Transcriptional and Physiological Responses of Wheat *Triticum aestivum* to a Combination of Abiotic and Biotic Stress

By

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Abstract

Plant responses to simultaneous biotic and abiotic stresses involve the activation of multiple signalling pathways that often interact in a synergistic or an antagonistic manner. Recent studies have shown that the plant response to a combination of stresses differ to those that occur when exposed to a single stress. The aim of the present study is to investigate the response of wheat (*Triticum aestivum*) to both salinity and aphid *Sitobion avenae* infestation, applied singly and in combination, at the physiological and transcriptional levels, to provide a better understanding of the impact of abiotic and biotic interactions and cross tolerance in wheat. These two forms of stress were selected since they are known to have a major impact on crop productivity. Wheat genotype 122-1 was shown to be tolerant to salt (160 mM NaCl) on the basis of biomass; accumulating high levels of Na^+ in the shoots and was partially resistant to aphids in terms of fecundity. Pretreatment of this genotype with salt significantly ($p<0.001$) reduced aphid fecundity (by 37%) relative to its control, indicating enhanced resistance to aphids. This positive interaction between salt and aphid stress was investigated at 6h and 24h post aphid infestation on the wheat transcriptome. Microarray analysis indicated common and specific gene expression patterns of the 61.290 transcripts differentially regulated in response to salt stress alone, aphid infestation alone and dual stress compared to the controls. Dual stress specifically and strongly increased the transcription level of the following genes assigned to jasmonate synthesis encoding lipoxygenase (LOX), abiotic stress (heat), miscellaneous enzyme families (acid and other phosphatases) at 6h, and secondary metabolism (phenylpropanoids) at 24h. Furthermore, based on functional classification analysis, several categories which were shown to be significantly activated by dual stress such as cytokinin hormone synthesis and MAP kinases signalling were not, however, significantly changed under either salt stress or aphid infestation alone. The current study demonstrated that jasmonate hormone signalling pathways antagonized those of salicylic acid under dual stress and aphid infestation at 6 h. Results suggests that the interaction between combined salinity and aphids stresses caused distinct alteration in gene expression patterns that could not be detected under either of the two stresses when applied individually. This study proposes that the activation of specific genes involved in the acquisition of defence/tolerance, such as those encoding cytochrome P450 and MYB domain transcription factor families, due to prior exposure to salt may enhance subsequent tolerance to aphids. The present study sheds light on candidate genes with putative functions in the crosstalk and the acquisition of cross tolerance and provides new insights on wheat response to multiple stress conditions. Such information is a prerequisite for enhancing crop tolerance to a broad-spectrum of stress.

In the Name of God, the Most Gracious, the Most Merciful.

This Thesis is dedicated to my family and parents

for their endless love, support and encouragement.

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Thana Khan

Table of Contents

List of Figures

List of Tables

List of Abbreviations

ABA	Abscisic acid
ABF	ABA-responsive element binding factor family
ABRE	ABA-responsive element family
AOS	Allene oxide synthase
BYDV	Barley yellow dwarf virus
CBF/DREB	C-repeat-binding factor /dehydration-responsive element binding
CDNA	Complementary DNA
CDPKs	Calcium-dependent protein kinases
CYP	Cytochrome P450
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
Dsm^{-1}	Decisiemens per meter
EC	Electrical conductivity
ERF1	Ethylene response factor 1
ET	Ethylene
FAO	Food and Agriculture Organization of the UN
HS	Heat shock protein
JA	Jasmonic acid
LEA	Late embryogenesis abundant protein
LOX	lipoxygenase
MAPK	Mitogen activated protein kinases
Misc	Miscellaneous
NHX1	Sodium/hydrogen exchanger 1 Na(+)/H(+) exchanger 1
Ns	Not significant
OP	Osmotic potential
OPR	12-oxophytodienoate reductase
PFIs	Phloem-feeding insects
PR	Pathogenesis-related protein
QRT-PCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RWC	Relative water content
SA	Salicylic acid
SOS	Salt overly sensitive pathways
SPAD	Special products analysis division (a division of Minolta)
SR	Stress responsive genes
TFs	Transcription factors
TW	Turgid weight
USP	Universal stress protein

List of Equations

CHAPTER 1

1 General Introduction

1.1 Enhancing crop tolerance to stress

There are several reasons that have driven the necessity for ensuring global food security thus increasing pressure on the demand for sustainable crop production (Takeda and Matsuoka, 2008). Firstly, the world population is increasing at an exponential rate, with conservative estimations forecasting that the population will grow to approximately 9-10 billion by 2050 (United Nations Population Division). Thus, one of the main challenges facing the world is the ability to provide sufficient amounts of food to feed an added 2.3 billion people (Chrispeels and Sadava, 2003: Edwards and Gatehouse, 2007). The FAO estimated that by 2050 food production must rise by 70%; this requires crop yields to increase by about 50% in a sustainable manner in order to meet the world food requirements (Ladeiro, 2012). Secondly, the world's existing land for crop cultivation is limited, and thus to increase the amount of food production there is either the option of increasing the agricultural foot print through utilization of uncultivated areas which causes negative impacts such as loss of environmental resources and natural habitat and contaminating soil and ground water or growing crops more efficiently (Ferry and Gatehouse, 2009). Thirdly, crops yield and quality are affected by climate change directly, and indirectly. It is predicted that the earth's surface temperature may rise by 3-5°C over the next 50-100 years, leading to various severe conditions (Newton *et al*., 2011). For instance, alteration in rainfall and temperature levels causes drought and heat waves which in turn increases hot arid areas. Also, rising sea levels lead to floods and widespread salinization. Therefore, climate change affects crop growing periods, reduces appropriate lands for agriculture and exacerbates the effects of biotic and abiotic damage, especially when occurring concurrently (Mittler and Blumwald, 2010: Atkinson and Urwin, 2012). Moreover, it is suggested that novel and unpredictable stress conditions are likely to be encountered by plants and occur in the agricultural system of many parts of the world, presenting new challenges in producing multiple stress-tolerant crops (Easterling *et al*., 2000; Newton *et al*., 2011).

A key solution to overcome these constraints is to improve crop plant tolerance to abiotic and biotic stress, as well as enhancing their adaptation to climate change through genetic manipulation and conventional breeding (Takeda & Matsuoka, 2008). The development of plant molecular genetics and the application of genetic engineering technologies have provided new awareness and approaches to address these challenges (Ronald, 2011). Over the last decade the incorporation of genetically engineered crops into cultivation practices and agronomic systems have significantly contributed to agricultural sustainability worldwide, and have proved to be an effective approach to global food security (Ferry and Gatehouse, 2009). Currently, however, limitation in improving tolerance to abiotic stress in cereal crops has been associated with the absence of efficient screening methods, availability of germplasm with desired traits, and lack of understanding of the underlying molecular basis of abiotic stress tolerance in plants (Powell *et al.,* 2012*;* Spiertz, 2012). Therefore, a pre-requisite for improving crop tolerance to stress is to provide targets and avenues for exploitation. To achieve this goal it is fundamental to understand the molecular regulatory networks induced by plants in response to different stress conditions and the interaction between combinations or multiple stresses.

Biotic and abiotic stresses have a huge impact on world agriculture, limiting plant growth and crop productivity. Environmental or abiotic stress such as heat, cold, drought, salinity and nutrient deficiency are suggested to reduce average potential yields by >50% for most major crop plants (Wang *et al*., 2003). Salinity is one of the most commonly occurring global abiotic stresses, which affects cultivation of crop species such as wheat (Prasch and Sonnewald, 2013). Further to this, crop plants must protect themselves from attack by biological or biotic stresses including various arrays of pests, pathogens and herbivorous insects (Atkinson and Urwin, 2012). Crop losses due to insect herbivores is estimated at 10-20% for major crops (Ferry *et al*., 2004), while other studies suggest that damage can be as high as 40% globally (Edwards and Gatehouse, 2007).

1.2 Wheat: (*Triticum aestivum*)

Wheat (*Triticum aestivum* L.) is globally one of the three most important cereals and grain crops and together with rice and maize dominates world agriculture. The cultivation of wheat (*Triticum* s) reaches far back into history as it was one of the first domesticated food crops. The majority of wheat is cultivated in the temperate climate zones of the world (Kawaura *et al*., 2006). Wheat has been the basic staple food for

8000 years and continues to be the major food grain crop consumed by humans (Curtis *et al*., 2002). World wheat production is now averaging nearly 600 million tonnes annually, yet, production measures are more critical due to greater impact of abiotic and biotic stresses. Future prediction is that the annual yield will need to increase by 2.5 percent per year to fulfil the demand for food due to rapid population growth by 2025 (Fig 1.1). Although wheat is cultivated on more areas than any other commercial crop; the amount of agricultural land is finite. Therefore, increasing wheat production will depend on exploiting the available cultivated land by producing higher yields per unit area, through improving cultivars and enhancing agricultural practices (Curtis *et al*., 2002).

Bread wheat has a large genome estimated at 16000 Mb (Lagudah *et al*., 2001) and is a hexaploid species (6x) which regularly forms 21 pairs of chromosomes (2n = 42) during meiosis, comprising three different ancestral genomes (termed A, B, and D). Each of these homoeologous groups normally contains 7 pairs of chromosomes (AABBDD) (Francki and Apples, 2002). It has been reported that wheat is considered as a model for the growth habits and genome structure of gramineous plants (Kawaura *et al*., 2006). The primary use of bread wheat is for bread manufacture and the whole grain product is a source of essential amino acids, minerals, vitamins, beneficial phytochemicals and dietary fibre components to the diet (Shewry, 2009).

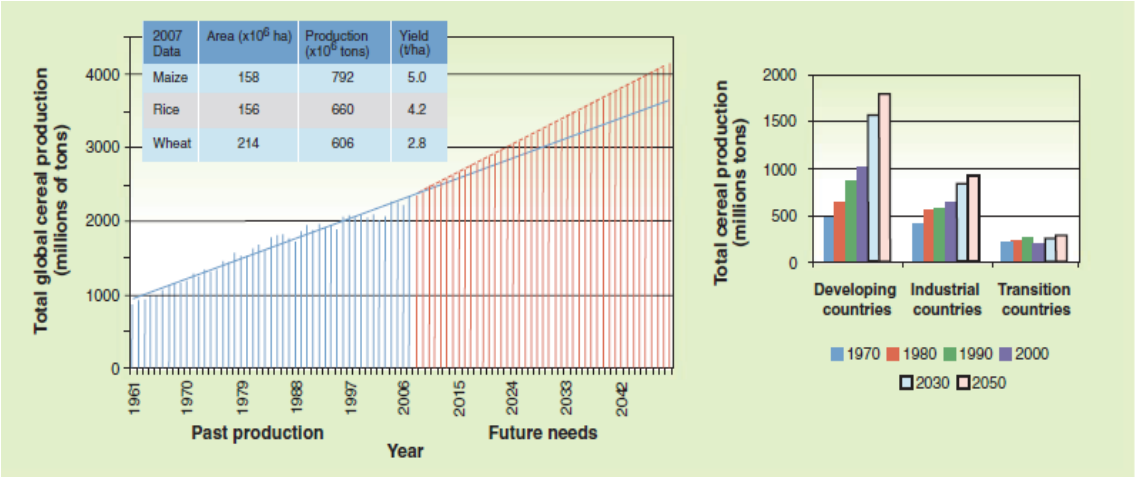


Figure 1.1 Cereal production targets. FAO: http://faostat.fao.org/
To meet predicted demands, production will need to rise > 4000 million metric tons by 2050 (red). The rate of yield increase must move from the blue trend line (32 million metric tons per year) to the red dotted line (44 million metric tons per year) to meet this demand, an increase of 37%.

1.3 Salinity (abiotic stress)

Soil salinity is one of the most devastating environmental stresses (Wang *et al*., 2009). It is estimated that about 20% of total cultivated lands and 33% of irrigated agricultural lands worldwide are affected by high salinity (Jamil *et al*., 2012). Furthermore, salinization areas are increasing at a rate of 10% annually for various reasons, including low precipitation, high surface evaporation, weathering of native rocks, irrigation with saline water, and poor agricultural practices (Jamil *et al*., 2012). It is estimated that more than 50% of the world's arable land could be salinized by the year 2050, unless some correction procedures are applied (Ashraf, 2009). Saline soil is generally defined as having electrical conductivity (EC) of saturation extract in the root zone of more than 4 dsm^{-1} (~40 mM NaCl) at 25°C and has 15% of exchangeable sodium. The yield of most crop plants is reduced at lower ECs (Munns, 2005). There are two types of salinity. Primary or natural salinity, which is caused by salty rain water near and around the coast as well as from contamination from rocks and oceanic salts (Turkan and Demiral, 2009). Secondary or human-induced salinity is caused by clearing poor drainage and irrigation (Munns 2005). Sodium chloride ions constitute the majority of the salt in the soil which are toxic to plant cells when present at high concentrations, both externally and internally.

1.3.1 Effects of salinity on plants

Plants are traditionally classified as glycophytes or halophytes according to their ability to grow in and withstand salinized environments (Turkan and Demiral, 2009). Glycophytes, which comprises most plants including the major crops, cannot tolerate salt stress as their growth is severely inhibited or even destroyed by 100-200 mmol L^{-1} NaCl, and they tend to exclude salt. By contrast, halophytes constitute the flora of high salinity environments as they can survive salinity in excess of 300 mmol L^{-1} and some can tolerate levels as high as 700-1020 mmol L^{-1} NaCl (Zhu, 2007). Halophytes have the ability to compartmentalize the high levels of salt accumulated in the cell into vacuoles, thus, protecting cytosolic enzymes from damage.

The main effect of salinity on plants is growth inhibition; other general symptoms of salt-induced damage which occurs during prolonged exposure include accelerated development, senescence and programmed cell death. Furthermore, photosynthesis declines and oxidative stress occurs due to salt induced increase in abscisic acid production which causes stomatal closure and inhibition of cell expansion (Zhu, 2007).

It has been suggested that salinity imposes two effects that cause two phases of plant growth reduction. Firstly, osmotic or water-deficit effects are caused by the presence of salt in the soil which inhibits the plant's ability to take up water, resulting in a reduced plant growth rate (phase 1). In this phase, plant cellular and metabolic responses resemble those of drought-affected plants and ions such as Na^+ or Cl^- do not accumulate in the growing tissues. Meristematic tissues and elongating cells are protected from salt by effectively excluding salt from the phloem and sequestering salt that arrives in the xylem within vacuoles. Also leaf growth has been shown to be more reduced than root growth in this first phase of plant response to salt. Secondly, an ion toxicity effect is caused by the presence of salt inside the plant (salt-specific), which leads to cell injury, dehydration, nutrient imbalance and inhibition of enzyme activity, resulting in a further growth reduction (phase 2) (Munns, 2005). In this second phase, salt entering the plant through the transpiration stream is stored in old leaves, resulting in high levels of Na^+ and Cl^- ions after prolonged periods. This may exceed the plant's ability to compartmentalize the excess amount of salt into vacuoles (Munns *et al*., 2006). Thus, it is important for the plant to maintain low sodium/potassium ratios by increasing a selective high-affinity potassium uptake in order to maintain cell turgor, membrane potential and enzyme activities. Failure to do so causes potassium deficiency, which inhibits growth (Zhu, 2007). Moreover, another important factor in regulating the expression and activity of potassium and sodium transporters is calcium. Increased calcium supply has a protective effect on plant under salt stress by sustaining potassium transport and potassium/sodium selectivity (Hasegawa *et al*., 2000; Hussain *et al*., 2010). Another subsequent salinity effect is the induction of oxidative stress which is considered as a secondary effect besides the two previous effects, osmotic and ionic which are considered as primary effects (Ashraf, 2004).

1.3.2 Mechanism of salt tolerance

Salt tolerance is defined as the ability to sustain plant growth in a soil environments affected by NaCl (Gregorio *et al*., 1997). Genetic analysis of plant responses to salt and drought has shown that maintenance of a low concentration of sodium in the cytoplasm is a key indicator of plant tolerance to salt (Zhu, 2002; 2007). Plants possess two main mechanisms to tolerate salt-specific effects (ion toxicity). The first is salt exclusion, which minimizes the entry of salt into the plant and lowers salt accumulation in leaves. Most plants exclude about 98% of the salt in the soil solution, allowing only 2% to be transported in the xylem to the shoots. It has been documented that cereal genotypes

showed contrasting rates of Na^+ uptake when growing in 50 mM NaCl. Bread wheat has been shown to exclude > 98% of the Na^+ in the soil solution from its tissue; the concentrations does not build up in leaves to more than 50 mM NaCl (Munns, 2005). The second mechanism is salt inclusion or tissue tolerance, which minimizes the concentration of salt in the cytoplasm by compartmentalizing the salt into vacuoles (Flowers, 2004). The salt tolerance index is usually determined by measuring the percent of plant biomass production in saline soil relative to plant biomass in non-saline soil, after exposure to salt for a prolonged period of time. Other parameters used for assessing salt tolerance include yield of crops in saline versus non-saline conditions and the percent of survival for slow-growing, long-lived, or uncultivated plant species.

Wheat, rice and maize, which are probably the three most important crops in the world, show different growth responses to salinity. Wheat (*Triticum aestivum*) shows more tolerance to salt among these crops species as many wheat cultivars maintain 50% growth in biomass under salinity conditions up to approximately 150 mM NaCl (Munns *et al*., 2006). Rice is more salt-sensitive, and many cultivars suffer a 50% reduction in growth at half the above salt concentration. Maize falls in between these two species in terms of salt sensitivity. Bread wheat is considered as a moderately salt tolerant crop as it is able to produce a reduced yield in the field with salinity up to levels of 100 mM NaCl (about 10 dS m^{-1}). In contrast, durum wheat (*Triticum turgidum* ssp. durum) is less tolerant than bread wheat (Munns *et al*., 2006).

1.3.3 Molecular responses to salinity

Evidence from molecular studies on the mechanism of abiotic stress responses and tolerance confirmed the complexity of plant adaptation to abiotic stess including salinity, which involves the interaction of various genes, proteins, metabolic and signalling pathways (Fig 1.2) (Zhu, 2000; Ashraf, 2009). Salt stress triggers a dynamic regulation of gene expression. For instance, salt has been shown to induce the activation of phosphorylation and kinase cascades followed by increased abundance of various transcription factors (Jamil *et al*., 2012). The latter regulate the expression of genes that are associated with several functional categories including, genes associated with transport to control salt uptake, genes that have osmotic or protective activity, and genes maintaining and accelerating plant development (Munns, 2005). Studies have identified genes and proteins conferring tolerance to salinity and drought, which have roles in the following activities: ion and water transporters such as SOS1 antiporter, Na^+/H^+

antiporter such as NHX1, encoding protein families (e.g. heat-shock proteins, chaperones, late embryogenesis abundant (LEA) protein), detoxifying enzymes, transcription factors (heat shock factor (HS), the C-repeat-binding factor /dehydration-responsive element binding protein (CBF/DREB) and ABA-responsive element binding factor/ABA-responsive element (ABF/ABRE) families and signalling cascades (salt overly sensitive pathways (SOS), kinases, phospholipases and mitogen activated protein kinases (MAPK) cascades). The latter MAPK activates transcription factors which lead to the accumulation of osmolyte and osmoprotectants (Jamil *et al*., 2012). Evidence from salinity microarray studies have demonstrated the following impacts on different biological processes after plant exposure to salt stress, a decrease in the expression level of transcripts involved in photosynthesis, energy metabolism and protein synthesis, and increase of those in transporters, osmoprotective, stress-signalling, hydrophilic and antioxidative response (Deyholos, 2010).

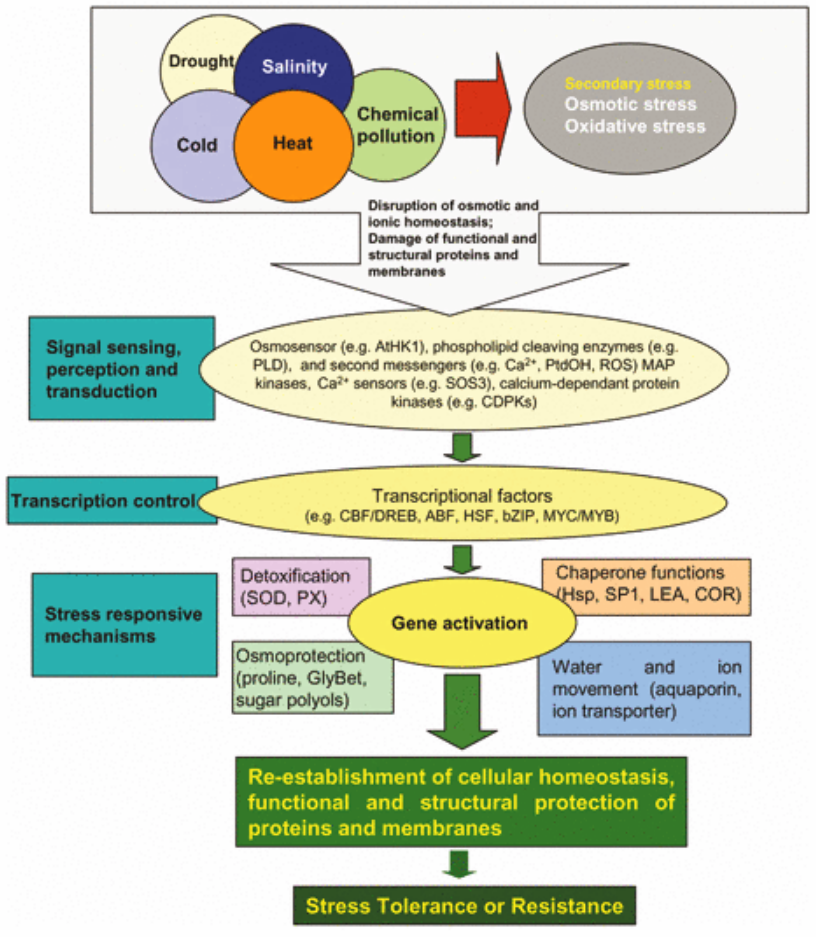


Figure 1.2 The complexity of the plant response to abiotic stress. (Gatehouse and Ferry personal communication).

1.4 The English grain aphid (*Sitobion avenae*)

Aphids (order Hemiptera: family Aphididae) are major pests of agriculture worldwide causing crop damage and growth reduction by removing photoassimilates, manipulating growth and nutrient partitioning, and vectoring plant viruses (Smith and Boyko, 2007). The English grain aphid *Sitobion avenae* is one of the most important insect pests causing substantial yield losses in wheat and other cereals (Liu, 2011). The main method to control this aphid is the application of chemical pesticides. However, chemical control causes negative impacts on agroecosystems and can lead to the evolution of insect resistance to pesticides. Many pest aphid species and several hundred other insect pests are considered resistant to insecticides (Smith and Boyko, 2007). Aphids are the largest group of insect phloem feeders which are specialized to consume phloem sap. During feeding, aphids use their stylets which are slender narrow piercing-sucking mouthparts to penetrate tissues through a primarily intercellular route including epidermal, mesophyll, and parenchyma cells towards vascular tissues to reach the phloem. Aphids are able to maintain feeding by ingesting phloem sap from a single sieve tube for a prolonged period of time, up to hours or even weeks (Thompson and Goggin, 2006). Comparatively little tissue damage is caused by this specific feeding mode (Ferry *et al*., 2002; De Vos *et al*., 2007). However, probing still causes cell wall and plasma membrane disruption, and penetration of epidermal, mesophyll, and parenchyma cells (Thompson and Goggin, 2006; Ferry *et al*., 2011). Also, the degree of injury occurring during probing varies considerably among phloem feeding insect species. Limited local induction of proteinase inhibitors and other wound-responsive transcripts have been observed in plant responses to phloem feeding insect infestation (Zhu-Salzman *et al*., 2004; Kempema *et al*., 2007). It has been suggested that the impact of aphids on their hosts causes the withdrawal of assimilates and infection of saliva which contains numerous enzymes such as oxidases, pectinase, and cellulases (Goggin, 2007).

1.4.1 Plant responses to insect herbivore

Plants use both constitutive (direct and indirect) and induced defence mechanisms against pathogen and herbivore attack. Constitutive defence is species-specific and includes physical barriers such as cell walls, suberin, callose and cuticle which act to prevent pathogen or arthropod access to tissue. Direct defence utilizes stored defence chemicals (allelochemicals) which act to deter herbivore colonization of the plant

(antixenotic effect) or to deter herbivore growth, development, fecundity, and survival (antibiosis effect). Indirect defence, on the other hand, activates the interactions with natural enemies' predators and parasitoids against damaging herbivores via releasing volatile organic compounds (VOCs) (Walling, 2008). Induced defence which has aspects common to all plants is activated by herbivores both locally and systemically. This includes several well characterized plant defence compounds produced via secondary metabolism, wound response (proteinase-inhibitors), and signalling pathways such as systemin, jasmonate, oligogalacturonic acid and hydrogen peroxide which change gene expression and activate volatiles synthesis (Fig 1.3) (Walling, 2000; Gatehouse, 2002).

1.4.2 Molecular responses to insect herbivore

In general insect feeding causes major tissue damage, and induces a wounding response which is mediated by jasmonic acid, resulting in the synthesis of defensive proteinase inhibitors and polyphenol oxidases (Ferry *et al* 2011); this is particularly true for chewing insects. Plant responses to aphid attack are similar to the pathogen response, which is known as a gene-for-gene interaction. Responses are based on aphid-derived elicitors and are mediated by the signalling molecule salicylic acid (SA) (Walling, 2000; Moran *et al*., 2002; Smith and Boyko, 2007). However, aphids can also induce the expression of genes that are up-regulated by wounding due to cross-talk. Moran and Thompson (2001) demonstrated that green peach aphid (*Myzus persicae*) feeding on *Arabidopsis* induced the expression of salicylic acid (SA) genes which are associated with response to pathogens, as well as genes involved in the jasmonic acid mediated response pathway. These results suggest the stimulation of response pathways involved in both pathogen and herbivore responses. Extensive gene reprogramming in the plant has been shown to occur in the plant responses to aphid herbivores (Moran and Thompson, 2001). Recent transcript profiling studies indicate that phloem feeding insects induce transcriptional reprogramming in their host plants which include cell wall modifications, reduced photosynthetic activity, manipulation of source–sink relations, and modification of secondary metabolism. Many of these responses appear to occur within the phloem tissue. Moreover, microarray and macroarray data have identified genes involved in oxidative stress, calcium-dependent signalling, pathogenesis-related responses, and signalling as key components of the induced response (Moran *et al*., 2002). Plant responses to these insects appear to be regulated in part by the salicylate, jasmonate, and ethylene signalling pathways (Thompson and Goggin, 2006). Genes

involved in plant defence against insects have been shown to encode products that are either toxic to insects such as proteinase inhibitors or have the capacity to produce toxins such as enzymes involved in secondary metabolism (Gatehouse, 2002).

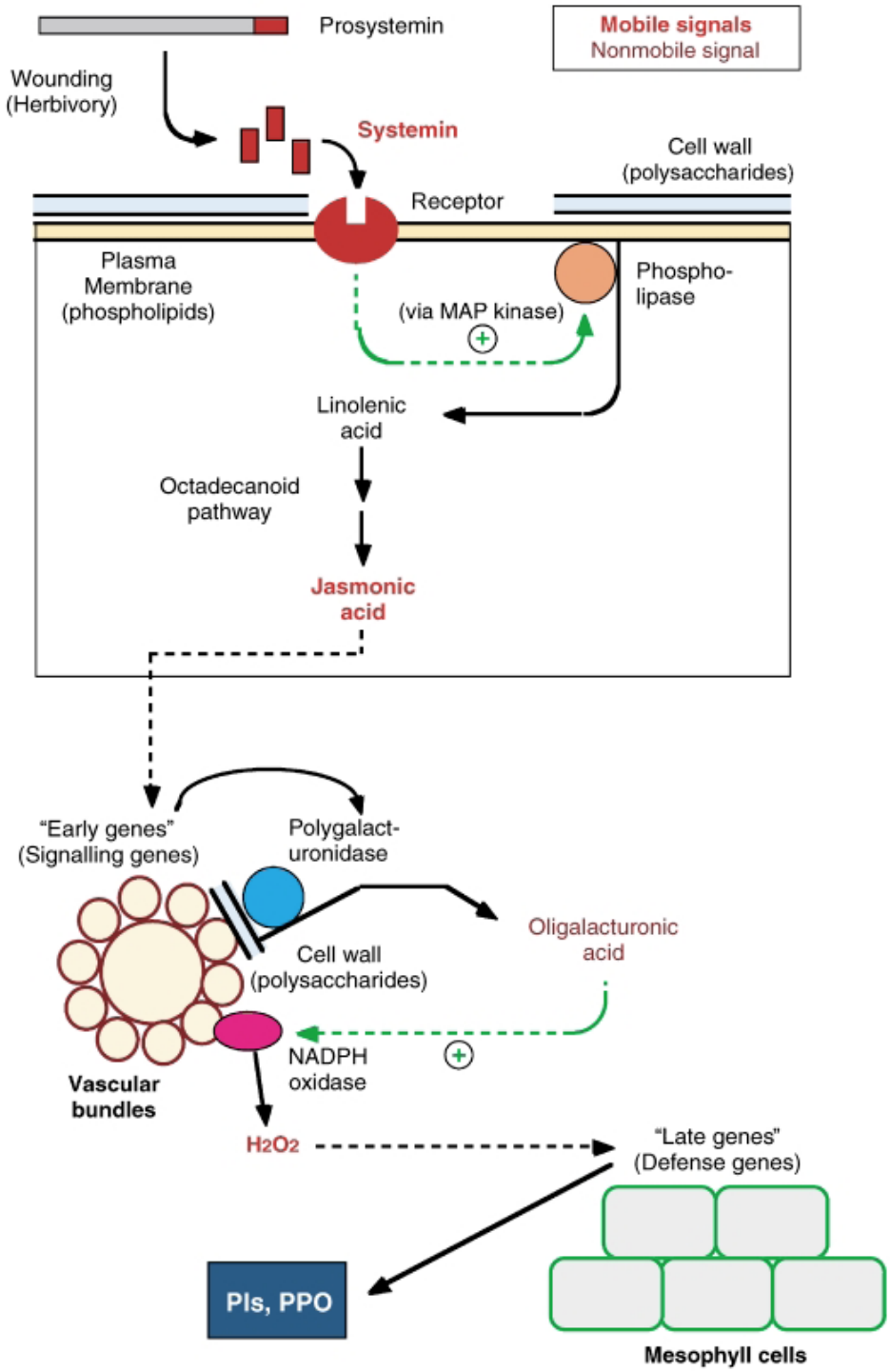


Figure 1.3 Schematic diagram of the signalling pathway necessary for local and systemic synthesis of the insecticidal proteins proteinase inhibitor (PI) and polyphenol oxidase (PPO) in the wounding response in tomato. Systemin is proposed to act as the systemic signal in this model, although evidence to suggest that jasmonate can also act systemically has been presented (Gatehouse, 2002).

1.5 Plant response to combinations of stress

Plants in their natural habitat and field conditions are often simultaneously exposed to various stress conditions (Prasch and Sonnewald, 2013) which, if severe,can adversely affect plant growth and crop productivity (Ahuia *et al*., 2010; Mittler and Blumwald, 2010). A growing body of research on plant responses to stress under field and laboratory conditions has revealed unique molecular changes in plants exposed to multiple stresses. These distinct responses are often different to plant responses to individual stress and cannot be detected from studies that applied either stress in isolation (Rizhsky *et al.,* 2004; Mittler, 2006; Atkinson and Urwin, 2012). For example, Mittler (2006) found that a combination of drought and heat stress heightened the total agriculture losses in the US compared to that caused by drought only, confirming the great severity of stress effects on plants when the two stresses are combined. Mittler also highlights the importance of studying the effect of different stress simultaneously which should be treated and considered as an entirely new set of stresses (Mittler, 2006). Other studies demonstrated opposing reactions elicited by one of the two combined stress. For example, a common plant adaptation to heat stress is opening stomata to reduce heat. However, when heat stress is combined with drought stress this response would be a disadvantage due to increased water loss (Rizhsky *et al.*, 2004). Likewise, under heat stress, an increase in transpiration requires more water uptake, but, in the presence of heavy metals this response would raise the uptake of heavy metals leading to more detrimental stress effects (Mittler and Blumwald, 2010). It is proposed that the cost of defence in terms of balancing resource allocation between growth/yield and stress defence is likely to be reduced if the plant utilizes specific genes and compounds that have roles in several different stress responses. For instance, studies identified and characterized some molecules such as those implicated in signalling pathways, transcription factors, effector proteins and secondary metabolites including flavonoids which are induced under biotic and abiotic stress and have been shown to confer resistance towards various stresses (Atkinson and Urwin, 2012). Despite increasing evidence of a specific stress response when subjected to a combination or multiple stress conditions (Rizhsky *et al*., 2004), little is known about the molecular mechanisms underlying plant responses to stress combinations (Mittler, 2006). It has been claimed that current techniques for developing and testing stress tolerance in plants by imposing each stress individually, while valuable, may be inadequate (Mittller and Blumwald, 2010). Such studies not only explain the effects of one stress on plants, but also eliminate the potential crosstalk and convergence points occurring between biotic

and abiotic stress signalling pathways. Moreover, it is proposed that to accurately characterize plant responses to multiple stresses, it is crucial to impose different stresses simultaneously and treat each set of stress combinations as an entirely new stress (Mittler, 2006).

1.5.1 Interaction between biotic and abiotic stresses

Plant responses to simultaneous biotic and abiotic stresses is complex, involving the expression of specific and sets of genes in common, as well as the activation of multiple signalling pathways that often interact in synergistic or antagonistic manners (Anderson *et al*., 2004: Asselbergh *et al*., 2008). There is increasing evidence to support the notion of crosstalk between signalling pathways and molecules such as calcium Ca^{2+}signalling, Ca^{2+} regulated proteins, MAP kinases and numerous transcription factors. These interactions generate signalling and regulatory networks that lead to various responses, enabling plants to adapt and acclimate to adverse environmental conditions (Fujita *et al*., 2006; Fraire-Velázquez *et al*., 2011; Huang *et al*., 2012). Studies that examined the consequences of abiotic stress with simultaneous effect of pathogen or herbivore attack reveal both positive and negative interactions, dependent on duration, nature, and intensity of each stress. For example, it has been reported that both high temperature and drought cause a negative interaction by reducing plant resistance to biotic invaders including pathogens, bacteria, virus, fungi, and nematodes (Atkinson and Urwin, 2012). However, a positive interaction between abiotic stress and pathogen infection has also been described. For instance, salt-induced osmotic stress enhances barley resistance to powdery mildew through the induction of antioxidant activity (Wiese *et al*., 2004). This positive interaction which leads to an increase in plant resistance and tolerance to subsequent stress after exposure to one specific stress is also known as cross tolerance (Pastori and Foyer, 2002 ; Tippmann *et al*., 2006; Shah *et al*., 2014), cross-protection (Sbehat *et al*., 1998) or cross adaptation (Alexieva *et al*. 2003). The latter authors proposed that this phenomenon occurs due to the fact that the first stress has already activated the defence systems thus enhancing plant resistance to the following unfavourable factors. For instance, bacterial and arbuscular mycrorrhizae have been shown to enhance abiotic stress tolerance in various crop species by producing antioxidants, increasing osmolyte production and improving abscisic acid (ABA) regulation (Atkinson and Urwin, 2012).

1.5.2 Crosstalk between hormone signalling pathways regulating biotic and abiotic responses

The phytohormones salicylic acid (SA), jasmonate/jasmonic acid (JA), ethylene (ET) and abscisic acid (ABA) are endogenous molecules of low molecular weight that primarily regulate protective and defence responses against biotic and abiotic stresses in plants (Fujita *et al*., 2006). ABA is a universal plant stress hormone regulating abiotic stress responses by triggering the expression of downstream abiotic stress-related genes and acts as a major internal signal enabling plants to survive adverse environmental conditions such as salt, drought and cold stress (Keskin *et al*., 2010). In addition, recent studies have shown that ABA plays an important role in disease susceptibility, resistance to pathogen infection, and interaction with other hormone-mediated biotic stress responses (Yasuda *et al*., 2008). By contrast, salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are major players in signalling pathways against biotic stress (Pieterse *et al*., 2001). It has been pointed out that the SA-mediated resistance is active against biotrophic pathogens, whereas JA or ethylene-mediated responses are mainly against nectrophic pathogens and herbivorous insects (Spoel and Dong, 2008).

These phytohormones are also key players in regulating the signalling pathways which can crosstalk either positively or negatively leading to synergistic or antagonistic responses respectively (Singh *et al*., 2011). This crosstalk provides a great regulatory mechanism for initiating resistance to various stresses encountered by plants, helping the plant to prioritize one response over the other (Atkinson and Urwin, 2012), and allows plants to favour either a stress response or a developmental process (Spoel and Dong, 2008). An example of hormone crosstalk is that ABA has been shown to inhibit the accumulation of SA and the expression of genes involved in basal resistance to pathogens (Yasuda *et al*., 2008). Another study showed that NaCl treatment suppressed the induction of systemic acquired resistance (SAR) by activating ABA-mediated signal transduction that inhibits the signal transduction upstream and downstream of SA (Yasuda *et al*., 2008). Also, ABA appears to negatively modulate the SA-dependent defence pathways in tomato plants against the necrotrophic fungus *Botrytis cinerea* and partly represses phenylalanine ammonialyase activity in healthy wild-type tomato plants (Yasuda *et al*., 2008).

Another studied example of hormonal crosstalk in plant defence is the interaction between SA and JA dependent pathways (Hunter, 2000). For example, the JA pathway

induced by insect attack can compromise defence against pathogens through inhibiting the SA pathway, and pathogen attack can compromise defence against insects through inhibiting the JA pathway. These results indicate a trade-off between SA and JA mediated defence responses since crosstalk between SA and JA dependent defence pathways may be a burden when enhanced pathogen resistance is associated with reduced insect resistance (Pieterse *et al*., 2001). However, this negative crosstalk is not constant as it appears to be determined by specific pathogen-plant-insect combinations as well as influenced by concentration, timing and nature of the stress (Singh *et al*., 2011). On the other hand, other studies suggested that pathogen infection may increase resistance to insect herbivores indicating a positive crosstalk (Hunter, 2000). For example, attack by the rust fungus *Uromyces rumicis* reduces growth, survival and fecundity of the Chrysomelid beetle *Gastrophysa viridula* (Hunter, 2000). Also, studies of *Rumex* indicate that pathogen resistance activated by herbivores is also active against other pathogen species under field conditions (Hunter, 2000). Schenk *et al*., (2000) confirmed the existence of coordinated plant defence responses in *Arabidopsis* by using microarray analysis, especially between the SA and JA pathways, which have been shown to interact antagonistically.

1.5.3 Other components involved in cross talk and cross tolerance

Recent molecular studies have identified and characterized the function of signals for genes involved in crosstalk between biotic and abiotic stress signalling cascades. For example, calcium and reactive oxygen species ROS act as second messengers within the early response to stress and might form the regulatory basis for developing such multiple tolerance mechanisms (Tippmann *et al*., 2006). Also, MAP-kinase cascades and WRKY transcription are considered promising candidates for common molecular players with key roles in mediating stress signalling crosstalk (Fujita *et al*., 2006; Fraire-Velázquez *et al*., 2011). Other key components include heat shock factors and small RNAs (Atkinson and Urwin, 2012). Capiati *et al* (2006) reported that a number of studies have shown that calcium-dependent protein kinases (CDPKs) are involved in the response to several environmental stresses, suggesting that these kinases could function as crosstalk mediators between signalling pathways leading to cross tolerance. The above components, besides phytohormones, represent examples of cross adaptation by increasing plant resistance to various unfavourable environmental factors (Alexieva *et al*., 2003).

1.6 Strategies for enhancing stress tolerance in plants

Despite the complexity of plant resistance and tolerance to stress, Atkinson and Urwin (2012) reported success in enhancing stress tolerance to environmental factors in some transgenic plants and crops. Different transgenic approaches including the expression of proteinase inhibitors and R gene-based resistance have shown to confer resistance to plant-parasitic nematodes in potato, rice, and banana plants (Fuller *et al*., 2008; Atkinson and Urwin, 2012). In addition, tolerance to both biotic and abiotic stresses has been achieved in maize through breeding programmes producing plants with the ability to tolerate drought and resist the parasitic weed *Striga hermontchica* (Atkinson and Urwin, 2012). These improvements have been achieved through manipulation of some key regulatory genes. For example, it is reported that over-expression of barley HVA1 (a late embryogenesis abundant gene LEA) in transgenic rice confers tolerance to drought and salt stress (Sabehat *et al*., 1998). It is also reported that constitutive expression of low levels of heat shock proteins (HSPs) can confer resistance to both high and low temperature stress (Sabehat *et al*., 1998). One strategy that provides a targeted approach involves manipulating the expression of transcription factors which specifically affect a subset of stress responsive genes (Harrison, 2012). The latter author also reported that the major crosstalk points that involve both ABA and ethylene signalling pathways have been considered as primary targets for manipulation to improve the response of crops to multiple stress conditions. Other important targets include gene products with protective activity against oxidative stress, which is induced by many abiotic stressors. It has been suggested that one mechanism that may confer resistance to many types of stress is the activity of the antioxidant pathways, including superoxide dismutase, peroxidase, catalase, and the ascorbate-glutathione cycle. The high activities of these enzymes under different kinds of stress may suggest that these proteins have a general role in the acquisition of tolerance by plants (Sabehat, 1998). Some studies demonstrated that manipulating the expression of a single gene conferred tolerance to other types of stress. For example, over-expression of the superoxide dismutase gene not only enhanced tolerance to oxidative stress in some transgenic plants such as tobacco, alfalfa, potato and cotton, but also enhanced tolerance to freezing stress, chilling injury and water deficit (Sabehat, 1998). However, there are still constraints in achieving the ultimate goal of improving stress tolerance in crops. Harrison (2012) pointed out that although studies on transgenic model plants such as *Arabidopsis* have eased the identification of key components in plant responses to stress, data are not readily or directly transferable to crop plants. Furthermore, Harrison

(2012), added that although functional genomics studies on cereal species have improved understanding of stress tolerance, applying and transferring such information from controlled conditions to the field will need more time and research.

1.7 Transcriptome analysis of multiple stress responses

Microarrays are the basis of global gene expression analysis and the most widely employed transcriptomic technique in functional genomics. Microarrays measure the abundance of transcripts presented by a pre-defined probe set and provide information on thousands of genes simultaneously. Microarrays have many advantages such as relatively high sensitivity, specificity, accuracy, throughput and cost-efficiency. Another great strength of microarray analysis is derived from the relative simplicity of its use. Therefore, this technology has been intensely utilized to identify stress-related genes and used as a screening tool in gene discovery, and thus has increased the knowledge of plant stress response and tolerance. Moreover, studies have revealed the efficiency of using microarrays in phenotype characterization associated with loss-of-function and overexpression of specific transcription factors or other direct regulators of transcript abundance. On the other hand, despite many technical and practical advantages provided by microarrays, like any analytical technique, it has some disadvantages. One inevitable constraint is that transcript abundance of a particular gene is not necessarily associated with gene product function due to post-transcriptional regulation. Furthermore, only a weak correlation has been observed in almost every direct comparison between proteomic (gene activity) and transcriptomic data (transcript abundance), as well as a weak relationship between stress physiology and microarray data. The latter limitation may arise because of in appropriate experimental design and methodology. For example, sampling (whole organ, tissue, cell) variation between laboratory and field growth conditions, level of stress imposition, stress magnitude and germplasm used in a specific experiment could greatly affect plant transcriptomic responses. However, identification of stress-related genes and determination of their putative biological function via microarrays analysis still provides valuable information, which eventually may facilitate achieving the ultimate goal of enhancing biotic and abiotic stress tolerance in plants, chiefly crops (Birch and Kamoun, 2000; Oktem *et al*., 2008; Deyholos, 2010; Jamil *et al*., 2012).

It has been reported that the majority of studies on plant molecular responses to multiple stresses have been derived from plants exposed to each stress in isolation and then comparing the gene expression patterns induced by either stress applied individually.

These studies considered genes that were commonly induced by two or more stresses as universal stress responses and as convergence points between the two stress signalling pathways. These genes in common were suggested to represent targets for improving stress tolerance in crop plants (Fujita *et al*., 2006; Mantri *et al*., 2010; Atkinson and Urwin, 2012). However, such studies do not explain the effect of multiple simultaneous stresses on plant responses, which have been shown to be different from the responses to individual stress (Rizhsky *et al*., 2004; Mittler, 2006). A few microarray analysis studies have actually examined the effects of two or more stresses simultaneously on plant molecular responses. These include, combined drought and heat on *Arabidopsis* and tobacco plants (Rizhsky *et al*., 2002, 2004), combination of two insect herbivores applied simultaneously and sequentially on tobacco (Voelckel and Baldwin, 2004), nematode infection and water stress on *Arabidopsis* plants (Atkinson and Urwin, 2013). Other such studies include double combination of different abiotic stresses (cold, heat, high light, salt, flagellin) on *Arabidopsis* plants (Rasumussen *et al*., 2013), combined light and heat stress on *Arabidopsis* (Nishizawa et al., 2006), simultaneous effect of heat, drought and virus stress on *Arabidopsis* (Prasch and Sonnewald, 2013), combined high light and high temperature on sunflower (Hewezi *et al*., 2008), combined water and nitrogen stresses on genome-wide expression profiling of maize (Humbert *et al*., 2013). The interactive effect of temperature, osmotic stress and the phytohormone abscisic acid (ABA) in the regulation of gene expression in *Arabidopsis* seedlings (Xiong *et al*., 1999), and fungal infection and drought on peanut plants (Luo *et al*., 2005) are further examples of where microarrays have been used to study the molecular responses of plants to multiple stresses. Collectively, these studies have demonstrated that specific gene expression patterns are induced by plants under a combination of stresses compared to single stress conditions and have confirmed that multiple stress responses of plants cannot be anticipated from studying single stress response. Therefore, additional studies are required in order to comprehensively understand the complexity of plant responses to multiple stress situations.

1.8 Aims and objectives

The aim of this study was to understand the fundamental bases of plant responses to multiple simultaneous stresses through investigating the effects of combined abiotic (salinity) and biotic (aphid infestation) stresses on wheat plant responses at the physiological and molecular levels, as well as their potential interactions in terms of crosstalk and cross tolerance. Such information is critical to create new avenues for

enhancing stress tolerance in crop plants through conventional breeding or genetic manipulation, leading to improved yields and contributing to global food security. This aim was achieved through the following objectives:

- To investigate the wheat plant response to salt treatment at the physiological level through characterization and screening of 14 wheat genotypes for their potential tolerance to salinity (Chapter 2).
- To investigate the wheat plant response to aphid infestation in the presence and absence of salt stress at the physiological level through evaluating and screening three wheat genotypes for their potential resistance to aphid infestation (Chapter 3).
- To investigate the response of the wheat plant to salt stress and aphid infestation, applied in combination and individually, at the molecular level through a comparative transcriptome analysis and functional characterization of differentially expressed genes using Affymetrix GeneChip Wheat Genome Arrays (Chapter 4).

To the best of my knowledge, no studies have yet investigated the genome-wide expression profile of wheat subjected to this specific set of stress combinations (salt stress and aphid infestation) under controlled conditions. A combination of salt stress and aphid infestation can represent and mimic conditions encountered by many plants and crops growing in the natural environment or cultured in fields, especially in arid and semiarid areas of the world.

1.9 Breakdown of chapters

This thesis is divided into five chapters:

- Chapter 1: General introduction gives overview/background of the field of study; defines the topic of research and presents the rationale of the study.
- Chapter 2: Characterizes and evaluates the responses of 14 wheat genotypes to salinity at the physiological level in order to screen the 14 genotypes for differences potential tolerance to salt. Based on results obtained, three wheat genotypes were selected for further analysis.
- Chapter 3: Characterizes and assesses the response of the three selected wheat genotypes to aphid infestation through conducting a bioassay to measure aphid fecundity as a resistance index. Plant physiological parameters in the presence

and absence of salt treatment were also measured. At the end of the experiment, one wheat genotype was selected for further investigation.

- Chapter 4: Investigates and analyses the wheat transcriptome of the selected genotype in response to salt and aphid infestation when applied individually and in combination. The selected wheat genotype 122-1 was exposed to 4 treatments: control, salt (pre-treatment), aphid infestation and dual stress (salt combined with aphid). Data were analysed by Robin, MapMan and PageMan software and genes up and down regulated under combined stress were identified as well as those associated with the individual stressors.
- Chapter 5: Discusses potential correlations between physiological and transcriptome responses examined in the present study in order to better understand the wheat response to stress. It also discusses the potential of using this approach to help inform wheat breeding programmes for enhanced tolerance to stress.

CHAPTER 2

2 Screening Wheat Genotypes for Salt Tolerance

2.1 Introduction

Salinity is one of the major abiotic stresses that reduce plant growth and crop productivity worldwide. It is estimated that 6% of the world's total agricultural land including 20% irrigated and 2% non-irrigated areas are salt-affected (FAO, 2005). Saline soil occurs naturally and as a result of land clearing and irrigation. Saline soil has been categorized to salinity, sodicity and alkalinity (Ussl, 2005). Most crops are glycophytes that cannot tolerate high salt stress whereas halophytes plants can tolerate high salinity level and constitute the flora of saline environment (Yokoi, 2002). Plants under salt stress may exhibit two phases of growth reduction; first, the salt in the soil surrounding the roots causes osmotic stress which restricts plant cells to uptake water resulting in a change in leaf water relations. Second, the salt inside the leaves causes ionic stress which reduces the photosynthetic capacity leading to leaf injury and death (Munns and Tester, 2008). Wheat (*Triticum aetivum*) is one of the most important food crops and is the most widely cultivated crop in the world (FAO, 2008). Wheat is characterized as moderately salt-tolerant crop compared to rice and maize which show less tolerance to salt. However, wheat yield is substantially reduced as salinity levels rise in soil to 100 mM NaCl. Therefore, improving wheat salt tolerance is fundamental to achieve high yield and increase food production in order to meet the projected demand of a growing population (Munns *et al*., 2006). Glycophytes such as bread wheat (*Triticum aestivum*) tolerate salt stress through utilizing two main mechanisms (i) excluding Na^+ from leaves and shoots to lower salt uptake, (ii) tolerating high internal Na^+ content which is also known as tissue tolerance (Colmer *et al*., 2005). The exclusion of toxic ions by wheat is considered as the primary selection criterion for salt tolerance. The uptake and accumulation of ions in plants are genetically regulated and are also affected by the environment (Ashraf, 2004).

Salt tolerance (ST) is a genetically and physiologically complex trait. Genetic studies on wheat have reported that ST is controlled by multiple genes (Genc *et al*., 2007). Moreover, differences in the salt tolerance among genotypes may occur at different growth stages (Ashraf and Akram, 2009). Ashraf, (2004) reported that 5000 spring

wheat lines showed different levels of tolerance to salt during their life cycle. Another screening study on 5,072 lines of wheat germplasm showed that there was more diversity between species than between ploidy levels. For example, tetraploid wheat exceeded hexaploid and diploid wheat in the proportion of tolerant lines and diversity (Munns *et al*., 2006). Identifying salt tolerant genotypes through discovering genetic diversity and using effective screening techniques is a prerequisite to improve salt tolerance. Several methods have been used to screen large numbers of genotypes for salinity tolerance in glasshouses or under controlled environments. Each screening method is applicable to a specific level of salinity, measures specific parameter and response (plant damage to very high salinity level, growth, yield and physiological mechanisms), requires specific length of salt treatment and shows a particular advantage (Munns and James, 2003).

Biomass in terms of shoot dry matter is a measurement of plant growth under saline relative to/versus control conditions and has often been used as selection/screening criteria for salt tolerance. Studies showed that biomass is more correlated with crop salt tolerance at early growth stages (Ashraf, 2004) and most likely relates to field (Munns and James, 2003) and to grain yield (Genc *et al* 2007). Biomass also has been used to assess potential tolerance in a large number of wheat genotypes under moderate salinity 50-150 mM NaCl (Munns and James, 2003) and high salinity level up to 250 mM NaCl (Martin *et al*., 1994). A study conducted by Ahmed *et al*., (2011) showed significant positive correlation between dry biomass and yield, indicating that total dry biomass along with yield can be good selection criteria under salinity stress. Therefore, plant biomass parameter was used in the present study as a salt tolerance index for screening 14 wheat genotypes under salinity level of 160 mM NaCl over a period of 3 weeks.

Osmotic adjustment is the physiological process which occurs in plants under salinity conditions to achieve osmotic balance through accumulation of high concentrations of either organic solutes, inorganic (ions) or both. Previous studies investigated the correlation of osmotic adjustment with growth in different plant species have showed conflicting results. For example, in grass species and cowpea salt tolerance was strongly associated with the higher capacity of osmotic adjustment, while other studies on different plant species have found little or no correlation (Ashraf, 2004). In addition, osmometer is used for determining the osmotic concentration of aqueous solutions such as determination the osmolality of plant saps. A small amount of aqueous solution (100µl or 50µl) is used to measure the freezing point. Based on this value the instrument

calculates the osmotic concentration (= osmotic pressure). Osmotic potential determines the ability of plant to take up water from the environment and to generate and maintain turgor pressure; the osmotic potential becomes more negative with increasing ion or solute concentration and the freezing point decreases with increasing solute concentration (Blum, 2011).

Chlorophyll content has been reported to associate with salinity tolerance and the reduction in chlorophyll content is due to the osmotic effect which increases the accumulation of abscisic acid (ABA). Increased chlorophyll content has been observed at low salinity and degraded at high salinity (Ashraf, 2004). Measuring chlorophyll content in leaves via a SPAD meter has been applied in many salinity screening studies. This non-destructive measurement of chlorophyll content has proved to be a practical and cost-effective method especially when screening large plant population for breeding programmes (Munns and James, 2003; Munns *et al*., 2006; El-Hendawy *et al*., 2005; 2007). Moreover, the effectiveness of the SPAD meter as a measuring method/tool in screening for salt tolerance has been examined in order to use it as an index for response of chlorophyll content to stress (Samdur *et al*., 2000).

Ion accumulation in plants exposed to salinity is considered as an indicator of salt tolerance (Veraplakorn *et al*., 2013). Most crop species including wheat have showen association between salt tolerance and ability to accumulate low ion content, a mechanism known as salt exclusion (Munns, 2008). Other plants that accumulate high level of Na^+ in leaf and show degrees of salt tolerance are likely to apply another mechanism known as tissue tolerance in order to cope with internal salt in plant (Zhu, 2007).

The objectives of the present study were to: (i) characterize different wheat genotypes for their response to salinity at the physiological level and to screen for their potential tolerance to salt; (ii) identify suitable wheat genotypes exhibiting tolerance to salt for subsequent studies to investigate the response to dual stress, and (iii) assess and evaluate some appropriate methods to screen wheat genotypes for salt tolerance. The underlying aim of the study was to improve our understanding of plant responses to simultaneous multiple stress conditions (wheat responses to a combination of salt and aphid infestation). Knowledge of the molecular mechanisms (transcriptome level) controlling abiotic and biotic interaction in plants will create avenues for enhancing crop plants tolerance to multiple stresses. Such an approach is an important step towards sustainable crop production and contributing to global food security.

2.2 Materials and methods

2.2.1 Plant material and growth conditions

Thirteen varieties of bread wheat (*Triticum aestivum* L.) and one variety of durum wheat (*Triticum turgidum*) with different levels of tolerance to salt were obtained from different sources (Table 2.1). The Indian landrace Kharchia is universally considered as highly salt tolerant and used as a standard for salt tolerance screening of wheat worldwide (El-Hendawy *et al*., 2007). Uniformly-sized seeds from each genotype were selected, washed with distilled water and germinated on a filter paper moistened with distilled water, in darkness at 25°C. After 2-3 days of germination, uniformly-sized seedlings were selected and transferred into pots (width 8 cm and height 7.5 cm) filled with silica sand (Carroll *et al*., 1994), with one seedling per pot, to provide 5 replicates per genotype for both control and NaCl treatments. Prior to transfer of seedlings, silica sand was washed with distilled water before filling the pots. Using sand enabled controlling the imposition of salinity and nutrient solution, easy removal of plants and harvest of clean undamaged root material. To prevent algae from growing on the sand surface each pot was covered with aluminium foil leaving a small hole in the middle to allow shoot emergence. Five pots were placed in each tray and small plastic plates were placed under each pot. Seedlings were irrigated with half strength Hoagland solution (pH 6) (Hoagland and Arnon, 1950) every other day for two weeks and afterwards with full strength. The experiment was conducted in controlled growth chambers with 23/18°C day/night temperatures, 18:6 h day:night length (photoperiod), and 250-300 μmol m^{-2} s^{-1} illumination. After 21 day/3 weeks of exposure to either 160 mM NaCl or control treatments, plants were harvested and separated into root and shoot. Samples were dried at 65°C for 48-72 h. Dried samples were then stored for ion analysis.

2.2.2 Treatments

When leaf three had fully emerged, two treatments were applied. Control treatment consisted of plants irrigated with non-saline Hoagland solution (pH 6) and salt treatment consisted of plants irrigated with saline Hoagland solution (pH 6). The salinity treatment was introduced to plants in an incremental manner over 4 days starting with 40, 80, 120, and 160 mM NaCl to reach a final concentration of 160 mM which coincided with the day after the plants were irrigated with the full strength nutrient solution. Salt-treated plants were exposed to 160 mM NaCl for 21 days whereas control

plants were grown under full-strength Hoaglands. Salt treatment was applied by supplying the nutrient solution with additional NaCl. In this way, the salt treatment was given homogeneously and more quantitatively than administering soil-grown plants with salty water or sea water.

Table 2.1 List of different wheat genotypes used in the experiment.

Genotype	ST traits	Source & reference
Triticum aestivum		
Drysdale	Moderate tolerant	Australia, El-Hendawy (2007)
Kharchia 65*	High leaf Na superior tolerant	Mexico, CIMMYT
Krichauff	Low leaf Na most sensitive	Australia, Y. Genc *et al*.(2007)
Yitpi	Low leaf Na	Australia, Y. Genc *et al*.(2007)
Yecora Rojo	High yielding in salt affected field	Saudi, Genc *et.al*. (2007)
122-1	High yield and low Na	Spain, S. Quarrie
123-5	High yield and high Na	Spain, S. Quarrie
116-2	High Na accumulation	Spain, S. Quarrie
118-1	Low Na accumulation	Spain, S. Quarrie
Claire	Not screened/no result	UK
Pasban 90	Salt tolerant	Mexico, CIMMYT
Shorawaki	Low Na superior tolerance	Mexico, CIMMYT
Chinese spring	Low Na superior tolerance	Mexico, CIMMYT
Triticum turgidum		
PBW 34	Intolerant	Mexico, CIMMYT

* Kharchia has been considered as the most salt tolerant and used as a standard for salt tolerance test of wheat worldwide (El-Hendawy et al., 2007).

2.2.3 Growth measurements

Non-destructive growth measurements including shoot height, number of leaves on main stem and number of tillers were made on the day prior to destructive harvesting. Shoot height (cm) was determined by measuring the height of the main shoot starting from the border of the plastic pot to the top of the main shoot. Measurement was not taken from the top of the soil, as the soil may condense with watering over time.

2.2.4 Chlorophyll content and leaf area

Leaf chlorophyll content was estimated on fully expanded leaf number 5 using a handheld chlorophyll SPAD meter (Opti-Sciences CCM-200) which provides an immediate and non-destructive estimation of chlorophyll content in leaves. Three readings were recorded from three different positions: at the base, middle and tip of the

leaf blade and the mean was calculated. Leaf area (LA) (cm^2) was determined on the same leaf (number 5) by measuring the width (W) and length (L) of the leaf blade. The following formula was applied for calculating leaf area according to (Gardner *et al*. 2003, 1985):

Equation 2-1: LA=W*L*0.75

Both parameters were measured one day before terminating the 21 days salt treatment.

2.2.5 Leaf water relations

Sampling leaves for water relations measurements

One day prior to the final harvest for measuring biomass, leaves were sampled for water content and osmolality applying two methods of sampling. First some genotypes were sampled by using segments from the same leaf 5 and 6 for measuring both parameters. Second, other genotypes were sampled by using one whole leaf for each parameter, leaf 6 and leaf 7 for osmolality and RWC respectively. Samples for osmolality were immediately preserved at -20°C while samples for relative water content were processed on the same day.

Relative water content

Leaf samples for relative water content (RWC) were cut into sections of about 5-10 cm^2, covered with foil and then placed in plastic bags on ice. The fresh weight (FW) was recorded then each sample was placed in a pre-weighed airtight (also oven proof) glass vial and immediately hydrated to full turgidity for 3-4 h under normal room light and temperature. After hydration, the samples were taken out of water and were dried of any surface moisture quickly and lightly with filter/tissue paper and immediately weighed to obtain fully turgid weight (TW). Samples were then oven dried at 80°C for 24h and weighed on the second day (after being cooled down in a desiccator) to determine dry weight (DW). All weighing was done to the nearest mg and RWC was determined by the standard method (Barr and Weatherley, 1962) and calculated using the following equation:

Equation 2-2: RWC (%) = [(FW-DW) / (TW-DW)] x 100

Where FW: sample of fresh weight, TW: sample of turgid weight and DW: sample of dry weight.

Leaf sap osmolality

Leaf samples for osmolality were stored at -20°C and prior to leaf sap extraction samples were snap/flash frozen in liquid nitrogen. The thawed leaf was squeezed to extract the sap which was collected with a pipette and placed in a small Eppendorf tube and kept on ice. The osmolality of leaf sap was determined using the Gonotec cryoscopic osmometer (Osmomat 030) and following the manufacturer's protocol. The measurement of osmolality is based on freezing point depression and determines the total aqueous solution.

Osmotic potential and adjustment

Readings of leaf sap osmolality and RWC were used to calculate osmotic potential and osmotic adjustment using the equations below (Zhang *et al*., 1999):

Osmotic potential (OP)

Equation 2-3: OP: Ψ_s (MPa) = - C (mosmol kg^{-1}) x R x T.

Where C: osmometer reading, R: 8.32 J mol^{-1} K^{-1} (gas constant), T: 298 (absolute temperature in K).

Equation 2-4: OP = Ψs (100) x RWC/100 (osmotic potential at 100% water saturation)

Osmotic adjustment (OA) was measured as the difference in OP between control/non-stressed and stressed leaves (MPa).

Equation 2-5: $OA = \Psi s\ (100)^{control} - \Psi s\ (100)^{stress}$

2.2.6 Ion content

For the determination of Na^+ and K^+ contents, 200 mg of dried sample was transferred into a beaker and 30 ml of digestion acid mixture (1 vol. of perchloric acid, approx. 60% w/w $HClO_4$, to 4 vol. of nitric acid, approx. 70% w/w HNO_3) was added. Beakers were covered with a watch glass and allowed to stand overnight. To start the oxidation, beakers were placed on a hot plate maintained at approx. 100°C. When the initial reaction subsided temperature of the hot plate was increased to 180-200°C and beakers were left on the hot plate until oxidation was completed. To volatilize all the perchloric acid the temperature was increased to 240°C and the watch glass was moved to one side

to allow evaporation. Beakers were then removed from the hot plate to cool down then 10 ml of approx. 2 M hydrochloric acid was added and brought to the boil and gently simmered for ~ 5 min. The watch glass was removed and rinsed with distilled water, collecting the washing in the same beaker. The content of the beaker was quantitatively transferred into a 50 ml graduated flask and diluted to 50 ml. The solution was filtered through a 9 cm Whatman filter paper No. 541, and the first few ml of filtrate solution was rejected while the remainder was retained (Fish & Food, Ministry.of Agriculture, 1974). Finally a blank determination of Na^+ and K^+ was carried out using a flame photometer (Jenway PFP7). The blank solution used consisted of a series dilution prepared from ion stock solution and diluted in 20 ml HCL 2M to make 5, 10, 15, 20 and 25 mg/ml of Na^+ and K^+ each.

2.2.7 Experimental design and statistical analysis

The experimental design was a randomized block design with one level of salinity (160 mM NaCl) and control treatments (no salt) using five replicates for each genotype and for each treatment. Statistical analysis was performed using the statistical software IBM SPSS statistics 19 and data were submitted to two way analysis of variance (2 way-ANOVA) to study the main effects (genotypes and treatments) and their interactions. Differences between the mean values were assessed by Tukey's multiple comparison tests at ($P < 0.05$) and relationships between individual variables were examined using simple linear correlations and regressions which were performed using excel statistical analysis and charts.

2.2.8 Ranking and scoring of genotypes for salt tolerance

In order to allow comparisons among genotypes, a salt tolerant genotype, Kharchia was chosen as a reference. Salt tolerance index (STI) was calculated for each parameter measured of each genotype based on the method described in Goudarzi andPakniyat (2008).

Equation 2-6: STI=Ps/Pc

Ps; the mean of the genotype under salt stress and Pc; the mean of genotype under control condition. The indices were then used to score and rank the genotypes according to the method used by El-Hendawy *et al*. (2007). Scores were assigned form the highest value to the lowest value (indicated by 1 to 5) of the following growth parameters: biomass, chlorophyll content (SPAD units), RWC, K^+ content and K^+/Na^+ ratio in

shoots. For instance, score number 1 for shoot height means that this genotype had the highest shoot height compared to others. Whereas, scores for other parameters such as leaf water relations and Na^+ content in shoot were assigned from the lowest value to the highest value (also indicated by number 1 to 5). For example, score number 5 for Na^+ content means that this genotype had the highest Na^+ content.

2.3 Results

Physiological characterization of 14 wheat genotypes at the vegetative stage in response to salinity at 160 mM NaCl for 21 days was determined by measuring some growth parameters. In general, results showed that there was a genetic variation between all tested genotypes under both control and salinity conditions. Also, a general trend of reduction in growth traits was caused by salinity.

2.3.1 Salinity effects on plant growth

The effect of salt stress on plant growth was determined by measuring different morphological traits such as: shoot height, leaf area and number of leaves and tillers. These parameters were measured in salt treated plants and compared with control plants. Generally, salinity had induced a significant decline in plant growth by the end of the experimental period. Analysis of variance (factorial analysis) revealed that the main effects of genotypes and salt treatment on plant growth were significant. However, genotype × treatment interactions had no significant effect on shoot height and leaf area whereas there was a significant effect on number of tillers and leaves.

Shoot height and leaf area

Wheat genotypes showed different shoot heights (SH) under both control and salinity conditions. Average of SH ranged from max 36 cm to min 22 cm under salt treatment in 122-1 and Claire genotypes, respectively. Whereas, SH average ranged from max 46 cm to min 29 cm under control conditions in Chinese Spring and Claire genotypes, respectively. After 21 days of salt stress a significant decrease (p<0.001) in shoot height was observed in salt treated plants compared to control. However, differences in SH between control and stressed plants were smaller in genotype 122-1 and greater in Chinese spring compared to other genotypes (Fig. 2.1a).

Genotypic variations in leaf area were also significant among genotypes under both salt and control treatments. Leaf area was also affected by salinity showing a significant reduction in comparison to control (p<0.001). Some genotypes exhibited more reduction in leaf area than others, for example, the greatest decline was observed in two genotypes 118-1 and Kharchia (34%), whereas, the lowest decrease in leaf area was observed in three genotypes Yecora rojo, Sharawaki and Claire (7%, 9% and 12%, respectively) (Fig. 2.1b).

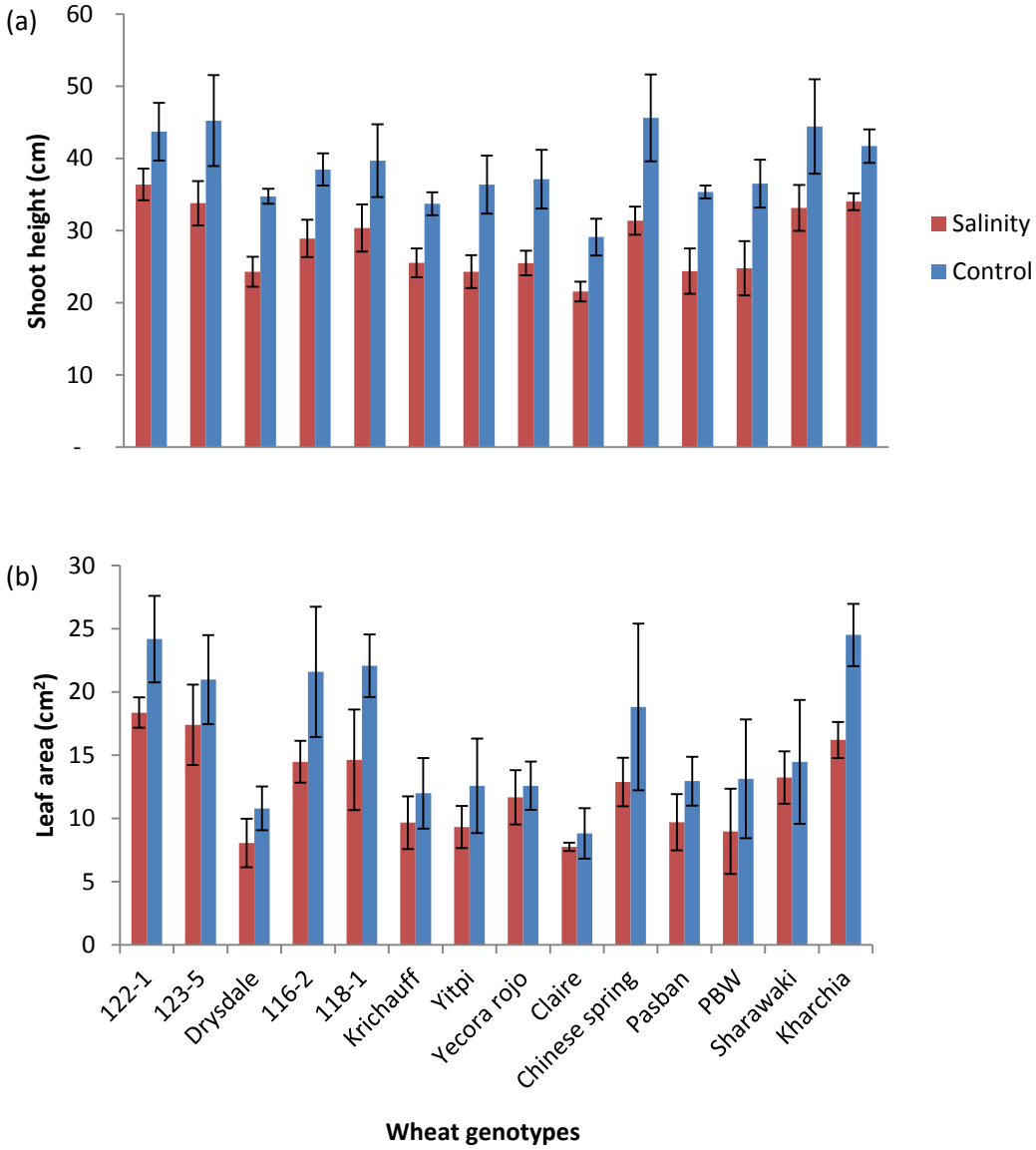


Figure 2.1 Effect of salinity on plant growth parameters
(a) Shoot height and (b) Leaf area measured as LA=W*L*0.75 of 14 wheat genotypes. Plants were grown in salinized conditions with 160 mM NaCl (red bars) and in control conditions (blue bars) for 21 days, values are means ± SD (n=5).

Tiller and Leaf numbers

Salinity significantly affected the number of leaves and tillers (p=0.004 and p=0.001, respectively). Number of tillers on control plants ranged from min 6 to max 12 tillers, whereas plants exposed to salt had lower tillers number ranging from min 2 tillers (recorded in Drysdale, 116-2, Claire) to max 6 tillers (in Pasban).

At the end of the experiment, there were 8 leaves on the main stem for all genotypes under control conditions except Yecora rojo which had 9 leaves. However, under salinity leaf number was reduced and there was a variation in number of leaves as 7, 8 and 9 leaves were observed on the main stem of different wheat genotypes (Table 2.2).

Table 2.2 Effect of salinity on leaf and tiller numbers of 14 wheat genotypes grown under 160 mM NaCl and control treatments for 21 days at the vegetative stage (values are means analysed by 2-way ANOVA, n=5).

	Leaf number		Tiller number	
Genotype	Control	Salt	Control	Salt
122-1	8	9	8	3
123-5	8	8	6	3
Drysdale	8	8	6	2
116-2	8	8	5	2
118-1	8	8	6	3
Krichauff	8	8	6	3
Yitpi	8	7	6	3
Yecora rojo	9	9	9	3
Claire	8	7	8	2
Chinese spring	8	8	12	5
Pasban	8	7	12	6
PBW	8	9	7	3
Sharawaki	8	7	10	5
Kharchia	8	8	6	3
Main effect	SED	Probability*	SED	Probability*
Genotype	0.156	0.000	0.436	0.000
Treatment	0.083	0.004	0.233	0.000
Genotype×treatment	0.220	0.002	0.616	0.000

2.3.2 Biomass parameters

Shoot and root dry weight

Wheat genotypes under control (no salt) conditions showed different shoot biomass production. Yecora rojo, 122-1, chinese spring, Kharchia and 118-1were the most vigorous genotypes producing shoot dry weight at 2.26, 2.23, 2.17, 2.10 and 2.03 (g) compared to others. Salinity (160 mM NaCl for 21 days) caused highly significant reductions in shoot dry weight of all the genotypes (p=0.001) compared with control (Fig. 2.2a). Among genotypes under salinity the highest shoot dry weight was obtained in 122-1 (0.966 g/plant), and the minimum in Drysdale (0.326 g/plant). However on a relative basis (i.e. compared to biomass in control conditions), the minimum reduction in shoot dry weight in response to applied salt treatment was recorded in 123-5 (53.9%). Under salinity the maximum root dry weight was produced by Kharchia (0.444 g/plant), whereas, minimum in Drysdale (0.093 g/plant). The highest reduction in root dry weight under salinity compared to control was recorded in Yitpi (74.6%), whereas the lowest in 123-5 (30.9%) (Fig. 2.2b).

Root/shoot ratio

There was a significant variation in fresh root/shoot ratio among genotypes (p = 0.001). However, salinity had no significant effect on dry root/shoot ratio (p = 0.945) and there was no significant interaction between genotypes and treatments (p = 0.130). Interestingly, some genotypes exhibited increased dry root/shoot ratio in salinized condition compared to control whereas others had decreased dry root/shoot ratio under salinity compared to control. Among genotypes exposed to salinity Kharchia scored the highest dry root/shoot ratio (0.051) whereas; both Claire and Chinese spring recorded the minimum ratio (0.025) (Fig. 2.2c).

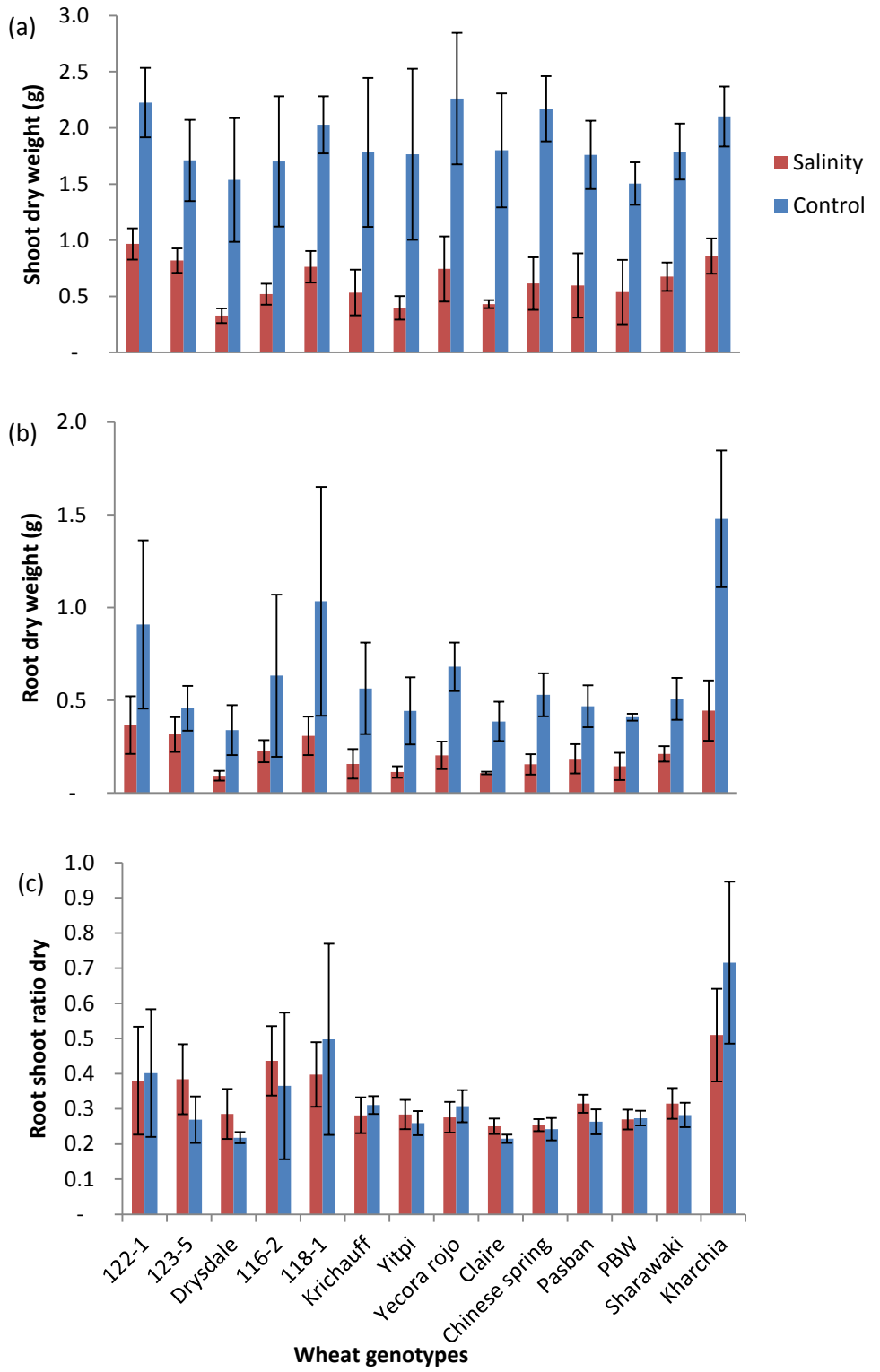


Figure 2.2 Effects of salinity on root, shoot and their ratio
(a) Shoot dry matter and (b) Root dry matter (c) Root/Shoot ratio of 14 wheat genotypes measured at vegetative stage after exposure to salinity by growing in salinized sand soil with 160 mM NaCl represented in red bars and control conditions represented in blue bars for 21 days (values are means ± SD of n=5).

2.3.3 Assessment of salinity tolerance

Total biomass

The level of tolerance to salinity across different wheat genotypes was determined according to previously described methods (Munns and James, 2003; Rivelli *et al*., 2002; Genc *et al*., 2007; El-Hendawy *et al*., 2005) which use a salinity tolerance index expressed as the percentage of total plant biomass in saline versus control treatments. Therefore, in the present study salt tolerance (ST) based on shoot dry matter was calculated according to the following equation:

Equation 2-7

$$\frac{\text{shoot dry matter (salt treatment)}}{\text{shoot dry matter (control)}} \times 100$$

The final biomass production after 21 d under salinity was recorded for all genotypes and ST was calculated (Fig. 2.3). As a result, the 14 wheat genotypes were classified into three levels of salt tolerance: tolerant genotypes maintained a high level of ST (> 40%), moderately tolerant genotypes showed moderate ST (28 - 40%) and sensitive genotypes showed a low level of ST (< 28%) (Fig. 2.3 & Table 2.3). Three genotypes 123-5, 122-1 and Kharchia produced the highest biomass (i.e. relative dry shoot matter) of 48%, 43% and 411%, respectively and were ranked as the most tolerant to salinity among other genotypes. Drysdale which known to show moderate tolerance was the most sensitive genotype producing the lowest biomass 21% under salinity conditions applied in this study.

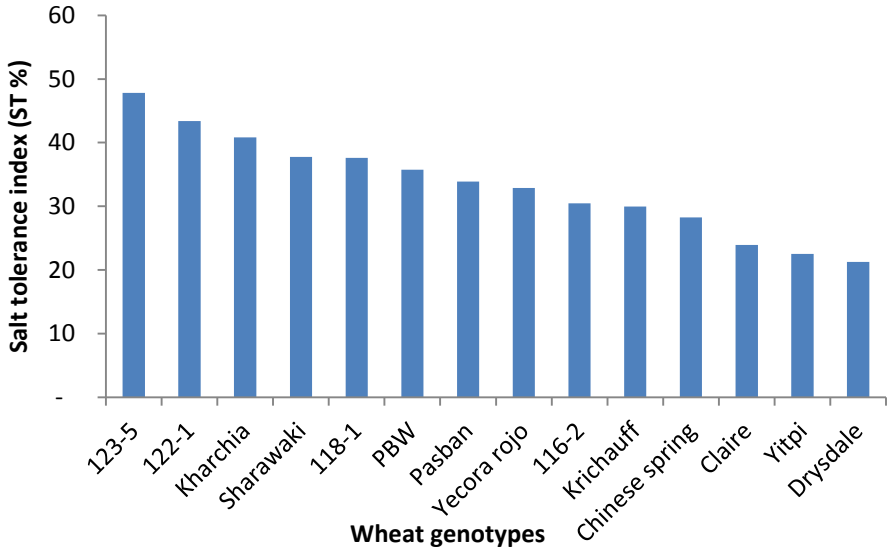


Figure 2.3. The range in potential salinity tolerance ST of 14 wheat genotypes (Relative shoot dry matter production under salinity as % of control) of wheat genotypes at vegetative stage grown under 160 mM NaCl and control conditions for three weeks (n=5).

Table 2.3 Ranking of wheat genotypes for their relative salt tolerance in terms of total biomass. Shoot dry matter and salt tolerance (ST) (determined as relative shoot dry matter production under salinity as % of control at final day of salt treatment 3 weeks), in 14 wheat genotypes at the vegetative stage.

	Shoot dry matter (g plant $^{-1}$)		Salt tolerance ST (%)	Genotype ranking	Tolerance degree
Genotype	Control	Salt			
123-5	0.8	1.7	48	1	Tolerant
122-1	1.0	2.2	43	1	Tolerant
Kharchia	0.9	2.1	41	1	Tolerant
Sharawaki	0.7	1.8	38	2	Moderate
118-1	0.8	2.0	38	2	Moderate
PBW	0.5	1.5	36	3	Moderate
Pasban	0.6	1.8	34	3	Moderate
Yecora rojo	0.7	2.3	33	3	Moderate
116-2	0.5	1.7	30	4	Moderate
Krichauff	0.5	1.8	30	4	Moderate
Chinese spring	0.6	2.2	28	4	Moderate
Claire	0.4	1.8	24	5	Sensitive
Yitpi	0.4	1.8	23	5	Sensitive
Drysdale	0.3	1.5	21	5	Sensitive
Main effect	SED	Probability*			
Genotype	0.110	*p <0.001*			
Treatment	0.059	*p <0.001*			
Genotype×treatment	0.155	*p=0.766* NS			

* Based on tests of between-subjects effects. Genotypes were arranged in descending order of salt tolerance based on shoot dry matter. SED, standard error; NS, non-significant.

2.3.4 Salinity effects on physiological parameters

To evaluate the effect of salt on physiological responses the following parameters: leaf chlorophyll content, leaf relative water content, leaf osmotic potential and leaf osmotic adjustment were measured. Salinity had significant effects on these parameters compared to control. However, effects of genotype and genotype × treatment interaction were only significant on chlorophyll content while no significant effect was observed for other parameters.

Chlorophyll content

Salinity decreased plant leaf chlorophyll content significantly ($p<0.001$) compared to control conditions with the exception of three genotypes 122-1, Yecora rojo and Sharawaki. The two former exhibited similar chlorophyll contents (26.5 and 25 SPAD units, respectively) compared to their corresponding control, whereas, the latter exhibited higher chlorophyll content (32.9 SPAD units) compared to control (Fig. 2.4a). To assess the relationship between chlorophyll content and salinity tolerance in the 14 tested wheat genotypes, the chlorophyll contents measured for leaf 5 at the end of the experimental period of 21 days were compared with biomass production calculated at 21 days. Chlorophyll content was negatively correlated with Na^+ concentration in shoot, showing high regression coefficient ($r^2 = 0.72$). In addition, chlorophyll content was positively correlated with salt tolerance ($r^2 = 0.18$) but the regression coefficient was low (Fig. 2.4b,c).

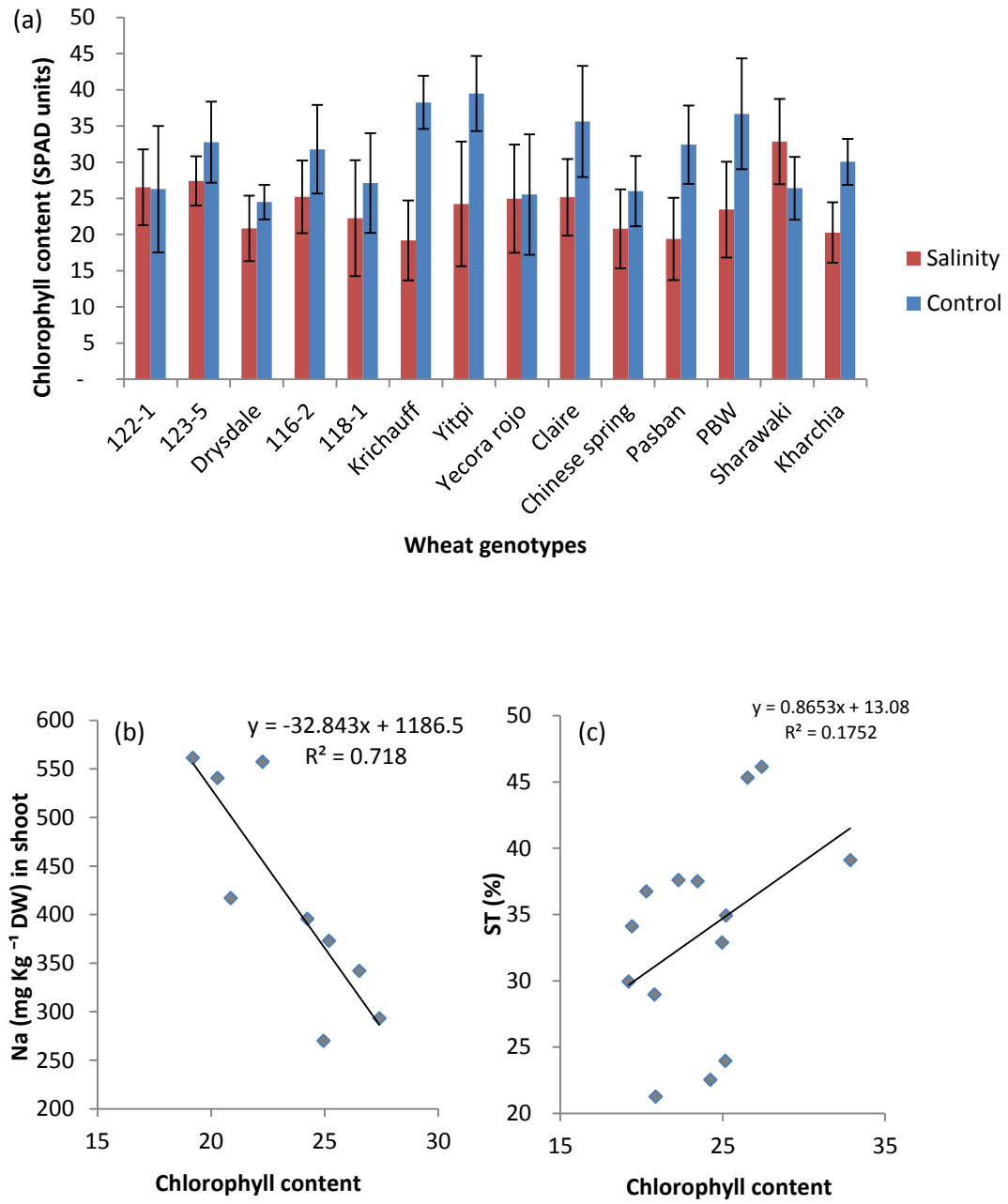


Figure 2.4 Effect of salinity on chlorophyll content
(a) Chlorophyll content (SPAD units) determined in leaf 5 of 14 wheat genotypes grown under control (blue bar) and salinity (red bars,160 mM NaCl for 21 days). Data are presented as the mean of 5 replicates±SD. Relationship between chlorophyll content in leaf number 5 and (b) Na concentration in shoot (p<0.001) and (c) ST (relative shoot dry matter production under salinity as % of control) measured after exposing wheat genotypes with 160mM NaCl for 21 days (p<0.05).

2.3.5 Water relations

Leaf relative water content

Relative water content (RWC) was significantly affected by salt treatment in all genotypes (p = 0.006). Neither genotype nor treatment x genotype interaction had significant effects on RWC (p = 0.473 and p = 0.688, respectively). Under control conditions, RWC differed among genotypes ranging from min 76% to max 106% for Yecora rojo and Pasban, respectively, but this variation was not significant (Fig 2.5). On the other hand, genotypes exposed to salt stress had lower water content in leaves compared to control. Yitpi had the lowest RWC at 81.9% whereas Pasban had the highest RWC at 103.1%. Relative water content showed a weak positive correlation with salt tolerance index of genotypes grown in saline condition (r^2 = 0.06). In addition, the pattern of reduction in RWC was not correlated with genotypes potential level of salt tolerance. For instance, in genotypes classified as tolerant, RWC was decreased by 8.6% at 160 mM NaCl relative to control. Similarly, in sensitive genotypes this magnitude of reduction in RWC was also observed. Moderate genotypes, however, exhibited the lowest reduction (5.4 %) in RWC. Interestingly, among all genotypes Yecora rojo plants treated with salt scored the highest water content in leaves compared to control plants (Fig. 2.5).

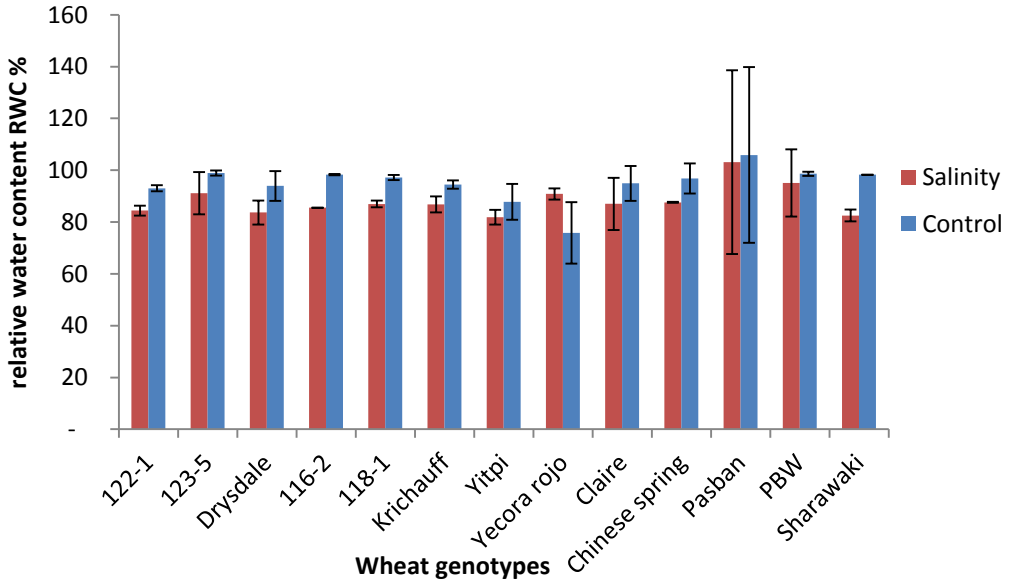


Figure 2.5 Effect of salinity on relative water contents
RWC % of different wheat genotypes after 21 days of growth in salinity at160 mM NaCl, red bars and control conditions, blue bars (values are means ± SD, n=5).

Leaf sap osmolality

Values for leaf sap osmolality were much higher in plants subjected to salt in comparison to controls. Salinity significantly ($p = 0.001$) increased solute concentration in leaf sap to a similar extent in all genotypes relative to control except Pasban which showed a slight increase in leaf sap osmolality under salt compared to control (Fig. 2.6a). Unexpectedly, statistical analysis showed that genotypes had no significant effect on this parameter and neither did the interaction between treatment and genotype. Among all genotypes, the lowest osmolality (0.548 mOsm/Kg H_20) was recorded in Pasban whereas the highest osmolality (1.601 mOsm/kg H_20) was recorded in genotype 118-1. Leaf sap osmolality was not correlated with salt tolerance index ($r^2 = 0.01$).

Osmotic potential Ψπ/Ψs

Salinity significantly increased solute concentration in leaf cells in all genotypes compared to control which resulted in less water and low osmotic potential ($p = 0.001$). Genotypes under salinity had lower osmotic potential (more negative value) due to the presence of solute in leaf sap and a low water content, whereas plants growing in control conditions had higher osmotic potential (less negative value) due to lower concentrations of solute and higher water content in leaf sap (Fig. 2.6b). The highest osmotic potential was observed in Pasban which also showed similarity to the control (-12.2 MPa) while line 118-1 exhibited the lowest osmotic potential (-34 MPa) among other genotypes under salt stress. Both factors, i.e. genotype and its interaction with treatment had no significant effect on this measured trait. Also, there was a weak negative correlation between osmotic potential and salt tolerance but not significant ($r^2 = 0.03$).

Osmotic adjustment

The degree of osmotic adjustment was estimated as the differences of osmotic potential Ψπ(100) between control and salt-treated plants. Results indicated that among the tested genotypes, 4 genotypes (PBW, 123-5, Yitpi and 118-1) maintained high osmotic adjustment (20.4, 18.8, 18.6 and 18 MPa, respectively) (Fig 2.6c) while genotype 116-2 retained the lowest osmotic adjustment at 7.5MPa. Other genotypes showed moderate levels of osmotic adjustment. However these differences were not statistically significant and the only significant effect was caused by treatment

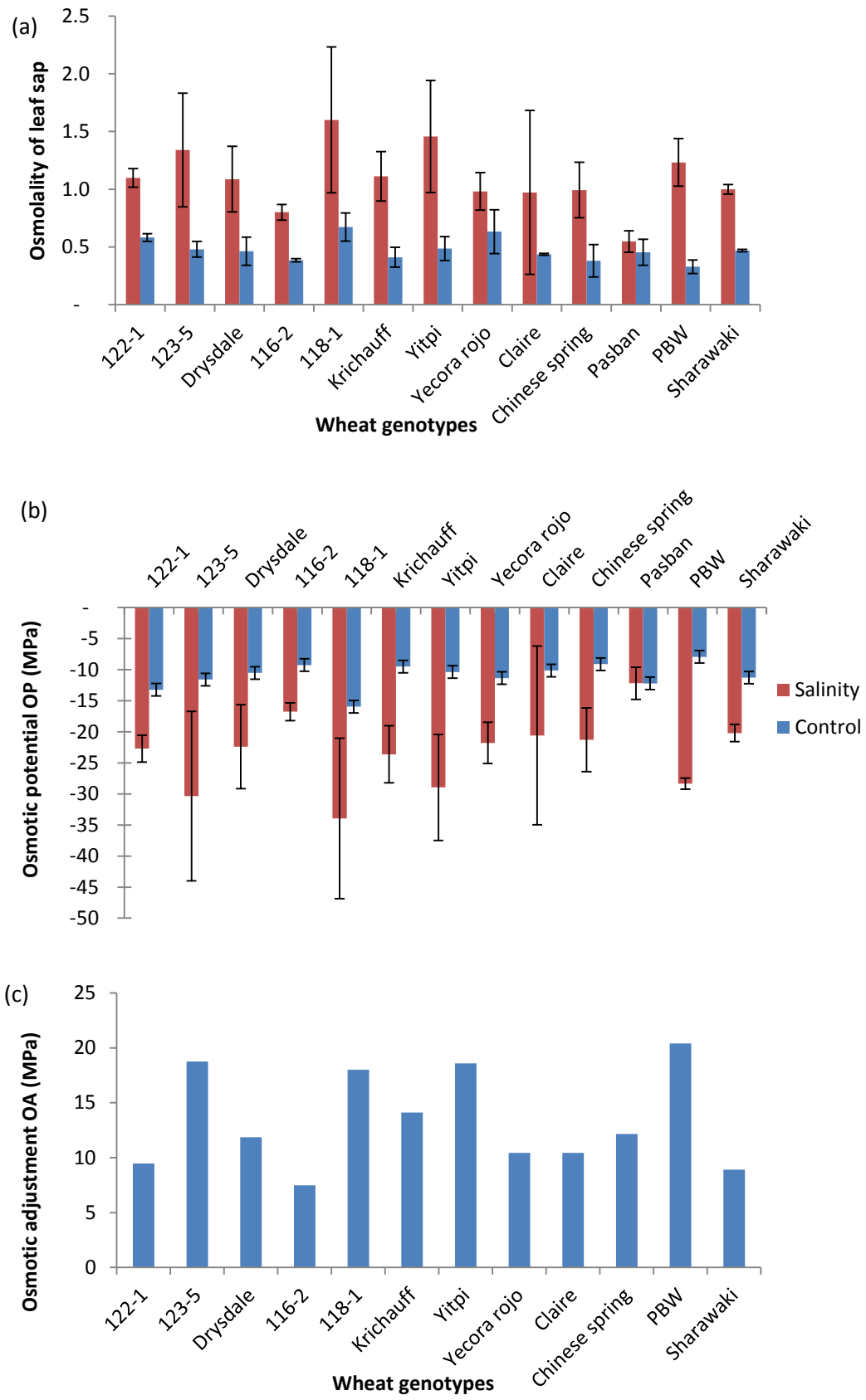


Figure 2.6 Effect of salinity on water relations
(a) Leaf sap osmolality (b) Osmotic potential at full turgor [Ψ π (100)] (c) The degree of osmotic adjustment [Ψπ(100)c - Ψπ(100)s, as the differences of Ψπ(100) between the control and salt-treated plants at 100% water saturation] measured in 14 wheat genotypes. Plants were grown under control conditions and salt treatment at 160 mM NaCl for 21 days (means ± SD, n=5).

as salinity significantly affected osmotic potential. Pasban was excluded from the comparison due to its negative value of osmotic adjustment. This parameter did not correlate with salt tolerance index (r^2 = 0.01, p=ns) but showed correlation with other parameters RWC and K^+ concentration under salt stress (r^2 = 0.12, p=0.10) and (r^2 = 0.29, p=0.025), respecively.

2.3.6 Effect of salinity on ion accumulation

Na^+ and K^+ concentration in shoot

Nine genotypes differing in their ability to tolerate salt stress were selected to measure ion concentration in whole shoots after 21 days of salinity treatment at 160 mM NaCl (Table S2.1). The effect of genotype, treatment and their interaction on Ion concentration were highly significant ($p < 0.001$). All nine genotypes showed different levels of Na content of which 3 moderate salt tolerance genotypes had the highest Na^+ content (average 553 mg/Kg) in shoot compared to other genotypes. The lowest Na^+ content was observed in a tolerant genotype 123-5 and Yecora rojo a moderate genotype (average 282 mg/Kg). The remaining genotypes with different degrees of salt tolerance had moderate Na^+ contents (average 382) (Fig 2.7a). Results indicate that there were large differences between genotypes in Na^+ accumulation which did not show a significant correlation with salt tolerance.

All nine genotypes maintained different levels of K^+ in shoot under salinity (Fig 2.7b). Two genotypes, 123-5 tolerant and 118-1 moderate accumulated higher levels of K^+ than other genotypes (345.33 and 339.12 mg/Kg, respectively). Yecora rojo (a moderate tolerant) accumulated lower K^+ content (232.35 mg/Kg) than other genotypes. The six remaining genotypes with different tolerance levels retained an average K^+ content of 296 mg/Kg. The effect of salinity on lowering K^+ accumulation in salt-treated plants compared to control plants was observed in all 9 genotypes. Differences in K^+ content between salt and control conditions varied among genotypes and the tolerant genotype 122-1 had the lowest difference compared to other genotypes. This genotype 122-1 was able to retain similar level of K^+ concentration under salt and control (Table S2.2) which may indicate that K^+ content was not affected by salinity conditions.

K^+/Na^+ ratio in shoot

The highest K^+/Na^+ ratio was found in shoots of two genotypes that were characterized in this study as salt tolerant (123-5 and 122-1), while the lowest K^+/Na^+ ratio was found in Kharchia and Krichauff which were characterized as moderate tolerant (Fig 2.7c). The relationship between Ion concentration in shoot and salt tolerance index are shown in (Fig 2.8). Data showed that Na^+ concentration was poorly correlated with salt tolerance and the linear regression coefficient was not high. However, K^+ and K^+/Na^+ contents showed significant correlation with salt tolerance. Furthermore, K^+ and K^+/Na^+ accumulation showed a positive relationship (r^2=0.28 and r^2=0.37, respectively) whereas Na showed a negative relationship with ST (r^2=0.05).

2.3.7 Assessing different measurements as screening tools for salinity tolerance

To evaluate the association of different parameters with salt tolerance ST and to assess the suitability of various physiological parameters for screening wheat genotypes for salt tolerance, all parameters measured in 14 wheat genotypes were ranked and scored based on the salt tolerance indices according to El-Hendawy *et al.* (2007). The relationship between the scores of physiological traits and biomass were further analysed using linear regression. In this study, results showed that some parameters for some genotypes were associated with their potential salt tolerance. For example, in terms of biomass parameter the salt tolerant genotypes 123-5 and 12-1 were ranked at the top for root dry weight. However some other physiological parameters could not be correlated with salt tolerance. For instance, water relations did not relate to salt tolerance. Moreover, salt tolerance for the three tolerant genotypes 123-5, 122-1 and Kharchia was associated with the Na exclusion measured in shoot. For some genotypes, however, the physiological trait of Na exclusion could not be correlated with salt tolerance. For instance, Drysdale was classified as the most salt sensitive according to its score on biomass, but Na^+ accumulation in shoots was scored as number one indicating that this genotypes had low Na^+ content in shoot according to score and ranking calculation applied (Table S2.3, S2.4, S2.5).

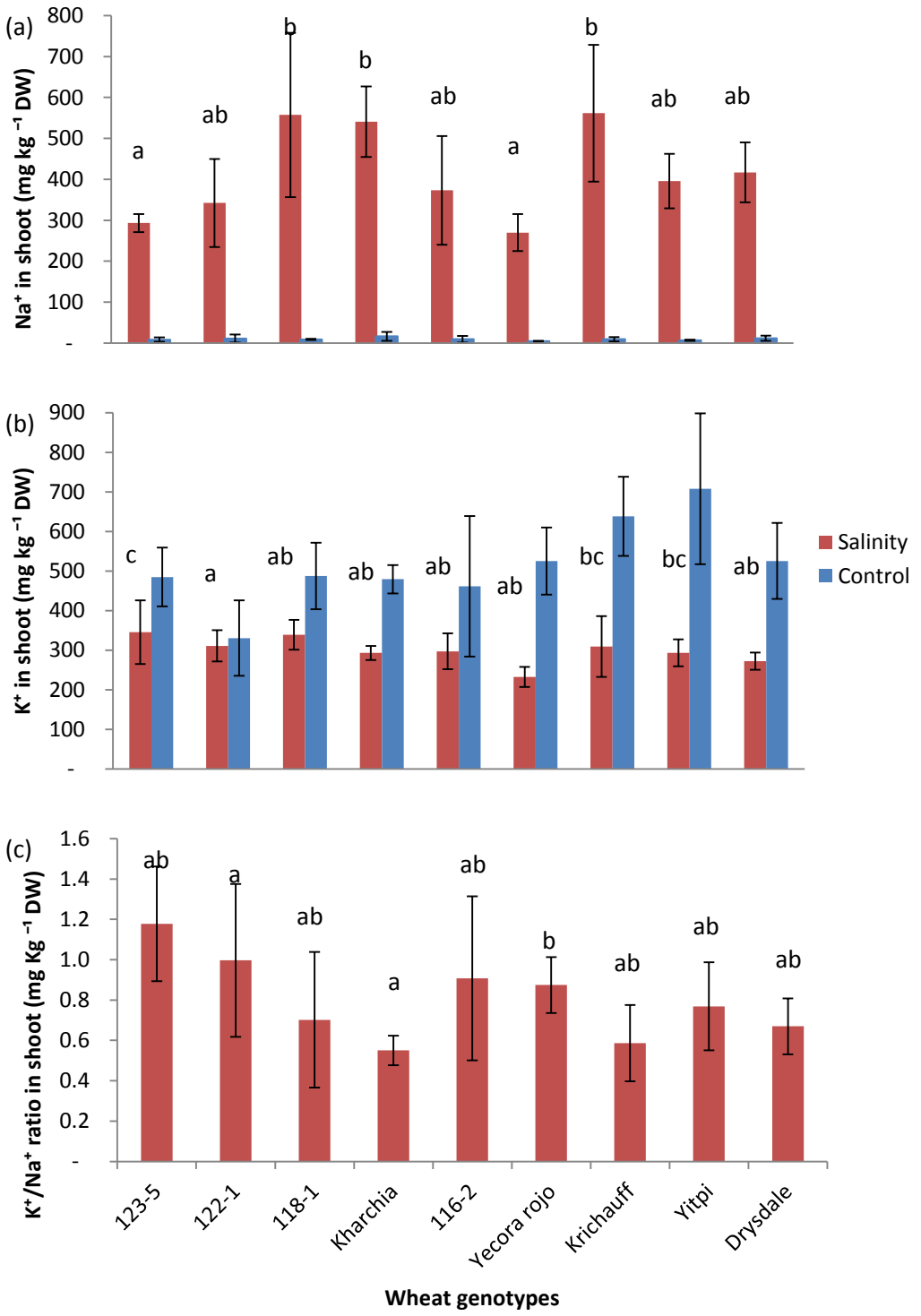


Figure 2.7 Effect of salinity on ions accumulation
(a) Na^+ content (b) K^+ content (c) K^+/Na^+ ratio in shoots of nine wheat genotypes measured after growing under salinity at 160 mM NaCl (red bars) and control (blue bars) treatments for 21 days. Different letters indicate significant differences among genotypes under salinity conditions at $P < 0.05$ (means ± SD, n=5).

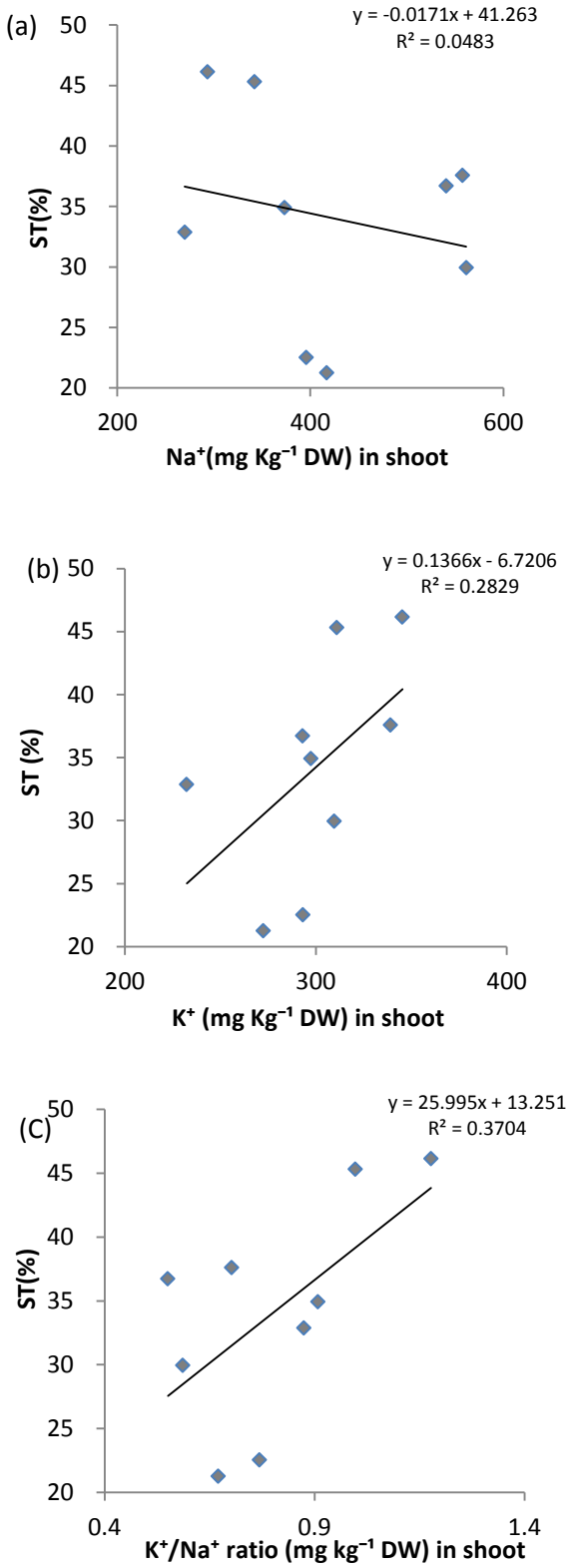


Figure 2.8 Relationship between salinity tolerance index and ions contents in shoots (a) Na^+, (b) K^+ and (c) K^+/Na^+ contents of nine wheat genotypes measured in shoots after growing for 21 days in 160 mM NaCl and control conditions (p=ns, $p \leq 0.05$ and $p<0.05$, respictively). All values are means (n = 5). Fitted linear regression is displayed on each figure.

2.4 Discussion

2.4.1 Biomass and plant growth

In the present study, salinity caused significant reduction in biomass based on shoot dry matter of 14 wheat genotypes. The average reduction in total biomass ranged from min 54% in 123-5 to max 79% in Drysdale after three weeks of exposure to 160 mM NaCl. This finding is in agreement with other studies that reported reduction in shoot weight in wheat under saline conditions (Rivelli *et al.,* 2002; Genc *et al*., 2007). The negative effect of salinity on plant growth may be due to two phases of stress. Firstly, the osmotic effect of salt outside the roots reduces the ability of root cells to take up water and mineral nutrients. Secondly, the toxic effect of salt accumulation over time in transpiring leaves causes leaf injury and death (Munns, 2005). There was a significant difference between genotypes in their growth responses to salinity indicating a clear genetic variation. This finding is in agreement with study conducted by Munns *et al*., (2006) who reported considerable genetic diversity amongst hexaploid and tetraploid lines of wheat based on survival at high salinity.

Under the experimental growth conditions used in the present study, results showed that biomass reduction of the following genotypes: Yecora rojo, Drysdale, Kharchia, Krichauff and Yitpi grown in soil were different from those grown in hydroponics (Genc *et al*. 2007). In addition the current study did not support the authors claim that there is an overall consistency in plant responses to salinity when grown in different conditions including hydroponic, field or soil assays. Furthermore, the present study did not find a relationship in plants performance in terms of vigorous growth between control and salinity conditions. For example, two genotypes Yecora rojo and Chinese spring which showed vigorous growth under control treatment among other tested genotypes, did not show however vigorous growth under salt treatment. This result is in contrast to Rivelli *et al*. (2002) who found that salinity reduced the growth of four genotypes to similar extent in control. In other words, genotype with high growth in control also showed high growth in salt compared to other genotypes. A possible explanation for these differences might be that not all genotypes showing high growth under control conditions behaved in a similar manner when exposed to salt treatment.

Salinity negatively affected the growth of wheat genotypes after exposure to 160 mM NaCl for 21 days. Among different measured parameters including shoot height, leaf area, number of leave and tillers; measurements of leaf area were well and highly

correlated with salt tolerance index ST. This finding is in agreement with Munns *et al*., (2006) who reported that measurements of whole plant leaf area expansion rates were well correlated with biomass production.

2.4.2 Measuring salt tolerance

In the present study, salt tolerance (ST) among wheat genotypes was evaluated using screening methods based on biomass production in salinity relative to biomass in non-saline conditions (Munns *et al*., 2006; Kausar *et al*., 2012). The advantages of using this method compared to other screening techniques are that it is more likely to relate to field performance (Genc *et al*., 2007) and it measures plant response to both osmotic stress and salt specific effects caused by salinity. However, this measurement of ST requires a control treatment and a relatively long term experiment in order to detect genetic differences between genotypes (Munns and James, 2003). The most salt tolerant genotypes in the present study were 123-5 and 122-1, which were significantly more tolerant than the Indian landrace Kharchia that is tolerant to sodic/saline soils (Munns *et al*., 2006). Moreover, results showed that there were differences in ST among tested genotypes which were ranked and classified as tolerant, moderate tolerant and sensitive to salinity. This finding supports other studies which reported significant genetic variation in salinity tolerance within bread wheat (Ashraf, 2004; Genc *et al*., 2007; Munns *et al*., 2006). The existence of such genetic variation may be explained by the physiological and genetic complexities of ST where possibly hundreds, of genes are involved (Shavrukov *et al*., 2011; Ashraf, 2004)

Under the experimental conditions used in the present study, results of salt tolerance for some genotypes which were previously screened are in agreement or disagreement with some previous studies. For example, genotypes 123-5 and 122-1 were found in the present study to be highly tolerant to salt and produced high biomass (46% and 45% respectively). A similar finding was obtained by Quarrie (unpublished) who recorded high yield in saline conditions for both genotypes. Kharchia, a landrace from India selected for salt tolerance was found to be also tolerant to salt in this study, with ST of 37%. This result is in consistent with other work (Genc *et al*., 2007) that found Kharchia to have relatively low salt tolerance. In the current study, Yecora rojo and Krichauff were ranked as moderate salt tolerant and Yitpi and Drysdale as sensitive to salt. Although, these results differ from Genc *et al*. (2007) findings, they are consistent with findings of El-Henday *et al*. (2005, 2007) who reported that Drysdale was more

sensitive at moderate and high salinity levels. It seems possible that the inconsistency of these results is due to utilizing different growth conditions, experimental length and salinity levels.

2.4.3 Na^+ accumulation and relationship with ST

To determine the mechanism of salt tolerance and assess the relationship between Na exclusion and salinity tolerance, a range of nine wheat genotypes were selected for this measurement. The Na^+ level in the whole shoot was compared with biomass production after 21 days of salinity treatment, by which time genetic differences in tolerance had appeared. Salinity resulted in an increased Na^+ accumulation in shoots to different extents, ranging from 269.83 mg/kg in Yecora rojo to 557.44 mg/kg in genotype 118-1. The experiment also detected significant genetic diversity among wheat genotypes. These findings are in agreement with those of Goudarzi and Pakniyat (2008), who reported variation in Na^+ accumulation in bread wheat and durum wheat cultivars in response to salt treatment.

Previous studies suggested that low salt accumulation in leaves indicates a mechanism known as salt exclusion which minimizes the entry of salt into the plant. In contrast, high salt accumulation in leaves refers to a mechanism known as tissue tolerance which minimizes the concentration of salt in the cytoplasm through compartmentalization of the salt in vacuoles or older leaves (Genc *et al*., 2007). Although some previous studies reported that Na^+ exclusion is correlated with salt tolerance in many species (Munns, 2005) and in wheat (Munns *et al*., 2006), in the present study small negative correlation (r = -0.22) or no clear relationship was observed between the levels of Na^+ content in shoot and salt tolerance based on biomass. For example, the three tolerant lines123-5 122-1 and Kharchia accumulated low Na. However, the sensitive genotype Drysdale also accumulated low content of salt in shoots. Additionally, the highest Na content in shoot was recorded in wheat genotype 118-1 that exhibited moderate tolerant to salt (Table S2.5). These findings support the study of Genc *et al*. (2007) who concluded that there was no relationship between Na exclusion and ST among wheat genotypes. The present findings further support the idea suggested by those authors that Na^+ exclusion and tissue tolerance varied independently, indicating that similar levels of ST may be achieved through different combinations of Na^+ exclusion and tissue tolerance mechanisms.

2.4.4 K^+ and K^+/Na^+ ratio

In the present study, salinity resulted in a significant decline in K^+ content in shoots for all genotypes compared to control/non-saline conditions. This might be explained by the high levels of Na^+ inhibiting K^+ up-take and consequently, causing a decrease in the K^+/Na^+ ratio. In addition, it has been suggested that plants under saline conditions are subjected to excessive amounts of Ion and mainly Na^+ occurs in the soil. As a result plants take up high amounts of Na^+ and the uptake of K^+ and Ca^{2+} is considerably reduced (Ulfat *et al*., 2007; Ashraf, 2004). Maintaining high K^+ content and high K^+/Na^+ selectivity in plants under saline conditions has been suggested as an important selection criterion for salt tolerance (Vazan and Rajabi, 2014). Results revealed that there were large differences between genotypes in K^+ accumulation and K^+/Na^+ discrimination (K^+:Na^+ ratio) in shoots. In addition, the main effects of independent factors i.e. genotype, treatment and their interaction on Ion content were highly significant. This clearly indicates the existence of genetic diversity of these traits among wheat genotypes. This finding is in agreement with the results obtained by Goudarzi and Pakniyat (2008), who reported variation of these traits in bread and durum wheat cultivars in response to salt.

Previous experiments on wheat have indicated that salt tolerance is associated with an enhanced K^+/Na^+ discrimination trait (Munns *et al*., 2006). In the current study, the relationship between both shoot K^+/Na^+ ratio and salt tolerance in wheat genotypes showed significant positive correlation (r = 0.61). Moreover, a similar positive relationship (r = 0.53) was detected between K^+ content and ST as well. Although these results are in agreement with other studies that have shown a positive effect of high K^+:Na^+ ratio to ST in durum wheat (Genc *et al*.,2007), they are in contrast with those of Munns and James, (2003) who found that K^+/Na^+ ratio showed small relationship with ST in different tetraploid lines of wheat. The most interesting finding in the current study was that K^+ content and K^+/Na^+ ratio were more correlated with ST than Na^+ content. Tammam *et al*. (2008) reported that high K^+/Na^+ ratio is more important for many species than simply maintaining a low concentration of Na^+. Therefore, it can be suggested that K^+/Na^+ ratio could be considered as a useful index for salt tolerance.

2.4.5 Leaf chlorophyll content

Chlorophyll content in leaves was measured by a non-destructive, rapid and easy technique using a hand held SPAD meter. This technique has been used to screen large numbers of genotypes and measures plant damage and tolerance to very high salinity levels. The effectiveness of the SPAD meter as a screening method has been examined in a number of studies (El-Hendawy *et al*., 2007; Munns and James, 2003). In the present study, results showed significant genotypic variation in SPAD units in wheat genotypes under salinity. Similar findings were obtained by El-Hendawy *et al.* (2007) and other studies which indicate that genetic differences in the rate of photosynthesis exist among different species and among cultivars within a single species (reviewed by Ashraf, 2004). Another important finding was that a significant negative relationship (r = 0.85) between SPAD units and high Na^+ accumulation was observed. This finding confirms that chlorophyll concentration is strongly negatively correlated with high Na level which caused and increased the percentage of dead leaf material (Fig. 1). This finding also corroborates the ideas of Munns and James (2003), who suggested that estimating chlorophyll concentration with a SPAD meter could be useful method to measure the ability of plants to tolerate the excessive amount of salt in their tissue i.e. the mechanism of tissue tolerance.

Salinity tolerance is related to the maintenance of net photosynthetic rate and stomatal conductance (Ashraf, 2004) and to the maintenance of leaf chlorophyll content (Ghogdi *et al*., 2012). Previous studies reported no or little association between plant growth and photosynthetic capacity in many species including wheat under salinity conditions (Ashraf, 2004). In agreement with previous studies the relationship between chlorophyll content and salt tolerance in this study showed a weak positive correlation (r = 0.42). Surprisingly, of the 14 wheat genotypes that exhibited lower chlorophyll content under salinity compared to control, three genotypes Sharawaki, Yecora rojo and 122-1 showed a different trend. Sharawaki was found to have higher chlorophyll content in salt than in control treatment, whereas both Yecora rojo and 122-1 maintained similar levels of chlorophyll content under both treatments. This finding is in consistent with Ashraf, (2004) who reported increased rate of photosynthesis at mild salinity levels in some species. A possible explanation for increased chlorophyll content is that leaves under salinity tend to be smaller in area but greener. This indicates that cell size and shape are affected and changed by salinity which may cause increase in the density of chloroplasts (Munns *et al*., 2006).

2.4.6 Leaf water relations

Relative water content

Relative water content represents a useful indicator of the state of water balance in plant and has been widely accepted as a reproducible and meaningful index of plant water status (Azadi *et al*., 2011). The results of this study indicate that leaf RWC was lower in salt treated than control plants for all genotypes with the exception of one genotype Yecora rojo which had higher RWC in leaf under salinity. Although these results are consistent with previous studies in terms of reduction in RWC under salinity (Farooq and Azam, 2006; Azadi *et al*., 2011), the increased of RWC in Yecora rojo was unexpected. However, a similar observation was reported by Revelli *et al*. (2002) in which RWC was increased significantly from 80% to 88% in the salt treatment in two lines with low-Na^+ accumulation. Similarly in this study, Yecora rojo had the lowest rate of Na^+ accumulation. The general reduction in RWC under salinity is explained by the low osmotic potential of soil solution in saline conditions which induces water deficit in plant tissue (El-Hendawy *et al*., 2007). Furthermore, the current study did not detect any genetic variation among genotypes and has shown the lack of consistent correlation ($r = 0.24$) between salinity tolerance and the criteria of RWC. These findings further support the idea reported by Ashraf (2004), after reviewing several reports, that measurements of water potential have little value in discriminating between salt tolerant and salt sensitive plants.

Osmotic adjustment

Osmotic adjustment in plants subjected to salt stress can occur by the accumulation of high concentrations of either inorganic ions or organic solutes (or both) (Nobile and Rogers, 1993). Furthermore, the accumulation of solute depends on the mechanism of salt tolerance. Thus, in plants in which salt exclusion is the major mechanism of salt tolerance, organic solutes and/or a variety of inorganic ions may be accumulated. In plants in which salt inclusion is the principal mechanism of tolerance, osmotic adjustment occurs due to accumulation of inorganic ions and particularly Na^+ and Cl^- ions at organ and tissue cellular levels (Nawaz *et al*., 2010). In this study no genetic variation in osmotic adjustment was detected among genotypes. There was no consistency across genotypes in osmotic adjustment and the degree of accumulation of K^+ and Na^+ in shoots showed positive correlation ($r = 0.53$) and little or no correlation

with osmotic adjustment (r = 0.29), respectively. This finding supports the idea that K^+ content in shoot and spikes is an appropriate index of osmotic adjustment (Tammam *et al*., 2008). Therefore, genotypes maintained high K^+ content in shoot also had high osmotic adjustment.

Prior studies have noted the importance of osmotic potential as an effective marker of salinity resistance in crop plants and a positive relationship of growth with the capacity of osmotic adjustment of different plant species has been reported (Ashraf, 2004). In wheat, previous studies showed that salt tolerant cultivars tend to have higher osmotic adjustment as compared with salt sensitive cultivar. In contrast, the present study did not detect a relationship (r = 0.07) between salt tolerance and osmotic adjustment. However, this finding is in agreement with other studies that found little or no correlation between these two parameters (Ashraf, 2004). Genotypes with high leaf osmolality (solute concentration) have higher capacities for osmotic adjustment and a strong relationship between these two parameter was found in this study (r = 0.91). Therefore, the relative ability of the plant organ to stimulate the accumulation of cytosolutes in its tissue (osmotic adjustment) will partially determine its tolerance to salinity (Tammam *et al*., 2008).

2.4.7 Assessing several types of measurements as screening tools for salinity tolerance

In order to evaluate the effectiveness of the parameters measured in this study as markers for salt tolerance in wheat, the relationship between these different parameters and the salt tolerance ST index were determined via linear regression analysis and correlation coefficient values (Table S2.6). This analysis will define which specific parameter is related directly with ST. Growth parameters including root and shoot dry weight, shoot height and leaf were strongly positively correlated with salt tolerance index (Table S2.7). Water relation parameters were weakly correlated with ST either positively or negatively (Table S2.8). Moreover, ion contents measured in the whole shoot of wheat genotypes showed weak correlations with ST index except for K^+ and K^+/Na^+ ratio that showed a positive significant correlation with ST (Table S2.9).

2.5 Conclusion

In the present study the response of 14 different wheat genotypes to salinity was investigated at the physiological level. Results demonstrate that there was genetic variation between different wheat genotypes in their response to salinity at the vegetative stage at 160 mM NaCl for 21 days. Among the genotypes tested 123-5, 122-1 and Kharchia appeared to be more tolerant to salt and Claire, Yitpi and Drysadle appeared to be more sensitive to salt than others based on relative shoot biomass production. The performance of genotypes 123-5, 122-1 and Kharchia during salinity treatment showed that these salt tolerant genotypes could be utilized through appropriate selection and breeding programs for future improvement in salinity tolerance of wheat genotypes. The tested parameters with the exception of leaf water relation parameters showed significant genotypic variation, indicating that the traits that have a significant genotypic variation may possibly be used as screening criteria. Moreover, parameters that showed significant positive relationship with salt tolerance, for instance root and shoot dry weight, shoot height, leaf area, root/shoot ratio and number of leaves could be also be considered as salt tolerance indexes. The data presented here demonstrate that there were large difference between genotypes in Na^+ accumulation and no relationship was detected between Na^+ exclusion and ST among genotypes. The result indicated that Na^+ exclusion and tissue tolerance varied independently and suggested that plant may utilize a combination of two different mechanisms known as Na exclusion and tissue tolerance, in order to achieve salt tolerance. Future research should not only focus on Na exclusion as the main mechanism of salt tolerance in bread wheat but also focus on investigating the mechanism of tissue tolerance and select for both traits in breeding program.

Based on results obtained from the current study, genotypes 123-5, 122-1 and Drysdale were selected for subsequent analysis. The two former genotypes showed best performance and were characterized as salt tolerant accumulating low and high Na content in shoot, respectively. Whereas Drysdale was the most sensitive genotype to salt accumulating high Na^+ content in shoot. These three genotypes were further characterized and screened for potential resistance and tolerance to the aphid *Sitobion avenae*. In addition, the salt-aphid-plant interactions on these genotypes were investigated at the physiological level in the next following chapter (Chapter 3). The present study provides information on screening methods and parameters used for salt tolerance in wheat. Such evaluation may facilitate the improvement of salt tolerance in

wheat to achieve the ultimate goal of sustainable agriculture and food security. Future steps and breeding programme towards reaching this goal should not only focus on one single trait but also identify and integrate other physiological, biochemical and generic traits.

CHAPTER 3

3 Consequences of Wheat Exposure to Salt on Aphid Performance

3.1 Introduction

The production of wheat (*Triticum aestivum* L.), one of the major Triticea crops, is threatened by both biotic and abiotic stresses, causing about 10-20% and 50% of crop loss, respectively (Ferry *et al*., 2004: Wang *et al*., 2003). Moreover, by 2020 it is estimated that wheat production will need to increase by 60% to meet projected demands (Cimmyt.org., 2014; Tolmay *et al*., 2001). These constraints, together with the increasing need to ensure food security and sustainable agriculture are a current challenge. A key solution is to improve existing cultivated lands and utilize uncultivated lands under suboptimal conditions with new improved crop cultivars. Therefore, current biotechnological and molecular approaches are important tools in discovering novel strategies for breeding new crop cultivars that exhibit a wide spectrum of tolerance/resistance to stress. Salinity is considered a major abiotic stress that affects 7% of the world's land and limits crop productivity, especially in arid, semi-arid area and irrigated lands (Rivelli *et.al*. 2002). Similarly, insect pest such as aphids can also significantly affect crop yields both by abstraction of nutrients and, more importantly, through the vectoring of viral and bacterial pathogens A major insect pest of wheat is the grain aphid *Sitobian avenae*, which is responsible for the transmission of barley yellow dwarf virus (BYDV) (Smith and Boyko, 2007).

3.1.1 Response of plants to stress

Plants in their natural habitat are exposed to many biotic and abiotic factors that affect growth performance via changes in physiological and molecular responses. Previous molecular studies indicated that under a combination of two different stresses plants respond differently to those exposed to each of the different stresses individually (Mittler, 2006). Studies on the interactions between abiotic and biotic stresses in plants revealed both positive and negative interactions as plant exposure to abiotic stress can alters plant's response to subsequent biotic stress (Chojak *et al* 2012). For example, some studies demonstrated that high temperature and drought caused negative interaction effects by reducing plant resistance against biotic invader pathogen including

bacterial, viral, fungal, and nematode (Atkinson and Urwin, 2012). Also it has been reported that drought and salinity have shown induced susceptibility in high plants (red pine, oak, citrus) to fungus and nematode (Tippmann *et al*., 2006). On the other hand, positive interactions between abiotic stress and pathogen have also been documented. For instance, salt-induced osmotic stress enhanced barley resistance to powdery mildew though the induction of antioxidant activity (Wiese *et al*., 2004). Also enhanced biotic stress tolerance in tobacco to mosaic virus was observed after plant exposure to sub-lethal abiotic stress (ozone, UV) (Yalpani *et al*., 1994), and non-lethal abiotic stress are known to induce the accumulation of defence transcripts, anti-microbial proteins and compounds, leading to enhanced disease resistance (Tippmann *et al*., 2006). Although the impact of individual forms of abiotic stress (e.g. drought, salinity, chilling) and biotic stress (e.g. pathogen infection and insect infestation) on plants has been extensively investigated, little is known about how a combination of these different stresses, applied simultaneously or sequentially, affects plant growth and subsequent productivity. To achieve a thorough understanding of plant-stress interactions, it is necessary to investigate in detail the plant response to stress, and in the case of biotic (aphid) stress, the response of the insect; thus it is important to investigate both sides of the interaction.

3.1.2 Effects of host plant stress on insect performance

Several studies have been carried out to investigate the effects of host plant abiotic stress on the subsequent performance of herbivorous insects following exposure to environmental stressors that include: drought (Simpson *et al*., 2012; Nguyen *et al*., 2007; Mody *et al*., 2009), elevated atmospheric CO_2 (Hughes and Bazzaz, 2001), elevated ozone and CO_2 (Holopainen, 2002), soil cadmium (Gao *et al*., 2012), ozone (Menendez *et al*., 2009), wounding and jasmonate (Brunissen *et al*., 2010), low temperature (Powell and Bale, 2005), and nitrogen fertilization (Levine *et al*., 1998). In contrast there have been few studies that have investigated the effects of salinity and insect or pathogen interactions, but with conflicting results. In one study on salinity and pathogen interactions, contrasting results were obtained indicating that plant resistance to pathogens may be enhanced or compromised by salt stress (Chojak *et al*., 2012). Again, with host plant exposure to salinity on insect performance, different studies have shown contrasting results, with negative, positive or unaffected insect performance and hence enhanced or decreased susceptibility of host plant to aphids. Plants exposed to deicing salt on the edge of motorways showed increased susceptibility to the green

apple aphid *Aphis pomi,* resulting in increasing aphid populations (Braun and Fluckiger, 1984). A similar study on the effect of de-icing salt concentrations in roadside soil on plant susceptibility showed that aphids *Rhopalosiphum padi* grew more rapidly and had greater fecundity on plants grown in soil taken from the verge of the carriageway (Spencer and Port, 1988; Martel, 1998). However, in contrast higher survival of the Japanese beetles *Popillia japonica* was observed on control unsprayed leaves and leaves sprayed with distilled water than on the salt-treated leaves as a direct effect of sodium (Stamp and Harmon, 1991; Martel, 1998). Another study on the effect of host plants irrigated with saline Hoagland solution (0-700 mM NaCl) on the aphid *Schizaphis graminum* showed that the population growth rate of *S. graminum* in wheat declined with the amount of salt accumulated by leaves (Araya *et al*., 1991). Similarly, the increased content of chlorine in the leaves of street trees grown in saline soil was accompanied by a decrease in the numbers of the lime aphid *Eucallipterus tiliae* L (Baczewsk *et al*., 2011).

3.1.3 Types of host plant resistance

Resistance to insects in plants has been identified by Snelling (1941) as the plant's ability to avoid, tolerate, or recover from infestation conditions that could severely damage other plants from the same species. Plant resistance against aphid infestation is achieved through utilizing three mechanisms, antixenosis, antibiosis and tolerance (Tolmay, 2001). The two latter mechanisms were evaluated in the present study. Antibiosis describes the negative influence of the plant on the biology of an insect attempting to use that plant as a host and measures the effect of a given plant on insect biology such as reducing fecundity (Tolmay, 2001). This may be expressed as reduced body size and mass, prolonged periods of development in the immature stages, reduced fecundity or failure to pupate or eclose. Many authors have reported antibiosis in wheat lines resistance to the Russian wheat aphid *Diuraphis noxia* (Tolmay, 2001). Tolerance, however, indicates the plant's ability to withstand or compensate for insect damage (Tolmay, 2001). Known components of this form of resistance include general plant vigour, compensatory growth, wound healing, mechanical support in tissues and organs and changes in photosynthetic partitioning. Environmental factors, however, may affect tolerance more than other types of resistance (Tolmay, 2001).

One of the most effective and preferred pest management strategies that have been used in controlling various agriculture pests for many years is utilizing host plant resistance

(Tolmay, 2001). Some advantages of using this strategy are reducing the use of pesticides that could cause health problems and environmental pollution, maintaining a sustainable agriculture system, reduced costs and less detrimental to the environment or natural enemies, non-target insets (Tolmay, 2001). However, to achieve this goal, a first step is to screen and identify resistant/tolerant cultivars and then investigate the mechanisms underlying this resistance before transferring these traits to other cultivars.

Many studies that reported/quantified antibiosis resistance towards the Russian wheat aphid *Diuraphis noxia* have measured aphid fecundity on wheat lines (reviewed in Tolmay *et al*., 1999). Moreover, studies showed that the nymph development time, longevity as well as fecundity are the most important indices in identifying the resistance of wheat varieties to aphids (Ozder, 2002). Three techniques were used to determine antibiosis to *D. noxia* in a wheat accession: embryo count technique, colony count technique and nymphs count technique. The latter method was used in the present study to measure potential antibiosis mechanisms in three wheat genotypes against the grain aphid *Sitobion avenae*. Reese *et al*. (1994) and Gao *et al*. (2008) suggested that yield, plant damage and plant survival, were important parameters for assessing tolerance mecchanism. For oats, rye and barley, crop damage and height as well as seedling survival were considered good indicators of tolerance, whereas in wheat and its wild relatives, the two parameters plant height and plant dry weight were considered as reliable measurements for quantifying tolerance to aphids (Reese *et al*., 1994). Therefore, the latter two parameters were used in the present study for the purpose of quantifying potential crop tolerance to the aphid *S. avenae* in three different wheat genotypes.

Host plant quality is known to affect herbivore performance and population dynamics. It has been hypothesized that plants under abiotic stress become more suitable as a food source for herbivorous insects (Koricheva and Larsson, 1998). Also it has commonly been noted that a factor inducing stress in plants also favours insect growth. It has been suggested that plants subjected to such stressful conditions become more susceptible to herbivorous insects owing to the plant's increased nutritional quality and/or reduced concentrations of defensive chemicals (Koricheva and Larsson, 1998). However, these authors, in reviewing and examining the results of a number of experimental studies on insect response to stress in trees found surprisingly little support for the stress hypothesis in its present general form. There are at least two possible explanations for the lack of support: (i) the concept of plant stress used by insect ecologists is too simple

and (ii) there are fundamental differences between different types of insects with respect to stress-induced changes in food quality. Insect performance has been reported to increase, decrease or remain unchanged in response to plant stress (Nykanen and Koricheva, 2004).

3.1.4 Response of sap-sucking insects to plant stress

There are many sources of variation in insect responses to plant stress, namely, variation related to insect traits, variation related to the host plant and variation related to experimental design. Focusing on sucking insects, studies have shown that the degree to which insects benefit from plant stress is associated with the mode of insect feeding. This is particularly important in respect of sucking insects (Koricheva and Larsson, 1998). Moreover relative growth rate (RGR) and reproductive potential of sucking insects increased on stressed plants, whereas, survival and colonization were not significantly affected; the timing of feeding had no effect on sucking insects. In terms of the type of stress, pollution increased the reproduction potential of sucking insects, whereas, water stress tended to decrease their population growth. Furthermore, with regard to duration and timing of stress treatment the reproduction potential of sucking insect was increased by simultaneous stress, whereas releasing plants from stress prior to bioassays tended to decrease their fecundity. In general, sucking feeding insects showed increased as well as decreased performance on stressed plants in different studies (Nykanen and Koricheva, 2004).

3.1.5 Cross-talk between signalling pathways

An increasing amount of evidence indicates that crosstalk between signalling pathways and components induced in response to biotic and abiotic stresses does exist and are widespread in plants. Cross tolerance is the positive outcome of crosstalk i.e. the interaction between different stresses in which plant resistance after exposure to a specific stress also operates against another form of stress (Pastori and Foyer, 2002). Studies have investigated this cross-tolerance in response to different biotic or abiotic stresses, and in response to a combination of these stresses (biotic and abiotic) at the molecular, physiological and ecological levels. For instance a study conducted by Yalpani *et al*., (1994) on tobacco plants subjected to ultraviolet light (UV) and ozone (O_3) resulted in enhance resistance towards virus attack through the acclimation of salicylic acid and pathogenesis-related proteins. Salinity has shown to stimulate

Arabidopsis resistance to *Botrytis cinerea* (fungus) (Mengiste *et al*., 2003), increased cold hardiness in potato and spinach seedlings (Ryu *et al*., 1995), induced wound-related genes which in turn enhanced tomato plant response to wounding stress locally and systemically (Dombrowski, 2003) and increased wheat seeds tolerance to subsequent temperature stress (Lei *et al*., 2005; Song *et al*., 2005). However, many issues and questions regarding this interaction/phenomenon have yet to be addressed. Thus, studies that highlight signalling, genes and pathways underlying cross talk and specifically cross-tolerance are urgently needed to increase our knowledge of how plants interact with their surrounding environment and what genes confer tolerance to different stresses (Mei and Song, 2010).

Aims and objectives

The underlying aim of this study was to improve our understanding of the effect of salinity on plant-aphid interactions as well as the interactions between aphid and salt stress on plant performance at the physiological level. This was achieved through the following objectives:

- To evaluate of *Sitobion avenae* performance on three wheat genotypes for potential resistance/tolerance and to investigate the effects of salt pre-treatment (plant exposure to salinity prior to aphid bioasay) on aphid *S. avenae* performance through measuring reproductive/fecundity parameters.
- To examine the effect of aphids and salinity in combination on plant performance through measuring several plant physiological/growth parameters.
- Testing the following two hypotheses:
  - Plant stress hypothesis which suggests that plant susceptibility to herbivorous insects increases under stressful environmental conditions.
  - Cross-tolerance hypothesis which suggests that plant resistance or exposure to a type of stress enhances plant's ability to respond to subsequent stress. By testing these hypotheses we will gain a better understanding of the interaction between biotic and abiotic stress in wheat.

3.2 Materials and methods

3.2.1 Plant material

Three wheat *Trititcum aestivum* L. genotypes were selected based on results decribed in chapter 2, which was conducted to screen 14 wheat genotypes for their relative tolerance to salinity (as described in chapter 2). The three selected genotypes 123-5, 122-1 and Drysdale were characterized as salt tolerant accumulating low levels of sodium Na^+ in the shoot; tolerant accumulating high levels of Na^+ in the shoot; and sensitive with high Na content in the shoot respectively. Seeds were kindly provided by Prof Steve Quarrie and Prof Peter Langridge from Adelaide University, Australia. These genotypes have not been assessed or evaluated for aphid resistance. Prior to planting, seeds were sown by germinating in the dark on moisted filter paper for 2 days under controlled environmental conditions. Single seedlings at 1-2 cm coleoptile stage of each wheat genotype were transferred and planted in plastic pots (width 8 cm and height 7.5 cm) containing silver sand and plants were grown in a growth chamber with 16 h light (22°C)/ 8 h dark (17°C) under 300 μmole m^{-2} s^{-1}. Hoagland's solution (Hoagland and Arnon, 1950) was used for irrigation; plants were watered with half-strength Hoagland solution every other day for the first two weeks then with full strength till the end of the experiment. Overall 90 (30 plants per genotype) potted plants were used in this experiment.

3.2.2 Insects (aphid culture)

The English grain aphid *Sitobion avenae* (F.) was used as the target insect and obtained from a laboratory culture which was established from a single female and maintained at 20°C, 55% R.H. under a 16:8 light: dark regime. The aphid colonies were reared on wheat *Triticum aestivum* L. cv. Claire and infested plants kept in 45 x 45 x 50 cm Prespex cages. New plants were supplied weekly to keep the colonies going on. Aphids were transferred to experimental plant using a fine paint brush.

3.2.3 Experimental design

The experiment was designed to investigate the effects of genotype (Study 1) and salt (Study 2) on aphid fecundity, measured as daily and accumulative nymph production over time.

Study 1: Screening for aphid resistance (bioassay): Three wheat genotypes were screened for potential aphid resistance and evaluate the two mechanisms of resistance to aphids (i) antibiosis, which studies the effect of the plant on aphid performance and (ii) tolerance, which studies the effect of the aphid on plant performance.

Antibiosis test: Aphid performance was assessed by measuring *S. avenae* fecundity (number of nymphs per adult aphid over a defined period of time) using the nymph count technique (Tolmay *et al*., 1999). Three reproductive parameters were measured: (i) total fecundity calculated as the total number of nymphs produced per genotype divided by total number of adults on the first day of the bioassay;(ii) daily fecundity measured as the number of nymphs produced per female per day; (iii) the cumulative mean of nymphs over 3 weeks. The fecundity of 20 adult aphids was estimated on ten plant replicates for each tested genotype. Low fecundity was used as the resistance index against aphid.

Tolerance test: Plant performance was assessed by measuring the following growth parameters: number of tillers, shoot height, root and shoot dry weight and the physiological parameter chlorophyll content at the end of the trial. Two treatments, non-infested plants (control) and aphid-infested plants (experimental) were used to determine tolerance. Ten plant replicates were distributed in a complete block design in growth champers under conditions described above. All data from the infested plants were expressed as a percentage of the non-infested control plants. High biomass based on relative shoot dry matter was used as the tolerance index towards aphids.

Study 2: The effects of salt stress on wheat –aphid interactions (cross-tolerance): Plant and aphid performance was assessed through measuring the effect of (i) the combination of both stresses (aphid infestation and salinity condition) on plant performance and (ii) wheat plants grown under salinity on aphid performance. This enabled the hypothesis of cross tolerance which states that induced plant resistance through exposure to one stress enhances their resistance to another stress to be tested. Measurements and parameters of aphid and plant performance were as described in study 1.

3.2.4 Bioassays

When all plants of the three wheat genotypes reached the 3- leaf stage, each genotype was divided into three groups (each of 10 plant replicates). Each group was then subjected to one of the three following treatments:

Control treatment: Control plants were neither infested with aphids nor treated with salt. These plants were irrigated with Hoagland's solution and although not infested, they were enclosed in ventilated bread bags to maintain the same conditions as plants in the other two treatments.

Salinity treatment: Salt was imposed incrementally to plants in four steps over four days (Steve Quarrie, personal communication) using saline Hoagland's solution with a series of concentrations starting with 40mM on day 1, 80mM on day 2, 120mM on day 3 and a final concentration 160 mM NaCl on day 4. This final concentration was maintained until the end of the experiment when salt-treated plants had been exposed to 160 mM NaCl for 5 weeks. This final concentration (160 mM NaCl) was maintained over the course of the experiment. For further details see chapter 2.

Aphid bioassays: At the 3-leaf stage plants were either exposed to salt treatment or non-saline Hogan's solution for 4 days. On the 5th day, when the plants were at the 4-leaf stage the aphid bioassays were started. Two adult apterous aphids were randomly chosen from the aphid colonies and placed on the leaf surface, one aphid per leaf, using a suitable fine paint brush. Plants were arranged in a block design in chambers under environmental growth room conditions 22:17°C day: night and 55% R.H. under a 16:8 LL: DD light regime. Following infestation plants were enclosed with ventilated bread bags to prevent aphid escape. The two adult aphids per plant were allowed to reproduce for 24 h and on the next day when reproduction commenced the adults and all nymphs produced, except for two, were removed. The remaining nymphs were permitted to develop through to adulthood (about two weeks); the bioassay trial started on the first day that these new adults produced nymphs. Adult aphid survival was monitored and reproduction was recorded daily over a period of 21 days. The duration of aphid infestation imposed on plants was 5 weeks.

3.2.5 Plant growth measurements and parameters

The number of tillers, shoot height, chlorophyll content and the dry matter of roots and shoots of the three selected wheat genotypes were measured at the end of the bioassay; this represented a time-frame of 5 weeks after the start of salinity treatment and 3 weeks after the start of aphid infestation. To determine the dry matter, the freshly harvested organs were separated; residual sand was washed from the roots, and dried in the oven at 80°C. The chlorophyll content was quantified in leaf 5 using a SPAD chlorophyll

meter and the mean of three readings from three different positions/spots on the leaf blade (base, middle and top) was determined.

3.2.6 Statistical analysis

Data collected form aphid and plant performance were analysed using two-way Analysis of Variance (ANOVA). Means were subsequently separated and compared by the Tukey post hoc test at $p < 0.05$ to detect statistical differences among the means. Analysis was conducted using SPSS software.

3.3 Results

The results are presented below both in terms of aphid performance and in terms of plant performance as follows:

3.3.1 Aphid performance

Aphid (*Sitobion avenae)* performance on the three different wheat (*Triticum aestivum)* genotypes 123-5, 122-1 and Drysdale was assessed in terms of total fecundity, daily fecundity, and reproduction rate to screen for their respective resistance/tolerance to aphids over a period of 21 days (designated study 1). A second study was carried out to investigate the effects of exposure of wheat to salt stress/salinity on the wheat-aphid interaction over the same period of time (designated study 2).

Effects of wheat genotype on total aphid fecundity

Screening for aphid resistance (Study 1.): Nymph production was used to screen the three wheat genotypes for a potential antibiosis resistance mechanism. Statistical analysis showed that aphid total fecundity, measured by the mean total number of offspring produced by each adult aphid over a period of 21 days was significantly different among the three wheat genotypes ($p = 0.001$). Aphids on Drysdale produced 50 nymphs per adult which was significantly higher than those produced on either genotype 123-5 ($p = 0.001$) or 122-1 ($p = 0.003$), while mean nymph production was 44 and 36 per adult for genotype 122-1 and 123-5, respectively. However the differences between these two wheat genotypes were not significant. These results suggest that Drysdale may exhibits low levels of antibiosis, and that the other two genotypes may

exhibit moderate to high levels of antibiosis; although not statistically significant, genotype 123-5 exhibited the highest levels of antibiosis (Fig. 3.1a).

Effects of salt on wheat-aphid interaction (Study 2): The performance of *S. avenae* was evaluated on these same three wheat genotypes grown in saline soil conditions in order to study the consequences of host plant exposure to salinity on subsequent aphid performance. Aphid fecundity was significantly negatively affected by salinity (p = 0.001) and wheat genotype (p = 0.001). However, the interaction between the two factors was not significant. Aphid fecundity was significantly reduced (p = 0.001) on salt-treated plants for all wheat genotypes relative to their respective control plants grown under non-saline conditions, with total fecundity being reduced by 30%, 37% and 29% on salt-treated plants 123-5, 122-1 and Drysdale respectively. Drysdale, which supported the highest level of nymph production in the presence of salt, differed significantly compared to 123-5 (p = 0.001) and 122-1 (p= 0.003). As seen for plants grown in the absence of salt, there were fewer nymphs produced on 123-5 compared to 122-1 under saline conditions, but again this difference was not statistically significant (Fig. 3.1a).

Effects of wheat genotype on daily aphid fecundity

The daily fecundity, measured as the number of nymphs produced per adult per day, was also recorded for *S. avenae* on the three wheat genotypes for the two studies. Results showed significant variation among genotypes (p = 0.001).

Screening for aphid resistance (Study 1): On genotype 123-5 *S. avenae* produced 2 nymphs per adult per day, whereas aphids on the two other genotypes (122-1 and Drysdale) produced 2.4 nymphs per adult per day. Genotype 123-5 thus had the lowest daily fecundity and differed significantly when compared with Drysdale (p= 0.001) but did not show significant difference when compared to 122-1 (p= 0.080) (Fig. 3.1b). The only significant difference occurred between 123-5 and Drysdale (p<0.05).

Effects of salt on wheat-aphid interaction (Study 2): Daily fecundity of *S. avenae* was significantly affected by salinity treatment (p < 0.001) and genotype (p < 0.001), however the interaction between the two factors was not significant. Daily nymph production was reduced on all wheat genotypes treated with salt compared to control plants with no salt. In the absence of salt adult aphids produced 2.0 ± 0.5, 2.4 ± 0.4 and 2.4 ± 0.4 nymphs per day on genotypes 123-5, 122-1 and Drysdale, respectively. In the

presence of salt, adults produced significantly lower numbers of nymphs, these being 1.4 ± 0.4, 1.5 ± 0.3 and 1.9 ± 0.2 nymphs per day for genotypes 123-5, 122-1 and Drysdale, respectively (Fig. 3.1b). Drysdale differed significantly from 123-5 and 122-1 genotypes while the two latter genotypes did not show significant difference in daily fecundity.

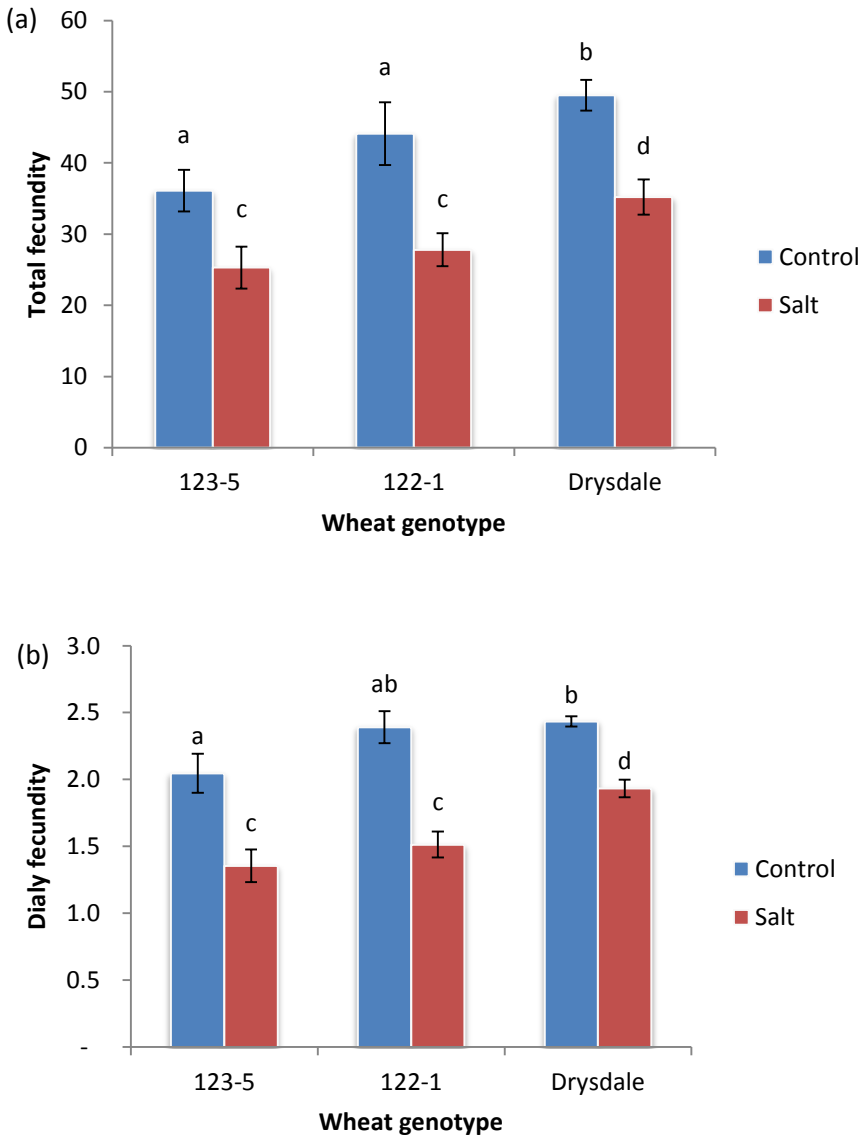


Figure 3.1 Reproduction parameters of *S. avenae*
(a) Total fecundity (measured as mean total number of nymphs produced per aphid over 21 days, mean ± SE; n=10) (b) Daily fecundity (nymphs per day; means ± SE; n=10) on three wheat *T. aestivum* genotypes. Aphids were reared under two treatment conditions on salt treated plants at 160 mM NaCl for 5 weeks (red bars) and on control plants with no salt (blue bars). Different letters indicate significant differences among genotypes under each treatment at $p<0.05$ according to Tukey's test after two-way ANOVA.

Effects of wheat genotype on reproductive rate

Nymph production over time. The rate and trend of *S. avenae* reproduction was observed and analysed over time for the three wheat genotypes. In the absence of salt, aphids on genotype 123-5 produced 0.9 ± 1.5 nymphs/adult/plant on day 1 (the first day of adult life). Fecundity increased thereafter as production peaked at 3.5 nymphs/adult/plant on days 5 and 7. However, fecundity decreased on day 8 to 2.2 nymphs/adult/plant, increased again to > 3 nymphs/adult/plant on days 9 and 11 and decreased thereafter to < 2 nymphs/adult/plant for the remaining reproduction period. Genotype 122-1 produced 2.5 ± 2.4 nymphs/adult/plant on day 1 and fecundity increased thereafter, peaking at 3 nymphs per day, which occurred on days 3 and 5. By day 8, fecundity decreased to 2.4 nymphs per day and continued to decrease thereafter to < 2.4 nymphs per day for the remaining of the production period. For Drysdale 1.6 ± 2 nymphs per day were produced on the first day of adult life. Fecundity increased thereafter as production peaked at 3.4 nymphs per day on days 5, 6 and 7. However, fecundity decreased on day 8 to 2 nymphs per day, increased slightly on days 9 and 10 (2.7 nymphs per day) and decreased thereafter for the remaining of the reproduction period (Fig. 3.2). The cumulative number of *S. avenea* nymphs produced on plants grown in the absence of salt was different among the three wheat genotypes. Results showed that *S. avenae* exhibited high, moderate and low cumulative number of nymphs on Drysdale, 122-1 and 123-5 respectively (Fig. 3.2). Drysdale differed significantly from 123-5 ($p < 0.001$) and 122-1 ($p=0.013$) genotypes while the two latter genotypes did not show significant difference in cumulative number of nymphs.

There was a strong negative correlation between the total number of nymphs produced daily over 21 days and the age of adult aphid feeding on control plants of genotypes 123-5, 122-1 and Drysdale. A comparison among the three wheat genotypes showed that the relationship was highly significant on 122-1 ($r^2 = 0.85$) followed by 123-5 ($r^2 = 0.42$) then Drysdale ($r^2 = 0.32$).

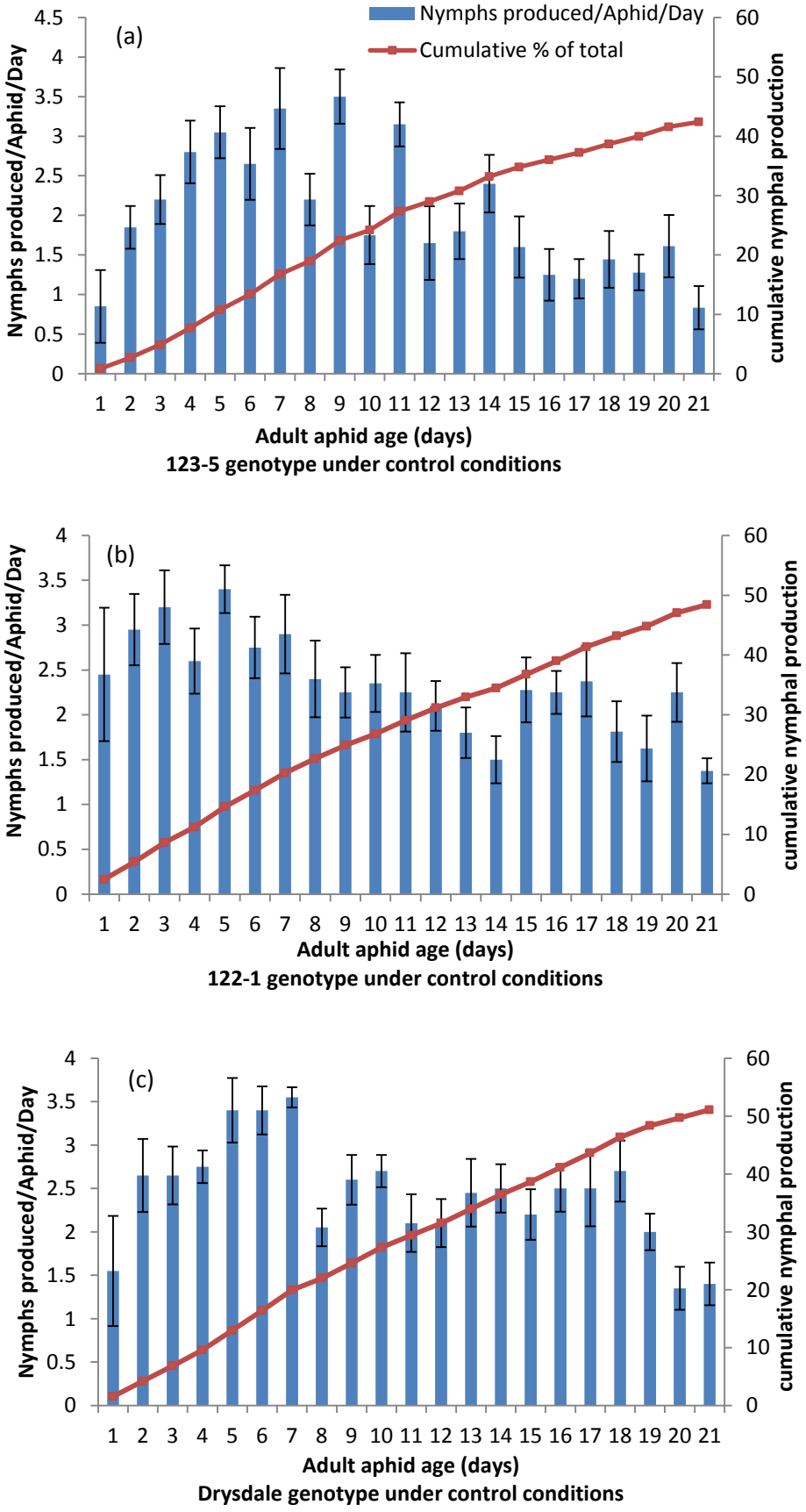


Figure 3.2 Nymph production over time and cumulative number of nymphs produced by *S. avenae* on three different wheat genotypes
(a) 123-5, (b) 122-1 and (c) Drysdale grown under control/non-saline conditions over a period of 21 days (numbers are means±SE, n=10).

Changes in *S. aveana* reproductive rate as a consequence of increased plant exposure to salt: Overall there was a decline in daily reproductive rate of *S. avenea* towards the end of the bioassay trial on the three wheat genotypes in both treatments, however salinity treatment caused a significant (p<0.05) reduction in daily reproduction rate of aphids compared to those feeding on control plants. Therefore, the increase of host plant exposure to salt over time was accompanied by a decrease in aphid reproductive ability (Fig. 3.3).

Total nymph numbers produced by adult aphids feeding on salt-treated plants were strongly negatively correlated with increasing the days of host plant exposure to salinity. There was a significant negative relationship between the total number of nymphs produced by adults feeding on salt-treated plants and days of host plant exposure to salinity. The correlation coefficients were $r^2 = 0.70$, $r^2 = 0.82$ and $r^2 = 0.87$ for wheat genotypes 123-5, 122-1 and Drysdale, respectively. Cumulative nymph numbers of *S. avenae* were significantly affected by salinity treatment (p = 0.001) and genotype (p = 0.001); however the interaction between the two factors was not significant (p = 0.611). Salt pre-treatment caused a reduction in cumulative nymph production for all wheat genotypes compared to control plants. This reduction was greater with increasing days of plant exposure to salt stress.

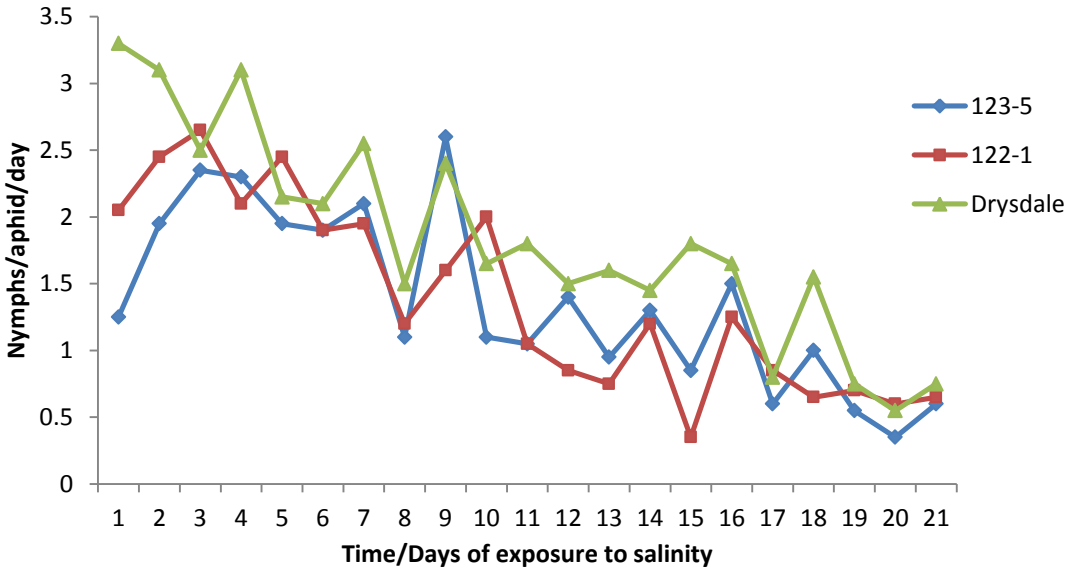


Figure 3.3 Influence of plant exposure to salinity over time on reproductive rate of *S. avenae* for three wheat genotypes (salt treated plants).
Aphids were monitored daily for reproduction/nymphs productions until the end of the bioassay. Blue line represents genotype 123-5; red line represents genotype 122-1 and green line represents Drysdale.

Drysdale differed significantly when compared with 123-5 (p = 0.001) and 122-1 (p = 0.013), however, the difference between the two latter genotypes was not significant (p = 0.525). Comparison among genotypes demonstrated that 122-1 exhibited a greater reduction in cumulative number of nymphs produced on plants grown under saline conditions. The reduction in cumulative number of nymphs caused by salinity treatment and the differences in cumulative number of nymphs between the presence and absence of salt was greater in 122-1 (indicated by the red arrow; Fig. 3.4) compared to the other two genotypes 123-5 and Drysdale.

The bioassay was terminated after 3 weeks from the onset of nymph reproduction and did not continue until all aphids die. This was because plants treated with salt started to show severe salinity effects. Therefore, in order to gain a potential observation and prediction, regression analysis was used. A negative correlation was detected between the total number of nymphs produced over 21 days and the age of adult aphids feeding on the three wheat genotypes. Using the regression function it was predicted that the increase in aphid age to 40 days, commencing from nymph production, resulted in a decrease in the abundance of nymph production by an average -0.52 and -3.40 total nymphs on control plants of 123-5 and 122-1 respectively. In contrast, on Drysdale nymph production was predicted to increase by an average 22.01 total nymphs. However, using the regression function it was predicted that increasing host plant exposure to salt for 40 days from the onset of nymphs production resulted in a decrease in the abundance of total nymphs production by an average -13.29, -20.96 and -21.17 total nymph on 123-5, 122-1 and Drysdale respectively (Table S3.1).

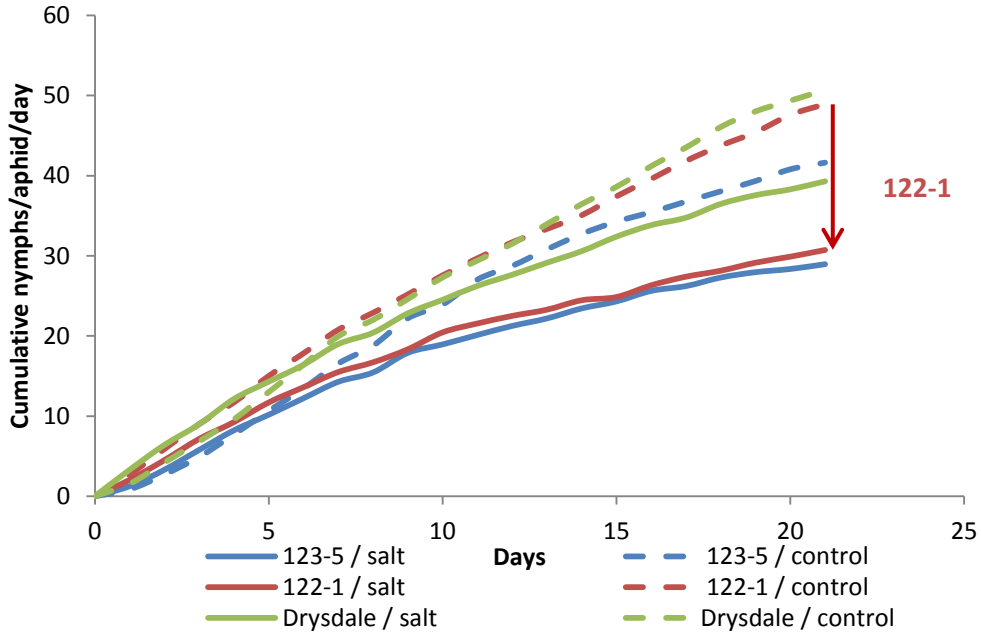


Figure 3.4 Cumulative number of nymphs produced by ***S. avenae*** over 21 days on three wheat genotypes
123-5 (blue line); 122-1 (red line) and Drysdale (green line). Plants were grown in saline at 160 mM NaCl and non-saline conditions over 5 weeks. Infestation with aphids started after 5 days of salinity treatment and reproduction started after 2 weeks from infestation and last for 3 weeks (n=10).

3.3.2 Plant performance

Plant performance was evaluated on three wheat genotypes 123-5, 122-1 and Drysdale by measuring specific growth and physiological parameters in order to screen these wheat genotypes for both potential tolerance to aphid and also to determine the basis of this tolerance utilised in plants against aphid infestation (Study 1). As stated above, a second study was carried out to determine the effects of combined aphid and salt stress on plant performance (Study 2). For clarity, results of plant performance obtained from the two studies will be compared among the three wheat genotypes under three treatments namely: (i) control, in which plants were grown in the absence of salt or aphid exposure; (ii) aphid infestation, in which plants were grown in the absence of salt but infested with aphids and (iii) dual stress i.e a combination of biotic and abiotic stress in which plants were exposed to both saline conditions and aphid infestation.

Growth parameters

Several growth parameters including number of tillers, shoot height, chlorophyll content, and root and shoot dry weight were measured at the end of the bioassay period

on three wheat genotypes which differed both in their levels of salt tolerance and sodium Na^+ content in shoot (see Chapter 2). Overall, statistical analysis revealed that the main effects of treatment and genotype on plant growth parameters for all three wheat genotypes were highly significant ($p \leq 0.001$). Results showed that there was a greater reduction in plant growth of all genotypes under conditions of dual stress (aphid infestation plus salt) compared to either aphid infestation alone or control treatments. These results suggest that salt is having the greatest impact. The interaction between treatment × genotype was only significant ($p< 0.05$) effects in terms of shoot height and dry root weight, with tiller number, chlorophyll content and dry shoot weight not being significant.

Effects of treatments between wheat genotypes on tiller number

Results showed that under aphid infestation genotypes 123-5, 122-1 and Drysdale produced on average 3, 3 and 4 tillers/plant respectively; this was not significantly different to control non-infested plants. However, tiller number was reduced significantly (p=0.001) to 2 tillers/plant for all genotypes when plants were exposed to the combination of salt stress plus aphid infestation, compared to individual aphid infestation and control treatments. Among wheat genotypes Drysdale differed significantly by producing more tillers than either 123-5 (p=0.006) or 122-1(p=0.025) under control conditions. Whereas, under aphid infestation the only significant difference in tiller number among wheat genotypes occurred between Drysdale and 123-5 (p=0.047) (Fig. 3.5a).

Effects of treatments between wheat genotypes on shoot height

Results showed that there was no significant difference between shoot heights of plants infested with aphid and control plants for all wheat genotypes. However, shoot height was reduced by exposure to dual stress in all genotypes and showed significant differences compared to those under aphid infestation (p=0.001) and control treatment (p=0.001). Furthermore, differences in shoot height among genotypes showed that 122-1 scored the maximum shoot height (49.9 cm in control, 46.5 cm in aphid infestation and 36.2 cm in dual stress) than other genotypes in all three treatments followed by 123-5 (shoot height was 44.7 cm in control, 39.9 cm in aphid infestation and 20.05 cm in dual stress); Drysdale scored the minimum shoot height (29 cm in control, 28.1 cm in aphid infestation and 19.9 cm in dual stress). Infested plants in the presence of salt

stress exhibited up to 55% reduction in shoot height of genotype 123-5, which was greater than reduction in shoot height for either 122-1 or Drysdale, which were up to 27% and 31% respectively. However, plants infested with aphids in the absence of salt exhibited lower reduction in shoot height, these being up to 11%, 7% and 3% in genotypes 123-5, 122-1 and Drysdale, respectively (Fig. 3.5b).

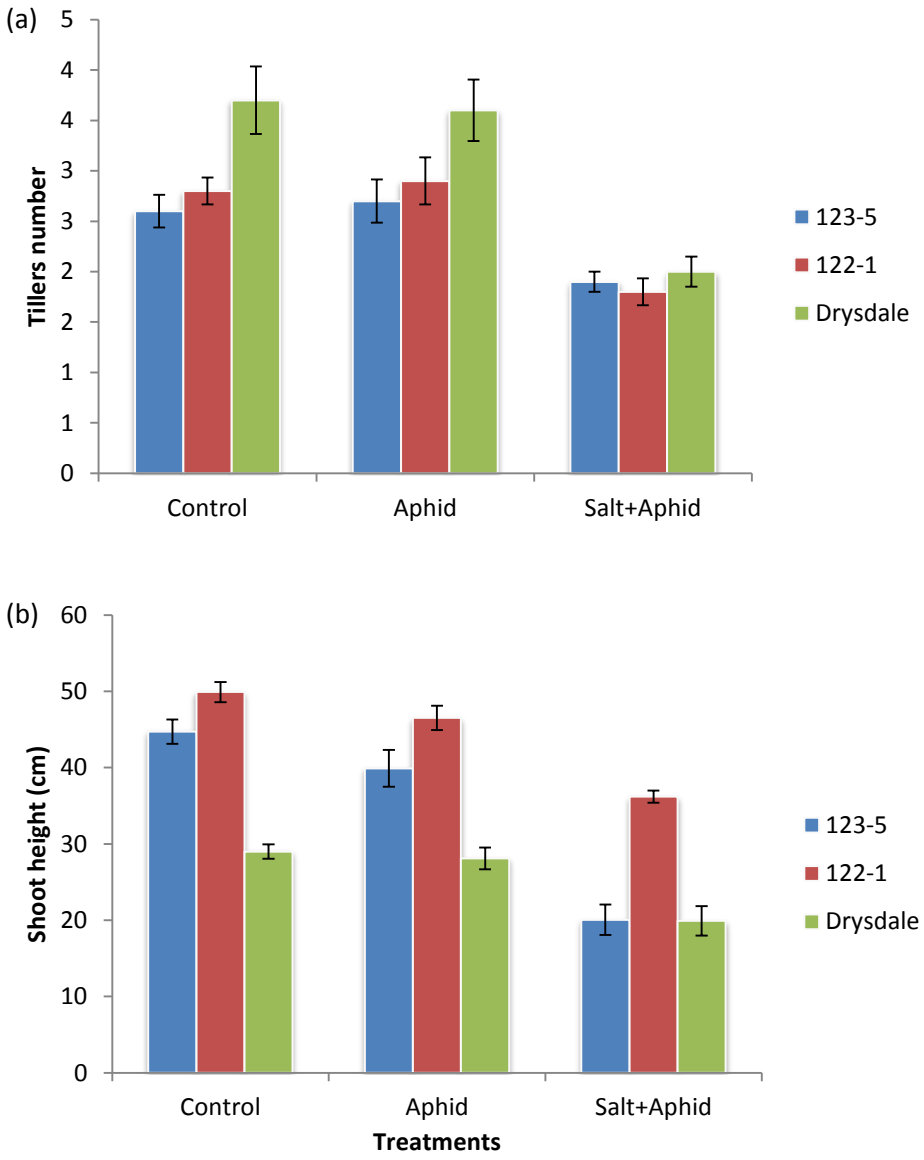


Figure 3.5 Measurements of plant growth parameters
(a) Tiller numbers and (b) Shoot height/cm of three wheat genotypes 123-5, 122-1 and Drysdale. Plants were grown under three treatments: control, aphid infestation and dual stress (salt + aphid). Salinity treatments lasted for 5 weeks and aphid infestation lasted for 3 weeks (means ± SE n=10).

Effects of treatments between wheat genotypes on root and shoot dry weight

Results showed that root dry weight for all genotypes was negatively affected by the dual stress and showed significant differences when compared to root dry weight of plants under control conditions (p = 0.001) and aphid infestation (p = 0.001). However, root dry weight did not differ significantly between plants under aphid infestation and plants under control conditions for all genotypes except for Drysdale, which exhibited significant variation (p =0.001). Under aphid infestation the only significant difference in root dry weight among genotypes was detected between 123-5 and Drysdale (p=0.006). Reduction in root dry weight caused by aphids alone was up to 4%, 2% and 25% in 123-5, 122-1 and Drysdale respectively. However the reduction in root dry weight was greatest in genotypes under salinity plus aphid than in those under aphid stress alone (p = 0.001), with reductions in root dry weight up to 75%, 70% and 73% for genotypes 123-5, 122-1 and Drysdale respectively. The maximum root dry weight was recorded in genotype 123-5 under aphid infestation whereas genotype 122-1 scored the maximum root dry weight under the dual stress (Fig 3.6a)

Similarly, shoot dry weight of all genotypes was reduced the greatest by dual stress and showed significant variation when compared to either control plants (p =0.001) or to aphid infestation (p=0.001). However differences between aphid infestation and control in shoot dry weight of wheat genotypes was only significant for Drysdale. Aphid infestation caused a slight reduction in shoot dry weight up to 10%, 4% and 18% in 123-5, 122-1 and Drysadle respectively. The presence of salinity during aphid infestation, however, caused more reduction up to 71%, 60% and 68% in 123-5, 122-1 and Drysdale respectively. Among genotypes 122-1 had the greatest shoot dry weight under aphid infestation and dual tress whereas, Drysdale had the lowest shoot dry weight in all treatments (Fig 3.6b).

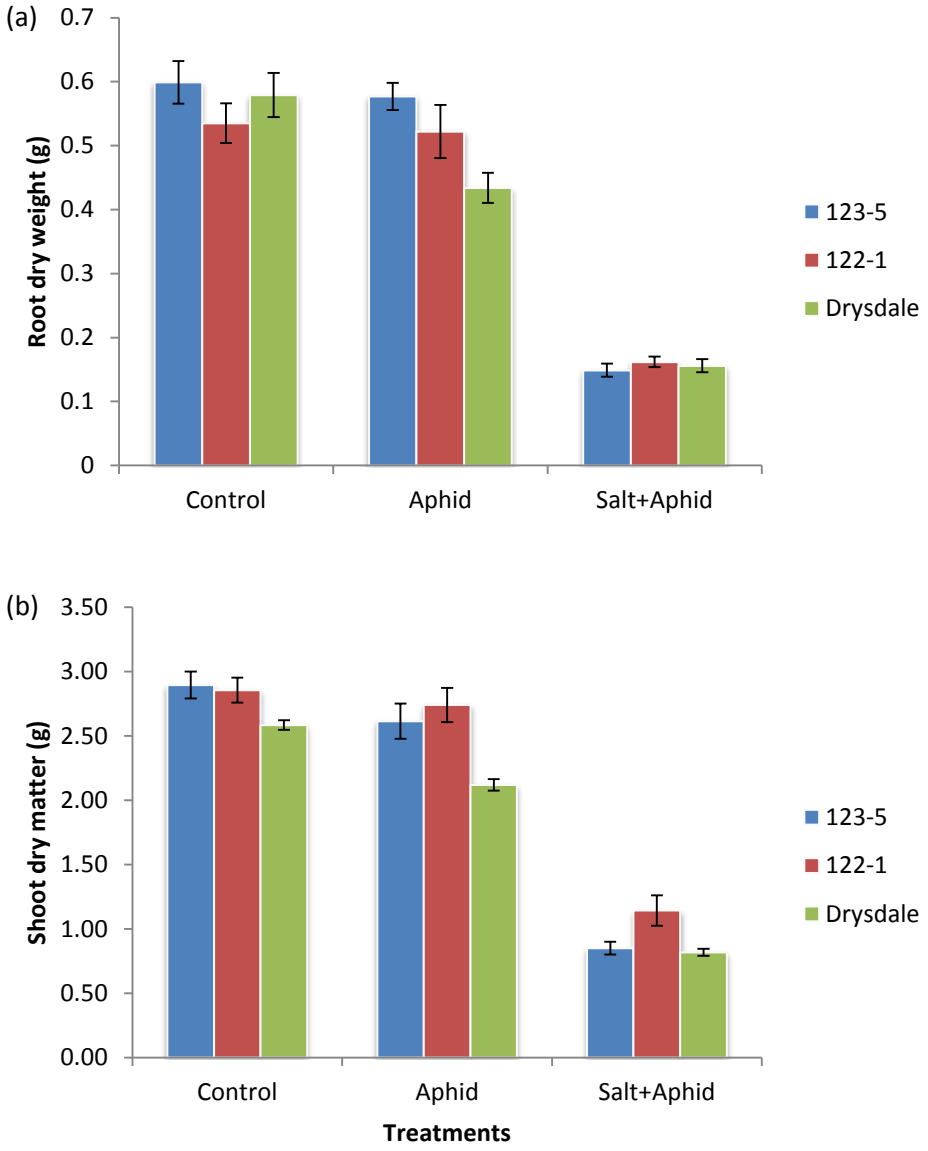


Figure 3.6 Measurements of plant growth parameters
(a) Root dry weight and (b) Shoot dry weight of three wheat genotypes 123-5, 122-1 and Drysdale. Plants were grown under three treatments: control, aphid infestation and dual stress (salt + aphid). Salinity treatments lasted for 5 weeks until the end of the bioassay and aphid infestation started 2 weeks after plant exposure to salt and lasted for 3 weeks (means ± SE n=10).

Effects of treatments between wheat genotypes on chlorophyll content

Chlorophyll content in leaf number five was measured at the end of the bioassay trail on the three wheat genotypes to evaluate plant performance after exposure to the three different treatments. The results were unexpected as they showed that the chlorophyll content was increased in all wheat genotypes under the two stress treatments compared to their respective controls (26.7 in 123-5, 24.9 in 122-1 and 28.99 SPAD units in Drysdale). Moreover wheat genotypes under the combination of salt and aphids had

higher chlorophyll content (33.5 in 123-5, 29.6 in 122-1 and 33.3 in Drysdale) than under aphids alone (27.8 in 123-5, 26 in 122-1 and 31.5 in Drysdale). However, these differences in chlorophyll content among treatments were not significant. Furthermore, the variation in chlorophyll content among wheat genotypes was only significant between genotype 122-1 and Drysdale (p=0.046) under aphid infestation, although, Drysdale in general was shown to have the maximum chlorophyll content among tested genotypes under all treatments (Fig. 3.7).

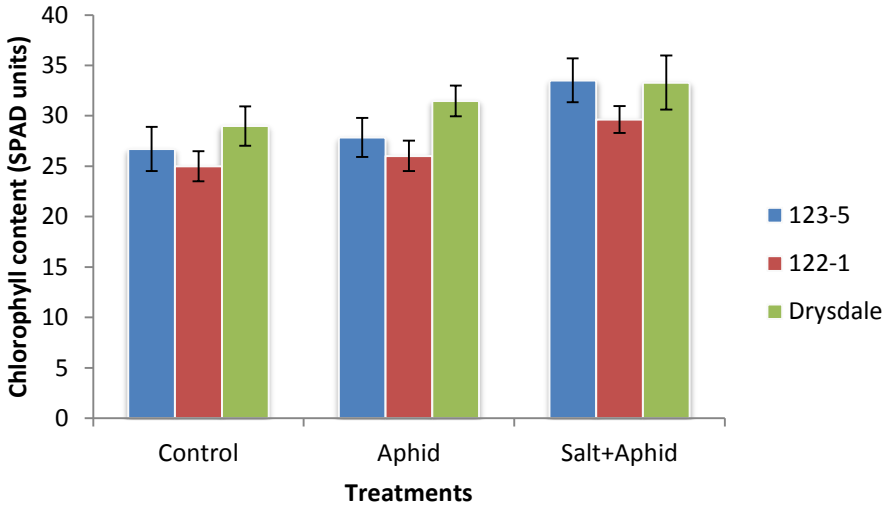


Figure 3.7 Chlorophyll concentration
Estimated with a SPAD meter in leaf 5 of three wheat genotypes 123-5, 122-1 and Drysdale under three treatments: control, aphid infestation and dual stress (salt + aphid). Salinity treatments lasted for 5 weeks and subsequent aphid infestation lasted for 3 weeks (means ± SE n=10).

Effects of treatments between wheat genotypes on biomass and aphid tolerance

Plant growth under aphid infestation was used as an index of potential tolerance to aphid infestation and was measured as biomass production calculated as the percentage of shoot dry weight under stress conditions relative to the control (Table S3.2). Results showed that in general, aphid infestation caused a slight reduction in plant growth by the end of the bioassay. Genotype 122-1 had the highest biomass, relative to its control (96 %) and was therefore considered the most aphids tolerant of the different genotypes, with Drysdale having the lowest biomass (82%) and therefore the most susceptible; genotype 123-5 showed 90% biomass production under aphids. As expected, plants infested with aphids in the presence of salt produced lower biomass, these being 40%, 29%, and 32% for genotypes 122-1,123-5, and Drysdale, respectively. These results

show that genotype 122-1 had the highest biomass production under both aphid infestation and dual stress, compared to the other genotypes and was therefore considered to be the most stress tolerant (Fig. 3.8).

Relationship between plant stress tolerance index and aphid fecundity

Data showed that there was no correlation (r^2=0.01) between plant tolerance index, measured by the percentage of shoot dry weight of plants grown under stress conditions relative to control, and *S. avenae* fecundity on three wheat genotypes grown under salinity conditions. However, there was a weak negative correlation (r^2=0.25) between tolerance index and *S. avenae* fecundity on plants grown under non-saline conditions i.e. in the absence of salt.

Relationship between aphid performance and plant performance in the presence and absence of salt

Wheat plants grown in the absence of salt showed a strong negative relationship between aphid performance (cumulative nymph production) and the growth parameters shoot height (r = - 0.57) and dry shoot weight (r = - 0.52). Moreover, a weak negative correlation was observed between aphid performance and chlorophyll content and dry root weight. However, tiller number showed a positive correlation with aphid performance (r = 0.32). Among these plant growth parameters, shoot height showed the highest correlation with *S. avenae* performance on the three different wheat genotypes. For plants grown in the presence of salt, there was a weak correlation between *S. avenae* performance with all plants growth parameters measured except for chlorophyll content and root dry weight where there was a positive correlation. Among these plant growth parameters, shoot dry weight showed the highest correlation with *S. avenae* performance on three different wheat genotypes (Table S3.3).

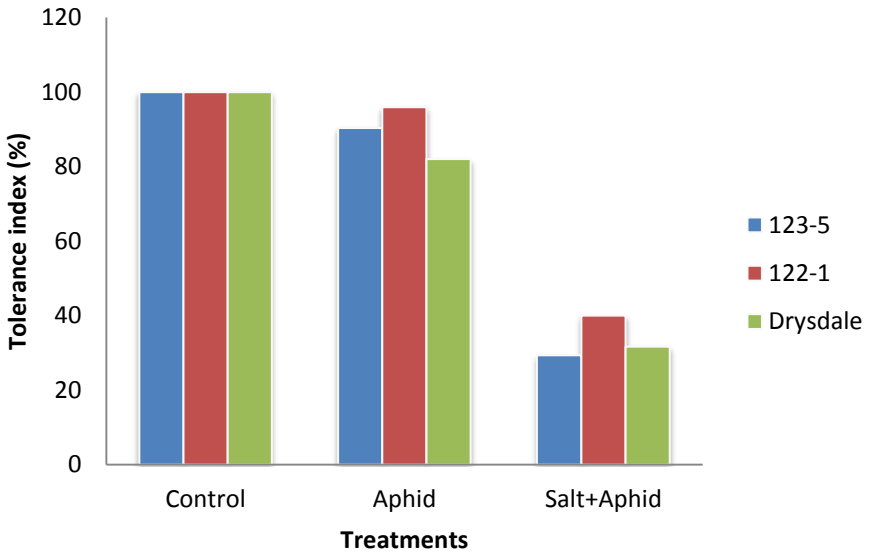


Figure 3.8 Tolerance index measured as relative biomass production which was calculated as the percentage of shoot dry weight in stress (aphid, salt+aphid) relative to control in three wheat genotypes 123-5, 122-1 and Drysdale.

3.4 Discussion

3.4.1 Evaluating aphid performance on wheat genotypes: screening for potential antibiosis

Antibiosis is defined as the negative influence of the plant on the biology of an insect attempting to use that plant as a host and measures the effect of a given plant on insect biology such as reducing fecundity (Tolmay *et al*., 1999). In the present study aphid fecundity, in terms of daily/cumulative nymph production, was used to screen for aphid performance as this technique is regarded as a sensitive measure of antibiosis (Tolmay *et al*., 1999), although Scott *et al*. (1991) claimed that the total colony counts may be a more realistic indicator of antibiosis than nymph counts. Furthermore, in the present study aphids were not confined to clip cages but were able to move and settle on different plant leaves as recommended by (Du Toit, 1992).

In the present study, three wheat genotypes 123-5, 122-1 and Drysdale were screened for resistance against the aphid *Sitobion avenae* under controlled environmental conditions using artificial infestation and the level of potential antibiosis was evaluated by measuring fecundity parameters using the nymph production over 21 days. *S. avenae* on 123-5 had the lowest fecundity compared to 122-1 and Drysdale, indicating that 123-5 may possess a high level of antibiosis towards *S. avenae*, rather than tolerance. In

contrast, *S. avenae* on Drysdale attained high fecundity, thus exhibiting low levels of antibiosis, while genotype 122-1 exhibiting intermediate levels of antibiosis resistance. Direct comparison of performance of *S. avenae* on these selected wheat genotypes with other studies has not been possible due to the lack of available data.

Factors that impact on the fecundity of *S. avenae* may include aphid-plant genotype, the particular aphid clone in question and the part of the plant where the aphids were placed (Ozder, 2002). Moreover, previous studies on aphids found that the population growth variation might be related to plant nutrition, leaf age, leaf surface and the presence of secondary compounds (Taheri *et al*., 2010). It has also been noted that *S. avenae* reproduced faster on wheat ears than on leaves (Ozder, 2002). All these factors may contribute to the differences in aphid fecundity found among the three wheat genotypes in this study.

In general, this study demonstrated that the reproductive performance of *S. avenae* were generally affected by wheat genotypes and suggesting a genetic basis. In terms of total fecundity both 123-5 and 122-1 genotypes differed significantly from Drysdale whereas the daily fecundity was only significantly different between 123-5 and Drysdale. This finding is in agreement with other studies that reported highly significant differences in fecundity among a selection of 29 cultivars of wheat *Triticum aestivum* against two aphid species, the green bug and the Russian wheat aphid (Castro *et al*. 2004, 2005) and in various wheat varieties that differed considerably in terms of their quality as host for the bird cherry-oat aphid (Taheri *et al*., 2010).

In this study, the wheat genotype Drysdale was the most preferred host for *S. avenae* and 123-5 was the least favourable. The high performance of the aphid on Drysdale mostly resulted from the highest number of nymphs produced on this genotype. Conversely, the poor performance of *S. avenae* on 123-5 was correlated with the lowest fecundity. Recent studies showed that fecundity, adult longevity and developmental time affect aphid performance (Taheri *et al*. 2010). The exact mechanism concerning the differences considered in this study is unknown and requires additional assessment.

3.4.2 Evaluating wheat performance under aphid infestation: screening for potential tolerance

Tolerance is defined as the plant's ability to withstand or compensate for insect damage (Tolmay *et. al*., 1999). Infestation by *S. avenae* did not affect plant shoot height, tiller

number or chlorophyll content for any of the genotypes screened as there was no significant variation between infested and control plants. However root and shoot dry weight were significantly affected (p=0.001) by aphid infestation as compared to control plants for Drysdale. This is in contrast with other studies that reported severe reduction in plant height in wheat in response to Russian wheat aphid (Du Toit, 1992; Scott *et al*., 1991). These authors regarded plant height as a reliable measure of tolerance contradicting the findings in this study. A possible explanation for this may be due to how the assays were carried out. In the present study all nymphs were removed on a daily basis (enabling the intrinsic rate (r_m values) of reproduction to be calculated) only leaving the two adults; however in other studies the nymphs were not removed allowing a very rapid build-up in aphid numbers. This procedure of keeping the infestation at a constant level has been reported in previous studies (Tolmay *et al*., 1999) and is considered to be more sensitive. However, the finding that tiller number was not influenced by aphid infestation is in agreement with a previous study examining wheat resistance against cereal aphids in the field which showed that there were no differences in the number of tillers per meter square between treated and un-treated plots (Khan *et al*., 2007). These authors proposed that the number of tillers is influenced by the genetic potential of the wheat genotype rather than the effect of aphid infestation. Based on the findings of the present study, the results showed that shoot dry weight was higher for 122-1 than for 123-5 and Drysdale, producing shoot dry weight of 96 %, 90% and 82% respectively at the end of the experiment under aphid infestation.

3.4.3 Aphid performance on wheat plants grown under salinity

To date relatively few studies have been carried out to investigate the effects of salt stress on the plant-insect interactions. Examples of such studies include the following aphid species the lime aphid *Eucallipterus tiliae* L. (Baczewska *et al*., 2011), *Aphis pomi* (Braun and Fluckiger, 1984), *Schizaphis graminum* (Araya *et al*., 1991) and *Rhopalosiphum padi* (Spencer and Port, 1988). Other examples include the Japanese beetles (Stamp and Harmon 1991), the leaf mining *Bucculatrix maritima* (Hemminga and Soelen, 1992), and the gall-forming insects *Eurosta solidaginis* (Martel, 1995), and *Epiblema scudderiana* (Martel, 1998). However, no such studies have investigated performance of the aphid *Sitobion avenae* on wheat plant under the effect of salt stress. The present study is the first study to investigate the influence of soil salinity on the reproductive capacity of aphids *S. avenae* feeding on leaves of three different wheat

genotypes that differ in their level of salt tolerance and sodium content in the vegetative tissues.

3.4.4 Variation in insect performance in response to salt treatments

Insect performance is very variable and has been reported to increase, decrease or remain unchanged in response to plant stress (Larsson 1989; Larsson and Bjorkman, 1993). For example, boring and sucking insects were found to perform better on stressed plants (Koricheva and Larsson, 1998). A few studies on the effect of salt stress to plants on insect performance have revealed conflicting results. The present study revealed that *S. avenae* performance in terms of fecundity was significantly reduced on wheat plants for all three genotypes growing in salinized-soil conditions at 160 mM NaCl for prolonged periods of time (5 weeks). This finding indicates that aphid performance was negatively affected in response to plants grown under saline conditions and is in agreement with Araya *et al*. (1991) who demonstrated that increased accumulation of salt in wheat and barley leaves decreased the population growth rate of the aphid *Schizaphis graminum* in proportion to the amount of salt accumulated by leaves. In addition, the present results are consistent with the study on the influence of soil salinity on abundance of lime aphid which showed that the increase content of chlorine was accompanied by a decrease in the number of aphids (Baczewska *et al*., 2011). In contrast, Braun and Fluckiger (1984) observed higher population densities of the green apple aphid *Aphis pomi* on NaCl-sprayed *Crataegus spp* plants and found higher amino acid and sugar concentrations in phloem of salt treated plants relative to control plants. Furthermore, another experiment which used different NaCl concentrations showed no significant effect on any life–history parameter of aphids *Rhopalosiphum padi* (Spencer and Port, 1988). Thus, previous studies have shown no consistent response of phloem-feeding insects to increased salinity conditions.

3.4.5 NaCl accumulation in host plants and aphid performance

Previous work has shown that Cl^- and Na^+ ions accumulation in plants following salinity treatment negatively influenced aphid performance and decrease the aphid population (Araya *et al*., 1991; Baczewska *et al*., 2011). In the present study Na^+ concentration in the plant was not measured, however, in Chapter 2, Na^+ content was quantified in the dry shoots of the same three wheat genotypes included in this study. Results showed that after exposure to 160 mM NaCl for 3 weeks, genotypes 122-1 and

Drysdale accumulated high Na^+ content in shoot whereas 123-5 accumulated low Na^+ levels in shoots. Moreover, it is speculated that by increasing the duration of plant exposure to salt up to 5 weeks, salt is likely to build up and accumulated even more in the shoots particularly of genotypes 122-1 and Drysdale. As a result, Na^+ concentration in shoot of these wheat genotypes may contribute to the reduction in *S. avenae* fecundity on plants treated with a combination of salt for 5 weeks and aphid infestation for 3 weeks.

Furthermore, it can be argued that Na^+ content in plants could play some role in reducing aphid fecundity as other studies reported that Na^+ and Cl^- appeared in the sieve tube/phloem sap which was collected from fully expanded leaves of NaCl treated barley plants through aphid stylet (Munns, 1988). However, these authors found that the Na^+ concentration in the sap plateaued and thereafter was not affected by the level or duration of exposure to salt (Munns, 1988). The level of Na^+ in the phloem was not measured in the present study, but it is likely to have affected aphid performance. However, measuring Na^+ content in the sap is needed to confirm the presence of salt and its effect on aphid. In addition, it has been reported that approximately 13-36% of the Na^+ and Cl^- imported into leaves through the xylem were exported by the phloem (Lohaus, 2007).

3.4.6 The plant stress hypothesis and insect performance

It has been hypothesized that plants under abiotic stress become more suitable as food for herbivorous insects (King *et al*., 2006). Moreover, it has been reported that changing soil physical and chemical characteristics as a result of salinity affects plant growth, causes physiological changes and alters plant nutrition which in turn creates conditions more or less favourable to insects feeding on them. Thus, the effect of abiotic and biotic stress on host plants alter food nutrients and balance which in turn negatively affect sap-sucking insects such as aphids when feeding on stressed plants (An Nguyen *et al*., 2007). Previous studies have shown that salinity can increase sugar content and the amino acids asparagine, glutamine and aspartic acid in the phloem exudate. These biochemical variations enhanced aphid development (Braun and Fluckiger, 1984). In addition, salinity induces several metabolic changes in plants, such as accumulation of proline and glycine-betaine, which was shown to increase both survival and reproduction in aphids (Araya *et al*., 1991). A study on the influence of soil salinity on the abundance of lime aphid on the leaves of lime trees growing along the roadside

showed that there was no nitrogen deficiency in the leaves and the increase of this element was accompanied by an increase in the number of aphids (Baczewska *et al*., 2011). In this study neither of the elements quantified in previous studies were measured, nevertheless, these conditions are to be expected by salinity treatment applied in this study; further analyses are required for confirmation. In the present study salt treated plants were not favoured by *S. avenea*. Therefore, this study did not support the plant stress hypothesis that ranks stressed plants as better hosts for insects.

3.4.7 Wheat plant response to a combination of aphid infestation and salt stress

Plant performance under the combination of aphid infestation and salt stress i.e dual stress was evaluated through measuring a range of growth and physiological parameters. Dual stress significantly affected plant performance compared to either aphid infestation alone, or the non-treated control plants for the three different wheat genotypes. The combination of salt and aphid infestation significantly reduced all plant parameters measured. Interestingly, plant performance for the 3 different genotypes, when evaluated in response to aphid infestation with no salt, was only significantly affected in terms of root and shoot dry weight. There are several possible explanations for this result. First, the severe effect of dual stress on plant performance is mainly caused by the prolonged exposure of plants to relatively high salinity (5 weeks from the onset of aphid infestation). Second, aphid infestation was initiated at very low levels (each plant was infested with only two aphids) and so over 3 weeks did not significantly contribute to the reduction in plant performance under dual stress. Third, when comparing these results with those obtained from the screening experiment conducted in chapter 2, in which wheat genotypes were exposed for only three weeks to the same salt concentration as opposed to 5 weeks, plant growth was more retarded in the present study. These differences in growth reduction can be explained by the two phases that causes growth reduction under saline conditions, as explained by Munns (2002).

In chapter 2 the three selected wheat genotypes were evaluated and screened for salt tolerance. Genotypes 123-5 and 122-1 were classified as salt tolerant, accumulating high and low Na^+ content in the shoots respectively, whereas Drysdale was classified as salt sensitive, but accumulating high Na^+ content in shoot. Therefore, the single salinity treatment on its own was not repeated in this chapter. When comparing plant performance, the results for all three genotypes showed that there was a greater reduction in plant growth under dual stress compared to either stress alone. However,

since no differences have been found in plant growth parameters measured under aphid infestation alone relative to control plants, it can be assumed that aphid infestation accounts for only a small, if any, plant growth reduction seen under dual stress, although stress to salinity may alter the plant's sensitivity to aphid infestation. It is the presence of salt stress that caused plant growth reduction and the prolonged duration of plant exposure to salinity treatment that last for 5 weeks increased this reduction compared to a period of 3 weeks in previous experiment.

This reduced plant performance seen in the present study under the combination of abiotic and biotic stress is in agreement with Chojak *et al*. (2012) who demonstrated that sequentially applied salt stress to cucumber (*Cucumis sativus*) plants inoculated with *Pseudomonas syringae* reduced plant growth and leaf expansion and decreased chlorophyll content. Interestingly, subsequent pathogen-induced stress did not change shoot, leaf or root growth. However, in the present study chlorophyll content was increased under stress treatment, but this was not statistically significant. Increased photosynthetic activity has been cited as evidence for the ability of plants to compensate for damage (Salt *et al*., 1996). However a decrease in the photosynthetic activity of leaf tissue has also been reported in aphid infested leaves (Salt *et al*., 1996).

In the present study there were no differences in the number of tillers between plants infested with *S. avenae* and their respective controls. This finding is in agreement with Khan *et al*. (2007) who screened wheat genotypes for resistance against cereal aphids and found no difference in tiller number between treated and un-treated plots. These authors suggested that tiller number is determined by the genetic potential of the genotype rather than the effects of infestation. However, aphid infestation did not affect tiller number in the present study, the dual stress did.

3.4.8 Interactions between biotic and abiotic stress and potential for cross-tolerance

The ability of plants to resist different stresses after exposure to one specific stress is known as cross-tolerance. Previous studies on this phenomenon reported that tomato plants treated with salt showed enhanced resistance to wounding due to accumulation of proteinase inhibitors (Dombrowski, 2003). In the present study the significant ($p \leq 0.05$) decline in aphid performance/fecundity on stressed plants after exposure to 160 mM NaCl in comparison to unstressed plants may be due to several reasons: Firstly, the indirect responses of insect infestation to environmental stress, Menedenz *et al*. (2008)

reported that plants adapt and acclimatise to salt stress through morphological and physiological changes, which may also negatively affect aphids. This is referred to as an indirect effect i. e. cross tolerance. Data from the present study showed that plant responses induced by salt stress may also enhance the resistance against aphids, thus supporting the hypothesis of cross-tolerance. Secondly, the direct effect of stress on insects, plants growing under salinity accumulate NaCl in their leaves, ranging from low levels of accumulation to high levels depending on both the ability of the plant to tolerate the salt and the mechanism used to cope with excessive salt in the soil (exclusion and/or inclusion) (Genc *et al*., 2007). Araya *et al*. (1991) reported that an aphid population of *S. graminum* in wheat decreased with increasing levels of salt accumulation in the leaves. The results of the present study are in agreement with those of Araya and colleagues in which the different wheat genotypes investigated were found to accumulate different concentrations of Na^+ in the leaves when exposed to 160 mM NaCl (previous experiment-data not presented); this is likely to account for the decline in aphid fecundity on genotypes growing in salt.

In terms of aphid performance, irrespective of the differences among the tested wheat genotypes in their response to salt treatment i.e. in their level of salt tolerance and in Na^+ content in the shoot, all showed a trend in terms of a reduction in *S. avenae* fecundity in the presence of salt compared to aphid fecundity in the absence of salt (irrespective of their mode of salt tolerance). However, Drysdale the most salt sensitive genotype with the highest Na^+ content was also the most susceptible to aphid infestation both in the presence and absence of salinity conditions. These findings indicate that Drysdale was more preferred and favoured by aphids than the other wheat genotypes 123-5 and 122-1 regardless of salinity/salt stress. In terms of plant performance, among the wheat genotypes investigated, 122-1 showed the highest relative shoots dry matter under aphid infestation and dual stress than the other genotypes 123-5 and Drysdale. Based on these results, genotype 122-1 was selected to investigate the molecular interactions between wheat and the cereal aphid *Sitobion avenae* and salt stress (see Chapter 4).

3.5 Conclusions

The present study was designed to determine the impact of salinity on plant-aphid interactions at the physiological level in three wheat *Triticum aestivum* genotypes through investigating the consequences of salt stress on both plant and aphid *Sitobion avenae* performance. Results from the present study demonstrate the following:

- There were differences between genotypes in aphid fecundity, genotype 123-5 which is salt tolerant and accumulate low Na^+ content in shoot was more resistant to aphid (low fecundity), genotype 122-1 which is salt tolerant and accumulate high Na^+ content in shoot was moderately resistant (moderate fecundity) and Drysdale which is salt sensitive and accumulate high Na^+ in shoot was susceptible to aphids (high fecundity) in both conditions of the presence and absence of salt.
- Plants under salinity conditions were not favoured and preferred by *S. avenae* all salt-treated plants experiences reduced aphid fecundity for both salt tolerant and sensitive genotypes. Therefore this result does not support the plant-stress hypothesis.
- Results support the cross-tolerance hypothesis. This is borne out in the present study where plants treated with salt, irrespective of whether they are salt tolerant (122-1 and 123-5) or not (Drysdale), caused significant reductions in aphid fecundity. Thus salt has a negative effect on aphid performance. Since the mode of salt tolerance in these genotypes differ (122-1 and Drysdale accumulates salt whilst 123-5 exclude salt), the effects on aphids is not due to salt *per se* but is plant-mediated. It is therefore important to understand the molecular basis of these effects. This is addressed in chapter 4 including one genotype 122-1 which produced the highest plant biomass under the dual stress and under aphid infestation relative to control. Affymetrix GeneChip wheat genome array was used to identify and characterize putative genes involved in such interactions and in cross tolerance.

This study contributes to our knowledge of the nature of plant responses to a combination of abiotic and biotic stresses. In addition, it sheds light on the influence of salt stress on plant-insect interactions. Such information is fundamental in providing opportunities for developing broad-spectrum stress tolerant crops. Further studies and additional assessment need to be conducted in order to know and confirm the exact

rational concerning/beyond the differences in aphids performance on three wheat genotypes in the presence and absence of salt considered in the present study.

CHAPTER 4

4 Gene Expression Profiles in Wheat under a Combination of Salt and Aphid Stresses

4.1 Introduction

Plants in their natural habitat as well as crop plants in the field are continuously exposed to various biotic and abiotic stresses which occur simultaneously, affecting growth and productivity (Mittler and Blumwald, 2010; Prasch and Sonnewald, 2013; Rasmussen *et al*., 2013). Evidence from recent molecular studies revealed that plant responses to a combination of different stress conditions are distinct, activating specific stress responses that cannot be directly extrapolated and detected from studying either stress individually (Rizhsky *et al*., 2004; Mittler, 2006; Atkinson and Urwin, 2012). For instance, transcriptome studies conducted by Rizhsky *et al*. (2002, 2004) on plants subjected to multiple abiotic stresses showed that in both tobacco (*Nicotiana tabacum*) and Arabidopsis (*Arabidopsis thaliana*), a combination of drought and heat stress induced a novel programme of gene expression, activating transcripts that are not induced by either stress individually. Similarly, microarray analyses by Voelckel and Baldwin (2004) revealed that exposure of native tobacco plants (*Nicotiana attenuate*) to sequential or simultaneous attacks by two herbivores, sap-feeding mirids (*Tupiocoris notatus*) and chewing hornworms (*Manduca sexta*), elicited a transcriptional response that is distinct from responses to each individual attack. Despite accumulating evidence, the majority of studies on plant stress factors have tested each of the different stresses in isolation (Rizhsky *et al*., 2004; Atkinson *et al*., 2013) and little is known about the molecular mechanisms involved in plant acclimation and adaptation to a combination of two different stresses or multiple stress conditions (Mittler, 2006). Therefore, there is a need to change the focus of plant stress research towards increasing understanding and knowledge of plant response and adaptation to multiple stress conditions (Atkinson and Urwin, 2012). Such information is vital for enhancing stress tolerance in plants and for breeding broad spectrum plant tolerance.

Plant response to multiple stresses is complex as a range of molecular mechanisms act together in a regulatory network. Cross talk refers to the interaction between two or more signalling pathways that could alter cellular responses (Taylor *et al*., 2003).

The effects of simultaneous biotic and abiotic stresses may interact both positively and negatively (Atkinson and Urwin, 2012) and is regulated by phytohormone signalling pathways that often interact synergistically or antagonistically. Other key components and common players involved in this cross-talk include: kinase cascades, transcription factors, reactive oxygen species and heat shock factors (Anderson *et al*., 2004; Fujita *et al*., 2006; Asselbergh *et al*., 2008b; Rasmussen *et al*, 2013). Many molecular components functioning as cross talk mediators between different signalling pathways, which lead to cross tolerance have been documented. Calcium-dependent protein kinases (CDPKs) which are involved in plant responses to various environmental stresses have been implicated in cross tolerance (Capiati *et al*., 2006). Jasmonic acid has long been known to be involved in enhancing resistance to herbivores and more recently has been shown to mediate the induction of wound-related genes in response to salt stress (Capiati *et al*., 2006). The accumulation of reactive oxygen species (ROS) scavenging enzymes in plants under different biotic and abiotic stresses may suggest a generalized role of ROS removal activity in mediating stress tolerance by plants (Sabehat *et al*., 1998; MEI and Song, 2010). Microarray technology is a powerful tool for studying the expression of hundreds of genes simultaneously (Volckel and Baldwin, 2004). It has been proven to be an effective method for identifying the molecular basis of plant stress responses (Deyholos 2010; Liu *et al*., 2012), and for studying the global analysis of gene expression in order to understand plant response to biotic and abiotic stresses.

4.1.1 Molecular responses to phloem-feeding insects

Although salinity and aphid stresses have been extensively studied individually, relatively little is known about how their combinations affect plants. Transcript profiling studies of plant responses to phloem feeding insects have been documented and reviewed (Thompson and Goggin, 2000). Phloem-feeding insects (PFIs) or piercing and sucking insects cause minimal amounts of damage during feeding on plant tissue; however, may trigger pathogen related response in a compatible reaction (Walling, 2000; Baldwin *et al*., 2001). Plants respond to aphids through the activation of plant defence signalling pathways which are regulated by both salicylate and jasmonate signalling molecules (Smith and Boyko 2007). Interestingly, under the case of plant-aphid compatible interactions, plants activate SA-dependent genes, while supressing the expression of JA-dependent genes (Giordanego *et al*, 2010). Transcript profiling studies showed that plant response to phloem-feeding insects is characterized by cell wall

modification, decrease in photosynthesis, manipulation of source-sink relations, and modification of secondary metabolism (Thompson and Goggin, 2006). These responses appear to be regulated in part by the phytohormones salicylate, jasmonate and ethylene. Moreover, transcripts induced by wheat plants in response to the Russian wheat aphid *Diuraphis. noxia* feeding, encodes proteins functioning in direct plant defence and signalling, oxidative burst, cell wall degradation, cell maintenance, photosynthesis and energy production (Botha *et al*., 2010, 2012).

4.1.2 Molecular responses to salt

Microarray studies on plant responses to salinity have been previously documented in rice, *Arabidopsis*, barley and poplar (Munns, 2005) as well as some cereals including rice, barley and maize (Jamil *et al*., 2011); several studies have been reported regarding gene expression profiles of wheat under salt stress (D'onofrio *et al*., 2004; Kawaura *et al*., 2006, 2008; Mott and Wang, 2007; Lu *et al*., 2011; Liu *et al*., 2012; Hussein, 2013). Plant responses to salinity involve utilization of various genes, proteins, metabolism and signalling pathways that function in a complex manner (Zhu, 2000; Ashraf, 2009). Some genes are associated with pathogen defence such as those involved in salicylic acid, jasmonic acid and ethylene signalling pathways, whilst other genes related to general abiotic stress response, such as dehydration, sugar transports, chaperonins and heat-chock proteins (Munns, 2005). Studies have also revealed that a considerable number of genes induced by salt stress were found to be regulated under cold and dehydration stress (Munns, 2005). Signalling genes induced by salt include mitogen protein kinases (MAPK) cascades which is activated by hyperosmolarily under salinity (Chinnusamy *et al*., 2004); this signal was suggested to lead to the induction of transcription factors followed by increase synthesis of osmolytes, osmoprotectants and detoxifying enzymes (Vinocur and Altman, 2005). Plants under saline conditions produce protective compounds which include osmolytes and osmoprotectants such as sugar and proline are produced by the plant (Jamil *et al*., 2011). Under these conditions plants also produce ROS detoxifying or scavenging enzymes such as superoxide dismutase, catalase and aldehyde dehydrogenase (Ashraf, 2009). Munns *et al*., (2006) reported and discussed a number of candidate genes conferring tolerance to salt and categorized them into three main functional categories: salt uptake and transport; osmotic or protective; and plant growth.

The overall aim of the present study is to develop an understanding of the physiological and molecular basis of the wheat response to a combination of salinity (abiotic) and aphid infestation (biotic) stresses compared with the individual stress, as well as an understanding of the potential interaction between these responses which may lead to cross tolerance. Such information is vital and provides a baseline for future attempts to breed broad-spectrum stress-tolerant crops.

The objectives were to use microarray analysis to: (1) investigate the interaction between salt and aphid stresses with respect to cross talk that could lead to cross tolerance through identifying putative genes; (2) investigate the influence and effect of pre-treatment with salt (abiotic stress) on plant-aphid interactions and identify related genes. These findings provide a foundation for the elucidation of the molecular basis and candidate genes associated with the wheat plant response to abiotic and biotic stresses applied singly and in combination, as well as the effect of salt stress on plant-aphid interactions. To the best of our knowledge this work comprises the first study in wheat to investigate differential gene expression in response to aphid infestation under conditions of abiotic (in this case saline) stress. The wheat genotype 122-1 was selected for study after being evaluated and characterized in two previous experiments in response to salinity (chapter 2) and aphid feeding (chapter 3) at the physiological level. This genotype was shown to be highly tolerant to salt (vigorous growth/high shoot dry matter relative to control under salinity at 160 mM NaCl) among 14 tested wheat genotypes and was shown to support moderate aphid fecundity in the presence and absence of salt among three tested wheat genotypes.

4.2 Methodology

4.2.1 Plant and insect material

Wheat (*T. aestivum*) genotype 122-1 was selected and used in this study after being characterized and evaluated for physiological responses to salinity and aphid infestation in two previous preliminary experiments (see Chapter 2 and 3). Genotype 122-1 showed a high degree of salt tolerance among other 14 wheat genotypes screened and showed moderate antibiosis resistance to aphids compared to the other two wheat genotypes. Wheat plants were grown in growth chambers under controlled environmental conditions (22°C/17°C at 16 h/ 8 h day/night). Seeds were germinated in petri dishes with moisture filter papers and the uniform seedlings were transferred and grown in silica sand, with one plant per pot. Half strength Hoagland's culture solution (Hoagland and Arnon, 1950) was used to water plants every other day for the first two weeks then full strength was used for irrigation until leaf number three was fully emerged (three leaf stage). Plants were grown until they reached three-leaf stage. Colonies of the cereal grain aphids *Sitobion avenae* were reared on wheat *T. aestivum*, cv. Clair and maintained at 20°C temperature inside an incubator. To maintain the aphid population new plant material was supplied every week and old plant material was removed after aphids had transferred and settled on the new plants.

4.2.2 Treatments and experimental design

When plants reached the three-leaf stage, a total of 128 plants were divided into four groups and labelled as: control, salt, aphid, or dual stress treatments; two time points were used, 6h and 24 h (Fig 4.1). The 128 plants representing 4 treatments×4 biological replicate×4 plants×2 time points were arranged in 4 chambers. Each biological replicate consisted of 4 individual plants arranged in one deep plastic tray with a small plastic dish underneath each pot. Each chamber contained 8 trays representing the four treatments for 6 h and 24 h. The experiment was arranged in a randomized complete block design in such a way that we obtained four biological replicates, each consisting of four plants for each of the four treatments and for each of the two time points (4×4×4×2).

Salt treatment

Thirty two plants were allocated for salt stress alone and a salinized full strength Hoagland's nutrient solution was used for watering these plants. The initial salt concentration was 40 mM NaCl; this was increased daily by an increment of 40 mM for four days to reach the desired final concentration of 160 mM NaCl. Salinity treatment was imposed on plants until the end of the experiment i.e. until the respective harvest time after 6 h and 24 h of aphid infestation. For consistency these plants were also covered with white nylon mesh (bread bags). Control plants were grown as above, but in the absence of NaCl.

Aphid infestation

Thirty two plants were assigned to aphid infestation alone and 20 apterous aphids (adults and nymphs) were randomly collected from the aphid colonies in small petri dishes using a fine camel hair brush. Each plant was infested by placing one small petri dish with the 20 aphids on the surface of the pot allowing the insects to transfer, climb and settle on the plant. Plants were covered with white nylon mesh (bread bags) to prevent the aphids from escaping. After infestation, plants were kept in the growth chambers until the respective harvest time. Control plants were grown under identical conditions, but in the absence of aphids.

Dual stress treatment

For the dual stress treatment, both salt stress and aphid infestation were combined and imposed on plants in a sequential manner. Plants were exposed to salt stress (160 mM NaCl) as described previously, and then infested with aphids as described previously on day five from commencing salt stress i.e. after 24 h of imposing the final concentration of 160 mM NaCl. Both stresses were then imposed simultaneously for 6 h and 24 h from the time of introducing aphids.

Sampling procedure

Prior to sample collection, all aphids on plants under aphid infestation and dual stress were carefully removed with a fine camel hair brush; and control plants and those under salt stress were also carefully brushed for consistency. Plants were harvested (6 h and 24 h post aphid infestation) by cutting at the shoot base, and then quickly wrapped in aluminium foil and immediately flash frozen in liquid nitrogen and stored at -80°C for the microarray analysis (128 samples).

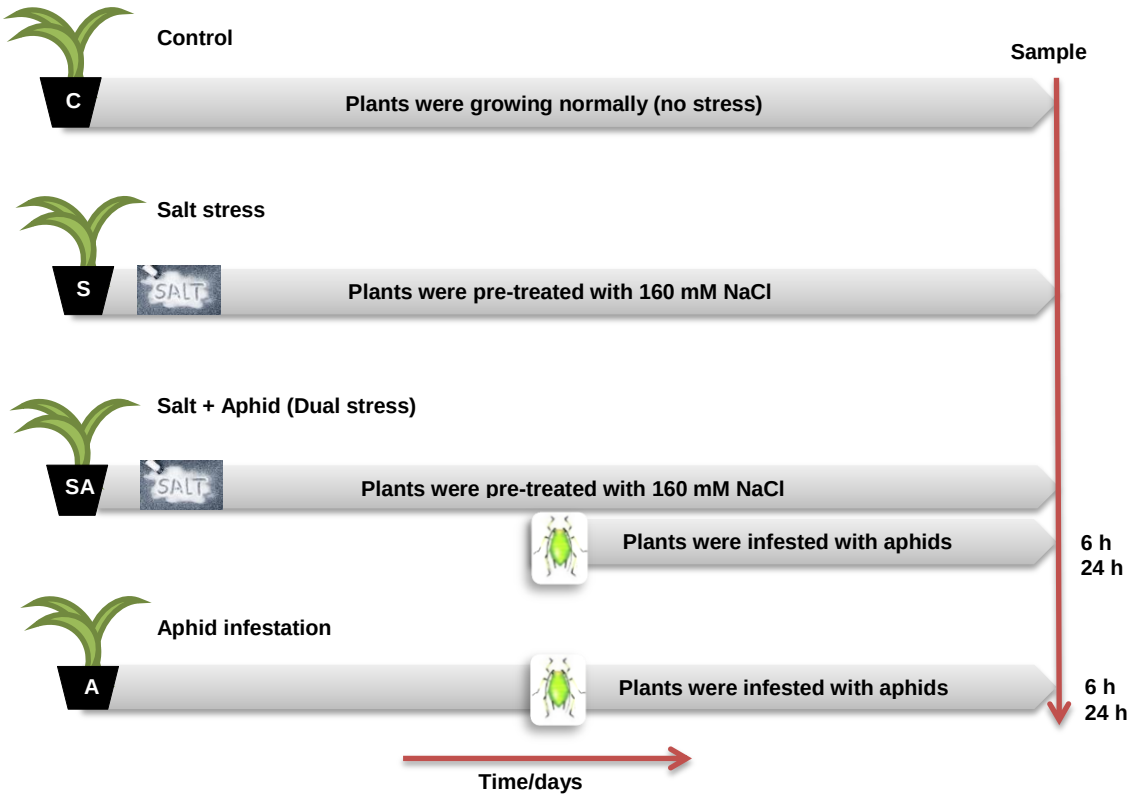


Figure 4.1 The experimental design for applying a combination of salinity and aphid infestation to wheat

4.2.3 RNA extraction and probe preparation

The frozen shoot samples were ground into fine powder in liquid nitrogen using a mortar and pestle (Diethylpyrocarbonate DEPC-treated water; autoclaved and dried). Shoots were ground in one batch at a time and then transferred to a 50-ml conical tube for storage at -80°C. Each sample (biological replicate) consisted of 4 individual whole shoots i.e. one sample was pooled from 4 plants. The total RNA was extracted using TRIzol reagent Plus RNA Purification Kit (ambion RNA by life technologies according to manufacturer's instructions (Invitrogen). Between 90-100 mg of shoot tissue powder was weighed in a microfuge tube and homogenized in 900-100 µl TRIzol using a microfuge pestle. Samples were incubated with TRIzol reagent for 5 min to allow for complete dissociation of nucleoprotein complexes and then inverted quickly 10 times. Exactly 180 µl chloroform was added per 900 µl TRIzol reagent and samples vortexed vigorously for 15 sec and incubated at room temperature for 2-3 min. Samples were centrifuged at 12000 xg for 15 min at 4°C. Exactly 400 µl of the colourless upper aqueous phase, which contains the RNA, was collected into a fresh RNase-free microfuge tube. Exactly 400 µl of 70% ethanol was added to obtain an EtOH concentration of 35% and was mixed well by vortexing. Samples were then processed for binding to spin cartridge and then processed for on-column Pure-link DNase treatment and finally samples were washed; 30 µl of RNase-free water was used for RNA elution.

4.2.4 Determination of RNA quality

RNA quality and concentration were determined using a NanoDrop spectrophotometer and Agilent's 2100 Bioanalyzer and Agilent RNA 6000 Nano Kit. RNA quality and integrity were tested by determination of OD 260/230 ratios of samples (to confirm ratio > 1.8 for each sample) and bioanalyzer checks. All samples were found to be of high RNA quality and hence used for microarray analysis.

4.2.5 Gene expression profiling

Microarray cDNA synthesis, labelling, hybridization, signal scanning and normalization of the array were carried out by Source BioScience UK Ltd, Nottiingham following protocols supplied by Affymetrix for analysing RNA samples. Affymetrix GeneChip Wheat Genome Arrays were used for transcriptomics analysis. Each wheat genechip array contains 61127 probe sets representing 55052 transcripts for all 42 chromosomes

in the wheat genome and were constructed using ESTs distributed across the wheat genome. Equal amounts of total RNA were collected (50 µl at 50 ng/µl using reduced EDTA buffer (0.1 mM EDTA, 10mM Tris HCl, pH8.0 to provide 2 µg per sample). Thus 24 total RNA samples (3 biological replicates; 4 treatments; 2 time points) were prepared for subsequent expression profiling using Affymetrix GeneChip Arrays.

4.2.6 Data analysis using RobiNA

For data analysis the experimental raw data Affymetrix CEL files were imported into Robin (Lohse *et al*., 2010). Since the Affymetrix CEL data format is uniform and does not require further processing or configuration the user can directly proceed to the quality assessment step. After importing the chip data, a variety of quality assessment methods were available to run to allow the user to get an overview of the quality of input data and subsequently exclude chips that show strong technical artefacts individually. For the analysis of differential expression and identifying significant differences of interest between the four treatments (at 6 h and 24 h) three biological replicates were used. Statistical analysis of differential gene expression was carried out using the linear model based approach developed by Smyth (2004). The obtained P values were corrected for multiple testing using the strategy described by Benjamini and Hochberg (1995) separately for each of the comparisons made. Genes that showed an absolute log2 fold-change value of at least 1 and a P-value lower than 0.05 were considered significantly differentially expressed.

MapMan analysis and annotation

For further analysis and putative annotation of differentially expressed genes, data files from Robin were imported into MapMan (version 3.5.1R2) software (Thimm *et al*. 2004, Usadel *et al*. 2005, http://gabi.rzpd.de/projects/MapMan/). Mapman introduced a hierarchical ontology different from GO terms that can be used for visualizing large data sets (expression profiles) onto metabolic pathways and other biological processes (Thimm *et al*., 2004). The ontology was originally built for the model species *Arabidopsis thaliana* (based on publicly available gene annotation from TIGR (The Insitute for Genomic Research) using the TIGR3 annotation and updating to the current TAIR8 version of the *Arabidopsis* genome and furthermore extended to cover also other plants. The mapping file for wheat *Triticum aestivum* (Taes_AFFY_0709) provided by MapMan was selected and loaded (available at Mapman website; mapman.gabipd.org).

Mapman uses a hierarchical ontology system, into which the wheat exemplary sequences were classified. MapMan BINs were assigned to each probe on the chip based on the wheat mapping file available in MapMan ontology. A total of 34 Mapman bins were used for the wheat MapMan classification and these were extended in a hierarchical manner into > 1,200 sub-bins. MapMan compares each stress treatment with the control treatment and the change of expression ratio of each gene is calculated as log2 fold change to generate the experimental file, which is then visualized at the pathway level (Thimm *et al*., 2004; Usadel *et al*., 2005). A cut off value of a 2-fold change is commonly used for microarray analysis (Smith *et al.*, 2010).

PageMan analysis

Pageman aims at providing a statistics-based overview of enriched functional categories from global omics responses. The microarray experimental data files were imported into PageMan (version 0.12) (Usadel *et al*. 2006, http://mapman.mpimp-golm.mpg.de/pageman) and the same mapping file (*Triticum aestivum* Taes-Affy-0709) for MapMan was used to identify functional categories with significant enrichment or depletion of up-regulated genes. A statistical analysis was performed using a Wilcoxon rank sum to test whether there were bins that were significantly and consistently behaving differently than the other bins in the MapMan ontology using the built-in function in MapMan. Also, within the PageMan package, a Wilcoxon test combined with Benjamin-Hochberg filtering was used to calculate P values for enriched categories. The obtained P values were transformed to z-scores and plotted as a heat map. Only significant functional categories are shown in the figure. A Wilcoxon rank sum test implemented in MapMan was used to extract bins whose gene members exhibited significantly different regulation compared to all other bins (for corrected p-value <0.1). The data are visualized by compressing the response of whole pathways (all of the genes in a sub-BIN or BIN) down to single-coloured rectangles.

4.2.7 QRT-PCR verification of microarray transcripts

To validate the results from the microarray experiment, 7 genes, which were identified as differentially regulated under the different treatments through microarray analysis were analysed using quantitative real-time PCR (Table S4.15). The same aliquots of RNA samples used for the hybridization of Affymetrix GeneChip wheat genome array were used for qRT-PCR. RNA samples for microarray hybridization were free from any

residual genomic DNA as it was already removed by on-column Pure-Link Dnase treatment. First strand cDNA was synthesized from 100 ng of total RNA with Oligo (dT) primer using SuperScript II Reverse Transcriptase in 20 µl final reaction volume containing: 1 µl Oligo dT primer, 100 ng of total RNA, 5 µl dNTP Mix, sterile distilled water to 12 µl, 5X first-strand buffer 4 µl, 0.1 M DTT 2 µl and superscript II RT (200 units) 1 µl. The cDNA was amplified using specific primers designed for the selected genes by Sigma Life Science Co. The RT-PCR primers designed for the seven target genes or genes of interest were evaluated for PCR amplification efficiencies by carrying out real-time PCR using five series dilution of cDNA template from biological replicate number 3 of control samples at 5 h and 24 h.

A 100% PCR efficiency (default value = 2) was used, Validation of Gene of Interest vs Endogenous Control for 1 time-point

Equation 4-1 ΔCT gene of interest - endogenous control

Equation 4-2 ΔΔCT (ΔCTtarget - ΔCTcalibrator)

The qRT-PCR was performed in 96-well plates using the SYBR Select Master Mix (Applied Biosystems) in a total of 20 µl reaction volume containing: SYBR master mix 10 µl, primer-forward 0.5 µl, primer-reverse 0.5 µl and RNAse-free water 8 µl and RT product (cDNA template) 1 µl. Then the QRT-PCR was performed on a thermo cycler using the following thermal cycling conditions/steps (profile): 59°C (*P5CS1*); 53°C (*SOS1*); 55.3°C (*UBQ10*) for 30 min and 95°C for 2 min. (Reverse-Transcription) followed by 40 PCR cycles, at 94°C for 15 sec, 59°C (*P5CS1*); 53°C (*SOS1*); 55.3°C (*UBQ10*) for 30 sec, plate read and 72°C for 1 min. All reactions were performed in quadruplicates.

Actin 2 (ACT2) was used as an endogenous control (reference housekeeping gene) and data were normalized based on this expression data of the internal reference gene. The PCR programme was carried out for each gene and 4 technical replicates and 3 biological replicates were used at each sampling point. The quantification of gene expression was performed using the relative quantification methods (ΔΔCT) (Livak and Schmittgen, 2001) and comparing data with the internal control.

4.3 Results

4.3.1 Global comparison of wheat transcriptome profiles

The impact of the aphid *S. avenae* infestation (A) and salt treatment (S) applied individually and in combination/dual stress (SA) on wheat global gene expression was investigated using Affymetrix GeneChip wheat genome array which contains 61,127 probe sets representing 55,052 transcripts for all 42 chromosomes in the wheat genome. A total of 61290 transcripts were differentially regulated in wheat genotype 122-1 in response to the three stress treatments (salt; aphid; dual stress) compared to control non stressed plants. Genes were considered as being highly regulated stress responsive genes if (i) their fold change expression difference between stress treatment vs. control treatment were ≥ 0.5 for up-regulated genes and ≤ -0.05 for down-regulated genes (values are log2 transformed fold change), and (ii) these differences were significant ($p < 0.05$) under at least one of the three stress treatments at 6 h and 24 h post aphid infestation. Results revealed that 285, 3056 and 1592 stress responsive transcripts were differentially regulated at 6h and 467, 1580 and 504 were differentially regulated at 24 h for salt, aphid and the dual stress, respectively. In general, there were more stress responsive transcripts suppressed than induced as part of the early response (i.e. 6 h post aphid infestation) to either aphid infestation or the dual stress. This is in contrast to the late response (i.e. 24 h post aphid infestation) where more stress responsive genes were induced than repressed. The results also showed an increase in gene expression between the early (6 h) and late response (24 h) for both of the two single stress treatments, but not in plants receiving the dual stress (both exposure to salt and aphid infestation). Furthermore, there was a trend of decreased gene suppression from 6h and 24h for all three stress treatments (Fig. 4.2). The highest number of stress responsive genes that showed strong up or down regulation (≥ 0.5 or ≤ -0.5 fold change expression difference) was induced by aphid infestation alone compared with those induced by either salt stress alone or the dual stress at both time points (i.e. 6 h and 24 h post aphid infestation). For instance, the number of genes activated by aphids was five times and two times the number of genes activated by salt stress alone and dual stress, respectively, at 6 h while the number of genes repressed was 18 times and two times the number of genes suppressed under salt stress alone and dual stress, respectively. The same trend of a pronounced effect in response to aphid infestation on the expression of stress responsive genes was also maintained after extending the duration of infestation

to 24 h as the number of genes induced by aphids was three times the number of genes activated under both salt stress alone and dual stress (Fig. 4.2).

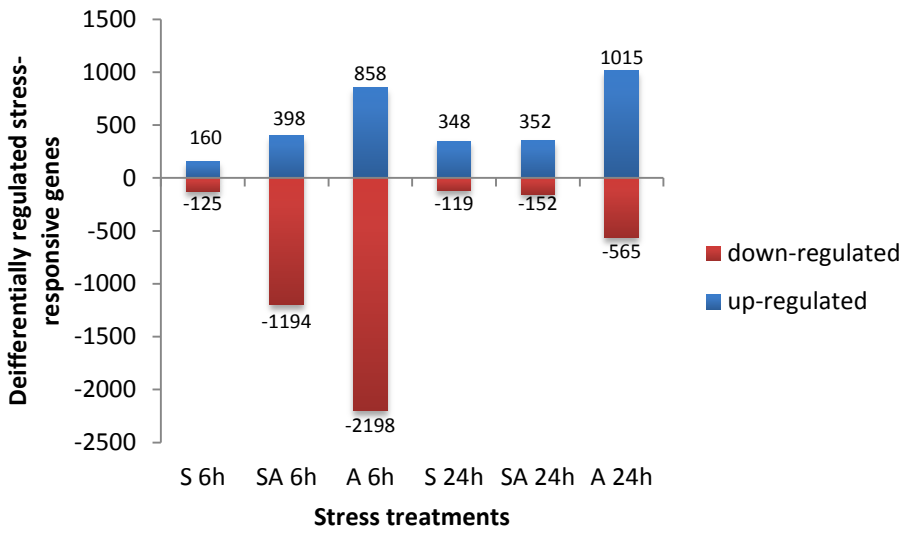
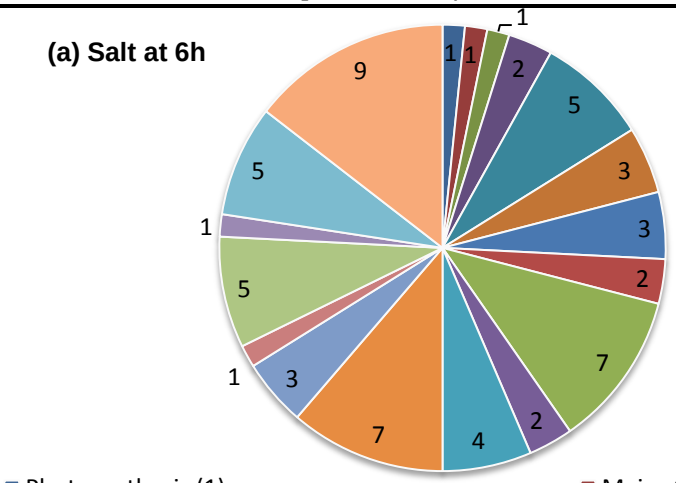


Figure 4.2 Distributions of differentially expressed stress responsive genes in wheat following exposure to three stress treatments at 6 h and 24 h post aphid infestation.
The numbers on the y axis represents numbers of up- and down-regulated genes (probes), and the letters on the x axis represents the three stress treatments: salt stress (S), dual stress (SA), aphid infestation (A). These genes were determined by selecting a threshold cut-off value of ≥0.5 and ≤-0.5 fold change expression difference between stress *vs* control treatments (on a log2 scale). Higher numbers of genes were repressed than activated after 6 h of aphid infestation in both dual stress and aphid infestation alone, while, higher numbers of genes were up-regulated than down-regulated by aphid infestation after 24 h in both dual stress and aphid infestation alone. Under salinity treatment alone more genes were up regulated than down regulated at both time points.

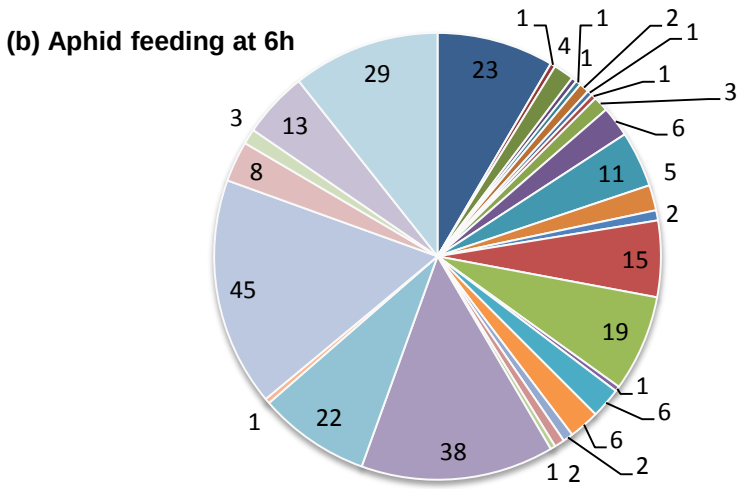
4.3.2 Functional categorization of stress responsive (SR) genes

The putative annotation and functions of strongly up regulated stress responsive (SR) genes were identified through MapMan ontology which classified and grouped these genes into 35 major bins and numerous sub-bins representing different biological functions. At 6 h under salt stress alone the highly up regulated SR genes were putatively involved in 18 major functional classes. The transport function category comprised most SR transcripts up regulated under salinity (9 transcripts) and other categories are illustrated in (Fig. 4.3a). Under aphid infestation alone, the highly up regulated SR genes were categorized into 29 major functional groups, most of these genes were involved in bin 29 representing protein (45 transcripts) and other categories

are illustrated in (Fig. 4.3b). The SR genes strongly up-regulated by dual stress were assigned to 22 main functional classes, and the bin for miscellaneous contained most transcripts (20 transcripts) and other categories are illustrated in (Fig. 4.3c). At 24 h the strongly up-regulated SR genes under salt stress were categorized into 21 functional groups, of these bin RNA comprised most transcripts (17) and other categories are illustrated in (Fig. 4.4a). Under aphid infestation alone a substantial number of SR genes were assigned to DNA synthesis chromatin structure and other categories are illustrated in (Fig. 4.4b). Under dual stress, most of the SR genes that are highly up-regulated were associated with miscellaneous bins and other categories are illustrated in (Fig. 4.4c). Bin number 35 for unknown or not assigned transcripts, had the highest number of transcripts and was not included in the pie chart. To identify the significantly altered bins (functional categories) in response to the three stress treatments, a Wilcoxon rank sum test was conducted via MapMan to compare the average responses of genes assigned to a specific bin with all the other bins. Results revealed that the number of the most significantly altered major bins varied between the three stress treatments and. Salt stress, aphid infestation and their combination at 6 h significantly altered 15, 17 and 19 bins respectively. In addition salt stress, aphid infestation and dual stress at 24 h significantly changed 17, 15 and 22 bins respectively (Table S4.1).



- Photosynthesis (1)
- Fermentation (1)
- Cell wall (5)
- Amino acid metabolism (3)
- Hormone metabolism (7)
- Stress.abiotic (4)
- RNA (3)
- Protein (5)
- Development (5)
- Major CHO metabolism (1)
- Mitochondrial/ATP synthesis (2)
- Lipid metabolism (3)
- Secondary metabolism (2)
- Stress.biotic (2)
- Misc (7)
- DNA (1)
- Signalling (1)
- Transport (9)



- Photosynthesis (23)
- Minor CHO metabolism (4)
- Fermentation (1)
- OPP pathway (1)
- Mitochondrial/ATP synthesis (3)
- Lipid metabolism (11)
- Metal handling (2)
- Hormone metabolism (19)
- Stress.biotic (6)
- Redox (6)
- Biodegradation of Xenobiotics (1)
- RNA (22)
- Protein (45)
- Cell (3)
- Transport (29)
- Major CHO metabolism (1)
- Glycolysis (1)
- Glyoxylate cycle (2)
- TCA cycle (1)
- Cell wall (6)
- Amino acid metabolism (5)
- Secondary metabolism (15)
- Tetrapyrrole synthesis (1)
- Stress.abiotic (6)
- Nucleotide metabolism (2)
- Misc (38)
- DNA (1)
- Signalling (8)
- Development (13)

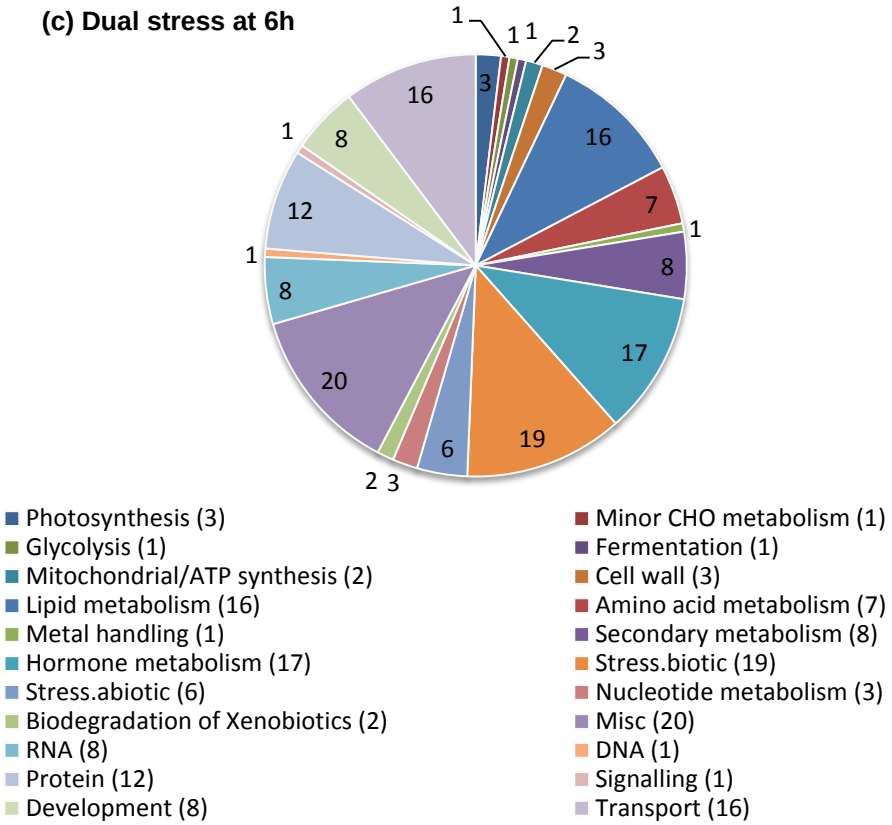
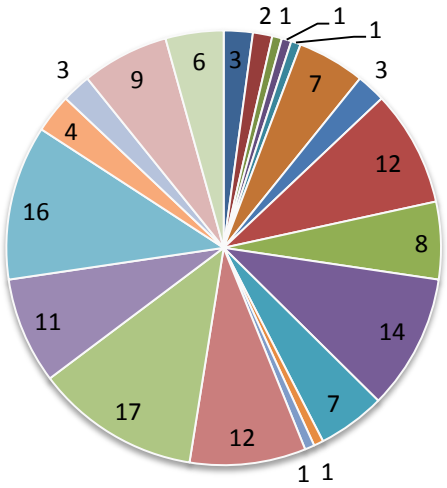


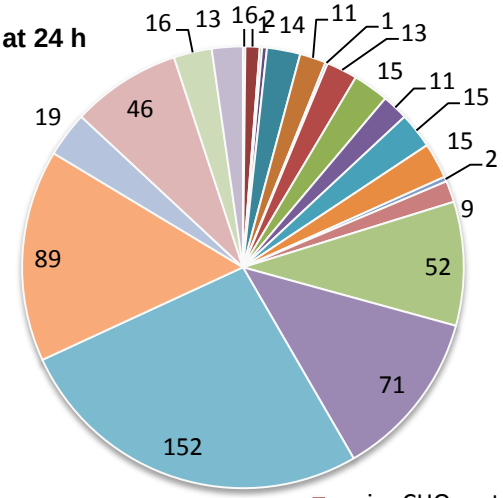
Figure 4.3 MapMan overview analysis identifying functional BINs with respective gene numbers differentially regulated at 6 h.
Strongly up-regulated stress responsive genes with high fold change (≥0.5 fold on log2 bases) at 6 h were used for the functional classification. The legend presents the MapMan defined parent BIN name and respective transcripts numbers in parentheses; the numbers of transcripts classified under each parent BIN are also presented as data labels. BINs representing Misc, hormone metabolism and transport comprised major groups under salt stress. BINs related to Misc, RNS, protein, transport and photosynthesis comprised most trancripts under aphid infestation. BINs representing Misc, hormone metabolism, stress biotic, lipid metabolism and transport had substantial numbers of genes under dual stress.

(a) Salt stress 24 h



- Photosynthesis (3)
- major CHO metabolism (2)
- gluconeogenese/ glyoxylate cycle (1)
- TCA / org. transformation(1)
- Cell wall (1)
- Lipid metabolism (7)
- amino acid metabolism (3)
- Metal handling (12)
- Secondary metabolism (8)
- Hormone metabolism (14)
- stress.biotic (7)
- stress.abiotic (1)
- redox (1)
- misc (12)
- RNA (17)
- DNA (11)
- protein (16)
- signalling (4)
- cell (3)
- development (9)
- transport (6)

(b) Aphid feeding at 24 h



- Photosynthesis (1)
- major CHO metabolism (6)
- glycolysis (1)
- mitochondrial/ATP synthesis (2)
- cell wall (14)
- lipid metabolism (11)
- N-metabolism (1)
- amino acid metabolism (13)
- secondary metabolism (15)
- hormone metabolism (11)
- stress.biotic (15)
- stress.abiotic (15)
- redox (2)
- nucleotide metabolism (9)
- misc (52)
- RNA (71)
- DNA (152)
- protein (89)
- signalling (19)
- cell (46)
- development (16)
- transport (13)

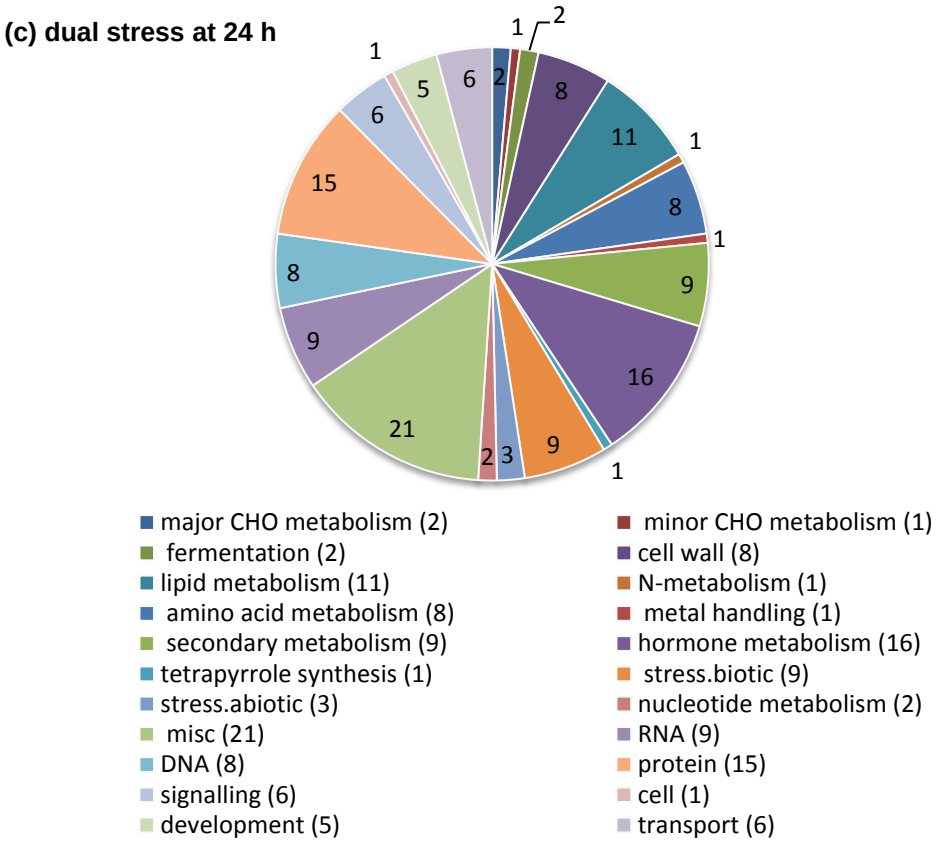


Figure 4.4 MapMan overview analysis identifying functional BINs with respective gene numbers differentially regulated at 24 h.
Strongly up-regulated stress responsive genes with high fold change (≥0.5 fold on log2 scale) at 24 h were used for the functional classification. The legend presents the MapMan defined parent BIN name and respective transcript numbers in parentheses; the numbers of transcripts classified under each parent BIN are also presented as data labels. BINs representing hormone metabolism protein and RNA comprised major groups under salt stress. BINs related to Misc, DNA, RNA, protein, and cell comprised most trancripts under aphid infestation. BINs representing Misc, hormone metabolism and protein had substantial numbers of genes under dual stress.

Commonly up regulated stress responsive (SR) genes

In order to provide information on the common and stress specific responses, the differentially expressed genes were analysed and illustrated in Venn diagrams which were generated via MapMan software selecting a threshold cut off value of: 0.5 fold change (on a log2 bases). Data revealed that firstly, after 6 h of aphid infestation, 32 genes were induced in common under all three stress treatments, dual stress shared more induced transcripts with aphid infestation alone (776 transcripts) than with salt stress alone (65 transcripts) and a small number of genes overlapped between the two individual stresses (7 transcripts). Moreover, dual stress, salt stress and aphid infestation specifically strongly induced 299, 101 and 1352 transcripts respectively at 6h (Fig 4.5a). Secondly, after 24 h of aphid infestation, a total of 25 differentially expressed transcripts were common to the three stress treatments, dual stress shared more induced transcripts with salt stress alone (85 transcript) than with aphid infestation alone (62 transcripts), and 29 induced genes were common between salt stress and aphid infestation. Moreover, each stress treatment, dual, salt and aphid resulted in 137, 218 and 1045 genes, respectively, to be highly differentially expressed at 24 h (Fig 4.5b).

Moreover, among all commonly differentially expressed genes under the three stress treatments, only 3 and 11 genes showed strong induction and high fold change (fold change ranging from 0.5 to ≥ 1 on $\log_2$ scale) at 6 h and 24 h and were considered as early and late stress responsive SR genes, respectively. Identification and annotation of these genes revealed that, firstly the three early SR genes were assigned to three different functional categories: biotic stress, miscellaneous and not assigned (unknown) encoding PR4 (pathogenesis-related 4), cytochrome P450 and putative uncharacterized protein respectively (Table S4.2). Secondly, the late stress responsive genes were putatively involved five biological processes as following: one gene for amino acid synthesis encoding sarcosine oxidase family protein; one gene for hormone gibberellin metabolism, 4 genes for DNA synthesis encoding replication protein, histone H4; one gene for development encoding nodulin MtN3 family protein and 4 unknown genes (Table S4.3).

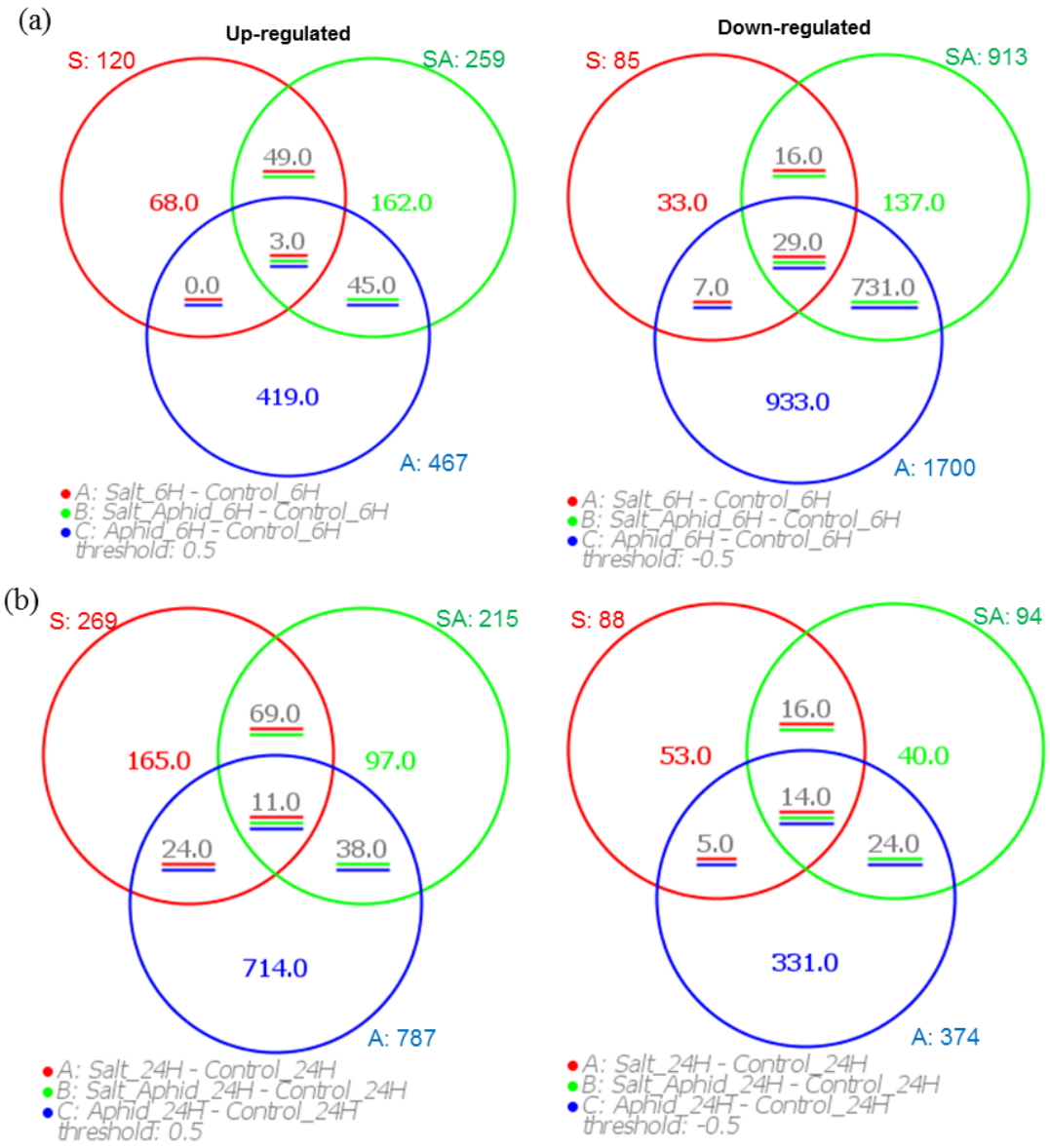


Figure 4.5 Venn diagram showing numbers of specific and common differentially regulated genes in wheat in response to stress treatments compared to control.
Salt stress (S, red circle); aphid infestation (A, blue circle); dual stress (SA, green circle). Numbers within circles show specific genes, numbers within intersections/overlap show common genes and numbers outside circles show the total number of genes for each stress treatment. A threshold value of ±0.5 fold change (based on $\log_2$ fold values) was chose to construct these diagrams via MapMan software. (a) Differential expression at 6 h post aphid infestation, dual stress shared more up regulated genes with salt stress alone than with aphid infestation alone, while more down regulated genes were common between dual stress and aphid infestation alone than those between dual stress and salt stress alone. Aphid infestation alone induced the highest number of specific up- and down-regulated genes followed by dual stress and lastly salt stress alone. Notably, no gene was commonly up regulated between salt stress and aphid infestation applied singly at this specific fold change threshold. (b) Differential expression at 24 h post aphid infestation. Dual stress shared more up regulated genes with salt stress alone than with aphid infestation alone, while more down regulated genes were common between dual stress and aphid infestation alone than those between salt stress alone and dual stress. Aphid infestation alone induced the highest number of specific up- and down-regulated genes followed by dual stress and lastly salt stress alone.

Among 1640 genes commonly induced by dual stress and salt stress treatments at 6 h, only 49 genes were strongly induced showing high fold change. These genes had functions in cell wall degradation, lipid metabolism, amino acid proline, hormone metabolism ethylene, heat abiotic stress, biotic stress, miscellaneous cytochrome P450, MYB4 transcription factor, protein posttranslational modification, Late embryogenesis abundant 14 (LEA14), development nodulin MtN3 family protein, transport and not assigned/unknown (Table S4.4). Among 8441commonly activated genes between dual stress and aphid infestation stress at 6 h, only 45 were strongly induced with high fold change. These were involved in the following: photosynthesis, mitochondrial electron transport ATP, lipid metabolism, amino acid asparagine, secondary metabolism phenylpropanoids, hormones metabolism (abscisic acid, ethylene, jasmonate lipoxygenase LOX), miscellaneous, protein, development and not assigned/unknown (Table S4.5). However, no strongly induced genes were common between the two single treatments salt stress and aphid infestation.

Both dual stress and salt stress strongly commonly induced 69 genes at 24 h. These were annotated to many functional groups namely, cell wall degradation, lipid metabolism, amino acid proline, metal handling, secondary metabolism phenylpropanoids, hormone metabolism (abscisic acid, ethylene, jasmonate lipoxygeneases), biotic stress, miscellaneous cytochrome P450, RNA processing and regulation of transcription MYB4, protein posttranslational modification and not assigned/unknown (Table S4.6). Both dual stress and aphid stress strongly commonly induced 38 transcripts. Some of these transcripts were annotated to beta 1,3 glucan hydrolases, glutathione S transferases, O-methyl transferases, secondary metabolism flavonoids.chalcones, phenylpropanoids lignin biosynthesis COMT, signalling calcium (calmodulin binding), early-responsive to dehydration 2, basic chitinase (ATHCHIB) (Table S4.7). Both salt stress and aphid stress strongly commonly induced 24 genes at 24 h. These were involved in miscellaneous O-methyl transferases, acid and other phosphatases, biotic stress PR-proteins, RNA regulation of transcription DNA methyltransferases and proliferating cellular nuclear antigen (PCNA1) (Table S4.8).

Specifically up regulated stress responsive (SR) genes

Dual stress, salt stress and aphid infestation at 6h caused 16, 14 and 17 transcripts, respectively, to be highly and uniquely expressed (at log2 fold change of ≥1 expression difference between stress treatments vs. control treatment) (Table S4.9, S4.10, S4.11,

respectively). Dual stress strongly increased the expression of 16 genes (fold change ranging from 1.02 to 1.51). These were involved in lipoxygenase (5 genes), heat abiotic stress (DNAJ heat shock N-terminal domain-containing protein), miscellaneous acid and other phosphatases (phosphoric monoester hydrolase) and not assigned/unknown functions. Salt stress strongly increased the expression of 14 genes (fold change ranging from 1.02 to 2.01) associated with amino acid metabolism, synthesis glutamate family proline, miscellaneous (nitrilases nitrile lyases berberine bridge enzymes reticuline oxidases troponine reductases, and cytochrome P450 CYP71B35), RNA regulation of transcription MYB domain transcription factor family, DHN4, LEA3 and assigned/unknown functions. Aphid infestation strongly increased the expression of 17 genes involved in PS light reaction photosystem, protein synthesis and 11 genes were not assigned to any functional category (unknown).

Moreover each stress treatment, SA, S and A showed strong specific up-regulation of 5, 14 and 193 genes respectively at 24 h (Log2 fold change of ≥1 expression difference between stress treatments vs. control treatment) (Supplementary Table S4.12, S4.13, S4.14, respectively). Dual stress specifically up regulated 5 genes involved in secondary metabolism, phenylpropanoids and not assigned/unknown functions. Salt stress uniquely up regulated 14 genes involved in amino acid metabolism aromatic tyrosine (aminotransferase), metal handling chelation and storage, and not assigned/unknown functions. Aphid infestation strongly increased the expression of 193 genes associated with cell wall modification and pectin esterases (PME), lipid metabolism phospholipid synthesis cyclopropane-fatty-acyl-phospholipid synthase, biotic stress PR-proteins, nucleotide metabolism (deoxynucleotide metabolism and ribonucleoside-diphosphate reductase), miscellaneous (beta 1,3 glucan hydrolases, O-methyl transferases, acid and other phosphatases, dynamin and GDSL-motif lipase), RNA processing (RNA helicase), RNA regulation (transcription DNA methyltransferases, nucleosome/chromatin assembly factor group, putative transcription regulator and SNF7), DNA (synthesis chromatin structure histone and repair), protein (synthesis ribosomal protein eukaryotic 40S subunit S6, posttranslational modification, degradation subtilizes, degradation ubiquitin E3 RING, cell (organization, division, cycle, vehicle transport), development unspecified and not assigned/unknown functions. Results have the following functional categories will be presented below: Signalling, transcription factor, hormones, redox regulation, and biotic and abiotic stress responses.

4.3.3 Signalling related genes

Quantitative analysis of global expression of genes associated with signalling revealed that at 6 h more genes were suppressed than up regulated under all stress treatments. The highest number of up regulated genes was induced by aphid infestation alone, while the highest number of down regulated genes was induced by dual stress. In contrast, at 24 h more genes were up regulated than repressed under both dual stress and salt stress alone, but not under aphid infestation alone. The highest number of up regulated genes was induced by dual stress, while the highest number of down regulated genes was induced by aphid infestation alone. Based on functional categorization analysis these differentially expressed genes were implicated in different signalling functions. At 6 h salt stress alone significantly down regulated genes related to receptor kinases signalling. Under both aphid infestation alone and dual stress more signalling categories were significantly down-regulated including receptor kinases, calcium signalling, G-proteins and lipids signalling, while sugar and nutrient physiology category was significantly up regulated. In addition, at 24 h both salt and dual stress significantly down regulated receptor kinases signalling while significantly up regulated calcium signalling category. Dual stress specifically significantly up regulated three categories namely: phosphinositides, G-proteins and MAP kinases. Under aphid infestation alone one category namely 14-3-3 proteins was specifically significantly up regulated while lipid signalling was significantly down regulated (Fig. 4.6a).

Based on Venn diagram analysis and identification of single candidate genes, the study identified many specific and common up-regulated signalling related transcripts in response to the three stress treatments. Among these at 6 h post aphid infestation, one transcript involved in sugar and nutrient physiology encoding phosphate-responsive protein (EXO) was specifically highly up-regulated (0.49 fold) in plants pre-treated with salt i.e. salt stress alone. Plants under dual stress highly induced (0.48 fold) one transcript associated with receptor kinases signalling encoding callus expression of rbcs 1011 (CES10). Aphid infestation alone specifically highly up-regulated twelve transcripts (with fold change ranging from 0.49 to 0.52) including one gene for sugar and nutrient physiology signalling encoding phosphate-responsive protein putative (EXO), one gene for calcium signalling encoding calmodulin 1 (CAM1) and ten genes for receptor kinases encoding protein kinase putative, cysteine-rich RLK 6 (CRK6), leucine-rich repeat family protein, strubbelig-receptor family 3 (SRF3). Dual stress and

aphid infestation alone commonly up regulated one gene for MAP kinases signalling encoding MAP kinase 7 (ATMPK7) (0.45 and 0.49 fold, respectively) (Fig. 4.6b).

In addition, among differentially expressed genes at 24 h, one transcript functioning in calcium signalling coding for IQ-domain 5 (IQD5) calmodulin binding was commonly highly up regulated by all stress treatments. Another two transcripts also functioning in calcium signalling and encoding the same gene product were commonly highly up regulated by dual stress (0.46, 0.56 fold) and aphid infestation (0.42, 0.86 fold). One transcript related to receptor kinases encoding leucine-rich repeat transmembrane protein kinase and three transcripts involved in calcium signalling encoding calcium-binding protein and calcium-binding pollen allergen were specifically highly induced in plants under salt stress alone. Under dual stress, plants specifically highly activated four transcripts involved in receptor kinases encoding polygalacturonase inhibiting protein 1 (PGIP1), protein kinase and strubbelig-receptor family 3 (SRF3), and G-proteins encoding *Arabidopsis thaliana* hopm interactor 7 (ATMIN7). The specifically highly up-regulated 24 transcripts (fold change ranging from 0.46 to 0.91) under aphid infestation alone include the following: one gene for sugar and nutrient physiology, five genes for calcium signalling, eight genes for G-proteins and ten genes for receptor kinases (leucine rich repeat XI, III and misc) (Fig. 4.6b).

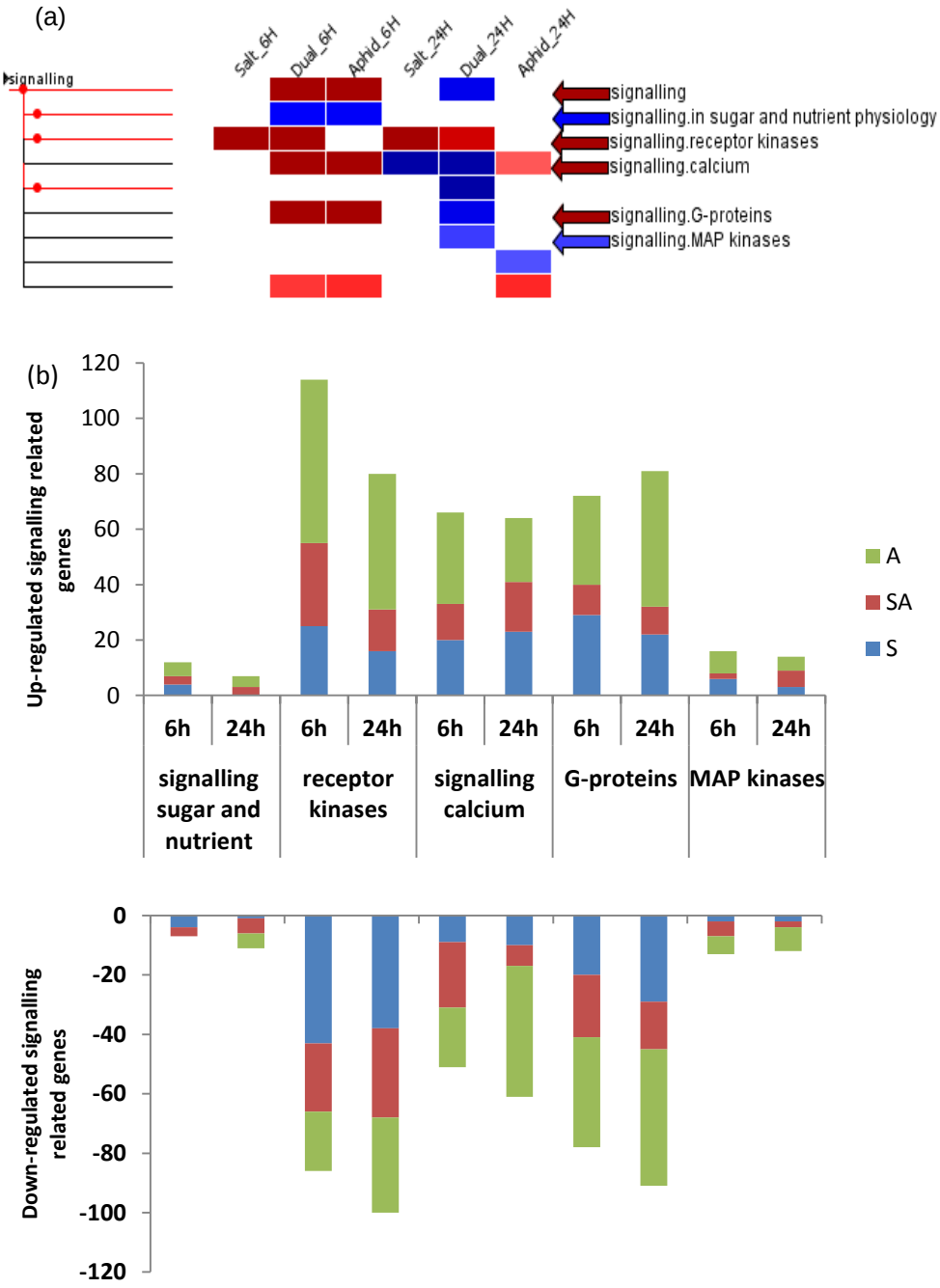


Figure 4.6 Changes in signalling functional category and distribution of related genes.
(a) A condensed PageMan display of coordinated changes of signalling functional category. The log2 fold change between each stress treatments and control were imported into PageMan. The data was subjected to a Wilcoxon test and the results were displayed in false-colour code. Bin and sub-bin coloured in red or blue are significantly down-regulated or up-regulated, respectively relative to the rest of the array. Significant activation of most signalling categories was triggered by dual stress at 24 h. (b) Numbers of specifically differentially expressed genes associated with signalling under salt stress S (blue bar), dual stress SA (red bar) and aphid infestation A (green bar) at 6 h and 24 h following aphid introduction. These include: sugar and nutrient physiology, receptor kinases, calcium signalling, G-proteins and MAP kinases. Data were obtained from Venn diagram and displayed in a bar chart. Among up-regulated genes, receptor kinases genes were abundant in aphid and dual stress at 24 h, and G-protein genes were abundant in salt at 6 h. Among down regulated genes, receptor kinases genes were abundant in salt stress at 6 h and dual stress at 24 h, and G-proteins genes were abundant in aphid infestation at 24 h.

4.3.4 RNA regulation of transcription factors (TFs)

Based on functional categorization analysis, results showed that the most notable observation was the significant trend of down regulation in the majority of RNA categories including TFs under the three stress treatments at 6 h. In contrast, at 24 h there was a significant trend of up regulation in the same categories under all stress treatments (Fig 4.7a). In addition, quantitative analysis showed that 65 and 95 transcripts were highly down regulated by the dual stress and aphid alone, respectively. This significant trend in reduction was diminished after 24 h of aphid infestation, as only a few TF related genes were shown to be highly suppressed by salt stress (4 genes), dual stress (5 genes) or aphid infestation (15 genes). In terms of up regulated TFs, aphids infestation highly activated (15 genes) at 6 h, while (55 genes) were highly up-regulated at 24 h. Dual stress highly induced the expression of three TFs candidate genes at both time points. Salt stress strongly induced two and 14 TFs putative genes at 6 h and 24 h, respectively (Fig 4.7b).

Based on Venn diagram and single candidate genes analysis, among genes differentially regulated at 6 h, two TFs genes were specifically highly induced by salt stress including one gene related to MYB domain transcription factor family encoding MYB domain protein 4 (MYB4) and one gene related to MADS box transcription factor family encoding APETALA1 (AP1) DNA binding transcription factor. Dual stress specifically highly up regulated one gene associated with C2C2 (Zn) DOF zinc finger family which encodes Dof-type zinc finger domain-containing protein. Aphid infestation alone specifically highly up regulated 23 TFs related genes involved in various categories. Of these were genes encoding the following: high-level expression of sugar-inducible gene 2 (HSI2), EIL1 (ethylene-insensitive3-like 1), myb domain protein (MYB55, MYB61, AtMYB19), NAC67, zinc finger family protein (AN1-like, B-box type). One putative MYB domain gene encoding MYB domain protein 4 (MYB4) was commonly highly activated by salt stress alone and dual stress but, interestingly, repressed by aphid infestation. One gene for HB Homeobox transcription factor family encoding *Arabidopsis thaliana* Homeobox 7 (ATHB-7) was commonly highly activated by aphid infestation alone and dual stress.

At 24 h post aphid infestation, salt stress specifically strongly induced 9 TFs transcripts encoding WRKY DNA-binding protein (WRKY41, WRKY18), myb domain protein 4 (MYB4), heat shock factor 4 (HSF4), zinc finger family protein (CCCH-type)**.** Dual

stress specifically activated 2 TFs genes encoding imbibition-inducible 1 (IMB1) and struwwelpeter (SWP). Aphid infestation alone specifically activated 53 TFs genes of which some encode zinc finger (CCCH type, GATA type, C2H2 type) family protein, proline-rich family protein, auxin response factor (ARF8, ARF6), basic helix-loop-helix (bHLH) family protein, anti- silencing function 1B (ASF1B), chloroplast nucleoid DNA-binding protein-related . Dual stress specifically highly up regulated two TFs genes encoding imbibition-inducible 1 (IMB1) and struwwelpeter (SWP). Both dual stress and salt stress alone strongly activated one TFs gene encoding myb domain protein 4 (MYB4) which was also suppressed by aphid infestation, similarly to what observed at 6 h. A common high activation of 2 TFs genes encoding chloroplast nucleoid DNA-binding protein-related was detected between dual stress and aphid infestation. The two single treatments shared strong activation of five TFs genes encoding DP-E2F-like 1 (DEL1), proliferating cellular nuclear antigen (PCNA1), decreased methylation 2DNA (MET1) and High mobility group B 6 (HMGB6).

Transcription factors involved in the crosstalk stress responses

MYB domain and MYB-related transcription factor family

The MYB-related category was significantly down regulated by salt at 6 h while MYB domain category did not show significant change in the present analysis. Seventy one transcripts and 32 transcripts were associated with MYB domain and MYB-related transcription factor family, respectively. Of these only 7 MYB domain transcripts and one MYB-related transcript displayed high fold change. The latter transcript encoding myb family transcription factor was strongly down regulated (-0.52 fold) by dual stress at 6 h. Of the 7 MYB domain transcripts with high fold change, 3 transcripts coding MYB61, AtMYB19 and MYB55 were strongly activated by aphid infestation at 6 h. Another transcript encoding MYB4 was strongly induced by salt and dual stress at both time points (6 h and 24 h). The two other transcripts putatively coding for pentatricopeptide (PPR) repeat-containing protein and MYP family transcription factor were both strongly down-regulated by aphids at 6 h and one transcript encoding trf-like 9 (TRFL9) was highly down regulated by aphid infestation at 24 h (Fig S4.1a,b).

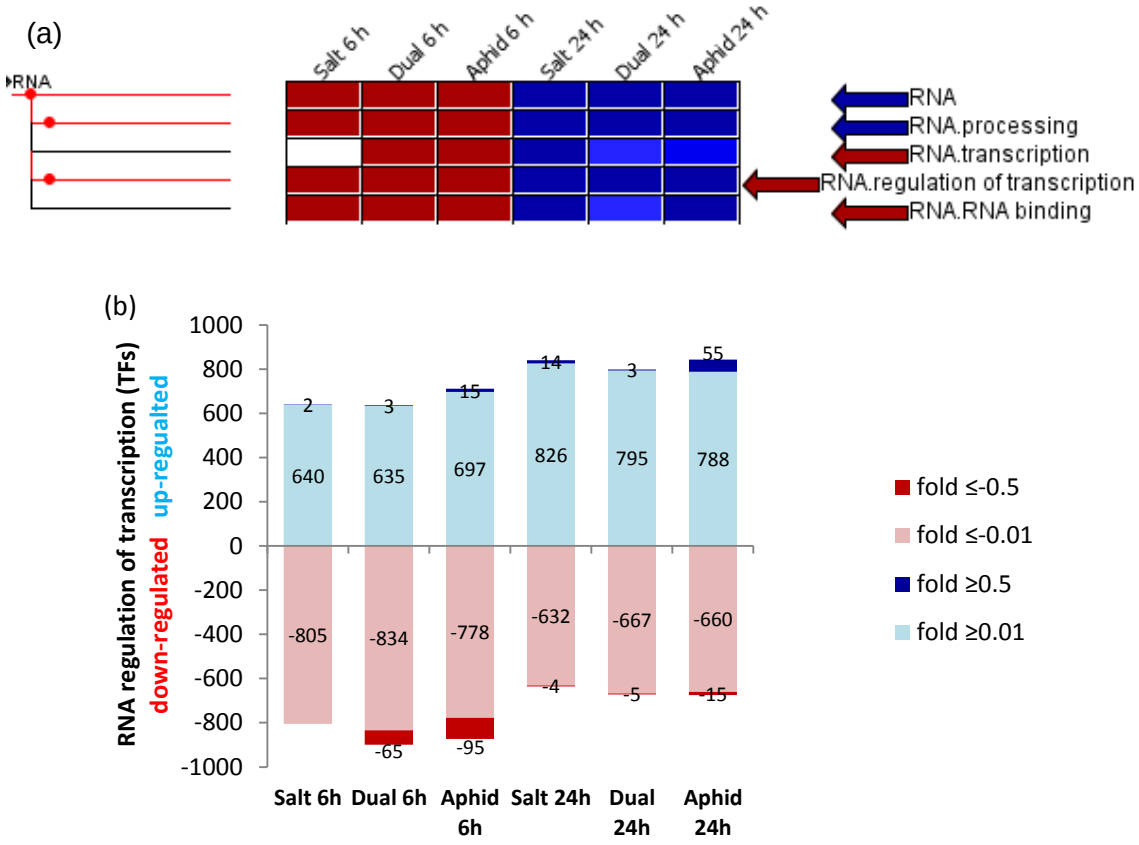


Figure 4.7 Changes in transcription factors TFs functional category and distribution of related genes.
(a) A condense PageMan display of coordinated changes of RNA functional category. The log2 fold changes between each stress treatments (salt, dual stress, aphid infestation) and control treatment were imported into PageMan for wheat. The data was subjected to a Wilcoxon test in PageMan and the results were displayed as false-colour coded. Bins coloured in red are significantly down-regulated relative to the rest of the array, whereas bins coloured in blue are significantly up-regulated. Significant suppression of all RNA categories genes was triggered by all three stress treatments at 6 h, whereas, significant activation was detected at 24 h. This shows a strong correlation between number of RNA and total number of genes differentially regulated in the analysis illustrated in figure 4.1. (b) Total number of up- and down-regulated genes (light blue and red respectively) involved in the regulation of transcription (TFs) in wheat under each stress treatments compared to control treatment at 6 h and 24 h after aphid introduction. Dual stress repressed the highest number of TFs at both time points, and the highest number of activated TFs was detected under aphid infestation alone at 6 h and under salt stress alone at 24 h. In terms of stress responsive genes (high fold change) associated with TFs, aphid infestation alone induced the highest number indicated as dark blue and red bars for strongly up- and down-regulated, respectively.

WRKY domain transcription factor family

Salt stress induced the highest number of WRKY related transcripts (29 transcripts) compared to the other stress treatments at 24 h, leading to significant up regulation of this category (p=0.011). Conversely, WRKY functional category did not show significant changes under dual stress and aphid infestation at either time points. Among the 39 transcripts associated with WRKY transcription factors only three genes encoding two WRKY18 and one gene encoding WRKY41, were highly induced by salt at 24 h, while, interestingly were highly supressed by dual stress at 6 h and aphid infestation at 24 h (Fig. S4.1c).

4.3.5 Regulation of genes involved in hormone metabolism

Based on Venn diagram analysis, results showed that among hormone related genes differentially expressed in wheat at 6 h post aphid infestation, 53 genes were commonly up regulated under all stress treatments at 6 h. Most of these genes were mainly involved in ethylene (16 genes) and jasmonate metabolism (13 genes), followed by abscisic acid ABA (9 genes), auxin (9 genes), gibberellin (4 genes) and cytokinin (2 genes) hormone metabolism. At 24 h post aphid infestation, 45 genes were commonly up regulated under all stress treatments. Most of these genes were involved in jasmonate and auxin hormone metabolism (10 genes for each), followed by ethylene (6 genes), gibberellin (6 genes), brassinosteroid (5 genes), ABA (4 genes) and cytokinin (3 genes). Interestingly, the analysis did not detect any salicylic acid related genes commonly up-regulated between the three stress treatments at both time points (Fig. 4.8). In addition, results revealed that at 6 h the number of commonly up-regulated hormone genes between dual stress and aphid infestation (115 genes) were higher than those between dual stress and salt stress (24 genes), and those between salt stress and aphid infestation (7 genes). However, at 24 h more genes were commonly up regulated between dual stress and salt stress (48 genes) than those between dual stress and aphid (39 genes), and those between salt and aphid (11 genes) (Fig. 4.8). Results relating to the following phytohormones will be presented below: abscisic acid, jasmonate, ethylene and salicylic acid.

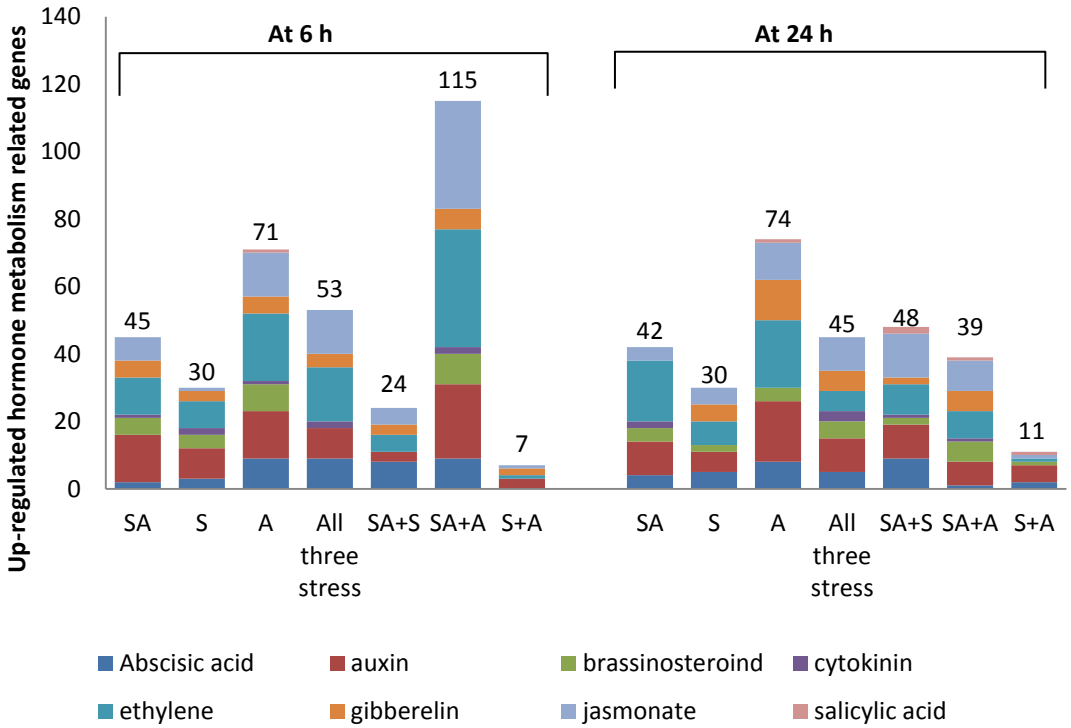


Figure 4.8 Distribution of up regulated genes related to different phytohormones metabolism. These genes were activated in wheat in response to three stress treatments: salt S, dual stress SA and aphid infestation A at 6 h and 24 h after aphid introduction and their transcript expression levels were compared to control treatment. Numbers above bars represent the total number of up regulated genes, labels on the x axis (All three stress, SA+S, SA+A, S+A) represent commonly up-regulated genes, and labels (SA, S, A) display specific up-regulated genes The data was obtained from a Venn diagram and presented in a stacked column to show the proportion of each phytohormone. Overall, four types of hormones namely abscisic acid, auxin ethylene and jasmonate constitute larger proportions than other hormones at both time points for all comparisons. The lowest number of commonly up regulated was between salt and aphid stress treatments applied alone. The most abundant specifically up regulated genes were: Auxin under dual stress and salt stress alone, ethylene under aphid stress alone at 6 h, while ethylene was the most abundant under the three stress treatment at 24 h.

Abscisic acid (ABA) hormone metabolism

Based on functional categorization analysis using the Wilcoxon rank sum test and visualization via PageMan, genes associated with abscisic acid (ABA) hormone metabolism and activation functions were significantly up-regulated by salt stress at 6 h and 24 h, but not by dual stress or aphid infestation. However, dual stress significantly up regulated two other subcategories associated with ABA hormone metabolism at 6 h (Fig S4.2). Of the putative ABA metabolism related genes, many genes were strongly up regulated showing at least 0.5 fold change (on a $\log_2$ scale) under one or more stress conditions at 6 h. Of these, two genes putatively encoding 9-cis-epoxycarotenoid dioxygenase 4 (NCED4) and *Arabidopsis thaliana* HVA22 homologue E (ATHVA22E) were specifically highly activated under dual stress. Aphid infestation specifically highly up regulated one gene coding for high-level expression of a sugar-inducible gene (HSI2). Two genes coding for protein HVA22 (0.78 fold) and 9-cis-epoxycarotenoid dioxygenase 2 (NCED2) (0.63 fold) were highly up regulated under salt stress. Two genes encoding putative 9-cis-epoxycarotenoid dioxygenase 4 (NCED4) were commonly highly induced by dual stress (0.61 and 0.70 fold) and aphid infestation (0.47 and 0.64 fold) at 6 h. In addition, specific and common strong induction of ABA metabolism related genes were also observed under different stress treatments at 24 h. Among these, one gene which putatively encodes protein HVA22 was highly up regulated under salt stress (0.68 fold). Both salt stress and dual stress highly up regulated one gene coding for protein HVA22 (0.69 and 0.53 fold, respectively). One gene encoding abscisic acid responsive elements-binding factor 2 (ABF2) was specifically highly up regulated under dual stress (0.47 fold) (Fig 4.9).

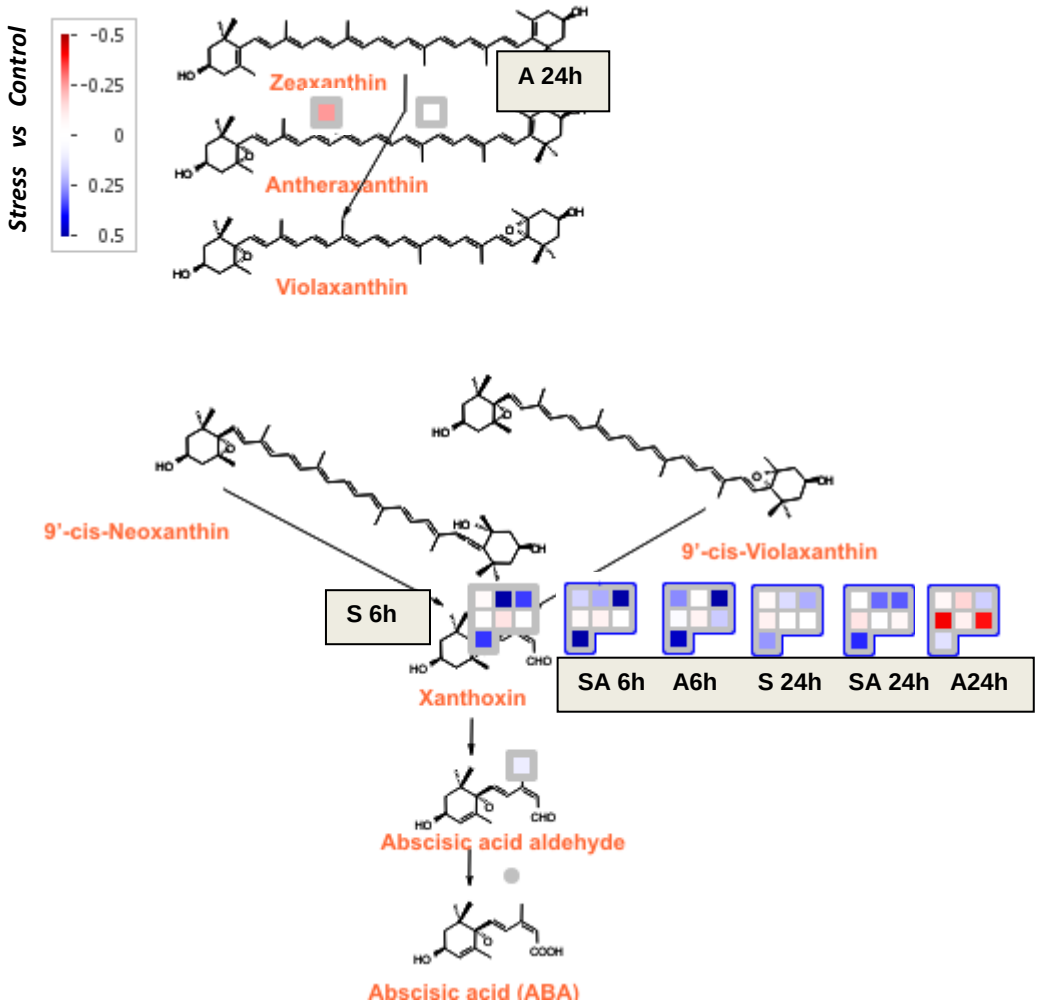


Figure 4.9 MapMan visualization of genes associated with abscisic acid (ABA) synthesis
(Transcripts encoding 9-cis-epoxycarotenoid dioxygenase). These genes were differentially regulated in wheat under salt stress alone S, dual stress SA, and aphid infestation alone at 6 h and 24 of aphid introduction. In the colour scale blue represents higher gene expression during stress treatment in comparison to control and red represents higher gene expression during control in comparison to stress treatment, and each symbol/ point represents one gene. These genes were strongly up regulated by all stress treatments under both time points except for aphid infestation alone at 24 h. The latter strongly down regulated these genes and another gene encoding zeaxanthin epoxidase.

Jasmonate (JA) hormone metabolism

Functional categories associated with jasmonate hormone metabolism were significantly regulated under almost all stress treatments at both time points. For instance, genes implicated in lipoxygenase synthesis-degradation were significantly up regulated by the three stress treatments at both time points except under aphid infestation at 24 h. Category for synthesis-degradation allene oxidase cyclase was significantly down-regulated under salt stress while it was significantly up-regulated by dual stress at 6 h. This category was also significantly up-regulated by both salt and dual stress at 24 h; interestingly, no significant change was detected under aphid infestation at both time points. The 12.Oxo.PDA-reductase category involved in jasmonate synthesis-degradation was significantly up-regulated by dual stress and aphid infestation at 6 h (Fig S4.2).

Both dual stress and aphid infestation at 6 h commonly strongly up regulated transcription of 8 genes involved in jasmonate synthesis, including those encoding lipoxygenase (5 genes) and 12-oxophytodienoate reductase (3 genes). Dual stress at 6 h specifically strongly increased the expression of ten genes encoding lipoxygenase (6 genes), allene oxide synthase (1 gene) and 12-Oxo-PDA-reductase (3 genes) which are associated with jasmonate synthesis. Two genes encoding lipoxygenase were highly up regulated under all stress treatments (Fig 4.10). In addition, at 24 h post aphid infestation, salt stress and dual stress highly induced 7 genes in common assigned to jasmonate synthesis encoding lipoxygenase. Other jasmonate synthesis related genes which exhibited strong induction under specific stress at 24 h were as follow: 2 genes encoding lipoxygenase (LOX5) and 12-oxophytodienoate reductase 2 (OPR2) were detected under dual stress; 2 other genes encoding lipoxygenase (LOX 4) and allene oxide synthase (AOS) were identified under aphid infestation (Fig 4.10).

Ethylene

Ethylene hormone metabolism category was significantly up regulated by dual stress at both time points and by aphid infestation at 6 h. Other sub-categories that showed significant alteration include the following: ethylene hormone synthesis-degradation was significantly up regulated by dual stress and aphid infestation at 6 h; ethylene hormone signal transduction was significantly up regulated by all three stress treatments

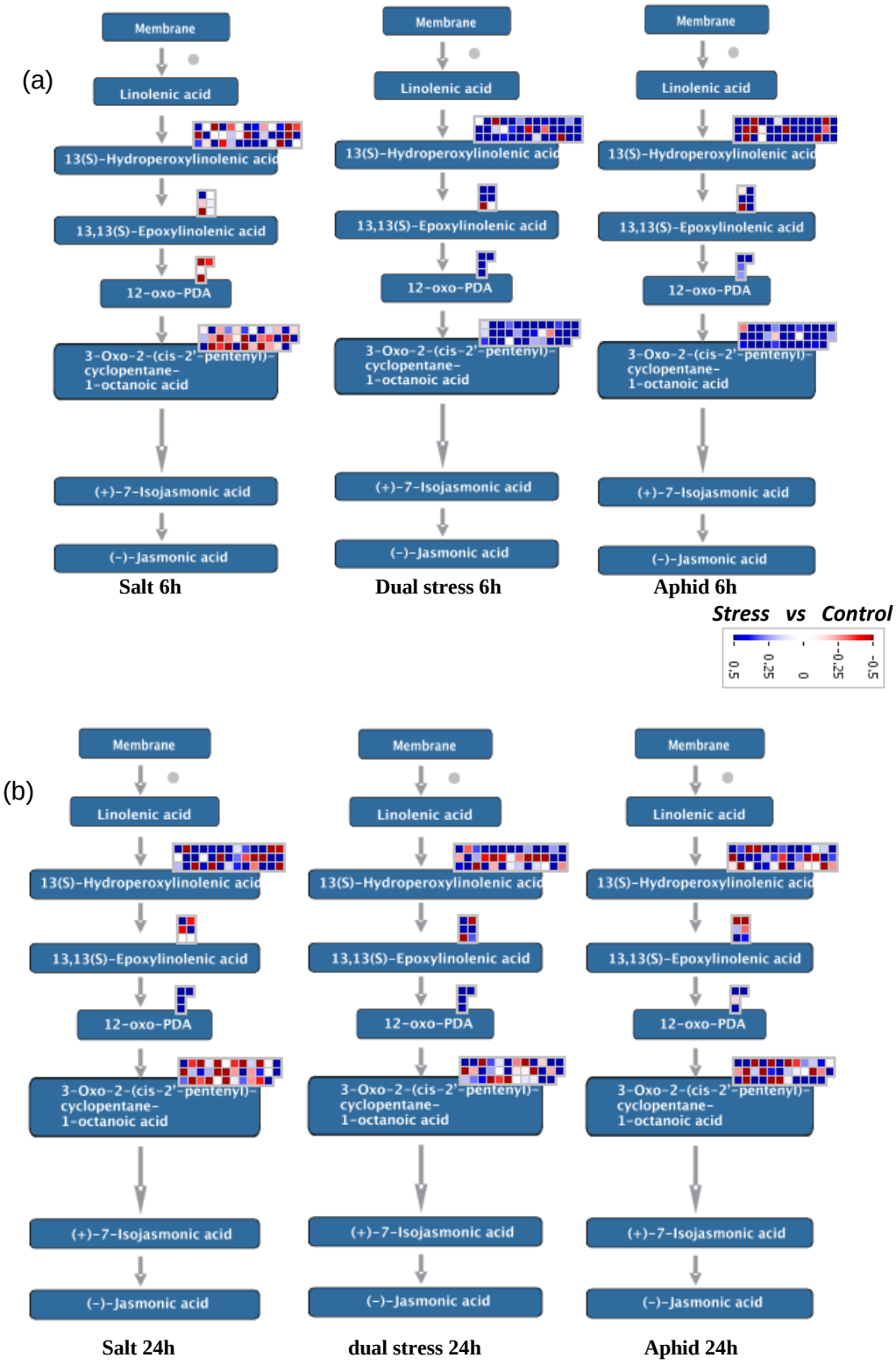


Figure 4.10 MapMan visualization of genes putatively involved in jasmonic acid synthesis (Transcripts encoding lipoxygenase, allene oxidase synthase, allene oxidase cyclase and 12-Oxo-PDA-reductase). These genes were differentially expressed in wheat under salt stress, dual stress, and aphid infestation at (a) 6 h and (b) 24 h after aphid introduction. Each point represents one gene and blue colour represents higher gene expression in stress treatment while red colour represents higher gene expression in control. Most of these genes were strongly up regulated by dual stress and aphid infestation, while more genes were strongly down regulated by salt stress at 6 h.

at 6 h and by dual stress at 24 h; two categories for ethylene synthesis-degradation (1-aminocyclopropane-1-carboxylate) and signal transduction were significantly down regulated by salt stress at 24 h (Fig S4.2). Among deferentially expressed genes at both time points, only 16 genes were strongly regulated under one or more stress treatments. Firstly, at 6 h salt stress and dual stress strongly induced 3 genes in common related to 1-aminocyclopropane-1-carboxylate oxidase. Putative oxidoreductase gene was strongly activated by both dual stress and aphid infestation. Salt stress specifically highly induced one gene encoding 1-aminocyclopropane-1-carboxylate oxidase. Aphid infestation specifically strongly up regulated 5 genes associated with the following: 1-aminocyclopropane-1-carboxylate oxidase, putative oxygenase related cluster, dehydration responsive element binding protein and universal stress protein. Secondly, at 24 h both salt stress and dual stress strongly induced 2 genes related to 1-aminocyclopropane-1-carboxylate oxidase. Salt stress specifically highly activated the expression of 2 genes involved in ethylene production encoding 1-aminocyclopropane-1-carboxylate oxidase, as well as 2 genes associated with signal transduction encoding ethylene-responsive factor-like transcription factor (ERFL1c) and ethylene-responsive element-binding factor.

Salicylic acid (SA)

Surprisingly, salicylic acid (SA) hormone metabolism category was significantly down-regulated by dual stress and aphid infestation at 6 h while no significant change was detected under the three stress treatments at 24 h (Fig S4.2). Both dual stress and aphid infestation at 6 h significantly decreased the expression of 7 and 5 genes, respectively. Of these, one gene encoding pentatricopeptide (PPR) repeat-containing protein-like was strongly suppressed under both dual and aphid stress (-0.57 and -0.69 fold, respectively). Another gene encoding the same protein was strongly suppressed under aphid infestation (-0.60 fold). At 24 h more SA related genes were induced than suppressed; however no significant change or high fold change was observed under the three stress treatments.

4.3.6 Redox regulation

The number of activated genes involved in redox regulation was higher under aphid infestation alone followed by the dual stress and then salt stress alone. When extending aphid infestation to 24 h a different expression pattern of genes involved in redox regulation was observed, as the number of activated genes associated with redox regulation was higher under dual stress followed by salt stress resulting in significant up regulation of this functional category. However, the suppression of redox regulation genes was higher under aphid infestation leading to significant suppression of this category at this time point (Fig S4.3). Among deferentially expressed genes, four transcripts including 2 genes associated with redox thioredoxin encoding thioredoxin h1 protein and disulfide isomerase 2 precursor protein were specifically strongly induced under aphid infestation at 6 h (0.53 fold) and 24 h (0.48 fold), respectively. Another two genes related to the sub-bins/categories, redox ascorbate and glutathione ascorbate encoding thylakoid ascorbate peroxidase and thylakoid-bound ascorbate peroxidase were also strongly induced by aphid infestation at 6 h (0.54 fold) and 24 h (0.48 fold), respectively. A gene associated with glutathione was highly suppressed (-0.56 fold) by aphid at 6 h while highly induced (0.56 fold) by salt at 24 h.

4.3.7 Genes related to biotic and abiotic stress responses

Based on functional enrichment categorization analysis, at 6 h post aphid infestation dual stress had significant impact on genes assigned to the stress functional category through significant activation of genes involved in biotic stress and abiotic cold stress functions. Salt treatment alone significantly up regulated genes associated with abiotic cold and wounding stress functions. Aphid infestation alone significantly up regulated genes related to biotic stress and cold abiotic stress functions. However, at 24 h post aphid infestation, the three stress treatments showed significant impact on genes implicated in the stress functional category. Dual stress significantly activated most of the biotic and abiotic related genes present in the sub-categories including those involved in respiratory burst, cold, drought/salt and light stress categories. Salt stress alone significantly up regulated genes involved in heat and cold stress responses. Aphid infestation alone significantly up regulated genes related to abiotic stress and light stress responses. The only significant repression was observed in heat abiotic stress category under dual stress and aphid infestation at 6 h (Fig. 4.11).

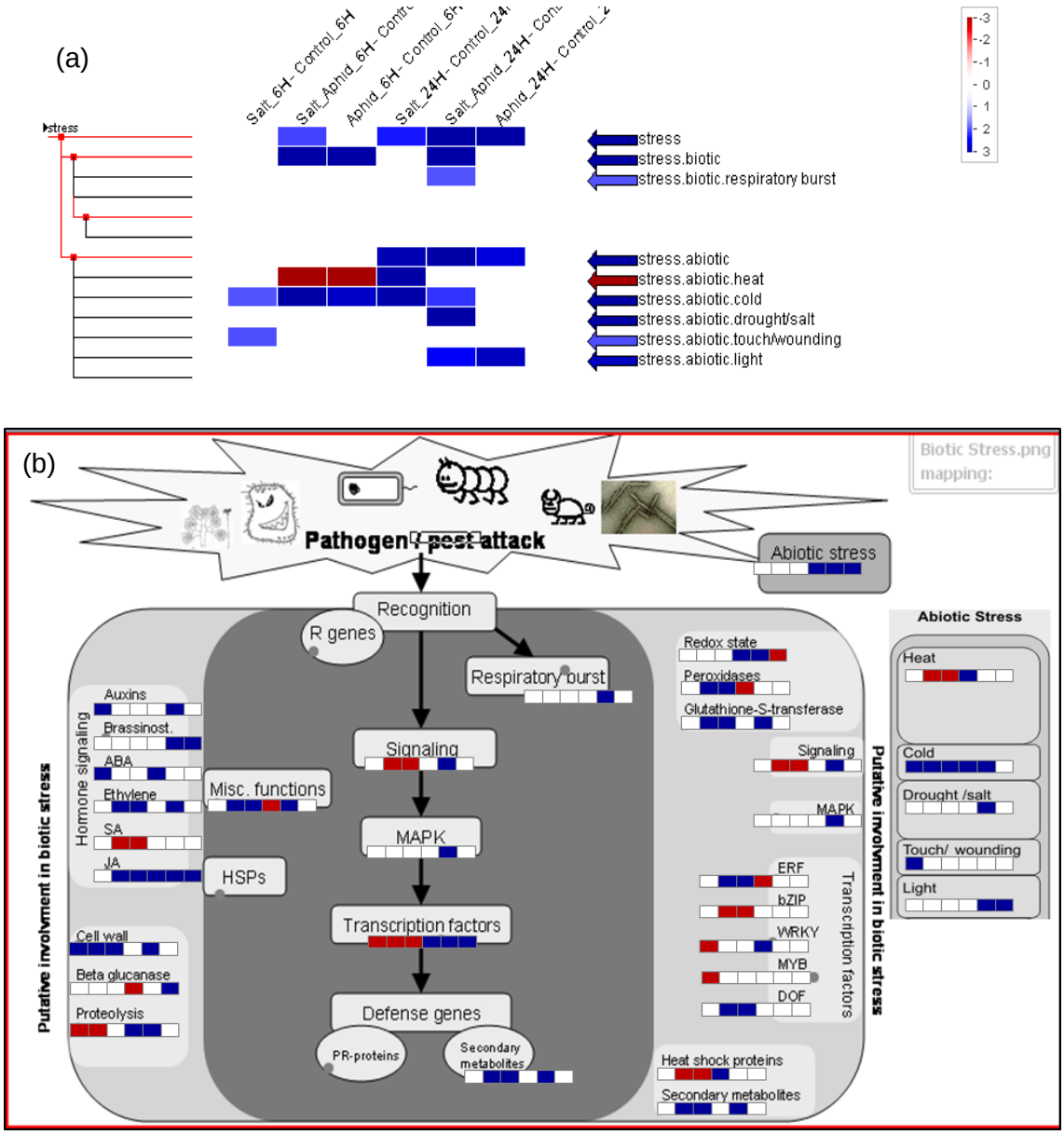


Figure 4.11 Significant changes in different stress functional categories.
(a) A condense PageMan display of coordinated changes of stress functional category including different sub-bins. The log2 fold changes between each stress treatments (salt, dual stress, aphid infestation) and control treatment were imported into PageMan for wheat. The data was subjected to a Wilcoxon test in PageMan and the results were displayed false-colour code. Bins and sub-bins coloured in red are significantly down-regulated and those coloured in blue are up-regulated relative to the rest of the array. Most significant activation of stress functional categories was caused by dual stress at 24 h. A significant suppression of abiotic heat stress was caused by dual stress and aphid infestation alone at 6 h. (b) Overview of stress pathways mapping putative genes involved in biotic stress (Pathogen/pest attack) and abiotic stress (heat, cold, drought/salt, touch/wounding and light). Data was extracted from Wilcoxon rank sum test which identify bins and sub-bins with significant changes relative to the rest of the array (blue for up regulated and red for down regulated) in response to three stress treatments compared to control at two time points. The following labels (S 6h, SA 6h, A6h, S 24h, SA 24h, A 24h) correspond to symbol/points arranged from left to right.

Biotic stress related genes

Among the differentially expressed biotic stress responsive genes at 6 h, only some were identified as up regulated stress responsive genes (≥0.5 and ≤-0.5 fold change on a log2 scale). The majority of these genes were strongly activated by dual stress (19 genes), while aphid infestation and salt treatment alone activated only 6 genes and 2 genes, respectively. At 24 h more biotic stress responsive genes were strongly activated under aphid infestation alone (14 genes) compared to those up regulated under dual stress (9 genes) and salt stress (7 genes) (Fig 4.12a).

Defence genes encoding Pathogenesis-Related Proteins (PR-proteins)

Family 1: Proteins of type PR. Among differentially expressed PR related genes only 9 transcripts were identified as stress responsive genes showing high fold change (≥ 0.5 on $\log_2$ scales). Of these, one transcript coding for leucine-rich repeat transmembrane protein kinase was highly induced by both aphid infestation(1 fold) at 6 h and salt stress (0.50 fold) at 24 h. Aphid infestation alone at 24 h increased the expression of 3 genes encoding disease resistance-responsive family protein (0.79, 0.46 and 0.47 fold). One transcript was highly induced by all stress treatments at 24 h (0.62 fold by salt, 0.49 fold by dual stress and 1.02 by aphid) and another transcript was induced by dual stress at 6 h (0.51 fold). This data indicate that overall the expression of PR genes was increased more at 24 h than at 6 h and that aphid infestation alone induced more PR genes than the other two treatments (Fig 4.12b).

Family 3: Chitinase. At 6 h post aphid infestation dual stress strongly induced the expression of 3 genes encoding basic chitinase (ATHCHIB), acidic endochitinase (CHIB1) and *Arabidopsis thaliana* chitinase class IV (ATEP3) (0.55, 0.55 and 0.48 fold change, respectively). Aphid infestation alone highly activated one gene encoding basic chitinase (ATHCHIB) (0.53 fold), whilst salt treated plants highly induced one gene encoding acidic endochitinase (CHIB1) (0.65 fold). In addition, at 24 h post aphid infestation three genes were strongly induced as follows: one gene for acidic endochitinase (CHIB1) was specifically induced by dual stress (0.56 fold), one gene for basic chitinase (ATHCHIB) was activated by both dual stress and aphid infestation (0.56 and 0.66 fold, respectively) and a chitinase putative gene was commonly induced by salt and dual stress (0.58 and 0.49 fold, respectively) (Fig 4.12b).

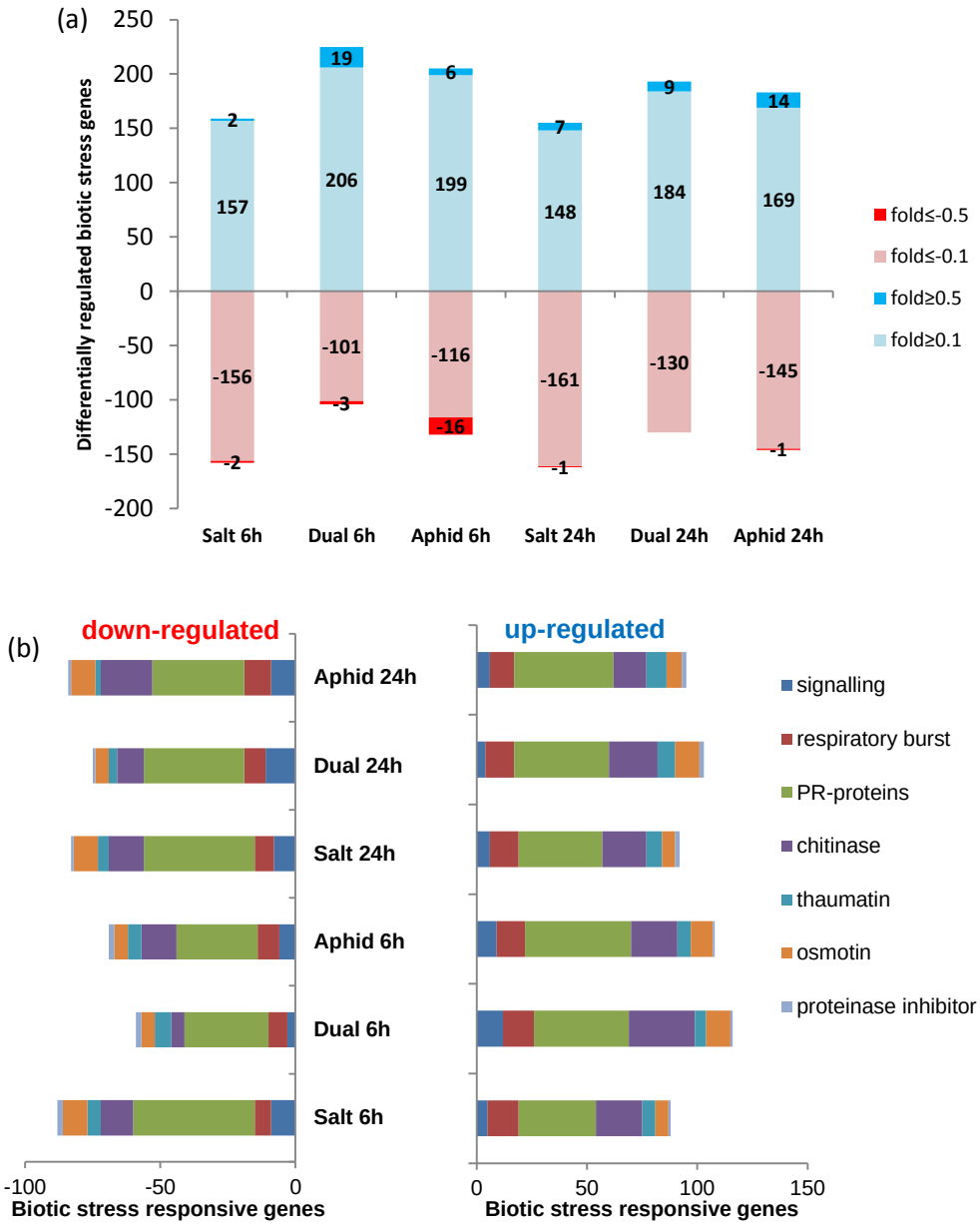


Figure 4.12 Distribution of differentially-expressed genes related to different biotic stress categories.
(a) Total number of differentially expressed biotic stress responsive genes in wheat under salt stress, dual stress and aphid infestation 6 h and 24 h after aphid introduction. The highest number of up regulated genes was triggered by dual stress at 6 h, while the highest number of down regulated genes was elicited by salt stress alone at 24 h. (b) Number of differentially expressed defence genes related to different biotic stress categories according to MapMan ontology classification. The transcript expression levels were compared to control treatment. Data is presented in a stacked column to display the proportion of each category. Two biotic categories for PR-proteins and chitinase constitute the largest proportions among other categories under all three stress treatments at both time points. Dual stress activated the highest number of these specific defence genes at both time points and down-regulated the lowest number at both time points.

Family 4: Thaumatin-like protein and osmotins. At 6 h post aphid infestation dual stress highly increased the expression level of three genes encoding ATOSM34 (OSMOTIN 34) (0.55, 0.56 and 0.66 fold). At 24 h, salt stress strongly induced one gene coding for thaumatin-like protein (0.47 fold), while two other genes coding for osmotin-like protein were highly induced by aphid infestation (Fig. 4.12b).

Family 5: Proteinase inhibitors. Three genes putatively encoding proteinase inhibitors were differentially expressed, but with relatively low fold change. The induction of these genes was greater at 24 h more than at 6 h (Fig. 4.12b).

Abiotic stress related genes

The present study identified 482 genes putatively implicated in abiotic stress and were associated with the following functional categories: abiotic stress 21 transcripts, heat 229 transcripts, cold 28 transcripts, drought/salt 89 transcripts, wounding 13 transcripts, light 3 transcripts and unspecified 99 transcripts (Fig. 4.13a).

Heat stress responsive genes. Among the 10 highly induced genes at 6 h, one gene encoding DNAJ heat shock N-terminal domain-containing protein was strongly up regulated by both dual stress and salt stress. Dual stress showed specific strong activation of one gene encoding a heat shock related- protein. Aphid infestation specifically caused high induction of 4 genes encoding the following: heat shock 70 kDa protein related cluster, Chloroplast heat shock protein 70, *Arabidopsis thaliana* DnaJ homologue 2 (ATJ2) and Heat shock protein 70 related clusters. At 24 h post aphid infestation the latter protein was strongly induced by both dual stress and aphid infestation. Salt stress highly up regulated a gene for DNAJ heat shock N-terminal domain-containing protein. Dual stress specifically highly up-regulated 2 genes coding for heat shock protein binding and heat-stress-associated 32 (HSA32). Aphid infestation specifically strongly activated 4 genes encoding the following: shepherd (SHD) ATP binding, *Arabidopsis thaliana* DnaJ homologue 3 (ATJ3), heat shock cognate 70 kDa protein (1HSC70-1) ATP binding and heat shock protein 91 (HSP91) (Fig. 4.13b).

Cold stress responsive genes. Cold abiotic stress was the most up-regulated transcripts among other abiotic stress functional categories under all stress treatments at both time points, except under aphid infestation at 24h. Among the 28 genes differentially regulated, the expression of 4 genes was strongly increased under one or more stress treatments. Of these, one gene encoding cold acclimation protein WCOR413 was highly

induced (0.61 fold) by dual stress at 6 h. Two genes encoding hypothetical protein and putative shock protein were strongly activated by aphids at 6 h (0.45 and 0.56 fold, respectively). Whilst a gene coding for universal stress protein (USP) was strongly up regulated by aphids at 24 h (Fig. 4.13b).

Drought/salt stress responsive genes. Among the 89 differentially expressed genes in this category, only 4 genes were highly up regulated. Dual stress at 6 h showed specific strong induction of 3 genes putatively encoding a hydrophobic protein, a low temperature protein and a salt responsive protein. Aphids at 24 h exclusively highly activated one gene coding for dehydration-responsive protein RD22-like (Fig. 4.13b).

Touch/wounding stress responsive genes. Salt stress, surprisingly, had a significant impact on the expression of touch/wounding related genes (p=0.044) through inducing the highest number (11 genes), compared to those induced under either dual stress or aphid infestation. The 13 genes involved in touch/wounding and encoding wound-responsive family protein and vein patterning 1 (VEP1) were differentially regulated but did not show high fold change (≥0.5 and ≤-0.5 fold change) under the three stress treatments at either time points (Fig. 4.13b).

Light stress responsive genes. Dual stress and aphid infestation at 24 h induced three genes assigned to light stress, leading to a significant alteration (p=0.014 and p=0.004, respectively). Two genes exhibited high fold change, one gene encoding UVB-resistance 8 (UVR8) was highly repressed by dual (-0.58 fold) and aphid stress (-0.83 fold) at 6 h. The third gene encoding UV-damaged DNA-binding protein 1A (DDB1A) was strongly induced by aphid infestation at 24 h (0.47 fold) (Fig. 4.13b).

Unspecified abiotic stress responsive genes. Among genes associated with the unspecified abiotic stress bin, only 15 genes exhibited high expression levels. Of the genes differentially regulated at 6 h, one gene encoding USP was up-regulated by all three stress conditions, but most highly by aphid infestation. Three genes encoding fatty acid alpha- oxidase were strongly induced by salt stress (0.75, 0.48, 0.49 fold). At 24 h post aphid infestation, five genes encoding a salt tolerant protein and universal stress protein (USP) family protein were up-regulated under the three stress treatments, but more strongly by aphid infestation. Two other genes encoding universal stress protein (USP) and osmotin-like protein precursor were highly activated by aphid infestation at 6 h (0.52 fold) and 24 h (0.55 fold) (Fig. 4.13b).

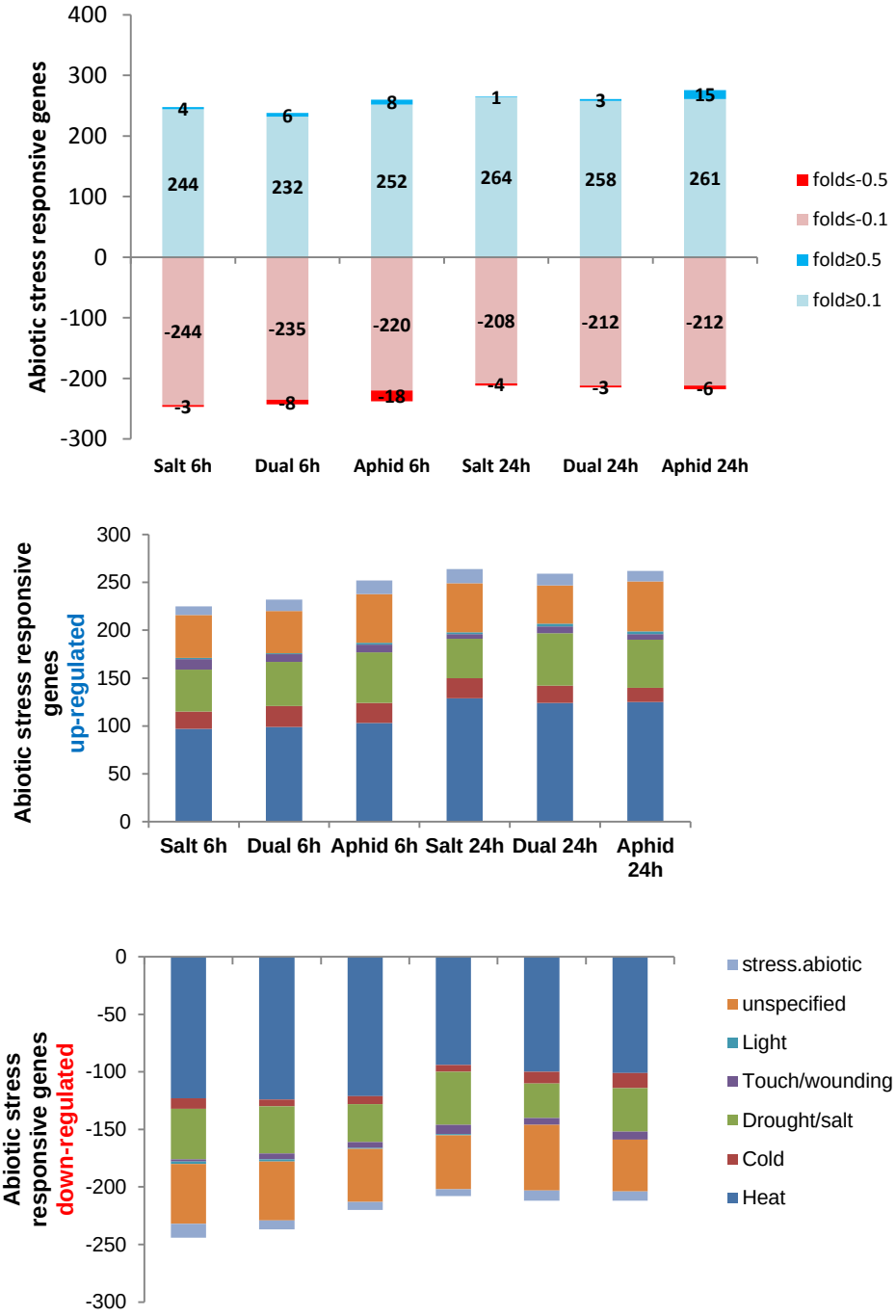


Figure 4.13 Distribution of differentially-expressed genes related to different abiotic stress categories.
(a) Number of total genes differentially expressed in wheat by salt stress, dual stress and aphid infestation at 6 h and 24 h after aphid introduction. The highest number of up regulated genes was triggered by aphid infestation at 24 h. The highest number of down regulated genes was elicited by salt stress at 6 h. (b) Number of genes related to different biotic stress categories according to MapMan ontology classification. The data is presented in a stacked column to display the proportion of each category. Heat, drought/salt and unspecified constitute large proportions among other categories under all three stress treatments at 6 h and 24 h. Salt stress and aphid infestation alone activated the highest number of these specific defence genes at 24 h and salt stress alone down-regulated the highest number at 6 h.

4.3.8 QRT-PCR for validation and confirmation of microarray data

To validate the results from the microarray analysis, eight differentially regulated genes, which represented up-regulated, unchanged, and down-regulated genes identified through the microarray studies, were selected and specific primers were designed for analysis using quantitative real-time PCR. These seven selected genes were involved in calcium signalling (calcium-binding protein, putative), redox thioredoxin protein disulfide isomerase (PDI-LIKE 1-1), redox dismutases and catalases copper/zinc superoxide dismutase 3 (CSD3), redox ascorbate and glutathione ascorbate GME (GDP-D-mannose 3',5'-EPIMERASE); GDP-mannose 3,5-epimerase/ NAD binding / catalytic, lipoxygenase (*LOX5*), allene oxide synthase (AOS); hydro-lyase/ oxygen binding and transport Major Intrinsic Proteins TIP GAMMA-TIP (Tonoplast intrinsic protein (TIP) gamma); water channel. A reference candidate gene actin 2 (*ACT2*) was selected as an internal standard (Table S4.15). Although actin was used as the internal standard in qPCR studies (Tenea *et al*., 2011), other studies suggest that ubiquitin or tubulin may be more reliable (Sirakov *et al*., 2009). However, this is highly unlikely to affect the results. Results showed that some of these selected genes showed good correlation with gene expression profiles obtained from the microarray data with respect to trends of regulation (Table S4.16 & Fig. S4.4). However, some inconsistencies between the qRT-PCR and microarray outputs were detected, but this may be due to the sensitivity of the methods used. RT-PCR depends on high quality template RNA that may be affected by extraction and storage, especially when the transcript level is low. In addition, both RT-PCR and microarray analysis are quantitative methods that may vary in opposite directions, creating slight inconsistency. In particularly 76% of the genes tested showed good correlation with the gene expression profiles, thus validating the microarray data.

4.4 Discussion

Plants are simultaneously exposed to multiple environmental stress factors that affect their survival, growth and reproduction. However, most studies of biotic and abiotic stress impacts on plants have addressed each stress individually, overlooking the influence of multiple stress interactions. Recently, researchers have changed their focus towards understanding the interaction between the response of plants to simultaneously biotic and abiotic stresses. Most previous published studies in this area have focused on pathogens (rusts, fungus/fungal, viruses, bacterial, etc.) as the biotic stress factor, and

drought, high temperature, heavy metals and wind as abiotic stress factors. Among various stress factors, salinity and insect herbivore attack are two common stress challenges encountered by many crop plants leading to yield loss and thus are considered as two major constraints on agricultural productivity. Although the impacts of salinity and herbivores on plants have been extensively studied individually, yet little is known about their effects in combination.

The present study was designed to investigate the impact of salinity and the aphid *Sitobion avenae* infestation applied in combination and individually on wheat gene expression profiling, highlighting the interactions between salt and aphid stress responses pathways. It was also designed to determine potential cross tolerance between these two different forms of stress. One of the main objectives of the present study was to identify putative genes and pathways associated with the interaction between abiotic and biotic stress. Therefore, the discussion will mainly focus on processes and genes significantly and exclusively affected by dual stress (i.e. interaction genes) as these candidate genes may play a key role in coordinating plant response to combined or multiple stress conditions. The biological processes associated with hormones, transcription factors and stress will be covered in the discussion.

In general the results showed that the numbers of stress-responsive genes highly differentially regulated in response to aphid infestation alone (3056 at 6 h and 1580 at 24 h) were far greater than those induced by either salt stress alone (285 at 6 h and 467 at 24 h), or by dual stress (1592 at 6 h and 504 at 24 h). More genes were suppressed than induced at 6 h post aphid infestation under both dual stress and aphid infestation alone, but not under salt stress alone. The general down regulation of transcripts under stress indicate that the plant hosts seem able to down-regulate these genes as an adaptive response to biotic attack, since a reduction in gene expression does not necessarily translate into loss of function (Botha *et al*., 2012). In contrast, at 24h more genes were induced in wheat plants than supressed under the three different stress treatments.

4.4.1 Common stress response

Venn diagrams revealed that there was a different degree of overlap between transcripts expressed in wheat plants during the three different stress conditions. When salt stress and aphid infestation were applied to plants in combination, the response of wheat plants to the dual stress was more similar to that of salt stress alone, than that of aphid infestation alone (Fig. 4.5). At both time points 6 h and 24 h, plants subjected to dual

stress shared more up regulated transcripts in common with plants exposed to salt alone (49 and 69 transcripts, respectively), compared to those exposed to aphid alone (45 and 38 transcripts, respectively). In contrast, in terms of down-regulated transcripts, the response of wheat plants to dual stress was more similar to that of aphid infestation alone than that to salt stress alone. The expression pattern of down-regulated genes under dual stress was more similar to that observed under aphid infestation alone (731 and 24 transcripts) than that under salt stress alone (16 and 16 transcripts) at both time points 6 and 24 h, respectively. This observation is consistent with Atkinson *et al*. (2013), who found that *Arabidopsis* gene expression under the effect of combined water deficit and nematode stress was more similar to that under water deficit alone than to that under nematode stress alone. This data suggest a higher profound impact of water deficit than nematode stress. It was also suggested that plant-parasitic nematodes have evolved mechanisms to minimize damage to plant tissue and thus avoid inducing standard plant defence systems (Atkinson and Urwin, 2012). Thus, when the two stresses occur together, the plant may prioritize a response to the potentially more damaging abiotic stress. The stylets of sap sucking feeders such as aphids may be comparable to fungal haustoria leading to a weak wound response (Dubey *et al*., 2013) and to the induction of defence-signalling pathways most commonly activated by pathogens (Walling, 2000; Moran *et al*., 2002). Moreover, salinity causes osmotic stress and ion-excess effects (Munns, 2005) therefore it could be suggested that the effect of salt stress on plant was more prominent than aphid infestation and the plant may prioritize acclimating response to potentially more damaging salinity.

The small number of differentially expressed transcripts in common between salt and aphid applied individually compared with the high number of transcripts specifically expressed under dual stress clearly indicate that the plant response to a combination of two stresses cannot be directly extrapolated and predicted from comparing plant response to each stress applied individually (Mittler, 2006). This finding supports the idea that testing stress-tolerant plants by imposing each stress factor in isolation may be inappropriate for developing stress tolerance in new varieties (Mittler and Blumwal, 2010; Atkinson and Urwin, 2012). The data presented in this chapter, suggest a more generalized and universal role of stress, indicating a putative involvement of some common genes in the crosstalk between pathways involved in responses to biotic and abiotic stresses. Interestingly, among the strongly and significantly up regulated genes were lipoxygenase (LOX5), pathogenesis-related protein (PR4) and cytochrome P450 (CYP71B38) which have been well documented to be involved in plant response to

stress and play key roles in regulating plant defence (Gatehouse, 2002; Ferry *et al*., 2004, 2006, 2011).

4.4.2 Specific dual stress (SA) responses

Functional categorization analysis showed that dual stress specifically and significantly (p<0.05) altered some major functional categories (bins) that were not significantly changed under either of the two single stresses. Results showed that plant specific response to dual stress at 6 h after aphid induction comprised significant down-regulation of genes involved in TCA transformation and mitochondrial electron transport fuctions, but significant up-regulation of large numbers of genes associated with stress (both biotic and abiotic functions), and tetrapyrrole synthesis function was unchanged. Furthermore plant specific responses to dual stress at 24 h of aphid introduction included significant up regulation of genes involved in seven functional categories namely: gluconeogenese/glyoxylate cycle, glycolysis, cell wall, secondary metabolism, *miscellaneous enzyme families*, signalling and transport. This finding reveals that a unique programme of gene expression is activated by the plant in response to dual stress. Similar findings have been demonstrated in transcriptome studies on plants subjected to multiple abiotic stresses. For example, in both tobacco (*Nicotiana tabacum*) and Arabidopsis (*Arabidopsis thaliana*) palnts, a combination of drought and heat stress induced a novel programme of gene expression, activating transcripts that were not induced by either stress individually (Rizhsky *et al*., 2002, 2004). Another microarray analysis has also revealed that exposure to multiple biotic stress (two species of herbivorous insect) elicited a transcriptional response that was distinct from each individual response (Voelckel and Baldwin, 2004).

4.4.3 Hormones and signalling

In the present study, based on functional categorization, dual stress strongly up regulated genes involved in abscisic acid (ABA), auxin, brassinosteroid, ethylene and jasmonate hormone metabolism which are documented to be elicited by phloem feeding insects during infestation and play key roles in regulating plant defence (Giordanengo *et al*., 2010). Also genes related to cytokinin metabolism were exclusively and significantly up regulated by dual stress. However, genes involved in salicylic acid synthesis were strongly and significantly down regulated. The activation of these genes under dual stress due to aphid infestation is in agreement with previous studies that

documented the involvement of the phytohormones JA, ET, ABA, and auxin in mediating greenbug aphid induced defence responses in near isogenic wheat lines (Reddy *et al*., 2013). Furthermore, based on analysis of single candidate genes, 10 transcripts mediating JA biosynthesis which occurs in the chloroplast and is mediated by lipoxygenase (*LOX*) (Reddy *et al*., 2013) were specifically and strongly up-regulated under dual stress at 6 h after aphid introduction including, six lipoxygenase *LOX* , 3 OPR (12-oxophytodienoate reductase and one allene oxide synthase AOS. After 24 h two transcripts putatively assigned to OPR and *LOX* were specifically and highly up-regulated by dual stress. This finding is consistent with studies documenting that plants under herbivore attack rapidly accumulate JA (Reddy *et al*., 2013) since *LOX*, a gene whose transcripts are associated with the JA signalling pathway, is strongly induced by foliar feeding of numerous insects (Smith and Boyko, 2007; Smith *et al*., 2010). For example, greenbug aphid showed increased expression of *LOX* related JA biosynthesis genes in near isogenic wheat lines (Reddy *et al*., 2013). Therefore, results suggest that the strong and significant up-regulation of transcripts encoding *LOX* genes observed under dual stress at both time points may be correlated with increased jasmonate levels.

The unexpected finding that dual stress significantly suppressed genes involved in salicylic acid metabolism at 6 h while they were unchanged at 24 h, contradicts other studies. Thompson and Goggin (2006) reported that phloem feeding insects elicit salicylic acid in addition to other signalling compounds including jasmonic acid and ethylene during infestation which may play key roles in regulating plant defence. It has also been shown that aphids induce salicylate accumulation in wheat, barley, soybean and tomato plants (Thompson and Goggin, 2006). Also in wheat, SA induction was observed in incompatible, but not compatible interactions with the Russian wheat aphid (Thompson and Goggin, 2006). *M. persicae* and *M. euphorbiae* feeding on tomato induced strong up-regulation of the salicylic pathway PR and PR4 genes (Couldridge, 2007). However, other studies were similar to the present findings; for instance, phloem-feeding aphids (*Myzus persicae*) on *Arabidopsis* leaves did not induce any measurable changes in salicylic acid levels (De Vos *et al*., 2005). Similarly no changes in SA levels were detected in aphid *Myzus nicotianae* infested *Nicotiana attenuate* plants (Thompson and Goggin, 2006).

A possible explanation for the decreased SA levels observed in the current study could be due to cross talk between phytohormones signalling pathways. It has been reported that plant defence against pathogen and herbivores are determined in part by the

coordinate regulation of plant hormone signalling pathways (SA, JA and ET) that can interact synergistically or antagonistically (Fraire-Velazquez *et al*., 2011*).* Also, previous studies have demonstrated the occurrence of negative crosstalk between SA and JA (Thompson and Goggin, 2006). For example, the phloem-feeding aphids *S. graminum, Macrosiphum euphorbiae* and *M. persicae* induced a strong up-regulation of the SA-dependent pathways and reduced the expression of JA-dependent genes. Studies have also suggested that aphids inhibit efficient plant defence conferred by JA-regulated genes via regulation of the SA genes (Moran and Thompson, 2001; Moran *et al*., 2002; Kempema *et al*., 2007). SA pathway was suggested to be less important in mediating resistance to Russian wheat aphid than the JA pathway (Smith *et al*., 2010).Therefore, the present study suggests that the strong and significant induction of JA hormone related genes triggered by aphid feeding in both dual stress and aphid infestation alone may antagonise SA hormone metabolism pathways leading to significant suppression of SA related genes. Although the significant activation of JA synthesis was also sustained at 24 h, the expression level of SA surprisingly remained unchanged under both dual stress and aphid infestation alone at 24 h. The antagonistic crosstalk between biotic and abiotic stress signalling pathways has been suggested to play a role in plant response to a combination of stresses (Atkinson *et al*., 2013).

Previous experimental data confirm that Jasmonic acid and ethylene operate synergistically to activate the expression of a subset of defence genes against necrotrophic pathogens and herbivorous insects (Fraire-Velazquez *et al*., 2011). Genes acting as point controls between these two pathways have been described, for example, ethylene response factor 1 (ERF1) is a positive regulator (Fraire-Velazquez *et al*., 2011). In the present study, both jasmonic acid and ethylene metabolism related genes were shown to be significantly up-regulated under dual stress and aphid infestation alone at 6 h and under dual stress at 24 h. Results also showed that genes involved in ethylene responsive element binding protein transcription factor family were significantly up-regulated under both dual stress and aphid infestation alone at 6 h which may be correlated with increased levels of ethylene hormone. This study also revealed that plants subjected to dual stress significantly activated genes involved in cytokinin hormone synthesis, whereas the single treatment with salt and aphid did not significantly change or affect the expression of these cytokinin genes. This difference may suggest that a combination of salt and aphid imposes on plants a different type of stress compared to salt and aphid alone, which requires the utilization and activation of cytokinin hormone genes. This finding is in line with Dubey *et al*., (2013) who were the

first to report the involvement of cytokinin in defence responses towards aphids and whiteflies.

4.4.4 Transcription factor TFs

Based on analysis of single candidate genes, one transcript putatively assigned to MYB domain transcription factor family encoding MYB domain protein 1 (MYB1) was strongly up regulated by both salt stress alone and dual stress, but not by aphid alone at 6 h and 24 h post aphid introduction. Increasing evidence suggests that transcription factors in the MYB superfamily play important roles in plant response and defence to various biotic and abiotic stressors (Zhang *et al*., 2011). Other studies have demonstrated that transcription factor MYB1 and MYB protein were among the salt-stress responsive genes commonly differentially regulated in shoots of five wheat lines including the salt tolerant wheat lines W4909 and W4910 (Mott and Wang, 2007). Also, dramatic increases in the transcription levels of *TaMyb1* genes (*Triticum aestivum* Myb transcription factor 1) occurred under hypoxia and was gradually increased in roots as the result of treatment with NaCl (Zhang *et al*., 2011). Results from the current study are also in line with other studies of combined stress, which found that MYB transcription factor gene family was identified as specifically elevated during a combination of drought and heat stress (Rizhasky *et al*., 2004). MYB domain transcription factor was significantly up regulated under both heat stress and salt stress in *Arabidopsis thaliana* (Matsuura *et al*., 2010). The present result is also consistent with that for wheat in which the majority of salt responsive MYB genes showed transient up and down regulation in *Triticum aestivum* cv. Chinese spring wheat (CS) subjected to 150 Mm NaCl solutions (Kawaura *et al*., 2008). It is likely that the high expression of MYB1 in wheat plants under salt stress and dual stress, irrespective of aphids feeding which failed to activate the same transcription factor when applied individually may be a consequence of plant acclimation and adaptation response to salinity as a result of pre-treatment with salt. Thus, this component may contribute to cross tolerance in dual stress. However, other different types of MYB (MYB61, MYB55, MYB19) were shown to be up-regulated by aphid infestation alone at 6 h. This finding is in agreement with that of Gutsche *et al*., (2009) who found that two genes encoding MYB transcription factors were differentially up regulated at three hours and three days in a tolerant barley line in response to *Diuraphis noxia* feeding.

Plant WRKY DNA-binding transcription factors are involved in plant pathogen interactions (Dubey *et al*., 2013). It has also been documented that the transcription factor WRKY (70, 6, 53) acts as a positive regulator of SA-dependent defences and a negative regulator of JA-dependent defences and plays a central role in determining the balance between these two pathways. For example, suppression of WRKY70 expression allows increased expression from JA-responsive genes (Fraire-Velazquez *et al*., 2011). In the current study the expression of three WRKY genes: one WRKY41 and two WRKY18 were highly down regulated for all treatments at both 6 h and 24 h except for exposure to salt at 24 h when they were highly up regulated. After aphid introduction, however other 2 WRKY18 and WRKY41 genes were exclusively highly up regulated under salt stress alone and were surprisingly/unexpectedly down regulated under dual stress and aphid infestation alone at 24 h of aphid introduction. Similar findings were obtained by Dubey *et al*. (2013), who found that although WRKY33 and WRKY3 expression were enhanced at all times/intervals measured, other WRKYs (21, 20, 1 and 35) were down regulated by two sap-sucking insects, the aphid *Aphis gossypii* and the whitefly *Bemisia tobacco* in cotton plants infested for 2 h and 24 h. This same study also reported that sap-sucking insects interact with plants by suppressing the defence-related transcription factors such as WRKY and other signalling MAP kinases involved in plant defence. This finding contradicts those of Smith *et al*. (2010) who detected higher expression level of WRKY transcripts associated with the SA signalling pathway as well as pathogenesis realted-1 PR1, PR4, PR5 than transcripts associated with JA signalling pathways in infested wheat plants expressing resistance to the Russian wheat aphid *D. noxia* (Yalpani *et al*., 1991; Smith *et al*., 2010). Moreover, aphid feeding was reported to stimulate WRKY expression (Voelckel *et al*., 2004; Smith *et al*., 2010), yet WRKY transcription factors were reported to supress JA (Smith *et al*., 2010). Therefore, based on results obtained from the current study and from previous studies, it could be suggested that the down regulation of WRKY transcripts observed under both dual stress and aphid infestation alone may partially explains the enhanced expression of JA signalling genes. The suppression of WRKY transcripts may also be correlated with the reduction of SA transcript levels at 6 h and the unchanged expression of SA-signalling genes at 24 h after aphid introduction.

4.4.5 Biotic stress response

Transcriptome analysis of wheat plants subjected to a combination of salt and aphid stress revealed a new pattern of biotic defence response in wheat plants compared to

single stress. Specific strong up regulation of transcripts involved in biotic stress responses were seen at 6 h under dual stress. These included: ATEP3 (*Arabidopsis thaliana* chitinase class IV), pathogenesis-related family protein, three transcripts encoding osmotin 34 (ATOSM34), basic chitinase (ATHCHIB), PR12, two transcripts for pathogenesis-related 4 (PR4), two transcripts coding for TLP, two transcripts encode WIR1B, 2 transcripts for PR1and PR13, disease resistance-responsive family protein. Transcripts encoding PR4 (pathogenesis-related 4) and acidic endochitinase (CHIB1) were highly up regulated by dual stress at 24 h. Transcripts encoding pathogenesis related (PR) proteins were also highly increased under the three stress treatments at both time points. The data indicate that transcripts encoding pathogenesis-related proteins were abundant and is consistent with the observation of changes in the expression profiles of many known genes, including pathogenesis related proteins (PR) in salt tolerant wheat germplasm lines in response to salt stress at electrical conductivity (EC) of 30 dS/m (Mott and Wang 2007). Furthermore, Mantri *et al*. (2010) showed that both the fungal pathogen *Ascochyta rabiei* and salinity up regulated PR in chickpea plants. The genes related to pathogen defence have been previously reported to be induced under salt stress (Munns, 2005) but their roles in salt stress adaptation still remain unknown.

4.4.6 Abiotic stress response

In this study a potential new type of defence response in wheat plants, induced specifically by dual stress was identified compared to individual stress. At 6 h, dual stress exclusively and highly up regulated two heat stress transcripts encoding heat shock protein-related and DNAJ heat shock N-terminal domain-containing protein. This dual stress also highly up regulated three transcripts involved in drought/salt stress coding for hydrophobic protein, putative low temperature and salt responsive protein, and also one cold stress transcript encoding cold regulated 413 plasma membrane (1COR413-PM1). At 24 h, this dual stress specifically and highly up regulated three heat stress transcripts encoding heat shock protein binding GRV2 (KATAMARI2), heat-stress-associated 32 (HSA32), and early-responsive to dehydration 2 (ERD2/HSP70T-1). Similarly, Mott and Wang (2007) finding showed that changes in expression profiles of many known genes involved in or affected by abiotic stresses were observed under salt, drought, cold and heat. Among those a cold related gene (WCOR518) was up regulated in salt tolerant wheat lines under salt stress (Mott and Wang 2007). This finding confirms the involvement of other abiotic stress genes or

pathways in response to dual stress, suggesting a general abiotic stress response. However, many genes revealed in this study need further investigation.

4.4.7 Growth vs defence

The ability of plants to grow and defend themselves against biotic and abiotic stress depends on their internal resources and based on the "growth-differentiation balance hypothesis", plants under stress must set a balance and prioritise between growth and the induction of defensive elements (Mewis *et al*., 2012). In the present study, a significant trend towards decreasing transcript levels of genes involved in cell organisation, cell division, cell cycle and vesicle transport functional categories was detected at 6 h post aphid introduction in plants subjected to both dual stress and aphid stress alone. However, after 24 h, this trend shifted from reduction to induction as significant up-regulation of genes associated with cell functional categories was observed under all threes stress treatments. This result is in line with the concept that aphid attack elicits a switch from growth to defence related transcriptional processes, and that stress specific changes occur largely in primary metabolism and signalling cascades (Voelckel and Baldwin, 2004).

4.5 Conclusion

The present study was designed to determine the effect of combined salt stress and aphid *S. avenae* infestation (dual stress) on the transcriptome responses of wheat compared with each of the two stresses applied individually. The identification of differentially expressed genes was determined using Affymetrix GeneChip Wheat genome array. Comparing gene expression profiles in wheat in response to the three stress treatments *vs* control treatment identified specific and common differentially expressed genes. The study demonstrated that wheat gene expression pattern in response to dual stress is different from the response to each of the two stresses applied individually. Thus the response to combined stressors cannot be extrapolated from responses to single stress. The study suggests that wheat plants under a combination of stress show unique alteration in the transcriptome. Careful examination of these genes revealed putatively annotated and novel genes which may have potential roles in cross tolerance. Validation of the roles of these genes and other candidate genes in response to dual stress requires functional confirmation experiments. For example, in order to

more fully understand the role of specific genes in the interaction response, future studies aimed at down regulations targeted genes could provide direct insight into the interaction response. Alternatively overexpression of wheat genes in model plant system could be used to evaluate their role in providing protection against aphid herbivores. In this study high numbers of expressed transcripts were identified with novel (not assigned) and unknown function. Elucidation of their function will provide additional information about putative genes and their expression patterns involved in wheat plant responses to stress. The present study not only confirms previous studies suggesting the activation of a specific and unique stress response by plants when subjected to a combination or multiple stresses compared to single stress, but also provides a new insight into plant response mechanism during the interaction of biotic and abiotic stress.

CHAPTER 5

5 General Discussion and Conclusion

5.1 General discussion

One major goal of plant science and breeding is to create broad spectrum stress-tolerant crops through transgenic or conventional breeding approaches. To achieve this goal, it is crucial to increase the level of understanding of the underlying mechanisms of plant responses to simultaneous stresses, which will provide targets and opportunities for manipulation. In the light of this, the present study was designed to investigate and determine the effects of combined salt stress and aphid infestation on the wheat plant responses at the physiological and molecular levels, as well as to investigate potential interactions in terms of crosstalk and cross tolerance. To the best of my knowledge there are no other published studies that have examined this particular set of stress combinations on the wheat transcriptome.

5.1.1 Plant-mediated effects of salinity on aphid performance (cross tolerance)

In the present study, the most important finding from investigating the consequences of plant exposure to salinity on aphid performance (Chapter 3) was that plants treated with salt, irrespective of whether they are salt tolerant (122-1 and 123-5) or not (Drysdale), caused significant reduction in the fecundity of aphids feeding on these plants compared to control untreated plants. This result suggests that salinity has a negative impact on aphid performance i.e. salt pre-treatment enhanced plant resistance to aphid infestation. Since the concentration of Na^+ in the shoots of the three wheat genotypes was different, it is proposed that the effects on aphids is not due to salt *per se*, but is plant-mediated. This positive interaction between abiotic and biotic stress is in accordance with the phenomenon of cross tolerance that allows plants to adapt/acclimate to a range of different stresses after exposure to one specific stress, since in the present study plants were pre-treated with salt prior to aphid infestation (Pastori and Foyer, 2000; Alexieva *et al*., 2003; Shah *et al*., 2012). This phenomenon has been well documented in several previous studies. For instance, salt stress was found to enhance tomato plant responses to wounding mechanically induced locally and systemically through the accumulation of proteinase inhibitors and the activation of other wound-related genes (Dombrowski, 2003).

Also, in barley, the salt-induced osmotic stress response was directly correlated with resistance to powdery mildew (Wiese *et al*., 2004).

Having found that the effects of simultaneous salinity and aphid infestation interact positively at the physiological level, this prompted a further investigation to identify genes and molecular mechanisms controlling this interaction. To this end, further investigations using microarray analysis were carried out on one selected salt tolerant genotype 122-1, using an Affymetrix Wheat Genome Arrays. Based on functional classification analysis, the results indicated that, pre-treatment with salt in both salt stress alone and dual stress caused significant activation of genes that were documented to be associated with cross tolerance. First, there was a significant increase in transcript levels of genes implicated in redox regulation (including thioredoxin) under both salt stress alone and dual stress, but not under aphid stress alone. Similarly, a previous study demonstrated that low-temperature pre-treatment can markedly increase the tolerance of barley seeds to high temperature which was correlated with the increase in ROS scavenging activity (Mei and Song, 2010). Also, ROS scavenging enzymes induced by heat treatment at 33°C was implicated in the cross tolerance of wheat seeds to salt stress (Lei *et al*., 2005). Tolerance to subsequent temperature stress in wheat seeds due to pre-treatment with NaCl solution at -0.8 MPa was also associated with increases in ROS scavenging enzymes activities such as, SOD, APX and CAT (Lei *et al*., 2005). Increases in ROS scavenging enzyme activities appear to be a common component in cross tolerance of seed germination in barley to temperature stress (Mei and Song, 2010). Moreover, redox poise (oxidant and antioxidant) has been shown to play a key role in mediating signalling between biotic and abiotic stress responses (Fujita *et al*., 2006) and in the acquisition of stress tolerance (Sabehat *et al*., 1998; Pastori and Foyer, 2002). Therefore, the significant activation of redox regulation genes detected in the present study may be salt-induced and may indicate the acquisition of cross tolerance to subsequent aphid infestation.

Secondly, the results indicated a significant increase of transcripts implicated in calcium signalling transcripts in plants under both salt stress alone and dual stress. Calcium is a key and universal signal transducer in signalling cascades as the cytosolic Ca^{2+} levels increase in plant cells in response to various harsh environmental conditions, including pathogen challenge, salt stress, osmotic stress, water stress, cold and wounding (Dey *et al*., 2010; Takahashi *et al*., 2011). Furthermore, calcium signalling is a major convergence point of signalling crosstalk between different stress pathways, including

salt stress, and has a major role in mediating cross tolerance (Tippmann *et al*., 2006; Velazquez *et a*l., 2011). Thirdly, the results revealed significant up regulation of genes involved in jasmonate hormone metabolism. This hormone, along with other regulator components like cytokinins and ABA have been shown to increase plant resistance to various unfavourable environmental factors again representing examples of cross adaptation (Alexieva *et al*., 2003). Also, mechanical wounding increases salt-stress tolerance in tomato plants through a mechanism that involves the signalling peptide systemin and the synthesis of jasmonic acid (JA) (Capiati *et al*., 2006). Collectively, it can be suggested that enhanced wheat resistance observed in this study against aphid infestation after exposure to salinity (salt pre-treatment) may be related to the significant up regulation of transcripts involved in ROS scavenging enzymes, jasmonate metabolic and may be mediated by calcium signalling Ca^{2+}. Furthermore, based on individual gene analyses, the results revealed that pre-treatment with salt strongly up regulated MYB domain transcription factors (TFs) in both salt alone and dual stress. Similarly, MYB TFs were specifically induced by the combination of drought and heat stress in *Arabidopsis,* but not by either stress individually (Rizhsky *et al*., 2004). Studies by Vannini *et al*., (2006, 2007) demonstrated that transgenic tomato and *Arabidopsis* plants expressing the rice OsMYB4 showed increased tolerance to abiotic and biotic stresses. MYB transcription factors may function as important mediators of stress responses, which involve complex activities crossing multiple stress signalling pathways (Fujita *et al*., 2006).

5.1.2 Genes with putative functions in crosstalk between salinity and aphid infestation

In the present study, microarray analysis indicated that under dual stress, significant up regulation of genes involved in the following functional categories were detected: signalling (sugar and nutrient physiology, phospinositides inositol-1,3,4-trisphosphate, calcium, G-proteins, MAP kinases), redox regulation and hormones metabolism (ABA, auxin, cytokinin, ethylene and jasmonate). Studies demonstrated that signalling compounds including reactive oxygen species, calcium, abscisic acid ABA, and salicylic acid (SA) are involved in crosstalk between different biotic and abiotic stress signalling pathways (Tippmann *et al*., 2006). The significant activation of genes involved in MAP kinases signalling under dual stress is consistent with a study that demonstrated the accumulation of MPK3 and MPK6 respective mRNAs in *Arabidopsis* upon challenging with biotic (bacterial pathogen) and exposure to abiotic (BTH, SA,

and 4-chloro-SA) stress (Velazquez *et al*., 2011). MAPK is a signalling cascade widely activated in response to abiotic and biotic stresses and has a crucial role in crosstalk between stress signalling pathways such as OsMPK5 kinase (Fujita *et al*., 2006; Velazquez *et al*., 2011). MAPK cascades also mediate ROS signalling (Fujita *et al*., 2006; Atkinson and Urwin, 2012). Collectively, it is proposed that the significant activation of genes associated with redox regulation, calcium, ABA and MAP kinases may mediate cross talk between salinity and aphid infestation responses pathways.

5.1.3 Molecular basis of salt tolerance in the wheat genotype 122-1

Results from the physiological characterization experiment (Chapter 2) revealed that wheat genotype 122-1 was characterized as salt tolerant on the basis of high shoot dry biomass relative to control under salinity (160 mM NaCl). Microarray results of the transcriptional response of this genotype to salt stress alone identified some potential genes and molecular mechanisms that may be involved in regulating the observed salt tolerance. Salt stress alone strongly and specifically activated sets of genes which were documented to be involved mainly in plant defence, including responses to abiotic stress. Among the specific and strongly induced genes under salt stress alone were genes functioning in ABA hormone metabolism. Studies have demonstrated that the hormone absicsic acid increased after drought and salinity stress (Munns and Cramer, 1996) and has been shown to regulate stomatal closure and also increase the production of compatible osmoprotectants and antioxidants (Tippmann *et al*.,2006).The significant and strong activation of antioxidative enzymes in the salt tolerant wheat genotype 122-1 is consistent with a study showing an increase in the accumulation and activity of antioxidant enzymes in salt tolerant species but not in salt-sensitive species, but not in salt-sensitive species (Tippmann *et al*., 2006). The induction of enzymes that detoxify reactive oxygen species (ROS) has been documented in plants grown under drought and salinity and shown to play an essential role in plant adaptation to salinity stress (Munns, 2005). Other important activities of the oxidative defence system are limiting photo-oxidative damage; protecting metabolic function in cells and preventing premature senescence (Foyer *et al*., 1994 in Munns *et al*, 2006). Previous studies provide evidence and suggested that the expression of antioxidant compounds could be used as potential selection criteria for breeding for salt tolerance in different crops (Ashraf, 2009).

The present study identified two genes encoding late embryogenesis abundant protein (LEA) and proline synthesis that play a role in the salt acclimation process. The

induction of late embryogenesis abundant protein (LEA) has been shown to confer either tolerance to dehydration or recovery on subsequent hydration (Munns, 2005). Proline is an osmoprotectant solute that accumulates under drought and salinity and shows a protective role against induced-osmotic effect (Munns, 2005). High proline accumulation enhances turgor maintenance, which in turn improves the rate of elongation of new leaves and roots (Munns *et al*, 2006). Over production of many of these solutes (proline) through gene transformation has increased growth of plants in saline-soil (Munns *et al*, 2006). Other genes highly expressed during salt acclimation were related to cold stress responsive genes and cytochrome P450. The up regulation of cold stress related genes observed in this study is in agreement with a study that demonstrated significant proportions of genes induced by salt stress were also induced by cold and dehydration stress (Munns, 2005). The significant activation of cytochrome P450 genes detected in this study under dual stress is in agreement with a study showing that cytochrome P450 in *Arabidopsis* was induced by various biotic and abiotic stresses, including salinity (Velazquez *et al*., 2011).

5.1.4 Molecular basis of wheat genotype 122-1 to aphid infestation

Physiological characterization of the response of wheat genotype 122-1 to aphid infestation in the absence of salt did not show any significant effects compared to controls. This might be due to the low number of aphids used in the study. However, this genotype showed the lowest reduction in shoot height, shoot and root dry weight under aphid infestation, compared to the two other genotypes tested. Results obtained from microarray analysis of wheat responses to aphid infestation alone showed that the most significantly altered genes by aphid infestation were those involved in photosynthesis, antioxidant and redox regulation, hormone metabolism, cell wall, cell functioning, secondary metabolism and transport functional categories. Whilst specific stress response genes were not significantly defferentially expressed as part of the early response (6 h), they were as part of the later response (24 h). The majority of the most significantly and strongly induced genes were unknown/not assigned. Similarly, results from a proteomic analysis of wheat responses to infestation by the aphid *Sitobion. avenae* conducted by Ferry *et al* (2011) from the Gatehouse group revealed that the majority of proteins altered by aphid infestation were involved in metabolic processes and photosynthesis. Other proteins identified were involved in signal transduction, stress and defence, antioxidant activity, regulatory processes, and hormone responses. These authors concluded that responses to aphid attack in wheat at the proteome level

were broadly similar to basal non-specific defence and stress responses. Therefore, it can be suggested that transcriptome analysis of wheat also showed basal non-specific defence and stress responses to aphid infestation, and are thus in agreement with Ferry *et al*. (2011).

5.2 Conclusions

This study demonstrated that at the physiological level, pre-treatment with salinity (160 mM NaCl) negatively affected aphid performance through a significant reduction in fecundity compared to those feeding on control palnts. Therefore, the imposition of salt enhanced wheat plant resistance to subsequent aphid infestation, indicating cross tolerance. Further investigation at the transcriptional level revealed that plant adaptation/acclimation to salinity due to pre-treatment with salt applied in salt stress alone and in dual stress prior to aphid infestation, was associated with significant increases in transcript levels of genes involved in defence, stress responses and detoxifying activity. Therefore, it is proposed that these genes may have a role in the acquisition of cross tolerance. For example, the increase in aphid tolerance caused by salt pre-treatment may be a result of increasing ROS scavenging enzyme activity. Data also suggest that MYB domain TFs activity may be necessary for the down-stream signalling events that lead to cross tolerance. Finally, data from the current study suggests that calcium signalling is likely to participate in the crosstalk that confers cross tolerance mechanism, by coordinating/interrelating responses to salt and aphid infestation.

This study represents the first investigation of the interaction between salt and aphid *S. avenae* infestation on the wheat at both physiological and molecular levels. The work provides new insights on genes and pathways unique and in common in the wheat response to salt and aphid applied in combination and individually. The work also sheds light on genes and pathways potentially involved in the cross talk and those that confers cross tolerance. These putative genes may be potential targets to develop crops with broad-spectrum tolerance to stress.

5.3 Future prospects

Since changes at the transcriptional level may not correlate with protein/enzyme activity levels, further investigations are necessary to confirm the identity of stress related genes and provide a comprehensive description of their functions. This may be achieved by:

- Employing a proteomic approach to identify potential proteins associated with wheat plant responses to combined salt and aphid stress.
- Integrating genetic approaches to confirm the function of some candidate genes with potential roles in controlling the observed stress interaction, such as the analysis of overexpression lines, loss-of-function mutants and gene silencing via RNA interferance.
- Further validation of microarray data using other reference genes such as ubiquitin and tubulin in addition to actin that was used in the present study.
- Further study the interactions i.e, "cross talk" between different hormone signally pathways using both wheat and *Arabidopsis* mutants

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Appendix I Supporting information

Table S2.1 Effect of salinity on Ion concentration in shoot (Na, K and K/Na ratio mg/kg^{-1} DW) of plants exposed to 160 mM NaCl treatment for 21 d. Values are means (n=5).

Genotype	Ion concentration (mg/kg $^{-1}$ DW)			Salt tolerance ST (%)	Tolerance degree
	Na^+	K^+	K/Na^+		
123-5	293.3	345.3	1.18	48	Tolerant
122-1	342.2	310.9	1	43	Tolerant
Kharchia	540.8	293.0	0.55	41	Tolerant
118-1	557.4	339.1	0.7	38	Moderate
Yecora rojo	269.8	232.4	0.87	33	Moderate
116-2	373.1	297.4	0.91	30	Moderate
Krichauff	561.4	309.6	0.59	30	Moderate
Yitpi	395.7	293.2	0.77	23	Sensitive
Drysdale	416.9	272.4	0.67	21	Sensitive
Main effect					
Genotype	*P*=0.000	*P*=0.000	*P*=0.006		
Treatment/salt	*P*=0.000	*P*=0.000	*P*=0.000		
Genotype × salt	*P*=0.000	*P*=0.000	*P*=0.007		

*Genotypes were arranged in descending order of salinity tolerance based on shoot dry matter at the vegetative stage.

Table S2.2 Differences in K content in shoot (mg/kg^{-1} DW) of wheat genotypes exposed to 160 mM NaCl and control conditions for 21 d. Values are means (n=5)

Genotypes	K^+ concentration		
	Salt	Control	Differences
123-5	345.33	484.9618	-139.63
122-1	310.91	330.6108	-19.70
118-1	339.12	487.7127	-148.60
Kharchia	293.04	479.2969	-186.25
116-2	297.37	461.7632	-164.40
Yecora rojo	232.35	525.1145	-292.77
Krichauff	309.64	638.4201	-328.78
Yitpi	293.22	707.6068	-414.39
Drysdale	272.41	525.4139	-253.00

Table S2.3 Scores among wheat genotypes for their relative salt tolerance on plant growth parameters, chlorophyll content (SPAD units) and biomass parameters at 21 days after exposing to 160 mM NaCl.

Genotypes	Growth parameters				Chlorophyll content	Biomass parameters		ST%
	Shoot* height	Leaf area	Number of leaves	Number of tillers	SPAD	Root dry weight	Root/shoot ratio	
123-5	3	2	3	1	3	1	1	1
122-1	1	3	1	3	2	3	4	1
Kharchia	1	5	4	1	4	4	5	1
Sharawaki	3	1	5	2	1	3	3	2
118-1	2	5	3	1	3	4	5	2
PBW	5	5	1	2	4	3	3	3
Pasban	5	4	4	2	5	3	2	3
Yecora rojo	5	1	3	5	2	4	4	3
116-2	3	5	3	1	3	3	2	4
Krichauff	2	3	3	2	5	4	4	4
Chinese spring	5	5	4	3	3	4	3	4
Claire	3	1	5	5	4	4	2	5
Yitpi	5	4	5	3	5	4	3	5
Drysdale	4	4	4	5	3	4	1	5

*Scores were assigned form the highest to the lowest for all above measurments.

Table S2.4 Scores among wheat genotypes for their potential salt tolerance on water relations parameters at 21 d after exposing plants to 160 mM NaCl.

Genotypes	Water relations			ST%
	RWC*	Leaf sap osmolality	osmotic potential	
123-5	4	3	3	1
122-1	4	2	2	1
Sharawaki	5	2	2	2
118-1	4	3	3	2
PBW	4	4	5	3
Pasban	3	1	1	3
Yecora rojo	1	1	2	3
116-2	5	2	2	4
Krichauff	4	3	3	4
Chinese spring	4	3	3	4
Claire	4	2	2	5
Yitpi	4	4	4	5
Drysdale	4	3	3	5

*Scores were assigned from the highest to the lowest (1 to 5) in RWC ans ST% while scores were assigned from the lowest to the highest for leaf sap osmolality and osmotic potential.

Table S2.5 Scores among wheat genotypes for their relative salt tolerance on Ion contents at 21 days after exposing plants to 160 mM NaCl.

Genotypes	Ion content in shoot*			ST%
	Na^+	K^+	K^+/Na^+ ratio	
123-5	1	2	1	1
122-1	1	1	1	1
Kharchia	1	3	3	1
118-1	5	2	3	2
Yecora rojo	4	4	3	3
116-2	2	3	3	3
Krichauff	4	4	3	3
Yitpi	4	5	3	4
Drysdale	1	4	3	4

*Scores were assigned form the highest to the lowest in K and K/Na ratio while scores were assigned from the lowest to the highest for Na.

Table S2.6 The correlation coefficients between salt tolerance index (ST) and various parameters measured for screening wheat genotypes for potential salt tolerance.

	Salt tolerance (ST%)	
Parameters	n	*r*
Growth and physiological parameters		
Root-DR	14	0.76***
Shoot-DW	14	0.85***
root/shoot-DW	14	0.55*
shoot height	14	0.76***
No of leaves	14	0.53*
No of tillers	14	0.30
Leaf area	14	0.81***
Chlorophyll content	14	0.42
Water relations parameters		
RWC	13	0.24
Osmometer reading	13	0.08
Osmotic potential v1	13	-0.08
Osmotic potential v2	13	- 0.08
OP (100) v1	13	-0.17
OP (100) v2	13	- 0.17
Ion content parameters		
Na^+ (g/kg)shoot	9	-0.22
K^+ (g/kg)shoot	9	0.53
K^+/Na^+ (g/kg)shoot	9	0.61*
Na^+/K^+ discrimination	9	- 0.51

Asterisks indicate the probability value associated to the coefficient: *$P < 0.05$; **$P < 0.01$; ***$P < 0.00$

Table S2.7 Correlation coefficients of growth and physiological parameters measured for screening wheat genotypes for salt tolerance n=14.

	ST%	*Root-DR*	*Shoot-DW*	*root/shoot-DW*	*shoot height*	*No of leaves*	*No of tillers*	*Leaf area*	*Chlorophyll content*
ST%	1.00								
Root-DR	0.76	1.00							
Shoot-DW	0.85	0.89	1.00						
root/shoot-DW	0.55	0.87	0.58	1.00					
shoot height	0.76	0.82	0.82	0.65	1.00				
No of leaves	0.53	0.30	0.46	0.10	0.22	1.00			
No of tillers	0.30	0.05	0.27	-0.14	0.27	-0.15	1.00		
Leaf area	0.81	0.89	0.87	0.75	0.94	0.32	0.12	1.00	
Chlorophyllcontent	0.42	0.11	0.24	0.02	0.37	- 0.04	0.02	0.33	1.00

Asterisks indicate the probability value associated to the coefficient: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

Table S2.8 Correlation coefficients of leaf water relation parameters measured for screening wheat genotypes for salt tolerance n=13.

	ST%	*RWC%*	*Osmometer reading*	*Osmotic potential v1*	*Osmotic potential v2*	*OP (100) v1*	*OP (100) v2*
ST%	1.00						
RWC	0.24	1.00					
Osmometer reading	0.08	- 0.43	1.00				
Osmotic potential v1	- 0.08	0.43	-1.00	1.00			
Osmotic potential v2	- 0.08	0.43	-1.00	1.00	1.00		
OP (100) v1	-0.17	0.29	- 0.98	0.98	0.98	1.00	
OP (100) v2	-0.17	0.29	-0.98	0.98	0.98	1.00	1.00

Table S2.9 Correlation coefficients of ion content parameters measured for screening wheat genotypes for salt tolerance n=9.

	ST%	*Na^+ (g/kg)shoot*	*K^+ (g/kg)shoot*	*K^+/Na^+ (g/kg)shoot*	*Na^+/K^+ discrimination*
ST%	1.00				
Na^+ (g/kg)shoot	-0.22	1.00			
K^+ (g/kg)shoot	0.53	0.32	1.00		
K^+/Na^+ (g/kg)shoot	0.61*	-0.83**	0.24	1.00	
Na^+/K^+ discrimination	-0.51	0.85***	-0.19	- 0.96***	1.00

Asterisks indicate the probability value associated to the coefficient: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$

Appendix II Supporting information

Table S3.1. Predicted number of total nymphs produced by *S. avenae* on three wheat genotypes using regression function in the presence and absence of salt (NaCl) treatment.

	Predicted number of total nymphs					
	Control treatment (no salt)			Salinity treatment		
Days	123-5	122-1	Drysdale	123-5	122-1	Drysdale
25	11.65	10.24	30.78	-0.25	-4.41	-2.09
30	3.54	1.14	24.94	-8.95	-15.45	-14.81
35	-4.58	-7.95	19.09	-17.64	-26.48	-27.53
40	-12.70	-17.05	13.25	-26.34	-37.51	-40.25
mean	-0.52	-3.40	22.01	-13.29	-20.96	-21.17

Predictions were based on the regression equation according to (Goa *et al*., 2012)

Table S3.2 Tolerance index measured as relative biomass production, which was calculated as the percentage of shoot dry weight under stress relative to control in three wheat genotypes 123-5, 122-1 and Drysdale under two stress treatments aphid infestation and dual stress (salt+aphid).

Genotype	Shoot Dry Weight (g)			Tolerance index (%)	
	Control	Aphid	Salt+Aphid	Aphid	Salt+Aphid
123-5	2.89	2.61	0.85	90	29
122-1	2.85	2.74	1.14	96	40
Drysdale	2.58	2.12	0.82	82	32

Table S3.3 Correlation between aphid *S. avenae* performance in terms of total number of nymphs/plant of three wheat genotypes and plant performance in terms of growth parameters measured under control and salinity conditions.

Plant performance	Aphid performance			
	Control plants		Salt-treated plants	
	Correlation coefficient	*P* value	Correlation coefficient	*P* value
Shoot height	-0.570	$P<0.05$	-0.039	ns
Tillers	0.318	ns	-0.092	ns
Chlorophyll content	-0.099	ns	0.186	ns
Dry root weight	-0.244	ns	0.088	ns
Dry shoot weight	-0.517	$P<0.05$	-0.238	ns

Ns; not significant at $P<0.05$

Appendix III Supporting information

Table S4.1 Significantly altered functional categories according to change in gene expression level in wheat plants under three stress treatments compared to control plants at two time points 6 h and 24 h. The results show bins codes, names and corresponding p-value (as calculated by MapMan Wilcoxon ran sum test) according to the MapMan gene ontology. Blue colour indicates significant up-regulation process whereas pink colour indicates significant down-regulation process.

Bin code	Bin name	Salt 6h	Dual 6h	Aphid 6h	Salt 24h	SA 24h	Aphid 24h
1	PS	0.196	0.207	<1E-20	8.39E-12	3.17E-09	1.79E-25
2	major CHO metabolism	0.191	0.086	0.133	0.044	1.27E-04	0.407
3	minor CHO metabolism	0.711	0.238	0.214	0.003	0.005	0.347
4	glycolysis	0.104	0.406	0.916	0.388	0.016	0.577
5	fermentation	0.960	0.585	0.992	0.987	0.705	0.998
6	gluconeogenese	0.014	0.008	0.003	0.667	0.018	0.633
7	OPP	0.077	0.117	0.758	0.064	0.368	0.858
8	TCA / org. transformation	0.056	0.009	0.441	0.163	0.106	0.037
9	mitochondrial electron transport	0.665	0.010	0.313	0.154	0.291	0.928
10	cell wall	0.015	0.031	1.21E-04	0.356	1.09E-04	0.146
11	lipid metabolism	0.334	0.583	0.898	3.32E-06	2.81E-13	0.412
12	N-metabolism	0.008	0.007	0.440	0.303	0.389	0.669
13	amino acid metabolism	0.599	0.867	0.977	5.54E-04	3.26E-04	0.157
14	S-assimilation	0.007	0.278	0.366	0.151	0.790	2.37E-04
16	metal handling	0.927	0.015	4.85E-07	4.94E-08	0.107	0.505
16	secondary metabolism	0.477	4.24E-12	3.67E-11	0.992	0.027	0.121
17	hormone metabolism	0.002	2.67E-12	4.94E-06	0.155	3.30E-08	0.026
18	Co-factor and vitamine metabolism	1.02E-04	0.179	0.072	0.379	0.821	1.45E-04
19	tetrapyrrole synthesis	8.96E-07	0.636	6.26E-06	0.802	0.066	3.85E-04
20	stress	0.712	0.038	0.101	0.021	8.63E-08	6.60E-04
21	redox.regulation	0.548	0.960	0.155	0.043	2.48E-04	4.89E-05
22	polyamine metabolism	0.002	0.666	0.482	0.003	0.038	0.566
23	nucleotide metabolism	7.46E-04	2.23E-07	2.96E-08	0.011	0.715	0.113

24	Biodegradation of Xenobiotics	6.66E-04	8.32E-04	0.001	0.305	0.291	0.155
25	C1-metabolism	0.151	0.967	0.798	0.676	0.470	0.841
26	misc	0.343	<1E-20	<1E-20	3.07E-05	4.09E-04	0.437
27	RNA	8.15E-10	1.50E-73	1.37E-51	<1E-20	5.77E-15	<1E-20
28	DNA	2.64E-05	2.51E-91	1.05E-79	<1E-20	<1E-20	<1E-20
29	protein	3.16E-32	7.01E-96	5.07E-40	<1E-20	<1E-20	<1E-20
30	signalling	0.141	3.70E-18	6.29E-07	0.397	0.011	0.150
31	cell	0.487	2.43E-29	6.31E-25	<1E-20	8.43E-13	3.98E-12
33	development	0.033	0.467	0.180	5.14E-05	0.001	0.001
34	transport	0.584	2.09E-08	8.47E-07	0.140	4.14E-08	8.69E-07
35	not assigned	<1E-20	<1E-20	<1E-20	7.81E-55	2.46E-122	1.36E-26

Wilcoxon's P value gives the probability of whether the average value of each indicator among genes in a functional group is significantly higher (blue) or lower (pink) tha the average values of all other genes in the whole genome set.

Table S4.2 Commonly up-regulated genes under all stress treatments at 6 h (threshold: 0.5 fold change)

BinCode	BinName	id	description	6h Salt	6h Dual	6h Aphid
20.1	stress.biotic	taaffx.128595.1.s1_at	pathogenesis-related protein 4 precursor (PR4)	**0.59**	**0.75**	**0.52**
26.10	misc.cytochrome P450	taaffx.50125.2.s1_at	CYP71B38 (cytochrome P450, family 71, subfamily B, polypeptide 38); oxygen binding	**0.52**	**0.59**	**0.53**

Table S4.3 Commonly up-regulated genes under all stress treatments at 24 h (threshold: 0.5 fold change)

BinCode	BinName	id	description	24h Salt	24h Dual	24h Aphid
13.1.5.2.41	amino acid metabolism.synthesis.serine-glycine-cysteine group.glycine.sarcosine oxidase	ta.7402.1.s1_at	Sarcosine oxidase family protein	**0.64** **0.019**	**0.50** **0.07**	**0.80** **0.005**
17.6.3	hormone metabolism.gibberelin.induced-regulated-responsive-activated	ta.11162.1.s1_at	GASA-like protein	**0.67** **0.005**	**0.69** **0.004**	**0.77** **0.002**
28.1	DNA.synthesis/chromatin structure	ta.3093.1.s1_a_at	replication protein, putative	**0.52**	**0.50**	**1.83**
28.1.3	DNA.synthesis/chromatin structure.histone	ta.10329.17.s1_x_at	histone H4	**0.75**	**0.73**	**2.10**
28.1.3	DNA.synthesis/chromatin structure.histone	ta.10329.9.s1_a_at	histone H4	**0.51**	**0.55**	**1.97**
28.2	DNA.repair	ta.3093.1.s1_a_at	replication protein, putative	**0.52**	**0.50**	**1.83**
33.99	development.unspecified	taaffx.33265.1.s1_at	nodulin MtN3 family protein	**0.78**	**0.89**	**0.70**

Table S4.4 Commonly up-regulated transcripts between salt stress and dual stress at 6 h (threshold: 0.5 fold change)

BinCode	BinName	id	description	6h		
				Salt	Dual	Aphid
10.6	cell wall.degradation	taaffx.44342.1.s1_at	peptidoglycan-binding LysM domain-containing protein	**0.71**	**0.66**	-0.05
11.8.2	lipid metabolism."exotics" (steroids, squalene etc).methylsterol monooxygenase	ta.13232.1.s1_at	sterol c4-methyl oxidase (SMO1-2); catalytic	**0.63**	**0.76**	0.02
13.1.2.2	amino acid metabolism.synthesis.glutamate family.proline	ta.7091.1.s1_at	P5CS2 (delta 1-pyrroline-5-carboxylate synthase 2); catalytic/ glutamate 5-kinase/ oxidoreductase	**1.23**	**0.95**	-0.39
17.5.1	hormone metabolism.ethylene.synthesis-degradation	ta.9107.1.s1_x_at	ethylene forming enzyme (EFE)	**0.58**	**0.54**	-0.14
17.5.1	hormone metabolism.ethylene.synthesis-degradation	ta.9107.2.s1_at	ethylene forming enzyme (EFE)	**0.50**	**0.54**	0.12
17.5.1	hormone metabolism.ethylene.synthesis-degradation	ta.9107.2.s1_a_at	ethylene forming enzyme (EFE)	**0.63**	**0.53**	0.13
20.2.1	stress.abiotic.heat	taaffx.87145.1.s1_at	DNAJ heat shock N-terminal domain-containing protein	**0.59**	**1.16**	-0.14
20.1	stress.biotic	ta.13785.1.s1_at	acidic endochitinase (CHIB1)	**0.65**	**0.55**	0.12
26.1	misc.cytochrome P450	taaffx.54157.2.s1_at	CYP71B30P (cytochrome P450, family 71, subfamily B, polypeptide 30 pseudogene); oxygen binding	**0.61**	**0.71**	0.31
27.3.25	RNA.regulation of transcription.MYB domain	ta.26049.1.s1_a_at	MYB4 (myb domain protein 4); transcription factor gb|CD454952	**1.58**	**0.76**	-0.05

29.4	transcription factor family protein.postranslational modification	ta.9516.2.s1_at	Protein phosphatase 2C, putative / PP2C, putative	**0.66**	**0.71**	-0.09
33.2	development.late embryogenesis abundant	ta.25026.1.s1_at	LEA14 (late embryogenesis abundant 14)	**0.59**	**0.72**	0.32
33.99	development.unspecified	taaffx.33265.1.s1_at	nodulin MtN3 family protein	**0.54**	**0.76**	0.08
34.99	transport.misc	taaffx.119486.1.s1_at	transporter-related	**0.64**	**0.79**	0.05
34.99	transport.misc	ta.4508.3.s1_a_at	transporter-related	**0.69**	**0.70**	0.07
34.99	transport.misc	ta.4508.3.s1_at	transporter-related	**0.75**	**0.72**	0.08
34.99	transport.misc	ta.4508.1.s1_a_at	transporter-related	**0.84**	**0.72**	0.11

Table S4.5 Commonly up-regulated transcripts between aphid infestation and dual stress at 6 h (threshold: 0.5 fold change)

BinCode	BinName	id	description	6h		
				Salt	Dual	Aphid
1.1.2.2	PS.lightreaction.photosystem I.PSI polypeptide subunits	taaffx.26397.1.s1_at	similar to (119)PSAN_HORVU gb|CA689283;	0.12	**0.50**	**0.63**
1.1.6	PS.lightreaction.NADH DH	taaffx.80571.1.s1_s_at	similar to (138)ATCG00430| Symbols: PSBG | Encodes a protein which was originally thought to be part of photosystem II but its wheat homolog was later shown to encode for subunit K of NADH dehydrogenase. similar to (172)NDHK_WHEAT gb|CA695140;	0.06	**0.53**	**0.72**
1.3.1	PS.calvin cyle.rubisco large subunit	taaffx.128414.24.a1_s_at	similar to (116)ATCG00490| Symbols: RBCL | large subunit of RUBISCO. similar to (115)RBL_SPIOL gb|CA722241;	-0.04	**0.90**	**0.70**
9.7	mitochondrial electron transport / ATP synthesis.cytochrome c oxidase	taaffx.1074.1.s1_at	similar to (94.7)ATMG01360| Symbols: COX1 | cytochrome c oxidase subunit 1. similar to (96.7)COX1_WHEAT gb|CK212638;	0.28	**0.99**	**0.74**
11.9.2.1	lipid metabolism.lipid degradation.lipases.triacylglycerol lipase	ta.17752.1.s1_at	similar to (84.3)AT4G18550| Symbols: | lipase class 3 family protein. gb|CA624076;	0.20	**0.73**	**0.58**
13.1.3.1	amino acid metabolism.synthesis.aspartate family.asparagine	ta.5645.2.s1_x_at	similar to (153)AT5G10240| Symbols: ASN3 | ASN3 (ASPARAGINE SYNTHETASE 3); asparagine synthase (glutamine-hydrolyzing) similar to (156)ASNS_ASPOF gb|CA707112;	0.12	**0.55**	**0.53**
13.1.3.1	amino acid metabolism.synthesis.aspartate family.asparagine	ta.5645.1.s1_x_at	similar to (485)AT5G65010| Symbols: ASN2 | ASN2 (ASPARAGINE SYNTHETASE 2); asparagine synthase (glutamine-hydrolyzing) similar to (516)ASNS_MAIZE gb|CA609920;	0.14	**0.51**	**0.51**
16.1.5	secondary metabolism.isoprenoids.terpenoids	taaffx.38062.1.a1_at	similar to (82.0)AT5G23960| Symbols: | terpene synthase/cyclase family protein | chr5:8092972-8095131 FORWARD gb|BJ252458;	0.00	**0.51**	**0.51**

16.2.1.10	secondary metabolism.phenylpropanoids.lignin biosynthesis.CAD	ta.6747.1.s1_at	similar to (436)AT4G37990| Symbols: ELI3, ELI3-2 | ELI3-2 (ELICITOR-ACTIVATED GENE 3). similar to (457)MTDH_MESCR gb|CA730395;	-0.22	**0.67**	**0.60**
17.1.1.1.10	hormone metabolism.abscisic acid.synthesis-degradation.synthesis.9-cis-epoxycarotenoid dioxygenase	ta.12813.1.s1_x_at	similar to (436)AT4G19170| Symbols: NCED4 | NCED4 (NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 4) | chr4:10481846-10483633 FORWARD gb|BQ172152;	0.35	**0.70**	**0.64**
17.5.1	hormone metabolism.ethylene.synthesis-degradation	ta.14087.1.s1_at	similar to (342)AT3G19000| Symbols: | oxidoreductase, 2OG-Fe(II) oxygenase family protein | chr3:6554010-6554993 REVERSEweakly similar to (154)FL3H_VITVI gb|BE446498;	-0.36	**0.78**	**0.58**
17.7.1.2	hormone metabolism.jasmonate.synthesis-degradation.lipoxygenase	taaffx.104812.1.s1_s_at	similar to (458)AT3G45140| Symbols: ATLOX2, LOX2 | LOX2 (LIPOXYGENASE 2) | chr3:16536422-16540218 FORWARDhighly similar to (820)LOX21_HORVU gb|BJ223744;	0.29	**1.06**	**0.79**
17.7.1.2	hormone metabolism.jasmonate.synthesis-degradation.lipoxygenase	ta.23763.1.s1_at	similar to (637)AT3G22400| Symbols: LOX5 | LOX5; lipoxygenase | chr3:7927018-7931174 FORWARDhighly similar to (757)LOX1_ORYSA gb|CK213159;	0.30	**1.15**	**0.56**
17.7.1.2	hormone metabolism.jasmonate.synthesis-degradation.lipoxygenase	ta.1967.1.s1_x_at	similar to (108)AT3G45140| Symbols: ATLOX2, LOX2 | LOX2 (LIPOXYGENASE 2) | chr3:16536422-16540218 FORWARDmoderately similar to (218)LOX21_HORVU gb|CK152466;	0.36	**0.96**	**0.69**
17.7.1.2	hormone metabolism.jasmonate.synthesis-degradation.lipoxygenase	ta.1967.2.a1_x_at	similar to (134)LOX21_HORVU gb|AJ614579;	0.39	**1.11**	**0.74**
17.7.1.2	hormone metabolism.jasmonate.synthesis-	ta.13650.1.a1_at	similar to (169)AT3G22400| Symbols: LOX5 | LOX5; lipoxygenase | chr3:7927018-7931174	0.43	**1.03**	**0.66**

	degradation.lipoxygenase		FORWARDweakly similar to (177)LOX3_ORYSA gb|CK211830;			
17.7.1.5	hormone metabolism.jasmonate.synthesis-degradation.12-Oxo-PDA-reductase	ta.1207.1.s1_at	similar to (517)AT1G76680| Symbols: OPR1 | OPR1 (12-oxophytodienoate reductase 1); 12-oxophytodienoate reductase | chr1:28781876-28783165 FORWARD gb|CA650490;	-0.07	**0.64**	**0.59**
26.23	misc.rhodanese	taaffx.12816.1.a1_at	similar to (90.9)AT2G17850| Symbols: | similar to unknown protein [Arabidopsis thaliana] (TAIR:AT5G66170.2); similar to unnamed protein product [Vitis vinifera] (GB:CAO48196.1); contains InterPro domain Rhodanese-like (InterPro:IPR001763) | chr2:7767087-7767869 REVERSE gb|BQ168997;	0.44	**0.55**	**0.58**
26.8	misc.nitrilases, *nitrile lyases, berberine bridge enzymes, reticuline oxidases, troponine reductases	taaffx.123816.2.s1_at	similar to (137)AT5G06060| Symbols: | tropinone reductase, putative / tropine dehydrogenase, putative | chr5:1824067-1825834 REVERSE gb|CA677017;	0.06	**0.58**	**0.64**
29.2.1.1.1.1.16	protein.synthesis.ribosomal protein.prokaryotic.chloroplast.30S subunit.S16	taaffx.4142.2.s1_at	similar to (84.3)RR16_WHEAT gb|CA672269;	-0.16	**0.50**	**0.60**
29.2.1.2.1.27	protein.synthesis.ribosomal protein.eukaryotic.40S subunit.S27	ta.13076.1.s1_at	similar to (132)AT5G47930| Symbols: | 40S ribosomal protein S27 (RPS27D) | chr5:19423649-19424555 REVERSEweakly similar to (130)RS27_HORVU gb|CA486547;	-0.18	**0.77**	**0.96**
29.2.1.2.2.10	protein.synthesis.ribosomal protein.eukaryotic.60S subunit.L10	ta.26752.1.a1_at	similar to (251)AT1G66580| Symbols: | 60S ribosomal protein L10 (RPL10C) | chr1:24842871-24844102 FORWARDmoderately similar to (247)RL10_MAIZE gb|CD491059;	0.04	**0.68**	**0.69**
33.99	development.unspecified	ta.26199.3.s1_a_at	similar to (81.3)AT3G52190| Symbols: PHF1	0.02	**0.50**	**0.68**

Table S4.6 Commonly up-regulated transcripts between salt stress and dual stress at 24 h (threshold: 0.5 fold change)

BinCode	BinName	id	description	24h		
				Salt	Dual	Aphid
10.6	cell wall.degradation	taaffx.44342.1.s1_at	similar to (94.4)AT5G62150| Symbols: | peptidoglycan-binding LysM domain-containing protein | chr5:24975551-24975859 FORWARD gb|CK212299;	**0.73**	**0.90**	0.08
11.9.2	lipid metabolism.lipid degradation.lipases	ta.601.1.a1_at	similar to (80.5)AT2G31100| Symbols: | lipase, putative | chr2:13263815-13265251 REVERSE gb|BE489046;	**0.67**	**0.83**	0.27
11.9.2.1	lipid metabolism.lipid degradation.lipases.triacylglycerol lipase	ta.9430.1.s1_at	similar to (171)AT1G06800| Symbols: | lipase class 3 family protein | chr1:2090108-2091442 REVERSEVAR1 gb|AJ614438;	**0.68**	**0.56**	-0.07
13.1.2.2	amino acid metabolism.synthesis.glutamate family.proline	ta.7091.1.s1_at	similar to (460)AT3G55610| Symbols: P5CS2 | P5CS2 (DELTA 1-PYRROLINE-5-CARBOXYLATE SYNTHASE 2); catalytic/ glutamate 5-kinase/ oxidoreductase | chr3:20635984-20639968 REVERSEhighly similar to (510)P5CS_ORYSA VAR1 COG5048 COG4886 COG5099 PRK08581 MopB_Res-Cmplx1_Nad11-M gb|CK194302;	**1.32**	**1.22**	0.10
15.2	metal handling.binding, chelation and storage	ta.618.1.s1_at	similar to (107)AT4G27590| Symbols: | copper-binding protein-related | chr4:13771230-13771796 FORWARD gb|BE490267;	**0.59**	**0.53**	0.21
16.2	secondary metabolism.phenylpropanoids	ta.9717.1.a1_a_at	similar to (91.3)AT4G35160| Symbols: | O-methyltransferase family 2 protein | chr4:16730994-16732813 REVERSEmoderately similar to (204)ZRP4_MAIZEVAR1 COG5048 gb|CK154440;	**0.80**	**1.33**	-0.17
17.1.3	hormone metabolism.abscisic acid.induced-regulated-responsive-activated	ta.16038.1.s1_at	similar to (150)AT5G50720| Symbols: ATHVA22E | ATHVA22E (Arabidopsis thaliana HVA22 homologue E) | chr5:20650668-20651728 REVERSEweakly similar to (155)HVA22_HORVU gb|CK215676;	**0.69**	**0.53**	-0.11

17.5.1	hormone metabolism.ethylene.synthesis-degradation	ta.9107.2.s1_a_at	similar to (84.3)AT1G05010| Symbols: ACO4, EAT1, EFE | EFE (ETHYLENE FORMING ENZYME) | chr1:1431418-1432694 REVERSEweakly similar to (111)ACCO1_ORYSA gb|BJ307565;	**0.79**	**0.58**	0.19
17.5.1	hormone metabolism.ethylene.synthesis-degradation	ta.9107.1.s1_x_at	similar to (253)AT1G05010| Symbols: ACO4, EAT1, EFE | EFE (ETHYLENE FORMING ENZYME) | chr1:1431418-1432694 REVERSEmoderately similar to (336)ACCO1_ORYSA gb|CK216168;	**0.78**	**0.55**	0.23
17.7.1.2	hormone metabolism.jasmonate.synthesis-degradation.lipoxygenase	ta.7830.1.s1_at	similar to (108)AT3G22400| Symbols: LOX5 | LOX5; lipoxygenase | chr3:7927018-7931174 FORWARDweakly similar to (117)LOXA_PHAVU gb|AJ613758;	**0.55**	**0.67**	-0.25
17.7.1.2	hormone metabolism.jasmonate.synthesis-degradation.lipoxygenase	taaffx.104812.1.s1_s_at	similar to (458)AT3G45140| Symbols: ATLOX2, LOX2 | LOX2 (LIPOXYGENASE 2) | chr3:16536422-16540218 FORWARDhighly similar to (820)LOX21_HORVU gb|BJ223744;	**0.79**	**0.77**	-0.04
17.7.1.2	hormone metabolism.jasmonate.synthesis-degradation.lipoxygenase	ta.1967.1.s1_x_at	similar to (108)AT3G45140| Symbols: ATLOX2, LOX2 | LOX2 (LIPOXYGENASE 2) | chr3:16536422-16540218 FORWARDmoderately similar to (218)LOX21_HORVU gb|CK152466;	**0.81**	**0.79**	0.02
17.7.1.2	hormone metabolism.jasmonate.synthesis-degradation.lipoxygenase	ta.13650.1.a1_at	similar to (169)AT3G22400| Symbols: LOX5 | LOX5; lipoxygenase | chr3:7927018-7931174 FORWARDweakly similar to (177)LOX3_ORYSA gb|CK211830;	**0.59**	**0.53**	0.07
17.7.1.2	hormone metabolism.jasmonate.synthesis-degradation.lipoxygenase	ta.1967.2.a1_x_at	similar to (134)LOX21_HORVU gb|AJ614579;	**0.76**	**0.71**	0.07
20.1	stress.biotic	ta.30739.2.s1_at	similar to (127)AT5G57625| Symbols: | allergen V5/Tpx-1-related family protein | chr5:23355091-23355803 FORWARDweakly similar to (135)PR13_HORVU gb|CA692019;	**0.52**	**0.56**	0.42
26.1	misc.cytochrome P450	ta.4306.1.s1_at	similar to (184)AT2G30770| Symbols: CYP71A13 | CYP71A13 (CYTOCHROME P450, FAMILY 71,	**0.70**	**0.75**	0.44

			SUBFAMILY A, POLYPEPTIDE 13); indoleacetaldoxime dehydratase/ oxygen binding | chr2:13116986-13119083 REVERSEmoderately similar to (288)C71C4_MAIZE gb|CA684557;			
26.19	misc.plastocyanin-like	ta.20591.2.s1_a_at	similar to (105)AT2G02850| Symbols: ARPN | ARPN (PLANTACYANIN); copper ion binding | chr2:826629-827719 REVERSEweakly similar to (132)BABL_LILLO gb|BE499625;	**1.57**	**1.35**	0.29
26.8	misc.nitrilases, *nitrile lyases, berberine bridge enzymes, reticuline oxidases, troponine reductases	ta.11025.1.a1_at	similar to (111)AT4G20860| Symbols: | FAD-binding domain-containing protein | chr4:11172737-11174329 FORWARDVAR1 gb|BQ168402;	**0.52**	**0.64**	-0.28
27.1.19	RNA.processing.ribonucleases	ta.4328.1.s1_x_at	similar to (174)PR1_ASPOF gb|BJ277748;	**0.58**	**0.58**	-0.09
27.1.19	RNA.processing.ribonucleases	ta.4328.1.s1_at	similar to (174)PR1_ASPOF gb|BJ277748;	**0.55**	**0.64**	0.19
27.3.25	RNA.regulation of transcription.MYB domain transcription factor family	ta.26049.1.s1_a_at	similar to (266)AT4G38620| Symbols: ATMYB4, MYB4 | MYB4 (myb domain protein 4); transcription factor | chr4:18053860-18054870 FORWARDmoderately similar to (441)MYB1_HORVUVAR1 gb|CD454952;	**0.71**	**0.65**	-0.41
29.4	protein.postranslational modification	ta.9516.2.s1_at	similar to (131)AT1G07430| Symbols: | protein phosphatase 2C, putative / PP2C, putative | chr1:2281148-2282653 REVERSE gb|BE444016;	**0.69**	**0.87**	0.00
29.4	protein.postranslational modification	ta.12348.1.a1_at	similar to (157)AT1G72770| Symbols: HAB1 | HAB1 (HOMOLOGY TO ABI1) | chr1:27394660-27396075 FORWARD gb|BQ172159;	**0.64**	**0.72**	0.18

Table S4.7 Commonly up-regulated transcripts between aphid infestation and dual stress at 24 h (threshold: 0.5 fold change)

BinCode	BinName	id	description	Salt	24h Dual	Aphid
10.6.3	cell wall.degradation.pectate lyases and polygalacturonases	ta.14588.2.s1_x_at	similar to (142)AT5G06860| Symbols: PGIP1 | PGIP1 (POLYGALACTURONASE INHIBITING PROTEIN 1); protein binding | chr5:2132374-2133435 FORWARDmoderately similar to (310)PGIP1_ORYSA gb|CD930954;	0.43	**0.56**	**0.58**
11.1.9	lipid metabolism.FA synthesis and FA elongation.long chain fatty acid CoA ligase	taaffx.113953.1.s1_s_at	similar to (395)AT1G64400| Symbols: | long-chain-fatty-acid--CoA ligase, putative / long-chain acyl-CoA synthetase, putative | chr1:23919465-23923344 REVERSE gb|CD892451;	0.16	**0.58**	**0.52**
12.4	N-metabolism.misc	ta.25705.2.a1_a_at	similar to (84.0)AT5G67220| Symbols: | nitrogen regulation family protein | chr5:26837502-26839152 REVERSE gb|CA695340;	0.25	**0.50**	**0.53**
13.1.6.5	amino acid metabolism.synthesis.aromatic aa.tryptophan	taaffx.13004.1.s1_at	similar to (334)AT3G57880| Symbols: | C2 domain-containing protein | chr3:21442175-21444496 REVERSE gb|BJ211609;	0.07	**0.57**	**0.79**
16.2.1.9	secondary metabolism.phenylpropanoids.lignin biosynthesis.COMT	ta.23042.1.s1_x_at	similar to (160)AT5G54160| Symbols: OMT1, ATOMT1 | ATOMT1 (O-METHYLTRANSFERASE 1) | chr5:21999301-22001393 FORWARDweakly similar to (200)OMT1_ORYSA gb|BE517350;	-0.04	**0.53**	**0.83**
16.8.2	secondary metabolism.flavonoids.chalcones	ta.7099.1.a1_at	similar to (193)AT5G13930| Symbols: CHS, TT4, ATCHS | ATCHS/CHS/TT4 (CHALCONE SYNTHASE); naringenin-chalcone synthase | chr5:4488764-4490037 FORWARDmoderately similar to (220)CHS2_SECCEVAR1 gb|CK155175;	0.49	**0.52**	**0.56**
20.2.1	stress.abiotic.heat	ta.23807.1.s1_x_at	similar to (115)AT1G56410| Symbols:	0.24	**0.73**	**0.56**

20.1	stress.biotic	ta.30501.1.s1_at	HSP70T-1, ERD2 | ERD2/HSP70T-1 (EARLY-RESPONSIVE TO DEHYDRATION 2); ATP binding | chr1:21120812-21122906 FORWARDweakly similar to (118)HSP7C_PETHY gb|CA699307; similar to (220)AT3G12500| Symbols: PR3, PR-3, CHI-B, B-CHI, ATHCHIB | ATHCHIB (BASIC CHITINASE); chitinase | chr3:3962508-3963952 REVERSEmoderately similar to (238)CHIQ_TOBAC gb|CK205943;	0.04	**0.56**	**0.66**
26.4	misc.beta 1,3 glucan hydrolases	ta.29552.3.s1_s_at	similar to (117)AT1G69295| Symbols: | beta-1,3-glucanase-related | chr1:26054155-26055506 REVERSE gb|CA645709;	0.18	**0.50**	**0.87**
26.6	misc.O-methyl transferases	ta.23042.1.s1_x_at	similar to (160)AT5G54160| Symbols: OMT1, ATOMT1 | ATOMT1 (O-METHYLTRANSFERASE 1) | chr5:21999301-22001393 FORWARDweakly similar to (200)OMT1_ORYSA gb|BE517350;	-0.04	**0.53**	**0.83**
26.9	misc.glutathione S transferases	ta.3628.1.s1_at	similar to (113)AT2G30870| Symbols: ERD13, ATGSTF4, ATGSTF10 | ATGSTF10 (EARLY DEHYDRATION-INDUCED 13); glutathione transferase | chr2:13148567-13149469 FORWARDvery weakly similar to (92.8)GSTF3_MAIZE gb|CF133144;	0.22	**0.54**	**0.89**
28.1.3	DNA.synthesis/chromatin structure.histone	ta.10329.17.s1_at	similar to (169)AT5G59970| Symbols: | histone H4 | chr5:24163578-24163889 REVERSEweakly similar to (169)H4_PEAVAR1 gb|BJ308545;	0.44	**0.64**	**1.97**
29.2.1.2.1.27	protein.synthesis.ribosomal protein.eukaryotic.40S subunit.S27	ta.13076.1.s1_at	similar to (132)AT5G47930| Symbols: | 40S ribosomal protein S27 (RPS27D) | chr5:19423649-19424555 REVERSEweakly similar to (130)RS27_HORVU gb|CA486547;	0.00	**1.23**	**2.07**
29.2.1.2.1.6	protein.synthesis.ribosomal	ta.12963.1.s1_at	gb|CA502685;	-0.05	**0.61**	**1.15**

29.2.1.2.2.10	protein.eukaryotic.40S subunit.S6 protein.synthesis.ribosomal protein.eukaryotic.60S subunit.L10	ta.26752.1.a1_at	similar to (251)AT1G66580| Symbols: | 60S ribosomal protein L10 (RPL10C) | chr1:24842871-24844102 FORWARDmoderately similar to (247)RL10_MAIZE gb|CD491059;	0.15	**0.93**	**1.71**
29.5.11.4.2	protein.degradation.ubiquitin.E3.RING	ta.12529.1.s1_x_at	similar to (170)AT5G60710| Symbols: | zinc finger (C3HC4-type RING finger) family protein | chr5:24428179-24432075 REVERSE gb|CK211211;	0.30	**0.52**	**0.65**
29.4	protein.postranslational modification	taaffx.2991.1.s1_at	similar to (466)AT5G25510| Symbols: | serine/threonine protein phosphatase 2A (PP2A) regulatory subunit B', putative | chr5:8882731-8884328 REVERSE gb|CA653919;	0.30	**0.52**	**0.72**
30.3	signalling.calcium	ta.6558.1.s1_x_at	similar to (145)AT3G22190| Symbols: IQD5 | IQD5 (IQ-domain 5); calmodulin binding | chr3:7831668-7833519 REVERSE VAR1 COG5048 PRK08581 COG4886 COG5099 MopB_Res-Cmplx1_Nad11-M gb|CK208447;	0.50	**0.56**	**0.86**
34.19.1	transport.Major Intrinsic Proteins.PIP	ta.2826.1.s1_at	similar to (183)AT4G35100| Symbols: PIP3A, PIP2;7, SIMIP, PIP3 | PIP3 (PLASMA MEMBRANE INTRINSIC PROTEIN 3); water channel | chr4:16708677-16709963 FORWARDweakly similar to (181)PIP24_ORYSA gb|AF366565.1;	0.48	**0.55**	**0.75**

Table S4.8 Commonly up-regulated transcripts between salt tress and aphid infestation at 24 h (threshold: 0.5 fold change)

BinCode	BinName	id	description	Salt	24h Dual	Aphid
11.3.7	lipid metabolism.Phospholipid synthesis.cyclopropane-fatty-acyl-phospholipid synthase	taaffx.24365.1.s1_at	similar to (96.7)AT3G23530| Symbols: | cyclopropane fatty acid synthase, putative / CPA-FA synthase, putative | chr3:8437479-8442604 FORWARD gb|CA720390;	**0.51**	-0.03	**1.64**
11.3.7	lipid metabolism.Phospholipid synthesis.cyclopropane-fatty-acyl-phospholipid synthase	ta.8082.1.a1_x_at	similar to (157)AT3G23510| Symbols: | cyclopropane fatty acid synthase, putative / CPA-FA synthase, putative | chr3:8428078-8433166 FORWARDCOG5048 VAR1 gb|BQ161248;	**0.51**	0.15	**0.79**
20.1.7	stress.biotic.PR-proteins	ta.3298.1.s1_at	similar to (159)AT1G65870| Symbols: | disease resistance-responsive family protein | chr1:24507287-24507856 FORWARD gb|CK215466;	**0.62**	0.49	**1.02**
26.13	misc.acid and other phosphatases	ta.23957.1.s1_at	similar to (814)AT5G50400| Symbols: ATPAP27, PAP27 | ATPAP27/PAP27 (purple acid phosphatase 27); acid phosphatase/ protein serine/threonine phosphatase | chr5:20540801-20543457 REVERSEhighly similar to (670)NPP_HORVU gb|BJ321521;	**0.60**	0.44	**1.69**
26.6	misc.O-methyl transferases	ta.11017.1.a1_at	weakly similar to (105)ZRP4_MAIZE gb|BQ168386;	**0.98**	-0.46	**1.52**
27.3.46	RNA.regulation of transcription.DNA methyltransferases	ta.9261.1.s1_at	similar to (397)AT5G49160| Symbols: METI, DDM2, DMT01, MET2, DMT1, MET1 | MET1 (DECREASED METHYLATION 2DNA) | chr5:19949727-19955412 FORWARDweakly similar to (103)CMT1_MAIZE gb|BT009495.1;	**0.62**	0.30	**1.63**
27.3.67	RNA.regulation of transcription.putative transcription regulator	taaffx.44615.1.a1_at	similar to (167)AT1G07370| Symbols: PCNA1 | PCNA1 (PROLIFERATING CELLULAR NUCLEAR ANTIGEN); DNA binding / DNA polymerase processivity factor | chr1:2263202-2264380	**0.52**	0.17	**1.36**

			FORWARDweakly similar to (187)PCNA_ORYSA gb|CK208222;			
28.1.3	DNA.synthesis/chromatin structure.histone	ta.28802.3.s1_at	similar to (166)AT5G02560| Symbols: HTA12 | HTA12; DNA binding | chr5:575435-576454 FORWARDweakly similar to (185)H2A5_ORYSA gb|BJ228498;	**0.62**	0.20	**1.48**
28.1.3	DNA.synthesis/chromatin structure.histone	ta.10329.36.s1_a_at	similar to (162)AT5G59970| Symbols: | histone H4 | chr5:24163578-24163889 REVERSEweakly similar to (162)H4_PEA gb|BJ316876;	**0.57**	0.45	**1.97**
28.1	DNA.synthesis/chromatin structure	ta.6719.1.s1_x_at	similar to (85.5)TOP2_PEAVAR1 COG5048 gb|BJ217169;	**0.67**	0.42	**1.74**
31.1	cell.organisation	ta.2157.1.a1_s_at	similar to (169)AT4G05190| Symbols: ATK5 | ATK5 (Arabidopsis thaliana kinesin 5); microtubule motor | chr4:2675336-2679480 FORWARDvery weakly similar to (96.3)KLP1_CHLRE gb|BG607913;	**0.53**	0.37	**1.15**
33.1	development.storage proteins	ta.28866.2.a1_s_at	similar to (142)AT4G37050| Symbols: PLP4, PLA V | PLA V/PLP4 (Patatin-like protein 4); nutrient reservoir | chr4:17457255-17459636 REVERSEvery weakly similar to (80.5)PAT5_SOLTU gb|CK193087;	**0.53**	0.01	**0.67**
33.2	development.late embryogenesis abundant	ta.5888.1.s1_s_at	gb|BQ807116;	**0.59**	0.02	**0.65**

Table S4.9 Specifically up-regulated transcripts by dual stress at 6h (threshold: 1.0 fold change)

BinCode	BinName	id	description	6h Salt	6h Dual	6h Aphid
17.7.1.2	hormone metabolism.jasmonate.synthesis-degradation.lipoxygenase	ta.23763.1.s1_at	similar to (637)AT3G22400| Symbols: LOX5 | LOX5; lipoxygenase | chr3:7927018-7931174 FORWARDhighly similar to (757)LOX1_ORYSA gb|CK213159;	0.30	**1.15**	0.56
17.7.1.2	hormone metabolism.jasmonate.synthesis-degradation.lipoxygenase	ta.13650.1.a1_at	similar to (169)AT3G22400| Symbols: LOX5 | LOX5; lipoxygenase | chr3:7927018-7931174 FORWARDweakly similar to (177)LOX3_ORYSA gb|CK211830;	0.43	**1.03**	0.66
17.7.1.2	hormone metabolism.jasmonate.synthesis-degradation.lipoxygenase	ta.1967.2.a1_x_at	similar to (134)LOX21_HORVU gb|AJ614579;	0.39	**1.11**	0.74
17.7.1.2	hormone metabolism.jasmonate.synthesis-degradation.lipoxygenase	taaffx.104812.1.s1_s_at	similar to (458)AT3G45140| Symbols: ATLOX2, LOX2 | LOX2 (LIPOXYGENASE 2) | chr3:16536422-16540218 FORWARDhighly similar to (820)LOX21_HORVU gb|BJ223744;	0.29	**1.06**	0.79
20.2.1	stress.abiotic.heat	taaffx.87145.1.s1_at	similar to (177)AT2G42750| Symbols: | DNAJ heat shock N-terminal domain-containing protein | chr2:17800481-17802496 FORWARD gb|CA594975;	0.59	**1.16**	-0.14
26.13	misc.acid and other phosphatases	ta.12413.1.s1_at	similar to (263)AT1G73010| Symbols: | phosphoric monoester hydrolase | chr1:27468441-27469841 REVERSECOG3883 gb|CA725003;	0.40	**1.15**	-0.02

Table S4.10 Specifically up-regulated transcripts by salt stress at 6h (threshold: 1.0 fold change)

BinCode	BinName	id	description	6h Salt	6h Dual	6h Aphid
13.1.2.2	amino acid metabolism.synthesis.glutamate family.proline	ta.7091.1.s1_at	similar to (460)AT3G55610| Symbols: P5CS2 | P5CS2 (DELTA 1-PYRROLINE-5-CARBOXYLATE SYNTHASE 2); catalytic/ glutamate 5-kinase/ oxidoreductase | chr3:20635984-20639968 REVERSEhighly similar to (510)P5CS_ORYSA VAR1 COG5048 COG4886 COG5099 PRK08581 MopB_Res-Cmplx1_Nad11-M gb|CK194302;	**1.23**	0.95	-0.39
26.1	misc.cytochrome P450	ta.21438.1.a1_at	similar to (130)AT3G26310| Symbols: CYP71B35 | CYP71B35 (cytochrome P450, family 71, subfamily B, polypeptide 35); oxygen binding | chr3:9642326-9644016 REVERSEweakly similar to (143)C71C4_MAIZE gb|CA670189;	**1.02**	0.12	0.04
26.8	misc.nitrilases, *nitrile lyases, berberine bridge enzymes, reticuline oxidases, troponine reductases	ta.11025.1.a1_at	similar to (111)AT4G20860| Symbols: | FAD-binding domain-containing protein | chr4:11172737-11174329 FORWARDVAR1 gb|BQ168402;	**1.07**	0.31	-0.78
27.3.25	RNA.regulation of transcription.MYB domain transcription factor family	ta.26049.1.s1_a_at	similar to (266)AT4G38620| Symbols: ATMYB4, MYB4 | MYB4 (myb domain protein 4); transcription factor | chr4:18053860-18054870 FORWARDmoderately similar to (441)MYB1_HORVUVAR1 gb|CD454952;	**1.58**	0.76	-0.05

Table S4.11 Specifically up-regulated transcripts by aphid infestation at 6h (threshold: 1.0 fold change)

BinCode	BinName	id	description	6h Salt	6h Dual	6h Aphid
1.1.1.1	PS.lightreaction.photosystem II.LHC-II	ta.30727.1.a1_at	similar to (405)AT5G01530| Symbols: | chlorophyll A-B binding protein CP29 (LHCB4) | chr5:209083-210242 FORWARDmoderately similar to (233)CB29_CHLREVAR1 gb|CK211113;	-0.09	0.33	**1.00**
1.1.1.2	PS.lightreaction.photosystem II.PSII polypeptide subunits	taaffx.28455.1.s1_at	similar to (214)ATCG00270| Symbols: PSBD | PSII D2 protein | chrC:32711-33772 FORWARDmoderately similar to (217)PSBD_SECCE gb|CA659945;	-0.22	0.05	**1.15**
1.1.2.2	PS.lightreaction.photosystem I.PSI polypeptide subunits	taaffx.12631.1.s1_at	similar to (337)ATCG00340| Symbols: PSAB | Encodes the D1 subunit of photosystem I and II reaction centers. | chrC:37375-39579 REVERSEmoderately similar to (351)PSAB_WHEAT gb|BJ248421;	-0.26	-0.11	**1.01**
1.1.2.2	PS.lightreaction.photosystem I.PSI polypeptide subunits	taaffx.26519.1.s1_at	similar to (137)ATCG00350| Symbols: PSAA | Encodes psaA protein comprising the reaction center for photosystem I along with psaB protein; hydrophobic protein encoded by the chloroplast genome. | chrC:39605-41857 REVERSEweakly similar to (137)PSAA_WHEAT gb|CA688032;	-0.30	-0.01	**1.03**
1.1.2.2	PS.lightreaction.photosystem I.PSI polypeptide subunits	taaffx.142789.1.s1_at	similar to (140)ATCG00350| Symbols: PSAA | Encodes psaA protein comprising the reaction center for photosystem I along with psaB protein; hydrophobic protein encoded by the chloroplast genome. | chrC:39605-41857 REVERSEweakly similar to (140)PSAA_WHEAT gb|CA671900;	-0.31	0.06	**1.17**
29.2.1.1.1.1.19	protein.synthesis.ribosomal	taaffx.128896.8.a1_at	similar to (122)ATCG00820| Symbols: RPS19 |	-0.25	-0.10	**1.11**

Table S4.12 Specifically up-regulated transcripts by dual stress at 24h (threshold: 1.0 fold change)

BinCode	BinName	id	description	Salt	24h Dual	Aphid
16.2	secondary metabolism.phenylpropanoids	ta.9717.1.a1_x_at	similar to (91.3)AT4G35160| Symbols: | O-methyltransferase family 2 protein | chr4:16730994-16732813 REVERSEmoderately similar to (204)ZRP4_MAIZEVAR1 COG5048 gb|CK154440;	0.43	**1.07**	-0.07
16.2	secondary metabolism.phenylpropanoids	ta.9717.1.a1_a_at	similar to (91.3)AT4G35160| Symbols: | O-methyltransferase family 2 protein | chr4:16730994-16732813 REVERSEmoderately similar to (204)ZRP4_MAIZEVAR1 COG5048 gb|CK154440;	0.80	**1.33**	-0.17
35.2	not assigned.unknown	ta.20231.1.a1_at	gb|CA676502;	0.87	**1.12**	-0.08
35.2	not assigned.unknown	ta.21267.1.s1_s_at	gb|CA694095;	0.76	**1.00**	0.43
35.2	not assigned.unknown	ta.14301.1.s1_at	gb|BU099360;	0.97	**1.10**	0.01

Table S4.13 Specifically up-regulated transcripts by salt stress at 24h (threshold: 1.0 fold change)

BinCode	BinName	id	description	Salt	24h Dual	Aphid
13.2.6.2	amino acid metabolism.degradation.aromatic aa.tyrosine	ta.21094.1.s1_at	similar to (366)AT5G53970| Symbols: | aminotransferase, putative | chr5:21927902-21929820 FORWARD gb|CD873115;	**1.31**	-0.23	0.36
15.2	metal handling.binding, chelation and storage	ta.5549.3.s1_at	similar to (146)NAS1_HORVU gb|CD878292;	**1.00**	-0.32	0.22

Table S4.14 Specifically up-regulated transcripts by aphid infestation at 24 h (threshold: 1.0 fold change)

BinCode	BinName	id	description	Salt	24h Dual	Aphid
10.7	cell wall.modification	taaffx.17141.1.s1_at	similar to (318)AT4G03210| Symbols: XTH9 | XTH9 (XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 9); hydrolase, acting on glycosyl bonds | chr4:1416107-1417197 FORWARDmoderately similar to (264)XTHA_PHAAN gb|CD866709;	0.16	0.28	**1.02**
10.7	cell wall.modification	taaffx.17141.1.s1_x_at	similar to (318)AT4G03210| Symbols: XTH9 | XTH9 (XYLOGLUCAN ENDOTRANSGLUCOSYLASE/HYDROLASE 9); hydrolase, acting on glycosyl bonds | chr4:1416107-1417197 FORWARDmoderately similar to (264)XTHA_PHAAN gb|CD866709;	0.35	0.47	**1.12**
10.8.1	cell wall.pectin*esterases.PME	taaffx.36894.2.s1_at	similar to (176)AT5G47500| Symbols: | pectinesterase family protein | chr5:19288489-19290072 REVERSE gb|BJ303490;	0.21	0.33	**1.06**
11.3.7	lipid metabolism.Phospholipid synthesis.cyclopropane-fatty-acyl-phospholipid synthase	ta.4726.1.s1_at	similar to (122)AT3G23510| Symbols: | cyclopropane fatty acid synthase, putative / CPA-FA synthase, putative | chr3:8428078-8433166 FORWARD gb|BJ270709;	0.48	-0.17	**1.20**
11.3.7	lipid metabolism.Phospholipid synthesis.cyclopropane-fatty-acyl-phospholipid synthase	taaffx.24365.1.s1_at	similar to (96.7)AT3G23530| Symbols: | cyclopropane fatty acid synthase, putative / CPA-FA synthase, putative | chr3:8437479-8442604 FORWARD gb|CA720390;	0.51	-0.03	**1.64**
20.1.7	stress.biotic.PR-proteins	ta.3298.1.s1_at	similar to (159)AT1G65870| Symbols: | disease resistance-responsive family protein | chr1:24507287-24507856 FORWARD gb|CK215466;	0.62	0.49	**1.02**
23.5.4	nucleotide metabolism.deoxynucleotide	ta.1010.1.s1_at	similar to (536)AT3G27060| Symbols: TSO2 | TSO2 (TSO MEANING 'UGLY' IN CHINESE);	0.24	0.31	**1.37**

	metabolism.ribonucleoside-diphosphate reductase		ribonucleoside-diphosphate reductase | chr3:9981208-9982294 REVERSEhighly similar to (547)RIR2_TOBACVAR1 COG5048 gb|BJ284296;			
23.5	nucleotide metabolism.deoxynucleotide metabolism	ta.6274.1.s1_s_at	similar to (631)AT2G21790| Symbols: R1, RNR1 | R1/RNR1 (RIBONUCLEOTIDE REDUCTASE 1); ribonucleoside-diphosphate reductase | chr2:9300609-9304660 FORWARD gb|BJ275177;	0.32	0.17	**1.42**
23.5	nucleotide metabolism.deoxynucleotide metabolism	ta.6274.1.s1_at	similar to (631)AT2G21790| Symbols: R1, RNR1 | R1/RNR1 (RIBONUCLEOTIDE REDUCTASE 1); ribonucleoside-diphosphate reductase | chr2:9300609-9304660 FORWARD gb|BJ275177;	0.42	0.22	**1.40**
26.13	misc.acid and other phosphatases	ta.23957.1.s1_at	similar to (814)AT5G50400| Symbols: ATPAP27, PAP27 | ATPAP27/PAP27 (purple acid phosphatase 27); acid phosphatase/ protein serine/threonine phosphatase | chr5:20540801-20543457 REVERSEhighly similar to (670)NPP_HORVU gb|BJ321521;	0.60	0.44	**1.69**
26.17	misc.dynamin	ta.7533.1.s1_a_at	similar to (308)AT1G53140| Symbols: | dynamin family protein | chr1:19802939-19806109 FORWARD gb|BJ320807;	0.27	0.18	**1.19**
26.28	misc.GDSL-motif lipase	ta.4207.2.s1_at	similar to (97.4)AT1G71691| Symbols: | GDSL-motif lipase/hydrolase family protein | chr1:26953057-26954835 REVERSE gb|BJ253228;	0.21	0.15	**1.11**
26.28	misc.GDSL-motif lipase	ta.4207.1.s1_a_at	similar to (390)AT1G71691| Symbols: | GDSL-motif lipase/hydrolase family protein | chr1:26953057-26954835 REVERSEweakly similar to (141)APG_BRANAVAR1 gb|BJ261209;	0.46	0.33	**1.35**
26.4	misc.beta 1,3 glucan hydrolases	taaffx.73807.1.a1_at	similar to (122)AT2G27500| Symbols: |	0.10	0.23	**1.11**

			glycosyl hydrolase family 17 protein | chr2:11759442-11760592 REVERSEvery weakly similar to (84.3)E13B_WHEAT gb|CD927227;			
26.6	misc.O-methyl transferases	ta.11017.1.a1_at	similar to (105)ZRP4_MAIZE gb|BQ168386;	0.98	-0.46	**1.52**
27.1.2	RNA.processing.RNA helicase	ta.3093.3.a1_at	similar to (165)AT5G61000| Symbols: | replication protein, putative | chr5:24566908-24569867 REVERSE gb|CN012478;	0.25	0.19	**1.60**
27.3.46	RNA.regulation of transcription.DNA methyltransferases	ta.15889.3.a1_a_at	similar to (108)AT1G69770| Symbols: CMT3 | CMT3 (CHROMOMETHYLASE 3) | chr1:26252159-26257182 REVERSEweakly similar to (119)CMT3_MAIZE gb|BJ213871;	0.17	0.06	**1.42**
27.3.46	RNA.regulation of transcription.DNA methyltransferases	ta.9261.1.s1_at	similar to (397)AT5G49160| Symbols: METI, DDM2, DMT01, MET2, DMT1, MET1 | MET1 (DECREASED METHYLATION 2DNA) | chr5:19949727-19955412 FORWARDweakly similar to (103)CMT1_MAIZE gb|BT009495.1;	0.62	0.30	**1.63**
27.3.62	RNA.regulation of transcription.Nucleosome/chromatin assembly factor group	ta.23439.1.s1_at	similar to (92.8)AT3G51880| Symbols: NFD1, HMGB1 | HMGB1 (HIGH MOBILITY GROUP B 1) | chr3:19258218-19259468 REVERSEweakly similar to (108)HMGL_IPONI gb|BJ221734;	0.20	0.18	**1.09**
27.3.62	RNA.regulation of transcription.Nucleosome/chromatin assembly factor group	ta.14587.1.s1_at	similar to (121)AT5G23420| Symbols: HMGB6 | HMGB6 (High mobility group B 6); transcription factor | chr5:7888715-7890114 REVERSE gb|CK207741;	0.40	0.29	**1.88**
27.3.67	RNA.regulation of transcription.putative transcription regulator	ta.2876.1.s1_x_at	similar to (207)AT1G07370| Symbols: PCNA1 | PCNA1 (PROLIFERATING CELLULAR NUCLEAR ANTIGEN); DNA binding / DNA polymerase processivity factor | chr1:2263202-2264380 FORWARDmoderately similar to (215)PCNA_ORYSA gb|BE417035;	0.34	0.15	**1.47**
27.3.67	RNA.regulation of	ta.7387.1.a1_at	similar to (147)AT1G04020| Symbols:	0.35	0.33	**1.10**

	transcription.putative transcription regulator		ATBARD1, BARD1 | ATBARD1/BARD1 (BREAST CANCER ASSOCIATED RING 1); transcription coactivator | chr1:1036609-1040044 FORWARDVAR1 gb|BJ306191;			
27.3.67	RNA.regulation of transcription.putative transcription regulator	ta.2876.2.a1_at	similar to (207)AT1G07370| Symbols: PCNA1 | PCNA1 (PROLIFERATING CELLULAR NUCLEAR ANTIGEN); DNA binding / DNA polymerase processivity factor | chr1:2263202-2264380 FORWARDmoderately similar to (231)PCNA_ORYSA gb|CK213813;	0.46	0.36	**1.77**
27.3.67	RNA.regulation of transcription.putative transcription regulator	taaffx.44615.1.a1_at	similar to (167)AT1G07370| Symbols: PCNA1 | PCNA1 (PROLIFERATING CELLULAR NUCLEAR ANTIGEN); DNA binding / DNA polymerase processivity factor | chr1:2263202-2264380 FORWARDweakly similar to (187)PCNA_ORYSA gb|CK208222;	0.52	0.17	**1.36**
27.3.71	RNA.regulation of transcription.SNF7	ta.7481.1.s1_a_at	similar to (295)AT5G44560| Symbols: VPS2.2 | VPS2.2 | chr5:17963743-17965449 FORWARD gb|CA642970;	0.34	0.37	**1.08**
28.1.3	DNA.synthesis/chromatin structure.histone	ta.22962.1.s1_at	similar to (140)AT3G54560| Symbols: HTA11 | HTA11; DNA binding | chr3:20207510-20208444 FORWARDweakly similar to (144)H2AV2_ORYSA gb|CN012324;	0.05	0.21	**1.36**
28.2	DNA.repair	ta.3093.1.s1_at	similar to (748)AT5G61000| Symbols: | replication protein, putative | chr5:24566908-24569867 REVERSEVAR1 gb|CD453942;	0.06	0.02	**1.27**
28.2	DNA.repair	ta.6986.1.s1_at	similar to (80.1)AT2G24490| Symbols: RPA2, ATRPA2, ROR1 | ATRPA2/ROR1/RPA2 (REPLICON PROTEIN A) | chr2:10405731-10407496 REVERSE gb|CB307828;	0.45	0.33	**1.82**
28.2	DNA.repair	ta.3093.1.s1_a_at	similar to (748)AT5G61000| Symbols: | replication protein, putative | chr5:24566908-24569867 REVERSEVAR1 gb|CD453942;	0.52	0.50	**1.83**

29.4	protein.postranslational modification	ta.6911.1.s1_at	similar to (494)AT1G20930| Symbols: CDKB2;2 | CDKB2;2 (CYCLIN-DEPENDENT KINASE B2;2); kinase | chr1:7292741-7294653 REVERSEmoderately similar to (489)CDC2D_ANTMA gb|CA702838;	0.17	0.08	**1.10**
29.4	protein.postranslational modification	ta.7655.1.s1_at	similar to (486)AT2G38620| Symbols: CDKB1;2 | CDKB1;2 (cyclin-dependent kinase B1;2); kinase | chr2:16159629-16160944 FORWARDmoderately similar to (484)CDC2C_ANTMA gb|CD894067;	0.19	0.15	**1.10**
29.2.1.2.1.6	protein.synthesis.ribosomal protein.eukaryotic.40S subunit.S6	ta.12963.1.s1_at	gb|CA502685;	-0.05	0.61	**1.15**
29.2.1.2.2.10	protein.synthesis.ribosomal protein.eukaryotic.60S subunit.L10	ta.26752.1.a1_at	similar to (251)AT1G66580| Symbols: | 60S ribosomal protein L10 (RPL10C) | chr1:24842871-24844102 FORWARDmoderately similar to (247)RL10_MAIZE gb|CD491059;	0.15	0.93	**1.71**
29.5.1	protein.degradation.subtilases	ta.28847.1.s1_a_at	similar to (216)AT1G66220| Symbols: | subtilase family protein | chr1:24674199-24677324 FORWARD gb|CA740446;	0.32	0.28	**1.62**
29.5.11.4.2	protein.degradation.ubiquitin.E3.RING	ta.7387.1.a1_at	similar to (147)AT1G04020| Symbols: ATBARD1, BARD1 | ATBARD1/BARD1 (BREAST CANCER ASSOCIATED RING 1); transcription coactivator | chr1:1036609-1040044 FORWARDVAR1 gb|BJ306191;	0.35	0.33	**1.10**
31.1	cell.organisation	ta.7750.1.a1_at	similar to (285)AT2G36200| Symbols: | kinesin motor protein-related | chr2:15186818-15192268 REVERSEmoderately similar to (293)K125_TOBACVAR1 gb|CK154620;	0.16	0.21	**1.30**
31.1	cell.organisation	ta.14540.1.a1_a_at	similar to (89.7)AT5G67270| Symbols: ATEB1C | ATEB1C (MICROTUBULE END BINDING PROTEIN 1); microtubule binding | chr5:26857474-26859210 REVERSE	0.17	0.31	**1.08**

			gb|BJ305221;			
31.1	cell.organisation	ta.2157.2.a1_a_at	similar to (96.7)AT4G05190| Symbols: ATK5 | ATK5 (Arabidopsis thaliana kinesin 5); microtubule motor | chr4:2675336-2679480 FORWARD gb|BQ169406;	0.41	0.41	**1.00**
31.1	cell.organisation	ta.2157.1.a1_s_at	similar to (169)AT4G05190| Symbols: ATK5 | ATK5 (Arabidopsis thaliana kinesin 5); microtubule motor | chr4:2675336-2679480 FORWARDvery weakly similar to (96.3)KLP1_CHLRE gb|BG607913;	0.53	0.37	**1.15**
31.2	cell.division	ta.7603.1.a1_at	similar to (569)AT5G48600| Symbols: ATCAP-C, SMC4, ATSMC3 | ATSMC3 (ARABIDOPSIS THALIANA STRUCTURAL MAINTENANCE OF CHROMOSOME 3); ATP binding | chr5:19719134-19726317 FORWARD gb|CA500427;	0.24	0.08	**1.14**
31.3	cell.cycle	ta.6253.1.s1_a_at	similar to (327)AT1G20610| Symbols: CYCB2;3 | CYCB2;3 (CYCLIN B2;3); cyclin-dependent protein kinase regulator | chr1:7135063-7137263 REVERSEhighly similar to (518)CCNB2_ORYSA gb|BQ238112;	0.14	0.11	**1.18**
31.4	cell.vesicle transport	ta.11282.1.s1_x_at	similar to (262)AT1G08560| Symbols: KN, ATSYP111, SYP111 | SYP111 (syntaxin 111); SNAP receptor | chr1:2709781-2710713 REVERSE gb|CA616162;	0.12	0.10	**1.06**
31.4	cell.vesicle transport	ta.11282.1.s1_at	similar to (262)AT1G08560| Symbols: KN, ATSYP111, SYP111 | SYP111 (syntaxin 111); SNAP receptor | chr1:2709781-2710713 REVERSE gb|CA616162;	0.33	0.21	**1.41**
33.99	development.unspecified	ta.25342.1.s1_x_at	similar to (108)AT2G42840| Symbols: PDF1 | PDF1 (PROTODERMAL FACTOR 1) | chr2:17833404-17834503 REVERSECOG3883	0.04	0.24	**1.21**

Table S4.15: Primers used for quantitative real-time PCR analysis

Probe id	Oligo Name	Sequence (5' to 3')
ta.2882.1.s1_s_at	CaBP	F: GAGTTCTGCGTCCTCATGGT
		R: GCGGAGAGAAGAAGAAACGA
ta.74.1.s1_at	PDI	F: TTATGACTTTGGCCACACCG
		R: CGAGCTCATCAAATGGCTTG
ta.6217.1.s1_at	CSD3	F: CAACAAGGATGGTGTTGCAG
		R: CACATCCAATTCTGGCTCCT
ta.3094.3.a1_x_at	GME	F: CATGAACGAGATGGCTGAGA
		R: CCATCCTTGAGCCTCATTGT
ta.28171.1.s1_at	LOX5	F: CCAACAGCATCTCCATCTGA
		R: TGCCAAATGCATGAGGATTA
ta.27217.1.s1_at	TaAOS	F: CTCTTCACCGGCACCTACAT
		R: GAAGTCGTTGAGCGTGTTGA
ta.22871.1.s1_s_at	TIP1	F: GGAGATCGTGATGACCTTCG
		R: CTGCTCAGTAGTCGGTGGTG
Endogenous control	ACT2	F: CAAATCATGTTTGAGACCTTCAATG
		R: ACCAGAATCCAACACGATACCTG

Table S4.16: Quantitative real-time PCR validation of expression patterns of 7 probes sets identified from the wheat microarray

Treatments	**Microarray log2-FC**							**qRT-PCR fold change**						
	CBP	**PDI**	**CSD3**	**GME**	**LOX**	**AOS**	**TIP**	**CBP**	**PDI**	**CSD3**	**GME**	**LOX**	**AOS**	**TIP**
Aphid 6h	0.06	-0.06	0.01	-0.03	0.13	0.02	0.12	1.35	-1.21	0.31	-1.46	-0.27	0.63	7.18
Salt 6h	0.05	0.01	0.03	-0.17	0.08	0.01	-0.02	0.45	-1.10	0.38	-1.39	0.36	0.38	4.58
Dual 6h	-0.06	-0.08	-0.04	-0.05	0.28	0.14	-0.06	-0.33	-0.49	0.36	-1.57	0.74	1.59	7.69
Aphid 24h	-0.12	0.13	-0.07	-0.03	0.01	0.04	0.04	-0.60	0.65	-1.26	-0.48	0.62	-0.51	3.55
Salt 24h	0.33	0.01	0.00	0.08	0.13	0.07	-0.14	2.25	0.57	-0.81	-0.64	1.51	0.34	1.42
Dual 24h	0.12	0.07	0.04	0.07	0.15	0.08	-0.03	-0.20	1.87	1.15	1.35	3.37	1.61	6.49

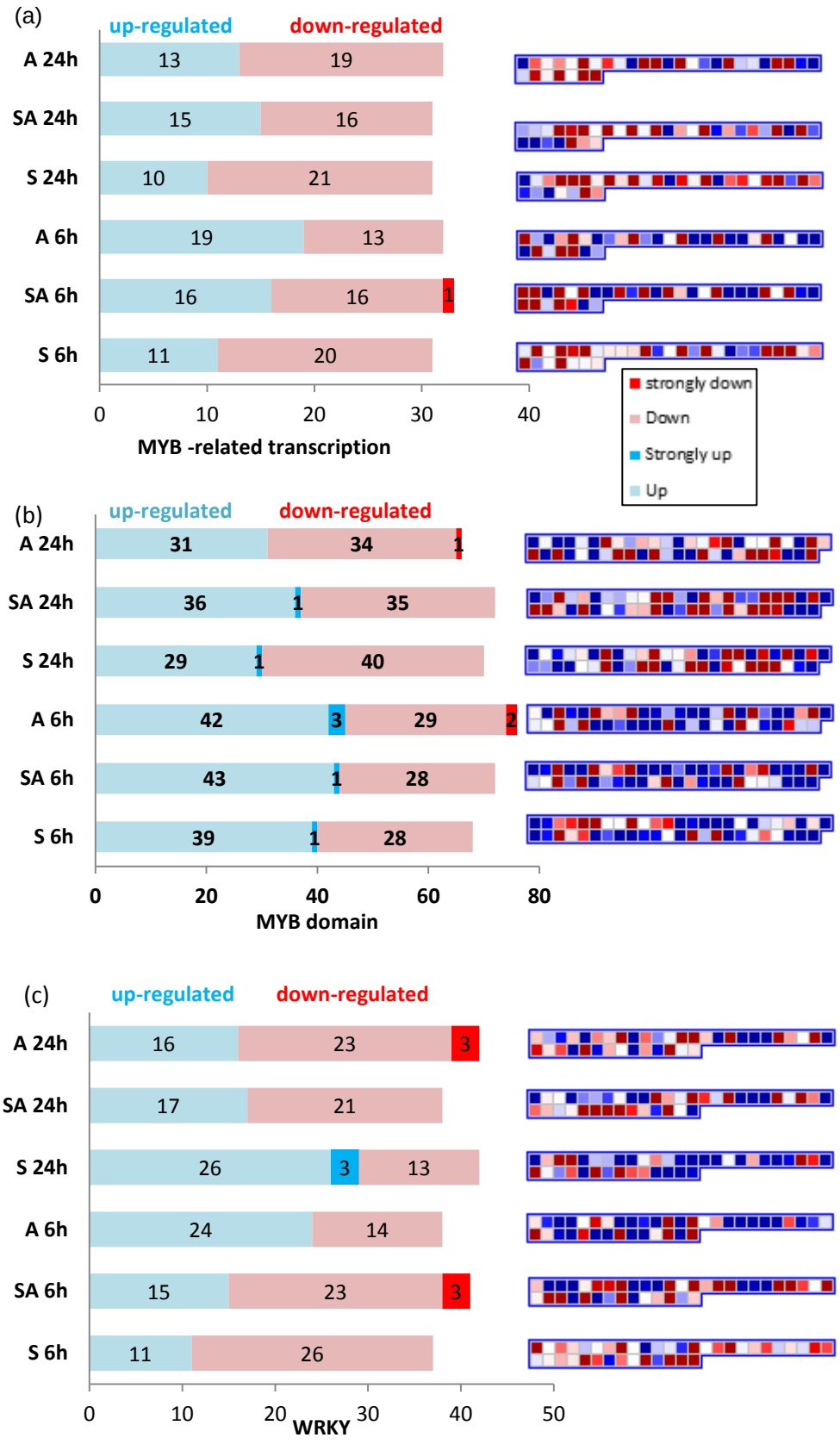

Figure S4.1: Number of differentially-regulated transcription factors TFs
(a) MYB-related transcription, (b) MYB domain (c) WRKY in wheat under stress treatments (salt S, dual stress SA, aphid A) compared to control (left). MapMan overview, (right) showing differences in transcript levels of TFs-related genes between stress and control treatments at 6 h and 24 h. Each point represents one gene. Blue represents higher gene expression under stress treatment while red represents higher gene expression under control.

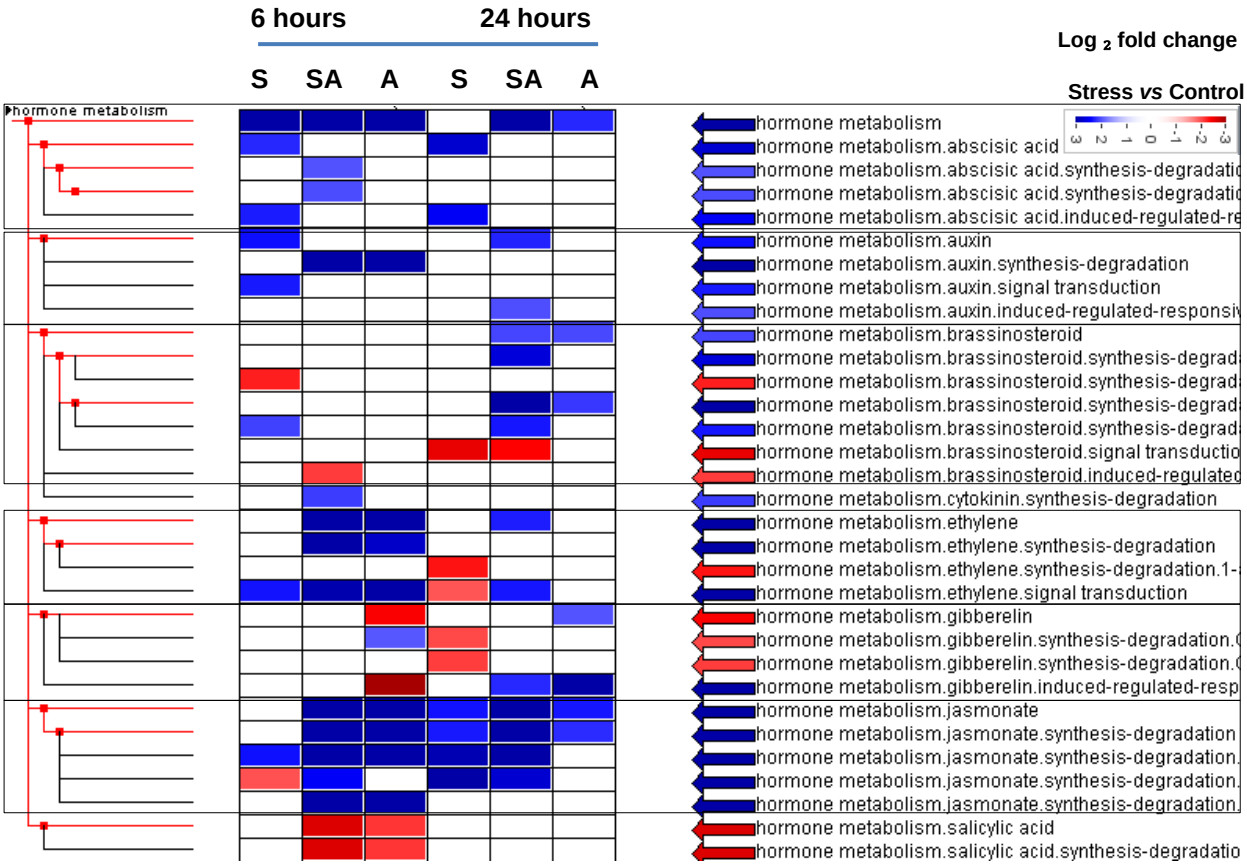

Figure S4.2: A condenses PageMan display of coordinated changes of hormone metabolism functional categories (bin and sub-bins).
The log2 fold changes between each stress treatment (salt, dual stress, aphid infestation) and control treatment were imported into PageMan for wheat at two time points 6 h and 24 h after aphid introduction. The data was subjected to a Wilcoxon rank sum test (statistical analysis) in PageMan and the results were displayed as false-colour code. Bin and sub-bin coloured in red are significantly down-regulated relative to the rest of the array, whereas bin and sub-bin coloured in blue are significantly up-regulated relative to the rest of the array. A predominant increase in the expression level of genes involved in hormone jasmonate metabolism at both time points, while a profound reduction in the expression level of genes associated with hormone salicylic acid metabolism at 6 h. A specific significant activation of genes related to cytokinine observed under dual stress at 6 h. Here, a highly saturated colour indicates a high absolute value, whereas smaller values are indicated by lower colour saturation. For the wilcoxon's test p-values, two different colours (blue and red) can be selected to distinguish between categories where the average of the signals for all the genes in a category increases or decreases.

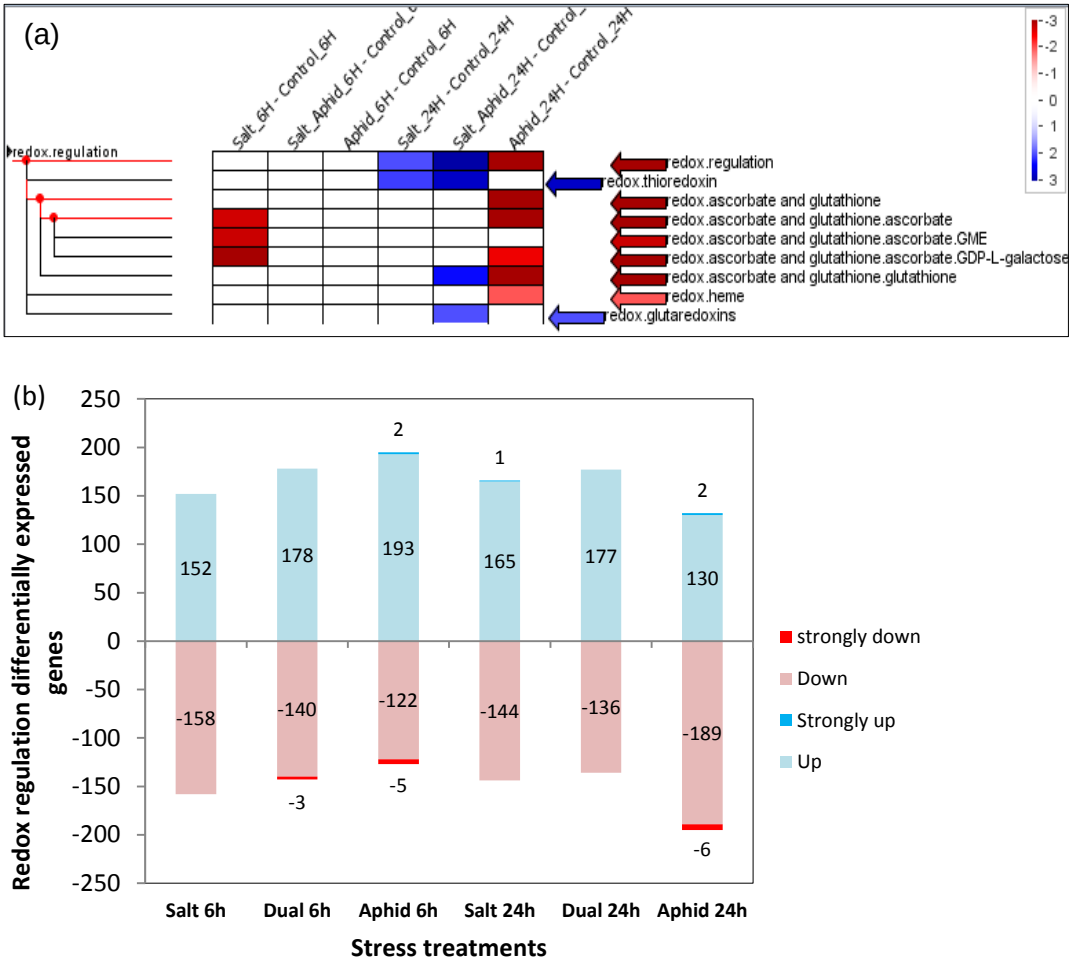

Figure S4.3: Distribution of differentially-expressed genes related to redox regulation category (a) A condense PageMan display of coordinated changes of redox regulation functional category. The log2 fold changes between each stress treatments (salt, dual stress, aphid infestation) and control treatment were imported into PageMan for wheat. The data was subjected to a Wilcoxon test in PageMan and the results were displayed as false-colour coded. Bins coloured in red are significantly down-regulated relative to the rest of the array, whereas bins coloured in blue are up-regulated. Significant suppression of genes involved in ascorbate was elicited by salt stress alone at 6 h, and in most redox regulation categories was triggered by aphid infestation alone at 24 h, whereas, significant activation of genes associated with thioredoxin was detected under both salt stress alone and dual stress at 24 h. Dual stress also significantly up regulated genes related to glutathione and glutaredoxin at 24 h. It can be seen that most activation of redox regulation genes in the analysis/comparison was induced by dual stress. (b) Number of total up- and down-regulated genes (light blue and red respectively) involved in redox regulation in wheat under each stress treatments compared to control treatment at two time intervals 6 h and 24 h after aphid introduction. Aphid infestation alone repressed the highest total number of redox genes at 24 h, and the highest total number of activated redox genes was detected under aphid infestation alone at 6 h however this change/alteration was not significant based on Wilcoxon.

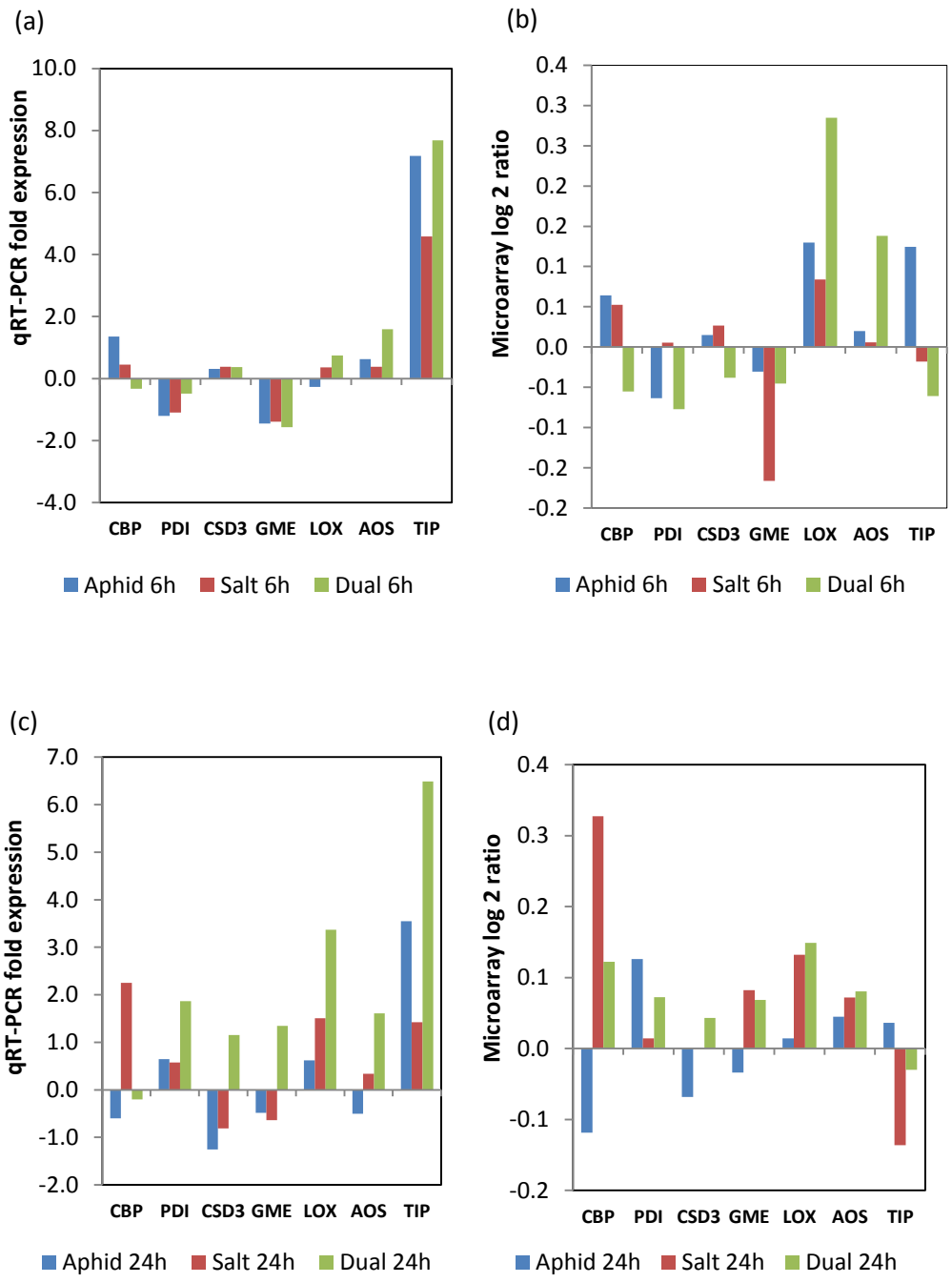

Figure S4.4: Validation of microarray results by quantitative real-time PCR (qRT-PCR). (a) and (c) represent the qRT-PCR data (relative expression, ΔCt) at 6 h and 24 h after aphid introduction respectively, and (b) and (d) are data from the microarray experiment at 6 h and 24 h after aphid introduction respectively. Overall, the data from the microarrays show relatively good correlation with the qRT-PCR data. Blue bars represent aphid infestation alone, red bars represent salt stress alone and green bars represent dual stress. 76% of the tested genes showed good correlation with gene expression profile obtained from microarray results.