphid's capacity for acting as a vector will be influenced by the way in which it chooses a host plant and the way in which it feeds on the host plant. How much dissemination of the virus is affected – positively or negatively – as a result of these three elements is dependent on the

individual virus and the manner in which it is transmitted. In terms of applied research, developing comprehension of the ways in which viral diseases are disseminated needs an appreciation of the constituents of the vector and how it behaves; the dissemination of vectors is fundamental in epidemiology (Ng and Perry, 2004).

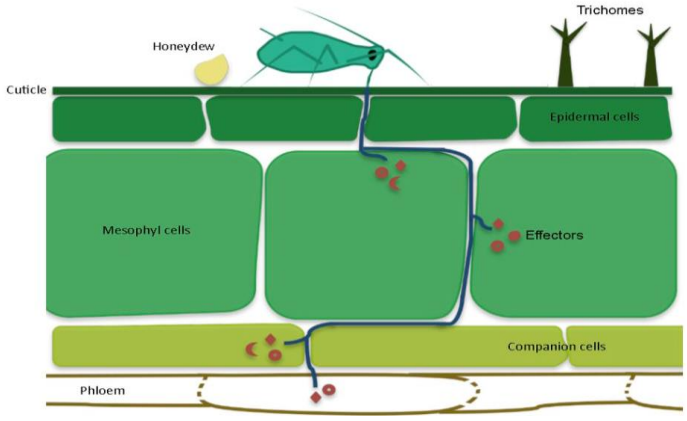


Figure 1-4. The pathway of the plant-aphid interface. The mouthparts of the aphid are not affected by plant defences such as wax and trichomes, and are able to penetrate the surface of the leaf.
The aphid stylets probe in order to find the phloem. Most cells along the stylet pathway are punctured, including the phloem cells. The aphid secretes saliva (which contains effectors) into the different cells and the apoplast. Honeydew is also secreted onto the surface of the leaf, which may act upon the plant's defence responses (Jaouannet *et al.*, 2014).

1.3.3 Current strategies for control of phytophagous insect pests

The harm caused by aphids is frequently combated by insecticides that are chemical-based, but these insecticides pollute the environment (Ali et al., 2018). Additionally, the use of these treatments over a long period has increased insects' capacity to resist them (Sonoda and Igaki, 2010; Xu *et al.*, 2017). The life of a grain aphid is short (Nalam *et al.*, 2019) but highly fecund, and they proliferate rapidly, which makes it hard to contain their infestation of plants (Xu *et al.*, 2017). Additionally, the insecticides used to control aphids are usually harmful to the various predators and parasitoids that specifically attack aphids, as well as to polyphagous predators, which indicates that a more cost-effective and environmentally friendly method of control may be to use crops that exhibit natural resistance (Stoger *et al.*, 1999). Furthermore, currently, consumers increasingly want organically grown food and crop produce that is free of residues, which drives a need to understand the natural mechanisms of insect resistance.

Genes of *Triticum turgidum*, *T. tauschii*, *T. speltoides*, *Secale cereale* (rye) and related species have been transferred across species effectively by natural crossing (Friebe *et al.*, 1995; Saidi and Quick, 1996). Through surmounting the obstacle of crossing between more distantly

related species, transgene technology provides additional places from which to source resistance genes and thereby provides an alternative method of long-lasting protection for crops (Gatehouse *et al.*, 1993). There have been many useful developments in the production of insect-resistant crops, such as those that express Bt endotoxins (Ferry *et al.*, 2004; Gatehouse *et al.*, 2011).

A particular and recent focus in research on genetic manipulation for the control of aphids is RNAi (plant-mediated RNA interference). Xu *et al.* (2014), for instance, utilised plant-mediated RNAi in wheat to target carboxylesterase (CarE) activity in the grain aphid via knockdown in gene expression, thus rendering the aphids less tolerant to the organophosphate pesticide phoxim and considerably less fecund.

1.4 Plant induced defence responses

Another approach currently being explored for crop protection is to enhance endogenous defence through improving our knowledge as to which genes in the crop are differentially expressed in response to aphid infestation. This strategy would form part of an integrated pest management method, which could be both cost-effective and effective.

A number of wound response and plant defence pathways are instigated by herbivores; such pathways create particular signals, or elicitors, that stimulate both synthesis of volatile compounds and changes in gene expression (van de Ven *et al.*, 2000; Gatehouse, 2002). Phloem-feeding insects that pierce or suck come into contact with plant cells on a continuous basis, activating defence mechanisms. Existing research suggests that the defence-alerting pathways that are induced by such insects are similar to those induced by pathogens of a bacterial, fungal or viral nature (Walling, 2000). The initial responses for plant defence against insects are illustrated in Fig. 1-5.

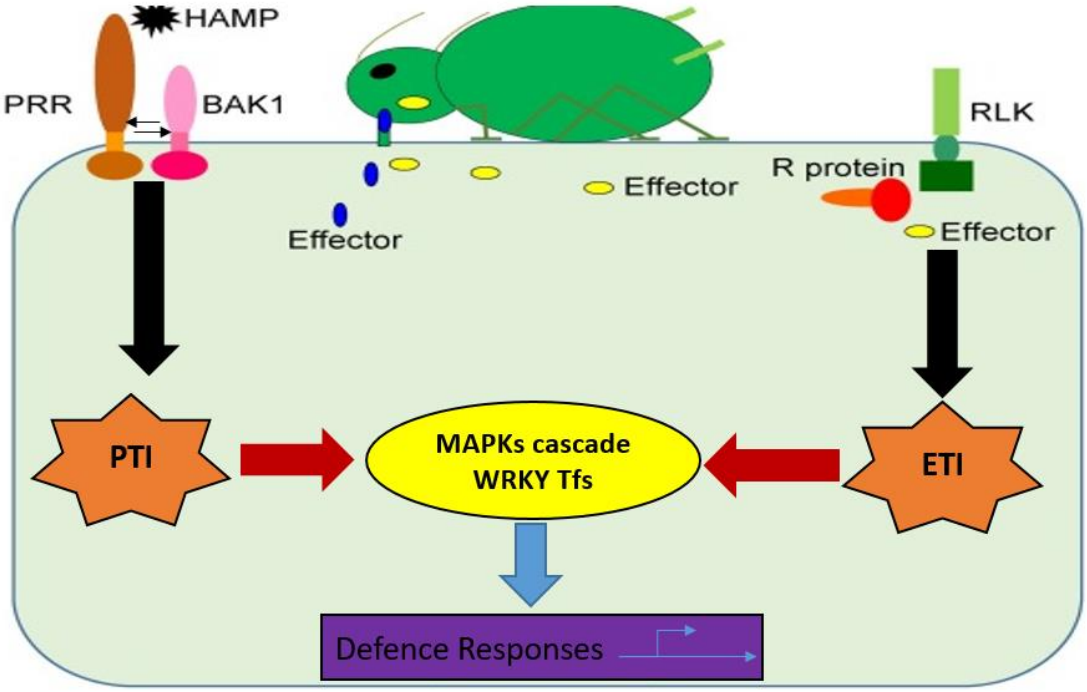


Figure 1-5. A model of plants' induced defence responses to aphids. Plant cells use plasma membrane-localised pattern recognition receptors (PRRs) to perceive conserved herbivore-associated molecular patterns (HAMPs) to activate pattern-triggered immunity (PTI). Effectors secreted into the host plant's cytoplasm interact with targets to modulate host cell processes. Aphid whole body extracts can stimulate plant defences. Bcl-2 homologous antagonist killer (BAK1), which functions as a co-receptor interacting with PRRs in PTI, is required for the induction of defences by aphid extracts. These include the formation of callose and reactive oxygen species (ROS) production. Aphid feeding also induces a signalling cascade, a MAPK pathway, which promotes the expression of genes to activate defences. In Effector-Triggered Immunity (ETI), effectors are recognised by the changes they cause to their target protein or by direct recognition. Plant can respond with more transcriptional changes which confer the Effector-Triggered Immunity (ETI). Changes in gene expression are mediated by the action of distinct transcription factors such as WRKY TFs, which in ETI can be triggered by effector activation of R-proteins. Figure adapted from Jaouannet *et al.* (2014); (Nalam *et al.*, 2019)

1.4.1 Recognition

Some of the systems whereby plants combat pathogens, which are described by the gene for gene model, which subsequently evolved into a multi-layered zig-zag model (Jones and Dangl, 2006), resemble the interaction between plants and herbivores (Hogenhout and Bos, 2011; Kaloshian and Walling, 2016). Following such models, the receptors in the plant that govern immunity detect elements that are derived from pests, a process that leads to immune reactions being evoked. Pattern recognition receptors (PRRs) have been developed by plants to detect molecular structures that are retained over a large group of organisms. Damage-associated molecular patterns (DAMPs) are endogenous molecules that the plant generates after infection; they are also recognised by PRRs as being elements that kick-start defensive reactions (Boller and Felix, 2009). Effector proteins can help pathogens to bypass such innate

immune responses because they subdue PTI when they are transported into the host cell. Disease resistance (R) proteins in certain plant genotypes detect pathogen effectors, which leads to effector-triggered immunity, or ETI (Jones and Dangl, 2006). Although some researchers consider the PTI/ETI model to be too simplified (Thomma *et al.*, 2011), conclusions have been able to be made concerning precise recognition in the interactions of plants and pathogens because relevant ligands and receptors have been identified molecularly: PTI is generally based on the non-specific detection of standard microbial molecules, while very pathogen-specific compounds are the trigger for ETI (Fig. 1-6) (Dodds *et al.*, 2006). One protein that induces PTI to aphids is the GroEL protein of the endosymbiotic bacterium *Buchnera aphidicola*, which has been identified in aphid saliva and is recognised by a multitude of plant hosts. The Bcl-2 homologous antagonist killer protein (BAK1), which is a co-receptor in PTI, is also needed in GroEL-induced resistance to aphids. GroEL induces callose deposition and an oxidative burst, suggesting that some molecular components are shared between plant defences elicited by pathogens and aphid-associated microbes (Chaudhary *et al.*, 2014).

The ways in which some highly resistant plants react to attack by aphids resemble the reactions to pathogens; this is called a gene-for-gene interaction, the reactions being founded on aphid-derived elicitors, the signalling molecule SA mediating the reaction (Smith and Boyko, 2007). Another reaction is that genes can be up-regulated from wounding as a result of cross-talk. Experiments by Moran and Thompson (2001) showed that genes induced by salicyclic acid (SA) were expressed when green peach aphids (*Myzus persicae*) fed on *Arabidopsis*; these genes are linked to reactions to pathogens, in addition to those genes that are engaged in the jasmonic acid mediated response pathway (see section 1.4.4). The experiment's outcomes indicate activation of pathways involved in responses to herbivores and pathogens. The plant's reactions to aphid herbivores demonstrate widespread reprogramming of gene expression (Moran and Thompson, 2001).

Insects, as opposed to pathogens, are extremely intricate organisms: they are multicellular, with diverse lifestyles and modes of behaviour. The plant may use the cues that result from these patterns to detect danger from herbivory and activate suitable defensive strategies (Erb *et al.*, 2012). Chemical defences such as defensins, various antimicrobial compounds and a broad span of enzymes accumulate in defensive reactions to hinder pathogenesis or combat consequent stresses in the plant cell (Freeman and Beattie, 2008). For example, plant chitinases, which are known to be involved in plant-pathogen interactions, show promise as a

low-cost biotech application for controlling fungal pathogens; furthermore, since insect cuticle is composed of chitin, this approach may also be viable for aphid control using chitinase-producing fungi such as *Trichoderma* as biological control agents (BCAs) (Chavan and Deshpande, 2013). Another approach is to express genes encoding these enzymes in plants for greater tolerance to a number of biotic stresses.

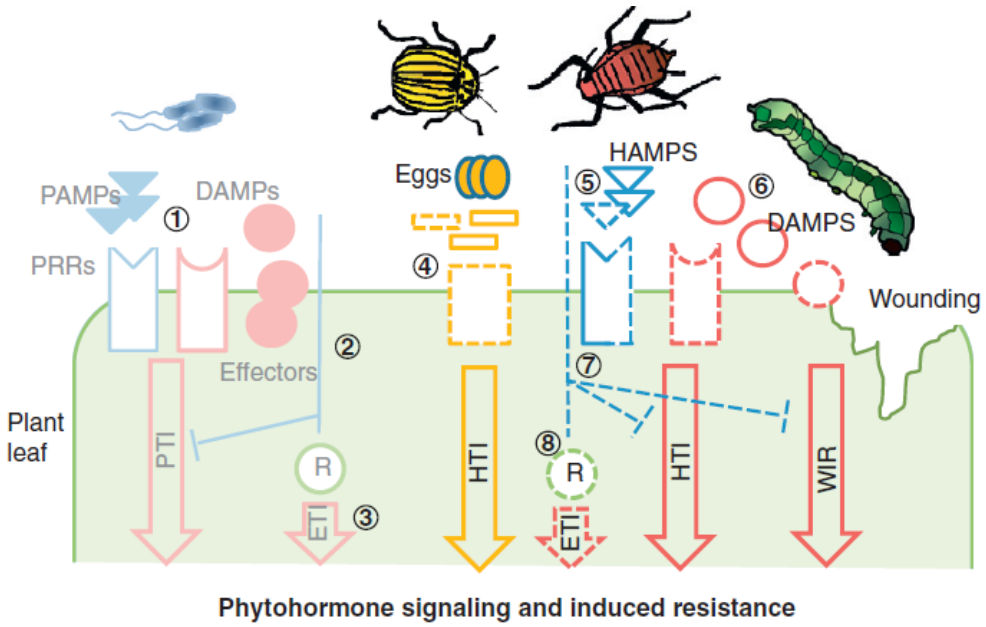


Figure 1-6. Plants' molecular recognition of pathogens and herbivores. **1**. Microbe-, pathogen- and damage-associated molecular patterns (MAMPs, PAMPs and DAMPs) are recognised by pattern recognition receptors (PRRs), resulting in PAMP-triggered immunity (PTI). **2**. PTI is suppressed by pathogen effectors. **3**. Effectors are recognised by resistance gene products, resulting in effector-triggered immunity (ETI). **4**. Unknown receptors recognise oviposition-associated compounds, which activate defensive responses. **5**. Receptors recognise putative herbivore-associated molecular patterns (HAMPs), which results in herbivore-triggered immunity (HTI). **6.** DAMPs are released by wounding, which also triggers wound-induced resistance (WIR). **7**. Suppression of HTI and WIR can be caused by insects' effector-like molecules. Broken lines denote uncharacterised elements (Erb *et al.*, 2012).

1.4.2 Insect Effectors

Insect effectors are instrumental in the majority of interactions between plants and insects, in adaptation to PTI (PAMP triggered immunity) and ETI (effector triggered immunity) (Elzinga and Jander, 2013; Rodriguez and Bos, 2013). With regard to aphids, scientists have discovered a number of possible salivary effector proteins that have the capacity to regulate plant defence, although only a small number have been characterised in terms of function (Wang *et al.*, 2015a). In aphid saliva, for example, effector proteins that have the ability to bind calcium have been identified; these may halt any sealing of the sieve element while feeding is taking place (Will *et al.*, 2007; Medina-Ortega and Walker, 2013). The process of colonisation is advanced by a number of effectors, although this only happens in particular interactions between the aphid and the host.

The C002 protein produced by aphids has been researched several times; being aphid-specific, it seems to be able to function as a salivary effector protein. In *Nicotiana benthamiana*, RNAi-silencing or over-expression of the C002 protein respectively lowered fitness or raised reproduction levels of aphids (Elzinga *et al.*, 2014). Studies with the potato aphid *Macrosiphon euphorbiae* have revealed that various aphid effectors can influence the interactions of plants and aphids. Expression of the effectors Me10 and Me23, which originate from the potato aphid, in *N. benthamiana* raised aphid fertility (Atamian *et al.*, 2013). Wang *et al.* (2015a) proposed that the Armet protein, which is widespread in animals, is also an aphid salivary effector protein.

With aphid candidate effectors being identified for a rising tally of aphid species (encompassing types that affect monocot and dicot plants), there is a growing urgency to produce tools that can be used to assay such proteins. As described earlier, successful identification of effector actions has been made with transgenic *Arabidopsis* lines and transient over-expression systems in *N. benthamiana* and tomato. Also, newer transient expression systems like the *P. fluorescens* Effector-to-Host Analyzer (EtHAn) system, in which there is engineering of non-pathogenic bacteria to express the *P. syringae* type III secretion system (TTSS), can be deployed to identify aphid candidate effectors in crops that come under a non-model heading – barley, wheat, and possibly even legumes (Jaouannet *et al.*, 2014).

A good method to work out which effectors are most useful in promoting susceptibility is to introduce high-throughput screening for the pertinent effector actions. In research that has

sought to discover *S. avenae* effectors, the structural sheath protein (SHP) has been shown to increase virulence on barley. To date, though, this is the only *S. avenae* effector identified (Yates and Michel, 2018).

1.4.3 Effector Triggered Immunity (R genes/ETI)

In the field of resistance (*R*) gene mediated defence, R-proteins recognise effectors called avirulence (*Avr*) gene products; these exist in pests and pathogens, and the consequence of this recognition is effector-triggered immunity (ETI) and increased resistance, as shown in Fig. 1-7 (Gururani *et al.*, 2012).

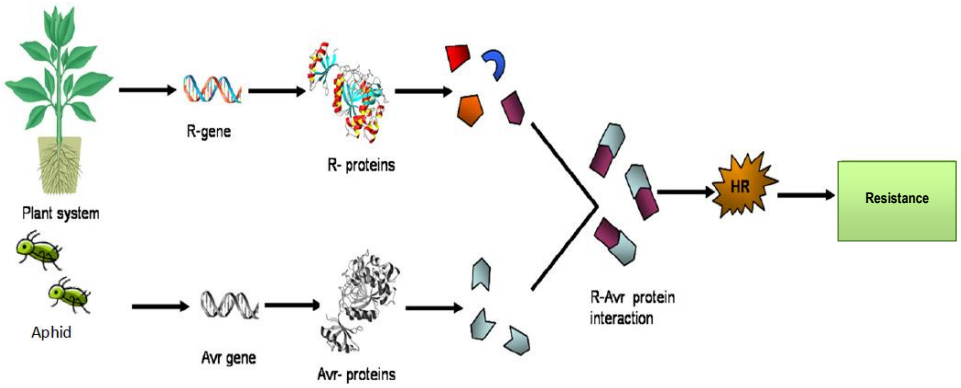


Figure 1-7. Schematic picture of *R*-gene mediated defence activated when plants and aphids interact. HR = hypersensitive response. Picture adapted from Gururani *et al.* (2012).

Eight types of plant *R*-gene have been identified. The majority of identified plant *R*-genes encode nucleotide-binding site – leucine-rich repeat (NBS-LRR) proteins and confer defences against pathogens or insects (Jones and Dangl, 2006; Hogenhout and Bos, 2011). There has been isolation of two aphid *R*-genes so far. One of these is the *Mi-1.2* gene, which was identified in tomato; it resists particular biotypes of *M. euphorbiae*. Melon was the source of the *Vat* gene; it confers enhanced resistance to the melon-cotton aphid (*Aphis gossypii*) (Smith and Chuang, 2014). Both genes come from the NBS-LRR resistance gene family. Although aphid effectors recognised by Mi-1.2 and Vat, as well as by the products of the mapped but not cloned genes APR, AKR and Rag, in plants have still to be identified, great strides have been taken in working out the mechanism of signalling related to *Mi-1.2* that helps tomato plants resist potato aphids (Nalam *et al.*, 2019). Particular genes engaged in ETI to microbes are needed for *Mi-1.2* in order to defend against the potato aphid (Bhattarai *et al.*, 2007).

There are multiple interactions between different transcription factors (TFs) and between TFs and additional nuclear proteins, such as co-activators/-repressors and elements of the general

transcriptional mechanisms to regulate the context-dependent expression of genes. One class of such TFs is the WRKY TFs (see below). Some WRKY factors negatively influence plant defence and others act positively; this implies that there is a link between these TFs and distinct regulatory complexes (Eulgem and Somssich, 2007; Shen *et al.*, 2007). The responses of plants subsequent to ETI signalling, with major transcriptional changes and especially the engagement of WRKY proteins, have been very involved in this procedure and are thought to be highly possible candidates for involvement of common molecular players, with vital functions in regulating stress signalling cross-talk (Fujita *et al.*, 2006; Shen *et al.*, 2007). The end-results of such signalling pathways are the plant's reactions to combinations of stresses.

Resistance can be classified either as qualitative or quantitative. "Qualitative", "major gene" and "vertical" resistance are alternative terms for *R*-based resistance; these terms imply extremely high levels of specific resistance. The drawback of this resistance form is that it is only activated by species housing the avirulence gene products (effectors). The defence, therefore, does not work on aphid biotypes that do not have the avirulence protein. Quantitative (polygenic; horizontal) resistance, on the other hand, is where several genes are involved in the observed resistance. This method is more durable but it is often not particularly powerful and it is harder to identify the genes that contribute to the resistance. An important line of investigation is to identify genes that are engaged in quantitative resistance in the wheat crop (Faris *et al*., 1999).

Aphid resistance genes in wheat

Sources of resistance to aphids are rare. The resistance that has been established as definite has been found in wild accessions or connected forms, or in unimproved landraces, making the procedure of building resistance into cultivated forms a long and drawn-out affair in terms of breeding. A total of 40,000 wheat accessions have been assessed for reactions to a strain of the Russian wheat aphid *Diuraphis noxia;* 300 have exhibited reactions that could be labelled as partly or fully resistant. Of these, only a handful – mostly in wheat-related crops – are presently being used in the breeding process and adopted into elite germplasm (Berzonsky *et al.*, 2003). A more recent development has aimed to find resistance to the highly virulent biotype 2 of the Russian wheat aphid. The number of accessions that were resistant totalled a mere 8%: these were from particular phylogenetic subgroups, which implies the prospective identification of previously undiscovered genes or alleles (Collins *et al.*, 2005; Peng *et al.*, 2009). Although numbers of resistant accessions are fairly high, resistance to aphids is normally dependent on just a few genes with a small number of resistance alleles. Further

genetic research is needed to discover if new resistance genes can be sourced from these accessions.

The future of worldwide food security will be hugely affected by expanding our knowledge of the wheat/*S. avenae* interaction, as insect control is essential to prevent crop loss. This will be more significant as climate change takes more effect and populations expand (Dogimont *et al.*, 2010). To date, relatively few studies have investigated the molecular responses of wheat to *S. avenae* infestation. Ferry *et al.* (2011) demonstrated that the response of commercial wheat, which exhibits only very low levels of resistance to this insect pest, was similar to the basal non-specific stress response at the proteome level. However, Guan *et al.* (2015) showed the presence of stress proteins and oxidative stress proteins in a resistant diploid line, but only in response to aphid infestation.

The present thesis focuses on identifying genes involved in the plant-aphid interaction to elucidate potential mechanisms which confer resistance to aphids. The variety of identified aphid-induced plant sequences to date suggests that many different pathways are involved and that defence is highly complex.

1.4.4 Role of plant hormones in signalling

Signalling pathways including ethylene, jasmonic acid (JA), salicylic acid (SA), abscisic acid, oligogalacturonic acid and hydrogen peroxide (Fig. 1-8) are used by plants to trigger induced defence systems, both at the site of attack and systemically. ABA is a plant stress hormone that is ever-present in plants; it stimulates abiotic stress reactions through activating how abiotic stress-related genes are expressed as they fulfil their function, and it performs as an internal signal that allows plants to withstand severe environmental effects such as extreme cold, drought and salt (Keskin *et al.*, 2010). Additionally, ABA has been demonstrated to perform vital tasks in connection with vulnerability to disease, as well as having the ability to help fight infection from pathogens and interact with a range of biotic stress reactions that are mediated by hormones (Yasuda *et al.*, 2008). By contrast, salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) have crucial roles in signalling pathways against biotic stress (Pieterse *et al.*, 2001). The observation has been made that SA-mediated resistance actively counteracts biotrophic pathogens, while JA, or reactions that are ethylene-mediated, principally combats necrotrophic pathogens and herbivorous insects (Spoel and Dong, 2008).

A further area of research into the hormonal cross-talk present in plant defence is how the pathways dependent on SA and JA can interact (Hunter, 2000). When plants are subjected to assaults from insects, activation of the JA pathway can adversely affect resistance to pathogens by stifling the SA pathway, while if there are assaults from a pathogen, the JA pathway can be stifled, thereby reducing defences against insects. Such findings suggest that there is a trade-off in the respective SA and JA reactions (Pieterse *et al.*, 2001). Such negative cross-talk does not always occur, though: it seems to depend on particular combinations of plants, insects and pathogens. It is also affected by the characteristics of the stress, as well as by its timing and its strength (Singh *et al.*, 2011). Alternative research, however, has posited that if plants are infected by a pathogen, opposition to insect herbivores may be enhanced, which implies positive cross-talk (Hunter, 2000). When the rust fungus *Uromyces rumicis* infects dock (*Rumex*), for example, the Chrysomelid beetle *Gastrophysa viridula*'s reproductive capacity, growth, and even its survival, are threatened (Hunter, 2000). Additionally, research into *Rumex* suggests that the opposition to pathogens that is triggered by herbivores is also effective against different types of pathogens in the field (Hunter, 2000). Microarray analysis by Schenk *et al.* (2000) showed that Arabidopsis contains integrated plant defences, particularly among SA and JA pathways, which can display positive interaction.

Aphid effectors are recognised via receptors; these trigger a signal transduction route that is regulated by a range of cellular messengers, mainly stress hormones, calcium and ROS (Moreno *et al.*, 2011; Lei *et al.*, 2014). Aphids, though, tend to regulate many hormonal signalling pathways in order to give an advantage to the insect. Whilst phytohormones are known to be involved in the defence response, their roles are not completely understood. Nonetheless, various studies have indicated that SA has a prominent function when aphid infestation begins; it mediates potential antagonistic cross-talk with JA-signalling pathways and consequent defence against insects (Coppola *et al.*, 2018). These phytohormones also have a central role in the cross-talk between abiotic and biotic stress signalling (Fujita *et al.*, 2006).

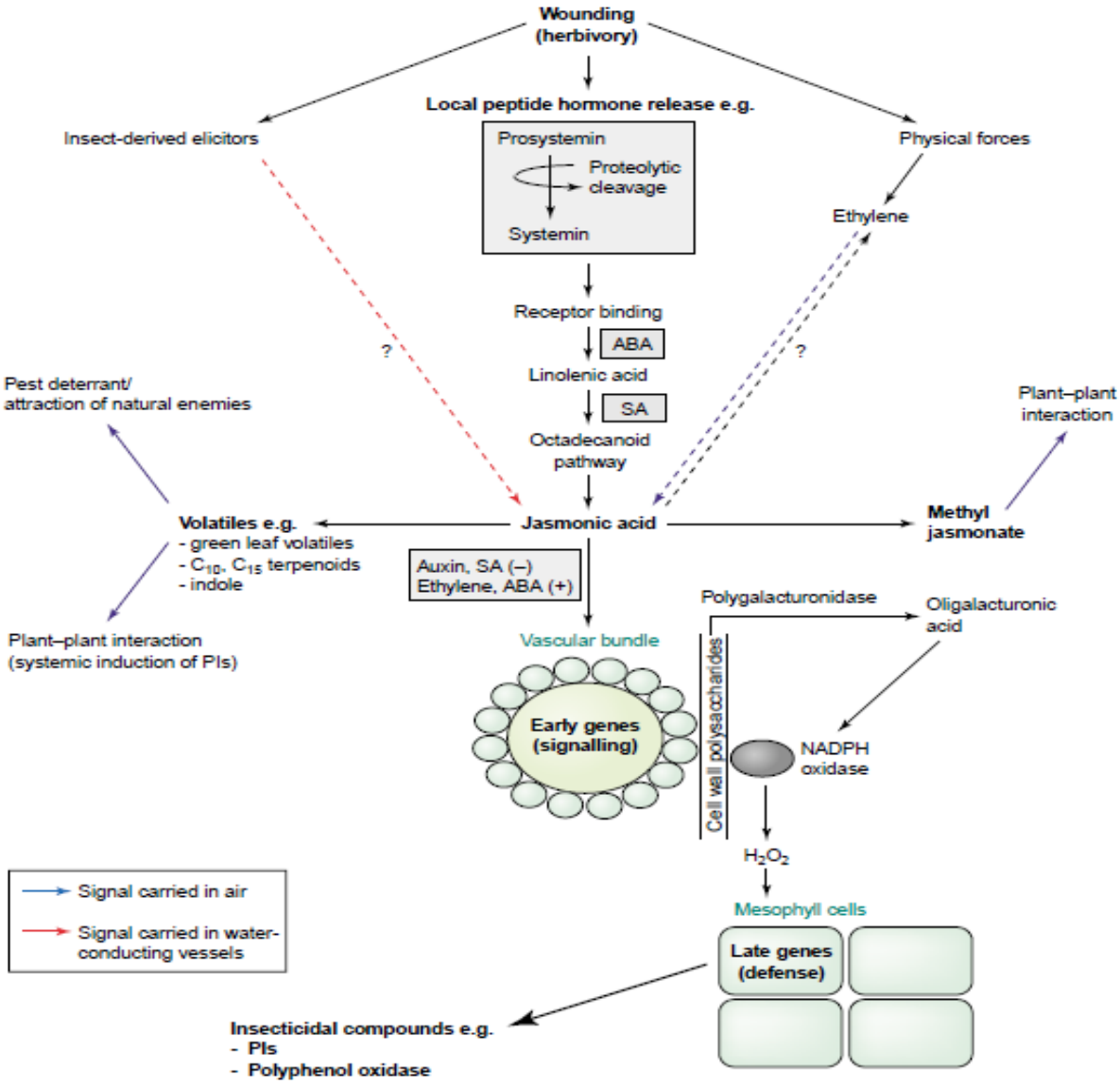


Figure 1-8. The wounding response; this is an overall view of the plant's response to wounding and the signalling molecules that are able to regulate it. Pathways that are needed for insecticidal proteins' local and systemic induction are illustrated. The abbreviations are: ABA, abscisic acid; SA, salicylic acid (Ferry *et al.*, 2004).

1.5 WRKY transcription factors: key regulators of plant processes

1.5.1 WRKY transcription factors

The initial stage in gene expression is transcription, which is modulated by transcription factors (TFs) that either activate or repress genes (Eulgem *et al.*, 2000). There is a domain in the TF that binds to a specific DNA sequence leading to formation of a transcriptional complex, thereby modulating gene expression (Mitsuda and Ohme-Takagi, 2009; Cui and Luan, 2012). TFs in wheat are classified into numerous groups depending on what the conserved domain is, such as the WRKY superfamily. WRKY (which are individual amino acid letters that represent tryptophan, arginine, lysine and tyrosine) transcription factors are part of an extended superfamily of WRKY-GCM1 TFs (Satapathy *et al.*, 2018). They constitute a group of plant-specific transcription factors that are involved in numerous biotic and abiotic stress reactions, in addition to plant development (Rushton *et al.*, 2010). Many of the WRKY family members have been identified and characterised in terms of function in numerous species, including Arabidopsis, rice, barley, cotton and soybean (Ding *et al.*, 2016).

Recently, the whole genome sequence of wheat has become available. A search of the genome has identified 171 TaWRKY genes (Ning *et al.*, 2017). Their products are comparable to 1113 WRKY TFs identified in 20 plant species representing the nine major evolutionary lineages of plants, as shown by several methods of analysis.

1.5.2 WRKY domain and W-box

WRKY proteins are particularly noteworthy for their WRKY domain, which is composed of around 60 amino acids and contains an extremely conserved WRKYGQK heptapeptide sequence and a zinc finger-like motif (Satapathy *et al.*, 2018). The arrangement of the zinc finger-like motif is either $CX_{4-5}CX_{22-23}HXH$ or $CX_7CX_{23}HXC$, where ‘X’ is any amino acid (Rushton *et al.*, 2010). The WRKY domain is the DNA-binding domain. WRKY TFs bind to W-box elements, which have the sequence TTGAC(C/T) (Eulgem *et al.*, 2000; Ciolkowski *et al.*, 2008; van Verk *et al.*, 2008). Nevertheless, the selectivity of binding of WRKY TFs to DNA depends on the flanking sequences that are beyond the W-box motif. Binding of WRKY proteins to their W-boxes is typical of reactions to biotic and abiotic stress (Satapathy et al., 2018).

WRKY TFs have been grouped into three classes, depending on the number of WRKY domains and the forms of zinc finger-like motifs. Group I contains proteins that possess two

WRKY domains, while one-domain WRKY proteins are allocated to group II or III, according to the form of zinc finger-like motif (Eulgem *et al.*, 2000). The zinc finger motif structure of C_2H_2 ($CX_{4\text{-}5}CX_{22\text{-}23}HXH$), together with the N-terminal WRKY sequence, is identified as a protein–protein interaction interface in Group I. Sequence-specific binding of WRKY proteins in Group I to target DNA sequences depends on the C-terminal WRKY domain, while the role of the N-terminal WRKY domain is yet to be clarified; this could enhance the affinity and/or specificity of binding to target sequences. Group II WRKYs, meanwhile, contain just one WRKY domain with a comparable C_2H_2 zinc finger motif (the same as the C-terminal domain of Group I), this having been recognised from phylogenetic analysis. A further sub-division into a total of five sub-groups (IIa–IIe), using the WRKY domains' phylogenetic relationships as a base, is as follows: IIa ($CX_5CX_{23}HXH$), IIb ($CX_5CX_{23}HXH$), IIc ($CX_4CX_{23}HXH$), IId ($CX_5CX_{23}HXH$) and IIe ($CX_5CX_{23}HXH$). Group III WRKY proteins have a solitary WRKY domain, which has the zinc finger motif structure C_2HC ($CX_7CX_{23}HXC$) (Eulgem *et al.*, 2000). Further phylogenetic analysis founded on comparisons between the WRKY gene sequences in Arabidopsis (*Arabidopsis thaliana*), rice (*Oryza sativa*) and other species has shown that the classification into groups is widely applicable (Wu *et al.*, 2005; Zhang and Wang, 2005).

1.5.3 The effects of stress on WRKY TF gene expression

There has been much documentation regarding the involvement of WRKY proteins in both biotic and abiotic stresses (Zhu *et al.*, 2013). An individual WRKY protein may regulate transcriptional reprogramming that is linked to many different plant processes; there are numerous inputs and outputs from the dynamic signalling network in which WRKY proteins function (Rushton *et al.*, 2010).

WRKY TFs have crucial functions in mediating various plant stress responses, but discovery of their participation in reactions to abiotic stress has not made as much progress as its biotic counterpart. However, one example of the role of WRKYs in abiotic stress is that of *AtWRKY75*, which is significantly induced under very low phosphate (Pi); at the same time, if the expression of *WRKY75* is suppressed, there will be an increased risk of Pi stress and a lower uptake of Pi in mutant plants (Devaiah *et al.*, 2007). More examples of the roles of WRKY proteins in abiotic stress include those involved in drought, salt and osmotic stress. When TaWRKY44 is over-expressed in tobacco, these transgenic lines exhibit improved tolerance to abiotic stress, either through successful eradication of ROS via triggering cellular antioxidant systems, or the galvanising of stress-linked gene expression (Wang *et al.*, 2015b).

In relation to biotic stress, WRKY proteins have been demonstrated to have vital functions in the resistance of plants to various pathogens. For example, the knockdown of the rice transcription factor OsWRKY45 reduces SA-induced resistance to fungal and bacterial pathogens (Wang *et al.*, 2015b).

WRKY proteins regulate gene expression of pathways linked to biotic and abiotic stress in either a positive or a negative way. Controlling WRKY-dependent signalling pathways is extremely intricate; the process includes transcriptional regulation, DNA-binding affinity and post-translational regulation (Ishihama and Yoshioka, 2012; Chi *et al.*, 2013). WRKY genes respond to stimuli of both an internal and an external nature, which amalgamate the signals and activate expression of target genes via binding to the W-boxes in their promoters (Eulgem and Somssich, 2007; Rushton *et al.*, 2010). WRKY gene promoters themselves can contain W boxes. WRKY transcription factors can attach to their own promoters and to the promoters of other WRKY transcription factor genes; this is known as auto-regulation or cross-regulation. Research using chromatin immunoprecipitation (ChIP) has shown that *Pc*WRKY1 protein of parsley (*Petroselinum crispum*) binds to the W boxes of its native promoter and the *PcWRKY3* promoter (Turck *et al.*, 2004). In Electrophoretic Mobility Shift Assays (EMSA), the W-box sequences upstream of the *AtWRKY60* gene promoter were recognised by *At*WRKY18 and *At*WRKY40, with both triggering *AtWRKY60* expression in protoplasts. This suggested that *At*WRKY18's and *At*WRKY40's target gene in ABA signalling may be *AtWRKY60* (Chen *et al.*, 2010). Experiments involving the ChIP-qPCR assay have indicated that by direct attachment to its own promoter, *At*WRKY3 may be able to control its own expression (Mao *et al.*, 2011).

Additionally, WRKY TFs are downstream in the chain from MAPK cascades and are phosphorylated by MAPKs, raising their propensity to attach to the promoters of the target genes that are engaged in the defence of plants and reactions to environmental stress (Shen *et al.*, 2012).

The modulation of WRKY TFs is a complex process, as WRKY TF/partner interactions make up a network that is dynamic and efficient. WRKY proteins modulate and regulate the expression of key genes in the defence of plants, thereby confirming them as highly suitable candidates to understand the connections between various stresses; this may be the underlying factor in the connection between *S. avenae* and nitrogen input.

A lack of nitrogen has a major effect on the leaf transcriptome, to the extent that there is an over-abundance of transcripts associated with cell wall formation, sugar and nutrient signalling, protein degradation and secondary metabolism. When there is a lack of nitrogen, there is a consequent re-configuration of leaf metabolism and gene expression; this activates defences that guard the metabolite-rich nitrogen-deficient leaves of barley against attack by *M. persicae* (Comadira *et al.*, 2015). Comparable patterns were seen in gene expression profiles of N-deficient barley leaves and Arabidopsis leaves infested with *M. persicae* (Comadira *et al.*, 2015). As an illustration, there was a major induction of transcripts encoding WRKY 18, 33, 40, 51 and 53 after nitrogen limitation in barley and after Arabidopsis leaves were infested by aphids. In contrast, although the information from the transcripts illustrated that nitrogen limitation led to greater amounts of flavonoid metabolism transcripts being present in barley, *M. persicae* feeding in Arabidopsis leaves stifled flavonoid metabolism (Comadira *et al.*, 2015). In each stress circumstance, there was an abundance of transcripts that encoded wall-associated kinases (WAKs) and DUF26 (domain of unknown function 26) kinases. These findings support the hypothesis that WAKs, DUF26 kinases and WRKY transcription factors have significant functions in basal resistance to aphids (Comadira *et al.*, 2015).

1.5.4 Future prospects for use of TFs in wheat breeding

Innovative technologies must be advanced to progress productivity by creating better genotyping and phenotyping methods and by increasing the diversity of genes in breeding germplasm (Tester and Langridge, 2010). Genetic engineering of transcription factors is a promising approach to the goal of developing crops that have improved agronomic traits, including tolerance to cold, heat, drought, and salt stress as well as tolerance to pests and pathogens. Transcription factors have vital functions, from perceiving stress signals to inducing expression of stress-responsive genes, so they have become potent tools for increasing the levels of tolerance to stress by manipulating intricate metabolic pathways in crops such as wheat (Hu and Xiong, 2014). For this to be successful, fundamental knowledge of the appropriate networks – at the physiological, biochemical and gene regulatory levels is crucial, as this will assist in our understanding of pleiotropic effects caused by the over-expression of transcription factors. A vital challenge in experiments involving genetic engineering is to limit transcription factor activity in order to restrict deleterious consequences. For example, over-expression of *DREB1/CBF* in transgenic rice lines caused

growth retardation under unstressed normal growth conditions, but tolerance under conditions of cold, drought and high salinity compared to the WT Ito *et al.* (2006).

Work with transgenic Arabidopsis has demonstrated that WRKY proteins from other species can transfer desirable characteristics. For example, transgenic Arabidopsis plants that over-express *TaWRKY2* or *TaWRKY19* exhibit better tolerance to salt, drought and/or freezing stresses than WT plants (Niu *et al.*, 2012). Qin *et al.* (2015) showed that *TaWRKY93* can positively regulate responses to abiotic stress, being able to increase levels of salinity, drought and low temperature stress tolerance by the augmentation of osmotic adjustment, retaining membrane stability and increasing transcription of stress related genes. Over-expression of *TaWRKY33* in Arabidopsis increased germination rates, promoted root growth under various stresses and resulted in enhanced tolerance to heat stress (He *et al.*, 2016).

Essentially, then, deciphering the functions of TF genes in wheat and their roles in ability to tolerate biotic stresses, as well as distinguishing the genes for TFs that are engaged in pathogen/pest responses, are vital current endeavours that are likely to contribute significantly in wheat breeding programmes to increase endogenous resistance, thereby decreasing losses (Hong *et al.*, 2018). Innovative breeding techniques, or transgenesis, may enable the expression of WRKYs to create wheat cultivars that exhibit increased tolerance to *S. avenae* under low nitrogen conditions, even in times when other growth conditions are at their optimum level.

1.6 Research Rationale

This study explores the potential beneficial effects of reduced nitrogen input in wheat using various complementary approaches. This includes use of molecular genetics to identify genes that confer resistance to aphids, with the eventually aim of producing crops with reduced nutrient input.

1.7 Research Hypotheses

It is hypothesised that:

1. Limiting nitrogen input in wheat increases aphid tolerance, but reduces plant growth.
2. A subset of WRKY transcription factor genes is differentially expressed in plants in response to growth at reduced nitrogen levels and aphid infestation.
3. Mutations in a differentially-expressed WRKY gene will affect the interaction between nitrogen supply and aphid fecundity.
4. Loss of binding by the one mutant line reduces the aphid population and negates the differentiation between population increases relative to nitrogen availability.

1.8 Project Aims

The present project seeks to investigate the interaction the between response of wheat to the grain aphid *Sitobion avenae* and reduced nitrogen availability, and to study the role of WRKY transcription factors in cross-talk via the regulatory network in this interaction (Fig. 1-9). The specific objectives are to:

1. Investigate the effect of reduced nitrogen availability at a physiological level on wheat-aphid performance.
2. Determine whether plant defence responses are primed at low nitrogen availability.
3. Assess the expression of *WRKY* genes during periods of single and dual abiotic and biotic stress.
4. Establish whether there is a role for TaWRKY transcription factors in the responses to nitrogen level and aphid infestation.
5. Evaluate the effects of mutations within the *WRKY* binding domain on aphid performance.
6. Identify which gene sequences a TaWRKY protein binds to using DNA-protein binding studies.
7. Determine how a mutation to a *WRKY3* gene affects the binding of the TaWRKY protein to W-box elements.

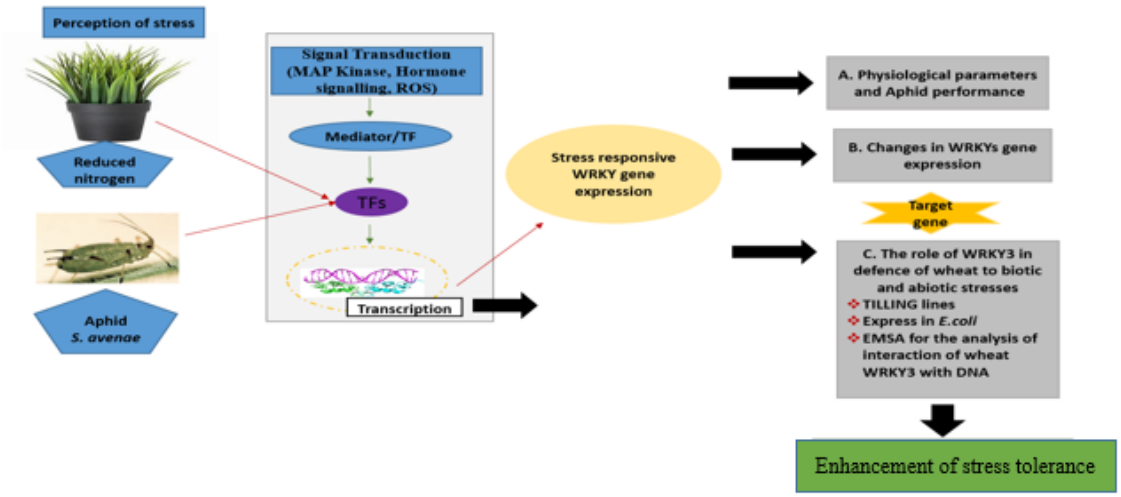


Figure 1-9. Experimental model of this research: to establish whether reducing nitrogen input increases tolerance to aphid infestation and to clarify the role of WRKY transcription factors in the response of hexaploid wheat to this interaction.

2 Chapter 2. Effects of Reduced Nitrogen Availability on Performance of Wheat and Subsequent Response to Aphid Infestation

2.1 Abstract

Food production must increase by 70% to feed an additional 2.3 billion people by 2050. Wheat (*Triticum aestivum*) is the third most produced crop after rice and maize in the world. During the growth and development of plants both biotic and abiotic stress result in great loss to crop productivity. However, many plants developed their responses to stress as they evolved. The aim of the present study is to investigate the response of wheat (*Triticum aestivum*) to both nitrogen input and aphid (*Sitobion avenae*) infestation, by using single and combined stresses, to understand the impact of stress and cross tolerance in wheat. Further experiments assessed the response of wheat to nitrogen single stress at the physiological level. Two wheat cultivars with different levels of tolerance to aphids (Cordiale and Grafton) were grown with different nitrogen levels. Changes were observed in both genotypes for all physiological parameters and were directly related to nitrogen input. High nitrogen availability (7.5 mM) significantly increased plant height, leaf area, chlorophyll content, NO_3^- ion accumulation and relative water content. However, the numbers of leaves and tillers did not differ significantly at different levels of nitrogen. Fecundity of aphids on the two wheat varieties was measured with different nitrogen inputs. Aphid fecundity was significantly reduced on plants grown with low nitrogen (2.25 mM), with total fecundity reduced to 20 and 31 nymphs per adult on Cordiale and Grafton, respectively. Increased nitrogen could change the quality and reduce the resistance of wheat to aphids.

Key words: wheat / abiotic / biotic / aphid performance

2.2 Introduction

2.2.1 Wheat nutrition: nitrogen

Wheat (*Triticum aestivum*) is one of the most important crops in the world with a total annual production of 722 million tonnes (Mt) of grain (FAO, 2015b). Environmental stresses can reduce the production of wheat, while crop yield can be increased by adding mineral fertilisers (Borlaug and Dowswell, 1993). Crop yields are becoming a growing area of concern and research effort, as there is an urgent need to increase wheat production in order to meet the demands of a growing population, while at the same time the amount of land available for agriculture is declining (Khan and Mohammad, 2016). One of the ways to increase crop yields is to increase levels of nitrogen available to crops.

Nitrogen (N) is one of the most important factors in the growth and development of plants and plant quality. There has been a tendency to maximise N fertilisation in order to increase crop yield to its maximum (Hirel *et al.*, 2007); however, the over- or under-use of N fertilisers is not cost effective. An increased nitrogen input significantly raises grain yield and protein concentration in wheat but it has negative environmental impacts. For example, the administration of too much N will result in it leaching into groundwater or running off the land in surface water, and the atmosphere can become polluted by ammonia volatilisation and NOx gases, which are a by-product of denitrification (Conley *et al.*, 2009). There is the greatest risk of this happening when fertilisation rates come close to or exceed the 'economic optimum level'. This level is calculated from the known response of the crop to N fertilisers (Delin and Stenberg, 2014). At the other end of the scale, if N fertilisers are under-utilised, then crop yields fall, meaning that their production is not cost effective, and populations dependent on the crop will be malnourished (Dawson *et al.*, 2008; Khan and Mohammad, 2016).

Therefore, it is necessary to develop improved cultivars so that yield can be increased while adhering to the requirements of food quality and safety, and protection of the environment. In this regard, minimising the amount of N input requires N-efficient crops that need less N fertiliser yet maintain current yields; this has become one of the most important objectives of applied research in agriculture, along with minimising the negative environmental effects (Tedone *et al.*, 2018). N use efficiency has been observed by several approaches in the literature, with different objectives (Moll *et al.*, 1982).

In this study, it was hypothesised that nitrogen content in leaves and plant performance would increase with increasing nitrogen availability, since reduced levels of nitrogen generally lead to decreased photosynthesis and root growth. Based on the above, the present study was initiated to consider plant productivity and the accumulation of nitrogen in relation to N input when two winter wheat genotypes (*Triticum aestivum*, Cordiale and Grafton) were grown with three nitrogen regimes: high (7.5 mM NO_3^-), moderate (5.25 mM NO_3^-) and low (2.25 mM NO_3^-). The aim was to determine whether improvements could be made to most physiological parameters with a standard nitrogen input. The finding was that growth is limited by low nitrogen levels with a range of adaptive responses.

2.2.2 Nitrogen-aphid interaction

The English grain aphid (*Sitobion avenae*) is the object of study. It is one of the most damaging pests of wheat (*Triticum aestivum*), and changing conditions in agriculture are leading to insect pests becoming a more serious threat to sustainable crop production globally (Castex *et al.*, 2018). Aphids are plant phloem sap feeders, causing damage to plants via depleting photoassimilates, manipulating growth and nutrient partitioning, and transmitting plant viruses (Thompson and Goggin, 2006).

Although phloem sap is composed mainly of amino acids and sugars, its composition varies depending on the species of plant, the stage of development of the plant, abiotic factors, for example the available levels of water and nitrogen, as well as temperature (Ponder *et al.*, 2000), and biotic stress such as aphid infestation (Sandström *et al.*, 2000). In response to environmental and biotic stress, plants have evolved a number of complex defence mechanisms (Fujita *et al.*, 2006).

It is still not entirely clear how aphid fecundity is affected by the levels of nitrogen available to a plant. Only a small number of studies have examined how nitrogen levels affect aphids (Jansson and Smilowitz, 1986) using N application rates that are actually utilised in practice, and just a few have directly investigated fecundity (Awmack and Leather, 2002). A study that investigated the fecundity of cereal aphids (Duffield *et al.*, 1997) on winter wheat with three different application levels of N (0, 190 and “Canopy Management” with 130–220 kg N/ha) found that as nitrogen levels increased, so did the population of *Metopolophium dirhodum*. Another studied example with wheat found that the lowest aphid population occurred at the lowest dose of urea fertiliser, and with increased application of urea the population increased (Wagan *et al.*, 2015). The link between aphids and N levels in

the soil comes from the fact that nutrient levels affect aphids' diet and the plant's response to attack, as they influence the composition of secondary metabolites. It is well known that increased inputs to crops affect the fecundity of herbivores via the nutritional value of host plants. When the influence of varying nitrogen fertiliser levels applied to different wheat cultivars on the fecundity of cereal aphids was examined, it was found that higher nitrogen fertiliser increased aphid population density by increasing fecundity and by decreasing the time to maturity (Aqueel and Leather, 2011).

The initial aim of this chapter was to study the link between nutrient availability (nitrogen) and aphids' performance on wheat. The level of resistance to aphids was assessed using the aphids' fecundity on the selected wheat cultivars (*Triticum aestivum,* Cordiale and Grafton) with varying nitrogen levels.

2.2.3 Aims and objectives

The aim of this study is to improve our understanding of plants' responses to simultaneous stress conditions and to study the effect of nitrogen availability stress on wheat-aphid performance interactions.

The specific objectives of this study:

1. To investigate the response of wheat to nitrogen treatment at a physiological level.
2. To investigate the response of wheat to nitrogen and aphid infestation (bioassay). This is assessed by measuring the aphids' fecundity on winter wheat (*Triticum aestivum*, Cordiale and Grafton) at three different nitrogen levels.

2.3 Materials and Methods

2.3.1 Experimental Design

The experiment was designed to investigate the effects of wheat response to reduced nitrogen availability on aphid fecundity. Three nitrogen regimes – 7.5 mM, 5.25 mM and 2.25 mM – were applied to two winter wheat genotypes, Cordiale and Grafton. Under each nitrogen condition, aphid tolerance was evaluated through aphid fecundity (Fig. 2-1).

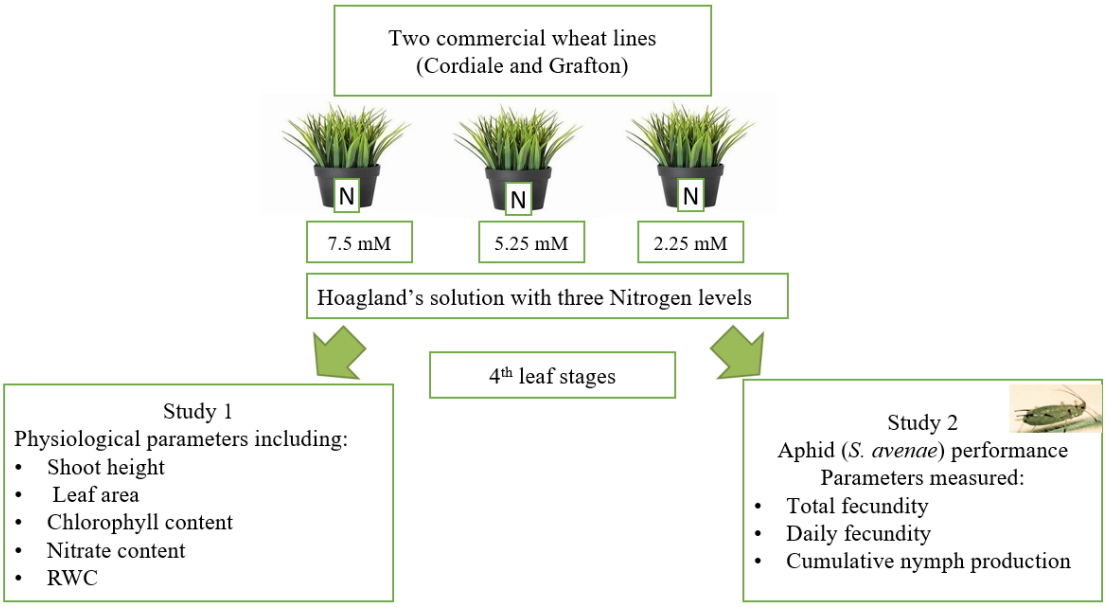


Figure 2-1. Experimental framework for applying nitrogen and aphid infestation stress to wheat, with three nitrogen regimes: standard condition which is 7.5 mM concentration and two lower levels of nitrogen, 5.25 and 2.25 mM.

2.3.2 Plant materials and growth conditions for physiological assessment

The experiment was conducted on two varieties of winter wheat, Cordiale and Grafton, supplied by KWS-UK. Silver sand was used to facilitate controlling the amount of nutrient solution, removal of plants and harvesting. Seeds were washed with deionised water and soaked in 30 ml deionised water in a screw cap centrifuge tube for 4 hours at room temperature. Seeds were transferred to petri dishes (15 seeds/dish) with two sheets of filter paper and supplied with deionised water for germination. The petri dishes were sealed with Parafilm and were kept at 28 °C to allow the seeds to germinate. After 2-3 days, the seeds were transferred to pots (diameter 9 cm and height 9 cm) with a piece of mesh at the base and

filled with silver sand (washed, dried and autoclaved) to avoid contamination. The treatment used for irrigation is described in section 2.3.4, Hoagland's solution and watering system. Four seedlings were sown in each pot to give four replicates per genotype for each nitrogen concentration. To inhibit algal growth, pots were covered with foil sheets after the 5th day, with holes for growth of plants. Plants were grown to the four leaf stage under the following conditions: fluorescent light 16 h light: 8 h dark, at 25 °C in light, 19 °C in darkness and 250-300 µmol m^{-2} s^{-1} illumination. Plants were harvested after 3 weeks and the effects of growth with different nitrogen levels were assessed.

2.3.3 Plant materials and growth conditions for aphid infestation

Single seedlings were grown to the three-leaf stage in sand under the following conditions: fluorescent light 16 h light: 8 h dark, at 25 °C in light, 19 °C in darkness and 250-300 µmol m^{-2} s^{-1} illumination. Plants were watered with half strength Hoagland's solution (see section 2.3.4) every two days for six weeks. Plants were transferred to a controlled environment chamber with 16 h light (22 °C) / 8 h dark (17 °C) with 300 µmol m^{-2} s^{-1} illumination. Wheat was grown to the four leaf stage and the bioassay was started. Aphids (*Sitobion avenae*) were obtained from a laboratory culture and maintained in a 45 x 45 x 50 cm Perspex cage at 20 °C, 55% R.H. under 16 h:8 h L: D light regime. New plants were supplied weekly. Two adult aphids were placed on the leaf number 2 with a paint brush. These adults were allowed to produce nymphs for 24 h then the adults were removed. The nymphs became adults after 10-12 d. Plants were exposed to aphids over 21 d, with reproduction recorded daily. Clip cages were used to allow nymphs to reproduce and for ease of counting them. Overall 36 potted plants (18 plants per genotype) were used in this experiment.

2.3.4 Hoagland's solution and watering system

The wheat plants were provided with nutrients via irrigation with half-strength modified Hoagland's solution (pH 5.5) (Hoagland and Arnon, 1950) for 3 weeks. Stock solution was prepared as shown in Table 2-1. Plants were supplied with 70 or 100 ml of solution every two days. N was supplied as $Ca(NO_3)_2.4H_2O$ and KNO_3 at high (7.5 mM), moderate (5.25 mM) and low (2.25 mM) concentrations (Table 2-2).

Table 2-1. The various chemicals utilised in the preparation of modified half-strength Hoagland's solution, with equivalent grams/litre. 1 litre of each main solution and 200 ml of each "micronutrient" were made. FeEDTA was dissolved with KOH in water, and the pH was adjusted to 5.5 using HCl (aq). Finally, $EDTA.2H_2O$ and $FeSO_4.7H_2O$ were added.

Chemical	Stock / M	g l^{-1} required
$Ca(NO_3)_2.4H_2O$	1	236.1
$CaC1_2$	1	111
KNO_3	1	101.1
K_2SO_4	1	87.13
KH_2PO_4	0.5	136.1
$MgSO_4.7H_2O$	1	246.5

To prepare Micronutrients:

Chemical	Stock Conc. / g l^{-1}	g / 200 ml required
H_3BO_3	2.86	0.572
$MnC1_2.4H_2O$	1.81	0.362
$ZnSO_4.7H_2O$	0.22	0.044
$CuSO_4.5H_2O$	0.051	0.0102
$NaMoO_4.2H_2O$	0.12	0.024
FeEDTA:		
KOH	56.1	11.22
$EDTA.2H_2O$	10.4	2.08
$FeSO_4.7H_2O$	7.8	1.56

Table 2-2. Volume (in cm^3) of each stock solution added per litre of final solution containing three different concentrations of nitrogen. 2.5 l of solution was made, so 2.5 times the given volumes were added to 2475 cm^3 deionised water.

Stock Solution	7.5 mM N	5.25 mM N	2.25 mM N
1 M $Ca(NO_3)_2.4H_2O$	2.5	1.75	0.75
1 M $CaCl_2$	0	0.75	1.75
1 M KNO_3	2.5	1.75	0.75
1 M K_2SO_4	0	0.75	1.75
0.5 M KH_2PO_4	1	1	1
1 M $MgSO_4.7H_2O$	1	1	1
Micronutrients	0.5	0.5	0.5

2.3.5 Physiological measurements

Chlorophyll content and leaf area

Chlorophyll content was measured by using a handheld chlorophyll meter (Opti-Sciences CCM-200, USA) from the middle of leaf number 3.

Leaf area (LA) of leaf number 3 was estimated according to Gardner *et al.* (2003) by measuring the width (W) and length (L) and following the equation:

$$LA= W*L*0.75.$$

Relative water content

After 20 days of growth, a sample consisting of leaf numbers 3 and 4 was used for measuring the fresh weight (FW). In order to obtain the turgid weight (TW), leaves were immersed in distilled water for 4 h at room temperature, and turgid weight (TW) was recorded after blotting off the excess water. Then the leaf samples were dried in an oven at 80 ºC for 24 h and weighed to give dry weight (DW). All the mass measurements were made using a balance (Barrs and Weatherley, 1962). Values of FW, TW and DW were used to calculate RWC, using the formula:

$$RWC\% = [(FW-DW) / (TW-DW)] X 100$$

Nitrate content

The nitrate content of leaves was measured using the Nitratest kit (Palintest).

Nitrate calibration curve: Distilled water was used to prepare three 1 ml samples with varying amounts of nitrate stock solution (4 mM, 2 mM and 1 mM KNO_3) in screw cap tubes. More distilled water was added to make the samples 20 ml each, and then one flat spoonful of zinc reductant and one nitrate tablet were added. The solution was mixed for 1 min and then 10 ml of clear solution was pipetted into a screw cap tube, and a crushed Nitricol tablet was added. After 10 to 15 min, the absorbance of each sample was read at 540 nm.

Plant extracts:

One gram of each leaf sample was prepared, with each sample comprising two leaves (numbers three and four). The leaves were sliced and then put into boiling tubes to which 9 ml distilled water was added. This was brought to boiling point and then left to cool. One millilitre of extract was prepared as for the nitrate calibration.

2.3.6 Data analysis

The data analysis was performed using IBM SPSS Statistics and data were submitted to two-way analysis of variance (2-Way ANOVA) to study the main effects of genotype and treatments and their interaction. To detect significant differences between treatments, means were compared by Tukey's post–hoc test at $p < 0.05$.

2.4 Results

2.4.1 Nitrogen effects on plant growth

Shoot height (SH)

Plant height (cm) was taken by measuring four plants per pot from the foil to the tip of the most extended leaf. Plants grown with 7.5 mM nitrogen were the tallest, with average plant height 38.5 cm in Cordiale (Fig. 2-2). Average heights were 34.5 cm in Cordiale and 24.5 cm in Grafton with 5.25 mM nitrogen. Minimum height was observed at 2.25 mM nitrogen with average shoot height 16.5 cm in Grafton. The effects of different levels of nitrogen were significant at p = 0.001. There was a significant interaction between the effects of wheat variety and nitrogen concentration [p = 0.038 (< 0.05)] on plant height.

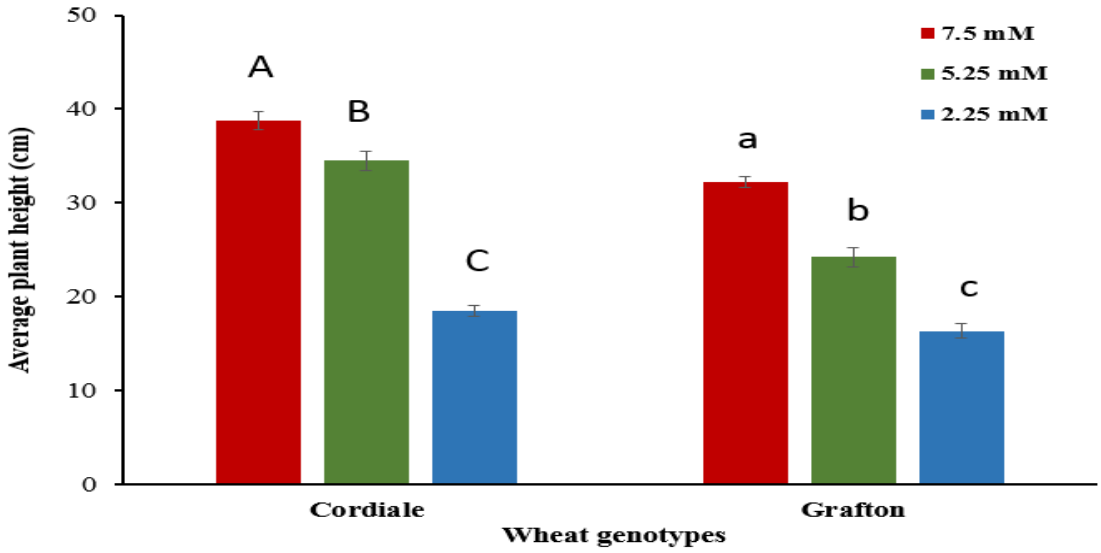


Figure 2-2. Effect of different nitrogen levels on plant height.
Wheat plants (Cordiale and Grafton) were grown for 21 d with three different levels of nitrogen: 7.5 mM, 5.25 mM and 2.25 mM. Data are shown as mean values ± SE (n = 4). For each cultivar, different letters represent statistically different means (Tukey test; p <0.05).

Leaf area (LA)

Leaf area is an important physiological determinant of crop yield. There were significant differences for both varieties among the nitrogen levels. The highest leaf area was observed in the genotype Cordiale (29.06 cm^2) compared with Grafton (24.4 cm^2) in the treatment with 7.5 mM N, whereas the lowest leaf areas in both Cordiale and Grafton were found with 2.25 mM N (7 cm^2 and 6 cm^2, respectively) (Fig. 2-3).

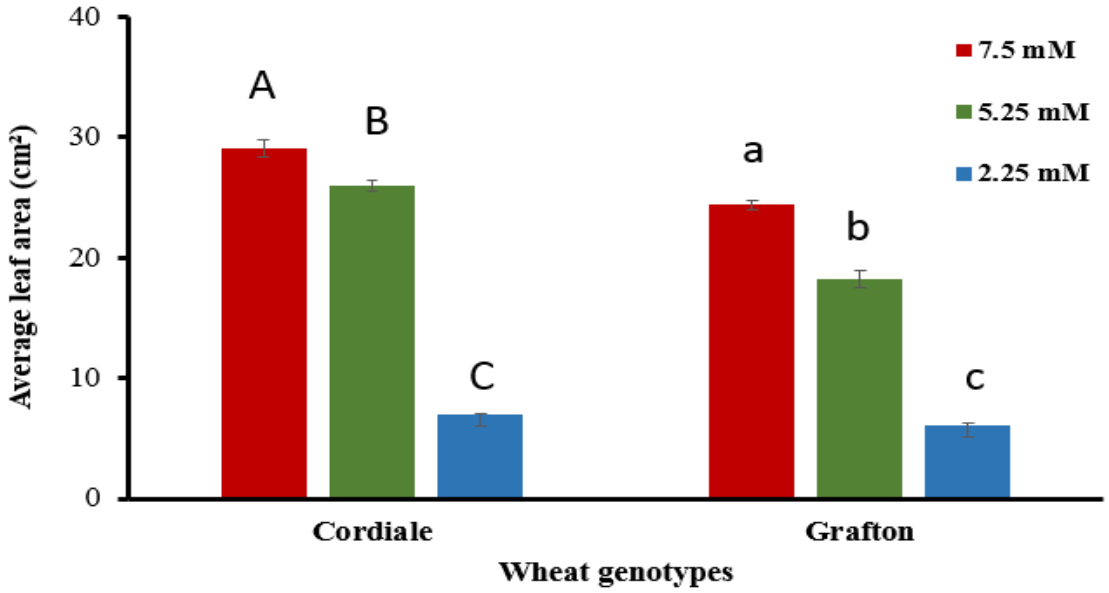


Figure 2-3. Effect of different nitrogen levels on leaf area.
Wheat plants (Cordiale and Grafton) were grown for 21 d with three different levels of nitrogen: 7.5 mM, 5.25 mM and 2.25 mM and. Data are shown as mean values ± SE (n = 4). For each cultivar, different letters represent statistically different means (Tukey test; $p < 0.05$).

Leaf number

Nitrogen gave no significant difference ($p > 0.05$) in the number of leaves with both genotypes. Furthermore, there was no interaction effect between wheat variety and nitrogen concentration [$F = 0.50$, $p = 0.615$ (>0.05)]. However, at low nitrogen availability leaf number was reduced for Cordiale. Mean numbers of leaves on control plants were 6.5 and 4.5 in Cordiale and Grafton respectively, whereas plants exposed to 5.25 mM nitrogen had mean values of 5.7 and 4.75 in Cordiale and Grafton respectively (Fig. 2-4).

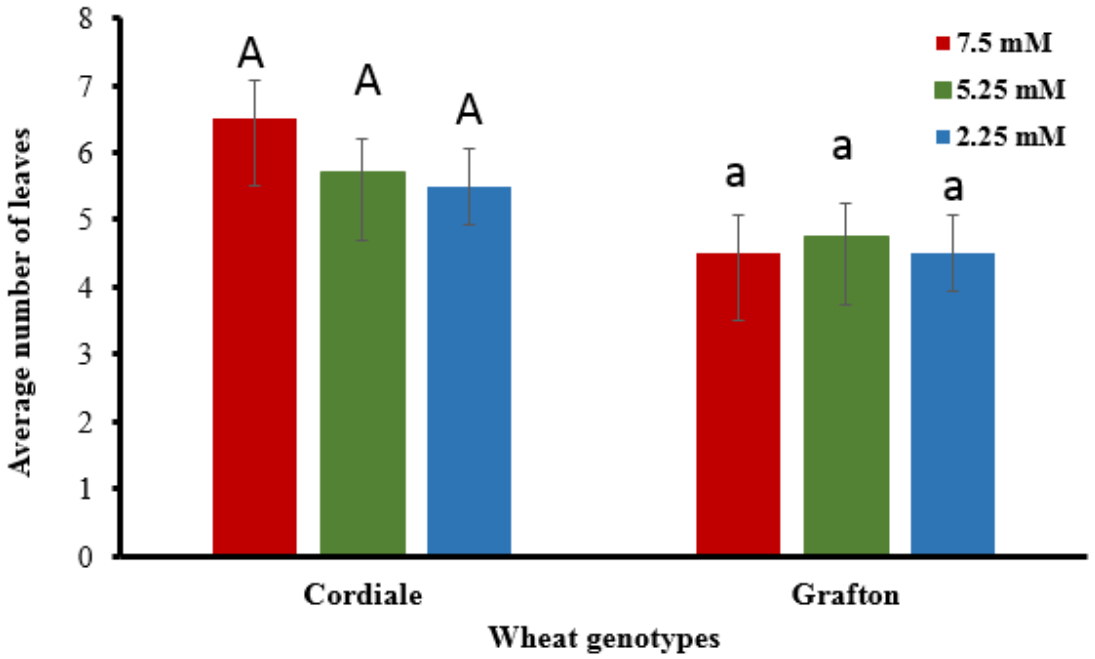


Figure 2-4. Effect of nutrients on leaf numbers of two wheat genotypes.
Wheat plants (Cordiale and Grafton) were grown for 21 d under three different levels of nitrogen: 7.5 mM, 5.25 mM, and 2.25 mM. Data are shown as mean values ± SE (n = 4). For each cultivar, means are not statistically different (Tukey test; $p > 0.05$).

Chlorophyll content

At day 21 the chlorophyll content per leaf increased significantly with increasing nitrogen input in both genotypes. Grafton had higher chlorophyll content with the average 16 SPAD units at standard nitrogen, while for Cordiale the corresponding value was slightly lower at 14 SPAD units. Chlorophyll contents were lower in Grafton and Cordiale (7.6 and 6 SPAD units, respectively) under low nitrogen levels (Fig. 2-5).

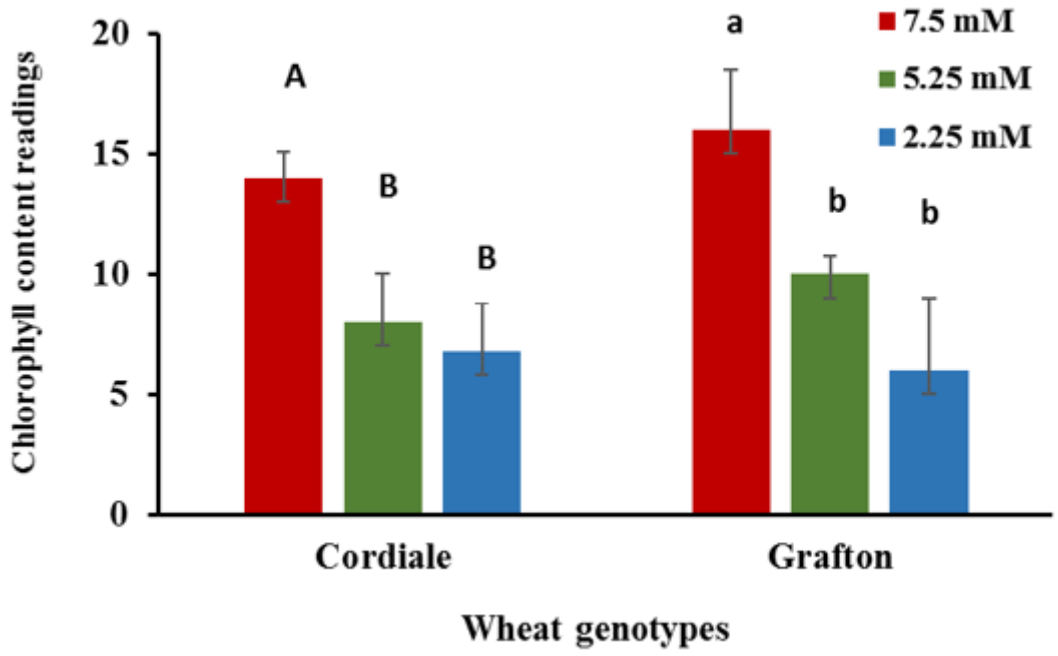


Figure 2-5. Effect of nitrogen supply on chlorophyll content.
Mean chlorophyll contents measured in leaf 4 of Cordiale and Grafton grown for 21 d with three different levels of nitrogen: 7.25 mM, 5.25 mM and 2.25 mM. Data are shown as mean values ± SE (n = 4). For each cultivar, different letters represents statistically different means (Tukey test; $p < 0.05$).

NO_3^- accumulation

Nitrogen treatment significantly affected ($p < 0.05$) nitrate concentration in leaves. The highest nitrogen input gave the highest nitrate content with average 8 and 7.5 mg g^{-1}, respectively, in Cordiale and Grafton. The nitrate content was 4.3 and 4 mg g^{-1} in Cordiale and Grafton, respectively, at 5.25 mM N. The nitrate content at 2.25 mM N was similar in Cordiale (2.25 mg g^{-1}) and Grafton (2.60 mg g^{-1}). At higher nitrogen levels (7.5 mM), wheat accumulates nitrogen in the greatest quantities and leaves have a dark green colour. At low nitrogen levels (2.25 mM) leaves were lighter green and nitrate content was decreased for both genotypes. The two varieties did not differ significantly in nitrate content ($p = 0.0775$) and the interaction was not significant ($p = 0.851$) (Fig. 2-6).

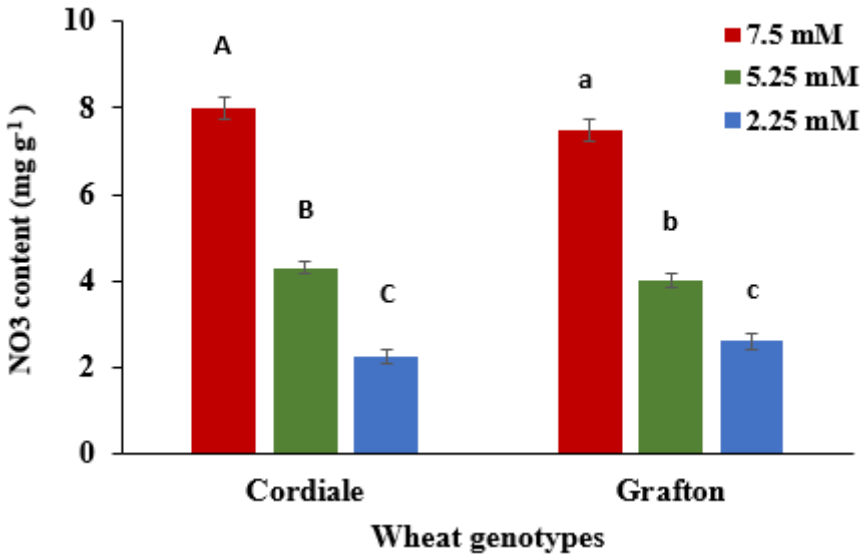
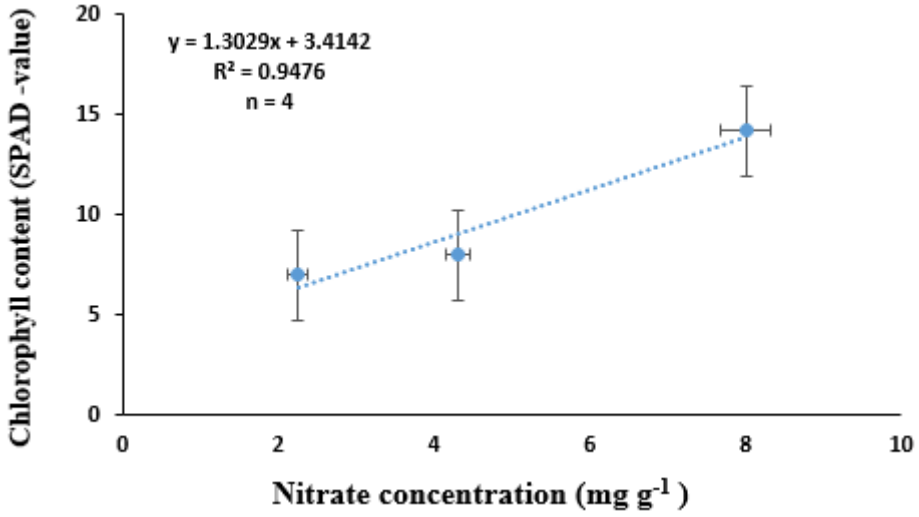


Figure 2-6. Effect of nitrogen supply on leaf nitrate content.
Plants (Cordiale and Grafton) were grown for 21 d with three different levels of nitrogen: 7.5 mM, 5.25 or 2.25 mM. Samples were taken from leaves 3 and 4. Data are presented as mean values ± SE (n = 4). For each cultivar, different letters represent statistically different means (Tukey test; $p < 0.05$).

Correlations between chlorophyll content and leaf nitrogen content are shown in Fig. 2-7a and b. There was a positive linear relationship between chlorophyll content index (CCI) and leaf nitrate in both Grafton and Cordiale ($R^2 = 0.998$ and $R^2 = 0.948$, respectively).

(a) Cordiale



(b) Grafton

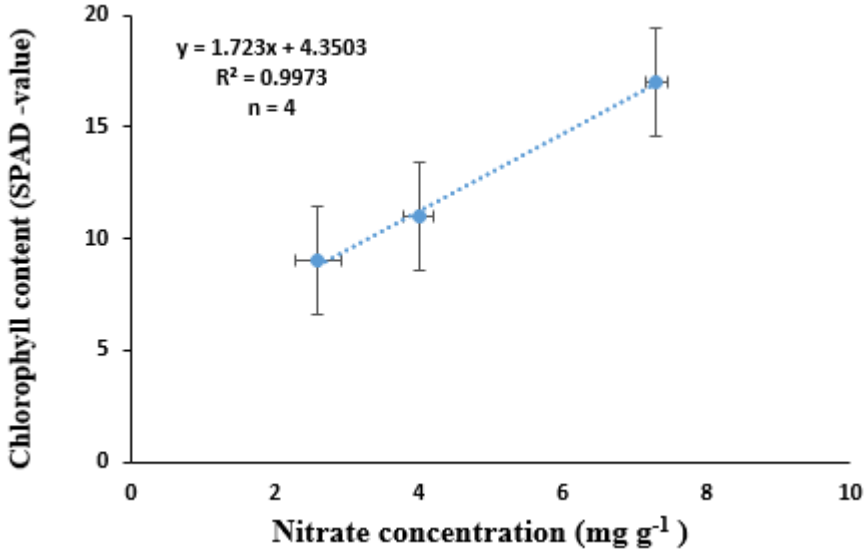


Figure 2-7. Relationship between chlorophyll content and nitrate concentration.
The linear correlation between chlorophyll meter reading SPAD in leaf number 3 and nitrate concentration in shoot number 3 and 4 of wheat genotypes (a) Cordiale and (b) Grafton. Values for individual plants are shown, according to the figure legend as mean values ± SE (n=4). Plants were grown with 7.5 mM, 5.25 mM and 2.25 mM N, for 21 d.

Relative water content (RWC)

With 7.5 mM nitrogen, RWC was 94.8% in Cordiale and 87.7% in Grafton. RWC showed a slight decrease with 5.25 mM N compared to 7.5 mM. RWC was higher in Cordiale (93.7%) than in Grafton (82%) The lowest RWC in leaves was observed with 2.25 mM N at 72% and 70% for Cordiale and Grafton, respectively. The RWC for Grafton was below that for Cordiale for all three nitrogen concentrations (Fig. 2-8). The relative water content increased significantly but slightly with increasing N input, for both genotypes [p = 0 .01 (< 0.05)]. Moreover, there is significant interaction between the effects of wheat variety and nitrogen concentration on RWC [p = 0.017 (p < 0.05)].

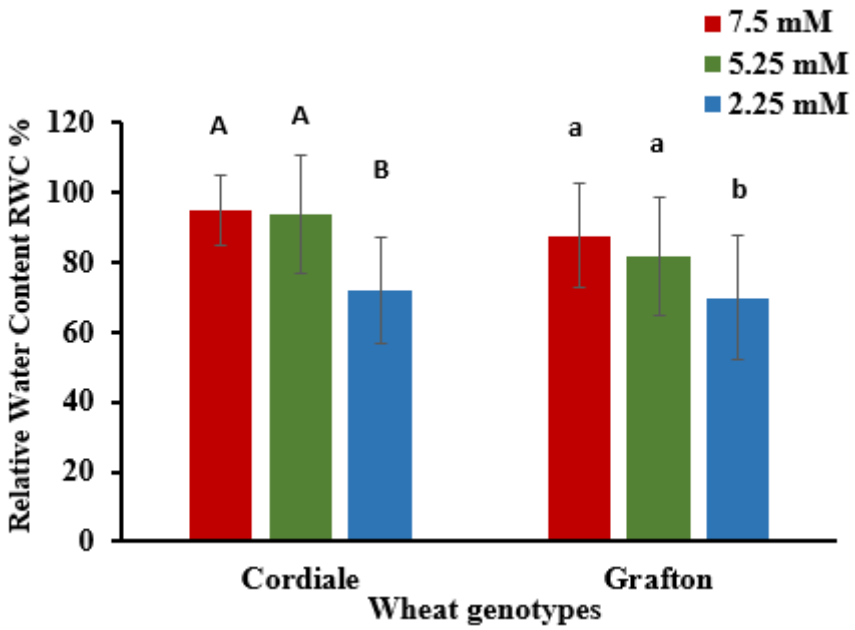


Figure 2-8. Effects of nitrogen supply on relative water content.
Wheat plants (Cordiale and Grafton) were grown for 21 d on Hoagland's solution containing 5.25 and 2.25 mM compared to 7.5 mM. Samples were taken from leaves 3 and 4. Data are represented according to the figure legends as mean values ± SD (n = 4). For each cultivar, different letters represent statistically different means (Tukey test; p < 0.05).

2.4.2 Performance of *S. avenae* on two wheat cultivars grown with different nitrogen levels

Aphid performance

Fecundity of aphids (*S. avenae*) on two winter wheat cultivars (Cordiale and Grafton) grown with different levels of nitrogen was studied in order to assess the effect of the nutrient on aphids' performance. Three different parameters were measured: aphids' total fecundity, daily fecundity and reproduction rate.

2.4.3 The influence of nitrogen input on aphid total fecundity

Total fecundity

The total fecundity of the aphids was measured by the mean total number of nymphs produced by each adult over 21 days. Total aphid fecundity was significantly different between the genotypes ($p < 0.004$) and nitrogen levels ($p < 0.0001$). However, the interaction between these two factors was not significant. The maximum nymph production occurred with high nitrogen input (7.5 mM) on Grafton, with total fecundity 60.8 nymphs, while that of Cordiale was 58 nymphs and this difference was significant ($p = 0.004$). For plants grown at 5.25 mM nitrogen, there were more nymphs produced on Cordiale, with total fecundity 54.5 nymphs, than on Grafton, and this difference was significant. Aphid fecundity was significantly reduced at 2.25 mM N ($p < 0.01$) for both wheat varieties relative to their growth on control plants (7.5 mM N), with total fecundity 20 nymphs and 31 nymphs on Cordiale and Grafton respectively (Fig. 2-9a).

Multiplication ratio

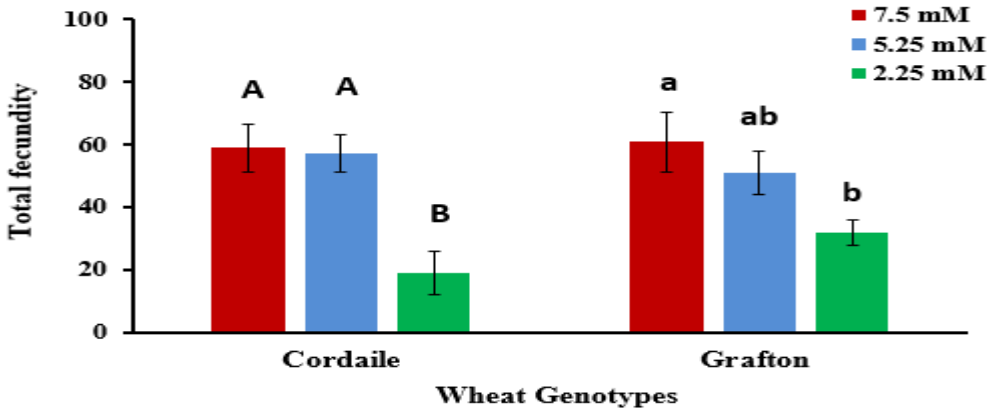
Aphid multiplication was the slowest on both cultivars at 2.25 mM nitrogen input ($p < 0.001$). By 21 days the mean number of aphids was 19 and 31.8 for Cordiale and Grafton, respectively, equivalent to an MR* of 10 and 16.4. The resistance of plants with a moderate N level was only moderate; the number of aphids increased, reaching 59 (MR = 29.5) and 50.8 (MR = 25.6) at 21 days on Cordiale and Grafton, respectively. However, with the control N treatment, by day 21, the number of aphids was 58 and 60.8 on Cordiale and Grafton, respectively. The high N level therefore resulted in the highest fecundity for both cultivars, with MRs of 30.4 and 30.9 respectively (Fig. 2-9b).

*MR= Ni/N0, where N0 was the initial number of aphids inoculated, and Ni was the number of aphids counted at each time point (He *et al.*, 2011).

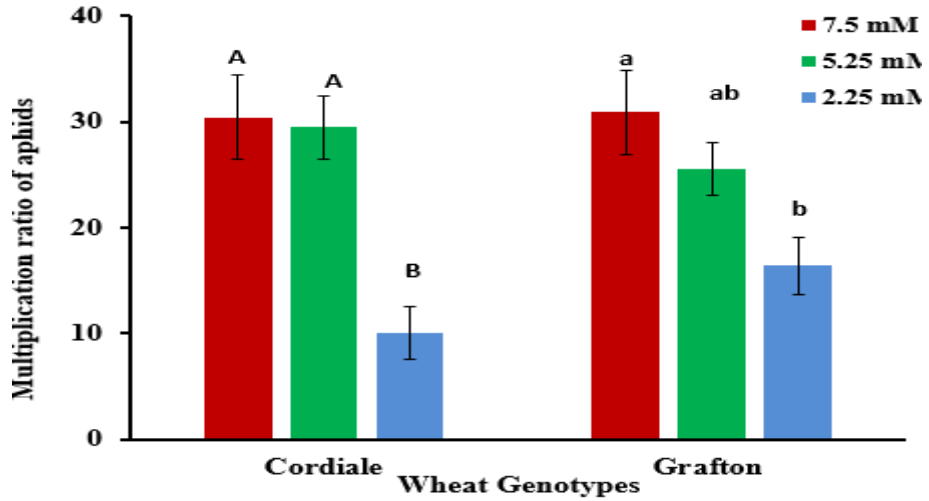
2.4.4 Daily aphid fecundity

The greatest number of nymphs produced per day was recorded on the two wheat cultivars which were treated with 7.5 mM nitrogen. Treatment with low levels of nitrogen (2.25 mM) reduced the daily nymph production. In the low nitrogen conditions, aphids produced an average of 9.1 and 5.5 nymphs per day on the six plants of genotypes Grafton and Cordiale, respectively, whereas aphids on the two other concentrations (7.5 and 5.25 mM) produced 16.5 and 16.9 nymphs per adult on Cordiale, and aphids on Grafton produced 17.4 and 14.5 nymphs per adult per day, respectively. On both varieties, aphid daily fecundity was therefore lowest at the lowest level of nitrogen ($p < 0.001$) (Fig. 2-10).

(a)



(b)



(c)

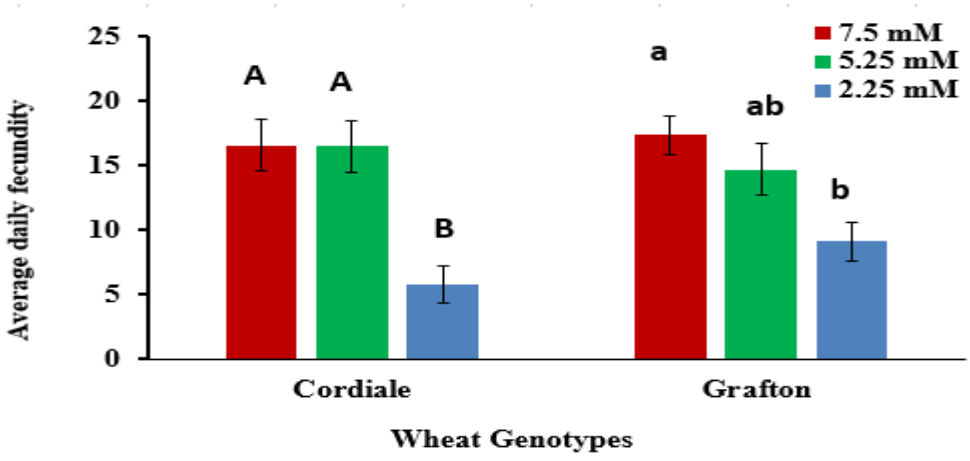
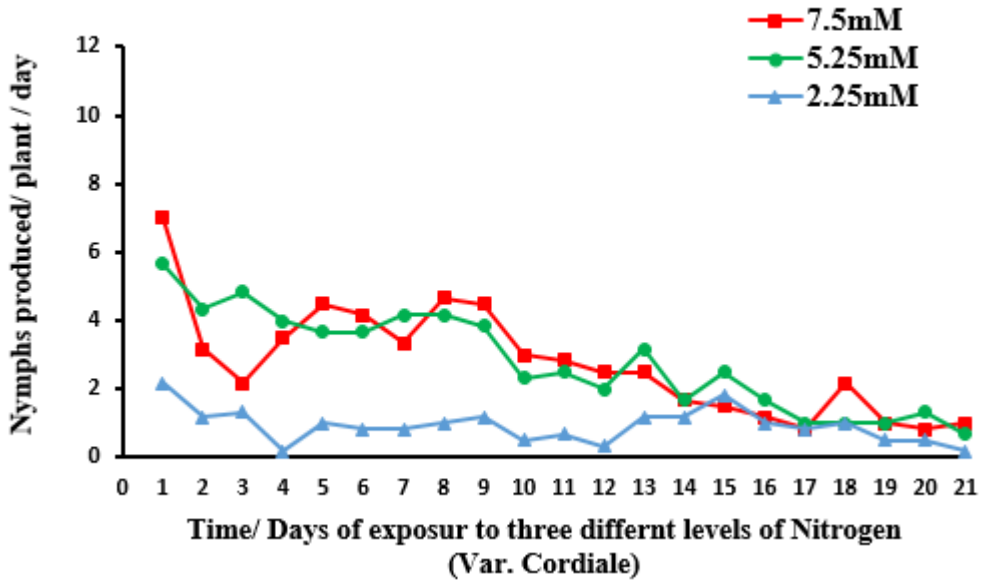


Figure 2-9. Aphid performance parameters on two genotypes, Cordiale and Grafton, grown with three nitrogen levels.(a) Total fecundity (measured as mean total number of nymphs per plant over 21 days, mean ± SE; n = 6). (b) Aphid multiplication ratio. MR: Multiplication ratio of aphids; mean ± SE; n = 6. (c) Mean daily fecundity (measured as mean total number of nymphs produced per day on 6 plants; mean ± SE; n = 6) on the two wheat genotypes.

2.4.5 Relative reproductive rate

The rate at which aphids reproduced was noted and analysed over time. On high-nitrogen Cordiale on day two the aphids produced 7 nymphs per plant. The rate of nymph production decreased then on day 9 increased to 4.5. By day 17 fecundity decreased to <1 per day and the low productivity continued over the remainder of the production period. On moderate nitrogen on day one the aphids produced 5.6 nymphs per plant, decreasing to 4.2 on day 8. Fecundity decreased on day 16 to 1.6 nymphs, then increased slightly on day 18 to 2.2 nymphs. On low nitrogen 2.2 nymphs per plant were produced on day 1. The production of nymphs decreased to <1.2 on most of days 2 to 21 (Fig. 2-10a). On Grafton grown on high nitrogen aphids produced 11.6 nymphs per plant on day 2 and fecundity increased slightly to 4.3 nymphs on day 3. However, fecundity decreased to 2.2 on day 12 and the fecundity on the last five days was < 1 nymph per day. At 5.25 mM nitrogen the aphids' fecundity on the first day was 5.3 nymphs. On days 5 and 7 they produced 2.8 nymphs and production continued to decrease to <1 nymph per day. On low nitrogen aphids produced 4.2 nymphs on the first day, decreasing to 2.8 on day 5 and decreasing thereafter to 1 or fewer from day 9 onwards (except day 19) (Fig. 2-10b).

(a)



(b)

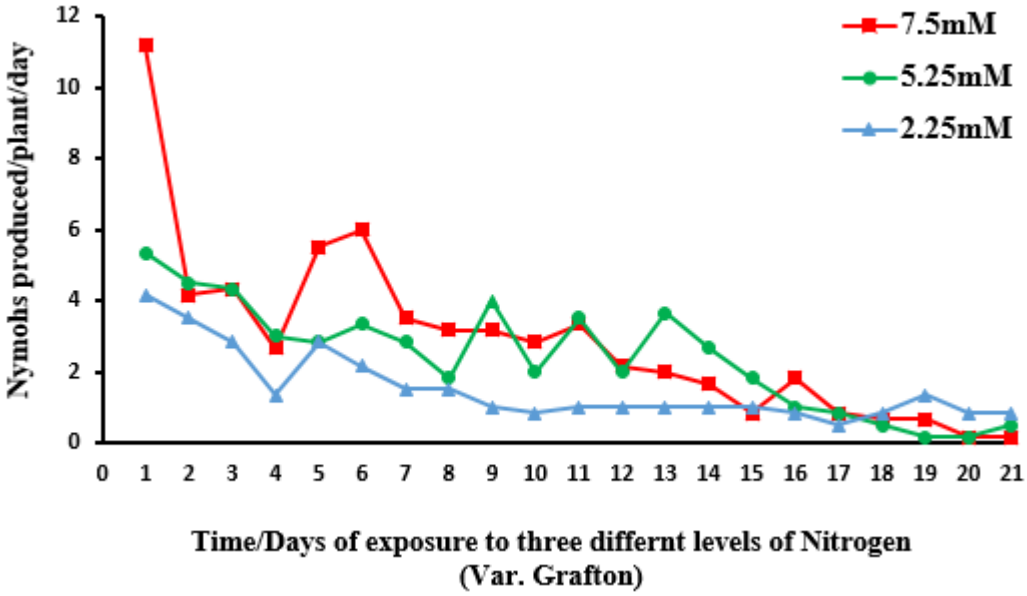


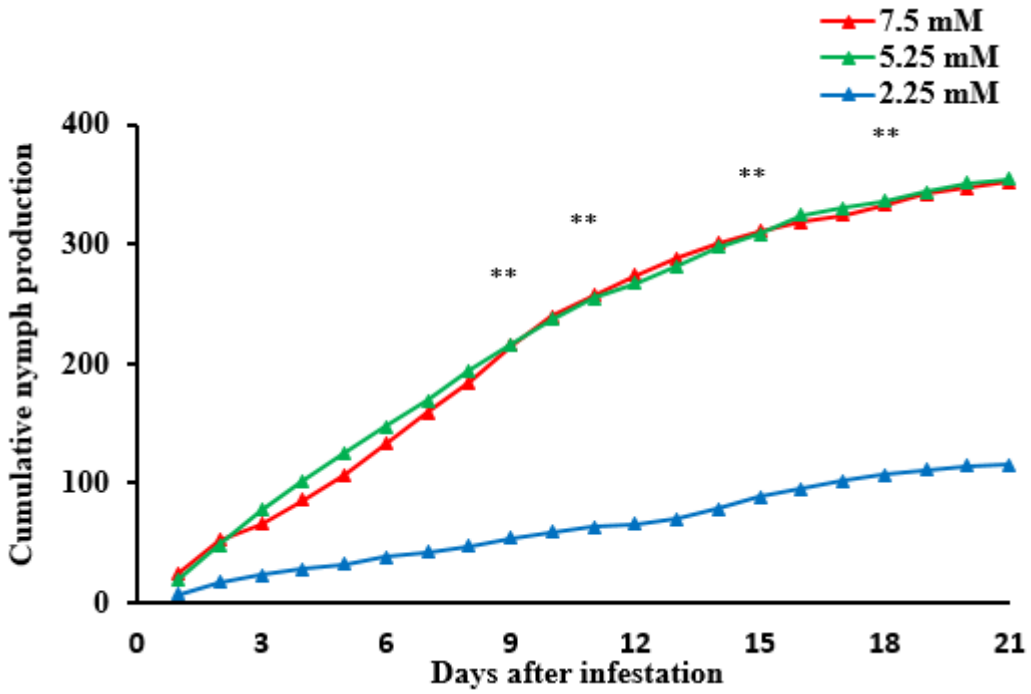
Figure 2-10. Aphid performance parameters on two genotypes, Cordiale and Grafton, grown with three nitrogen levels. (a and b) Aphids were monitored daily for reproduction/nymph production until the end of the bioassay on the two wheat genotypes at three nitrogen levels. Columns headed by different letters for the same cultivar are significantly different (Tukey test, $p < 0.05$).

2.4.6 The influence of nitrogen input on cumulative nymph production

The cumulative nymph production of aphids was significantly affected by nitrogen treatment ($p = 0.001$); however, the interaction between nitrogen treatment and wheat genotype was not significant. The total number of nymphs produced by adults was strongly and positively correlated with the increasing number of days that the host plant was grown. The rate of nymph production was lowest at 2.25 mM, so fewer were produced over time. Increasing nitrogen levels (5.25 and 7.5 mM) caused an increase in cumulative nymph production for both genotypes. The difference was greater with increasing age of plants (Fig. 2-11).

Three weeks after the nymphs started to reproduce the bioassay was terminated. The reason for this was that the different nitrogen inputs were clearly having a large effect. Regression analysis was therefore employed to obtain predictions of aphid numbers at later dates. There was a positive correlation between the total number of nymphs produced in the three weeks and the ages of the adult aphids feeding on the two wheat genotypes. Using quadratic regression (Gao *et al.*, 2012), it was predicted that an increase in aphid age to 30 days, commencing from nymph production, would have resulted in an increase in the abundance of nymphs by an average 22 and 24 total nymphs on Cordiale and Grafton, respectively, at 7.5 mM N. At moderate N levels (5.25 mM) nymph production was predicted to increase by an average 19.20 and 17.5 total nymphs on Cordiale and Grafton, respectively. However, using the regression function it was predicted that the decrease in nymph production at low levels of N (2.25 mM) would have resulted in a decrease in the total number of nymph by an average –4.6 and –8.0 total nymphs on Cordiale and Grafton, respectively.

(a) Cordiale



(b)Grafton

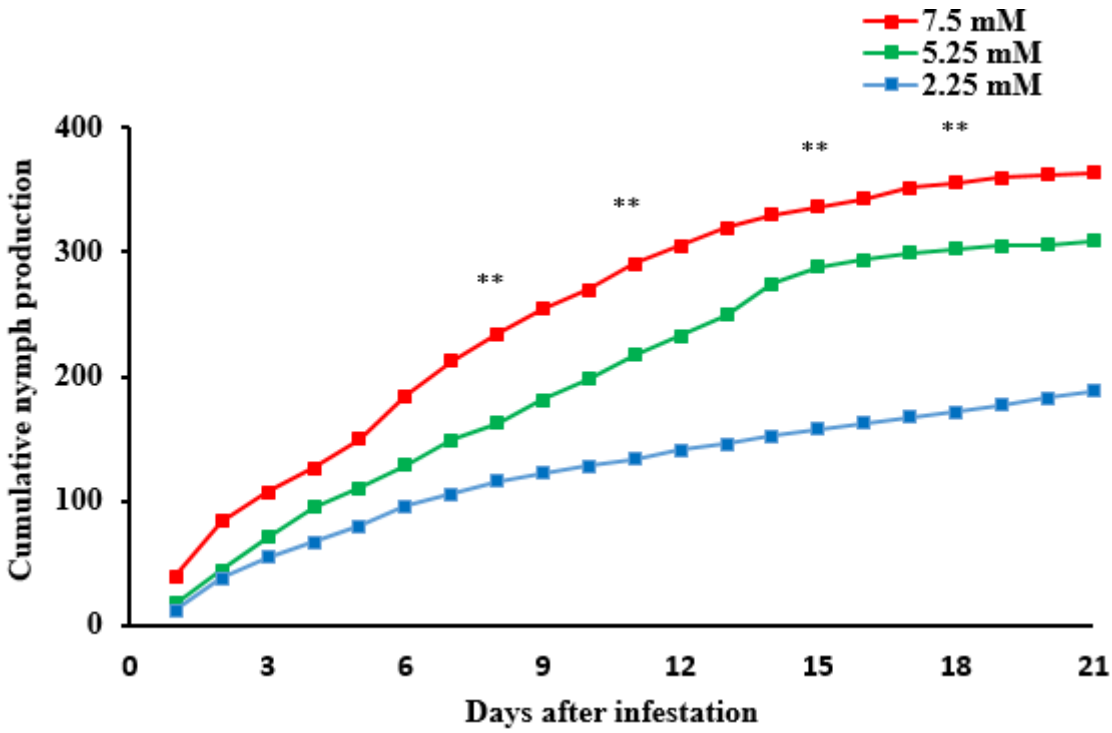


Figure 2-11. Cumulative numbers of nymphs produced over 21 days on Cordiale (a) and Grafton (b) with different levels of nitrogen: 7.5 mM (red line), 5.25 mM (green line) and 2.25 mM (blue line); ** indicates significant differences in treatment means at $P < 0.01$ according to Tukey's test. (n = 6).

2.5 Discussion

Nitrogen input affects plant growth through physiological parameters. However, in plants infested with aphids, it was observed that low nitrogen levels can have a positive effect, depending on the defence response and according to the environmental conditions. In accordance with this, a low nitrogen input can induce a higher level of insect resistance. The main determinant of fecundity in herbivorous insects is the quality of the host plant's phloem sap (Awmack and Leather, 2002) and several studies have reported that applying nitrogen can affect the development and reproduction of cereal aphids. Bird cherry-oat aphids (*Rhopalosiphum padi*) were kept in clip cages on hydroponically grown barley seedlings with 8 mM NH_4NO_3 or without nitrogen (Ponder *et al.*, 2000). Those plants with no nitrogen input had much lower concentrations of non-essential amino acids and consequently, the aphids that fed on them increased at a much lower rate. Similarly, Wagan *et al.* (2015) found that high infestation of wheat with Russian wheat aphids is correlated with the high use of nitrogenous fertilisers.

2.5.1 Plant nitrate content

This study investigated the effect of nutrient input on plant growth and nitrate distribution in the shoot. The results showed that there was a significant difference in nitrate content among the three levels of nitrogen, with higher nitrate correlating with high nitrogen input. This might elucidate the relationship between plant growth and nitrate accumulation. This is in agreement with the results obtained by Chen *et al.* (2004), who reported that plants grown with a high nitrate supply exhibited higher nitrate concentrations.

2.5.2 Leaf chlorophyll content

One of the aims of the experiment was to assess correlation between chlorophyll content and nitrogen status in leaves. The coefficient of determination (r^2) between chlorophyll content in SPAD units and nitrogen concentration (Fig. 2.7) was high for both the Cordiale and Grafton cultivars ($r^2 = 0.948$ and $r^2 = 0.998$, respectively) across all N levels This finding confirms that level of N is strongly and positively correlated with chlorophyll content. A previous study reported the correlation between nitrogen concentration and chlorophyll SPAD 502 readings ($r^2 = 0.693$) in tobacco leaves (Kowalczyk-Jusko and Koscik, 2002). This is in agreement with the findings of other researchers (Richardson *et al.*, 2002; Chang and Robison, 2003; Van den

Berg and Perkins, 2004), who observed a linear relationship between chlorophyll and N content in sugar maple ($r^2 = 0.64$), green ash ($r^2 = 0.73$) and in mangrove leaves ($r^2 = 0.95$).

The amount of nitrogen in leaves is correlated with the colour of the leaf. In the present study, leaves of plants grown with high N levels were dark green, whereas they were light green at low N levels. This indicates that the colour of the leaves is affected by the N input because N is required for synthesis of chlorophyll and proteins and the formation of chloroplasts (Bojović and Marković, 2009).

2.5.3 Relative water content

Relative water content (RWC) plays an important role in physiological functions and growth. It is a measure of the total amount of water in a plant (Waraich and Ahmad, 2010). N input greatly increased growth and development. Moreover, the results of this study regarding leaf RWC have shown that plants grown under high N conditions have higher RWC. The results of the present study are in agreement with Waraich and Ahmad (2010), who reported increases in RWC of wheat at two nitrogen supply levels from 83% to 93% and from 87% to 92%. However, decreases in RWC were recorded from 80% to 77% with low N levels. This finding is consistent with previous studies in which reduced RWC was seen as a result of water deficit (Gollojeh and Ranjbar, 2012). However, opposite results have been presented – that a decreased RWC in response to water deficit causes a decreased phytosynthetic rate via reduced stomatal conductance to CO_2 and photosynthetic metabolism (Ribaut *et al.*, 2009). RWC measurements are used to assess the amount of water and to predict the resistance level in plants water deficit, which may depend on the type of plants and the environmental conditions (Van Loon, 1981; Gollojeh and Ranjbar, 2012)

2.5.4 Aphid fecundity

The present experimental results demonstrated the influence of available nitrogen on aphid performance over 21 days. Two commercial winter wheat varieties (Cordiale and Grafton) were screened for aphid performance by using a technique to measure antibiosis via the nymph count (Tolmay *et al.*, 1999). Recent studies showed that aphid fecundity and developmental time affected aphid performance (Taheri *et al.*, 2010). There were significant differences in fecundity of aphids with different nitrogen levels. Differential nitrogen levels were used to identify putative defence responses in commercial winter wheat to aphid

feeding. The study provides a novel, interesting finding for future attempts to improve the resistance of wheat by changing the environmental stress on aphids.

For a number of years, the preferred method for controlling a variety of plant pests has been plant resistance (Tolmay, 2001). This strategy has the advantage of requiring less pesticides, which are known to be injurious to human health and to the environment, costs are therefore reduced and it facilitates the maintenance of a sustainable agriculture system that also does not target non-detrimental insects (Tolmay, 2001). Plant resistance to insects can be described as one plant's ability to either tolerate and avoid or recover from an insect attack that would greatly damage another plant of the same species (Snelling, 1941). A plant uses three mechanisms for resistance against aphids: tolerance, antixenosis and antibiosis (Tolmay, 2001). There are several reports of wheat lines that are resistant to the Russian wheat aphid *Diuraphis noxia* (Tolmay, 2001). Several aspects of this type of resistance have been identified, such as the general vigour of the plant, the ability to quickly heal wounds, compensatory growth, mechanical support in tissues and organs and alterations to photosynthetic partitioning. Tolmay (2001) reports that tolerance to aphids can be more affected by environmental factors than other kinds of resistance.

A low nitrogen supply could adversely affect fecundity of aphids via nutrient deprivation, a beneficial effect on plants' ability to resist aphids by antibiosis or other means, or both. Antibiosis is defined as a host plant's negative impact on the biology of an insect that tries to use it as a host. The measure for antibiosis resistance used in many studies examining host plant resistance to the Russian wheat aphid *Diuraphis noxia* has been aphid fecundity (reviewed in Tolmay *et al*., 1999). In this research, the fecundity of the aphids, measured as daily/cumulative nymph production, was used to investigate whether antibiosis might be occurring; this is a technique supported by previous studies (Tolmay *et al*., 1999). However, research has shown that measures other than fecundity, for example nymph longevity and development time, are also important indices for determining wheat lines' resistance to aphids (Ozder, 2002). Some researchers, e.g. Scott *et al.* (1991), propose using the total colony counts of all aphids produced on a single plant as a more accurate measure of antibiosis than nymph counts.

Increased nitrogen input to crops led to a positive effect on the growth and fecundity of *S. avenae*. Fecundity and growth rate of aphids are influenced by the nutrient levels of the host plant, moreover, Cisneros and Godfrey (2001) found that plant quality could change because of increasing nitrogen in plants, leading to an increase in the damage caused by aphids in

cotton. This is in accordance with a study by Bentz *et al.* (1995), which observed that increasing nitrogen levels increases the concentration of protein-nitrogen in the leaves.

This study demonstrated that on both wheat genotypes *S. avenae* had the lowest fecundity at a low level of nitrogen; it can thus be hypothesised that plants at this level exhibited high levels of antibiosis resistance. On the other hand, aphids on both genotypes growth with moderate and standard nitrogen levels experienced high fecundity, suggesting that the host plants exhibited intermediate and low levels of antibiosis. Moreover, previous studies on aphids found that low population growth was related to increased antibiosis resistance levels under salinity stress(Khan, 2014).

Total aphid fecundity decreased with low nitrogen levels to 20 and 31 nymphs per adults on Cordiale and Grafton, respectively. Increased nitrogen had the opposite effect, increasing the fecundity of *S. avenae*. The results were in accordance with those of Duffield *et al.* (1997), which showed that increasing nitrogen levels cause an increase in the natural population of *S. avenae*. In general, many studies have reported that nitrogen increases herbivores' performance on crops under controlled conditions and it increases the rate of nymph reproduction (Vereijken, 1979; Zhou and Carter, 1991; Honek, 1992; Thompson *et al.*, 1993). In contrast to these studies, the number of aphids on cotton did not appear to be affected by fertiliser conditions (Slosser *et al.*, 1997). Another study showed that the rate of multiplication of *Rhopalosiphum padi* increased with high levels of nitrogen, compared to low nitrogen input in wheat (Khan and Port, 2008). The findings of this study are in agreement with other studies that reported that the link between plant nitrogen and aphid infestation is complex, and transcripts associated with common signalling pathways involved in resistance to aphids were up-regulated under low nitrogen (Comadira *et al.*, 2015).

In this study, abiotic stress interacts positively with grain aphid stress. The herbivore *S. aveane* had a reduced ability to feed on wheat leaves stressed by low nitrogen supply, which may have contained a higher level of defence. Similarly, drought stress decreased the growth of *Spodoptera exigua* on tomato leaves, as it enhanced resistance levels (English-Loeb *et al.*, 1997). Different stresses in combination could be considered a positive or negative interaction and the effect of abiotic stress on insects or pathogens depends on the timing, nature and severity of the stress (Atkinson and Urwin, 2012).

A low level of nitrogen resulted in the lowest aphid fecundity. Correspondingly, the highest level of nymph production occurred with high levels of nitrogen. This factor may be useful for reducing the rate of increase of aphids. We can conclude that lower nitrogen availability

limits aphid populations. In addition, several previous studies have found that population growth in aphids may be linked to plant nutrient requirements, leaf age and leaf surface (Finlay and Luck, 2011).

2.6 Conclusions

Increasing crop productivity to meet global needs for food on existing agricultural land needs a high rate of application of nitrogen fertilisers (McLellan *et al.*, 2018). N assimilation in plants is needed for growth and grain yield. This study compared different levels of N input and their effect on the physiological parameters of wheat crops. In the present study, the nitrogen availability affected the physiological characteristics and growth of wheat. Two commercial winter wheats (Cordiale and Grafton) had maximum resistance to aphids at the lowest levels of nitrogen input. Different growth measurement parameters, including the height of the plant, leaf area, chlorophyll content, RWC and leaf area, showed a significant and positive relationship with N input for the growth of wheat for 21 days. Based on this, the wheat genotype Cordiale appeared to have a more positive relationship between N input, and plant height, leaf area, chlorophyll content and N content, which could also be considered as N tolerance.

Based on the above, those aiming to improve crop yields should therefore focus on improving the control of nutrient management and developing more resistant crops or pesticides via breeding. The main aim of this study was to use a combination of nitrogen availability (abiotic) and *S. avenae* (biotic) stress. This provided us with a starting point for building a picture of the defence response to aphids, and how this may be linked to nitrogen. Based on this, future research should focus on investigating the mechanism of wheat tolerance and N input for breeding programmes. Two wheat genotypes were characterised to determine the effect of nitrogen availability on the plant aphid *Sitobion avenae*'s performance over 21 days. The data presented here show the effect of N on plant-aphid performance in genotypes Cordiale and Grafton through fecundity. Reduced levels of N caused significant reduction in aphid fecundity. The plants were treated with low nitrogen levels to be more resistant to aphids; however, with a high nitrogen content, the number of aphids increased, making the plant more susceptible. These plants had high nitrogen accumulation.

Future studies and additional assessment need to use a single stress and a combination of stresses to link this phenotype to the molecular basis of these effects. We were able to narrow down our target (TaWRKY in this study) and focus on only one gene. This knowledge can be used to inform a directed strategy for plant breeding for enhanced tolerance via identification and verification of molecular markers. This will be achieved by identifying differentially expressed genes under both stresses at the transcriptional level underpinned by the availability

of the wheat genome. The results of the investigation into the gene expression profile in wheat variety Cordiale in response to nitrogen availability and aphid infestation will be presented in Chapter 3.

3 Chapter 3. Changes in WRKY Gene Expression in Response to Nitrogen Availability and Aphid Infestation

3.1 Abstract

The English grain aphid (*Sitobion avenae*), a phloem-feeding insect, is one of the most damaging pests of wheat (*Triticum aestivum*), which is the third most produced crop globally. Wheat productivity is significantly reduced by biotic and abiotic stress. Like most plants, wheat has endogenous mechanisms to protect against insect attack. This chapter investigates the link between reduced nitrogen availability (abiotic stress) and *Sitobion avenae* infestation (biotic stress), focusing on specific transcription factors (TFs), which are known to play important roles in the defence response in plants. WRKY TFs have been shown to be involved in the induced defence of barley; however, there is little information on the role of these TFs in wheat in responses to nitrogen levels and aphid infestation. The winter wheat cultivar Cordiale was grown in the presence of varying levels of nitrogen. At the 3rd to 4th leaf stage, the plants were infested with aphids, after which shoots were collected and analysed for the expression of *TaWRKY 3, 8, 19, 37, 45* and *46*. RT-qPCR showed a positive correlation between nitrogen availability and *WRKY* gene expression. Expression of the same genes increased overall during the initial stages of infestation by *S. avenae*, reversing the suppression of gene expression induced by the biotic stress factor. At the lowest nitrogen availability the speed and magnitude of this response were both greater than for the optimal condition. These studies allow a more comprehensive understanding of defence dynamics in wheat, suggesting that low nitrogen conditions may prime the defence of wheat against insect attack via the regulation of WRKY transcription factors, whilst aphid infestation results in an increase in the expression of defence genes. Of particular interest was the differential expression of *WRKY* genes under reduced nitrogen availability and aphid stress.

Key words: wheat / nitrogen / *Sitobion aveanae* / transcription factor WRKY

3.2 Introduction

From the results described in Chapter 2, it can be seen that limiting nitrogen input in wheat increases aphid tolerance, but that a low nitrogen input reduces the growth of the plant. Crop production can be achieved through the control of nutrient management and by developing more resistant crops or using pesticides. Here, the effects of cross-talk between biotic and abiotic stress responses in wheat, whereby the response of the regulatory network of transcription factors in the presence of one or more initial stresses may prime a plant to mount a more rapid and prolonged response to subsequent stress, are reviewed with an emphasis on explaining the molecular mechanisms involved (Bi *et al.*, 2007; Rejeb *et al.*, 2014).

Pest damage to wheat can be caused by phloem-feeding aphids such as *Sitobion avenae,* due to their propensity to vector numerous viruses, damage plants by depleting photoassimilates, and manipulate growth and nutrient partitioning (Thompson and Goggin, 2006). Changing agricultural conditions are leading to insect pests becoming a more serious threat to sustainable crop production (Hilder and Boulter, 1999; Tito *et al.*, 2018), and therefore understanding the molecular basis of endogenous tolerance to aphid infestation will help mitigate shortfalls in crop yields.

Transcription factors (TFs) are involved in the regulation of gene expression, including the responses of the plant to environmental factors and biotic stress (Eulgem *et al.*, 2000; Singh *et al.*, 2002). In response to abiotic stresses in plants such as drought, salinity and cold, expression of WRKY genes was shown to be differentially both increased and decreased (Niu *et al.*, 2012; Zhu *et al.*, 2013). In contrast, by analysing the expression of WRKY genes in response to aphid infestation in barley and *A. thaliana*, only up regulation was shown (Comadira *et al.*, 2015). Thus, the focus of this project is to identify whether there is a regulatory link between reduced nitrogen and aphid infestation through WRKY TFs, which play a role in the defence response in plants.

WRKY proteins are defined by the conserved amino acid sequence WRKYGQK at the N-terminal end with a zinc-finger motif showing a binding site for the DNA cis-acting element W box (C/T TGAC). They have a regulatory function in response to many stress factors, which result in the concerted activation of a large variety of genes (Zhu *et al.*, 2013). Cross-talk by WRKY TFs regulates the expression of other TFs such as MYBs, which regulate the ABA response (Rushton *et al.*, 2012). Okay *et al.* (2014) have identified ninety-two WRKY family members in wheat. The genetics of TaWRKY are not well documented and few have

been identified as having specific roles in relation to biotic and abiotic stress. WRKY orthologues in wheat and rice are similar to those in *Arabidopsis* (Niu *et al.*, 2012). It is thus helpful to study homologues using the best characterised model plants such as *Arabidopsis*, because little is known about functions of WRKY genes and domain sequences in wheat. Six wheat orthologues of Arabidopsis WRKY genes were selected based upon their known response to multiple abiotic stresses such as drought and cold, and biotic stresses such as fungi in *Arabidopsis*.

The cross-talk regulates the expression of WRKY genes by binding of WRKY proteins to the W-box elements in the promoter regions (Eulgem *et al.*, 2000). Previous work demonstrates that TaWRKY1 and TaWRKY33 bind to drought-related cis-acting elements, allowing TFs to bind within the promoters (He *et al.*, 2016). However, little is known about the roles of most WRKY transcription factors with regard to nitrogen and resistance to aphids in wheat.

In the present study, wheat variety Cordiale was analysed by RT-qPCR to identify WRKY transcription factors (TFs) regulated by a single stress (nitrogen) or a dual stress (aphids and nitrogen), including the selection of target genes associated with reference genes used to standardise mRNA levels between various samples, the expression levels of which were assessed for their consistency across different tissues following stress. Here we provide more knowledge of six TaWRKY TFs from the expression profiles in RT-qPCR regarding the response to nitrogen availability and aphid infestation.

3.2.1 Aim and objectives

To identify wheat genes which are linked to tolerance towards both reduced nitrogen input and *S. avenae* infestation, focusing on WRKY transcription factors (TFs). This was achieved by the following objectives:

1. Selection of wheat WRKY genes homologous to known WRKY stress response genes in Arabidopsis.
2. Assessing the expression status of WRKY genes during periods of single and dual stress, to determine the regulatory link between nitrogen input and the wheat response to aphid infestation.
3. Demonstrate priming of plant defence response under low nitrogen availability.

3.3 Materials and methods

3.3.1 Experiment Design

For this study, wheat variety Cordiale plants were grown to the 4th leaf stage in the presence of 7.5, 5.25 or 2.25 mM available nitrogen. Replicated plants were infested with 20 *S. avenae* adults, while non-infested plants (control T0) were kept in a separate bread bag. Leaves were harvested at 0, 3, 6, and 9 h post-infestation and leaves from the control plant were collected at the same time points. Four individual plants were used as biological replicates (Fig. 3-1).

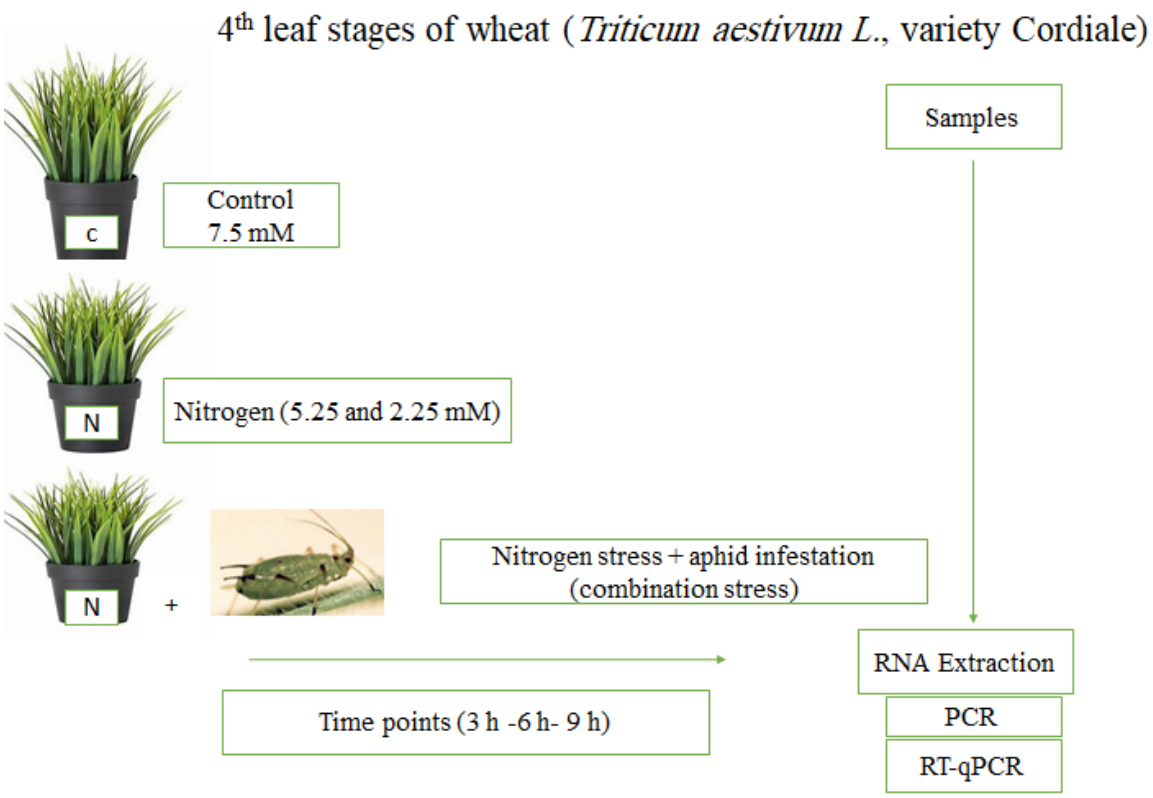


Figure 3-1. Experimental design used to assess the effect of biotic and abiotic stress on the expression of WRKY transcription factor genes in Cordiale.

3.3.2 Plants for Gene Expression

Wheat variety Cordiale supplied from KWS-UK was used for the gene expression analysis. Seeds were soaked in distilled water for 4 h and germinated on wet filter paper for 2 d at 28 ºC then transferred to pots containing washed silver sand. Plants were supplied with three different levels for nutrient treatment (Hoagland's containing 7.5, 5.25 and 2.25 mM nitrogen, see Chapter 2.3.4) and harvested at the 4th leaf stage. The room used for growing the wheat plants was maintained at 25 ºC with 16 h: 8 h light: dark conditions. Twelve plants were

assessed for each level of nitrogen stress. To see the effect of aphid on the plant over time at the 4th leaf stage, the plants were sampled at four different time points (0, 3, 6 and 9 h) after infestation. Thirty-six plants were assigned to aphid infestation and 20 adult aphids were randomly placed on each pot using a fine paint brush, and the plants were covered with bread bags to isolate individual plants. The T0 plant was grown under identical conditions, but without aphids.

3.3.3 RNA Extraction

Leaf tissue was frozen in liquid nitrogen on collection and subsequently freeze dried. 100 mg tissue was ground to a fine free-flowing powder using a Qiagen TissueLyser II. Total RNA was extracted using the PureLink™ RNA Mini Kit following the manufacturer's instructions. RNA concentration and purity were determined by analysing samples with a Nanodrop spectrophotometer (Labtech, ND-1000). The A_{260}, A_{280} and A_{230} values of the RNA samples were measured. Samples with A_{260}:A_{280} ratios between 1.8 and 2.0 were taken forward for cDNA synthesis. RNA was stored at -80 °C to prevent degradation.

3.3.4 cDNA Synthesis

cDNA synthesis was performed by reverse transcribing 1000 ng RNA using the SensiFAST cDNA synthesis kit (Bioline, Catalogue Number: BIO-65054). Reactions were performed in a final reaction mixture volume of 20 µl following the manufacturer's instructions. To ensure accurate temperature regulation the reactions were performed in a thermal cycler at 25 ºC for 10 min, then 42 ºC for 15 min, and 85 ºC for 5 min. The cDNA obtained was kept at -20 ºC until required for PCR.

3.3.5 Polymerase Chain Reaction (PCR)

a. Primers

Primers for 6 *TaWRKY* genes were used (Table1). Primer3 Plus software was used to design the RT-qPCR primers. Primers for WRKY3, WRKY8 and WRKY37 had previously been designed using the same software, and had successfully been used for qPCR on wheat-derived cDNA (Edwards, personal communication). The primer sequences for WRKY19 and WRKY45 were described previously (Bahrini *et al.*, 2011; Niu *et al.*, 2012). Primers for WRKY46 were identified using the wheat sequence and Primer3/BLAST software (https://primer3plus.com/) Wheat elongation factor 1 alpha 1 (*EF*) was used as a reference

gene and the primer sequences have previously been described elsewhere (Metz *et al.*, 1992). All primers were ordered from Sigma Life Science with concentrations of 100 µM.

Table 3-1. List of primers designed to amplify 6 different WRKY cDNAs plus the reference cDNA (EF1α) for real-time q-PCR

Gene	Accession number	Specific primers for Real time PCR		Expected size (bp)
		Forward primer (5'-3')	Reverse primer (5'-3')	
TaWRKY3	EU665432	GTGCTGGACGACGGATACAA	TAGCTCCTGGGATGAAGGCT	79bp
TaWRKY8	DQ323885	CCTACTTCCGGTGCTCCTTC	CGCCACGAGTATGGTCTTGT	83bp
TaWRKY19	EU665430	AGGGAAGCATACGCATGACGTGC	GGCGAGATCGTTCAGAATGGCTG	160bp
TaWRKY37	EU665452	GCCAGAAGGCAGTTAAGGGT	CTTAACTGGACAGCTCGCCT	77bp
TaWRKY45	EF397613	CATGAGGAGCTTGGAGGACG	AGGCCTTTGAGTGCTTGGAG	80pb
TaWRKY46	EF368365	CGAGCACAACCAACCAACAG	GTGGACAGACACATCACCGT	72bp
EF1 Reference gene	M90077	ACCTGAAGAAGGTCGGCTACAA	ATCTGGTCAAGCGCCTCAAG	139bp

b. Taq Polymerase PCR and Gel Electrophoresis

50 µl PCR reactions used 25 µl PCR Master Mix (2X) (Thermo Scientific), 1 µM forward primer, 1 µM reverse primer, 1 µl template DNA and nuclease-free water to 50 µl. Reactions were run in a thermal cycler at 95 °C for 3 min, followed by 40 cycles with 95 °C for 30 s. The annealing temperature was tested by a gradient PCR arranged from 60º to 58 ºC for 30 s and 72 °C for 10 s, with a final extension at 72 °C for 10 min. PCR products were separated by electrophoresis on 1% agarose (Melford, Ipswich, UK) and gels were made depending on the size of the product by using 1x TAE buffer with 0.5 µg/ml ethidium bromide (Fisher Scientific). The samples were run at 100 V in a tank of 1x TAE with 15 µl of each sample mixed with 5 µl loading dye (5X). The standard was 5 µl of 100 Hyperladder (Bioline).

3.3.6 Quantitative real-time PCR

The cDNAs from Cordiale were used as templates, and qRT-PCR was performed using the Qiagen Rotor Gene Platform, and 2x Rotor Gene SYBR Green PCR Master Mix solution (Qiagen BIO-98020). 50 µl reactions containing 25 µl 2x SYBR Green, 2 mM forward primer, 2 mM reverse primer, 2 mM template cDNA and 19 µl RNase-free water were run using the standard conditions of 95 ºC for 60 s, 40 cycles of 95 ºC for 5 s, and annealing and extension at 60 ºC for 10 s. In order to validate the primers, a standard curve based on serial dilution of cDNA was made to determine primer annealing efficiencies, the presence of primer dimers was assessed and the production of a single PCR product was verified.

3.3.7 Distance analysis

A distance tree was constructed based on comparisons of amino acid sequences of nineteen WRKY genes in wheat (*Triticum aestivum*) and sixty-seven AtWRKY proteins. All the TaWRKY gene sequences have been submitted to GenBank with accession numbers (http://www.ncbi.nlm.nih.gov/genbank). WRKY domain sequences were identified and aligned using the NCBI multiple sequence alignment tool. The tree was constructed from the aligned sequences using the UPGMA method in MEGA7 with default settings.

3.3.8 Data Analysis

Relative expression was calculated according to the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001):

1. ΔC_T = C_T (Target gene) - C_T (EF1α reference gene)

2. $\Delta\Delta C_T$ = ΔC_T (Test condition) - ΔC_T (Control condition)

3. Fold change = $2^{-\Delta\Delta C_T}$

Four biological replicates with three technical replicates were used for each time point. The average C_T values (number of cycles required to achieve critical fluorescence) for the EF1α reference gene and the target gene were calculated from the C_T value for each technical replicate for that biological sample, to give a set of C_T values. The mean ΔC_T value (equation 1) was then calculated for each biological replicate, and then for each condition (by taking the mean of the three biological replicates under that condition). This was carried out for all treatments and the control condition. The ΔC_T for the control was then subtracted from the ΔC_T of each treatment condition to give a $\Delta\Delta C_T$ value for the treatment condition (equation 2). The $2^{-\Delta\Delta C_T}$ value (equation 3) represents the change in gene expression of the target gene, taking into account changes in overall levels of gene expression, which may vary from plant to plant regardless of treatment. For normalisation the elongation factor 1 alpha-subunit (EF1α) gene was used as the reference gene as it has been shown to be appropriate for this function. The ΔC_T values were calculated for each gene in all treatments and in the control condition. Standard deviations of these values were then utilised to create a range of ± 1 standard deviation of the mean ΔC_T. This range was then used to calculate a range of gene expression ± 1 standard deviation either side of the mean fold change.

3.4 Results

3.4.1 Analysis of sequence similarity

To gain a better understanding of the role of wheat WRKY TFs, domain consensus sequences were obtained by a BLAST search from wheat and compared with the model plant Arabidopsis, because WRKY genes are well characterised in this species. For this, Arabidopsis WRKY protein sequences were taken from GenBank. This information was used to generate a distance tree for selected wheat and Arabidopsis WRKY proteins (Fig. 3-2).

WRKY proteins belonged to three different groups depending on the number of WRKY domains and the zinc-finger structure. WRKY proteins in group I had two WRKY domains. Groups II and III had one WRKY domain. The WRKY domains of group I and II had the same zinc finger motif (C_2H_2 motif); in contrast, group III had a C_2HC motif (Okay *et al.*, 2014).

In order to explore regulation of WRKY genes by nitrogen and aphids, we evaluated the relationships of the sequences of WRKY proteins regulated by stress to previously reported WRKY proteins (Fig. 3-2). Six WRKY genes were selected for expression analysis. WRKY3, a group II WRKY gene, showed homology to AtWRKY13 (Li *et al.*, 2016) (Fig. 3-2). The function of AtWRKY13 is not well understood, and little information is available. WRKY8 from group II, which showed homology to AtWRKY40, has been shown to be a central negatively regulated protein in the ABA signalling pathway (Chen *et al.*, 2012). Expression of TaWRKY19 has previously been shown to differ in response to abiotic stress including drought, cold and salinity. It showed homology to AtWRKY58 and to proteins with two WRKY domains that have N-terminal and C-terminal WRKY domains from group I (Niu *et al.*, 2012). TaWRKY37 in group II showed homology to AtWRKY4, which plays a role in plant immunity (Pandey and Somssich, 2009). However, full sequences for TaWRKY45 and TaWRKY46, both in group II, showed no direct homology with WRKY proteins from Arabidopsis. TaWRKY45 has been shown to play a role in disease resistance in wheat to pathogens such as *Fusarium* head blight ((Bahrini *et al.*, 2011).

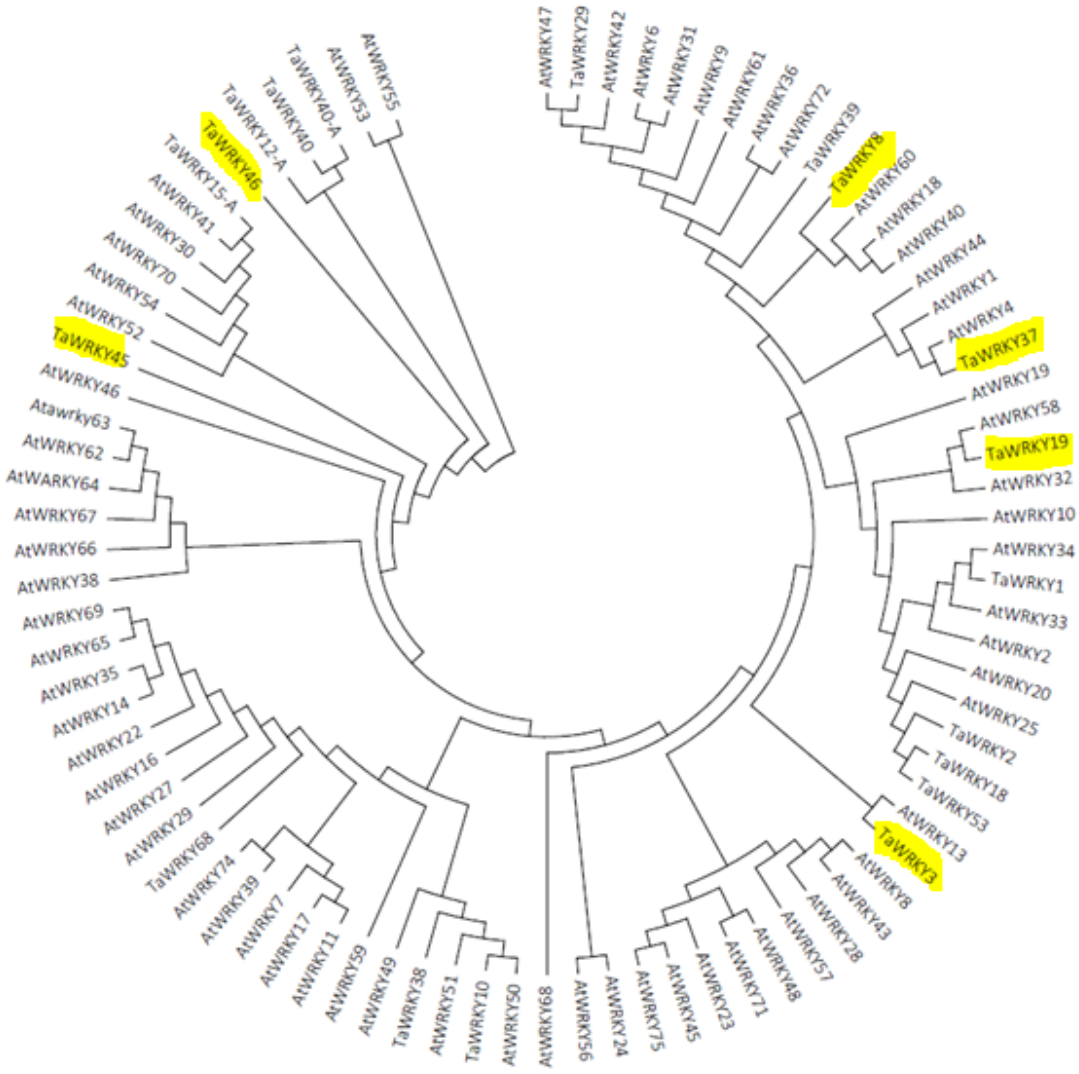


Figure 3-2. Distance tree of WRKY TFs in *A. thaliana* and *T. aestivum* constructed using the UPGMA method. The amino acid sequences are selected to evaluate lineages of WRKY TFs. Products of genes tested in this thesis are highlighted in yellow.

3.4.2 Verification of primers for qPCR

To ensure accurate quantification of gene expression it is vital that primers only amplify the target amplicon and give abundant product at an annealing temperature of 60 °C. All primer pairs used in this study gave products of the expected size for each gene. The PCR products for the reference gene elongation factor 1 alpha-subunit (TaEF1α) (139 bp) and for TaWRKY3 (79 bp), TaWRKY8 (83 bp), TaWRKY19 (160 bp), TaWRKY37 (77 bp), TaWRKY45 (80 bp) and TaWRKY46 (72 bp) started to appear from 58º to 60 ºC, after 40 cycles. Obviously, there are seven PCR products between the reference and the six TaWRKY genes, according to the corresponding size of these products and the specific annealing temperature (Fig. 3-3).

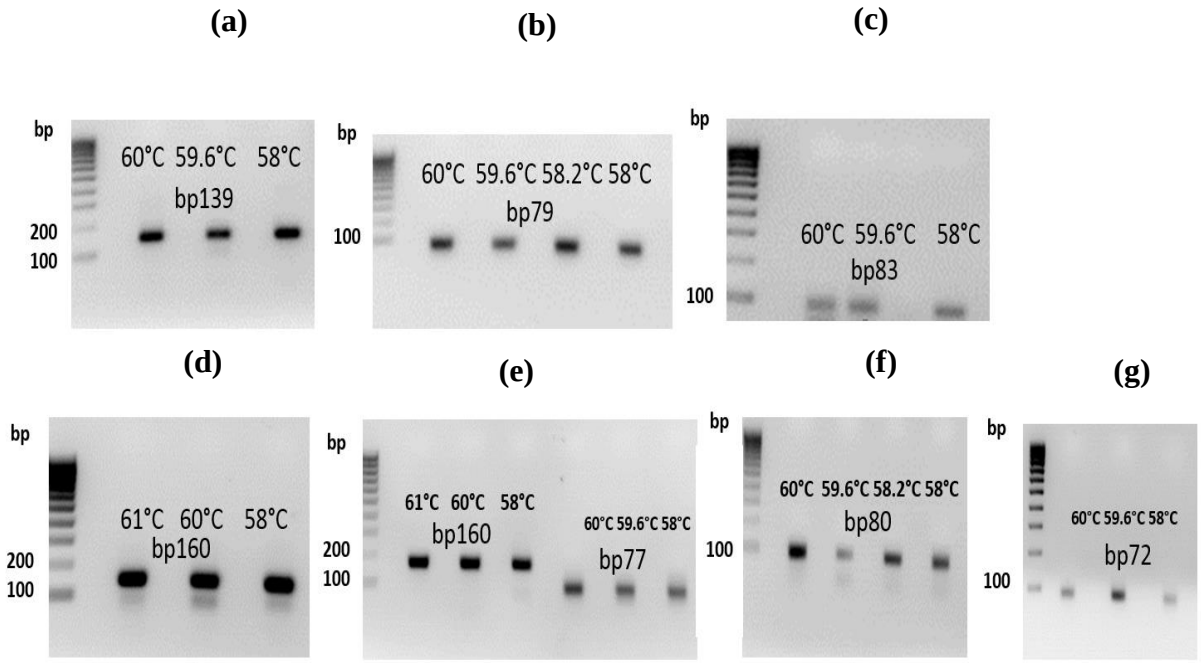


Figure 3-3. Agarose gels run at 100 V for 45 min of amplified DNA in samples of *Triticum aestivum* var. Cordiale using reference gene (TaEF) and 6 WRKY genes. (a) Elongation factor 1 alpha-subunit (TaEF1α) (b) TaWRKY3 (c) TaWRKY8 (d) TaWRKY19 (e) TaWRKY19 and 37 (f) TaWRKY45 (g) TaWRKY46. Primers were tested with different annealing temperatures as shown.

3.4.3 Confirmation that TaEF1α is a suitable reference gene

It is essential to employ an endogenous control in quantitative analysis. In this case, the control was employed to make sure that comparisons could be made between different samples by testing the total amount of relevant cDNA present. TaEF1α was tested in this study for use as the endogenous control in order to normalise the gene expression analysed and to check the efficiencies of reverse transcription in the different samples from gene expression studies. It has previously been used to analyse WRKY expression for studying viral infections in cereals (Jarošová and Kundu, 2010) (Fig. 3-4).

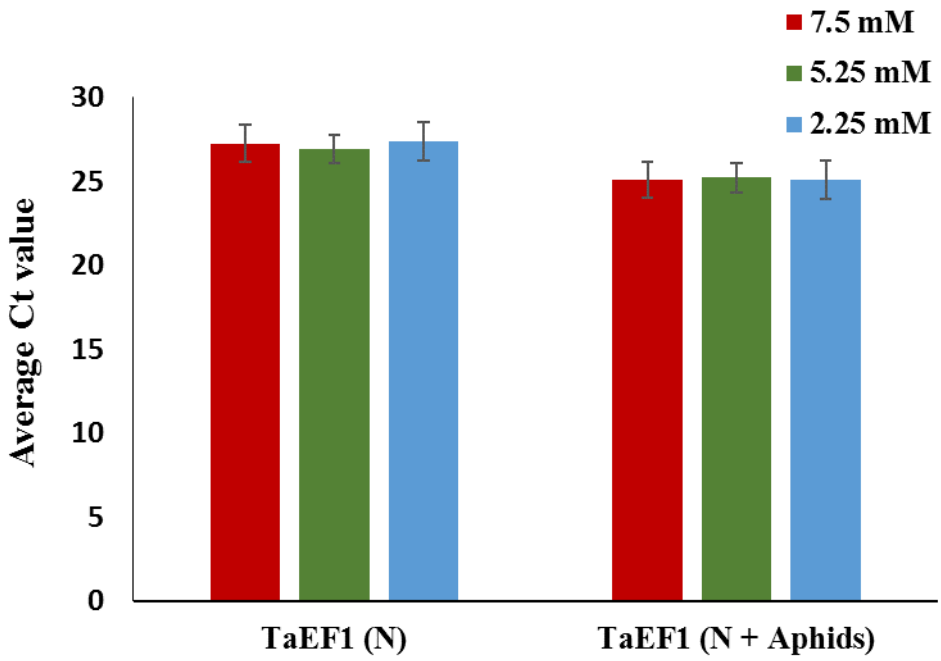


Figure 3-4. Elongation factor 1 alpha-subunit was tested for its suitability for gene expression across nitrogen inputs at 7.5 mM, 5.25 mM and 2.25 mM, and for dual nitrogen and aphid infestation stress. The data is represented as mean Ct values ± SE (n=4).

3.4.4 Effect of reduced nitrogen on WRKY gene expression in wheat variety Cordiale

The expression of six WRKY TFs in wheat plants grown with reduced nitrogen availability was determined by using qRT-PCR. WRKY3, WRKY8 and WRKY19 were identified from the GenBank NCBI database (https://www.ncbi.nlm.nih.gov/genbank/) as being evolutionarily close to AtWRKY13, AtWRKY40 and AtWRKY58, which play important roles in responses to biotic or abiotic stress. TaWRKY37 and TaWRKY45 are known to regulate stress tolerance to improve plant performance under different abiotic stresses and biotic stress respectively. Overall, the results show down-regulation of WRKY gene expression in response to the lowest level of nitrogen availability (Fig. 3-5), whereas the response of the six genes was more complex at the intermediate nitrogen availability (5.25 mM) with two of the six genes showing possible up regulation, one gene showing marginal down regulation and the remaining three being down regulated more than two fold.

The expression of WRKY3 at 5.25 mM nitrate was only marginally less, <0.25 fold, than the expression observed in plants grown with the optimal (7.5 mM) nitrogen availability. However, at the lowest level of nitrate availability (2.25 mM) expression of this gene was reduced by 4-fold compared to the optimum. At 5.25 mM nitrate, WRKY8 seemed to be expressed at a greater level, > 1.5 fold, than the optimum, whereas at 2.25 mM nitrate expression was reduced by 4.5 fold The expression of WRKY19 was down-regulated less, <0.5 fold, in low nitrogen conditions, but there was little difference at 5.25 mM from the high nitrogen level. Both levels of nitrogen (5.25 mM and 2.25 mM) resulted in similar down-regulation of WRKY37 and WRKY46 genes but the difference seen with WRKY46 was greater than with WRKY37. The expression of WRKY45 showed down-regulation under both low and intermediate nitrogen conditions, but the change with low nitrogen was less than with intermediate nitrogen (Fig. 3-5).

The link between gene expression and nitrogen conditions is that each gene is expressed differently from the others, with gene expression responding to the level of nitrogen input. WRKY8 and WRKY19 were the only ones whose expression appeared to increase at 5.25 mM, although this was only a very small change.

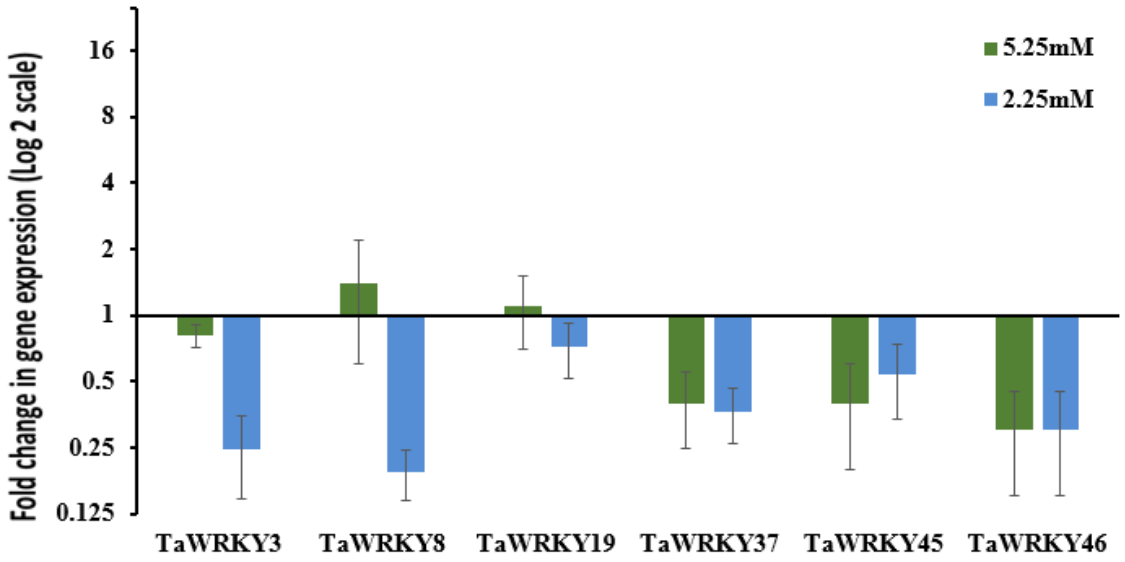


Figure 3-5. WRKY expression profiles relative to TaEF1α in response to different levels of nitrogen availability (5.25 mM and 2.25 mM), normalised to expression at 7.5 mM N in Cordiale wheat. Error bars represent standard deviations.

3.4.5 Time course of expression of WRKY genes induced during simultaneous stress from aphid infestation and reduced nitrogen availability

The expression of the six target WRKY genes was investigated at 3, 6 and 9 hours after aphid infestation at 7.5, 5.25 and 2.25 mM nitrogen availability. Overall, the expression of WRKY genes was up-regulated 3 hours after infestation. Expression either continued to increase or plateaued through the sixth hour after infestation and then decreased at the ninth hour. Each WRKY gene displayed individual expression characteristics within the time frame and these will be described below.

***Triticum aestivum WRKY* gene expression increases on infestation**

Expression of TaWRKY3

Expression of WRKY3 at 7.5 and 5.25 mM nitrate responded in a similar manner. Gene expression increased from 3 hours after infestation to a maximum at 6 hours and then returned to near-basal levels by 9 hours after infestation. Although the response was similar the magnitude of response was greater at 5.25 mM for all time points with relative expression levels being typically approximately twice those at 7.5 mM N. At the lowest level of nitrogen, 2.25 mM, the expression of WRKY3 was initially 4-fold lower than in non-infested plants at 7.5 mM N. After 3 hours of infestation the relative expression of this WRKY gene increased 64-fold resulting in a 16-fold higher expression compared to the reference condition. This magnitude of response was the greatest seen for this gene in the experiment. High expression was then maintained for longer than for the other two conditions (Fig. 3-6).

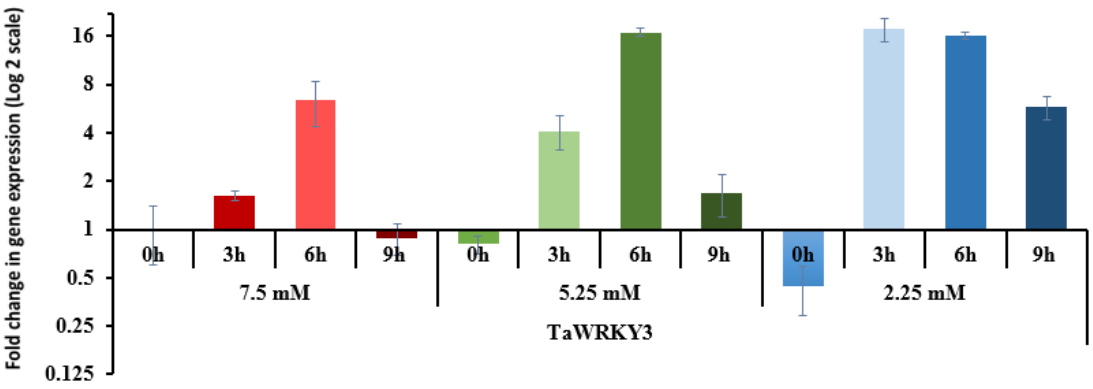


Figure 3-6. Results of qRT-PCR analyses of TaWRKY3 gene expression profiles relative to TEF1α under three different nitrogen conditions (7.5 mM, 5.25 mM and 2.25 mM) in winter wheat at different time points before (0 h) and after *S. avenae* infestation (3 h, 6 h, 9 h). All values are relative to time 0 h at 7.5 mM.Error bars represent standard deviations.

Expression of TaWRKY8

The expression of WRKY8 increased in response to aphid infestation at all nitrogen levels. At 7.5 mM nitrogen, maximum expression of this gene was 9 hours after exposure to aphids. At 5.25 mM nitrogen, expression of TaWRKY8 occurred sooner and it was expressed at a higher level 6 hours after infestation. The lowest level of nitrogen resulted in the same rapid onset of gene expression. The response was highest 3 hours and 9 hours after infestation, but the relative expression of this gene decreased slightly at 6 hours (Fig. 3-7).

(a)

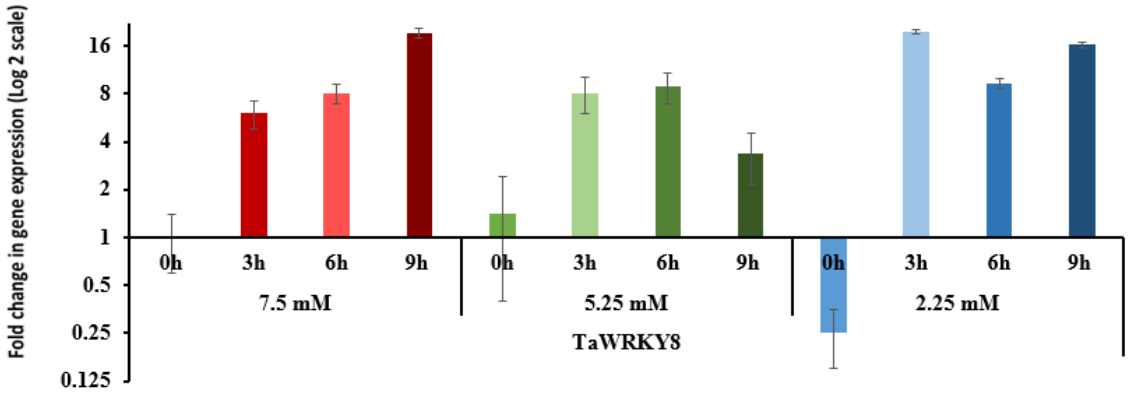


Figure 3-7. Result of qRT-PCR analyses of TaWRKY8 gene expression profiles relative to TEF1α under three different nitrogen conditions (7.5 mM, 5.25 mM and 2.25 mM) in Cordiale wheat at different time points before (0 h) and after *S. avenae* infestation (3 h, 6 h, 9 h). All values are relative to time 0 h at 7.5 mM. Error bars represent standard deviations.

Expression of TaWRKY19

WRKY19 showed little expression change at all nitrate conditions. At 7.5 mM nitrogen, expression of this gene decreased at 3 hours after infestation then increased to a maximum at 9 hours. The response was greater at 5.25 mM for 3 hours infestation with expression twice, but the expression of this WRKY decreased at 9 hours after infestation, that of the optimal conditions. At the lowest level of nitrogen maximum expression of WRKY19 was 3 hours after infestation. Expression of WRKY19 decreased at 6 hours then returned to increase at 9 hours after infestation (Fig. 3-8).

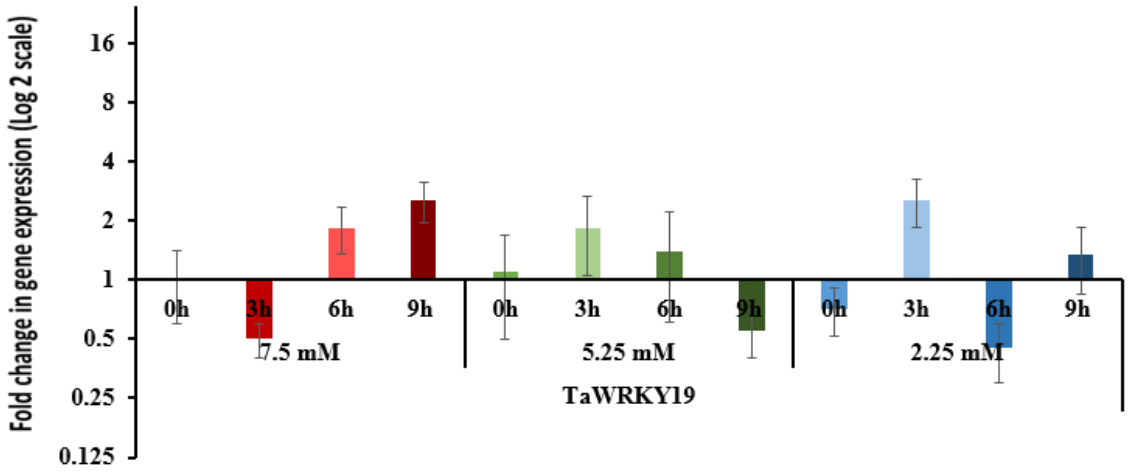


Figure 3-8. Results of qRT-PCR analyses of TaWRKY19 gene expression profiles relative to TEF1α under three different nitrogen conditions (7.5 mM, 5.25 mM and 2.25 mM) in Cordiale wheat at different time points before (0 h) and after *S. avenae* infestation (3 h, 6 h, 9 h). All values are relative to time 0 h at 7.5 mM. Error bars represent standard deviations.

Expression of TaWRKY37

Relative expression of WRKY37 increased in response to aphid infestation. At high nitrogen gene expression increased from 3 hours after infestation to a maximum 16-fold at 9 hours after infestation. Although the response was similar at 2.25 mM, the magnitude of response was greater for 3 hours and 6 hours with expression levels being higher than at 7.5 mM. Expression also returned to a lower level 9 hours after infestation. With the moderate level of nitrogen, 5.25 mM, the expression of WRKY37 was slightly down-regulated. The expression of this gene was up-regulated after 3 hours of infestation, but decreased to basal levels at 9 hours of infestation compared to the reference condition (Fig. 3-9).

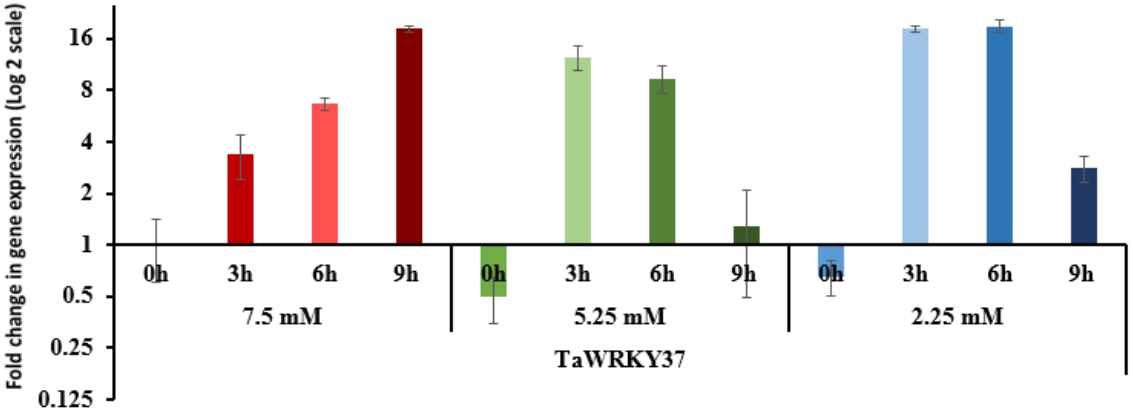


Figure 3-9. Result of qRT-PCR analyses of TaWRKY37 gene expression profiles relative to TEF1α under three different nitrogen conditions (7.5 mM, 5.25 mM and 2.25 mM) in Cordiale wheat at different time points before (0 h) and after *S. avenae* infestation (3 h, 6 h, 9 h). All values are relative to time 0 h at 7.5 mM. Error bars represent standard deviations.

Expression of TaWRKY45

WRKY45 showed very different expression patterns between the different levels of nitrogen over time. With 7.5 mM nitrogen, the relative expression of WRKY45 increased at 6 hours and 9 hours after infestation. At 5.25 mM nitrogen, relative expression increased at 3 hours after exposure to aphids. Expression then returned to basal level at 9 hours after infestation. Under the lowest level of nitrogen, 2.25 mM, the maximum expression of this gene was after 3 hours of infestation compared to the reference condition (Fig. 3-10).

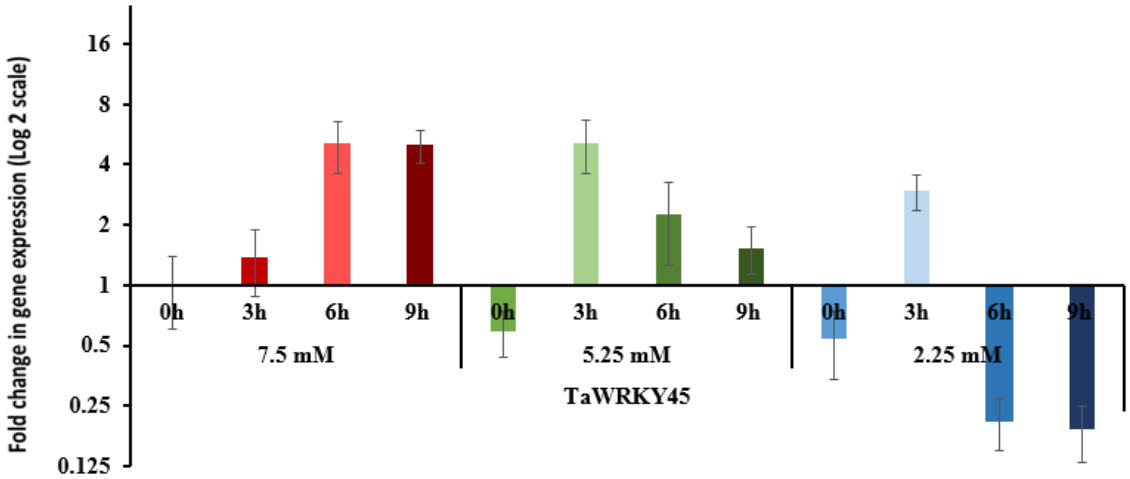


Figure 3-10. Result of qRT-PCR analyses of TaWRKY45 gene expression profiles relative to TEF1α under three different nitrogen conditions (7.5 mM, 5.25 mM and 2.25 mM) in Cordiale wheat at different time points before (0 h) and after *S. avenae* infestation (3 h, 6 h, 9 h). All values are relative to time 0h at 7.5 mM. Error bars represent standard deviations.

Expression of TaWRKY46

Relative expression of TaWRKY46 increased over time at 5.25 and 2.25 mM. With 7.5 mM, there was a similar rapid increase in gene expression, but the magnitude of the response was higher for the 3 hours and 6 hours time points. Expression then returned to the basal level at 9 hours after infestation. At 5.25 mM nitrogen relative expression was greater than at 7.5 mM. Expression also decreased slightly after 9 hours infestation. At the lowest level of nitrogen, the expression of WRKY46 was lower at time zero than in the reference conditions. After 6 hours infestation the relative expression of this WRKY gene increased 16-fold compared to the reference conditions (Fig. 3-11).

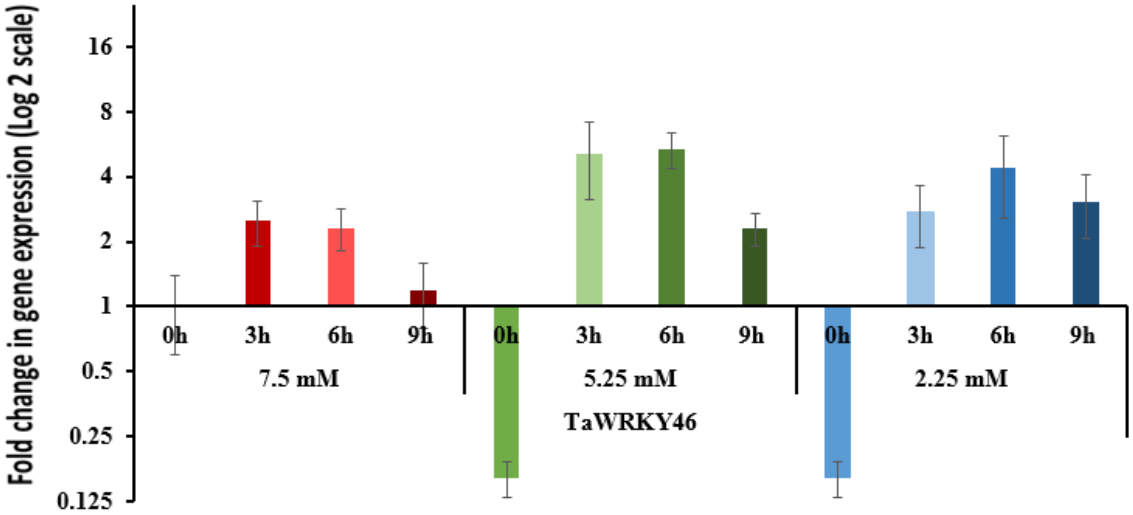


Figure 3-11. Results of qRT-PCR analyses of TaWRKY46 gene expression profiles relative to TEF1α under three different nitrogen conditions (7.5 mM, 5.25 mM and 2.25 mM) in winter wheat at different time points before (0 h) and after (*S. avenea*) infestation over time (3 h, 6 h, 9 h). All values are relative to time 0h at 7.5 mM. Error bars represent standard deviations.

3.5 Discussion

The WRKY gene sequences for bread wheat (*T. aestivum*) were downloaded from GenBank, the NCBI database (https://www.ncbi.nlm.nih.gov/genbank/). From the results of alignments and distance trees, six genes from *Triticum aestivum* coding for WKRY transcription factors were chosen for expression analysis during nitrogen availability and aphid infestation stress. Complete sequences of WRKY domains of these six genes were obtained with accession numbers as shown in Table 3-1. The distance tree revealed that most WRKY TFs belonged to groups I, II, and III. TaWRKY3, TaWRKY8 and TaWRKY37 were identified in groups IIa, IIb and IIc respectively, shown to be relatives to group I, and TaWRKY19 was found to be a close relative of group I. TaWRKY45 and TaWRKY46 were identified in group III. Recent investigations have revealed that most WRKYs in these studies function in drought, salinity, cold and fungi in many species. For example, TaWRKY19 responds to drought and salt stress in transgenic Arabidopsis plants (Niu *et al.*, 2012). Similarly, AtWRKY57 responds to drought in Arabidopsis (Jiang *et al.*, 2012). Moreover, TaWRKY45 was closely homologous to AtWRKY54, identified as group II, and this responds to fungi (Bahrini *et al.*, 2011).

In this study, WRKY gene expression in wheat leaves from plants grown with differing nitrogen levels and infested with *S. avenae* was standardised to expression of the elongation factor 1 alpha-subunit (TaEF1α) gene based on its consistency for use as an endogenous control for WRKY gene expression (Fig. 3-4). As hypothesised, it was found that infestation by aphids and reduced nitrogen levels resulted in differential expression of the WRKY genes. A reduction in available nitrogen levels led to down-regulation of expression of most of the WRKY genes studied, whereas most were up-regulated over time in response to aphid infestation. This was consistent with the results in response to low levels of nitrogen, as it is believed that this level of nitrogen plays an important role in the defence against aphid infestation. Considering the large genome of wheat and the relatively little that is known about how responses to nitrogen stress and interactions with phloem-feeding insects affect the plant, the identification of nitrogen-aphid tolerance mechanisms is still challenging. The data demonstrated that the relationship between the two stresses is complex.

3.5.1 WRKY genes responding to nitrogen input

The low level of nitrogen (2.25 mM) resulted in down-regulation of all the WRKY genes tested (Fig. 3-5). These results suggest that down-regulation of WRKY genes with low nitrogen treatment may lead to an up-regulation of defence response genes. This would be consistent with the 'growth versus defence' hypothesis, where a decrease in nutrient availability results in an increase in expression of defence-related genes, thereby potentially conferring resistance or increased tolerance to insects. Reduced growth was seen in Chapter 2. It is expected that WRKY proteins play roles as activators of signalling pathways by directly or indirectly regulating the expression of genes, which provide of plants growth conditions; growth is therefore limited and a large number of growth genes are down-regulated, prompting tissue senescence. Several members of the WRKY transcription factor family are believed to be involved with senescence (Eulgem *et al.*, 2000). For example, TaWRKY53 coordinates the expression of a wide of WRKY and other stress induced genes (Van Eck *et al.*, 2014). Moreover, senescence has been seen to increase with limited nitrogen levels, which also decrease the transcription of many genes responsible for the synthesis of amino acids, proteins, nucleotides, and chlorophyll (Peng et al., 2007).

In aphid-infested plants, there was a similar rapid onset of expression of the WRKY3 gene at all nitrogen levels, but the magnitude of the response (64-fold) was higher with 2.25 mM nitrogen than with the other two conditions. Results for WRKY8 were similar to those for WRKY3 under the reduced nitrogen conditions. In uninfested plants, WRKY37 was down-regulated 3-fold at the lowest nitrogen levels and up-regulated in response to infestation at all nitrogen levels; similar patterns were obtained for WRKY46, but up-regulation in response to infestation was much less. Gene expression of WRKY45 was lower with reduced nitrogen availability and there was less change in expression in response to aphid infestation. However, WRKY19 showed little or no difference in expression at moderate and low nitrogen conditions and little response to aphid infestation.

As a result of cross-talk via the regulatory network of transcription factors, the presence of one or more initial stresses may prime a plant to mount a more rapid and prolonged response to subsequent stress. In this case, it is necessary to consider how the reduced WRKY gene expression with low levels of nutrients would prime the defence of wheat (Fig. 3-12). Although there is still much to learn about the molecular basis of priming, some studies have advanced our knowledge of this field and it is now understood that the establishment of a primed state is influenced by the accumulation of latent defence-related transcription factors

(Van der Ent *et al.*, 2009), MAP kinases (Beckers *et al.*, 2009) and secondary metabolites and volatile organic compounds (Heil and Bueno, 2007). Typically, for plants grown under conditions of reduced nitrogen availability, priming takes the form of accelerated defence-related gene expression, which is observed when primed plants are under attack by a pathogen or insect. This response increases the plant's resistance to the attacking organism and therefore has a potential for use in agriculture. Priming is a mechanism that plants can use effectively and quickly to counter attacks, and it is used as a response to both biotic and abiotic stresses (Conrath *et al.*, 2006).

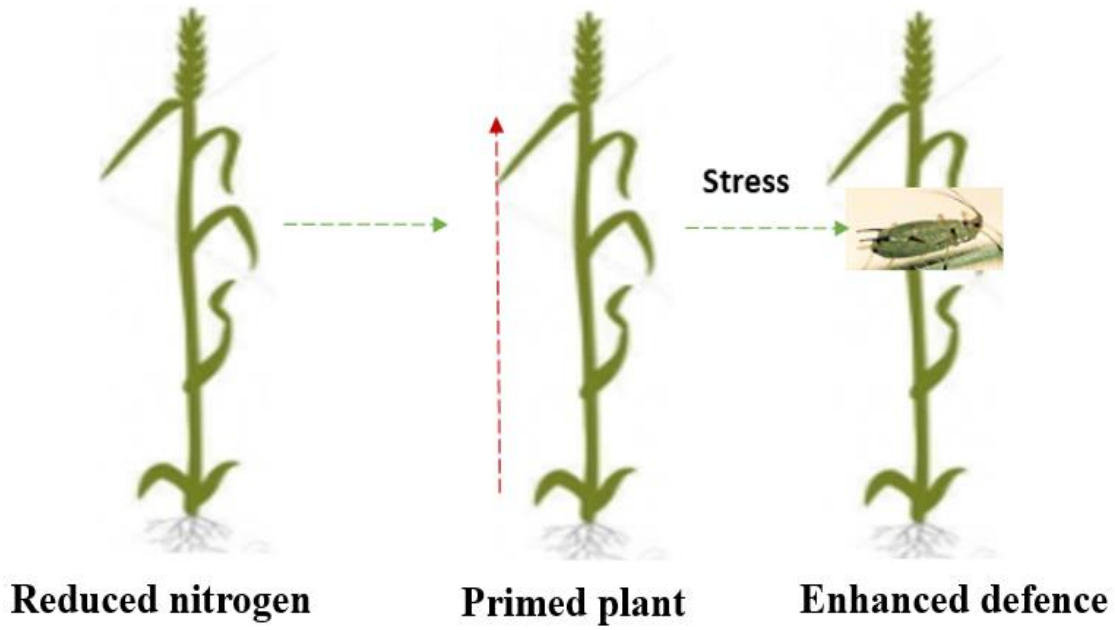


Figure 3-12. Priming or defence activating molecules.

3.5.2 WRKY genes responding to aphid infestation

Little is known about the molecular response in wheat (*Triticum aestivum L.*) to phloem sap feeding by insects. This plays a key role in the expression of defence-related genes and therefore in explaining the molecular basis of endogenous tolerance to aphid infestation to help mitigate shortfalls in global crop yields. Here, I provide an overview to explain the role of WRKY TFs in controlling tolerance to aphids. Chapter 2 described the response of wheat seedlings grown under different nitrogen availabilities and how this affected the population dynamics of *S. avenae*. In the present chapter, the differential expression of WRKY genes under these two stress conditions is reported. From this it can be concluded that there is increasing evidence that TF genes play an important role in the regulation of gene expression in plant defence responses against phloem-feeding insects (Fig. 3-13).

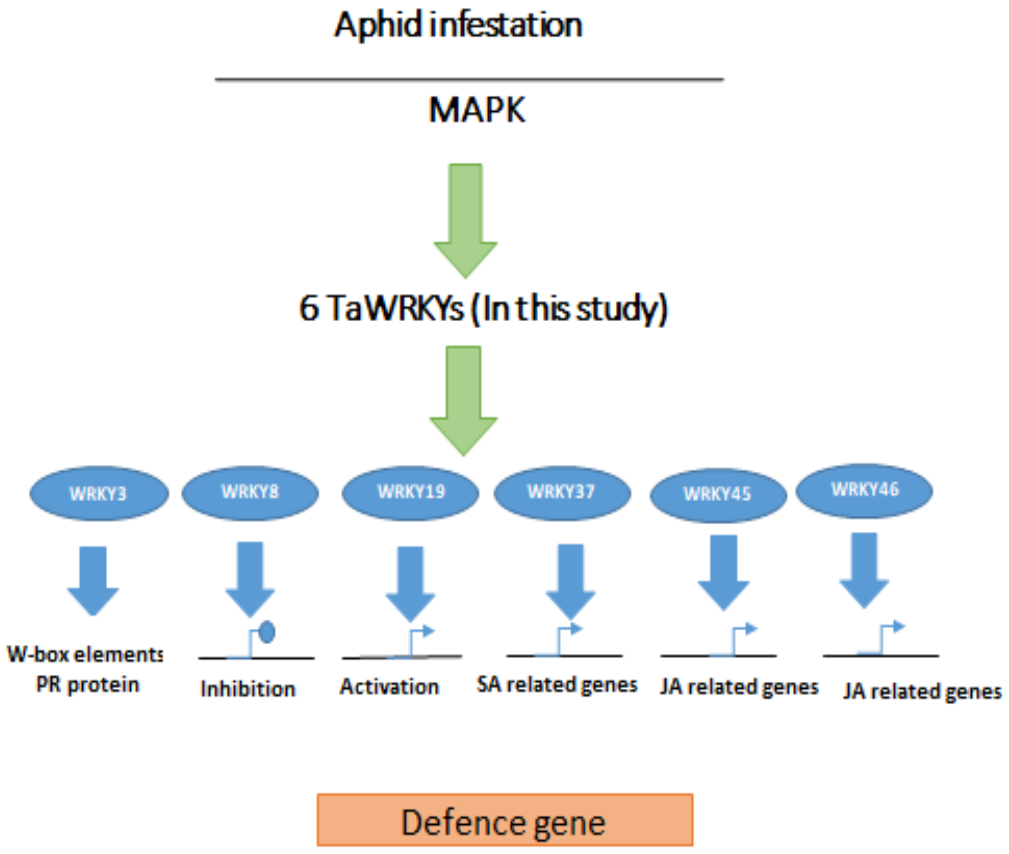


Figure 3-13. Outline of possible signalling pathways involving six WRKY TFs and their effects on defence gene expression, in response to aphid infestation. Adapted from several studies using phylogenetic *AtWRKY* analysis.

Compared to the gene expression seen in plants subjected to nutrient stress, aphid stress resulted in an overall up-regulation of the set of WRKY transcription factor genes studied. A multitude of transcriptional responses are activated at 3, 6 and 9 hours after aphid feeding. The different time points of aphid probing cause increased transcriptional changes and activate more genes. An interesting expression pattern was seen at two time points (3 h or 6 h) after aphid infestation, where the genes were generally more up-regulated than at 9 h infestation. This observation is consistent with Khan (2014), who used microarrays to show that wheat WRKY genes were up-regulated after 6 h of aphid infestation. These data suggest that the expression of various genes was triggered by the damage caused by aphids feeding on plants under different nitrogen conditions.

Plants' responses to aphids indicate that aphids activate plant defence signalling pathways due to salicylate and jasmonate signalling molecules (Smith and Boyko, 2007). TFs are known to play a direct role in plant-aphid interaction, and they may also mediate defence responses indirectly by controlling the development and growth of plants through photosynthesis, the formation of cell walls, and carbon metabolism. A number of factors could be responsible for this and future work will be required to better understand it. It is also possible that low nitrogen conditions with aphid infestation increase the expression of WRKY genes in response to aphids. This would be the first example of such a finding about a crop's response to the effect of a combination of nitrogen and aphid stresses.

Increased expression of pathogenesis-related (PR) genes, which are related to defence against pathogens, has been demonstrated in response to green peach aphid *(Myzus persicae)* feeding in Arabidopsis (Moran and Thompson, 2001). Furthermore, the fungus *Ascochyta rabiei* and salinity increased the expression of PR genes in chickpea plants (Mantri *et al.*, 2010). Previous studies have shown high expression of WRKY genes associated with activation of SA, PR and JA signalling pathways in wheat plants infested with the aphid *Diuraphis noxia* (Smith *et al.*, 2010). Ballini *et al.* (2013) observed an increased expression of the rice defence-related marker gene *Pi1* after *Magnaporthe oryzae* infection at high nitrogen levels, and this was linked to an increased number of cells invaded by the fungus, which triggered the defence mechanism. A multitude of transcriptional responses are activated at 3, 6 and 9 hours after aphid feeding. The different time points of aphid probing cause transcriptional changes and activate more genes.

3.6 Conclusion

This work shows that nitrogen and aphids induce different gene expression changes in wheat and this is linked to reduced nitrogen conditions. It is therefore possible to evaluate how a limited nitrogen supply under controlled conditions relates to well-known decreased levels of insect performance in plants (Chapter 2), and how changes in gene expression play an important role in the plant's induced defence response (Chapter 3).

To improve understanding of the molecular mechanisms that confer resistance, it is necessary to build on the current results and increase our understanding of the role that WRKY proteins play in controlling resistance. In order to achieve this, the results from nitrogen stress and aphid infestation were compared. This study has provided evidence that there are functional links between WRKY gene expression, aphid infestation and nitrogen availability, using interspecific homologies and studies of gene expression to identify TFs that are likely to be directly responsible for tolerance and involved in regulating the cross-talk between responses to biotic and abiotic stress (Fig. 3-13). This knowledge can be used to inform a directed strategy for plant breeding for enhanced tolerance via identification and verification of molecular markers. This can be achieved by identifying differentially expressed genes at both the transcriptional and proteomic levels underpinned by the availability of wheat EST databases and the rice and wheat genome projects.

The gene expression profiles demonstrate that TaWRKY3 is a stress-inducible wheat transcription factor, and it responds differently to aphids at high and low nitrogen levels. These data suggest that it may be useful to study the role of this gene in the response to interacting stresses by using TILLING lines with mutated WRKY genes. Evidence from TILLING lines could show whether TaWRKY3 enhances nitrogen and aphid stress tolerance. Studies of over-expression in wheat or in transgenic *Arabidopsis* plants could also be informative.

4 Chapter 4. TaWRKY3 is a key regulator in aphid defence (TILLING lines and LC/MS-MS)

4.1 Abstract

Limiting nitrogen input in wheat (*Triticum aestivum*) increases aphid tolerance, but reduces yield. Although direct damage caused by phloem-feeding aphids such as *Sitobion avenae* is minimal, their propensity to vector numerous viruses means they are one of the most damaging pests of wheat. Changing agricultural conditions are leading to insect pests becoming a more serious threat to sustainable crop production and therefore understanding the molecular basis of endogenous tolerance to aphid infestation can help mitigate shortfalls in global crop yield. WRKY transcription factors that regulate gene expression play important roles in the response to biotic and abiotic stresses. Previous work demonstrated that the expression of *TaWRKY3* changed in response to aphid and reduced nitrogen stress. To investigate the role of WRKY3 in the stress response, TILLING lines with mutations in the *WRKY3* gene were grown at different nitrogen concentrations and were also infested with aphids. In contrast to the wild-type plants, aphid fecundity on one mutant line showed no difference between high and low nitrogen levels, suggesting that WRKY3 plays a role in the link between nitrogen stress and aphid tolerance. Accumulation of jasmonic acid, salicylic acid and abscisic acid increased as a result of aphid infestation. Maximum concentrations of each phytohormone were identified at different times after infestation. The results suggest that low nitrogen conditions may prime the defence of wheat against insect attack as a result of cross-talk via a regulatory network of WRKY transcription factors. These results provide new knowledge and valuable resources that should be useful in the effort to produce crops with reduced nutrient input.

Key words: Wheat TILLING lines / WRKY3/ Bioassay /Jasmonic acid/ Salicylic acid/ Absisc acid

4.2 Introduction

The gene expression data collected in Chapter 3 identify TaWRKY3 as a target involved in the interaction between reduced nitrogen conditions and aphid infestation. The aim of the present chapter is to verify the role of TaWRKY3 in the plant's defence against biotic and abiotic stress. The understanding of this function in wheat will be important for this project. Due to the difficulties in generating transgenic wheat to either over- or under-express TaWRKY3, TILLING lines carrying mutations within the WRKY3 gene were selected. Although the TILLING lines may carry mutations in other genes, they are available immediately and are commonly used as a source of novel gene rearrangements.

WRKY proteins are a set of plant-specific protein transcription factors that contain a conserved WRKYGQK DNA-binding domain of 60 amino acids. They are commonly differentially expressed in response to stress. They control the transcription of the target genes by binding to promoter regions that contain a DNA element called the W-box with the core sequence TTGAC***Y*** (where ***Y*** is C or T) (Eulgem *et al.*, 2000). In this chapter, the importance of the conserved sequences in and adjacent to the WRKYGQK sequence within one WRKY domain was tested.

Mutations are beginning to be used in wheat to develop its nutritional value and to generate additional variability in genes in order to improve wheat's adaption to any environment (Krasileva *et al.*, 2017). To better understand the function of the WRKY3 gene in common wheat, *WRKY3* mutants were selected from hexaploid wheat in a Targeting Induced Local Lesions IN Genomes (TILLING) population (Avni *et al.*, 2014; Krasileva *et al.*, 2017). The TILLING method is useful for both functional genomics and crop improvement (Chen *et al.*, 2012). It has been developed for several crops such as maize, barley, soybean and rice. In wheat, TILLING has been applied to both tetraploids and hexaploids. The seeds of wheat var. Cadenza and mutants in the *WRKY3* gene supplied by the John Innes Centre. The effects of *WRKY3* mutations on responses to stress from low nitrogen input and aphid infestation were investigated.

Aphid fecundity was recorded at different nitrogen availabilities. Resistance to aphids depends on the specific combination of nitrogen levels and aphids. Plants have evolved complex network mechanisms of regulatory and hormone-mediated pathways, which are

thought to be regulated via the transcriptional activation of a complex regulatory network in the cell nucleus (Wang *et al.*, 2015).

Expression of TaWRKY3 is strongly induced by aphid infestation of plants grown at reduced nitrogen levels (Chapter 3). Because WRKY proteins are linked to responses to phytohormones, concentrations of three important phytohormones that are known defence signalling compounds were measured: jasmonic acid (JA), salicylic acid (SA) and abscisic acid (ABA). These hormones may have a central role in controlling defence gene expression and inducing the resistance response in wheat. Many studies have investigated cross-talk between their signalling pathways in crops, but few have conducted studies on mutants. The hormones were experimentally analysed using LC-MS/MS, in order to establish how insect behaviour is influenced by the JA, SA and ABA status of the host plant. Phytohormone levels were measured in leaf tissue of two mutants and compared with levels in wild-type plants (Forcat *et al.*, 2008), using the facility at Newcastle University. The response to wounding and herbivore-specific signals supports the hypothesis that WRKY3 helps plants to differentiate mechanical wounding from herbivore attack, mediating a plant's herbivore-specific defenses. Differences in responses to single and multiple elicitations indicate an important role of WRKY3 in potentiating and/or sustaining active JA levels during continuous insect attack.

This study is the first detailed analysis of the effects of mutations within the DNA binding domain of WRKY3 on the development and fecundity of cereal aphids (*S. avenae*) at low and high levels of nitrogen input. It provides new insights into the processes potentially involved in plants' defence against aphids.

4.2.1 Aim and objectives

To determine whether TaWRKY3 has a role in the stress response. This was achieved through the following objectives:

1. Evaluate the effects of mutations within the WRKY binding domain (TILLING lines) on aphid performance (bioassay) under optimal and sub-optimal nitrogen availability.
2. Quantify changes in phytohormone production as a consequence of mutations within the WRKY gene (see Fig. 4-1).

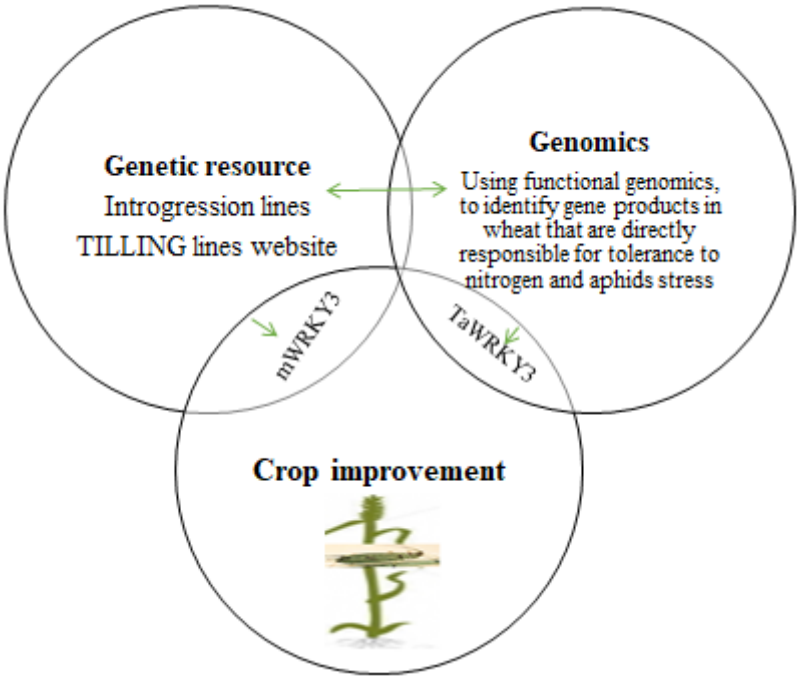


Figure 4-1. Scheme of the overlap between plants of TILLING lines and WRKY3 mutation and transcription. Molecular genetics was used to identify genes that could be valuable in either non-transgenic (TILLING) or transgenic approaches for cultivating crops with improved tolerance and potential for high yield.

4.3 Material and Methods

4.3.1 Experiment design

The experiment was designed to investigate the role of wheat WRKY3 in responses to reduced nitrogen availability and aphid stresses (see Fig. 4-2).

Study (1): Use of TILLING lines with the WRKY3 mutation to investigate the interactions between the effects of mutations in plants and nutrients on aphid fecundity, measuring the daily and accumulative nymph production over time. This is similar to the first bioassay presented in Chapter 2.

Study (2): To investigate the involvement of jasmonic acid, salicylic acid and abscisic acid in wheat resistance to aphids, using LC/MS-MS as a quantitative method to detect the hormones in wild-type and mutant plants.

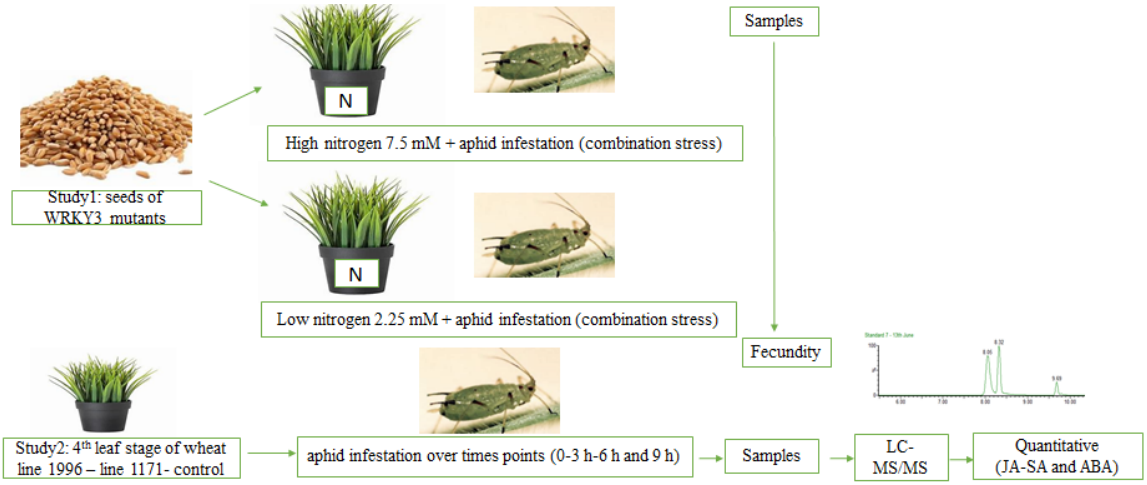


Figure 4-2. Flow chart to study the interactions of WRKY3 directly, through three approaches. 1. The mutants of WRKY3 in wheat were tested to study the effect of nutrients on mutant plants' populations of the aphid *S. avenae* (bioassay). 2. Changes in endogenous phytohormones (JA, SA and ABA) were measured at different time points of the infestation.

4.3.2 Plant material and treatments

Seven TILLING lines (0259, 0202, 1449, 1171, 1231, 1996 and 0877) containing mutations within WRKY3 and the wild type cultivar (Cadenza) were selected for this study, from the wheat TILLING database (http://www.wheat-tilling.com/) and supplied by Germplasm Resources Unit, John Innes Centre (UK). Plants were grown from the seeds supplied and seeds for experimentation were harvested from those plants. Plants were grown to the 4th leaf

stage with high (7.5 mM) and low (2.25 mM) nitrogen levels. Plants were irrigated 3 times with 70 ml weekly as described in section 2.3.3 and infested with aphids. Overall 64 (8 plants per line) potted single seeds were planted for use in this experiment.

4.3.3 Insect bioassay (aphid infestation)

Grain aphid (*Sitobion avenae*) bioassays against the TILLING lines were performed and analysed in exactly the same way as described in section 2.3.6.

4.3.4 LC/MS-MS

a. Preparation of standard solution

The internal standard was prepared with high-purity jasmonic, salicylic acid and abscisic acid (more than 98.5%). The standard stock (400 µg ml^{-1}) was obtained by dissolving 10 mg of individual standard in deionised water (DW). Mixed working solutions of SA, ABA and JA standards over the concentration range 0.031–10 µg ml^{-1} were prepared from stock solutions with water. The standard solutions used were 0, 12.5, 25, 50, 75, 100, 125 and 150 nM and the volume injected was 200 µl. The solutions were stored at 4 ºC (Huang *et al.*, 2015).

b. Sample preparation

Wheat lines 1171, 1996 and the control (wild type Cadenza) were grown in soil until the 4th leaf stage and infested with aphids (20 adults + nymphs). Samples were taken at 0 h, 3 h, 6 h and 9 h after infestation. Overall 24 plants (two replicates of four plants each per line, including the control) were assigned to aphid infestation. The extraction and analysis of plant hormones was performed with two biological replicates. The leaves were harvested from the plants, frozen in liquid nitrogen and freeze dried, then ground with liquid nitrogen. The tissue powder (20 mg) was added to 400 µl of 10% methanol + 1% acetic acid and then internal standards were added to this suspension (Forcat *et al.*, 2008). The samples were transferred to a microcentrifuge tube and kept on ice for 30 min before being centrifuged at 4 ºC for 10 min. The pellet was collected and re-extracted with 400 µl of 10% methanol + 1% acetic acid. This was kept on ice for 30 min, then centrifuged and the supernatants pooled. Two extractions achieved 90-95% recovery (Forcat *et al.*, 2008).

c. LC-MS/MS conditions

150 µl samples were submitted to LC/MS-MS carried out on a Acquity Uplc BEH 1.7 µm C18 100 mm x 2.1 column, at 35 ºC. The solvent gradient used was 100% A (94.9% H_2O: 5% CH_3CN: 0.1% CHOOH) to 100% B (5% H_2O: 94.9% CH_3CN: 0.1% CHOOH) for 20 min. Solvent B was held at 100% for 5 min then the solvent returned to 100% A for 5 min equilibration prior to the next injection and held at 100% A for 10 min equilibration (Forcat *et al.*, 2008).

The MS was operated in the negative mode using a Zspray ESI probe (Waters) as the ion source. Optimal conditions were determined using the Electrospray Ionisation Intellistart feature of mass spectrometry software both by infusing standards into the MS by syringe pump and injecting standards into a 200 µl/min flow of 50% Solvent A/50% Solvent B.

The optimised conditions were as follows: temperature 200 °C, ion source gas 7 bar, ion spray voltage 4 V, core voltage 33 V, CAD gas setting 5; the DP (-25 V), EP (-9) and CEP (-2) were held constant for all transitions with source offset 50 V. Data were acquired and analysed using Masslynx version 4.1 software (Waters Inc.).

4.3.5 Data analysis

Data from the aphid bioassay were analysed using the general linear model of Minitab 17. Data were submitted to a two-way analysis of variance (ANOVA). Mean separation was assessed by Tukey's multiple comparison tests at $P < 0.05$. The influences of infestation and genotype on phytohormone concentrations were analysed using ANOVA and significant differences between time points were determined by Tukey's test at $P < 0.05$.

4.4 Results

4.4.1 Wheat TILLING platform

There are two wheat TILLING populations available: Cadenza and Kronos. The winter wheat Cadenza was selected in order to link the research to all my winter wheat studies. The Wheat TILLING website (http://www.wheat-tilling.com) and BLAST searching with the TaWRKY3 sequence were used to obtain mutant alleles in this gene which could be utilised to investigate the role of WRKY3 in the aphid-induced defence response. Several missense mutations that are predicted to affect WRKY3 protein activity were identified and selected. Mutations of interest are shown in Table 4-1. Het/Hom in Table 4-1 identifies whether the sequenced individual in the original population was heterozygous or homozygous for the particular SNP position (the position of the EMS mutation). If the WT sequence was found in less than 15% of the sequence reads, then the SNP is called as homozygous in the mutant line. Otherwise the SNP is called heterozygous. The selected mutants with the positions of changes in the amino acids are shown in Fig. 4-3.

Table 4-1. Selected WRKY3 mutations. Het/Hom refers to whether the sequenced individual in the original population was heterozygous or homozygous for the particular SNP position (the position of the EMS mutation). The mutations are in the homeologous *WRKY3* genes on chromosomes 2A, 2B and 2D.

WRKY name	Database gene	Line	het/hom	Genome	Amino Acid change
WRKY3	IWGSC_CSS_2DL_scaff_99906833	0259	Hom	D	R176W
WRKY3	IWGSC_CSS_2DL_scaff_99906833	0202	Hom	D	V164I
WRKY3	IWGSC_CSS_2DL_scaff_99906833	1449	Hom	D	G82D
WRKY3	IWGSC_CSS_2AL_scaff_6437167	1171	Hom	A	R139W
WRKY3	IWGSC_CSS_2AL_scaff_6437167	1231	Hom	A	G82E
WRKY3	IWGSC_CSS_2BL_scaff_7976862	1996	Het	B	Y160D
WRKY3	IWGSC_CSS_2BL_scaff_7976862	0877	Het	B	G90D

(a)

TaWRKY3 (EU665432)

MEGGSQLGACLPSLYALDPYASPPLLAPLPNQHKLHQLPLVLQEQPGNH
GVMFSSDHGGGLYPLLPGIPFCHSAAACEKSTGFAPLGGTGEAGTSAARA
GNEFASATTTTTASCHGPSSWWKGAEKGKMKVRRKMREPRFCFQTRSE
VDVLDDGYKKWRKYGQKVVKNSLHPRSYYRCTHSNCRVKKRVERLSEDC
RMVITTYEGRHTHTPCSDDDAGGDHTGSCAFTSF

(b)

TaWRKY3	GFAPLGGTGEAGTSAARAGNEFASATTTTTASCHGPSSWWKGAEKGKMKVRRKMR
	EPRFCFQTRSEVDVLDDGYKWRKYGQVVKNSLHPRSYYR
Line 0877	GFAPLGGTPEAGTSAARAGNEFASATTTTTASCHGPSSWWKGAEKGKMKVRRKMR
	EPRFCFQTRSEVDVLDDGYKWRKYGQVVKNSLHPRSYYR
Line 1231	EFAPLGGTGEAGTSAARAGNEFASATTTTTASCHGPSSWWKGAEKGKMKVRRKMR
	EPRFCFQTRSEVDVLDDGYKWRKYGQVVKNSLHPRSYYR
Line 1996	GFAPLGGTGEAGTSAARAGNEFASATTTTTASCHGPSSWWKGAEKGKMKVRRKMR
	EPRFCFQTRSEVDVLDDGYKWRKDGQVVKNSLHPRSYYR
Line 1171	GFAPLGGTGEAGTSAARAGNEFASATTTTTASCHGPSSWWKGAEKGKMKVRRKMR
	EPWFCFQTRSEVDVLDDGYKWRKYGQVVKNSLHPRSYYR
Line 0259	GFAPLGGTGEAGTSAARAGNEFASATTTTTASCHGPSSWWKGAEKGKMKVRRKMR
	EPRFCFQTRSEVDVLDDGYKWRKYGQVVKNSLHPRSYYW
Line 1449	GFAPLGDTGEAGTSAARAGNEFASATTTTTASCHGPSSWWKGAEKGKMKVRRKMR
	EPRFCFQTRSEVDVLDDGYKWRKYGQVVKNSLHPRSYYR
Line 0202	GFAPLGGTGEAGTSAARAGNEFASATTTTTASCHGPSSWWKGAEKGKMKVRRKMR
	EPRFCFQTRSEVDVLDDGYKWRKYGQIVKNSLHPRSYYR

Figure 4-3. (a) The protein sequence of TaWRKY3 (GenBank EU665432). The black oval indicates the WRKY domain, while the amino acids in red ovals form the zinc finger. Available mutations in Cadenza are highlighted in blue, while those in Kronos are green.
(b) Comparison of TaWRKY3 with the seven TILLING mutants. The red letters show changes in amino acids caused by the mutations. The mutated amino acids are highlighted in blue in the TaWRKY3 sequence.

4.4.2 Performance of *S. avenae* on TILLING lines with high and low nitrogen availability

Aphid performance

Aphid performance on the seven different TILLING lines of winter wheat (Cadenza) and control were assessed under high and low nitrogen levels, in terms of fecundity. Four different parameters were measured: total fecundity, daily fecundity, reproduction rate and cumulative number of nymphs over three weeks.

4.4.3 Effect of wheat on total fecundity

a. Total fecundity

The total fecundity of the aphids was measured by the mean total number of nymphs produced by both adults per plant over 21 days. The results showed that aphids' total fecundity was highly significantly different between the mutant lines ($p < 0.001$) and nitrogen treatments ($p < 0.001$), except that total aphid fecundity on mutant line 1996 showed no difference between high and low nitrogen levels. At 7.5 mM nitrogen, aphids on lines 1231, 0259 and 0877 produced averages of 48.5, 45 and 40 nymphs, significantly more than on lines 1171 ($p < 0.01$) and 0202 ($p <0.01$). For plants grown at 2.25 mM nitrogen, there were fewer nymphs produced on line 1171 than on plants at 7.5 mM nitrogen ($p < 0.001$), with total fecundity reduced to 14.8 nymphs per adult. Aphids on lines 0202 and 1449 produced 32.8 and 26 nymphs at 7.5 mM nitrogen ($p < 0.001$), while total aphid production at 2.25 mM nitrogen was 23.6 and 15.6 nymphs, respectively. There were no differences between these two nitrogen levels for line 1996, with total fecundity 26 nymphs per adult at both levels (Fig. 4-4 a).

b. Multiplication ratio

Nymphs produced by two adult aphids were counted every day over a period of 21 days. Multiplication ratio (MR*) was calculated.

*MR= Ni/N0, where N0 was the initial number of aphids inoculated, Ni the number of aphids at the final time point (He *et al.*, 2011).

Aphid multiplication was slowest at 2.25 mM available nitrogen input ($p < 0.001$).The multiplication ratios on lines 1171 and 1449 were 17.5 and 13.4, respectively. The resistance of *WRKY3* mutant plants at 7.5 mM nitrogen was moderate. The most susceptible of the lines were 1231 and 0259 with MR values of 24.6 and 23. On lines 0877 and 0202 at 7.5 mM nitrogen, the MR values were 20 and 16.5, respectively, while the resistance of both lines at 2.25 mM nitrogen was moderate and the MR values were 14.6 and 12.5 respectively on day 21 (Fig. 4-4b).

C. Daily aphid fecundity

The average number of nymphs produced per plant per day was recorded on Cadenza and the seven *WRKY3* mutants. There were significant effects of nitrogen availability and mutant genotype ($p < 0.001$). All mutant plants grown with low nitrogen (2.25 mM) had lower average daily nymph production than the control plants, except that mutant line 1996 showed no difference between high and low nitrogen levels. In the high nitrogen conditions (7.5 mM), aphids produced 9.2, 8.5 and 5 nymphs per adult per day on genotypes 1231, 0259 and 1449, respectively. However, at low levels of nitrogen (2.25 mM) for these genotypes, adults produced significantly lower numbers of nymphs: 6.2, 4.2 and 3 nymphs per day, respectively. Aphids on the two lines 0877 and 0259 produced 7.7 and 8.5 nymphs per day at 7.5 mM nitrogen. There were significant differences on lines 1231, 1171 and 0259 - between the two nitrogen levels ($p < 0.001$). Overall, daily fecundity was lower at the low level of nitrogen, except that line 1996 produced 5 nymphs per day at both nitrogen levels (Fig. 4-5).

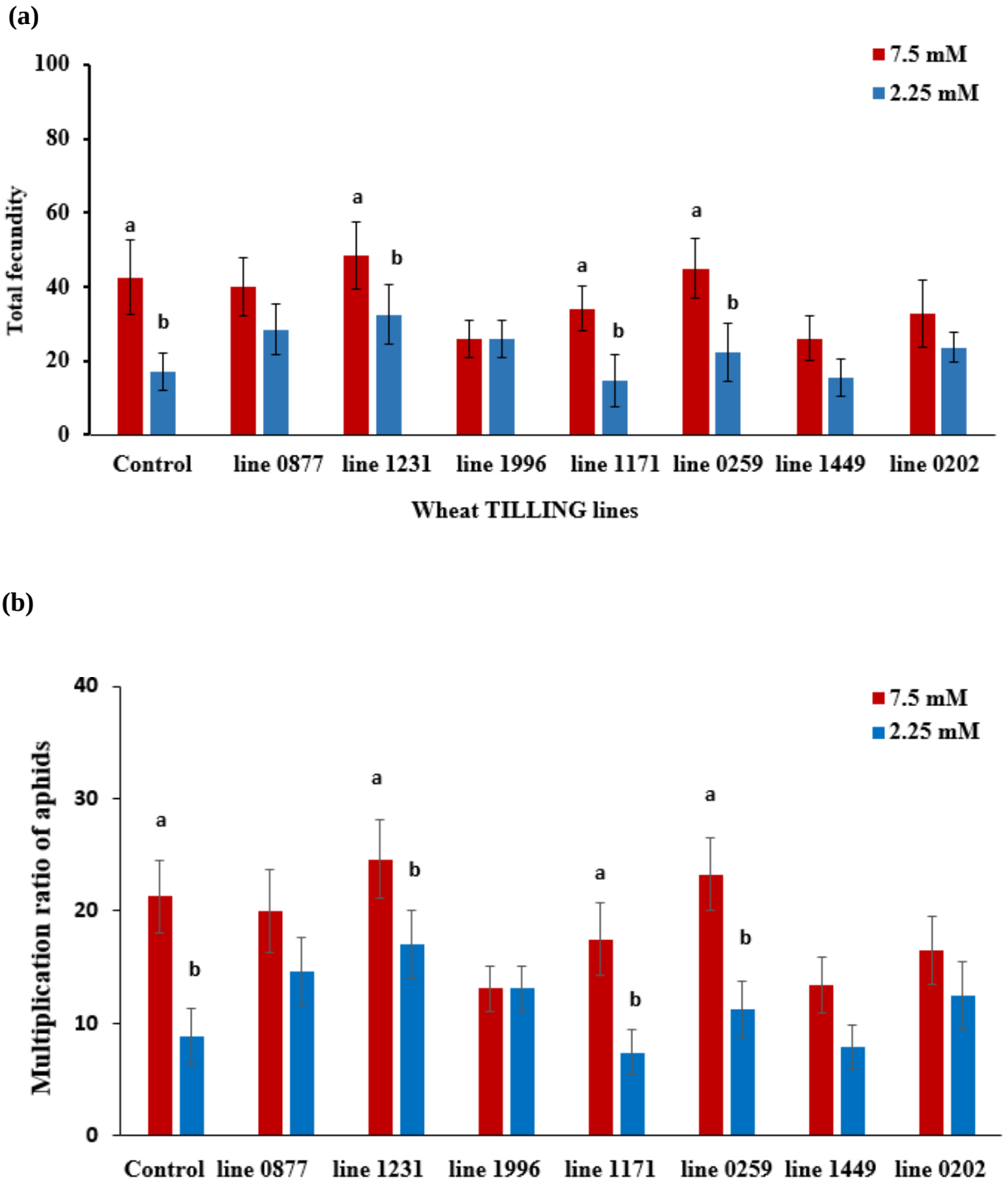


Figure 4-4. Aphid performance parameters on seven wheat TILLING lines of WRKY3.
(a) Total fecundity (measured as mean total number of nymphs per plant 21 days after infestation, mean ± SE; n = 4).
(b) Aphid multiplication ratio on seven wheat TILLING lines of WRKY3.
Aphids were recorded on plants grown at two nitrogen levels for 5 weeks. Columns headed by different letters are significantly different (2-Way ANOVA to study the main effects of genotype and treatments followed by Tukey´s test, $p < 0.05$).

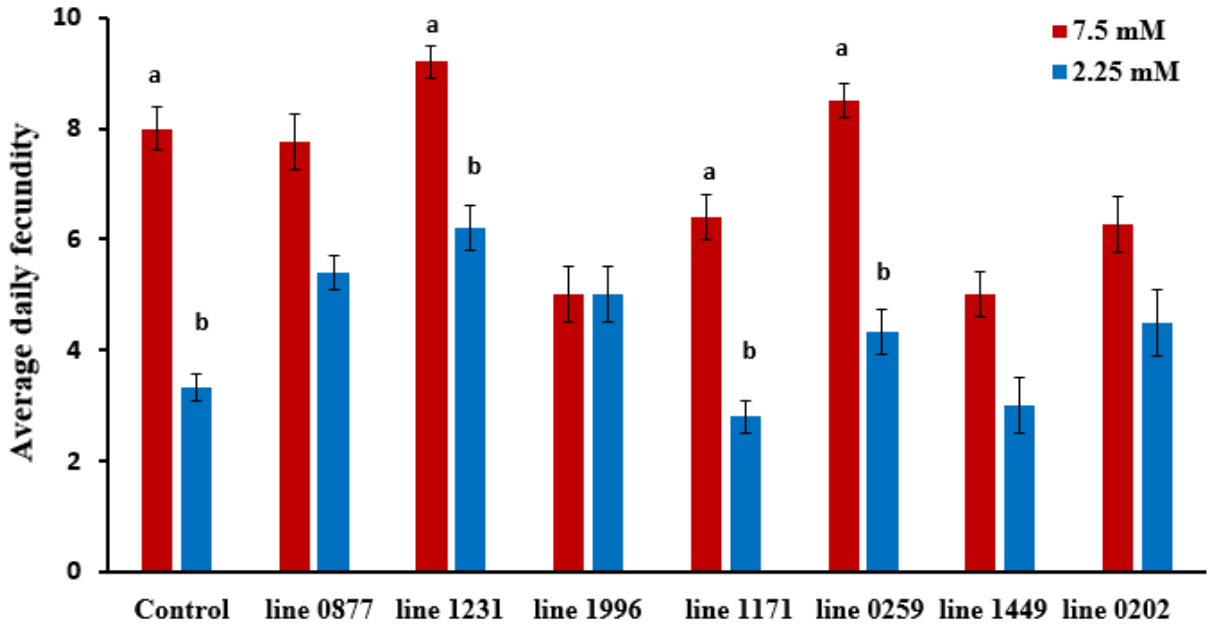


Figure 4-5. Aphid daily fecundity (number of nymphs produced per day on four plants of each line; means ± SE) on seven wheat TILLING lines of WRKY3.
Aphids were recorded on plants grown at two nitrogen levels for 21 days. Columns headed by different letters are significantly different (2-Way ANOVA to study the main effects of genotype and treatments followed by Tukey´s test, $p < 0.05$).

4.4.4 Relative reproductive rate

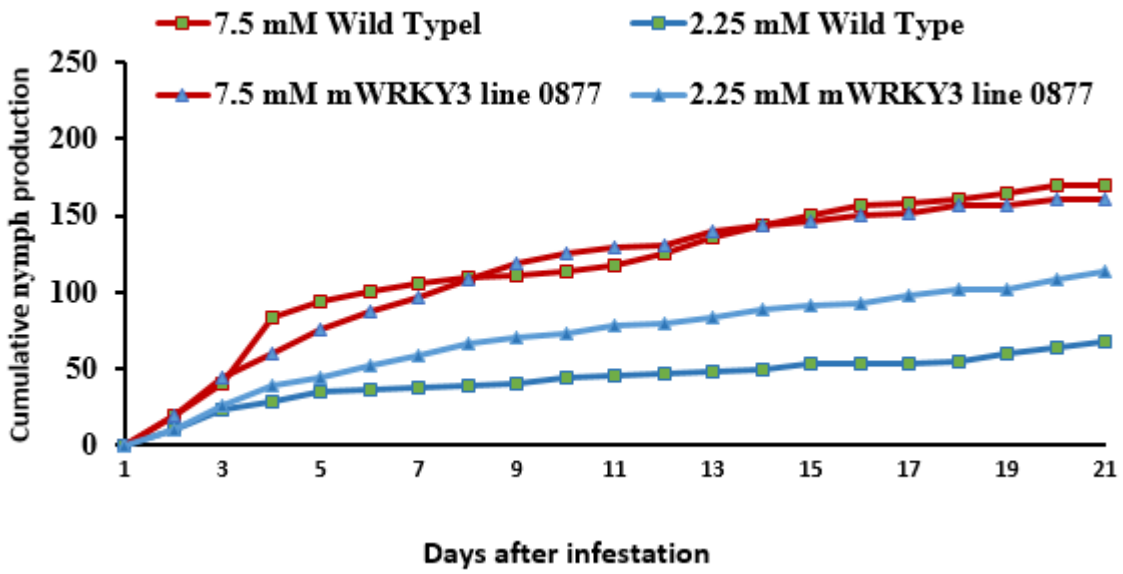
The rate at which aphids reproduced was noted and analysed over 21 days in the *WRKY3* mutant plants. At the high nitrogen level (7.5 mM), aphids on control plants on day one produced 16 nymphs, then fecundity decreased to 5 nymphs on days 7 and 8, and decreased further on day 18 to 2 nymphs. Fecundity was lower on plants grown at low nitrogen (2.25 mM), where aphids produced 6 nymphs on day 1 and the rate of nymph production was lower at 1 nymph on days 6 and 7, increased again to 7 nymphs on day 16 and decreased to 1 nymph on day 21. Aphids on genotype 0877 grown with 7.5 mM nitrogen produced 18 nymphs on day one, then fecundity decreased to 11 nymphs on day 8 and continued to decrease to 5 per day on day 17. However, fecundity was lower on plants grown at 2.25 mM nitrogen, with a production of 3 nymphs on days 9 and 14 and thereafter 2 nymphs on day 21. For line 1231 more than 50 nymphs were produced on the first day at 7.5 mM and numbers decreased to 5 nymphs on days 4 and 5. By day 15, fecundity increased to 7 nymphs and decreased to 2 nymphs on day 19. At 2.25 mM nitrogen, aphids produced 3 nymphs on day 1, increasing to 4 nymphs on day 18. On genotype 0259 aphids produced 45 nymphs on day 1 and fecundity decreased to 8 nymphs on days 4 and 5, then decreased on day 10 to 2 nymphs. At 2.25 mM nitrogen aphids produced 20 nymphs on day 1 and fecundity decreased to 1 on day 7 then increased again to 3 nymphs on days 13 and 15. For line 1171 at 7.5 mM, 15 nymphs were produced on the first day and thereafter the number decreased to 5 nymphs on day 6, increased again to 12 nymphs on day 12 and decreased to 4 on day 21. At 2.25 mM nitrogen aphids produced 4 nymphs on day 1; the number decreased to < 2 nymphs over time for 21 days. On line 0202 at 7.5 mM nitrogen, aphids produced 45 nymphs on day 1, thereafter decreasing to 9 nymphs on days 12 and 15, and decreasing again to 6 nymphs on day 20. At 2.25 mM nitrogen, aphids produced 30 nymphs on day 1, decreasing to 6 nymphs on day 17. For genotype 1449, aphids on plants grown at 7.5 mM N produced 20 nymphs on the first day. Fecundity decreased to 4 on day 6, again decreasing to 2 nymphs on day 15. However, aphids produced 10 nymphs on day 1 at 2.25 mM, decreasing to 1 nymph on day 18. Aphids on plants of genotype 1996 grown with 7.5 mM nitrogen produced 20 nymphs on day 1, decreasing to 10 nymphs on day 13 and decreasing to 2 nymphs on day 21. At 2.25 mM nitrogen, aphids produced 27 nymphs on day 1, decreasing to 3 nymphs on days 15 and 16 and increasing again to 5 nymphs on day 18 (Fig 4-6).

4.4.5 Cumulative increase in aphid population on wheat TILLING lines

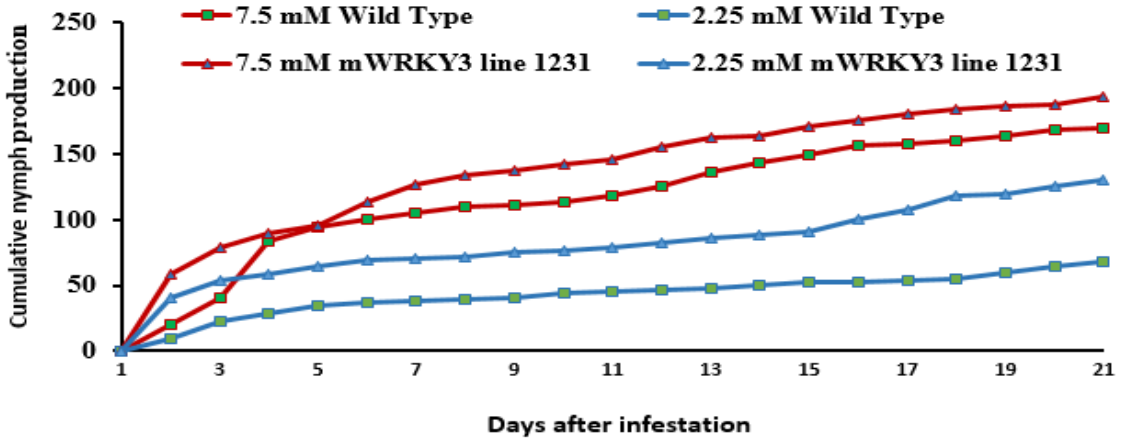
The cumulative nymph production of aphids was significantly affected by nitrogen availability ($p < 0.001$). The total number of nymphs produced by aphids feeding on N-treated plants was strongly positively correlated with the increasing number of days that the host plant was grown. Four TILLING lines (0877, 1171, 0202 and 1449) exhibited a reduction in the cumulative number of nymphs at the high nitrogen level of 7.5 mM, in contrast to the control (see Figure 4-5). The rate of nymph production in line 0259 at 7.5 mM changed over time similarly to the control. However, there were no differences observed in the total nymph production on the line 1996 plants, under both nitrogen conditions (see Figure 4-5g).

The aphid bioassay showed that TILLING lines 0877, 1231, 0259, and 0202 supported greater aphid growth associated with reduced nitrogen treatment (2.25 mM). In addition, nymph production was higher than on control plants, which showed a higher level of resistance to aphids.

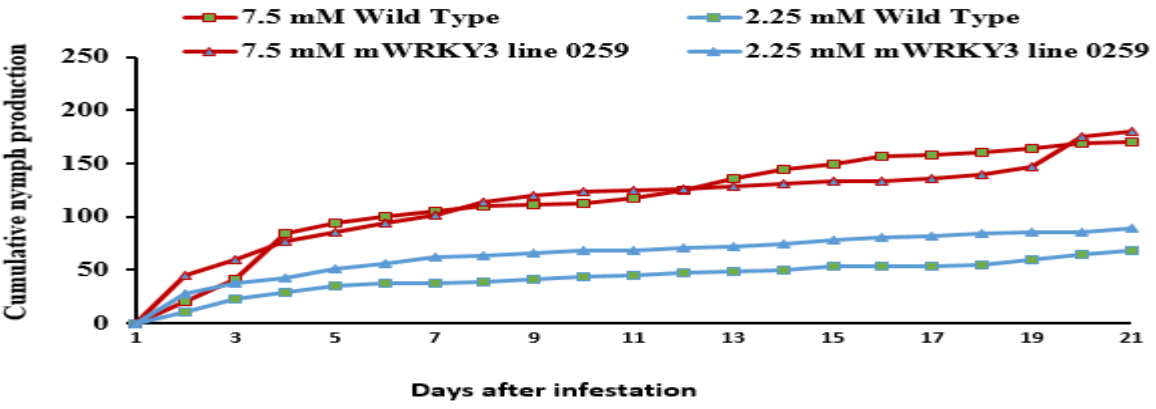
(a)


7.5 mM Wild Typel
2.25 mM Wild Type
7.5 mM mWRKY3 line 0877
2.25 mM mWRKY3 line 0877
Cumulative nymph production
250
200
150
100
50
0
1
3
5
7
9
11
13
15
17
19
21
Days after infestation

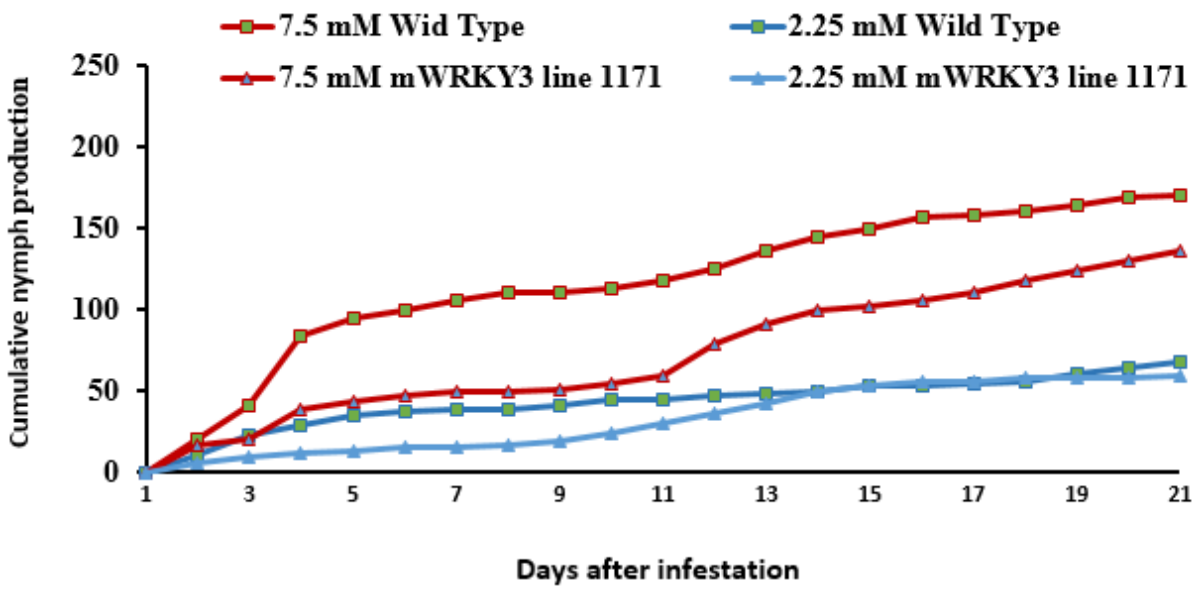
(b)


7.5 mM Wild Type
2.25 mM Wild Type
7.5 mM mWRKY3 line 1231
2.25 mM mWRKY3 line 1231
Cumulative nymph production
250
200
150
100
50
0
1
3
5
7
9
11
13
15
17
19
21
Days after infestation

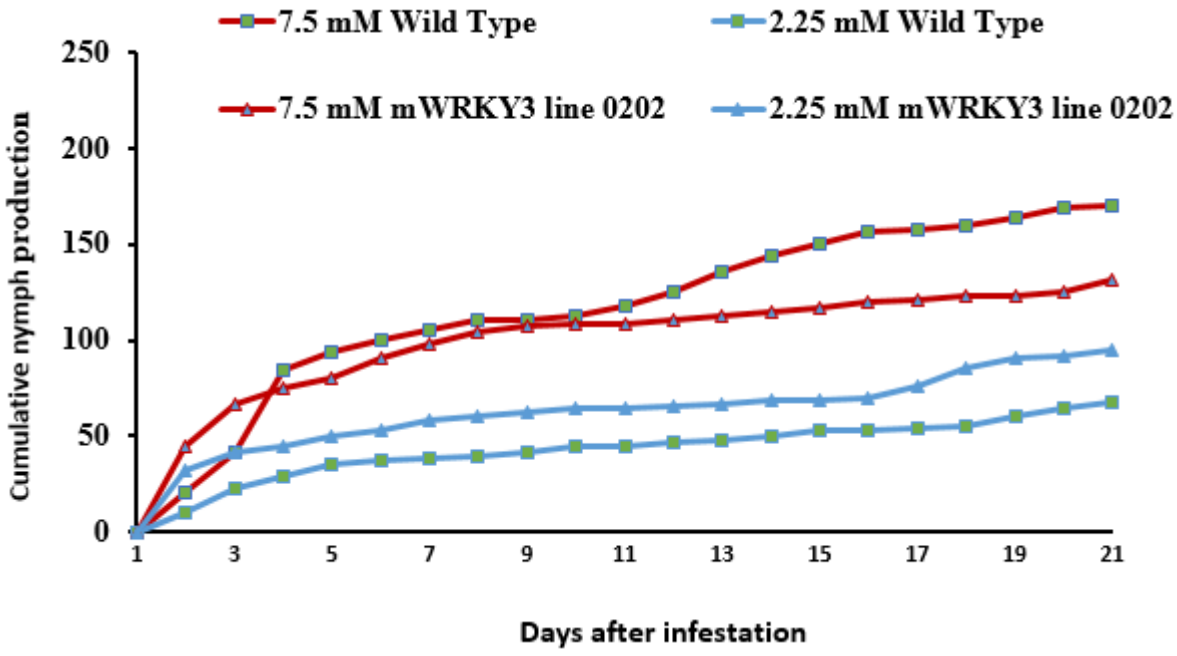
(c)


7.5 mM Wild Type
2.25 mM Wild Type
7.5 mM mWRKY3 line 0259
2.25 mM mWRKY3 line 0259
Cumulative nymph production
250
200
150
100
50
0
1
3
5
7
9
11
13
15
17
19
21
Days after infestation

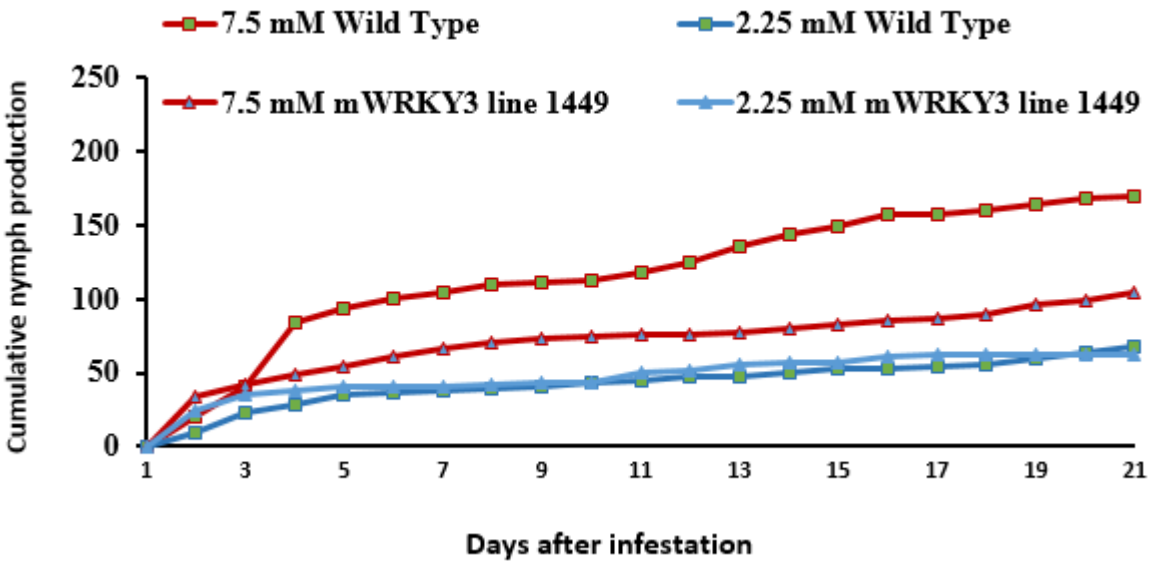
(d)


7.5 mM Wid Type
2.25 mM Wild Type
7.5 mM mWRKY3 line 1171
2.25 mM mWRKY3 line 1171
Cumulative nymph production
250
200
150
100
50
0
1
3
5
7
9
11
13
15
17
19
21
Days after infestation

(e)


7.5 mM Wild Type
2.25 mM Wild Type
7.5 mM mWRKY3 line 0202
2.25 mM mWRKY3 line 0202
Cumulative nymph production
250
200
150
100
50
0
1
3
5
7
9
11
13
15
17
19
21
Days after infestation

(f)



(g)

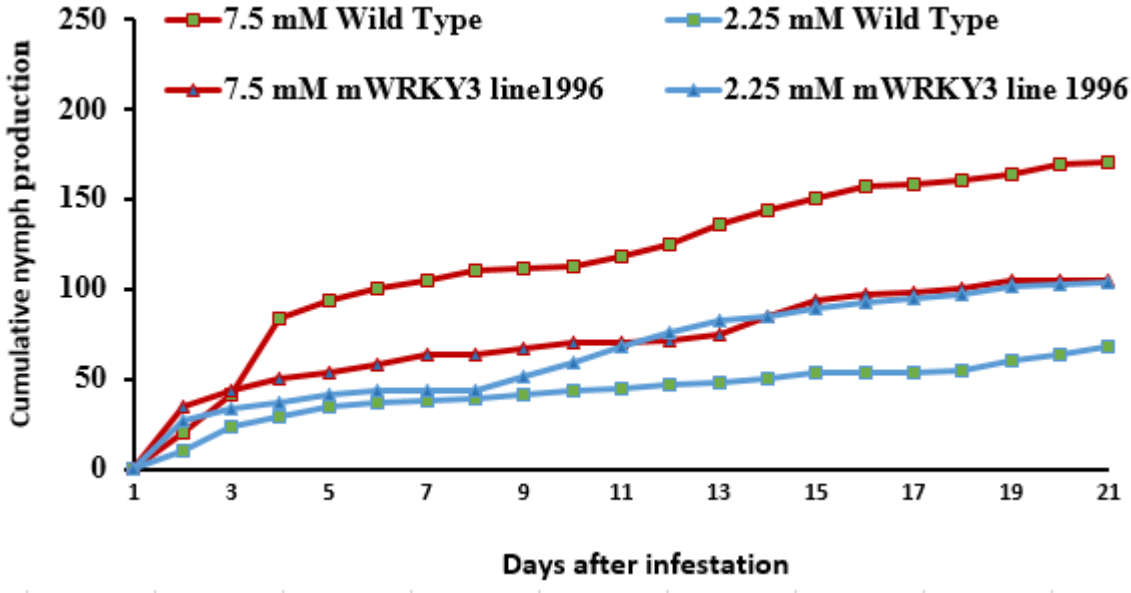


Figure 4-6. Cumulative numbers of nymphs produced over 21 days on the four plants of each of the seven WRKY3 TILLING mutant lines and wild type with two levels of nitrogen availability: 7.5 mM (red line) and 2.25 mM (blue line) n= 4 (a) line 0877, (b) line 1231, (c) line 0259, (d) line 1171, (e) line 0202, (f) line 1449 and (g) line 1996.

4.4.6 The phytohormone extraction method

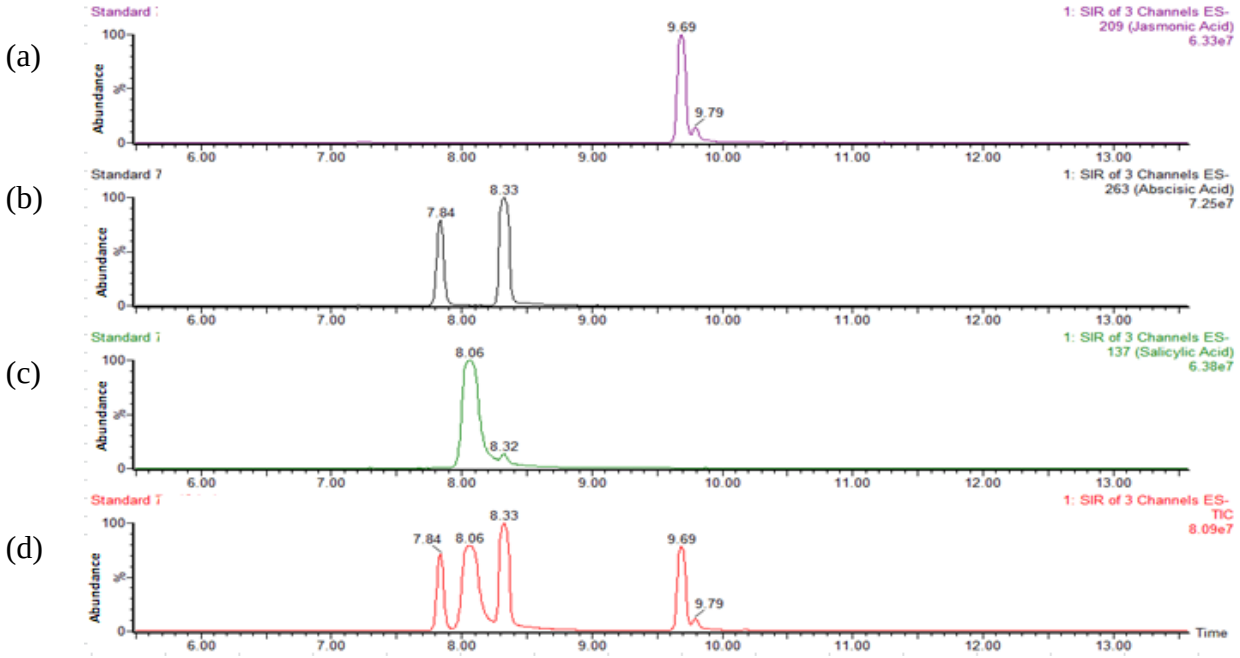
a. Optimisation of chromatography and mass spectrometry conditions

C18 100 mm x 2.1 columns were used for the separation and detection of the target compounds. It was found that a good separation of the phytohormone compounds SA, ABA and JA was achieved. Each compound was identified using selected ion monitoring (SIM) and Multiple Reaction Monitoring (MRM) scan modes. Identification of the compounds was based on appropriate Multiple Reaction Monitoring (MRM) of ion pairs for endogenous JA, SA and ABA using the following mass transitions: JA 209 > 59, SA 137 > 93, ABA 263 > 153 (Fig. 4-6) (Huang *et al.*, 2015).

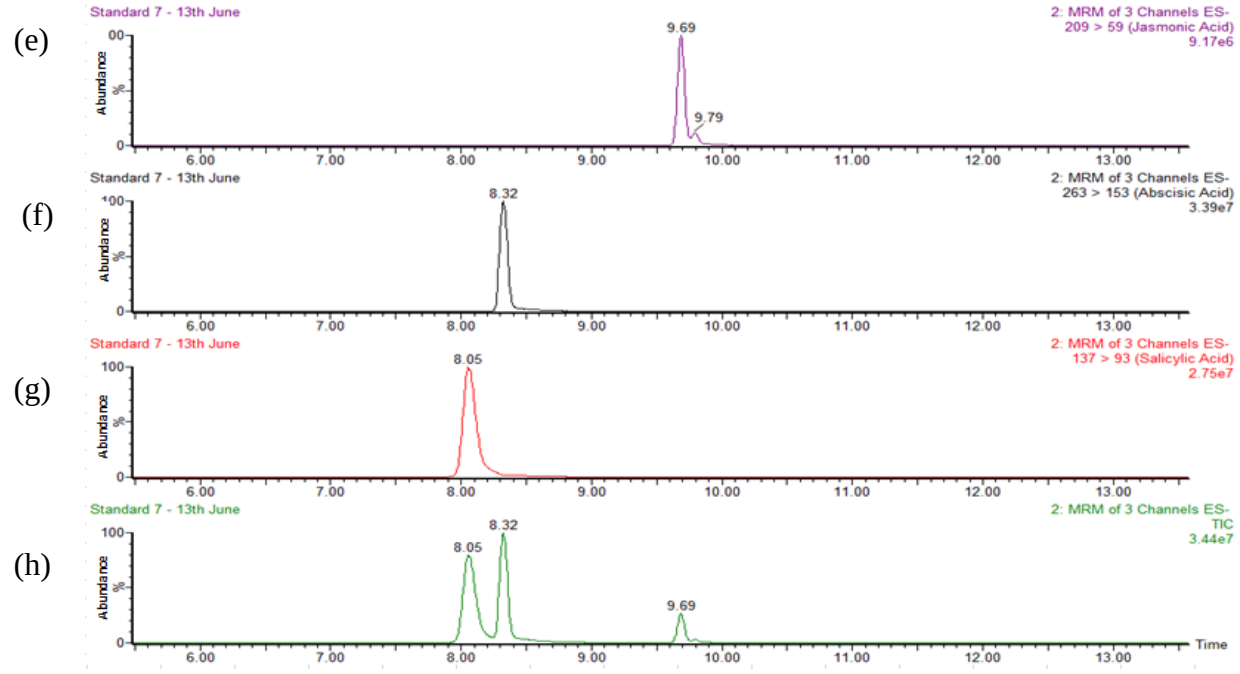
Table 4-2. MS parameters for analysis of three compounds (standard)

Compounds	Retention time (min)	Identification (m/z)	Quantification (m/z)
Salicylic acid	8.06	137 >93	137
Jasmonic acid isomer 1	9.69	209 >59	209 > 59 (209)
Abscisic Acid	8.34	263 > 153	263

MRM transitions for SA and JA were consistent with SIM scans, giving comparable retention times. Major ions of each compound obtained from standard solution are shown in Table 4-2. Two peaks were observed for ABA using SIM mode. However, only the peak eluting at approximately 8.32 min could be seen in MRM mode. Therefore, this peak was assumed to be the major isomer of ABA. SA and ABA were quantified using selective ion monitoring (SIM) at 137 and 263, respectively, whereas JA was quantified using the MRM described above (Fig. 4-7).



Time (min)



Time (min)

Figure 4-7. SIM (a, b, c and d) and MRM (e, f, g and h) chromatograms from 150 µl of standard solution of hormones: (a) and (e), jasmonic acid isomer 1; (b) and (f), abscisic acid; (c) and (g), salicylic acid; (c) and (h), total ion.

b. Sample stability

Sample analysis was carried out using the same conditions. In each case a greater number of peaks were observed in both SIM and MRM modes, with all compounds eluting within 15 min (Fig. 4-8). Therefore, identification of each compound was based on the retention time of the standard compounds (Fig. 4-9).

JA could not be detected in the samples using SIM mode. Therefore, quantification was based on the corresponding MRM signal, whereas both SA and ABA were quantified using the SIM signal (Table 4-3).

Table 4-3. MS parameters for analysis of three compounds (samples)

Compounds	Retention time (min)	Identification (m/z)	Quantification (m/z)
Salicylic acid	8.06	137 > 93	137
Jasmonic acid isomer 1	9.62	209 > 59	209 > 59 (209)
Abscisic Acid	8.37	263 > 159	263
Jasmonic acid Isomer2	7.92	209 > 59	209
Jasmonic acid Isomer4	10.09	209 > 59	209

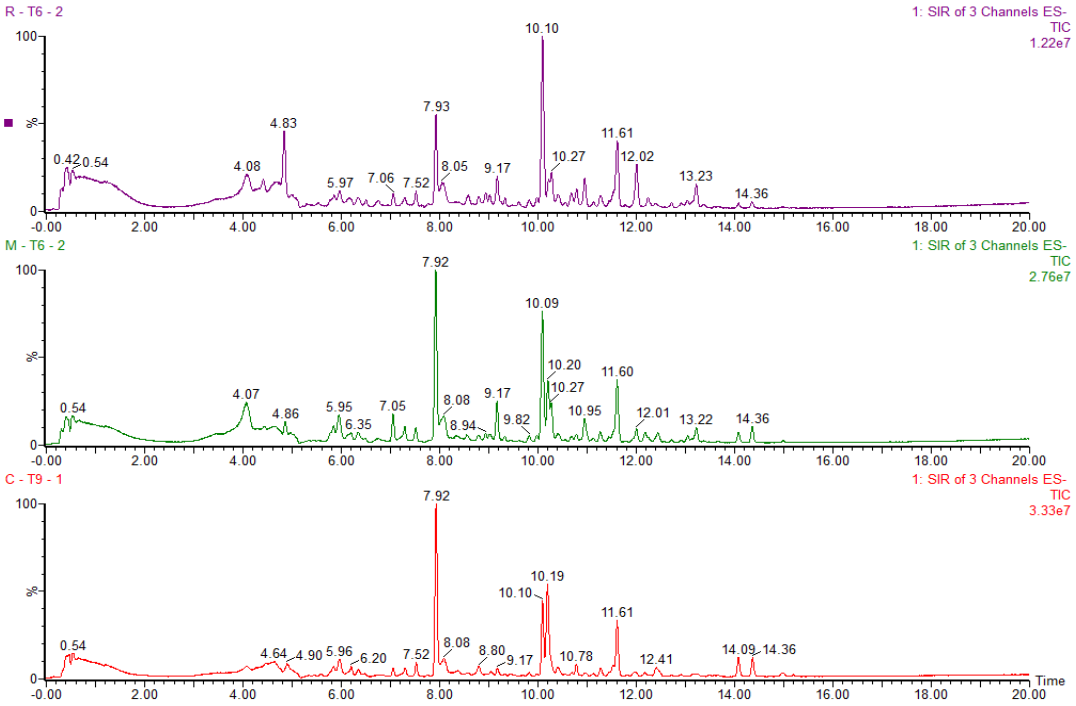
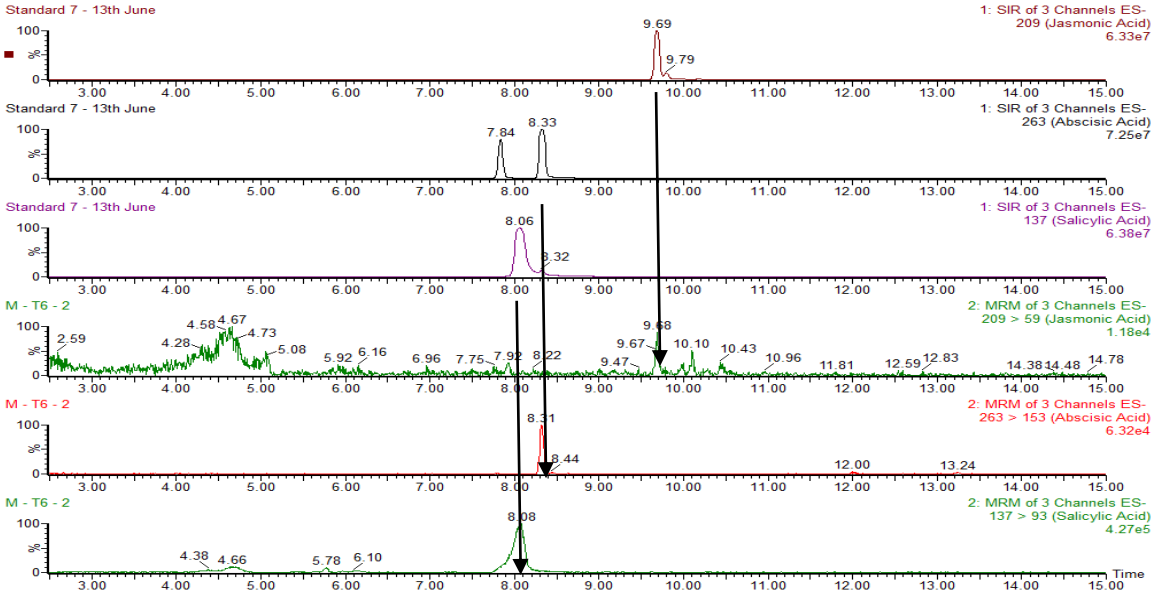


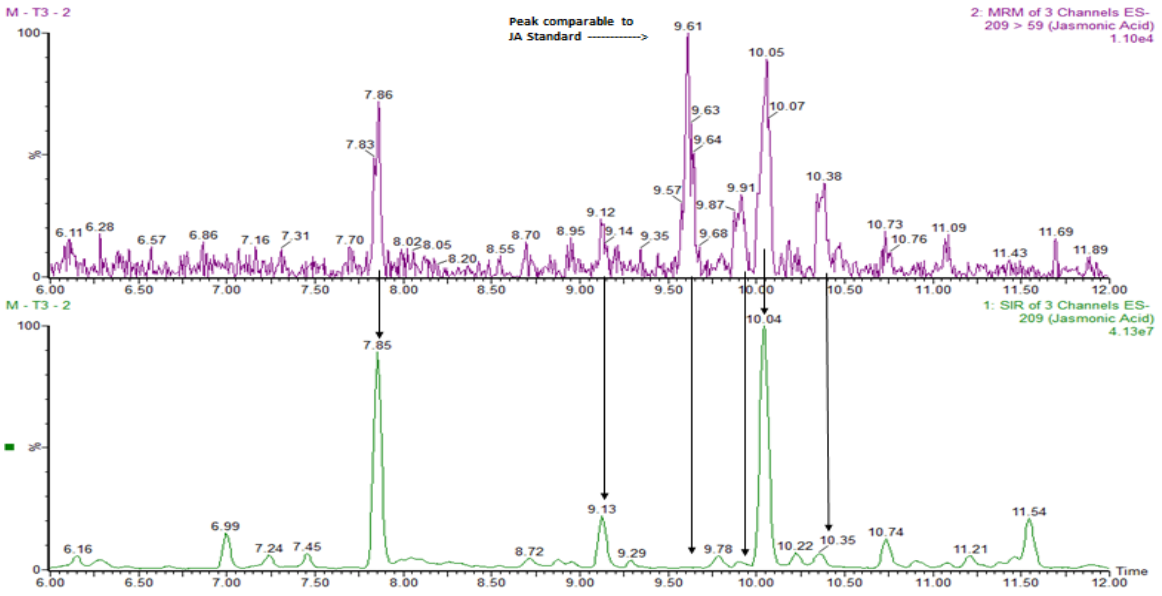
Figure 4-8. Typical chromatograms of defence chemicals of wheat TILLING lines 1171, 1996 and control leaves infested by *S. avenae*.



Time (min)

Figure 4-9. Identification (MRM) of each compound was based on the retention time of the standard compounds as shown by SIR. Top three chromatograms are SIR chromatogram as standards; bottom three are MRM chromatograms for line 1996 at 6 h as an example.

Isomers of jasmonic acid (Fu *et al.*, 2012) could be detected in the samples using MRM mode. There were much greater concentrations of isomers 2 and 4 than of isomer 1 in all samples (Fig. 4-10).



Time (min)

Figure 4-10. MRM chromatograms showing peaks comparable to jasmonic standard solution for line 1996 at 3 h.

4.4.7 Phytohormones

a. Sample analysis

The aim of this study was to investigate the effect of herbivory on concentrations of the plant hormones JA, SA and ABA in wild-type and mutant plants, to help elucidate the molecular link between WRKY proteins and aphid resistance. Hormone concentrations were measured after the application of aphid infestation over time at the 4th leaf stage.

As can be seen in Fig. 4-11a, the changes in SA level after exposure to aphids were striking ($p < 0.05$). With aphid infestation, the concentration of SA in mutant line 1996 plants increased to 29.6 nM and 30.5 nM and 31.5 nM at 3, 6 and 9 h after infestation. The concentration of SA decreased in mutant line 1171 plants to 21.5 nM and 20 nM in infested plants at 6 and 9 h. The SA concentrations at different periods of infestation in the mWRKY3 line 1996 plants (from 22.6 nM to 31.5 nM) differed greatly from the control.

The highest concentrations of ABA were found in the mWRKY3 line 1171 plants at 0, 3 and 9 h and were significantly greater than in the control plants ($p < 0.05$) (Fig. 4-11b). After

infestation with *S. avenae*, the ABA concentration in line 1171 plants fell to 1.3 nM at 6 h after infestation. The concentrations of ABA also were similar with exposure to aphids in line 1996 and control plants, which had similar concentrations of SA at all-time points.

Changes in the concentration of JA isomer 1 in leaves infested by *S. avenae* are shown in Fig. 4-11c. The JA isomer 1 concentration increased greatly after 9 hour infestation in the mWRKY3 line 1996 (up to 5.9 nM), similarly to the control. In contrast, the concentration of JA isomer 1 fell significantly in the mWRKY3 line 1171 between 0 and 3 h after infestation, then remained constant.

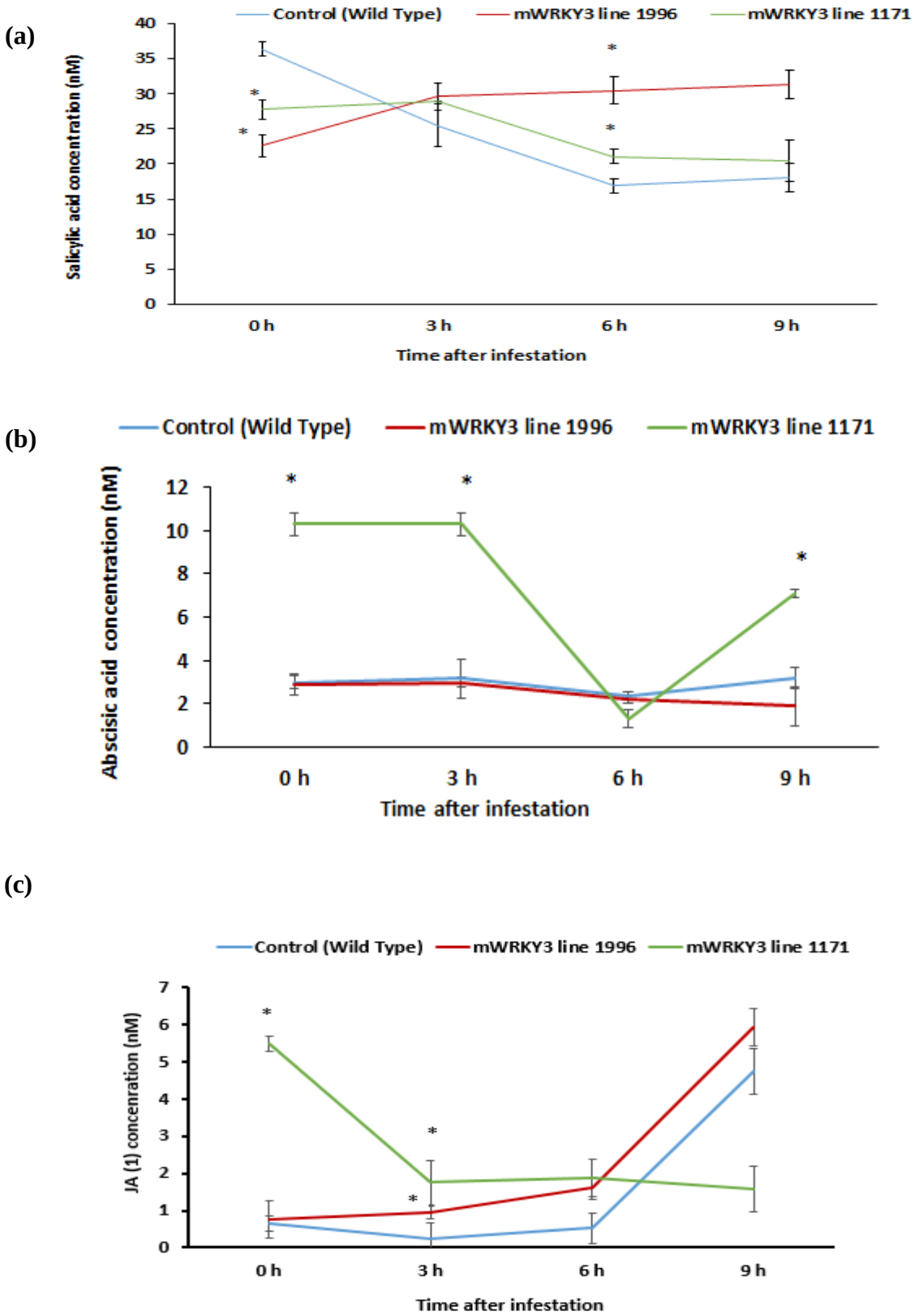


Figure 4-11. Changes in concentrations of salicylic acid (a), abscisic acid (b) and jasmonic acid (c) in the two mutant wheat lines (1171 and 1996) and Cadenza control incubated with *Sitobion avenae*. * represents significant difference compared with control at $P < 0.05$ level.

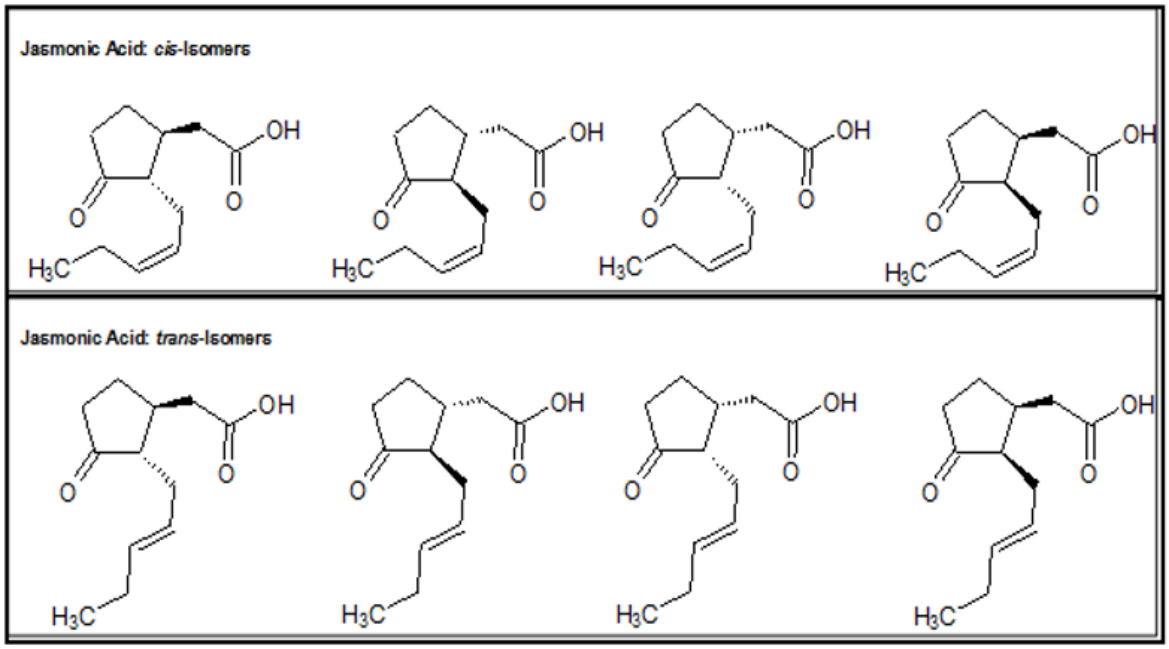
b. Jasmonic acid isomers

There are eight isomers of jasmonic acid (Fig. 4-12a), of which the naturally occurring ones are (-)-jasmonic acid and (+)-7-iso-jasmonic acid (Fu *et al.*, 2012). Isomers of jasmonic acid were identified by comparing a daughter ion scan of the standard compound (Fig. 4-12b) to the daughter ion scans obtained from potential isomer peaks observed during sample analysis (Fig. 4-12c-d). Several characteristic fragments were observed (m/z = 59; 83; 163; 165 and 191) that indicate the observed peaks are due to JA isomers. The isomers were quantified in all samples using daughter ion scans (Fig. 4-12). Levels of isomers 2 and 4 (Table 4-4) were significantly higher than others. Therefore, isomers 2 and 4 are probably (-)-JA and (+)-7-iso-JA. However, it was not possible to determine the identities of the isomers conclusively.

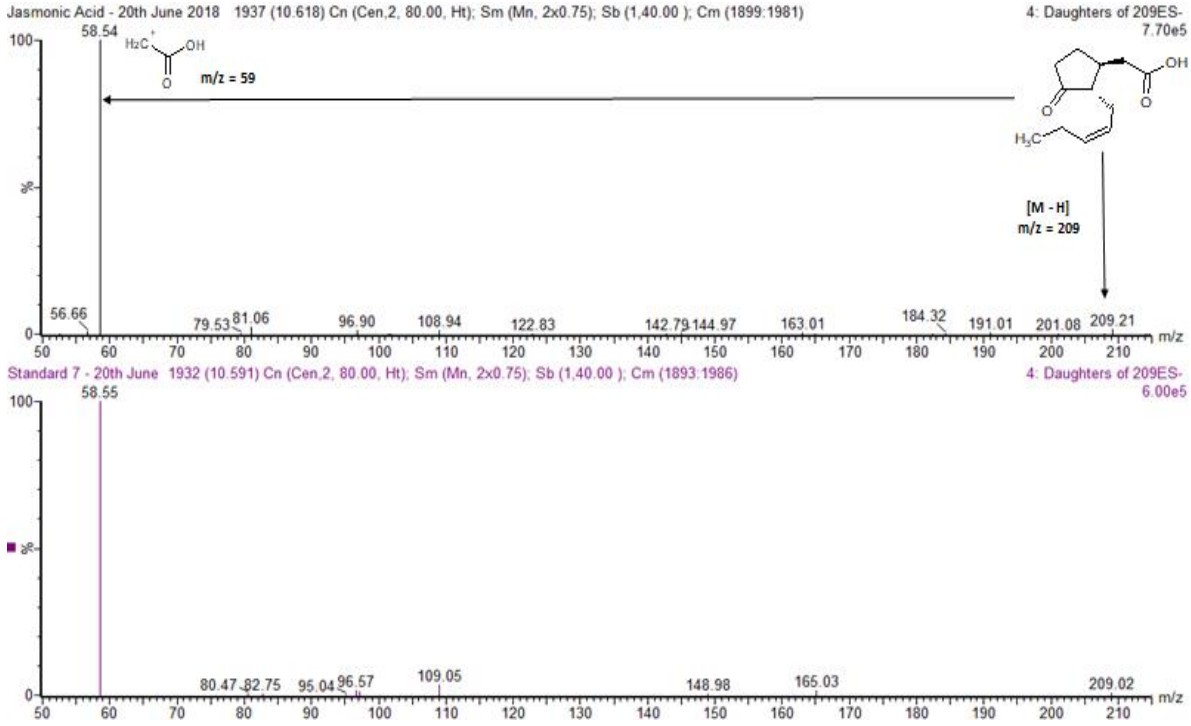
Table 4-4. Analysis of levels of stereoisomers of jasmonic acid in wheat infested by *S.avenae* at 3, 6, and 9 hours. Hormone extraction reproducibility was tested with two biological replicates of extracts. The numbers in parentheses are the standard errors based on the two biological replicates.

Infection of *S. avenae*	Compounds					
	Jasmonic acid Isomer 2			Jasmonic acid Isomer 4		
	Mean Concentration (nM)			Mean Concentration (nM)		
	Control	Line 1171	Line 1996	Control	Line 1171	Line 1996
T0 h	67 (±7)	33 (±5.8)	218 (±10)	47 (±19.4)	264 (±15)	121.7 (±7.2)
T3 h	174 (±9.2)	135 (±12)	236 (±23)	113.4 (±24)	286.9 (±16)	245.4 (±25)
T6 h	105 (±10)	39.4 (±3)	149 (±5.7)	50.22 (±22)	75.07 (±17)	144.2 (±15)
T9 h	211 (±17)	83 (±6)	60 (±2.9)	105.7 (±28)	196.6 (±20)	103.4 (±19)

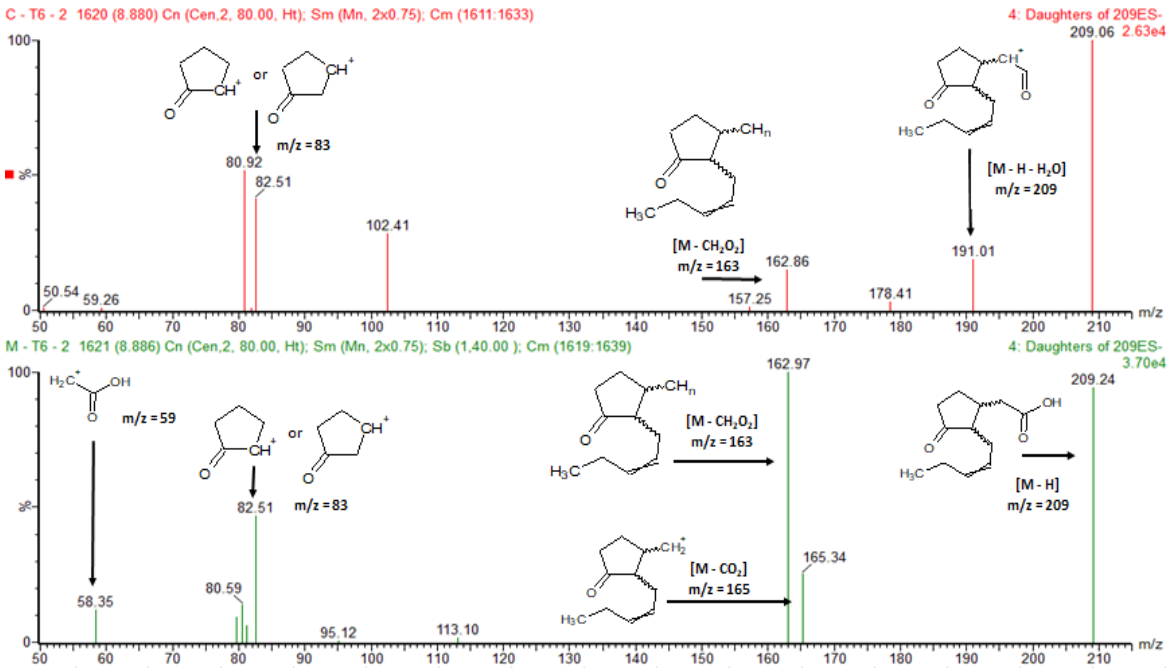
(a)



(b) Jasmonic Standard Spectra



(c) Isomer 2



(d) Isomer 4

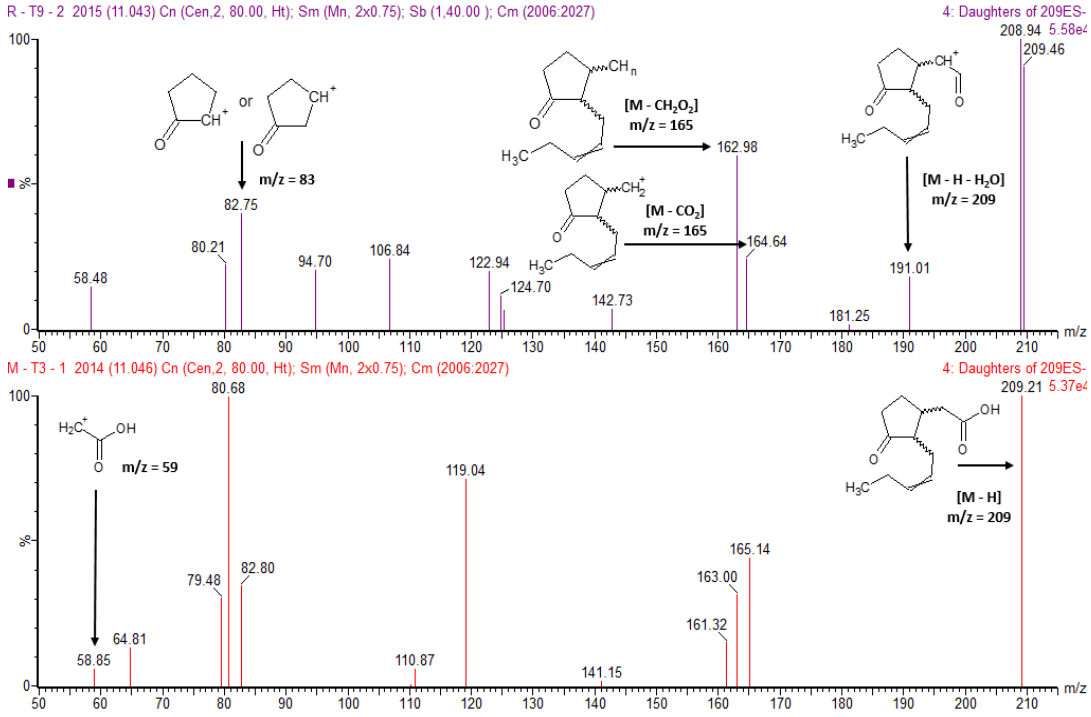


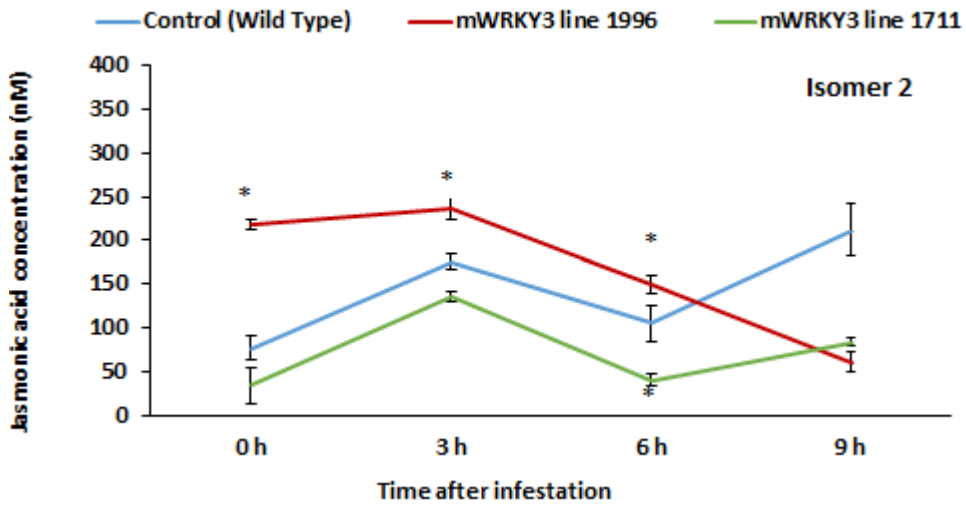
Figure 4-12. (a) The structures of the eight stereoisomers of jasmonic acid. (b) Isomers of jasmonic acid were identified by comparing a daughter scan of the standard compound with daughter scan spectra of m/z = 209 fragments of jasmonic acid isomers. Examples shown are (c) Control and line 1996 at 6 h and (d) Line 1171 at 9 h and line 1996 at 3 h.

The concentration of JA isomer 2 was significantly higher ($p < 0.05$) in the line 1996 plants than in the other two genotypes, but decreased at 6 and 9 hour after exposure to aphid infestation. At the early stage of infestation in the control and line 1171 plants, the level of JA isomer 2 increased ($p < 0.05$). The values were 174 nM and 135 nM, respectively, at 3 h and 211 nM and 83 nM, respectively, at 9 h after infestation, but lower at 6 h (Fig. 4-12a).

The highest level of JA isomer 4 in the line 1171 plants occurred sooner and was higher (up to 286 nM) at 3 h after infestation than in the other two genotypes ($p < 0.05$), then decreased to a much lower level 6 h after infestation, then increased at 9 h. The changes in the level of JA isomer 4 in the line 1996 plants were remarkably higher than in the control, and the peak value was 245 nM at 3 h, then it decreased at 6 h, when it was slightly higher than that in the control (Fig. 4-12b).

Fig. 4-13 compares the changes in concentration of the five phytohormones tested (ABA, SA, JA Isomers 1, 2 and 4) in each genotype. The highest concentrations were those of JA Isomers 2 and 4, which were from less than 100 nM to 286 nM in all three lines (Fig. 4-13a – b and c).

(a)



(b)

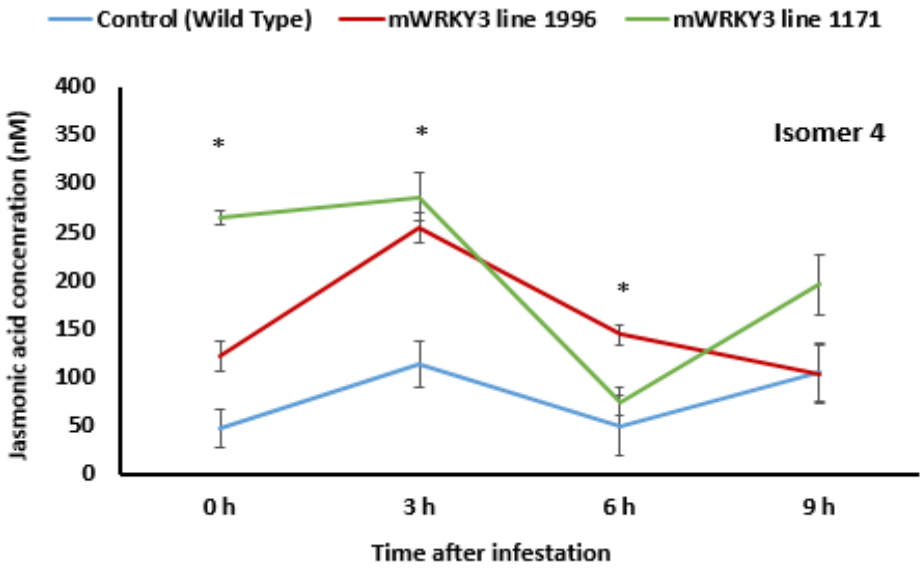
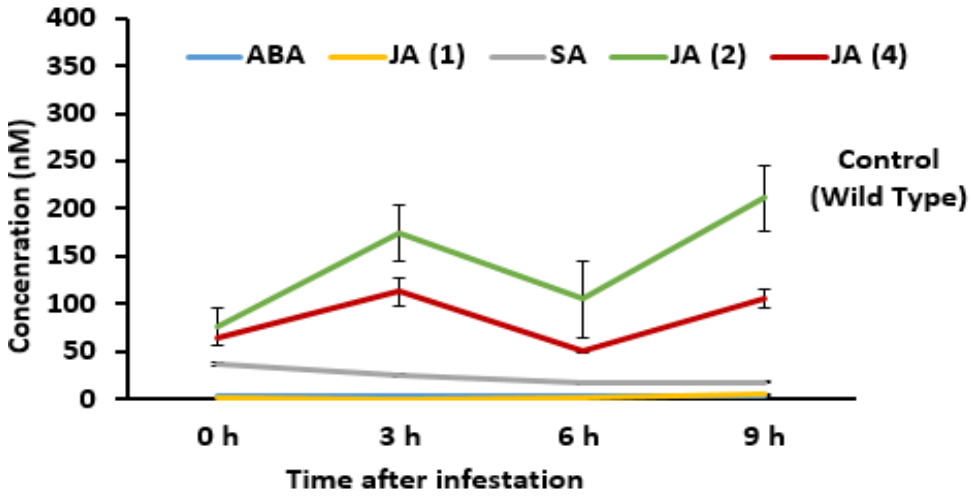
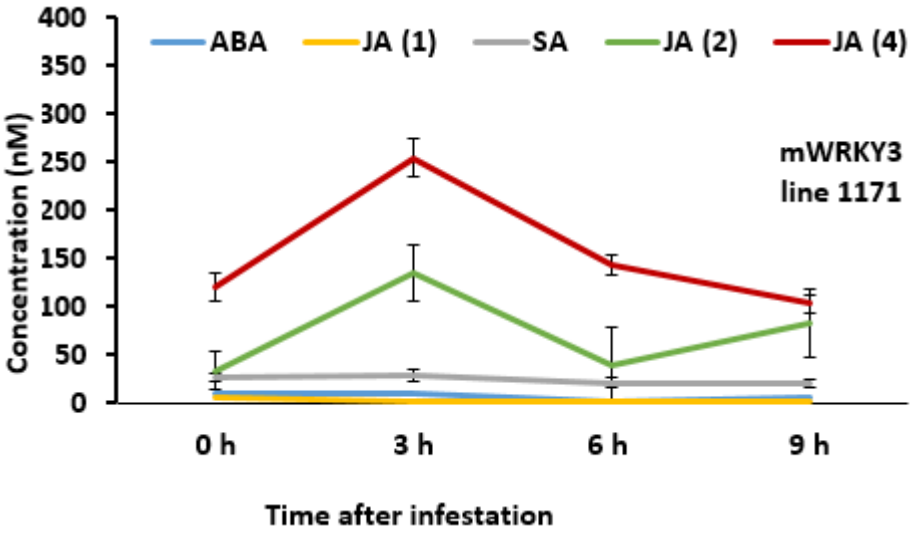


Figure 4-13. Changes in concentrations of (a) jasmonic acid isomer 2 and (b) jasmonic acid isomer 4 in lines 1171 and 1996 and Cadenza control incubated with *Sitobion avenae*. Data are represented as mean values ± SE (n = 2). * represents significant difference from the control determined by Turkey's test at $P < 0.05$.

(a)



(b)



(c)

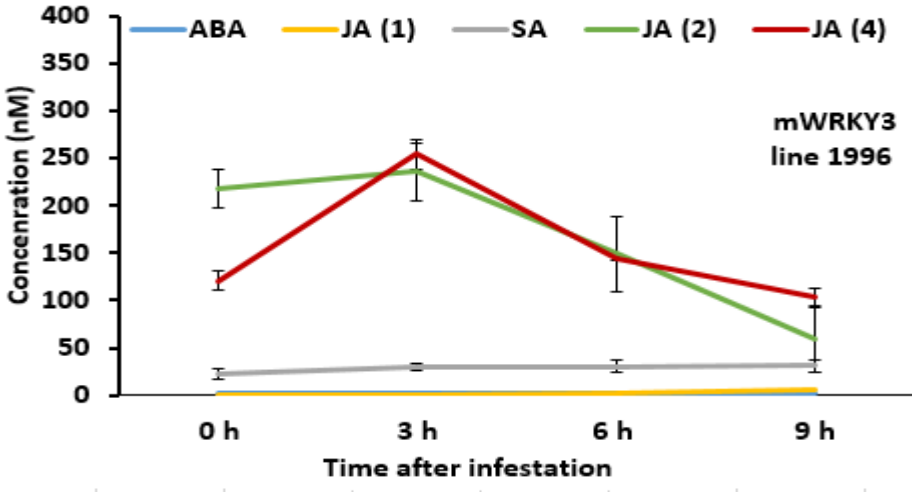


Figure 4-14. Changes in concentrations of the five compounds in the wheat Cadenza as a control (a), mWRKY3 line 1996 (b) and line 1711 (c) incubated with *Sitobion avenae*. Data are presented as mean values ± SE (n = 2).

All compound measurements gave highly reproducible quantitation as determined by the mean concentrations of hormone. Table 4-5 summarises the patterns of changes.

Table 4-5. Recovery of five signal phytohormones from leaf samples at different periods of infestation. (+ and – show increases and decreases respectively for each line and time compared to WT at 0 h).

Line	Time				Summary
	0 h	3 h	6 h	9 h	
WT	JA isomer 1 JA isomer 2 JA isomer 4 SA ABA	JA isomer 1 JA isomer 2 + JA isomer 4 + SA + ABA +	JA isomer 1 JA isomer 2 + JA isomer 4 - SA - ABA -	JA isomer 1 JA isomer 2 + JA isomer 4 - SA - ABA +	The levels varied greatly after 3 h of infestation
1171	JA isomer 1 JA isomer 2 - JA isomer 4+ SA + ABA +	JA isomer 1 JA isomer 2 + JA isomer 4 + SA + ABA +	JA isomer 1 JA isomer 2 + JA isomer 4 - SA - ABA -	JA isomer 1 JA isomer 2 + JA isomer 4 - SA - ABA +	The levels varied greatly after 3 h of infestation
1996	JA isomer 1 JA isomer 2 + JA isomer 4 - SA - ABA +	JA isomer 1 JA isomer 2 - JA isomer 4 + SA - ABA +	JA isomer 1 JA isomer 2 - JA isomer 4 + SA+ ABA -	JA isomer 1 JA isomer 2 - JA isomer 4 + SA + ABA -	The levels varied greatly after 9 h of infestation

4.5 Discussion

This study on WRKY3 and the mutant lines is the first study of its kind conducted on a member of the WRKY subgroup II, which is implicated by the results in Chapter 3 in tolerance of wheat to *Sitobion avenae*, to evaluate the effects of mutations within the WRKY domain. To establish whether WRKY3 has a role in the stress response TILLING lines with mutations to the *WRKY3* gene were grown under different nitrogen concentration and were also infested with aphids. Aphid fecundity on one mutant line showed no difference between high and low nitrogen levels. The results suggest that WRKY3 TFs plays a role in the link between nitrogen stress and aphid tolerance in wild type plants and possibly a central role in the cross-talk between environmental nitrogen and aphids and hormone signalling.

Collectively, the results suggest that there is an association between mutations in the WRKY domain of *TaWRKY3* and the production of two isomers of JA and that these isomers may coordinate responses to *Sitobion avenae* infestation under reduced nitrogen conditions. Plants change transcription factors in response to changes in growing conditions (Ferry *et al.*, 2011). Aphid fecundity (Fig. 4-5) was higher with 7.5 mM than with 2.25 mM nitrogen. One mutation in line 1996 was in the conserved WRKY domain and therefore this is almost certainly non-functional.

Furthermore, the low nitrogen conditions may prime the defence of wheat against insect attack. Aphid fecundity, along with the difference in concentration of plant hormones assayed directly by LC/MS-MS between mutant lines and WT, may contribute to the resistance of wheat to insect attack as a result of cross-talk via a regulatory network of WRKY transcription factors.

In addition, this study measured the concentrations of three phytohormones in two mutant lines (1171 and 1996), which have substitutions R139W and Y160D respectively, and in wild type plants infested with *S. avenae* at different time points (0 h, 3 h, 6 h and 9 h). In line with the role they play in response to stress from aphids it was found in this study that signalling mechanisms may be mediated by the stress hormones salicylic acid (SA), jasmonic acid (JA) and abscisic acid (ABA).

4.5.1 Aphid fecundity

This experiment was designed to assess how varying levels of nitrogen input applied to mutants of wheat, a common host plant of *S. avenae*, affected the performance of this aphid species. The major effect of nitrogen availability was on aphid fecundity. Karowe and Martin (1989) hypothesised that insect numbers are affected by the quality of the phloem sap, in which nitrogen content varies, due to a link between nitrogen input and phloem quality. Thus the most important finding from this investigation relates to the effect of lowering nitrogen availability on aphid fecundity (also observed in Chapter 2). A higher supply of nutrients may promote the reproduction of aphids by retaining photosynthates and amino acids, while low levels of nitrogen may limit aphid reproduction due to the reduced quantity of amino acid and sugars available for the aphids (Zebitz and Kehlenbeck, 1991).

Increased nitrogen inputs to crops have a positive effect on the growth and fecundity of *S. avenae* (Aqueel and Leather, 2011). This higher aphid fecundity may be due to the plant's quality when grown at 7.5 mM nitrogen; such plants are healthy and the phloem sap, which the aphids feed on, is high quality (Zebitz and Kehlenbeck, 1991). It has been observed in many studies performed in controlled conditions that plant performance increases with increasing levels of nitrogen (Aqueel and Leather, 2011). Similarly to our results, Lu *et al.* (2007) found that aphid populations were higher under optimal conditions because aphids assess the plant's quality by probing the leaf tissue.

The decrease in aphid fecundity with reduced nitrogen input at 2.25 mM is in agreement with other studies which reported that the link between plant nitrogen and aphid infestation is complex and acclimation to low nitrogen confers aphid resistance (Comadira *et al.*, 2015) and that nitrogen input can have a strong effect on aphid fecundity (Gash, 2012). Low levels of nitrogen have a large impact on the leaf transcription profile. Effects of plants in reducing aphid fecundity have been associated with antibiosis reactions in the nymph, which may account for aphid performance (Tolmay *et al.*, 1999). It has been previously observed that the link between signalling pathways and triggers is involved in cross tolerance between stresses (Foyer *et al.*, 2016).

The WRKY3 mutant line 1996 is notable in that there were similar numbers of aphids on this mutant line at both nitrogen levels. In particular, it can be proposed that the mutation in line

1996 within the WRKY binding domain reduces the rate at which aphid population increases and negates the differentiation between population increases relative to nitrogen availability. In the WRKYGQK sequence, Tyr 160 is important in binding to DNA as shown by the effect of a mutation to Asp (Chapter 5).

The results show that pathways involved in resistance mechanisms may be affected by the relationship between host plants and aphids under different nitrogen treatments. As with the wild-type plants, a nitrogen level of 7.5 mM increased the fecundity of all TaWRKY3 mutant lines except line 1996. However, fecundity was lower on some of the mutant lines than on the wild-type.

In order to improve the spatial and temporal management of aphids, it would be useful to explore how they affect the composition of phloem, as represented by leaf exudates, under different environmental conditions, with different levels of nitrogen and in different plant varieties, with consequential effects on the number of nymphs (Sandström *et al.*, 2000; Khan and Port, 2008).

4.5.2 Contrasting mechanisms of SA-JA and ABA defence against insects

In this experiment, it was possible to identify and quantify JA, SA and ABA in extracts of leaves by LC/MS-MS from two mutant wheat WRKY3 lines (1171 and 1996) and control (wild type) plants. Concentrations of these hormones were found to change markedly after *S. avenae* infestation. Levels of ABA were highest in leaves of line 1171. Aphid also performed more poorly on line 1171 than on wild-type plants. It has been proposed that in Arabidopsis ABA accumulation is beneficial to aphids because aphid feeding elicits an ABA-dependent decoy response (Hillwig *et al*., 2016) that could interfere with effective defences and make plants more suitable for aphid colonisation. However, the patterns of ABA accumulation observed here suggest that this hypothesis does not apply to the interaction between wheat and *S. avenae*.

The study results also demonstrate that after aphid infestation, SA levels rose in line 1996, suggesting that SA is produced in response to aphid damage. The lowest aphid fecundity was observed on line 1171 plants. Salicylic acid can also cause physiological effects in plants. For

example, research has revealed that it may act as a key signal molecule generated by systemic acquired resistance (Pieterse and van Loon, 1999).

Jasmonic acid can also cause a variety of physiological and morphological effects. When plants are attacked by pathogens or herbivores, they are able to put their defence systems into motion by triggering changes in endogenous JA levels (Huang *et al.*, 2015). Jasmonates are known to stimulate a number of defensive and physiological processes and the amino acid conjugates of jasmonic acid are involved in several plant developmental processes (Farmer and Ryan, 1992). Jasmonic acid is synthesised by the octadecanoid pathway. The structure of volicitin, an octadecatrienoate conjugated to an amino acid, suggests that the octadecanoid pathway interacts with elicitor molecules in herbivore-damaged plants (Farmer and Ryan, 1992). In the plants in this study, higher levels of total JA were produced when aphids were feeding on the wheat leaves in both mutant plants, compared to the control, and this may explain the lower aphid numbers on the mutant plants during the early stage of infestation (Fig. 4-12). The results are consistent with other evidence that JA plays a significant role in the interaction of plants with insects, but differ from the generalisation that the basal resistance to necrotrophic organisms is founded on JA-related mechanisms, while biotrophic or hemibiotrophic basal resistance is based on SA-related mechanisms (Segarra *et al.*, 2006). However, the results indicate a role of JA in the functions of WRKY3; changes in JA due to the action of WRKY proteins will allow a model of wheat defence response to be built, elucidating any molecular link.

4.6 Conclusions

The mutant WRKY lines identified provided additional insights into the coordination of the high/low nitrogen responses of plants. A putative low-nitrogen regulatory element may reduce the aphids' fecundity in response to low nitrogen levels in the plant. A better understanding of the complex regulatory network of the nitrogen-aphid response will help to develop transcription factor strategies to improve low nitrogen use.

In the current project, a 'loss of function' mutation was identified in WRKY3 mutant line 1996. The mutant and wild type lines were grown with high and low nitrogen levels, and the effect of the nutrient supply on aphids' performance on these mutant lines was analysed. There was no difference in performance between high and low nitrogen levels in mutant 1996. This result provides evidence that WRKY3 plays a role in the interaction between nitrogen stress and aphid tolerance in wild type plants.

The mutation found in TILLING line 1996 is expected to affect binding to the W-box element in target gene promoters. Gel shift experiments and protein-DNA interaction assays were used to analyse interactions of wheat WRKY transcription factors with the W-box element in several promoters in Chapter 5.

5 Chapter 5. WRKY transcription factors require the archetypal WRKYGQK domain to bind to promoter cis-elements

5.1 Abstract

WRKY transcription factors play an important role in induced plant defence by regulating gene expression in response to external stimuli. WRKY transcription factors contain a region binding to one particular DNA element – the W-box. In the present study, genes encoding wild-type TaWRKY3 and the mutated form found in TILLING line 1996, which has a tyrosine to aspartate substitution in the invariant WKRYGQK sequence, were successfully expressed in *E. coli* in order to test WRKY3 and the mutant form for their binding to W-box elements (*TaPR1-23* in the downstream flanking region, *PcPR1-1* promoter and synthetic W-box repeated sequence). DNA binding was disrupted by the mutation in the TILLING line, indicating that this sequence is required for proper DNA binding. The regulation of PR gene expression is important for the activation of plant defence responses; the present work provides evidence that TaWRKY3 may regulate this important response by binding to the W-box element in the promoter. This will enhance our understanding of the molecular basis of the mode of action of WRKY3 in wheat.

Key words: Parsley promoters /*TaWRKY* transcription factors/ W-box element/ DNA-binding

5.2 Introduction

Changing agricultural conditions are leading to insect pests becoming a more serious threat to sustainable crop production (Castex *et al.*, 2018). Therefore, it is important to understand the increase in aphid fecundity under high nitrogen (standard) conditions, believed to be controlled at least partly by WRKY genes. Better understanding of the molecular basis for this link could help allow optimal nitrogen availability without compromising tolerance to aphids.

WRKY transcription factors (TFs) are involved in the regulation of gene expression in plants in response to biotic and abiotic stresses (Eulgem *et al.,* 2000). WRKY proteins are defined by the conserved amino acid sequence WRKYGQK at their N-terminal end and by a zinc-finger and have a regulatory function which helps with the concerted activation of a large variety of genes (Zhu *et al.,* 2013). The expression of target genes is modulated by DNA-binding transcription regulators and other components for normal development and proper response to environmental stimuli. The W-box sequence TTGAC is required for specific DNA binding of WRKY proteins to DNA (de Pater *et al.*, 1996; Rushton *et al.*, 1996; Wang *et al.*, 1998; van Verk *et al.*, 2008).

The promoter region for *PR1-1* has been identified in many monocotyledon plant species including wheat and also in dicotyledon species such as parsley. The regulation of *PR1-1* gene expression is important for the activation of plant defence responses (Van Loon and Van Strien, 1999). The present work demonstrates that TaWRKY3 may regulate this important response by binding to the W-box element in these promoters. As the WRKY family is large within a given species, and as these proteins bind to the W-box, some specificity may be granted by additional nucleotide sequences flanking W-box elements in promoters.

In Chapter 3, the *TaWRKY3* gene was identified as being involved in the link between nitrogen availability and *S. avenae* fecundity. Gene expression profiles using qPCR analysis during periods of single and dual stress gave evidence for the priming of plant defence responses under low nitrogen availability. Chapter 4 analysed the function of WRKY3, using TILLING lines with mutations in WRKY3, and it evaluated the effects of mutations within the WRKY binding domain on aphid performance. Aphid fecundity on a line with a mutation in the WRKYGQK sequence (line 1996) showed no difference between high and low nitrogen levels, suggesting that WRKY3 plays a role in the link between nitrogen stress and aphid tolerance in wild type plants. In order to examine this further, protein-DNA interaction assays

were used. WRKY3 wild type and the mutant protein in line 1996 were tested for their binding abilities *in vitro* to different W-box elements and promoter sequences containing a W-box. This analysis revealed clear differences in binding.

Moreover, it was demonstrated through the DNA-protein assay that TTGAC acts as a binding site for WRKY3 in the *cis*-sequence under study. This binding site, referred to as the WT-box, is rich in promoters of the genes up-regulated to WRKY proteins. A non-radioactive Electrophoretic Mobility Shift Assay (EMSA) was used in the present study to assess the binding activity of WRKY3. In order to elucidate the binding sites of transcription factors, DNA sequences were end-labelled with biotin [5' TAGCATATGCTA 3'].

5.2.3 Aim and Objectives

To investigate the impact of a mutation in the WRKY domain, specifically how it affects binding to W-box. This was achieved by the following objectives:

1. Purification of recombinant wild-type and mutant WRKY3.
2. Investigation of TaWRKY3 binding to synthetic and whole promoter W-box elements. The whole promoter W-box elements are from the parsley *PR1-1* and wheat *PR1-23* genes. The parsley sequence tested is the W2 sequence, which was one of the first sequences shown to bind to WRKY proteins (Rushton *et al.*, 1996) and was used by Ciolkowski *et al.* (1998) to study the DNA binding specificity of Arabidopsis WRKY proteins.
3. Investigation of how the mutation in TILLING line 1996 affects the binding of TaWRKY3 to W-box elements.

5.3 Materials and methods

5.3.1 Experimental design

Proteins for electromobility shift assays (EMSAs) were expressed in *E. coli* and purified. All experiments were performed using total soluble *E. coli* proteins with pETSUMO constructs containing an N-terminal 6xHis tag and SUMO fusion protein. EMSAs were performed to verify DNA binding specificity of TaWRKY3 proteins following the manufacturer's instructions for LightShift™ Chemiluminescent EMSA kit (Thermo Fisher Scientific) (Fig. 5-1).

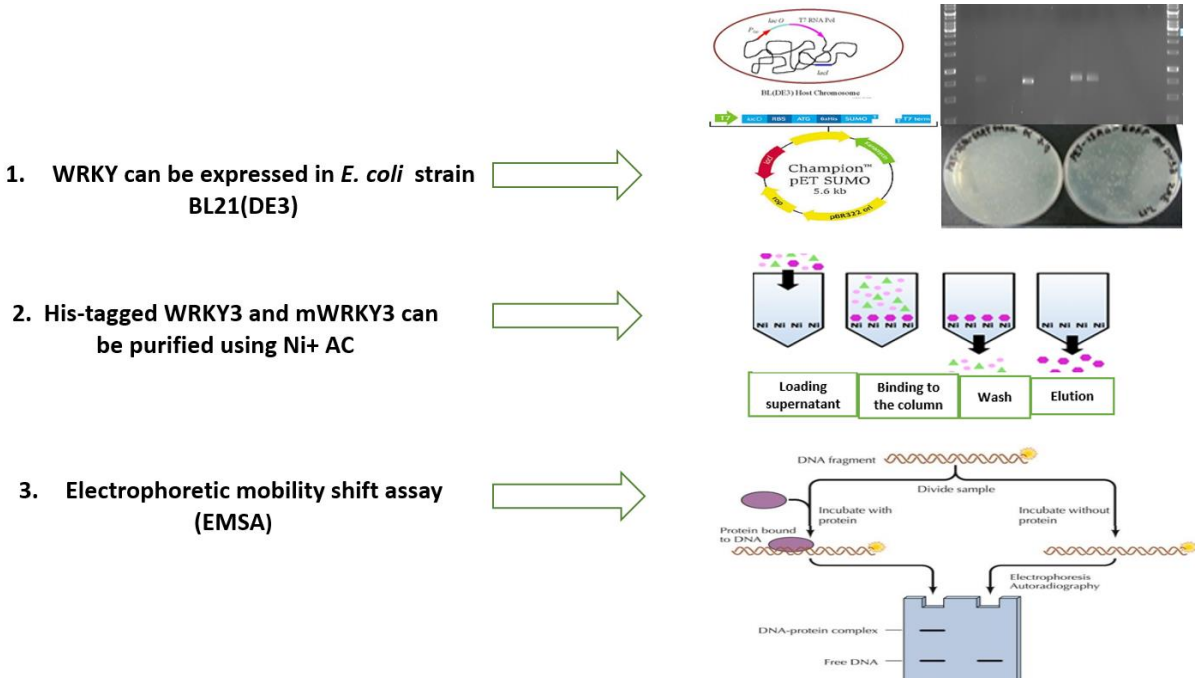


Figure 5-1. Experimental framework used to assess the binding of recombinant transcription factor proteins to promoter elements. Recombinant WRKY3 and mutant expressed in bacteria have identical binding properties. 1. Recombinant proteins were produced in *E. coli*. 2. WRKY proteins were isolated using NIAC under native conditions as described in section 5.3.5. 3. EMSA of WRKY binding to DNA fragments containing each of the W-box sequences indicated by chemiluminescence in section 5.3.6.

5.3.2 Cloning

a. pET SUMO plasmid construction

Ligation reactions were set up as follows: 2 µl pET SUMO vector (25 ng μl^{-1}) (Invitrogen) was added to 2 µl of fresh PCR product , 1 µl 10x Ligation Buffer, 4 µl nuclease-free water and 1 µl T4 DNA ligase to a final volume of 10 µl. This was mixed and incubated at 15 ºC overnight.

Table 5-1. Primers for cloning of TaWRKY3 genes

Gene	Specific primers for cloning	
	Forward primer (5'-3')	Reverse primer (5'-3')
TaWRKY3 (CDS)	AGATTCTTGTACGACGGTATTAG	TAGTTATTGCTCAGCGGTGG

b. *E. coli* Transformation

The Mach1™-T1R *E. coli* strain was used for the production of plasmid DNA, and strain BL21 (DE3*) was used for protein expression. A similar protocol was used for both. Chemically competent *E. coli* cells were put on ice. 2 µl of vector was added and the cells were incubated for 30 min. The cells were heat shocked at 42 ºC for 1 min then transferred to ice. 250 l of S.O.C. medium (Super Optimal broth with Catabolite repression) was added and incubated with shaking at 37 ºC for 1 h 30 min. 100 µl was spread onto LB agar plates containing 50 µg ml^{-1} kanamycin antibiotic and they were left overnight to incubate at 37 ºC.

Putative transformants were screened for the presence and directionality of *TaWRKY3* by colony PCR. 48 µl of the PCR High Fidelity Mixture (Invitrogen) was added with either 1 µl of SUMO Forward primer and 1 µl of the WRKY3 reverse primer or 1 µl of T7 reverse primer and 1 µl of the WRKY3 forward primer to give a final volume of 50 µl. Ten colonies were resuspended individually in 50 µl PCR reaction mix and incubated for 10 min at 94 ºC to lyse the cells followed by 30 cycles of amplification and incubation at 72 ºC for 10 min for the final extension. This was done to confirm that the gene is in the correct orientation and in frame with the N-terminal tag by agarose gel electrophoresis. Sequence analysis was performed in order to confirm that the wild-type and mutant coding sequences were in the correct orientation and in frame with the N-terminal tag.

For long-term storage, single colonies were picked from the plates and suspended in 10 ml LB liquid broth with the antibiotic kanamycin. They were kept overnight at 37 ºC with shaking.

250 µl of the culture was then mixed with 250 µl 50% glycerol and the stock was stored at -80 ºC.

5.3.3 Protein Expression

All constructs to be expressed as protein were transformed into *E. coli* strain BL21 (DE3*) (5.3.2b Transformation). A stock culture (0.5 ml) was added to 10 ml LB liquid medium containing 50 µg ml^{-1} kanamycin and 1% glucose and the culture was grown overnight at 37 ºC with shaking. 1 ml of this culture was added to 50 ml fresh LB with kanamycin and grown at 37 ºC with shaking. The optical density at 600 nm of the culture was checked with a spectrophotometer. Once 0.6 was reached, the culture was split into two samples and IPTG (Fisher Scientific) was added at a final concentration of 1 mM to one culture, giving one induced and one uninduced culture. Post-induction samples were taken at regular time points. After 12 hours' growth at 37 ºC, the cells were collected by centrifugation at 4600 x *g* for 10 min at 4 ºC. For each time point, the pellet was resuspended in 1.5 ml BugBuster HT Protein Extraction Reagent (Merck) for 30 min with gentle shaking then centrifuged at 16,000 × *g* for 20 min at 4 °C. The supernatant containing the soluble protein fraction was transferred to a fresh tube with 20 µl 2x SDS-PAGE loading buffer (5.3.4 a. SDS-PAGE). This could then be used in the protein purification system. The insoluble fraction (pellet) was resuspended in 80 µl 1x SDS-PAGE loading buffer. For long-term storage at -20 ºC, all samples were heated to 100 ºC for 5 min. 15 µl of the supernatant and the pellet were loaded onto an SDS-polyacrylamide gel and electrophoresis was performed.

5.3.4 Protein Analysis

a. SDS-PAGE

Polyacrylamide gels for electrophoresis were prepared with stacking gels containing 5% acrylamide in 0.125 M Tris-HCl, pH 6.8, 0.1% ammonium persulphate, 0.01% TEMED. Separating gels contained 6% or 10% acrylamide in 0.375 M Tris-HCl, pH 8.8, 0.1% ammonium persulphate, 0.005% TEMED.

All samples were run for 1 hour at 80 V then at 120 V for 1 hour in a tank containing 1x running buffer (25 mM Tris, 192 mM glycine).

b. Coomassie Blue staining

Gels were stained overnight in Coomassie Blue Stain (0.25% Brilliant Blue, 50% methanol, 10% glacial acetic acid) and destained for an hour in Destain (40% methanol, 70% glacial acetic acid).

c. Western Blotting

Gels were blotted onto 0.45 µm nitrocellulose membranes for 1 hour at 50 mA. All blocking, probing and washing was carried out in 1x PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4 with adjusted pH 7.4) with shaking, as follows.

- Membranes were soaked in 5% Semi-skimmed milk in PBS buffer with 1% Tween 20 for 1 hour.
- The membranes were probed with 1:2000 primary anti-His Tag antibody raised in mouse (ThermoFisher) in PBS overnight at 4 ºC with shaking.
- The membranes were washed with 1x PBS and 0.1% Tween20 for 5 min (x3).
- 1:2000 diluted peroxidase-conjugated goat anti-mouse secondary antibody (Bio-Rad) was applied and the membrance was shaken gently for three hours at room temperature.
- The membrane was washed with 1x PBS for 5 min (x3).
- Substrate solution (Chemiluminescent Western Blot Detection) (ThermoFisher) was prepared by mixing equal volumes of Luminol/Enhancer Solution and Stable Peroxide Solution, added to the membrane and incubated for 1 min. X-ray film (Kodak) was exposed to the membrane for 5 to 20 min then developed, washed and fixed.

5.3.5 Protein Purification

All experiments were performed using total soluble *E. coli* proteins (Fig. 5-2).

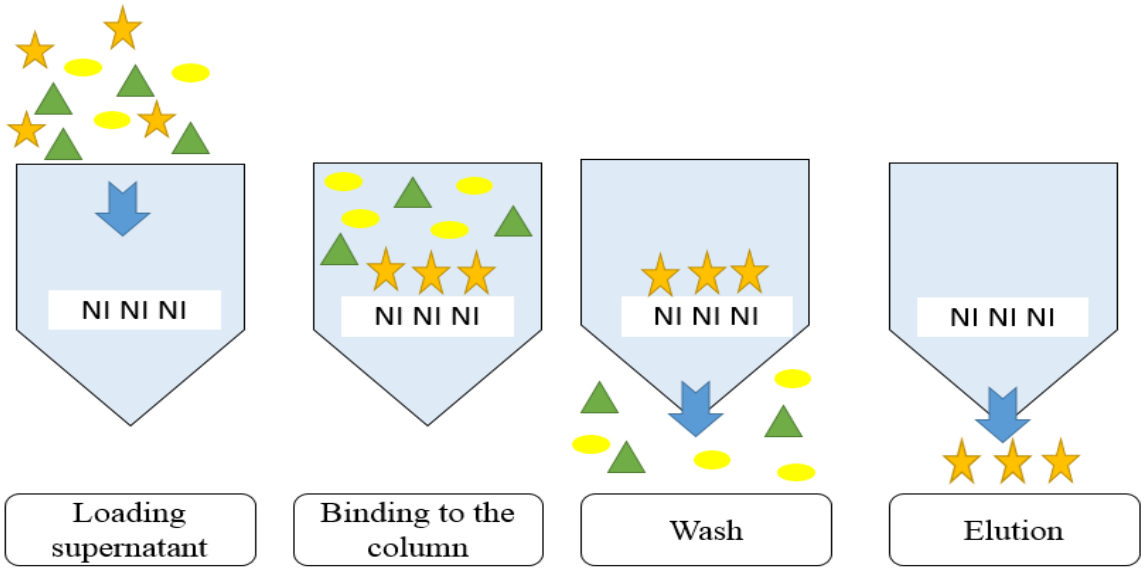


Figure 5-2. Schematic of Nickel Ion Affinity chromatography. Total protein including recombinant protein with 6xHis tag is added to the columns containing Ni^{2+} ions. The recombinant protein binds to the column. All non-specifically bound proteins can be removed by washing with 25 mM imidazole. The recombinant protein is eluted with 250 mM imidazole.

Protein was isolated using Nickel Ion Affinity chromatography (Thermo Scientific) using the following protocol:

Buffers for native conditions:

- Equilibration Buffer: 20 mM sodium phosphate, 300 mM sodium chloride (1x PBS) with 10 mM imidazole; pH 7.4
- Wash Buffer: PBS with 25 mM imidazole; pH 7.4
- Elution Buffer: PBS with 250 mM imidazole; pH 7.4

Buffers for denaturing conditions:

- Equilibration Buffer: PBS with 8 M urea and 10 mM imidazole; pH 7.4
- Wash Buffer: PBS with 8 M urea and 25 mM imidazole; pH 7.4
- Elution Buffer: PBS with 8 M urea and 250 mM imidazole; pH 7.4

HisPur Ni-NTA spin columns (3 ml) were used for purifications. Columns were centrifuged for 2 min at 700 x *g* and the fraction was collected in a centrifuge tube. The bottom plug was placed in the column and the prepared protein extract was added. It was mixed with an end-over-end mixer for 30 minutes at room temperature. The bottom plug was removed and the column was centrifuged at 700 × *g* for 2 minutes, collecting the flow-through in a centrifuge tube. The resin was washed with two resin-bed volumes of wash buffer and centrifuged at 700 × *g* for 2 minutes and the fraction was collected in a centrifuge tube. This step was repeated two more times collecting each fraction in a separate centrifuge tube. His-tagged proteins were eluted from the resin by adding one resin-bed volume of elution buffer and centrifugation at 700 × *g* for 2 minutes. This step was repeated two more times, collecting each fraction in a separate tube. The eluted protein could then be directly analysed by SDS-PAGE.

5.3.6 In Vitro Protein-DNA Binding Assay

The electrophoretic mobility shift assay was performed using LightShift™ Chemiluminescent EMSA (Thermo Fisher Scientific) with purified WRKY3 and mWRKY3 (5.3.5) (Fig. 5-3).

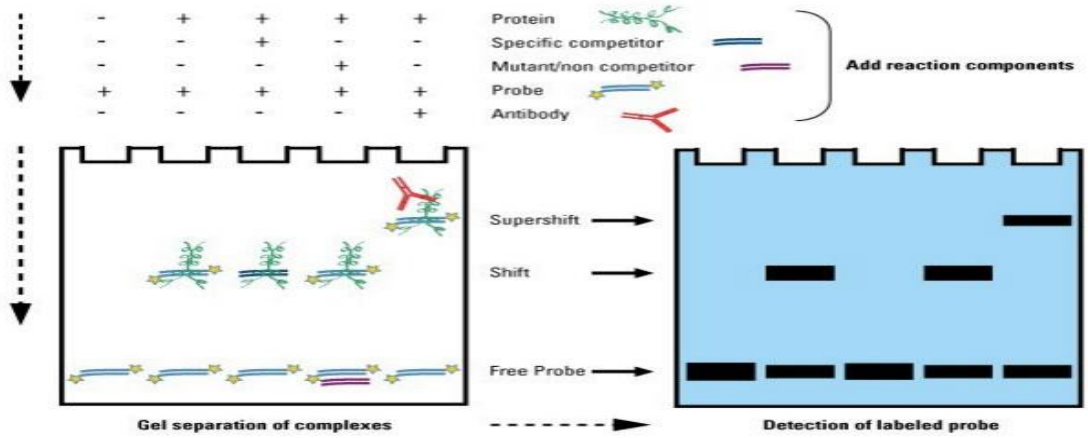


Figure 5-3. There are 3 steps in a gel shift assay: (1) binding reactions, (2) electrophoresis, (3) probe detection (ThermoFisherScientific, 2018).

Procedure Protocol

a. Lightshift EMSA Optimisation to Anneal Complementary Pairs of Oligonucleotides

The DNA probes (Table 5-2) were prepared by annealing complementary oligonucleotides containing the 60 bp biotin end-labelled sequences. Probe was prepared at a concentration of 1 pmol μl^{-1} with forward and reverse sequences in a 1:1 molar ratio and the final concentration of 1 pmol μl^{-1} was achieved by adding buffer containing 10 mM Tris-HCl, 1 mM EDTA, and 50 mM NaCl (pH 8.0). Oligonucleotides were annealed by incubating the tube in a water bath for 5 min. It was then left at room temperature to cool and the probes were stored at -20 °C.

Table 5-2. Probes for gel shift assay

Probes	Oligo (5'-3')	Primer sequence with probe sequence	
		Forward primer (5'-3')	Reverse primer(5'-3')
PcPR1-1 promoter and Synthetic	TAGCATATGCTA	[Btn] TTATTCAGCCATCAAAAGTTGACCAATAAT TTATTCAGCCATCAAAAGTTGACCAATAAT	[Btn]ATTATTGGTCAACTTTTGATGGCTGAATAA ATTATTGGTCAACTTTTGATGGCTGAATAA
TaPR1-23 gene and synthetic	TAGCATATGCTA	[Btn]CGCTTCCTCACGCCATCGTTGACCCCCGCT CGCTTCCTCACGCCATCGTTGACCCCCGCT	[Btn]AGCGGGGGTCAACGATGGCGTGAGGAAGCG AGCGGGGGTCAACGATGGCGTGAGGAAGCG
Wb synthetic	TAGCATATGCTA	[Btn] CGTTGACCTTGACCTTGACTTT CGTTGACCTTGACCTTGACTTT	[Btn] AAAGTCAAGGTCAAGGTCAACG AAAGTCAAGGTCAAGGTCAACG
mWb synthetic	TAGCATATGCTA	[Btn] CGTTGAACTTGAACTTGAATTT CGTTGAACTTGAACTTGAATTT	[Btn] AAATTCAAGTTCAAGTTCAACG AAATTCAAGTTCAAGTTCAACG

b. Ta*PR1-23* gene of the downstream fragment

Primers were designed and synthesised according to the Ta*PR1-23* (HQ700377.1) downstream flanking sequence in wheat (Table 5-2). The sequences are identical to a region of the wheat *PR1-23* gene containing one W-box, which was selected as follows. The At*PR1* gene from GenBank (with accession number NM_127025.3) was submitted to Ensembl wheat genome Blast (https://plants.ensembl.org/). The region containing the most similar WRKY sequence in wheat (Ta*PR1-23*) was identified (TGACV1-scaffold-457095-5DS:39.367-39.995). Predicted *Oryza sativa* (as the most similar species to wheat) transcription factor binding sites in the flanking region (37367 to 39369) were located using the PlantPAN 2.0 program (http://plantpan2.itps.ncku.edu.tw) (Fig. 5-4).

Figure 5-4. Nucleotide sequence of the downstream flanking region of the Ta*PR1-23* (HQ700377.1) gene. The one potential candidate W-box is the sequence 1484-1489, which is a predicted binding site for *Oryza sativa* WRKY proteins identified by using the PlantPAN (Plant Promoter Analysis Navigator) 2.0 program (http://plantpan2.itps.ncku.edu.tw). The W-box is highlighted.

c. DNA-binding protein reagents

Following the EMSA manufacturer's instructions (Thermo Fisher Scientific) the control Epstein Barr nuclear antigen (EBNA) system was generated using a binding reaction.

The WRKY3 and mutant test was optimised by altering other components and noting how they affected the shift assay (see Table 5-3).

Table 5-3. Three reactions for the TaWRKY3 and mTaWRKY3 were performed

Component	Reactions			
	1	2	3	4 & 5
Ultrapure Water	12 µl	8 µl	8 µl	10 µl
1X binding buffer	2 µl	2 µl	2 µl	2 µl
50% Glycerol	1 µl	1 µl	1 µl	1 µl
5mM $MgCl_2$	1 µl	1 µl	1 µl	1 µl
50 ng/µl poly (dI.dC)	1 µl	1 µl	1 µl	1 µl
0.05% ng/µl	1 µl	1 µl	1 µl	1 µl
0.5 nM Unlabeled Target DNA	--	--	2 µl	--
2 µg Protein Extract (Bradford assay was used)	--	4 µl	4 µl	4 µl
0.5 nM Biotin End- Labeled	2 µl	2 µl	2 µl	--
Total Volume	20 µl	20 µl	20 µl	20 µl

All the reactions were incubated for 20 min at room temperature. 5 µl of 5x Loading Buffer was added to each 20 µl binding reaction, and 20 µl of each sample was loaded on a 5% polyacrylamide gel in 0.5x Tris Borate EDTA buffer (TBE) cooled to 4 ºC. The gel was run for 1 hour at 100 V.

Nylon membrane was soaked in 0.5x TBE for 10 min with gentle shaking. Next the membrane was blocked after sandwiching the gel, using 0.5x TBE at 380 mA for 1 hour cooled at 6 ºC.

Membranes were cross-linked using a commercial UV light with 254 nm bulbs at 120 MJ cm^{-2} for 45 s.

d. Detection of biotin-labelled DNA by chemiluiminescence

The membrane was blocked with 20 ml blocking buffer and incubated for 20 min with shaking. The membrane was probed with 100 µl stabilised streptavidin-horseradish peroxidase conjugate in 30 ml blocking buffer at 1:300 dilution for 20 min with gentle shaking. Three washes were performed 5 times for 5 min each in 20 ml of 4X wash buffer with gentle shaking. 30 ml of substrate equilibration buffer was added to the membrane for 5 min with gentle shaking. The substrate solution was made using 6 ml Luminol to 6 ml stable peroxide; this was added to the membrane for 5 min on a flat surface. Membranes were placed in a film cassette and exposed to X-ray film for 15 s to 2 min. The film was developed and fixed using Kodak reagents.

5.4 Results

5.4.1 Amplification of WRKY Coding Sequences by PCR

To generate the material required to construct the recombinant expression vectors the coding sequences for both TaWRKY3 and mTaWRKY3 were amplified using PCR. Bands of the PCR product the expected size (695 bp) were found after agarose gel electrophoresis (Fig. 5-5).

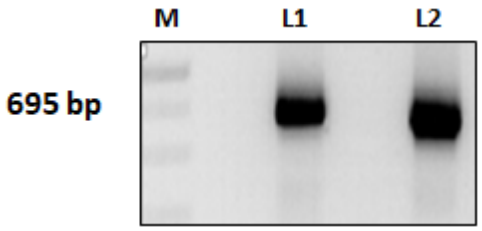


Figure 5-5. Agarose gel (0.8%) electrophoresis of amplified PCR products was performed with both WRKY3 and mWRKY3 with full-length sequences 695 bp. M, marker 1 kb DNA Ladder. L1 and L2 represent products from WRKY3 and mWRKY3.

5.4.2 Cloning of WRKY3 and mWRKY3 sequences

The fresh PCR (amplification) products were ligated with a 1:1 molar ratio of vector:gene to create the best ligation efficiency (5.3.2a). The genes were cloned into pET SUMO vector and transformed into Mash1-T1R chemically competent *E. coli* (5.3.2b).

Colony PCR was used to confirm that TaWRKY3 and mTaWRKY3 coding sequences were the expected size (Fig. 5-6).

Transformed clones were sequenced to verify the integrity and orientation of the WRKY3 coding sequence. Furthermore, the sequencing data confirmed correct orientation of the cloned coding sequence with the SUMO fusion and the N-terminal 6xHis tag (Fig. 5-7). Sequencing data showed the 695 bp fragment corresponding to both the wild type and mutant forms.

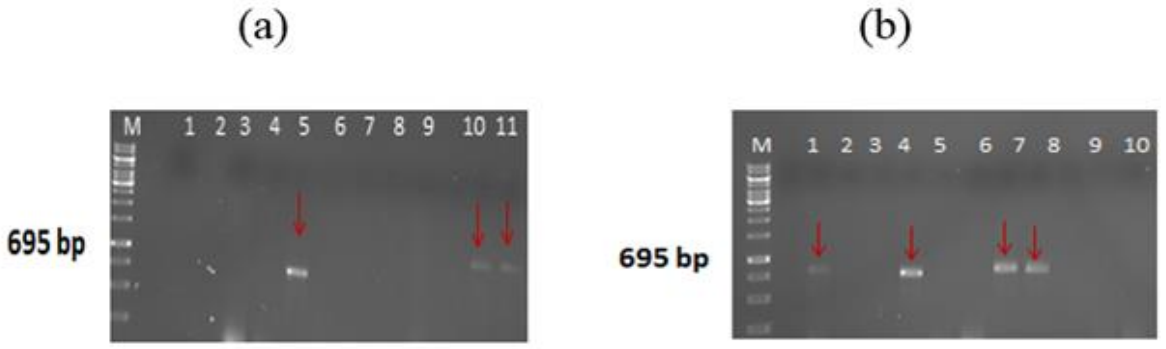


Figure 5-6. Colony PCR products of (a) WRKY3 and (b) mWRKY3 full coding sequences (expected size 695 bp) on 0.8% agarose gels.

Figure 5-7. (A) TaWRKY3 expression cassette cloned into pET SUMO, also showing the position of the mutation T/G (* = TILLING line 1996) and 6x His tag. (B) Sequence alignment showing 100% identify between cloned insert (Query) and WRKY3 (Sbjct), in reverse orientation; (C) sequence alignment showing 99% identity between cloned insert (Query) and mWRKY3 (Sbjct), in forward orientation. Black rectangle shows position of the mutation in the cloned sequence. (D and E) Sequences coding for the fusion proteins in the pET SUMO vector. *TaWRKY3* and *mTaWRKY* sequences up to 695 bp are highlighted in grey with the start codon in red. The His tag and SUMO forward priming sites are shown in boxes. Sequences coding for the SUMO fusion protein are in yellow. The T/G mutation is shown in red.

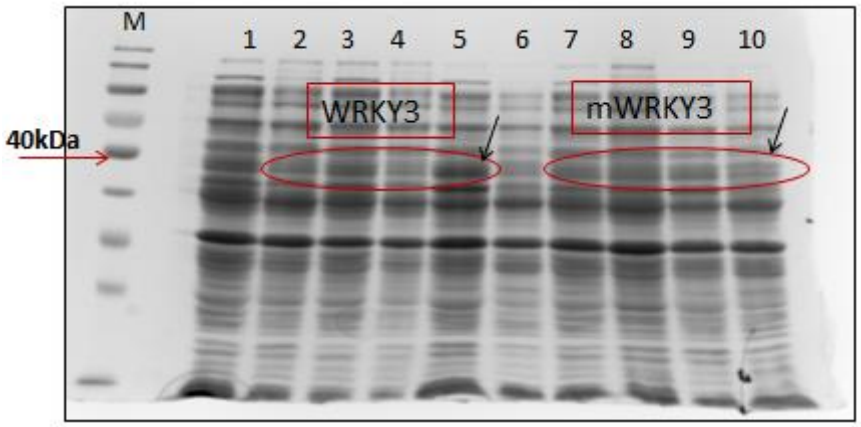
5.4.3 Heterologous Expression of WRKY3 and mWRKY3 in BL21 (DE3*) *E. coli*

The expression of WRKY3 and mWRKY3 in transformed *E. coli* lines containing pETSUMO vector was initiated by the addition of 1 mM IPTG to bacterial cultures. The resulting proteins were analysed by SDS-PAGE. Native WRKY3 (and hence mWRKY3) is a 25 kDa protein. Due to the fusion with the SUMO region and the addition of a 6xHis-tag the recombinant proteins had an increased molecular mass of 38 kDa (Fig. 8A).

Anti-His Tag antibodies were used to confirm the expression of proteins by western blot analysis (5.3.4). Figure 5-8.B shows that when the control BSA fusion protein (internal control for antibody binding) and TaWRKY3 and mTaWRKY3 lanes were probed with an anti-His Tag antibody, a band of the correct size was present for the control (70 kDa),

TaWRKY3 (38 kDa) and mutant (38 kDa), suggesting that the correct fusions have been made (Fig. 5-8B).

(A)



(B)

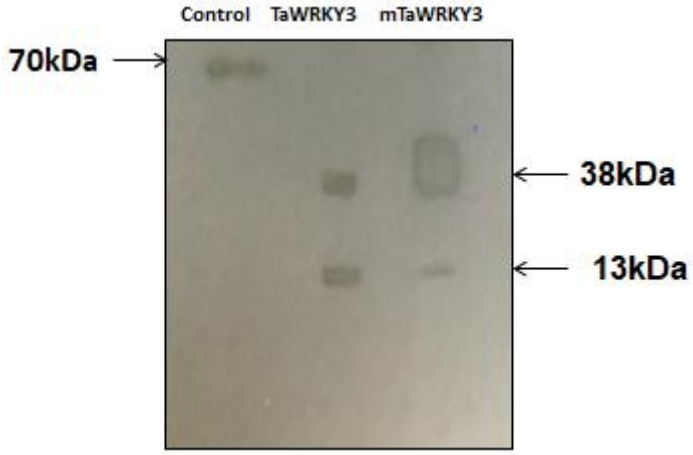


Figure 5-8. (A) SDS PAGE gel of protein in eight cultures of *E. coli* strain lane BL21 (DE3*) containing pET SUMO with WRKY3 and mWRKY3. Total extract (lane1), uninduced cultures (lanes 2, 4, 7, 10) and induced cultures (lanes 3, 5, 8, 9). (B) Western blotting of TaWRKY. Western blotting with anti-His antibody shows the control BSA fusion protein with the molecular mass of 70 kDa, and TaWRKY3 and mTaWRKY3 recombinant proteins at approximately 38 kDa with the N-terminal peptide containing the 6x His-tag and SUMO fusion protein at approximately 13 kDa.

5.4.4 Purification of *WRKY3* and *mWRKY3*

An assay was designed to test *in vitro* whether TaWRKY3 and mTaWRKY3 specifically bound to DNA. The His-tag WRKY3 and mWRKY3 proteins were purified using NI-NTA under both native and denaturing conditions (data only shown for native conditions). The presence of the recombinant protein in the elution fractions demonstrates the correct translation of the His-tag and binding to the nickel affinity column. The purified protein should be in native condition for use in electrophoresis mobility shift assays (Fig. 9A). The eluted proteins can be directly analysed by western blotting (Fig. 9B). Protein yield was measured via a Bradford assay (Bio-Rad) and the amount of protein was adjusted to 2 to 4 µg.

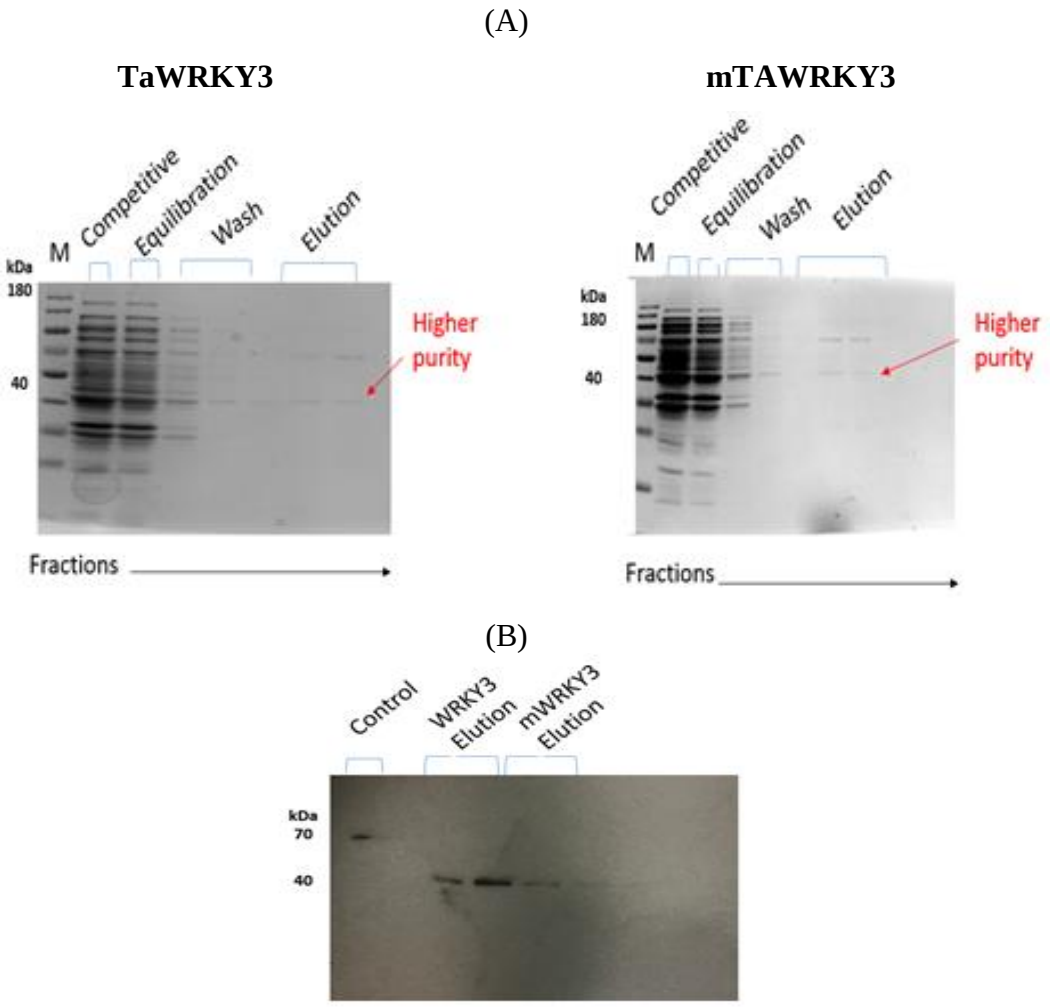


Figure 5-9. (A) Purification of WRKY3 and mWRKY3 using NIAC under native conditions. His-tagged protein was present in Competitive-total cell lysate and was detected in elution. Three stages of the purification. (1) Equilibration - run off after incubation with His-tag, (2) wash - flow through after washing (3) elution - His tag elution. (B) The eluted WRKY3 and mWRKY3 are detected with anti-His antibody by western blotting.

5.4.5 DNA binding specificity of *TaWRKY* protein

To test whether WRKY3 could directly bind to specific DNA sequences and to determine how the mutation in TILLING line 1996 affects this binding the recombinant proteins were used in an electrophoretic mobility shift assay (EMSA). Recombinant proteins were incubated with DNA sequences representing repeating W-box elements or 30 bp regions of promoters from parsley *PR1-1* and wheat *PR1-23* genes containing one W-box.

a. Control Epstein Barr nuclear antigen (EBNA) system

The light shift chemiluminesecent EMSA kit optimised the control protocol for shift assay; three reactions were subjected to electrophoresis, transferred and detected. The results of the control EBNA system are shown in Fig. 5-10. Each line was generated through binding reactions prepared with biotin or unlabelled EBNA DNA.

Figure 5-10. Electrophoretic mobility shift assays (EMSAs) were performed as described in the manufacturer's instructions for the control system. (1) Biotin labeled band was detected by chemiluminescent nucleic acid detection module, no protein extract for DNA to bind; an unshifted probe band. (2) The biotin labeled DNA probe was incubated with EBNA Extract containing target protein to effect binding and shift of the labeled DNA. (3) Biotin labeled DNA + EBNA Extract + unlabeled EBNA DNA, giving an unshifted probe band.

b. DNA Binding of Wheat WRKY Factors

DNA fragments containing the W-box (TTGAC) element in the region of the promoter sequences from parsley (Pc*PR1-1*), wheat (Ta*PR1-23*) and synthetic oligonucleotides containing three tandem TTGAC repeats or mutants with TTGAA were used as probes for mobility gel shift assays (Table 5-2).
In the first set of experiments, an oligonucleotide was synthesised with end labelled biotin and an unlabelled probes annealed to generate double-stranded oligonucleotide (5.3.6 a. Anneal complementary). WRKY3 and mWRKY3 proteins purified by HisPur Ni-NTA spin columns (5.4.4 protein purification) are shown in Fig. 5-9A, and the quantity of these proteins was measured using a Bradford assay with 2 to 4 µg purified protein.

The protein-DNA interactions were separated by electrophoresis and exposed to X-ray film. In the case of WRKY3, binding of the recombinant protein to the oligonucleotides resulted in a retardation of the migration of the labelled probe through the polyacrylamide gel. This was observed by a shift in the detected band from the bottom of the resulting image to a position higher up the gel. Furthermore, the binding activity increased the intensity of the related band in all cases by competition with high amounts of protein and probe. Figure 5-11 A and B show that a recombinant wild type TaWRKY3 protein was able to bind cis-DNA elements from wheat Ta*PR1-23* (A) and parsley *PR1-1* promoter (B), whereas the single amino acid mutation in mTaWRKY3 resulted in complete loss of binding to both promoter regions.

In Figure 5-11 C and D, in order to analyse TaWRKY3 binding to the W-box, synthetic single stranded oligonucleotides with three TTGAC repeats or TTGAA repeats for the mutant W-box within 22 bp were annealed to generate double strand oligonucleotides and end labelled by biotin. The WRKY3 protein bound to this element. The mutant W-box, however, also bound to WRKY3, so that WRKY proteins may be able to bind to more than one sequence. The mWRKY3 line 1996 did not bind to either oligonucleotide region (Wb and mWb).

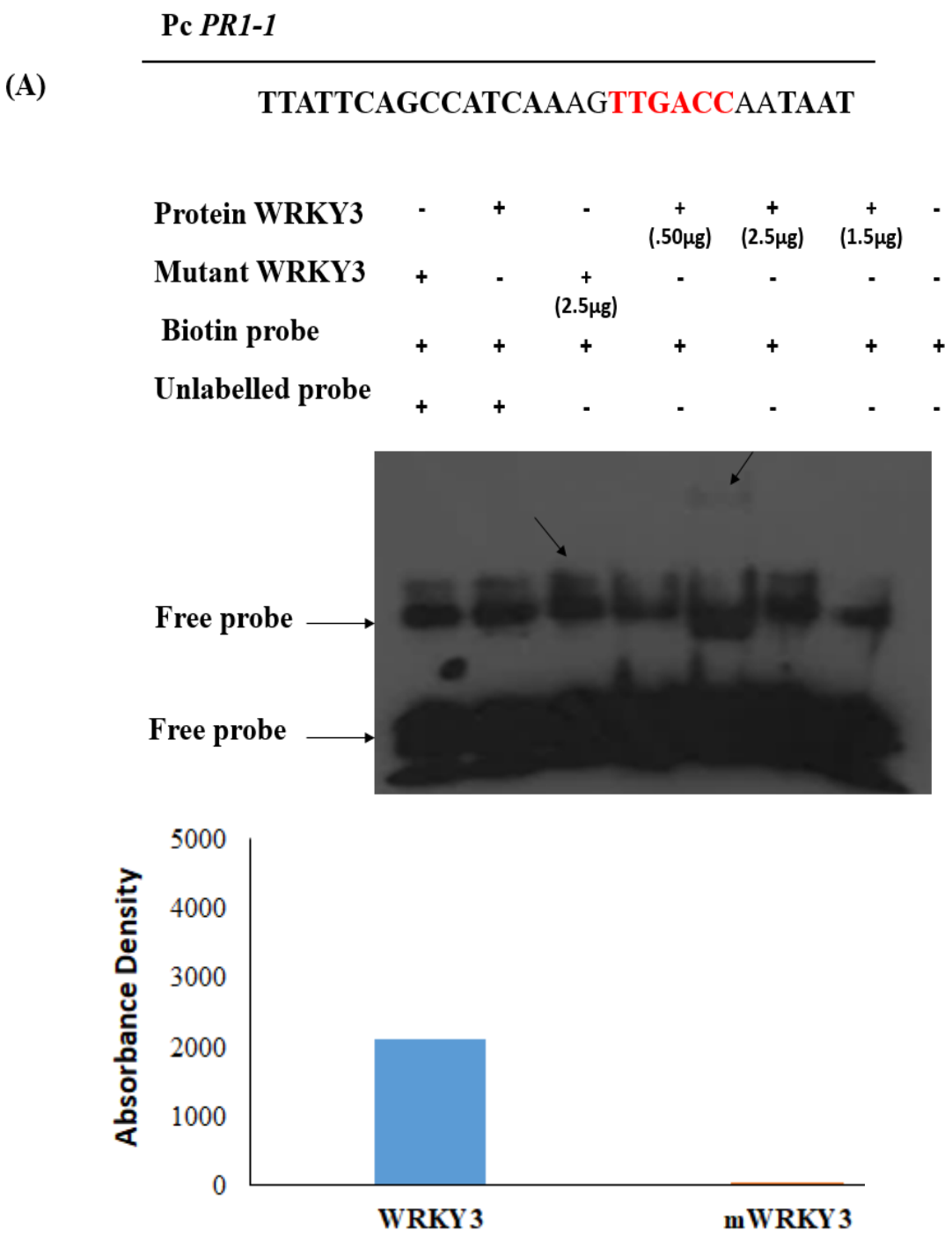


Figure 5-11. Binding site preference of TaWRKY3 to W-box**.**
(A) Sequence of the parsley Pc*PR1-1* promoter regions containing the W-box (in red letters). Biotinylated DNA fragment, expressed (TaWRKY3 or mTaWRKY3) protein, unlabelled competitor DNA. Absorbance densities were measured by densitometry using Image Lab software (Bio-Rad).

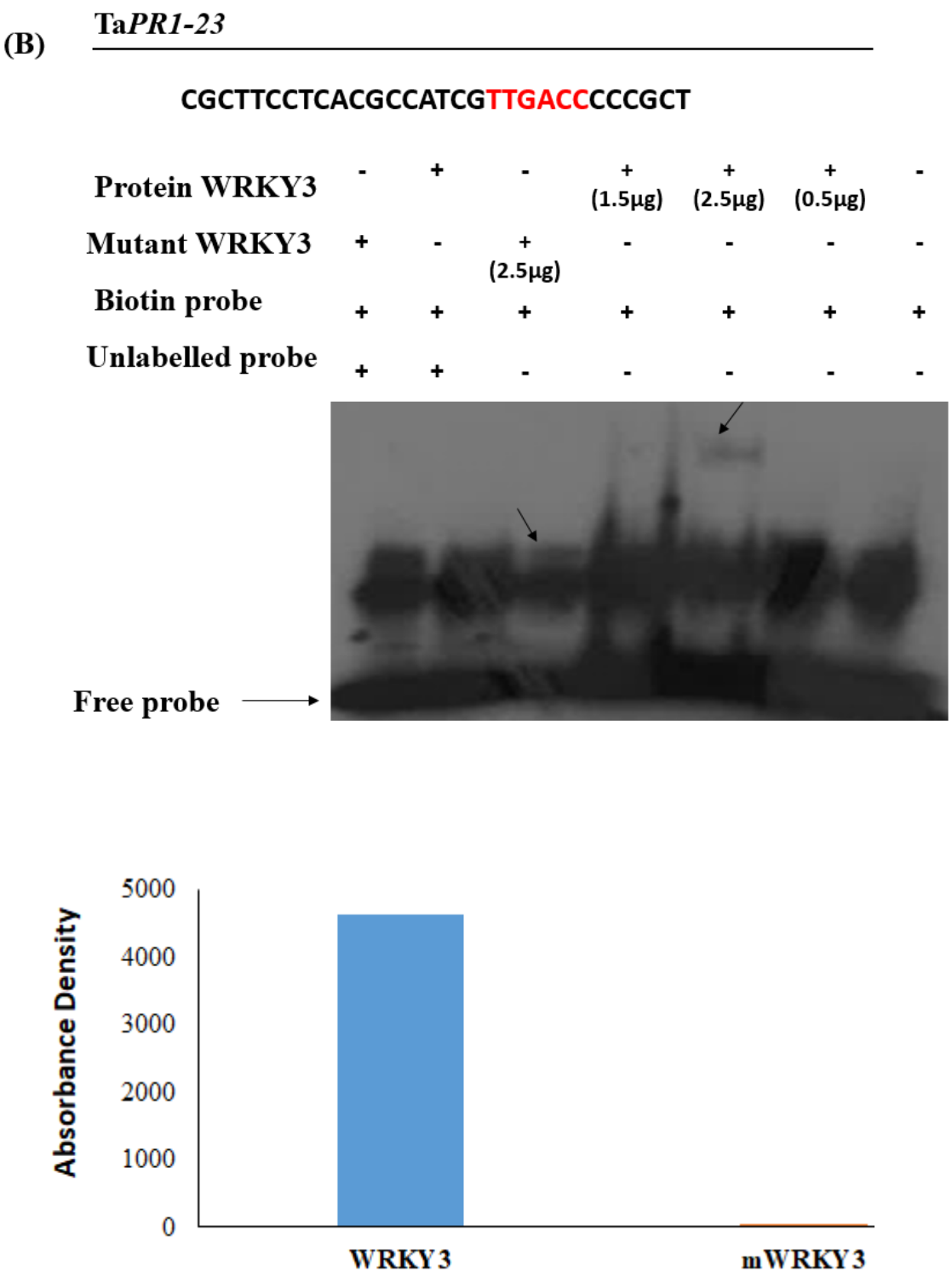


Figure 5-12. Binding site preference of TaWRKY3 to W-box**.**
(B) Sequence of the wheat Ta*PR1-23* containing the W-box (in red letters). Biotinylated DNA fragment, expressed (TaWRKY3 or mTaWRKY3) protein, unlabelled competitor DNA. Absorbance densities were measured by densitometry using Image Lab software (Bio-Rad).

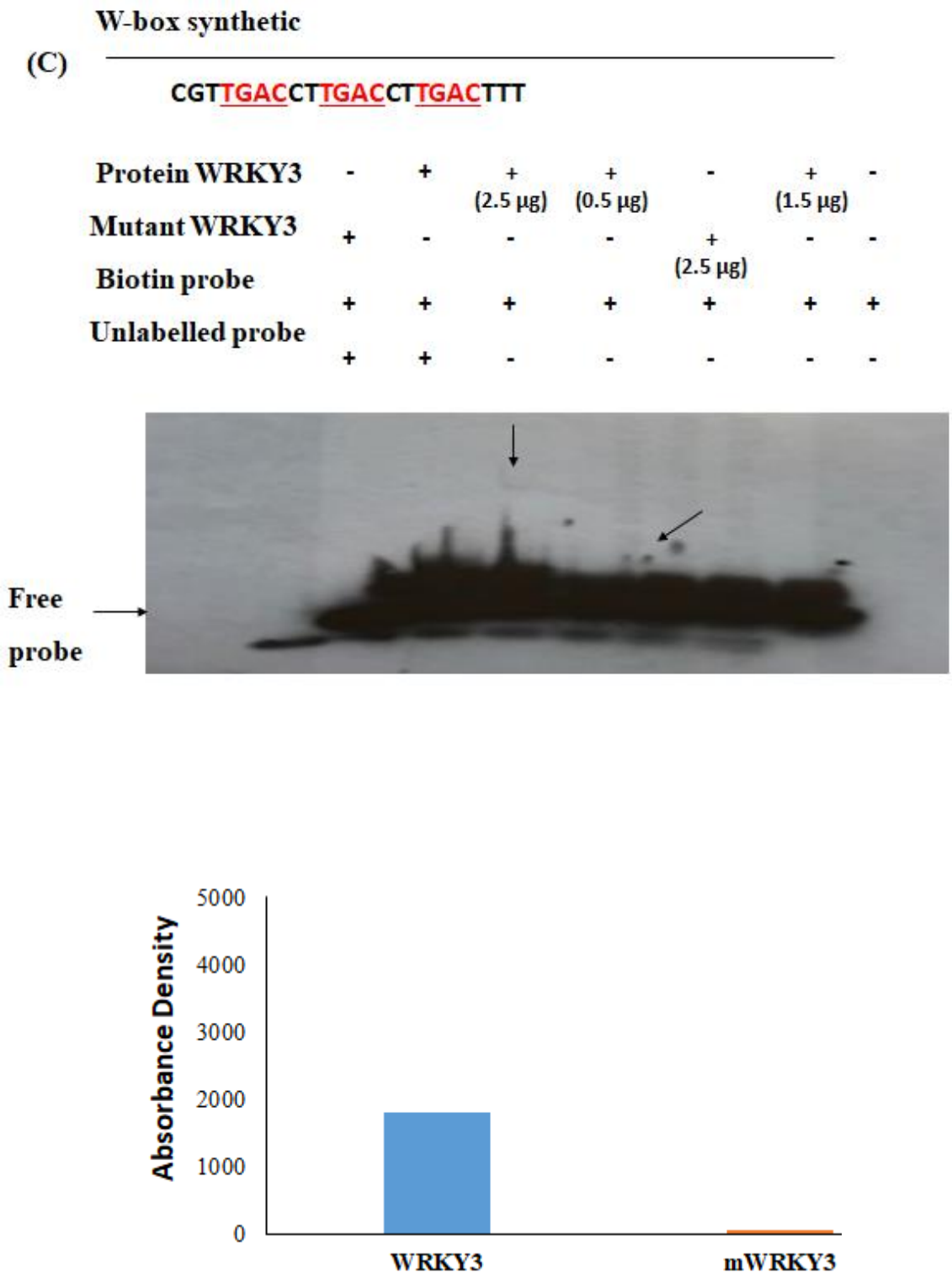


Figure 5-13. Binding site preference of TaWRKY3 to W-box**.**
(C) Sequence of the W-box oligonucleotide containing three tandem W-box repeats (in red letters). Biotinylated DNA fragment, expressed (TaWRKY3 or mTaWRKY3) protein, unlabelled competitor DNA. Absorbance densities were measured by densitometry using Image Lab software (Bio-Rad).

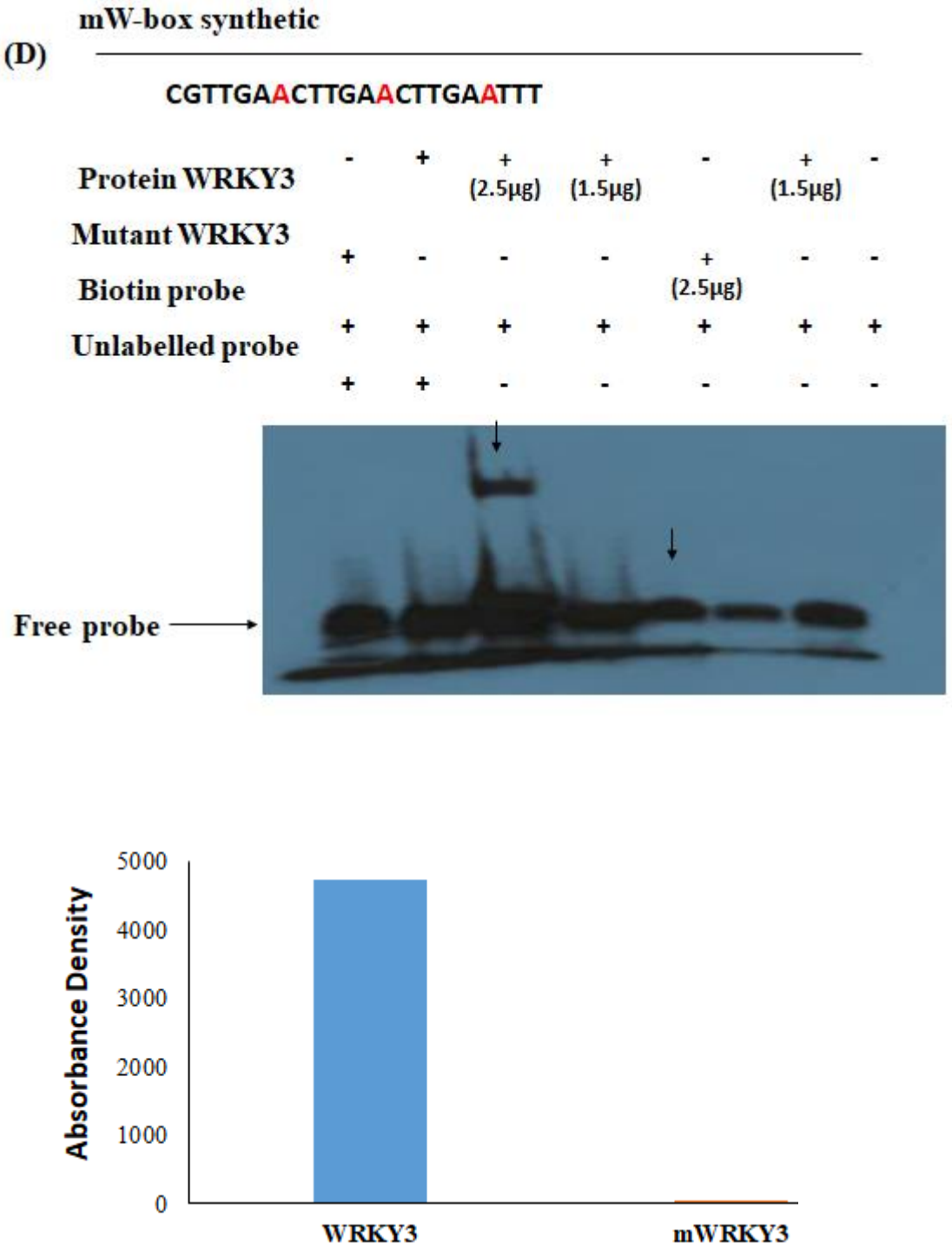


Figure 5-14. Binding site preference of TaWRKY3 to W-box**.**
(D) Sequence of the mW-box synthetic sequence containing three tandem W-box repeats with a C to A substitution (in red letters). Biotinylated DNA fragment, expressed (TaWRKY3 or mTaWRKY3) protein, unlabelled competitor DNA. Absorbance densities were measured by densitometry using Image Lab software (Bio-Rad).

5.5 Discussion

Several species of plant have been shown to contain DNA-binding proteins containing the WRKY domain (Dong *et al.*, 2003). The interactions between DNA and proteins are important for the regulation of gene expression and key biological processes. For transcriptional regulation, TFs bind to specific DNA sequences. This results in genes being either expressed or repressed (Satapathy *et al.*, 2018). However, further research is required on DNA-protein docking as this interaction is still relatively unexplored and it therefore remains a challenge in the field of structural bioinformatics.

5.5.1 A modified method

The pET SUMO cloning vector was selected to implement the expression system with a 6xHis tag at the N-terminus. This was intended to help purify the proteins, which was believed to be essential for EMSA. The vector was successfully transformed into BL21 (DE3*) cells to detect the expression of the WRKY3 protein after 1 mM IPTG and 1% glucose induction, which was performed with the soluble *E. coli* proteins. It is important to obtain WRKY3 as a soluble protein, because attempts to refold WRKY proteins from inclusion bodies have been unsuccessful (Ciolkowski *et al.*, 2008). In order to use the recombinant WRKY3 and mWRKY3, after native protein extraction and purification (via Ni-NTA column), binding in EMSA was used to detect the complex of protein with its promoter cis-element, namely the W-box. A labelled DNA probe (biotinylated) from Lightshift EMSA Optimisation module was used – the system can detect 0.5 nmol of the probe to assess the binding specificity of the transcription factors (Table 5-3).

5.5.2 Function of TaWRKY3 in DNA-binding

The results of Chapter 4 show that TaWRKY3 plays an important role in aphid resistance; the effects of mutations within the WRKY binding domain on aphid performance were evaluated. It can be concluded that the mutation in line 1996 reduces aphid populations and negates the differentiation between population increases relative to nitrogen availability. From this it can be predicted that the mutation in the TILLING line 1996 affects the binding of TaWRKY3 to W-box elements.

In this study, recombinant wild-type and mutant *TaWRKY3* protein were examined in an *E. coli* assay system in order to determine whether they were able to bind to promoter regions.

WRKY proteins have been found to bind to the W-box and the WRKYGQK sequence is very important for DNA-binding activity.

The gel shift assay showed that TaWRKY3 can bind to the conserved W-box element, TTGAC (Fig. 5-11). In contrast, the mutation found in the TILLING line disrupted binding to the W-box, showing that the replacement of Tyr 160 with Asp decreases the DNA-binding activity of TaWRKY3 (Fig. 5-11). The mutation changes residue tyrosine (Tyr – Y), which is polar with ability to form hydrogen bonds, to aspartic acid (Asp – D), which is a negatively charged amino acid that makes salt bridges (Karadaghi, 2019). Amino acid residues were found within WRKY proteins that may be important for formation of the correct zinc-finger structure and DNA binding was reduced significantly if each of the amino acid residues in the WRKYGQK sequence was replaced with alanine (Hayashi *et al.*, 2001).

This is the first study of its kind in which the involvement of TaWRKY3 binding to W-boxes has been observed. The mutant WRKY3 line 1996 reduces aphid populations and negates the differentiation between population increases relative to nitrogen availability (Fig. 4-5g in Chapter 4), and the mutation in its WRKY sequence results in a dramatic loss of binding to W-box elements. Thus, the binding change implies that TaWRKY3 is a negative regulator of tolerance, and it may act to initiate the response to aphid infestation (Fig. 2-6a in Chapter 2), most likely by binding to the W-boxes in target genes (Fig. 5-12). Evidence for such a function includes the reduced level of basal resistance to insects under high nitrogen levels and the increased expression of TaWRKY3 in the short term. When the mutant 1996 line was infested with aphids, aphid fecundity was similar with low and high nitrogen levels. A number of previous studies have reported genes taking on the role of negative regulators as part of the defence against pathogens (Zeng *et al.*, 2004; Wang *et al.*, 2006). For example, the rice gene *spotted leaf11*, which encodes a U-box/armadillo repeat protein, is a negative regulator of plant cell death and defence (Zeng *et al.*, 2004). Similarly, in Arabidopsis, Wang *et al.* (2006) reported that WRKY58 is a negative regulator of defence. When plants were treated with suboptimal concentrations of benzothiadizole (BTH), it was observed that a mutation in *WRKY58* increased resistance to a pathogen (Wang *et al.*, 2006). It is thought that there are two possible functions of these negative regulators: preventing unnecessary activation of defence responses when suboptimal concentrations of signal molecules are present, or turning off SAR when a pathogen attack has been successfully overcome (Wang *et al.*, 2006).

Niu *et al.* (2012) showed that TaWRKY2 and TaWRKY19 proteins bind to the W-box sequence TTGACC. They identified W-box sequences from the promoter regions of *STZ*, *DREB2A*, *RD29B*, *Cor6*.6 and *RD29A* and found that TaWRKY2 or TaWRKY19 could bind specifically to some of these sequences by gel shift assay (Niu *et al.*, 2012). Moreover, it has been reported that in *Arabidopsis* WRKY33 is a pathogen-inducible transcription factor and is phosphorylated by MAPKs MPK3 and MPK6. Phosphorylation of AtWRKY33 by MPK3 or MPK6 was necessary for its activity, but performing an electrophoresis mobility shift assay with AtWRKY33 showed that phosphorylation did not affect its binding to the W-box (Mao *et al.*, 2011).

5.5.3 Significance of TaWRKY3 binding to *PR1* genes

There have been a number of cases where W-box elements adjacent to each other have been noted in gene promoters (Eulgem, 1999; Yang *et al.*, 1999; Yu *et al.*, 2001; Chen and Chen, 2002; Zhang *et al.*, 2004). For example, in *PcWRKY1*, the effect on transcription of the presence of these multiple sites seems to be a synergistic one (Eulgem, 1999), while Hv-*WRKY38*, found in barley, requires two W-boxes that are closely adjacent for DNA binding to be efficient (Mare *et al.*, 2004).

This study used a DNA regions derived from the Pc*Pr1-1* and Ta*PR1-23* genes and synthetic tandem W-box repeats; this made it possible to demonstrate that the sequence environment that the W-box elements are embedded into is able to affect protein binding. Previous researchers have found closely adjacent W-box elements in several gene promoters (Ciolkowski *et al.*, 2008). Based on the results, the recombinant wild type TaWRKY3 was able to bind to single W-box elements in parsley and wheat genes.

WT and *WRKY3* mutant plants exhibit different levels of tolerance to different stresses; this could be a reflection of the specificity of the WRKY protein with regard to DNA binding and the regulation of downstream genes. *PR* genes, which are defence marker genes, are known to enhance tolerance to stress (Zuo *et al.*, 2007). The wild type WRKY3 protein binds to the W-box in the *PR1-23* gene and DNA binding is disrupted by the mutation found in the TILLING line. From the effect of the mutation on aphid fecundity, it is hypothesised, therefore, that TaWRKY3 is a negative regulator of resistance. Maybe WRKY3 represses transcription of *PR1-23*. Negative regulation by WRKY proteins has been reported previously: pathogen-induced Arabidopsis WRKY7 is a transcriptional repressor and

enhances plant susceptibility to *Pseudomonas syringae* (Kim *et al.*, 2006). This results suggest that WRKY7 is a negative regulator of plant defence.

Studied defence-related genes such as *PR* genes and most of the defence-regulated *WRKY* genes from *Arabidopsis* are rapidly induced in pathogen-infected plants (Dong *et al.*, 2003). Regulation of *PR1* gene expression is an important part of the activation of plant defence responses (de Pater *et al.*, 1996; Rushton *et al.*, 1996); the present work suggests that TaWRKY3 may regulate this important response by binding to the W-box element in the promoter.

Within the 2100 bp sequence of the Ta*PR1-23* (HQ700377.1) gene downstream flanking region there are distinct regions containing relevant cis-acting elements. The one potential candidate W-box among the relevant elements is the sequence TCGTTGACCC (1484-1489) (Fig. 5-4), which has a binding site for *Oryza sativa* WRKY proteins identified by using the PlantPAN 2 program. Figure 5-11B shows that this W-box element is able to bind to WRKY3, whereas the single amino acid mutation in mTaWRKY3 results in a complete loss of binding. Moreover, WRKY binding is not caused by just the presence of the W-box element in the DNA region. The results clearly show that TaWRKY3 can bind to W-box element in both promoters and to a sequence that differs by single base from the consensus W-box sequence. However, this research has also demonstrated that we need better knowledge of DNA elements and the cognate DNA-binding factors required for transcription regulation in wheat.

5.6 Conclusions

In summary, the EMSA assays indicate that WRKY3 can effectively bind to several sequences containing a W-box, including sequences in *PR1* genes. Studies on a mutant of WRKY3 show that it has lost the ability to bind to these sequences. These results combined suggest that the WRKY3 transcription factor is important for a signal transduction pathway that leads from elicitor perception to *PR1* gene activation. Carrying out further work to understand which genes are under the control of which WRKY protein will allow a model of wheat defence response to be built, elucidating any molecular link between nitrogen input and aphid tolerance.

6 Chapter 6. General discussion

This study examined the effects of reduced nitrogen input and grain aphid (*Sitobion avenae*) infestation to determine how commercial winter wheat cultivars Cordiale and Grafton responded to a combination of stresses. WRKY-type transcription factors (TFs) regulate gene expression and are involved in stress responses across the plant kingdom; this study provides the first evidence to link WRKY TFs with the stress responses seen under conditions of reduced nitrogen and aphid infestation in commercial winter wheat. Furthermore, this study investigated the potential cross-talk interactions via a regulatory network involving WRKY TFs in response to stress. Further research needs to be done to clarify WRKY TF targets in wheat, but it has been shown that in Arabidopsis WRKY proteins regulate the expression of many genes associated with defence. Moreover, there is evidence that AtWRKY70 behaves as a molecular switch that influences the balance between JA- and SA-mediated signalling (Eulgem and Somssich, 2007). These phytohormones are known to regulate gene expression in response to various pathogens in dicotyledonous plants, for example Arabidopsis. Attack by biotrophic pathogens generally induces SA-mediated signalling, whereas there is a close association between JA-mediated signalling and attack by necrotrophic pathogens; both responses have been found to act antagonistically against each another in dicotyledonous plants. In contrast, it has been proposed that in rice, a model monocotyledonous plant, JA and SA activate a common defence system (Tamaoki *et al.*, 2013). WRKY proteins could potentially serve as signalling molecules that are an essential part of a plant's defence against pathogens via these pathways. Pandey and Somssich (2009) have reported that the differential expression of WRKY genes can have a direct influence on the resistance of Arabidopsis to disease. A line where the expression of *AtWRKY3* and *AtWRKY4* had been knocked out was shown to be much more susceptible to *Botrytis cinerea*, whereas over-expressing *AtWRKY4* resulted in higher levels of susceptibility to *Pseudomonas syringae*. This illustrates how one WRKY protein can regulate a plant's defence against a number of pathogens, in both a negative and positive way.

The grain aphid is one of the major wheat pests in many regions, causing direct damage by feeding and indirect damage by the transmission of plant viruses (Zhang *et al.*, 2013; Scorsetti *et al.*, 2017). Plant responses to aphid feeding are rapid, and these feeding-induced resistance

responses are seen in wheat. During this response *WRKY* genes are regulated. To establish a role for WRKY TFs in the defence response of wheat, TILLING lines with mutations in the *TaWRKY3* gene were selected. During bioassays these lines supported different populations of aphids, demonstrating the effect that these mutations have on the *in vivo* function of the WRKY3 transcription factor.

6.1 Effects of reduced nitrogen on aphid performance

Chapter 2 assessed how varying the availability of nitrogen to the commercial winter wheat varieties Cordiale and Grafton affected the performance of *S. avenae*. The results showed that increased nitrogen input had a positive effect on the growth and fecundity of *S. avenae*. Under control conditions (7.5 mM) nitrogen increased herbivore performance, and at the lowest nitrogen levels (2.25 mM) insect fecundity was significantly reduced. Similar findings were suggested by Gash (2012), who demonstrated that aphid performance was influenced by differences in nitrate accumulation. This finding is also consistent with other studies which reported that the protein-nitrogen contents increased with increased nitrogen levels (Aqueel and Leather, 2011).

The relationship between aphid performance and host plant nitrogen input is complex, as the responses to these factors are largely controlled by different hormone signalling pathways. However, the low nitrogen conditions may prime the defence of wheat against insect attack as a result of cross-talk via a regulatory network involving WRKY transcription factors. In addition, Comadira *et al.* (2015) showed that leaf transcriptome profiles are affected by nitrogen deficiency, with transcripts associated with sugar and nutrient signalling, protein degradation and secondary metabolism being over-represented in leaves. Relatively little is known about how the response to nitrogen stress and interactions with phloem feeding insects (PFIs) affect the plant. As a result, further investigations using RT-qPCR analysis were carried out on the variety Cordiale, which was subjected to a combination of reduced nitrogen availability and aphid infestation.

6.2 TaWRKYs are involved in the interaction between nitrogen and aphids in wheat

In Chapter 3, RT-qPCR showed a correlation between low nitrogen availability and reduced *WRKY* gene expression in Cordiale, with 6 *WRKY* genes responding in a dose-dependent manner. These experiments showed that reduced levels of nitrogen result in the down-regulation of all *WRKY* genes investigated (Fig. 3-5). In contrast, analysis of the expression of *WRKY* genes in response to aphid infestation over time showed an increased expression of most *WRKY* genes tested (Fig. 3-6). *WRKY3*, for example, showed down-regulation under low nitrogen (2.25 mM) but up-regulation during aphid infestation at all nitrogen availabilities. Compared to the other two levels of nitrogen, the lowest level of nitrogen resulted in the same rapid increase of gene expression but the magnitude of the response was higher for all time points during the infestation. In addition, the response was maintained for a longer period, staying above the basal level throughout the assay period.

Plant have developed a wide range of defence responses to pathogens, pests and sub-optimal growing conditions (Deslandes *et al.*, 2002). The present results suggest that the down-regulation of *WRKY* genes during low nitrogen treatment may lead to an up-regulation of defence response genes, whilst aphid infestation also results in an increase in expression of defence genes. In previous studies using microarray-based analysis, Foyer *et al.* (2014) showed that N-deficient barley plants were resistant to aphids and that the resistance may be the result of the activation of similar signalling cascades. The data identified high levels of WAK (wall-associated kinase) and WRKY transcription factor transcripts in the low N transcriptome and the transcriptome from plants exposed to aphids. Another study comparing the aphid induced transcriptome and that induced by salinity and *Sitobion avenae* infestation showed that among 39 transcripts of WRKY genes identified, transcripts representing 2 different WRKY genes (TaWRKY18 and TaWRKY41) were strongly up-regulated by salt stress at 24 h but down-regulated by aphid stress at 24 h and by the combined stresses at 6 h (Khan, 2014)

In the present study, the regulation of plant WRKY gene expression was different between the two stresses, but the results did suggest that WRKY3 was involved in the wheat defence against both biotic and abiotic stress (Chapter 4 and 5). Further investigation of this dual

response would be a priority in the continuing efforts to improve understanding of aphid tolerance in wheat.

6.3 Investigation of the Role of TaWRKY3 using TILLING lines (TILLING – EMSA)

Currently, mutagenesis is being used to target wheat transcription factor (TF) genes of interest to improve crop adaptation to changing environments. Most mutant lines resulting from insertional methods are similar to knockout mutants, and as such lose gene function, and thus will not produce the range of mutations needed for crop improvement (Chen *et al.*, 2012). In Chapter 4, when plants were grown under dual stresses, aphid populations were greater on most wheat lines with mutations to the *WRKY3* gene than on WT plants, showing that most of the TILLING lines exhibited increased susceptibility to *S. avenae* at reduced levels of nitrogen. Uniquely, the TILLING mutant line 1996 was also more resistant than WT at high nitrogen plants and showed no difference in aphid populations at different nitrogen levels, suggesting that WRKY3 may play a role in the cross-talk between tolerance to different stresses in WT plants. The mutation in TILLING line 1996 is the only one in the highly conserved and essential WRKY gene sequence and so is the most likely to eliminate the WRKY3 function. The effect of the mutation was confirmed when wild-type and mutant recombinant WRKY proteins were used in protein-DNA binding assays with known W-box elements, as it eliminated binding to the W-box.

In the present study, three compounds involved in plant signalling pathways (jasmonic acid, salicylic acid and abscisic acid) were extracted from wheat leaves (WT and TILLING mutant lines 1171 and 1996) after they were infested with aphids; these compounds were investigated via LC/MS-MS-based analysis. The role of JA in the defence response of these plants was examined the concentration of JA isomer 1 increased in WT plants and mutant line 1996 at 9 h of aphid infestation. However, the concentration in the line 1171 mutant was initially high and decreased at 3 h aphid infestation. The comparatively high concentrations of jasmonic acid isomers 2 and 4 suggest that these are the main naturally occurring isomers, (-)-jasmonic acid and (+)-7-iso-jasmonic acid and indicate a significantly up-regulated defence response at 0, 3 h and 9 h of aphid infestation. Differences in the patterns in the mutant lines suggest an association between mutations in the WRKY domain of WRKY3 and the production of these two isomers of JA. On the other hand, levels of SA increased in TILLING line 1996 after

aphid infestation (see Table 4-5). WT plants had decreased concentrations of SA at 6 h and 9 h of aphid infestation. The results reported in this study with regard to JA and SA are broadly in line with previous research; for example, Huang *et al.* (2015) reported that in *Ulmus pumila* leaves the levels of SA and JA increased after the plant had been infested by the gall-aphid *Tetraneura akinire*. The study found that the maximum values for SA and JA were in the early stage and middle stage of gall development, respectively. Dubey *et al.* (2018) studied promoters of Arabidopsis genes whose expression had been found to respond to aphids and whiteflies. They found that promoters of two genes that responded to aphids also responded to JA but not to SA and that there was a positive correlation between the presence of conserved cis-regulatory elements and the activation of promoters by phytohormones (Dubey *et al.*, 2018). JA-dependent plant defences are generally thought to be activated by necrotrophic pathogens and chewing insects, whereas SA-dependent defences are often triggered by biotrophic pathogens. JA and SA signalling usually act antagonistically, but synergism between these two phytohormones has also been observed. These responses to pathogen attack require large-scale transcriptional reprogramming, including those of TF families such as *WRKY* genes (Pandey and Somssich, 2009).

Chapter 5 investigated the binding of TaWRKY3 specifically to the W-box (TTGACC) of *TaPR1-1* and *PcPR1-1* promoters and to synthetic sequences. The interesting aspects of this study include investigating the binding of TaWRKY3 to the W-box in the downstream flanking region of the wheat *PR1-23* (HQ700377.1) gene with recombinant WRKY TFs. The target promoters of WRKY3 and consequently the pathways it regulates can be used to identify genes under the transcriptional regulation of this important TF. However, the mutation of the WRKY3 line 1996 in the invariable WRKYGQK sequence reduced DNA binding to W-box elements. A number of previous studies have demonstrated that DNA-binding proteins containing a WRKY domain are involved in the transcription regulation of different sets of genes (Ciolkowski *et al.*, 2008; Niu *et al.*, 2012), giving a regulatory network that is activated in response to external stress factors.

With past developments in wheat breeding, yields have risen, but they have now started to level off; with the increasing demand due to rises in the global population, it is therefore essential to find new ways to develop varieties with higher yields. One of the ways to do this is through the genetic manipulation of elite wheat varieties (Alotaibi *et al.*, 2018). The use of

dicot promoters in wheat and rice has been investigated, but they have been found to be ineffectual (Alotaibi *et al.*, 2018). Recent findings by Alotaibi *et al.* (2018) identified two promoters that can be used to drive transgene expression in elite wheat cultivars. Such promoters offer new possibilities for expressing genes in transgenic wheat. It is common to employ constitutive promoter studies on plants when the genes of interest need to be over-expressed.

6.4 Research limitations

Transformation

To analyse the role of the *TaWRKY3* gene in plants, an attempt was made to express wild-type and mutant forms of this gene in Arabidopsis. Unfortunately, expression was not seen in the transformation experiments, so that it was not possible to test whether they affect the ability of Arabidopsis to resist nitrogen and aphid stresses. In any case, however, it is difficult to predict what will occur in plants through transgenic experiments, as the TaWRKY may not function correctly in *Arabidopsis* and the orthologous WRKY protein in the *Arabidopsis* (AtWRKY13) may mask the action of the recombinant molecule.

As no *Arabidopsis* plants were obtained from transgenic experiments to investigate the protective role of expressing a WRKY gene with known involvement in stress responses, no further analysis could be undertaken to examine either nitrogen or *S. avenae* infestation stress tolerance in transgenic plants. There was insufficient time available to analyse the transformants generated in this experiment, and this could therefore form part of future work.

6.5 Conclusions

The studies carried out in this thesis have given insights into the roles played by WRKY proteins, in particular providing evidence that WRKY3 regulates the link between reduced nitrogen availability and aphid infestation tolerance. Most notably, the results indicate that loss of WKRY3 binding to W-box sequences causes a large decrease in fecundity of aphids at high nitrogen, implying that WRKY3 moderates wheat plants' response to aphids when they are well supplied with nitrogen. This knowledge has the potential to help improve global food production to meet the demands of the world's increasing population. This current study opens new avenues of investigation, particularly with WRKY3 and the possible interaction between stresses, to elucidate mechanisms that confer resistance to insects without reducing yields (see Fig. 6-1).

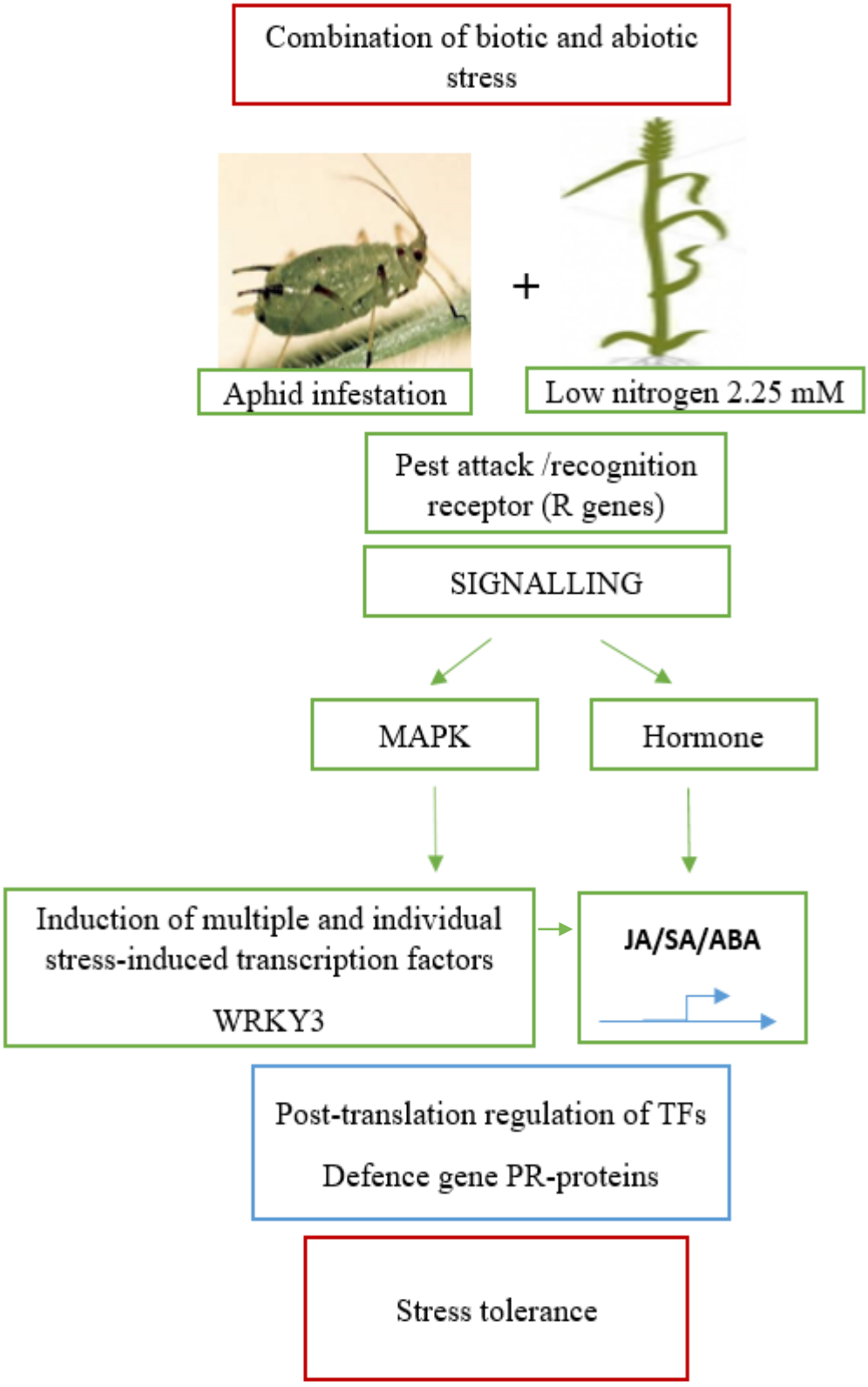


Figure 6-1. Outline model of the interaction network linking responses to reduced nitrogen and aphids to TaWRKY3 and other defence genes.

6.6 Future work: suggestions

The mechanisms by which reduced nitrogen availability influences aphid performance remain largely unknown, particularly the consequences of WRKY TF interactions for wheat and agricultural productivity more generally. Future studies that would extend the findings reported here by studying the link between nitrogen and aphids in wheat through the proposed interaction involving WRKY3 include:

- Investigating whether expression of selected WRKY genes in transgenic plants confers multiple stress tolerance, i.e. whether they enhance tolerance to nitrogen and aphid stress in transgenic Arabidopsis plants.
- Quantifying the activity of the plant's immune system by measuring callose deposition in TILLING mutant plant line 1996 under different nitrogen growth conditions and in response to aphids. If the mutation directly affects the resistance response, mutant plants can be expected to show different cellular responses relative to the wild-type with regard to callose deposition, which are likely to correlate with differences in aphid fecundity.
- Determining which genes are under the transcriptional control of WRKY3, and whether expression of these genes responds directly to aphids. This can be done by testing how TFs are involved in a plant's response to aphids, for example through GSL *glucan synthase-like* genes, which code for callose synthases (Jacobs *et al.*, 2003). Assessing the changes in WRKY expression in response to a variety of insects would enable us to determine which genes play specific roles, and which are differentially expressed in response to multiple stresses. This may help to assign more detailed functions to both the WRKYs, and the genes under their transcriptional regulation.
- Using the system of Virus Induced Gene Silencing (VIGS). This has the potential to create regulated knockdowns of target genes to provide useful functional information, and, if combined with transcriptome level analysis in the form of microarrays or RNA-Seq, may start to build a knowledge base of the molecular-level effects of specific WRKY proteins in wheat, especially *TaWRKY3*, as this gene was identified as being likely to play a key role in the response to both low nitrogen and aphid infestation stresses.

References

Adenle, A.A., Azadi, H. and Manning, L. (2018) 'The era of sustainable agricultural development in Africa: Understanding the benefits and constraints', *Food Reviews International*, 34(5), pp. 411-433.

Agarwala, B.K., Das, J. and Rosenheim, J. (2012) 'Weed host specificity of the aphid, Aphis spiraecola: Developmental and reproductive performance of aphids in relation to plant growth and leaf chemicals of the Siam weed, Chromolaena odorata', *Journal of insect science*, 12(1).

Ali, A., Desneux, N., Lu, Y. and Wu, K. (2018) 'Key aphid natural enemies showing positive effects on wheat yield through biocontrol services in northern China', *Agriculture, Ecosystems & Environment*, 266, pp. 1-9.

Alotaibi, S.S., Sparks, C.A., Parry, M.A.J., Simkin, A.J. and Raines, C.A. (2018) 'Identification of Leaf Promoters for Use in Transgenic Wheat', *Plants*, 7(2), p. 27.

Aqueel, M.A. and Leather, S.R. (2011) 'Effect of nitrogen fertilizer on the growth and survival of Rhopalosiphum padi (L.) and Sitobion avenae (F.)(Homoptera: Aphididae) on different wheat cultivars', *Crop Protection*, 30(2), pp. 216-221.

Atamian, H.S., Chaudhary, R., Cin, V.D., Bao, E., Girke, T. and Kaloshian, I. (2013) 'In planta expression or delivery of potato aphid Macrosiphum euphorbiae effectors Me10 and Me23 enhances aphid fecundity', *Molecular Plant-Microbe Interactions*, 26(1), pp. 67-74.

Atkinson, N.J. and Urwin, P.E. (2012) 'The interaction of plant biotic and abiotic stresses: from genes to the field', *Journal of experimental botany*, 63(10), pp. 3523-3543.

Avni, R., Zhao, R., Pearce, S., Jun, Y., Uauy, C., Tabbita, F., Fahima, T., Slade, A., Dubcovsky, J. and Distelfeld, A. (2014) 'Functional characterization of GPC-1 genes in hexaploid wheat', *Planta*, 239(2), pp. 313-324.

Awmack, C.S. and Leather, S.R. (2002) 'Host plant quality and fecundity in herbivorous insects', *Annual review of entomology*, 47(1), pp. 817-844.

Bahrini, I., Ogawa, T., Kobayashi, F., Kawahigashi, H. and Handa, H. (2011) 'Overexpression of the pathogen-inducible wheat TaWRKY45 gene confers disease resistance to multiple fungi in transgenic wheat plants', *Breeding science*, 61(4), pp. 319-326.

Ballini, E., Nguyen, T.T. and Morel, J.-B. (2013) 'Diversity and genetics of nitrogen-induced susceptibility to the blast fungus in rice and wheat', *Rice*, 6(1), p. 32.

Barrs, H.D. and Weatherley, P.E. (1962) 'A re-examination of the relative turgidity technique for estimating water deficits in leaves', *Australian Journal of Biological Sciences*, 15(3), pp. 413-428.

Beckers, G.J.M., Jaskiewicz, M., Liu, Y., Underwood, W.R., He, S.Y., Zhang, S. and Conrath, U. (2009) 'Mitogen-activated protein kinases 3 and 6 are required for full priming of stress responses in Arabidopsis thaliana', *The Plant Cell*, 21(3), pp. 944-953.

Bentz, J.-A., Reeves Iii, J., Barbosa, P. and Francis, B. (1995) 'Within-plant variation in nitrogen and sugar content of poinsettia and its effects on the oviposition pattern, survival, and development of Bemisia argentifolii (Homoptera: Aleyrodidae)', *Environmental Entomology* 24 (2), pp. 271-277.

Berzonsky, W.A., Ding, H., Haley, S.D., Harris, M.O., Lamb, R.J., McKenzie, R.I.H., Ohm, H.W., Patterson, F.L., Peairs, F. and Porter, D.R. (2003) 'Breeding wheat for resistance to insects', *Plant Breeding Reviews*, 22, pp. 221-296.

Bhattarai, K.K., Li, Q., Liu, Y., Dinesh-Kumar, S.P. and Kaloshian, I. (2007) 'The Mi-1-mediated pest resistance requires Hsp90 and Sgt1', *Plant Physiology*, 144(1), pp. 312-323.

Bi, Y.-M., Wang, R.-L., Zhu, T. and Rothstein, S.J. (2007) 'Global transcription profiling reveals differential responses to chronic nitrogen stress and putative nitrogen regulatory components in Arabidopsis', *BMC genomics*, 8(1), p. 281.

Blackman, R.L. and Eastop, V.F. (2000) *Aphids on the world's crops: an identification and information guide*. John Wiley & Sons Ltd.

Bojović, B. and Marković, A. (2009) 'Correlation between nitrogen and chlorophyll content in wheat (Triticum aestivum L.)', *Kragujevac Journal of Science*, 31, pp. 69-74.

Boller, T. and Felix, G. (2009) 'A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors', *Annual review of plant biology*, 60, pp. 379-406.

Borlaug, N.E. and Dowswell, C.R. (1993) 'Fertiliser: To nourish infertile soil that feeds a fertile population that crowds a fragile world', *Fertiliser News*, 38, pp. 11-20.

Castex, V., Beniston, M., Calanca, P., Fleury, D. and Moreau, J. (2018) 'Pest management under climate change: The importance of understanding tritrophic relations', *Science of the Total Environment*, 616, pp. 397-407.

Chang, S.X. and Robison, D.J. (2003) 'Nondestructive and rapid estimation of hardwood foliar nitrogen status using the SPAD-502 chlorophyll meter', *Forest Ecology and Management*, 181(3), pp. 331-338.

Chaudhary, R., Atamian, H.S., Shen, Z., Briggs, S.P. and Kaloshian, I. (2014) 'GroEL from the endosymbiont Buchnera aphidicola betrays the aphid by triggering plant defense', *Proceedings of the National Academy of Sciences*, 111(24), pp. 8919-8924.

Chavan, S.B. and Deshpande, M.V. (2013) 'Chitinolytic enzymes: an appraisal as a product of commercial potential', *Biotechnology progress*, 29(4), pp. 833-846.

Chen, B.-M., Wang, Z.-H., Li, S.-X., Wang, G.-X., Song, H.-X. and Wang, X.-N. (2004) 'Effects of nitrate supply on plant growth, nitrate accumulation, metabolic nitrate concentration and nitrate reductase activity in three leafy vegetables', *Plant Science*, 167(3), pp. 635-643.

Chen, C. and Chen, Z. (2002) 'Potentiation of developmentally regulated plant defense response by AtWRKY18, a pathogen-induced Arabidopsis transcription factor', *Plant physiology*, 129(2), pp. 706-716.

Chen, H., Lai, Z., Shi, J., Xiao, Y., Chen, Z. and Xu, X. (2010) 'Roles of Arabidopsis WRKY18, WRKY40 and WRKY60 transcription factors in plant responses to abscisic acid and abiotic stress', *BMC plant biology*, 10(1), p. 281.

Chen, L., Huang, L., Min, D., Phillips, A., Wang, S., Madgwick, P.J., Parry, M.A.J. and Hu, Y.-G. (2012a) 'Development and characterization of a new TILLING population of common bread wheat (Triticum aestivum L.)', *PLoS One*, 7(7), p. e41570.

Chen, L., Song, Y., Li, S., Zhang, L., Zou, C. and Yu, D. (2012b) 'The role of WRKY transcription factors in plant abiotic stresses', *Biochimica et Biophysica Acta (BBA)-Gene Regulatory Mechanisms*, 1819(2), pp. 120-128.

Chi, Y., Yang, Y., Zhou, Y., Zhou, J., Fan, B., Yu, J.-Q. and Chen, Z. (2013) 'Protein–protein interactions in the regulation of WRKY transcription factors', *Molecular plant*, 6(2), pp. 287-300.

Ciolkowski, I., Wanke, D., Birkenbihl, R.P. and Somssich, I.E. (2008) 'Studies on DNA-binding selectivity of WRKY transcription factors lend structural clues into WRKY-domain function', *Plant Molecular Biology*, 68(1), pp. 81-92.

Cisneros, J.J. and Godfrey, L.D. (2001) 'Midseason pest status of the cotton aphid (Homoptera: Aphididae) in California cottonIs nitrogen a key factor?', *Environmental Entomology*, 30(3), pp. 501-510.

Clough, S.J. and Bent, A.F. (1998) 'Floral dip: a simplified method forAgrobacterium-mediated transformation ofArabidopsis thaliana', *The plant journal*, 16(6), pp. 735-743.

Collins, M.B., Haley, S.D., Peairs, F.B. and Rudolph, J.B. (2005) 'Biotype 2 Russian wheat aphid resistance among wheat germplasm accessions', *Crop science*, 45(5), pp. 1877-1880.

Comadira, G., Rasool, B., Karpinska, B., Morris, J., Verrall, S.R., Hedley, P.E., Foyer, C.H. and Hancock, R.D. (2015) 'Nitrogen deficiency in barley (Hordeum vulgare) seedlings induces molecular and metabolic adjustments that trigger aphid resistance', *Journal of Experimental Botany*, 66(12), pp. 3639-3655.

Conley, D.J., Paerl, H.W., Howarth, R.W., Boesch, D.F., Seitzinger, S.P., Havens, K.E., Lancelot, C. and Likens, G.E. (2009) 'Controlling eutrophication: nitrogen and phosphorus', *Science*, 323(5917), pp. 1014-1015.

Conrath, U., Beckers, G.J.M., Flors, V., García-Agustín, P., Jakab, G., Mauch, F., Newman, M.-A., Pieterse, C.M.J., Poinssot, B. and Pozo, M.J. (2006) 'Priming: getting ready for battle', *Molecular Plant-Microbe Interactions*, 19(10), pp. 1062-1071.

Coppola, M., Manco, E., Vitiello, A., Di Lelio, I., Giorgini, M., Rao, R., Pennacchio, F. and Digilio, M.C. (2018) 'Plant response to feeding aphids promotes aphid dispersal', *Entomologia Experimentalis et Applicata*, 166(5), pp. 386-394.

Cui, X. and Luan, S. (2012) 'A new wave of hormone research: crosstalk mechanisms', *Molecular plant*, 5(5), pp. 959-960.

Dawson, J.C., Huggins, D.R. and Jones, S.S. (2008) 'Characterizing nitrogen use efficiency in natural and agricultural ecosystems to improve the performance of cereal crops in low-input and organic agricultural systems', *Field Crops Research*, 107(2), pp. 89-101.

de Pater, S., Greco, V., Pham, K., Memelink, J. and Kijne, J. (1996) 'Characterization of a zinc-dependent transcriptional activator from Arabidopsis', *Nucleic Acids Research*, 24(23), pp. 4624-4631.

DEFRA (2015) 'Department for Food and Rural Affairs. 2015.', *Agriculture in the United Kingdom 2014*.

Delin, S. and Stenberg, M. (2014) 'Effect of nitrogen fertilization on nitrate leaching in relation to grain yield response on loamy sand in Sweden', *European Journal of Agronomy*, 52 (B), pp. 291-296.

Deslandes, L., Olivier, J., Theulières, F., Hirsch, J., Feng, D.X., Bittner-Eddy, P., Beynon, J. and Marco, Y. (2002) 'Resistance to Ralstonia solanacearum in Arabidopsis thaliana is conferred by the recessive RRS1-R gene, a member of a novel family of resistance genes', *Proceedings of the National Academy of Sciences of the USA*, 99(4), pp. 2404-2409.

Devaiah, B.N., Karthikeyan, A.S. and Raghothama, K.G. (2007) 'WRKY75 transcription factor is a modulator of phosphate acquisition and root development in Arabidopsis', *Plant physiology*, 143(4), pp. 1789-1801.

Diaz, C., Saliba-Colombani, V., Loudet, O., Belluomo, P., Moreau, L., Daniel-Vedele, F., Morot-Gaudry, J.-F. and Masclaux-Daubresse, C. (2006) 'Leaf yellowing and anthocyanin accumulation are two genetically independent strategies in response to nitrogen limitation in Arabidopsis thaliana', *Plant and Cell Physiology*, 47(1), pp. 74-83.

Ding, L., Wang, K.J., Jiang, G.M., Biswas, D.K., Xu, H., Li, L.F. and Li, Y.H. (2005) 'Effects of nitrogen deficiency on photosynthetic traits of maize hybrids released in different years', *Annals of Botany*, 96(5), pp. 925-930.

Ding, W., Fang, W., Shi, S., Zhao, Y., Li, X. and Xiao, K. (2016) 'Wheat WRKY type transcription factor gene TaWRKY1 is essential in mediating drought tolerance associated with an ABA-dependent pathway', *Plant molecular biology reporter*, 34(6), pp. 1111-1126.

Dodds, P.N., Lawrence, G.J., Catanzariti, A.-M., Teh, T., Wang, C.-I.A., Ayliffe, M.A., Kobe, B. and Ellis, J.G. (2006) 'Direct protein interaction underlies gene-for-gene specificity and coevolution of the flax resistance genes and flax rust avirulence genes', *Proceedings of the National Academy of Sciences*, 103(23), pp. 8888-8893.

Dogimont, C., Bendahmane, A., Chovelon, V. and Boissot, N. (2010) 'Host plant resistance to aphids in cultivated crops: genetic and molecular bases, and interactions with aphid populations', *Comptes Rendus Biologies*, 333(6-7), pp. 566-573.

Domènech, L. (2015) 'Improving irrigation access to combat food insecurity and undernutrition: A review', *Global Food Security*, 6, pp. 24-33.

Dong, J., Chen, C. and Chen, Z. (2003) 'Expression profiles of the Arabidopsis WRKY gene superfamily during plant defense response', *Plant molecular biology*, 51(1), pp. 21-37.

Dubey, N.K., Mishra, D.K., Idris, A., Nigam, D., Singh, P.K. and Sawant, S.V. (2018) 'Whitefly and aphid inducible promoters of Arabidopsis thaliana L', *Journal of genetics*, 97(1), pp. 109-119.

Duffield, S.J., Bryson, R.J., Young, J.E.B., SylvesterBradley, R. and Scott, R.K. (1997) 'The influence of nitrogen fertiliser on the population development of the cereal aphids Sitobion avenae (F) and Metopolophium dirhodum (Wlk) on field grown winter wheat', *Annals of Applied Biology*, 130(1), pp. 13-26.

Elzinga, D.A., De Vos, M. and Jander, G. (2014) 'Suppression of plant defenses by a Myzus persicae (green peach aphid) salivary effector protein', *Molecular Plant-Microbe Interactions*, 27(7), pp. 747-756.

Elzinga, D.A. and Jander, G. (2013) 'The role of protein effectors in plant–aphid interactions', *Current Opinion in Plant Biology*, 16(4), pp. 451-456.

English-Loeb, G., Stout, M.J. and Duffey, S.S. (1997) 'Drought stress in tomatoes: changes in plant chemistry and potential nonlinear consequences for insect herbivores', *Oikos*, pp. 456-468.

Erb, M., Meldau, S. and Howe, G.A. (2012) 'Role of phytohormones in insect-specific plant reactions', *Trends in plant science*, 17(5), pp. 250-259.

Eulgem, T., Rushton, P.J., Robatzek, S. and Somssich, I.E. (2000) 'The WRKY superfamily of plant transcription factors', *Trends in plant science*, 5(5), pp. 199-206.

Eulgem, T., Rushton, P.J., Schmelzer, E., Hahlbrock, K. and Somssich, I.E. (1999) 'Early nuclear events in plant defense: rapid gene activation by WRKY transcription factors', *EMBOJ*, 18(17), p. 4.

Eulgem, T. and Somssich, I.E. (2007) 'Networks of WRKY transcription factors in defense signaling', *Current opinion in plant biology*, 10(4), pp. 366-371.

FAO, IFAD and WFP (2015b) '*The state of food insecurity in the world.*', Rome: FAO.

FAO (2017c) '*The state of food and agriculture: leveraging food systems for inclusive rural transformation.* ', Rome: FAO.

FAO (2019) 'Fertilizer Use to Surpass 200 Million Tonnes in 2018', *Food and agriculture orgnization.*

Farmer, E.E. and Ryan, C.A. (1992) 'Octadecanoid-redived signals in plants', *Trends in cell biology*, 2(8), pp. 236-241.

Fereres, A., Gutierrez, C., Del Estal, P. and Castanera, P. (1988) 'Impact of the English grain aphid, Sitobion avenae (F.)(Homoptera: Aphididae), on the yield of wheat plants subjected to water deficits', *Environmental Entomology*, 17(3), pp. 596-602.

Ferry, N., Edwards, M.G., Gatehouse, J.A. and Gatehouse, A.M.R. (2004) 'Plant–insect interactions: molecular approaches to insect resistance', *Current Opinion in Biotechnology*, 15(2), pp. 155-161.

Ferry, N. and Gatehouse, A.M.R. (eds.) (2009) *Environmental impact of genetically modified crops*. Wallingford.: CABI.

Ferry, N., Stavroulakis, S., Guan, W., Davison, G.M., Bell, H.A., Weaver, R.J., Down, R.E., Gatehouse, J.A. and Gatehouse, A.M.R. (2011) 'Molecular interactions between wheat and cereal aphid (Sitobion avenae): Analysis of changes to the wheat proteome', *Proteomics*, 11(10), pp. 1985-2002.

Finlay, K.J. and Luck, J.E. (2011) 'Response of the bird cherry-oat aphid (Rhopalosiphum padi) to climate change in relation to its pest status, vectoring potential and function in a crop–vector–virus pathosystem', *Agriculture, ecosystems & environment*, 144(1), pp. 405-421.

Forcat, S., Bennett, M.H., Mansfield, J.W. and Grant, M.R. (2008) 'A rapid and robust method for simultaneously measuring changes in the phytohormones ABA, JA and SA in plants following biotic and abiotic stress', *Plant methods*, 4(1), p. 16.

Foyer, C.H., Rasool, B., Davey, J.W. and Hancock, R.D. (2016) 'Cross-tolerance to biotic and abiotic stresses in plants: a focus on resistance to aphid infestation', *Journal of experimental botany*, 67(7), pp. 2025-2037.

Foyer, C.H., Verrall, S.R. and Hancock, R.D. (2014) 'Systematic analysis of phloem-feeding insect-induced transcriptional reprogramming in Arabidopsis highlights common features and reveals distinct responses to specialist and generalist insects', *Journal of Experimental Botany*, 66 (2), pp. 495-512.

Freeman, B.C. and Beattie, G.A. (2008) 'An overview of plant defenses against pathogens and herbivores', *The Plant Health Instructor*, doi: 10.1094/PHI-I-2008-0226-01.

Friebe, B., Zhang, W., Raupp, J.W., Gill, B.S. and Porter, D.R. (1995) 'Non-homoeologous wheat-rye chromosomal translocations conferring resistance to greenbug', *Euphytica*, 84(2), pp. 121-125.

Frink, C.R., Waggoner, P.E. and Ausubel, J.H. (1999) 'Nitrogen fertilizer: retrospect and prospect', *Proceedings of the National Academy of Sciences*, 96(4), pp. 1175-1180.

Fu, J., Chu, J., Sun, X., Wang, J. and Yan, C. (2012) 'Simple, rapid, and simultaneous assay of multiple carboxyl containing phytohormones in wounded tomatoes by UPLC-MS/MS using single SPE purification and isotope dilution', *Analytical Sciences*, 28(11), pp. 1081-1087.

Fujita, M., Fujita, Y., Noutoshi, Y., Takahashi, F., Narusaka, Y., Yamaguchi-Shinozaki, K. and Shinozaki, K. (2006) 'Crosstalk between abiotic and biotic stress responses: a current view from the points of convergence in the stress signaling networks', *Current Opinion in Plant Biology*, 9(4), pp. 436-442.

Gao, H.-H., Zhao, H.-Y., Du, C., Deng, M.-M., Du, E.-X., Hu, Z.-Q., Hu, X.-S. and Ni, X. (2012) 'Life table evaluation of survival and reproduction of the aphid, Sitobion avenae, exposed to cadmium', *Journal of Insect Science*, 12(1).

Gao, J., Guo, H., Sun, Y. and Ge, F. (2018) 'Differential accumulation of leucine and methionine in red and green pea aphids leads to different fecundity in response to nitrogen fertilization', *Pest management science*.

Gardner, F.P., Pearce, R.B. and Mitchell, R.L. (2003) *'Physiology of crop plants'*, Jodhpur.Scientific.Publishers.

Gash, A.F.J. (2012) 'Wheat nitrogen fertilisation effects on the performance of the cereal aphid Metopolophium dirhodum', *Agronomy*, 2(1), pp. 1-13.

Gatehouse, A.M.R., Ferry, N., Edwards, M.G. and Bell, H.A. (2011) 'Insect-resistant biotech crops and their impacts on beneficial arthropods', *Philosophical Transactions of the Royal Society of London B: Biological Sciences*, 366(1569), pp. 1438-1452.

Gatehouse, A.M.R., Shi, Y., Powell, K.S., Brough, C., Hilder, V.A., Hamilton, W.D.O., Newell, C.A., Merryweather, A., Boulter, D. and Gatehouse, J.A. (1993) 'Approaches to insect resistance using transgenic plants', *Phil. Trans. R. Soc. Lond. B*, 342(1301), pp. 279-286.

Gatehouse, J.A. (2002) 'Plant resistance towards insect herbivores: a dynamic interaction', *New phytologist*, 156(2), pp. 145-169.

Gaur, A., Biggs, T.W., Gumma, M.K., Parthasaradhi, G. and Turral, H. (2008) 'Water scarcity effects on equitable water distribution and land use in a major irrigation project—case study in India', *Journal of irrigation and Drainage Engineering*, 134(1), pp. 26-35.

Gollojeh, K.S. and Ranjbar, A. (2012) 'Evaluation of physiological characteristics and yield components of chickpea genotypes under rain-fed condition', *Current Opinion in Agriculture*, 1(1), p. 13.

Guan, W., Ferry, N., Edwards, M.G., Bell, H.A., Othman, H., Gatehouse, J.A. and Gatehouse, A.M.R. (2015) 'Proteomic analysis shows that stress response proteins are significantly up-regulated in resistant diploid wheat (Triticum monococcum) in response to attack by the grain aphid (Sitobion avenae)', *Molecular breeding*, 35(2), p. 57.

Guerrieri, E. and Digilio, M.C. (2008) 'Aphid-plant interactions: a review', *Journal of Plant Interactions*, 3(4), pp. 223-232.

Gururani, M.A., Venkatesh, J., Upadhyaya, C.P., Nookaraju, A., Pandey, S.K. and Park, S.W. (2012) 'Plant disease resistance genes: current status and future directions', *Physiological and molecular plant pathology*, 78, pp. 51-65.

Hayashi, S., Kojima-Suzuki, H., Morikami, A. and Nakamura, K. (2001) 'Role of conserved residues of the WRKY domain in the DNA-binding of tobacco WRKY family proteins', *Bioscience, biotechnology, and biochemistry*, 65(11), pp. 2428-2436.

He, G.-H., Xu, J.-Y., Wang, Y.-X., Liu, J.-M., Li, P.-S., Chen, M., Ma, Y.-Z. and Xu, Z.-S. (2016) 'Drought-responsive WRKY transcription factor genes TaWRKY1 and TaWRKY33 from wheat confer drought and/or heat resistance in Arabidopsis', *BMC plant biology*, 16(1), p. 116.

He, J., Chen, F., Chen, S., Lv, G., Deng, Y., Fang, W., Liu, Z., Guan, Z. and He, C. (2011) 'Chrysanthemum leaf epidermal surface morphology and antioxidant and defense enzyme activity in response to aphid infestation', *Journal of Plant Physiology*, 168(7), pp. 687-693.

Heil, M. and Bueno, J.C.S. (2007) 'Within-plant signaling by volatiles leads to induction and priming of an indirect plant defense in nature', *Proceedings of the National Academy of Sciences*, 104(13), pp. 5467-5472.

Herms, D.A. and Mattson, W.J. (1992) 'The dilemma of plants: to grow or defend', *The quarterly review of biology*, 67(3), pp. 283-335.

Hilder, V.A. and Boulter, D. (1999) 'Genetic engineering of crop plants for insect resistance–a critical review', *Crop protection*, 18(3), pp. 177-191.

Hirel, B., Le Gouis, J., Ney, B. and Gallais, A. (2007) 'The challenge of improving nitrogen use efficiency in crop plants: towards a more central role for genetic variability and quantitative genetics within integrated approaches', *Journal of Experimental Botany*, 58(9), pp. 2369-2387.

Hoagland, D.R. and Arnon, D.I. (1950) 'Cirular347 The water-culture method for growing plants without soil', Berkeley, *California Agricultural Experiment Station*.

Hogenhout, S.A. and Bos, J.I.B. (2011) 'Effector proteins that modulate plant–insect interactions', *Current opinion in plant biology*, 14(4), pp. 422-428.

Honek, A. (1992) 'Nitrogen fertilization and abundance of the cereal aphis Metopolophium dirhodum and Sitobion avenae (Homoptera, Aphididae)', *Zeitschrift fiir Pflanzenkrankheiten und Pflanzenschutz* 98 (6), pp. 655-660.

Hong, Z., Shikai, L., Changyou, W. and Wanquan, J. (2018) 'The role of transcription factor in wheat defense against pathogen and its prospect in breeding', *Journal of Plant Biology and Crop Research,*, 1, pp. 1005.

Hu, H. and Xiong, L. (2014) 'Genetic engineering and breeding of drought-resistant crops', *Annual review of plant biology*, 65, pp. 715-741.

Huang, Z.-h., Wang, Z.-l., Shi, B.-l., Wei, D., Chen, J.-x., Wang, S.-l. and Gao, B.-j. (2015) 'Simultaneous determination of salicylic acid, jasmonic acid, methyl salicylate, and methyl jasmonate from Ulmus pumila leaves by GC-MS', *International Journal of Analytical Chemistry*, 2015, p. 698630.

Hunter, M.D. (2000) 'Mixed signals and cross-talk: interactions between plants, insect herbivores and plant pathogens', *Agricultural and Forest Entomology*, 2(3), pp. 155-160.

Influentialpoints.com (2018) 'Sitobion avenae English grain aphid.' Available at: http://influentialpoints.com/Gallery/Sitobion_avenae_English_Grain_aphid.htm (Accessed: 6 April 2019).

International Wheat Genome Sequencing, C. (2014) 'A chromosome-based draft sequence of the hexaploid bread wheat (Triticum aestivum) genome', *Science*, 345(6194), p. 1251788.

Ishihama, N. and Yoshioka, H. (2012) 'Post-translational regulation of WRKY transcription factors in plant immunity', *Current opinion in plant biology*, 15(4), pp. 431-437.

Ito, Y., Katsura, K., Maruyama, K., Taji, T., Kobayashi, M., Seki, M., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2006) 'Functional analysis of rice DREB1/CBF-type transcription factors involved in cold-responsive gene expression in transgenic rice', *Plant and Cell Physiology*, 47(1), pp. 141-153.

Jacobs, A.K., Lipka, V., Burton, R.A., Panstruga, R., Strizhov, N., Schulze-Lefert, P. and Fincher, G.B. (2003) 'An Arabidopsis Callose Synthase, GSL5, Is Required for Wound and Papillary Callose Formation', *The Plant Cell*, 15(11), pp. 2503-2513.

Jansson, R.K. and Smilowitz, Z. (1986) 'Influence of nitrogen on population parameters of potato insects: abundance, population growth, and within-plant distribution of the green peach aphid, Myzus persicae (Homoptera: Aphididae)', *Environmental Entomology*, 15(1), pp. 49-55.

Jaouannet, M., Rodriguez, P.A., Thorpe, P., Lenoir, C.J.G., MacLeod, R., Escudero-Martinez, C. and Bos, J.I.B. (2014) 'Plant immunity in plant–aphid interactions', *Frontiers in plant science*, 5, p. 663.

Jarošová, J. and Kundu, J.K. (2010) 'Validation of reference genes as internal control for studying viral infections in cereals by quantitative real-time RT-PCR', *BMC Plant Biology*, 10(1), p. 146.

Jiang, Y., Liang, G. and Yu, D. (2012) 'Activated expression of WRKY57 confers drought tolerance in Arabidopsis', *Molecular plant*, 5(6), pp. 1375-1388.

Jones, J.D.G. and Dangl, J.L. (2006) 'The plant immune system', *Nature*, 444(7117), pp. 323-329.

Kaloshian, I. and Walling, L.L. (2016) 'Plant Immunity: Connecting the dots between microbial and hemipteran immune responses', in Czosnek, H. and Ghanim, M. (eds.) *Management of insect pests to agriculture*.: Cham: Springer, pp. 217-243.

Karadaghi, S.A. (2019) 'The 20 amino acids and their role in protein structures'. Available at: https://proteinstructures.com/Structure/Structure/amino-acids.html. (Accessed: 10 April 2019).

Karowe, D.N. and Martin, M.M. (1989) 'The effects of quantity and quality of diet nitrogen on the growth, efficiency of food utilization, nitrogen budget, and metabolic rate of fifth-instar Spodoptera eridania larvae (Lepidoptera: Noctuidae)', *Journal of insect physiology*, 35(9), pp. 699-708.

Keskin, B.C., Yuksel, B., Memon, A.R. and Topal-Sarıkaya, A. (2010) 'Abscisic acid regulated gene expression in bread wheat ('Triticum aestivum'L.)', *Australian Journal of Crop Science*, 4(8), pp. 617-625.

Khan, (2014) *Transcriptional and physiological responses of wheat Triticum aestivum to a combination of biotic and abiotic stress.* PhD thesis. Newcastle University

Khan, F.U. and Mohammad, F. (2016) 'Application of stress selection indices for assessment of nitrogen tolerance in wheat (Triticum aestivum L.)', *Journal of Animal and Plant Sciences*, 26(1), pp. 201-210.

Khan, M. and Port, G. (2008) 'Performance of clones and morphs of two cereal aphids on wheat plants with high and low nitrogen content', *Entomological science*, 11(2), pp. 159-165.

Kim, K.-C., Fan, B. and Chen, Z. (2006) 'Pathogen-induced Arabidopsis WRKY7 is a transcriptional repressor and enhances plant susceptibility to Pseudomonas syringae', *Plant physiology*, 142(3), pp. 1180-1192.

Kowalczyk-Jusko, A. and Koscik, B. (2002) 'Possible use of the chlorophyll meter [SPAD-502] for evaluating nitrogen nutrition of the Virginia tobacco', *Electronic Journal of Polish Agricultural Universities. Series Agronomy*, 5 (1), p. 4.

Krasileva, K.V., Vasquez-Gross, H.A., Howell, T., Bailey, P., Paraiso, F., Clissold, L., Simmonds, J., Ramirez-Gonzalez, R.H., Wang, X. and Borrill, P. (2017) 'Uncovering hidden variation in polyploid wheat', *Proceedings of the National Academy of Sciences*, p. 201619268.

Lei, J., Finlayson, S., Salzman, R., Shan, L. and Zhu-Salzman, K. (2014) 'BOTRYTIS-INDUCED KINASE1 modulates Arabidopsis resistance to green peach aphids via PHYTOALEXIN DEFICIENT4', *Plant Physiology*, 156(4), pp. 1657-1670.

Lesk, C., Rowhani, P. and Ramankutty, N. (2016) 'Influence of extreme weather disasters on global crop production', *Nature*, 529(7584), pp. 84-87.

Li, W., Wang, H. and Yu, D. (2016) 'Arabidopsis WRKY transcription factors WRKY12 and WRKY13 oppositely regulate flowering under short-day conditions', *Molecular plant*, 9(11), pp. 1492-1503.

Livak, K.J. and Schmittgen, T.D. (2001) 'Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method', *methods*, 25(4), pp. 402-408.

Lu, Z.-X., Yu, X.-P., Heong, K.-l. and Cui, H.U. (2007) 'Effect of nitrogen fertilizer on herbivores and its stimulation to major insect pests in rice', *Rice Science*, 14(1), pp. 56-66.

Mantri, N.L., Ford, R., Coram, T.E. and Pang, E.C.K. (2010) 'Evidence of unique and shared responses to major biotic and abiotic stresses in chickpea', *Environmental and experimental botany*, 69(3), pp. 286-292.

Mao, G., Meng, X., Liu, Y., Zheng, Z., Chen, Z. and Zhang, S. (2011) 'Phosphorylation of a WRKY transcription factor by two pathogen-responsive MAPKs drives phytoalexin biosynthesis in Arabidopsis', *The Plant Cell*, 23(4), pp. 1639-1653.

Mare, C., Mazzucotelli, E., Crosatti, C., Francia, E. and Cattivelli, L. (2004) 'Hv-WRKY38: a new transcription factor involved in cold-and drought-response in barley', *Plant molecular biology*, 55(3), pp. 399-416

McLellan, E.L., Cassman, K.G., Eagle, A.J., Woodbury, P.B., Sela, S., Tonitto, C., Marjerison, R.D. and van Es, H.M. (2018) 'The nitrogen balancing act: tracking the environmental performance of food production', *BioScience*, 68(3), pp. 194-203.

Medina-Ortega, K.J. and Walker, G.P. (2013) 'Does aphid salivation affect phloem sieve element occlusion in vivo?', *Journal of experimental botany*, 64(18), pp. 5525-5535.

Metz, A.M., Timmer, R.T., Allen, M.L. and Browning, K.S. (1992) 'Sequence of a cDNA encoding the α-subunit of wheat translation elongation factor 1', *Gene*, 120(2), pp. 315-316.

Mitsuda, N. and Ohme-Takagi, M. (2009) 'Functional analysis of transcription factors in Arabidopsis', *Plant and Cell Physiology*, 50(7), pp. 1232-1248.

Moll, R., Kamprath, E. and Jackson, W. (1982) 'Analysis and interpretation of factors which contribute to efficiency of nitrogen utilization 1', *Agronomy Journal*, 74(3), pp. 562-564.

Moore, F.C. and Lobell, D.B. (2015) 'The fingerprint of climate trends on European crop yields', *Proceedings of the National Academy of sciences*, 112 (9), pp. 2670-2675.

Moran, P.J. and Thompson, G.A. (2001) 'Molecular responses to aphid feeding in Arabidopsis in relation to plant defense pathways', *Plant Physiology*, 125(2), pp. 1074-1085.

Moreno, A., Garzo, E., Fernandez-Mata, G., Kassem, M., Aranda, M.A. and Fereres, A. (2011) 'Aphids secrete watery saliva into plant tissues from the onset of stylet penetration', *Entomologia Experimentalis et Applicata*, 139(2), pp. 145-153.

Morrison, W.P. and Peairs, F.B. (1998) 'Response model concept and economic impact', in Quisenberry, S.S. and Peairs, F.B. (eds) *Response model for an introduced pest—the Russian wheat aphid. Lanham, MD: Entomological Society of America*, pp. 1-11.

Nalam, V., Louis, J. and Shah, J. (2019) 'Plant defense against aphids, the pest extraordinaire', *Plant Science*, 279, pp. 96-107.

Ng, J.C.K. and Perry, K.L. (2004) 'Transmission of plant viruses by aphid vectors', *Molecular Plant Pathology*, 5(5), pp. 505-511.

Ning, P., Liu, C., Kang, J. and Lv, J. (2017) 'Genome-wide analysis of WRKY transcription factors in wheat (Triticum aestivum L.) and differential expression under water deficit condition', *PeerJ*, 5, p. e3232.

Niu, C.F., Wei, W., Zhou, Q.Y., Tian, A.G., Hao, Y.J., Zhang, W.K., Ma, B., Lin, Q., Zhang, Z.B. and Zhang, J.S. (2012) 'Wheat WRKY genes TaWRKY2 and TaWRKY19 regulate abiotic stress tolerance in transgenic Arabidopsis plants', *Plant, cell & environment*, 35(6), pp. 1156-1170.

Noctor, G. and Mhamdi, A. (2017) 'Climate change, CO2, and defense: the metabolic, redox, and signaling perspectives', *Trends in plant science*, 22(10), pp. pp. 857-870.

Okay, S., Derelli, E. and Unver, T. (2014) 'Transcriptome-wide identification of bread wheat WRKY transcription factors in response to drought stress', *Molecular genetics and genomics*, 289(5), pp. 765-781.

Ono, K., Terashima, I. and Watanabe, A. (1996) 'Interaction between nitrogen deficit of a plant and nitrogen content in the old leaves', *Plant and cell physiology*, 37(8), pp. 1083-1089.

Pandey, S.P. and Somssich, I.E. (2009) 'The role of WRKY transcription factors in plant immunity', *Plant physiology*, 150(4), pp. 1648-1655.

Peng, J.H., Bai, Y., Haley, S.D. and Lapitan, N.L.V. (2009) 'Microsatellite-based molecular diversity of bread wheat germplasm and association mapping of wheat resistance to the Russian wheat aphid', *Genetica*, 135(1), pp. 95-122.

Peng, M., Bi, Y.-M., Zhu, T. and Rothstein, S.J. (2007) 'Genome-wide analysis of Arabidopsis responsive transcriptome to nitrogen limitation and its regulation by the ubiquitin ligase gene NLA', *Plant molecular biology*, 65(6), pp. 775-797.

Petersen, G., Seberg, O., Yde, M. and Berthelsen, K. (2006) 'Phylogenetic relationships of Triticum and Aegilops and evidence for the origin of the A, B, and D genomes of common wheat (Triticum aestivum)', *Molecular phylogenetics and evolution*, 39(1), pp. 70-82.

Pieterse, C.M.J., Ton, J. and Van Loon, L.C. (2001) 'Cross-talk between plant defence signalling pathways: boost or burden?', *AgBiotechNet*, 3(june), pp. 1-8.

Pieterse, C.M.J. and van Loon, L.C. (1999) 'Salicylic acid-independent plant defence pathways', *Trends in plant science*, 4(2), pp. 52-58.

Ponder, K.L., Pritchard, J., Harrington, R. and Bale, J.S. (2000) 'Difficulties in location and acceptance of phloem sap combined with reduced concentration of phloem amino acids explain lowered performance of the aphid Rhopalosiphum padi on nitrogen deficient barley (Hordeum vulgare) seedlings', *Entomologia Experimentalis et Applicata*, 97(2), pp. 203-210.

Qin, Y., Tian, Y. and Liu, X. (2015) 'A wheat salinity-induced WRKY transcription factor TaWRKY93 confers multiple abiotic stress tolerance in Arabidopsis thaliana', *Biochemical and biophysical research communications*, 464(2), pp. 428-433.

Rahimifard, S., Stone, J. and Trollman, H. 11(2) (2018) 'Global food security: the engineering challenges'. *International Journal of Sustainable Engineering*, ,11(2), pp. 77-78.

Rejeb, I.B., Pastor, V. and Mauch-Mani, B. (2014) 'Plant responses to simultaneous biotic and abiotic stress: molecular mechanisms', *Plants*, 3(4), pp. 458-475.

Ribaut, J.-M., Betran, J., Monneveux, P. and Setter, T. (2009) 'Drought tolerance in maize', in *Handbook of maize: its biology*. Springer, pp. 311-344.

Richardson, A.D., Duigan, S.P. and Berlyn, G.P. (2002) 'An evaluation of noninvasive methods to estimate foliar chlorophyll content', *New phytologist*, 153(1), pp. 185-194.

Rodriguez, P.A. and Bos, J.I.B. (2013) 'Toward understanding the role of aphid effectors in plant infestation', *Molecular Plant-Microbe Interactions*, 26(1), pp. 25-30.

Rufin, P., Levers, C., Baumann, M., Jägermeyr, J., Krueger, T., Kuemmerle, T. and Hostert, P. (2018) 'Global-scale patterns and determinants of cropping frequency in irrigation dam command areas', *Global Environmental Change*, 50, pp. 110-122.

Rushton, D.L., Tripathi, P., Rabara, R.C., Lin, J., Ringler, P., Boken, A.K., Langum, T.J., Smidt, L., Boomsma, D.D. and Emme, N.J. (2012) 'WRKY transcription factors: key components in abscisic acid signalling', *Plant biotechnology journal*, 10(1), pp. 2-11.

Rushton, P.J., Somssich, I.E., Ringler, P. and Shen, Q.J. (2010) 'WRKY transcription factors', *Trends in plant science*, 15(5), pp. 247-258.

Rushton, P.J., Torres, J.T., Parniske, M., Wernert, P., Hahlbrock, K. and Somssich, I. (1996) 'Interaction of elicitor-induced DNA-binding proteins with elicitor response elements in the promoters of parsley PR1 genes', *The EMBO journal*, 15(20), pp. 5690-5700.

Saidi, A. and Quick, J.S. (1996) 'Inheritance and allelic relationships among Russian wheat aphid resistance genes in winter wheat', *Crop science*, 36(2), pp. 256-258.

Sandström, J., Telang, A. and Moran, N.A. (2000) 'Nutritional enhancement of host plants by aphids—a comparison of three aphid species on grasses', *Journal of Insect Physiology*, 46(1), pp. 33-40.

Satapathy, L., Kumar, D., Kumar, M. and Mukhopadhyay, K. (2018) 'Functional and DNA–protein binding studies of WRKY transcription factors and their expression analysis in response to biotic and abiotic stress in wheat (Triticum aestivum L.)', *3 Biotech*, 8(1), p. 40.

Schenk, P.M., Kazan, K., Wilson, I., Anderson, J.P., Richmond, T., Somerville, S.C. and Manners, J.M. (2000) 'Coordinated plant defense responses in Arabidopsis revealed by microarray analysis', *Proceedings of the National Academy of Sciences*, 97(21), pp. 11655-11660.

Schleussner, C.-F., Deryng, D., Müller, C., Elliott, J., Saeed, F., Folberth, C., Liu, W., Wang, X., Pugh, T.A.M. and Thiery, W. (2018) 'Crop productivity changes in 1.5 C and 2 C worlds under climate sensitivity uncertainty', *Environmental Research Letters*, 13(6), p. 064007.

Scorsetti, A.C., Pelizza, S., Fogel, M.N., Vianna, F. and Schneider, M.I. (2017) 'Interactions between the entomopathogenic fungus Beauveria bassiana and the Neotropical predator Eriopis connexa (Coleoptera: Coccinellidae): Implications in biological control of pest', *Journal of Plant Protection Research*, 57(4), pp. 389-395.

Scott, R.A., Worrall, W.D. and Frank, W.A. (1991) 'Screening for resistance to Russian wheat aphid in triticale', *Crop science*, 31(1), pp. 32-36.

Segarra, G., Jáuregui, O., Casanova, E. and Trillas, I. (2006) 'Simultaneous quantitative LC–ESI-MS/MS analyses of salicylic acid and jasmonic acid in crude extracts of Cucumis sativus under biotic stress', *Phytochemistry*, 67(4), pp. 395-401.

Shen, H., Liu, C., Zhang, Y., Meng, X., Zhou, X., Chu, C. and Wang, X. (2012) 'OsWRKY30 is activated by MAP kinases to confer drought tolerance in rice', *Plant molecular biology*, 80(3), pp. 241-253.

Shen, Q.-H., Saijo, Y., Mauch, S., Biskup, C., Bieri, S., Keller, B., Seki, H., Ülker, B., Somssich, I.E. and Schulze-Lefert, P. (2007) 'Nuclear activity of MLA immune receptors links isolate-specific and basal disease-resistance responses', *science*, 315(5815), pp. 1098-1103.

Shiferaw, B., Smale, M., Braun, H.-J., Duveiller, E., Reynolds, M. and Muricho, G. (2013) 'Crops that feed the world 10. Past successes and future challenges to the role played by wheat in global food security', *Food Security*, 5(3), pp. 291-317.

Singh, K.B., Foley, R.C. and Oñate-Sánchez, L. (2002) 'Transcription factors in plant defense and stress responses', *Current opinion in plant biology*, 5(5), pp. 430-436.

Singh, P.K., Chaturvedi, V.K. and Singh, H.B. (2011) 'Cross talk signalling: an emerging defense strategy in plants', *Current Science*, 100(3), pp. 288-289.

Slosser, J.E., Pinchak, W.E. and Rummel, D.R. (1997) ‘Cotton aphid response to nitrogen fertility in dryland cotton’. *Proceedings of the Beltwide Cotton Conferences*, 2, pp. 1051-1053.

Smith, C.M. and Boyko, E.V. (2007) 'The molecular bases of plant resistance and defense responses to aphid feeding: current status', *Entomologia Experimentalis et Applicata*, 122(1), pp. 1-16.

Smith, C.M. and Chuang, W.P. (2014) 'Plant resistance to aphid feeding: behavioral, physiological, genetic and molecular cues regulate aphid host selection and feeding', *Pest management science*, 70(4), pp. 528-540.

Smith, C.M., Liu, X., Wang, L.J., Liu, X., Chen, M.-S., Starkey, S. and Bai, J. (2010) 'Aphid feeding activates expression of a transcriptome of oxylipin-based defense signals in wheat involved in resistance to herbivory', *Journal of chemical ecology*, 36(3), pp. 260-276.

Socolow, R.H. (1999) 'Nitrogen management and the future of food: lessons from the management of energy and carbon', *Proceedings of the National Academy of Sciences*, 96(11), pp. 6001-6008.

Sonoda, S. and Igaki, C. (2010) 'Characterization of acephate resistance in the diamondback moth Plutella xylostella', *Pesticide biochemistry and physiology*, 98(1), pp. 121-127.

Spoel, S.H. and Dong, X. (2008) 'Making sense of hormone crosstalk during plant immune responses', *Cell host & microbe*, 3(6), pp. 348-351.

Stoger, E., Williams, S., Christou, P., Down, R.E. and Gatehouse, J.A. (1999) 'Expression of the insecticidal lectin from snowdrop (Galanthus nivalis agglutinin; GNA) in transgenic wheat plants: effects on predation by the grain aphid Sitobion avenae', *Molecular Breeding*, 5(1), pp. 65-73.

Taheri, S., Razmjou, J. and Rastegari, N. (2010) 'Fecundity and development rate of the bird cherry-oat aphid, Rhopalosiphum paid. (L) (Hom: Aphidiade) on six wheat cultivars', *Plant Protection Science*, 46(2), pp. 72-78.

Takeda, S. and Matsuoka, M. (2008) 'Genetic approaches to crop improvement: responding to environmental and population changes', *Nature Reviews Genetics*, 9(6), pp. 444-457.

Tamaoki, D., Seo, S., Yamada, S., Kano, A., Miyamoto, A., Shishido, H., Miyoshi, S., Taniguchi, S., Akimitsu, K. and Gomi, K. (2013) 'Jasmonic acid and salicylic acid activate a common defense system in rice', *Plant signaling & behavior*, 8(6), p. e24260.

Tedone, L., Ali, S.A., Verdini, L. and De Mastro, G. (2018) 'Nitrogen management strategy for optimizing agronomic and environmental performance of rainfed durum wheat under Mediterranean climate', *Journal of Cleaner Production*, 172, pp. 2058-2074.

Tester, M. and Langridge, P. (2010) 'Breeding technologies to increase crop production in a changing world', *Science*, 327(5967), pp. 818-822.

ThermoFisherScientific (2018) 'Gel shift assays -EMSA' Available at: https://www.thermofisher.com/uk/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/pierce-protein-methods/gel-shift-assays-emsa.html (Accessed 10 April 2019).

Thomma, B.P.H.J., Nürnberger, T. and Joosten, M.H.A.J. (2011) 'Of PAMPs and effectors: the blurred PTI-ETI dichotomy', *The plant cell*, 23(1), pp. pp. 4-15.

Thompson, G.A. and Goggin, F.L. (2006) 'Transcriptomics and functional genomics of plant defence induction by phloem-feeding insects', *Journal of Experimental Botany*, 57(4), pp. 755-766.

Thompson, G.B., Brown, J.K.M. and Woodward, F.I. (1993) 'The effects of host carbon dioxide, nitrogen and water supply on the infection of wheat by powdery mildew and aphids', *Plant, Cell & Environment*, 16(6), pp. 687-694.

Tito, R., Vasconcelos, H.L. and Feeley, K.J. (2018) 'Global climate change increases risk of crop yield losses and food insecurity in the tropical Andes', *Global change biology*, 24(2), pp. e592-e602.

Tolmay, V.L., van der Westhuizen, M.C. and van Deventer, C.S. (1999) 'A six week screening method for mechanisms of host plant resistance to Diuraphis noxia in wheat accessions', *Euphytica*, 107(2), pp. 79-89.

Turck, F., Zhou, A. and Somssich, I.E. (2004) 'Stimulus-dependent, promoter-specific binding of transcription factor WRKY1 to its native promoter and the defense-related gene PcPR1-1 in parsley', *The Plant Cell*, 16(10), pp. 2573-2585.

Uauy, C., Paraiso, F., Colasuonno, P., Tran, R.K., Tsai, H., Berardi, S., Comai, L. and Dubcovsky, J. (2009) 'A modified TILLING approach to detect induced mutations in tetraploid and hexaploid wheat', *BMC plant Biology*, 9(1), p. 115.

van de Ven, W.T.G., LeVesque, C.S., Perring, T.M. and Walling, L.L. (2000) 'Local and systemic changes in squash gene expression in response to silverleaf whitefly feeding', *The Plant Cell*, 12(8), pp. 1409-1423.

Van den Berg, A.K. and Perkins, T.D. (2004) 'Evaluation of a portable chlorophyll meter to estimate chlorophyll and nitrogen contents in sugar maple (Acer saccharum Marsh.) leaves', *Forest Ecology and Management*, 200(1-3), pp. 113-117.

Van der Ent, S., Van Hulten, M., Pozo, M.J., Czechowski, T., Udvardi, M.K., Pieterse, C.M.J. and Ton, J. (2009) 'Priming of plant innate immunity by rhizobacteria and β-aminobutyric acid: differences and similarities in regulation', *New Phytologist*, 183(2), pp. 419-431.

Van Eck, L., Davidson, R.M., Wu, S., Zhao, B.Y., Botha, A.-M., Leach, J.E. and Lapitan, N.L.V. (2014) 'The transcriptional network of WRKY53 in cereals links oxidative responses to biotic and abiotic stress inputs', *Functional & integrative genomics*, 14(2), pp. 351-362.

Van Loon, C.D. (1981) 'The effect of water stress on potato growth, development, and yield', *American Potato Journal*, 58(1), pp. 51-69.

Van Loon, L.C. and Van Strien, E.A. (1999) 'The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins', *Physiological and molecular plant pathology*, 55(2), pp. 85-97.

Van Verk, M.C., Pappaioannou, D., Neeleman, L., Bol, J.F. and Linthorst, H.J.M. (2008) 'A novel WRKY transcription factor is required for induction of PR-1a gene expression by salicylic acid and bacterial elicitors', *Plant Physiology*, 146(4), pp. 1983-1995.

Vereijken, P.H. (1979) *Feeding and multiplication of three cereal aphid species and their effect on yield of winter wheat*. PhD thesis. Wagenigen University

Wagan, T.A., Brohi, R., Hamada, C., Tunio, S.K. and Wagan, Z.A. (2015) 'Effect of different nitrogen fertilizer levels on aphid population and its natural enemies in winter grown wheat', *Wudpecker Journal of Agriculture Resarch*, 4(4), pp. 61-65.

Walling, L.L. (2000) 'The myriad plant responses to herbivores', *Journal of Plant Growth Regulation*, 19(2), pp. 195-216.

Wang, D., Amornsiripanitch, N. and Dong, X. (2006) 'A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants', *PLoS pathogens*, 2(11), p. e123.

Wang, W., Dai, H., Zhang, Y., Chandrasekar, R., Luo, L., Hiromasa, Y., Sheng, C., Peng, G., Chen, S. and Tomich, J.M. (2015a) 'Armet is an effector protein mediating aphid–plant interactions', *The FASEB Journal*, 29(5), pp. 2032-2045.

Wang, W., Vinocur, B. and Altman, A. (2003) 'Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance', *Planta*, 218(1), pp. 1-14.

Wang, X., Zeng, J., Li, Y., Rong, X., Sun, J., Sun, T., Li, M., Wang, L., Feng, Y. and Chai, R. (2015b) 'Expression of TaWRKY44, a wheat WRKY gene, in transgenic tobacco confers multiple abiotic stress tolerances', *Frontiers in plant science*, 6, p. 615.

Wang, Y., Wang, H., Ma, Y., Du, H., Yang, Q. and Yu, D. (2015c) 'Identification of transcriptional regulatory nodes in soybean defense networks using transient co-transactivation assays', *Frontiers in plant science*, 6, p. 915.

Wang, Z., Yang, P., Fan, B. and Chen, Z. (1998) 'An oligo selection procedure for identification of sequence-specific DNA-binding activities associated with the plant defence response', *The Plant Journal*, 16(4), pp. 515-522.

Waraich, E.A. and Ahmad, R. (2010) Physiological responses to water stress and nitrogen management in wheat (Triticum aestivum L.): evaluation of gas exchange, water relations and water use efficiency.*Fourteenth International Water Technology Conference (IWTC 14). Cairo, Egypt*, pp. 731-748.

Will, T., Tjallingii, W.F., Thönnessen, A. and van Bel, A.J.E. (2007) 'Molecular sabotage of plant defense by aphid saliva', *Proceedings of the National Academy of Sciences*, 104(25), pp. 10536-10541.

Wu, K.-L., Guo, Z.-J., Wang, H.-H. and Li, J. (2005) 'The WRKY family of transcription factors in rice and Arabidopsis and their origins', *DNA research*, 12(1), pp. 9-26.

Xu, L., Duan, X., Lv, Y., Zhang, X., Nie, Z., Xie, C., Ni, Z. and Liang, R. (2014) 'Silencing of an aphid carboxylesterase gene by use of plant-mediated RNAi impairs Sitobion avenae tolerance of Phoxim insecticides', *Transgenic research*, 23(2), pp. 389-396.

Xu, L., Hou, Q., Zhao, Y., Lu, L., Li, B., Ni, Z. and Liang, R. (2017) 'Silencing of a lipase maturation factor 2-like gene by wheat-mediated RNAi reduces the survivability and reproductive capacity of the grain aphid, Sitobion avenae', *Archives of insect biochemistry and physiology*, 95(3), p. e21392.

Yang, P., Chen, C., Wang, Z., Fan, B. and Chen, Z. (1999) 'A pathogen-and salicylic acid-induced WRKY DNA-binding activity recognizes the elicitor response element of the tobacco class I chitinase gene promoter', *The Plant Journal*, 18(2), pp. 141-149.

Yasuda, M., Ishikawa, A., Jikumaru, Y., Seki, M., Umezawa, T., Asami, T., Maruyama-Nakashita, A., Kudo, T., Shinozaki, K. and Yoshida, S. (2008) 'Antagonistic interaction between systemic acquired resistance and the abscisic acid–mediated abiotic stress response in Arabidopsis', *The Plant Cell*, 20(6), pp. 1678-1692.

Yates, A.D. and Michel, A. (2018) 'Mechanisms of aphid adaptation to host plant resistance', *Current opinion in insect science*, 26, pp. 41-49.

Yu, D., Chen, C. and Chen, Z. (2001) 'Evidence for an important role of WRKY DNA binding proteins in the regulation of NPR1 gene expression', *The Plant Cell*, 13(7), pp. 1527-1540.

Zebitz, C.P.W. and Kehlenbeck, H. (1991) 'Performance ofAphis Fabae on Chocolate Spot Disease-Infected Faba Bean Plants', *Phytoparasitica*, 19(2), pp. 113-119.

Zeng, L.-R., Qu, S., Bordeos, A., Yang, C., Baraoidan, M., Yan, H., Xie, Q., Nahm, B.H., Leung, H. and Wang, G.-L. (2004) 'Spotted leaf11, a negative regulator of plant cell death and defense, encodes a U-box/armadillo repeat protein endowed with E3 ubiquitin ligase activity', *The Plant Cell*, 16(10), pp. 2795-2808.

Zhang, M., Zhou, Y., Wang, H., Jones, H.D., Gao, Q., Wang, D., Ma, Y. and Xia, L. (2013) 'Identifying potential RNAi targets in grain aphid (Sitobion avenae F.) based on transcriptome profiling of its alimentary canal after feeding on wheat plants', *BMC genomics*, 14(1), p. 560.

Zhang, X., Davidson, E.A., Mauzerall, D.L., Searchinger, T.D., Dumas, P. and Shen, Y. (2015) 'Managing nitrogen for sustainable development', *Nature*, 528 (7580), pp. 51-59.

Zhang, Y., Fan, J., Francis, F. and Chen, J. (2018) 'Molecular characterization and gene silencing of Laccase 1 in the grain aphid, Sitobion avenae', *Archives of insect biochemistry and physiology*, 97(4), p. e21446.

Zhang, Y. and Wang, L. (2005) 'The WRKY transcription factor superfamily: its origin in eukaryotes and expansion in plants', *BMC evolutionary biology*, 5(1), p. 1.

Zhang, Z.-L., Xie, Z., Zou, X., Casaretto, J., Ho, T.-h.D. and Shen, Q.J. (2004) 'A rice WRKY gene encodes a transcriptional repressor of the gibberellin signaling pathway in aleurone cells', *Plant Physiology*, 134(4), pp. 1500-1513.

Zhou, X. and Carter, N. (1991) 'The effects of nitrogen and fungicide on cereal aphid population development and the consequences for the aphid-yield relationship in winter wheat', *Annals of applied biology*, 119(3), pp. 433-441.

Zhu, X., Liu, S., Meng, C., Qin, L., Kong, L. and Xia, G. (2013) 'WRKY transcription factors in wheat and their induction by biotic and abiotic stress', *Plant Molecular Biology Reporter*, 31(5), pp. 1053-1067.

Zuo, K.-J., Qin, J., Zhao, J.-Y., Ling, H., Zhang, L.-D., Cao, Y.-F. and Tang, K.-X. (2007) 'Over-expression GbERF2 transcription factor in tobacco enhances brown spots disease resistance by activating expression of downstream genes', *Gene*, 391(1), pp. 80-90.