PLANT-APHID INTERACTION: LOCAL AND SYSTEMIC EFFECTS ON PLANT PHYSIOLOGY AND GENE EXPRESSION

By

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Abstract

Aphids are economically important pests of glasshouse and temperate crops. By investigating the effects of aphid feeding on plant performance and by understanding the host-pest relationship, novel defence strategies can be devised. The effect of *Rhopalosiphum padi* (the bird cherry-oat aphid) feeding on young *Hordeum vulgare* (barley) plants was investigated. Particular emphasis was placed on changes within the sieve element (SE) because aphids are phloem-feeding insects.

Aphid infestation significantly reduced host plant growth rate. High performance liquid chromatography showed a local elevation of leaf calcium levels in infested leaves. Calcium, sulphate and magnesium levels were all elevated systemically by aphid feeding, whilst nitrate levels decreased. Aphid feeding increased the duration of phloem sap exudation from severed stylets but did not alter exudation rate.

Electrical penetration graph studies demonstrated that the duration of SE sap ingestion was reduced, and the time aphid stylets spent in pathway through the leaf increased, on pre-infested plants. The time taken for aphids to locate the SE did not increase, however, suggesting that the inhibitory effect was phloem-localised.

In an attempt to elucidate the transcriptomic response to aphid attack, microarrays were performed on infested and uninfested barley plants. Gene expression changes in the first and second leaves were compared to determine the local and systemic effects, respectively. The differentially expressed genes have putative roles in defence, hormone signalling, cell wall remodelling, metabolism, transport and regulation of transcription and translation. The general response was a local suppression and systemic induction of plant defences.

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AA	Amino Acid
AAP	Amino Acid Permease
ABA	Abscisic Acid
AFLP	Amplified Fragment Length Polymorphism
ANOVA	Analysis of Variance
AsA	Ascorbic Acid
ATP	Adenosine Triphosphate
AVR	Avirulence
BAC	Bacterial Artificial Chromosome
BLAST	Basic Local Alignment Search Tool
BPH	Brown planthopper
CC	Companion Cell
CP	Cell Penetration
DEPC	Diethylpyrocarbonate
dsRNA	Double stranded RNA
EDTA	Ethylenediaminetetraacetic Acid
EPG	Electrical Penetration Graph
ER	Endoplasmic Reticulum
EST	Expressed Sequence Tag
ET	Ethylene
ETI	Effector Triggered Immunity
FC	Fold Change
GA	Gibberellic Acid
GFP	Green Fluorescent Protein
HR	Hypersensitive Response
JA	Jasmonic Acid
LCM	Laser Capture Microdissection
LMPC	Laser Microdissection coupled with Pressure Catapulting
LRR	Leucine Rich Repeat
MAPK	Mitogen Activated Protein Kinase
MIAME	Minimum Information About a Microarray Experiment
MP	Movement Protein
NBS	Nucleotide Binding Site
NCAP	Non-Cell-Autonomous Protein
NGS	Next-Generation Sequencing
NiR	Nitrate Reductase
NO	Nitric oxide
NP	Non-probing
PCA	Principle Component Analysis
PCD	Programmed Cell Death
PCR	Polymerase Chain Reaction
PD	Plasmodesma(ta)
Pd	Potential drop
PFI	Phloem Feeding Insect
PM	Plasma Membrane

PPR	Pentatricopeptide
PPU	Pore Plasmodesma Unit
PR	Pathogenesis-related
QC	Quality Control
R gene	Resistance gene
RIN	RNA Integrity Number
ROS	Reactive Oxygen Species
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SA	Salicylic Acid
SE	Sieve Element
SE-CCC	Sieve Element-Companion Cell Complex
SEL	Size Exclusion Limit
siRNA	Short Interfering RNA
SSH	Suppression Subtractive Hybridisation
TAQ	*Thermus aquaticus*
TF	Transcription Factor
XTH	Xyloglucan Endotransglycosylase/Hydrolase

1 Plant-aphid interaction

1.1 Introduction

Aphids are major crop pests and cause yield losses in both agricultural and horticultural production systems (Blackman and Eastop, 2007). There are over 4000 aphid species, found most abundantly in temperate regions where they colonize around a quarter of the local plant species (Dixon *et al*., 1987). Over 100 of these species are prevalent enough to be considered of significant economic importance and they contribute 26% of the 45 major insect pests on the six main food crops (maize, wheat, potatoes, sugar beet, barley and tomatoes) of these regions (Dedryver *et al*., 2010). Aphids are therefore a target for crop defence strategies, and understanding their lifecycles, feeding adaptations and the effects they elicit in their host plants is key to reducing the damage they inflict.

In a susceptible relationship, aphids maintain an intimate and prolonged interaction with the host plant phloem, withdrawing nutrients and injecting saliva (Prado and Tjallingii, 1994). The current study focuses on the morphological, physiological and transcriptomic effects of aphid infestation on *Hordeum vulgare* (barley) plants, with a specific interest in the phloem response and phloem-mediated systemic defence responses. This introduction will discuss the structure and function of the plant phloem and the lifecycle and anatomical adaptations of aphid pests, specifically *Rhopalosiphum padi*, the bird cherry oat aphid, which is the model organism of choice in this study. The challenges of adopting phloem sap as a food source, and the hurdles aphids must overcome to survive, will be discussed, especially with regard to plant defences and the ability of aphids to reprogramme them. Finally, aphid pest-

control strategies will be introduced, as well as the functional genomic resources that are being employed to advance them.

1.2 The plant phloem: a transport superhighway

1.2.1 Structure of plant vasculature

Evolution of the vascular system, comprised of the phloem and xylem, of contemporary flowering plants has facilitated colonisation of the land from their beginning as single-celled organisms (Van Bel, 2003). The long-distance transport of nutrients, water and signalling molecules has enabled cell and organ specialization as well as significant increases in organism size; metabolites are carried over many metres in trees for example. Phloem translocation is the primary method of transportation of sugars from photosynthesising leaves (sources) to the areas of the plant where they are required (sinks)(Fisher, 2000). The xylem, which consists of lignified, anucleate, non-living cells, transports water and micronutrients, such as metals and cations, up from the roots via transpiration (Franks and Brodribb, 2005, Shabala, 2007). Phloem

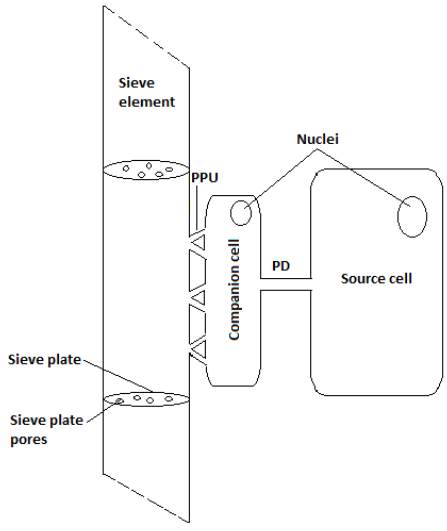


Figure 1-1 Diagram of SE-CCC showing sieve-plate, sieve-plate pores, plasmodesmata (PD) and pore-plasmodesma units (PPU). Created from the references within section 1.2.1

tissue is comprised of several different cell types (Figure 1-1). The main sap-conducting cells are the sieve elements (SEs). SE cells range from 40-500 µm in length and 10-100 µm in diameter depending on plant species and developmental stage (Pritchard, 2007). Individual sieve elements are connected longitudinally by sieve plates, which are interspersed with sieve-plate pores. Throughout the course of evolution, SE cells have followed a trend of increasing simplification, losing much of their function in gymnosperms and becoming entirely enucleate in angiosperms. This necessitates dependence on the associated companion cells (CCs) for almost all developmental, regulatory and maintenance functions (van Bel and Knoblauch, 2000). These cells are generally referred to collectively as the SE-CC complex (SE-CCC). Surrounding the SE-CCC are phloem parenchyma cells, which perform a support role, as well as a nutrient-transfer role in certain areas of the plant (Chinnappa *et al*., 2013).

1.2.2 Short distance movement of solutes through plasmodesmata (PD)

Solutes have been shown to travel between some cells through PD: membrane-lined pores that allow cytoplasmic continuity between adjacent cells (Lucas and Lee, 2004). They are the gateway to the symplasmic network formed by living phloem cells and are important regulators of molecular transport into and out of the phloem (Slewinski *et al*., 2012). The SE and associated CC are connected by specialised secondary PD, termed pore plasmodesma units (PPUs). PPUs are structurally distinct from other PD as a single pore on the SE side branches into multiple channels on the CC side (Oparka and Turgeon, 1999).

PD have a complex structure and form long plasma membrane (PM)-lined bridges, the lumen of which are occupied by appressed endoplasmic reticulum (ER), termed the desmotubule

(Brecknock *et al*., 2011). It has been speculated that transient gating of the cytosolic sleeve is imposed by tension (Oparka, 2004) applied by myosins arranged perpendicularly to the pore cylinder and visible as spokes in sections of PD (Radford and White, 2011, Ding *et al*., 1992).

The spokes connect spirally arranged actin filaments on the desmotubule to proteins in the PM (Ding *et al*., 1992) and subdivide the cytoplasmic sleeve into channels of 3-4 nm in diameter (Figure 1-2). PD are dynamic structures, exhibiting changes in permeability and positional frequency in response to changing requirements of the plant.

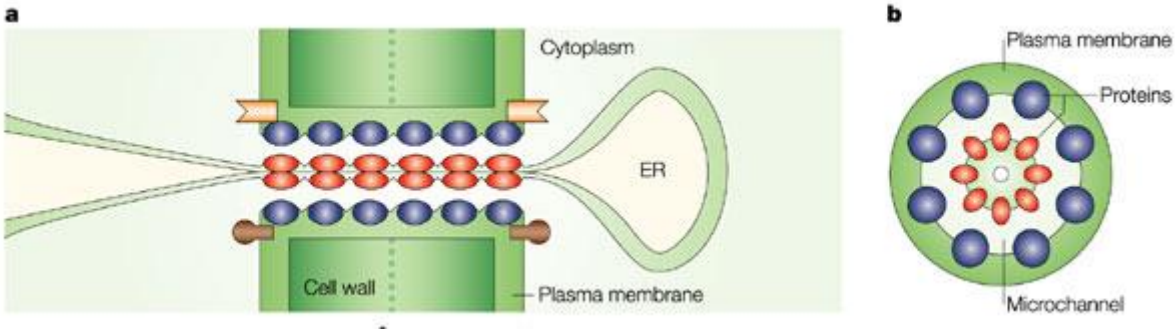


Figure 1-2 Schematic representation of a plasmodesma in (a) and cross-section (b) showing the arrangement of the cytoplasmic channel formed by the plasma membrane and ER and the proteins that divide this space. Taken from Lucas and Lee (2006)

The biogenesis of primary PD occurs during the formation of the cell plate during cell division. Secondary PD are biosynthesised *de novo* post-cytokinetically during cell expansion to maintain PD density across the growing cell wall and undergo degeneration at selected cell–cell interfaces in particular cell types where cytoplasmic isolation is necessary (Faulkner *et al*., 2008). Altering the frequency of PD between cells and fine tuning plasmodesmal permeability at particular cell junctions and developmental stages allows higher plants to maintain dynamic control over the formation of symplasmic domains (Oparka, 2004).

PPUs have been shown to have size exclusion limits (SEL) of up to 60 kDa (Stadler *et al*., 2005), which is substantially greater than that reported for PD connecting mesophyll or epidermal cells (Wolf *et al*., 1989). Mature SEs retain minimal function and are enucleate, therefore a large proportion of the SE solutes and all of the macromolecules are probably CC-derived and enter the SE via PPUs (Liesche and Schulz, 2013). By regulating molecular transport into and out of the phloem, PD and PPU facilitate integration of localised cellular communication into a long-distance signalling network, of which RNA is an integral part (Spiegelman *et al*., 2013).

Evidence of active intercellular trafficking of large macromolecules such as non-cell autonomous proteins (NCAPs) and some RNA species has been recorded (Lucas and Lee, 2004). Non-cell autonomous RNA including messenger RNA (mRNA), short interfering RNA (siRNA) and microRNA associated with systemic signalling have been suggested to traffic between cells via PD (Ruiz-Medrano *et al*., 1999, Voinnet *et al*., 1998).

Studies of viral RNA movement between cells provided the first direct experimental evidence that PD mediate the trafficking of proteins and nucleic acids (Gilbertson and Lucas, 1996). The plasmodesmatal SEL has been shown to alter in plants transgenically expressing a Tobacco Mosaic Virus movement protein (MP)(Wolf *et al*., 1989). A broad class of virally-encoded MPs, which are essential for the cell-to-cell spread of infection, has now been characterised (Gilbertson and Lucas, 1996, Wolf *et al*., 1989, Deom *et al*., 1992). The first evidence of intercellular movement of plant endogenous RNA via PD was from microinjection assays with the KNOTTED1 (KN1) homeodomain transcription factor. The KN1 protein moves from cell to cell via PD and facilitates movement of its own mRNA into neighbouring cells (Lucas, 1995). Another endogenous protein, CmPP16, is able to modify

the SEL of PD and thus facilitate movement of RNA molecules for which it has the capacity to bind, including its own mRNA. The *CmPP16* gene has some sequence homology to viral MPs (Xoconostle-Cazares *et al*., 1999, Kragler *et al*., 2000).

PD probably traffic NCAPS and ribonucleoprotein complexes by either a gate-open/gate-closed (GO/GC) pathway (Figure 1-3) whereby the openness of the PD, as determined by binding of 'gate-open' or 'gate-closed' proteins, dictates which molecules are able to pass through PD by passive diffusion (Lucas and Lee, 2004). Alternatively, passage of macromolecules may be by way of a selective trafficking mechanism in which carriers and/or chaperones deliver NCAPS or RNA–protein complexes to plasmodesmatal docking proteins, where binding induces microchannel dilation and selective trafficking into neighbouring cells, probably via an actin/ER network (Wright *et al*., 2007, Radford and White, 1998).

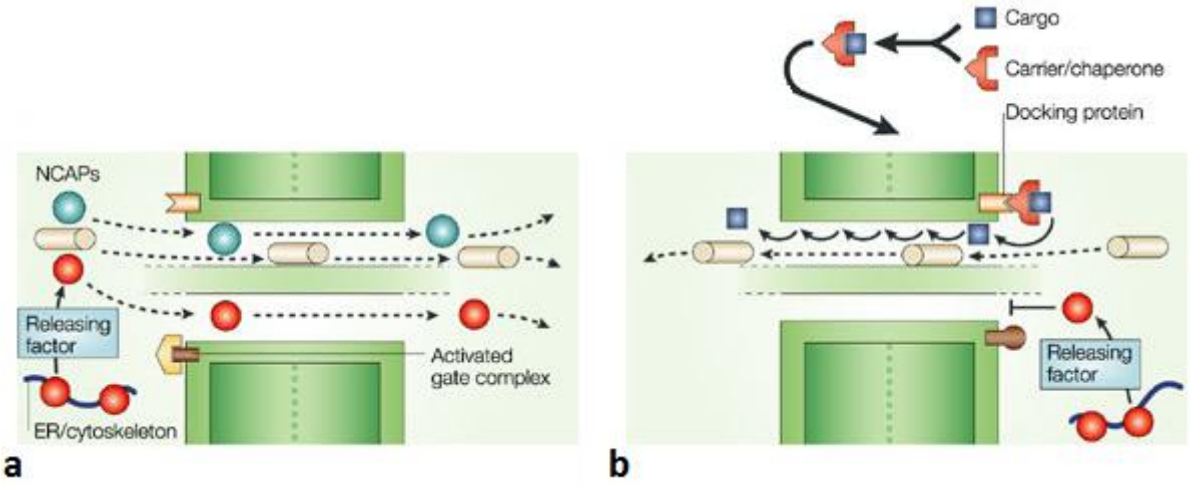


Figure 1-3 Schematic representation of: a) Gating model for the cell-to-cell movement of non-cell-autonomous proteins (NCAPs) through microchannels which dilate in response to the binding of a 'gate-open' protein (GO) to its cognate plasmodesmal gating receptor; and b) Plasmodesma-selective NCAP pathway. Carriers and/or chaperones deliver cargo (proteins or RNA–protein complexes) to a plasmodesmal docking protein, where binding induces microchannel dilation, followed by selective trafficking into neighbouring cells. From Lucas and Lee (2004).

Limitation of movement through PD is therefore regulated by either the binding of a 'gate-closed' protein or by the absence of a carrier/chaperone protein (Figure 1-4). Symplasmic isolation of cells can be achieved by the occlusion of PD. Callose, a β-1,3 glucan polymer, is an important regulator of PD size-exclusion limit as its reversible deposition at the plasmodesmal orifice can restrict or completely inhibit plasmodesmal trafficking (Radford and White, 2001, Levy *et al*., 2007). In fact a more extreme example of this activity can be observed during the formation of sieve-plate pores. Callose deposition around PD precedes the degeneration of appressed ER within them and disintegration of adjacent regions of cell wall. The callose plugs are eventually degraded to leave open pores characteristic of mature SEs (Lucas and Lee, 2004).

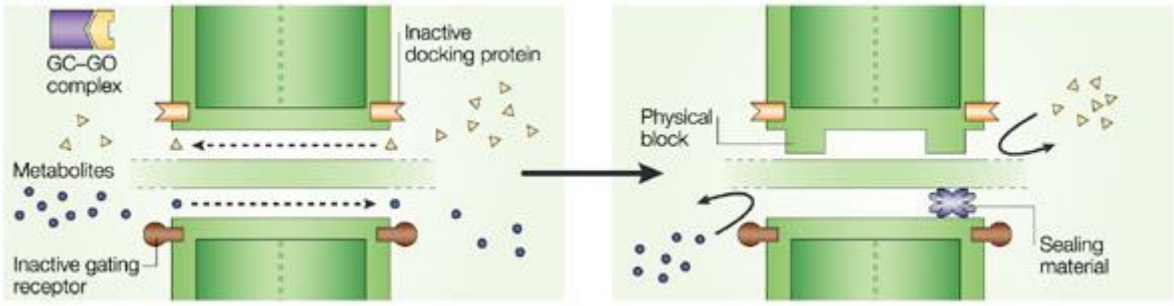


Figure 1-4 Restricted trafficking occurs by either removal of a gate-open protein (GO), through interaction with a gate-closure protein (GC), by carrier release from the plasmodesmal docking protein, or by occlusion of plasmodesmal microchannels. From Lucas and Lee (2006).

The importance of this system for regulating movement of macromolecules is exemplified by the systemic spread of RNA silencing, whereby small interfering RNA (siRNA) can move across graft junctions and travel throughout the plant and have a systemic action in target

cells (Dunoyer *et al.*, 2010). This mechanism regulates gene expression in a non-cell-autonomous way but does not spread into symplasmically isolated guard cells, implying that PD mediate the transport of nucleic-acid based gene-silencing signals (Voinnet *et al.*, 1998).

1.2.3 Long-distance transport via the phloem

In young sink leaves, the collection phloem is immature and does not function in transport. During leaf maturation, sink-to-source transition occurs and the minor vein phloem becomes able to carry sap (Turgeon, 2006). During this process the SE becomes enucleate, vacuolar membranes disappear, Golgi bodies and mitochondria decline in number and the remaining cytoplasmic constituents are concentrated in a parietal layer, forming a central conduit for flowing sap. The lateral ends of the SE become interspersed with sieve-plate pores, which eventually allow movement of sap between adjacent SEs. At this stage the biochemistry of the CC also alters to allow uptake and transport of sugars, amino acids and other phloem mobile solutes (Pritchard, 2007).

Phloem transport can be usefully divided into three processes: phloem loading; translocation and unloading.

1.2.3.1 Phloem loading

Photosynthate is loaded into the sieve-tube system of the collection phloem, which is localised to the minor veins of mature leaves. Solutes move into the SE via the CC, which, in turn, receives solutes from the mesophyll. Movement of solutes into the CC can take one of two pathways: apoplasmic, moving across plasma membranes and travelling part of the route through the cell wall continuum; or symplasmic, moving intercellularly through interconnecting plasmodesmata (Pritchard, 2007). Examples of both types of loading can be

found in different plant species, although it has been suggested that the evolutionary trend has been towards the apoplasmic route, which gives plants increased control over solute transport (Goggin *et al*., 2001). In most species PD are lost following sink-to-source transition, consistent with a switch to apoplasmic loading. The absence of PD is not always indicative of this form of loading, however, as plants with open symplasmic pathways have been observed to have a higher turgor pressure in the SE than the mesophyll, a state which requires some degree of controlled apoplasmic loading. Similarly, higher sucrose levels have been observed in the SE than in the mesophyll which may indicate apoplasmic loading, although another hypothesis, known as the 'polymer trap,' has been proposed whereby sugars are polymerised subsequent to entry into the SE, preventing backward diffusion via PD (Turgeon, 1996). This would explain movement of solutes against the concentration gradient during symplasmic loading.

Apoplasmic phloem loading is mediated by solute transporters, the specificities and expression patterns of which impose strict qualitative control on phloem sap composition (Lalonde *et al*., 2003a, Lalonde *et al*., 2003b, Sauer, 2007). However the transporters and channels involved are only known for a few solutes, detailed below.

Plant sucrose transporters belong to the major facilitator superfamily of transport proteins (Marger and Saier, 1993). The molecular physiology of these transporters has previously been reviewed (Sauer, 2007) and will briefly be discussed here. The most extensively studied of the sucrose transporter genes is *SUT1* (*Arabidopsis* homologue *SUC2*), which has been identified in many plant species, including potato, tobacco and tomato (Kuhn *et al*., 1999). The SUT1 protein has been found to transport ^{14}C-labelled sucrose in a pH-dependent manner, and downregulation of the coding gene (or *SUT1*) led to an accumulation of sucrose

in source leaves (Kuhn *et al*., 1996, Burkle *et al*., 1998). SUT4 is also involved in sucrose loading into minor veins (Weise *et al*., 2000). The *Arabidopsis* SUC3 protein is localised to the SE, as well as to numerous sink cells and tissues, such as guard cells, trichomes, germinating pollen, root tips and the developing seed coat and is strongly induced upon wounding of *Arabidopsis* tissue (Meyer *et al*., 2004). The *SUC1* homologue, *SUT2*, shows increased expression in phloem tissues (Barker *et al*., 2000, Vilaine *et al*., 2003).

Movement of solutes within the plant can be studied using a tracer such as green fluorescent protein (GFP). GFP is found in the photoorgans of the jellyfish *Aequorea victoria* and has been cloned and expressed in organisms such as *Escherichia coli*, *Caenorhabditis elegans,* algae and pigs (Chalfie *et al*., 1994). By coupling GFP to a protein of interest, the movement of that protein can be tracked by exciting a cell or tissue with a light source and recording the fluorescence of GFP. Sucrose loading and unloading can be visualised by attaching GFP to the *AtSUC2* promoter, which regulates expression of the CC-specific *AtSUC2* sucrose–H^+ symporter gene (Imlau *et al*., 1999). In species where sucrose is not the primary sugar produced, other transporters have been isolated; for example a mannitol/proton symporter in celery (Noiraud *et al*., 2001a, Juchaux-Cachau *et al*., 2007) and a sorbitol/proton symporter in common plantain (Ramsperger-Gleixner *et al*., 2004).

Due to the pH dependent nature of these symporters, ATPases are required to pump protons out of the cytosol in order to generate the proton motive force necessary to energise them. The plasma membrane ATPase family contains 12 members, several of which have been localised to the SE-CCC (DeWitt and Sussman, 1995).

1.2.3.2 Translocation

Sap movement throughout the plant is via pressure-driven bulk flow (van Bel *et al*., 2002). The hydrostatic pressure required to drive solution through the phloem is generated by the loading of solutes into the collection phloem. Loading of sucrose across the SE-CCC plasmalemma lowers the SE water potential and facilitates osmotic water inflow through aquaporins (Patrick *et al*., 2001). The pressure generated elevates turgor pressure and drives the phloem sap out of the leaf via the leaf lateral veins, from where it enters the main translocation stream (Gould *et al*., 2005).

Pressure differences can be measured along the phloem and turgor is higher near sources than at sinks. These pressure differences, along with the properties of the sieve tube and the phloem sap, determine the rate and direction of transport (Fisher and Cash-Clark, 2000). The osmotically driven turgor differences along the tube provide the driving force and the sieve tube system provides resistance to flow, at various levels. For example, resistance of membrane barriers; plasmodesmatal conductance and density; the diameter, number and openness of sieve-plate pores; and the number of functional sieve elements are all determinants of sap flow rate (Pritchard, 2007). Models of sap flow are based on the Münch hypothesis or pressure/mass flow hypothesis and modifications of it (De Schepper *et al*., 2013). The theory states that a high sugar concentration at the source draws water in by osmosis. This generates a high turgor pressure, which pushes solution through the SE towards the sink. The concentration gradient, and thus the turgor pressure, is maintained by unloading of sugars at the sink. The basic assumption is that flow through the phloem system can be compared to the flow through a tube as described by the Hagen-Poiseuille

relationship (van Bel and Hafke, 2005). This relates the rate of flow to the properties of the tube (length, radius) and of the solution (viscosity).

1.2.3.3 Unloading

Sap moves throughout the plant towards sink tissues, which are regions of the translocation stream where solutes are taken up for use in various processes. For example, developing leaves, fruits, seeds, growing roots and storage tissues are all sinks. In these regions, efficient unloading is necessary in order to maintain sufficient turgor differences and thus maintain flow. Similar to phloem loading, cells in sink tissues can take up solutes either by symplasmic delivery, through open PD, or apoplasmically across membranes (Oparka, 1990). Unloading at a specific sink may be exclusively apoplasmic, for example in seeds, or may show developmental or spatial variation, as in growing roots and fruits (Zhang *et al*., 2004).

The pathway of sucrose through the plant tissue can be visualised using GFP as a marker. Sucrose was found to be unloaded symplasmically from the phloem of *Arabidopsis* into sink tissues, such as the seed coat, the anther connective tissue, cells of the root tip, and sink leaf mesophyll cells. GFP was trafficked through PD between the sieve element, companion cells and sink mesophyll cells. The size exclusion limit of PD can change during organ development (Imlau *et al*., 1999).

1.2.4 Phloem as a nutrient delivery system

Three main types of sugar are known to be transported in SE sap: sucrose, raffinose-family oligosaccharides (e.g. raffinose, stachyose, verbascose, ajugose), and polyols (e.g. mannitol, sorbitol) (Noiraud *et al*., 2001b). The dominant sugar in most species is sucrose (Smith and Milburn, 1980, Gould *et al*., 2004). It has been measured in concentrations as high as

150mM in willow (Weatherley *et al*., 1959) and 900mM in maize (Ohshima *et al*., 1990). In some species, however, mannitol or sorbitol is more abundant (Moing *et al*., 1997). Reducing sugars, such as glucose and fructose, on the other hand are absent from the phloem, or are found only in trace amounts, probably due to a need to minimise oxidative damage (Raven, 1991).

SE sap also contains high concentrations of free amino acids (Dinant *et al*., 2010a). Concentrations have been found to vary greatly with species, for example in *Arabidopsis*, amino acid concentrations are around 49mM (Zhu *et al*., 2005), whereas in rice they can be as high as 600mM (Fukumorita and Chino, 1982). Amino acid concentration has also been found to be elevated in the SE following drought (Ponder *et al*., 2000) and to vary diurnally in *Arabidopsis* (Gattolin *et al*., 2005).

The major cations present in SE sap are potassium, calcium, magnesium and sodium; phosphate, sulphate, chloride, nitrate, bicarbonate and malate are the main anions (Hayashi and Chino, 1986). Micronutrients such as iron, copper, manganese, zinc, boron and molybdenum, are also present (Douglas, 2003), as are the vitamins thiamine and ascorbic acid (AsA) (Dinant *et al*., 2010b).

Phloem sap constituents are not uniform and can vary between species, in varying environmental conditions, diurnally (Gattolin *et al*., 2008) and in different regions of the plant. For instance, a diurnal pattern in root growth was attributed to variation in delivery of carbohydrate to growing regions with varying irradiance (Muller *et al*., 1998). However, these types of changes are not observable in many plant species as they are buffered by releases from carbohydrate stores in order to maintain sufficient turgor pressure in the SE (Slewinski, 2011). Plant developmental stage has also been shown to affect phloem

contents. For example, SE amino acid concentration was found to vary in oats and barley during development, falling during maturation before rising again in older plants (Weibull, 1987). Further to this, dual phloem systems have been discovered in the *Cucurbitaceae* (cucurbits) and metabolomic and proteomic analyses indicate that these may have independent functions (Zhang *et al.*, 2012b). The cucurbit fascicular phloem or bundle phloem is contained within the vascular bundles and has a major role in sugar transport, whilst the peripherally situated extrafascicular phloem transports other solutes as well as /performing signalling and defence-related role (Zhang *et al.*, 2010a).

1.2.5 Phloem as a mediator of whole plant communication

Both small molecules and macromolecules are trafficked long-distance in the phloem (Turgeon and Wolf, 2009). Many have been shown to influence gene expression or other cellular processes in the cells at their final destination resulting in altered development, physiology or defence processes (Turnbull and Lopez-Cobollo, 2013). Having a non-cell-autonomous function makes these molecules important as long-distance signals and the current knowledge of phloem-delivered signalling molecules is summarised below.

1.2.5.1 Phytohormones and small molecule signalling

Phytohormones have been detected in both phloem and xylem sap (Hirose *et al.*, 2008, Rahayu *et al.*, 2005, Thorpe *et al.*, 2007, Gowing *et al.*, 1993b). This implies a role in long-distance signalling (Gowing *et al.*, 1993a, Jackson, 1997). Auxin (Swarup *et al.*, 2001, Golan *et al.*, 2013), cytokinins (Kamboj *et al.*, 1998, Hirose *et al.*, 2008), gibberellins, abscisic acid (ABA), 1-aminocyclopropane-1-carboxylic acid (the precursor of ethylene), jasmonic acid

(JA)(Thorpe *et al*., 2007), and salicylic acid (SA)(Shulaev *et al*., 1995) have all been identified in the phloem.

Nitric oxide (NO) was detected in phloem sap of *V. faba* (Gaupels *et al*., 2008b). NO is a small uncharged molecule that easily crosses cell membranes, acting as an important signalling messenger (Seabra *et al*., 2014). It has been shown to respond to abiotic stress such as inducing stomatal closure during drought (Garcia-Mata and Lamattina, 2009), Na^+ sequestration during salt stress (Zhao *et al*., 2007), inducing ROS scavenging enzymes during heat stress (Uchida *et al*., 2002), and functioning as a messenger in gene-for-gene defence responses during disease infection (Floryszak-Wieczorek *et al*., 2007, Delledonne, 2005). NO has also been shown to alter plant gene expression (Arasimowicz and Floryszak-Wieczorek, 2007). The dicarboxylic acid, azelaic acid, has also been found in *Arabidopsis* phloem sap and acts in systemic resistance (Jung *et al*., 2009).

1.2.5.2 Protein translocation

The phloem is enriched in proteins involved in redox control, stress responses, calcium and G-protein signalling and flowering (Giavalisco *et al*., 2006). Some phloem-mobile proteins appear to have a role in defence responses following insect or microbial attack. For example, in solanaceous species, tissue damage caused by chewing insects results in the systemic induction of protease inhibitors (Korth and Dixon, 1997). The phloem-mobile signal appeared to be systemin, an 18-mer polypeptide that is produced in the CC (Howe, 2004b). Systemin binds a membrane associated receptor, leading to upregulation of the mitogen-activated protein (MAP) kinase (Holley *et al*., 2003) and jasmonate signalling pathways (Pearce *et al*., 1991). As systemin triggers these signalling cascades within vascular cells, signals are then relayed to surrounding tissues where defence genes are upregulated

(Stratmann and Ryan, 1997). As a result of such signaling activities, the basic defences of the phloem sap are enhanced by additional chemical weapons (Mewis *et al*., 2005).

1.2.5.3 RNA trafficking

Various mRNAs, siRNAs, miRNAs, rRNAs and tRNAs, with a variety of purported roles, have been identified in the phloem of several plant species (Asano *et al*., 2002, Vilaine *et al*., 2003, Hedrich *et al*., 2008, Wolf *et al*., 2007, Sasaki *et al*., 1998, Gaupels *et al*., 2008a, Dinant *et al*., 2008). Hundreds of mRNAs, which encode proteins including transcription factors, stress response proteins, metabolic enzymes, transporters or other structural components, have been identified in the phloem (Divol et al., 2005b, Deeken et al., 2008). Transcripts are important signalling molecules as they can transmit a signal in non-functional form. If the protein products were transported, they may interact with substrate in the SE sap during translocation and cause undesired or deleterious effects. The importance of phloem mRNA is highlighted by the absence of RNases in SE sap (Sasaki *et al*., 1998, Doering-Saad *et al*., 2002).

The subset of mRNAs present in the phloem sap appears distinct from those present in other tissues or organs (Hedrich *et al*., 2008). Some of these transcripts have been found in distantly located sink organs (Kehr and Buhtz, 2008, Haywood *et al*., 2005, Kim *et al*., 2001) suggesting that they may have a role in long-distance signalling (Kehr and Buhtz, 2008, Kehr, 2009). In situ hybridization experiments have demonstrated the long-distance movements of mRNA through grafted tissue (Ruiz-Medrano *et al*., 1999) and mRNA moving in the SE can have phenotypic effects distant from its source (Haywood *et al*., 2005, An *et al*., 2004, Corbesier *et al*., 2007, Turnbull *et al*., 2002). An example of this long-distance activation of genes is the translocation, in *Arabidopsis*, of mRNA of the FLOWERING LOCUS (T) gene to the

shoot apex and activation of other genes involved in the initiation of flowering (Huang *et al*., 2005).

Interestingly, some RNAs present in phloem sap are also able to cross host-parasite junctions. For example, several mRNAs were found to move from tomato to the dodder *Cuscuta pentagona* (Westwood *et al*., 2007, Sinha *et al*., 2008). This presents the potential to use gene-silencing technologies to improve crop resistance against parasites by exploiting the interspecific trafficking of small interfering RNAs to target vital functions in the parasite. The use of dsRNA or RNA interference expressed *in planta* has already proved successful against the western corn rootworm and the cotton bollworm (Roberts *et al*., 2007, Chen *et al*., 2007).

A range of RNA-trafficking proteins have also been isolated from SE sap (Lough and Lucas, 2006, Li *et al*., 2011b). In pumpkin sap, for instance, at least 82 proteins annotated as RNA-binding were described (Lucas *et al*., 2009). Some of these proteins were proposed to be involved in the unfolding and refolding of RNAs trafficking through PD between CC and SE (Lough and Lucas, 2006). The importance of this selective trafficking is highlighted in *Arabidopsis*, where the transcript profile of phloem sap does not reflect the profile of CC, indicating that not all transcripts of the CC are transported into the SE (Hedrich *et al*., 2008). Similarly, in melon, only a small fraction of mRNAs were able to translocate across graft junctions (Wolf *et al*., 2007). This suggests that, besides long distance signalling, other, local functions may be undertaken by these RNAs.

1.2.6 Studying the phloem

1.2.6.1 Localising phloem constituents in situ

Genes and proteins can be localised using such techniques as *in situ* hybridization and reporter gene visualization (Kerk *et al*., 2003). *In situ* hybridisation involves labelling DNA, RNA or oligonucleotides either radioactively, fluorescently or with biotin. A section of target tissue is taken and the labelled probe is added. The reporter molecules can then be visualised (Engler *et al*., 2001).

In situ hybridisation has been used to localise mRNA within the phloem system. For example, the mRNA of an abundant rice phloem thioredoxin protein (TRX h), was examined by taking serial cross sections of rice shoots containing the labelled mRNA (Ishiwatari *et al*., 2000). It was found to be localised mainly in the CC in mature leaves and roots but in various cell types in immature leaves and root tips. This allowed observation of mRNA accumulation across developmental stages of the vascular bundle. Similarly, ISH was used to confirm the SE origin of cDNA generated from exuding SE sap from *Ricinus communis* (Doering-Saad *et al*., 2006). *In situ* RNA hybridization experiments can thus provide gene expression data at high resolution. The technique is not suitable for the study of large numbers of genes, however.

1.2.6.2 Phloem enriched samples

In a limited number of plant species, it is possible to separate the vascular tissue from the surrounding parenchyma tissues manually. For example, the petioles of celery (*Apium graveolens*) plants have been exploited in this manner, to determine the transcriptome of these phloem-enriched samples by expressed sequence tag (EST) sequencing and cDNA macroarray hybridisation (Vilaine *et al*., 2003). The same approach was used to investigate

the systemic phloem response of celery to infestation by *M. persicae* (Divol et al., 2005a). Vascular strands have also been excised from *Arabidopsis* rosette leaves in preparation for enzymic protoplast isolation (Ivashikina *et al*., 2003).

1.2.6.3 Single cell sampling

Certain cell types, such as natural single-cell suspensions, low-complexity aggregations of cells such as pollen, and surface tissues such as trichomes, can be identified and separated relatively easily by dissection, homogenization, abrasion, or other physical means (Nelson *et al*., 2008). Isolating particular cell types from organised plant tissue is more complicated and there are several methods of achieving this. One technique is fluorescence-activated cell sorting (Birnbaum *et al*., 2005). This relies on being able to separate the cells and to visualise and distinguish these cells from those of surrounding tissues. The first step requires the production of suspensions of plant protoplasts. Some protoplasts can be identified and sorted at this stage, based on endogenous properties, such as size and chlorophyll content (Galbraith, 2010). Others require labelling to aid detection. These protoplasts can be induced to express markers such as GFP and are then subjected to fluorescence-activated sorting (Sheen *et al*., 1995). The sorting process can be rapid, providing 1000 or more purified protoplasts per second. A concern with this method in transcriptomics experiments is that gene expression may change during protoplast preparation (Grosset *et al*., 1990, Birnbaum *et al*., 2005), however it has recently been suggested that only a small subset of genes are activated during protoplasting and the overall gene expression profile is largely unaltered (Rogers *et al*., 2012). Zhang *et al*. (2008)successfully isolated nuclei of *Arabidopsis* CC by transgenically expressing nuclear-targeted GFP in the CC then purifying the nuclei by

fluorescence-activated sorting. Using this technique they were able to extract CC-specific RNA for microarray analysis and identify genes expressed in this cell type.

An alternative approach is laser-capture microdissection (LCM). One laser-based method employs a laser to adhere cells of interest to a thin film, which is subsequently removed, exposing the target cells (EmmertBuck *et al*., 1996). A second method uses laser to excise cells, which may be procured by laser pressure catapulting (laser microdissection coupled with laser pressure catapulting; LMPC). Despite recent advances, LCM remains expensive and time-consuming (Curran *et al*., 2000) and is a destructive technique (Asano *et al*., 2002).

Another important method of sampling from individual cells is microsampling. Microsampling techniques involve direct sampling of cellular contents or marked protoplasts using glass microcapillaries (Brandt *et al*., 1999). Only small samples can be obtained by this method, however; single cells contain on average 150-760fg mRNA (Dresselhaus *et al*., 1994). This can be overcome to an extent by sampling from multiple cells of the target type.

1.2.6.4 SE sap sampling

Sampling from the SE differs from other cell sampling methods as, although a single cell type is being sampled from, the SE is a series of interlinked cells rather than discrete entities and sampling from living plants is possible. This makes sampling more challenging but also gives the advantage of being able to sample over time to increase sample volumes. Collection is complicated by calcium-mediated phloem defence mechanisms such as forisome or callose-mediated sealing of sieve plates following SE damage, preventing further exudation (Furch *et al*., 2007). Various approaches have been devised to overcome this problem, such as selecting species with a reduced sealing response (Milburn, 1970) and using calcium chelators to inhibit the calcium-mediated response (King and Zeevaart, 1974).

The most basic method of sampling SE sap is by collecting exudate from an incision in the plant stem. For example, sap will freely exude from a wound in the bark of *R. communis* (Milburn, 1970). However, these types of phloem-exudate samples have been shown to be contaminated by the contents of other cell types. For example Ruiz Medrano *et al.* detected the presence of Rubisco mRNA after 30 cycles of RT-PCR (1999). A second method is EDTA-mediated sap extraction. A stem or petiole is severed and immersed in 3mM EDTA solution, which, as a calcium chelator, prevents calcium-mediated sealing of the sieve tubes (King and Zeevaart, 1974). Sap will therefore flow freely out into the solution. This technique is a relatively quick and easy method for collecting phloem-enriched samples. However EDTA collection does not allow the estimation of sample volumes, and so concentrations, and instead delivers information of solute ratios (Tetyuk *et al*., 2013). In addition this method can introduce artefacts into data, in the form of changes in sugar and amino acid composition (Girousse *et al*., 1991) and the introduction of contaminative proteins (Gaupels *et al*., 2008c). This may be because EDTA removes calcium anchors of the plasma membrane, causing cells to become leaky (van Bel and Hess, 2008). Because of the low copy number of phloem transcripts, even small changes in RNA concentration will provide misleading results. In order to avoid this and to detect subtle changes in phloem composition, a purer sample is required.

Aphids and other PFIs insert inert chitin stylets directly into the phloem and sap, under high osmotic pressure, is passively removed. This has been exploited by researchers in a method termed stylectomy (Kennedy and Mittler, 1953). Stylectomy uses aphids as a tool to extract sap by severing the stylets with a microcautery device while the insects feed (Figure 1-5).

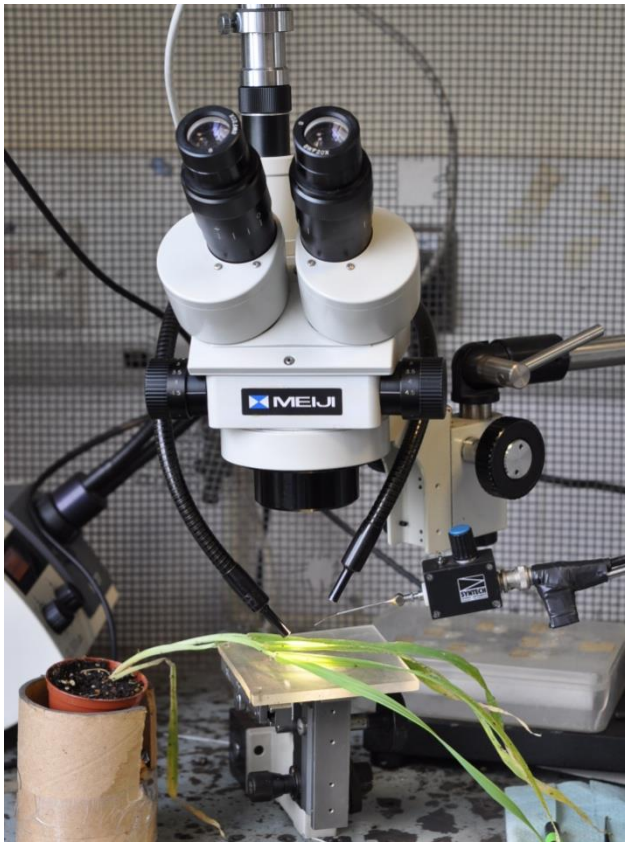


Figure 1-5 Stylectomy set-up showing microscope, light source and sharpened tungsten needle attached to micromanipulator.

The inert chitin stylets remain in the plant and sap flows out and can be collected under oil or using a pulled glass microcapillary (Figure 1-6). Sap quantities in the range of 1-2nl per minute can be obtained using this method (Doering-Saad *et al.*, 2002). The solute profile and the osmotic pressure of the SE sap can be measured (Gattolin *et al.*, 2008, Pritchard, 1996).

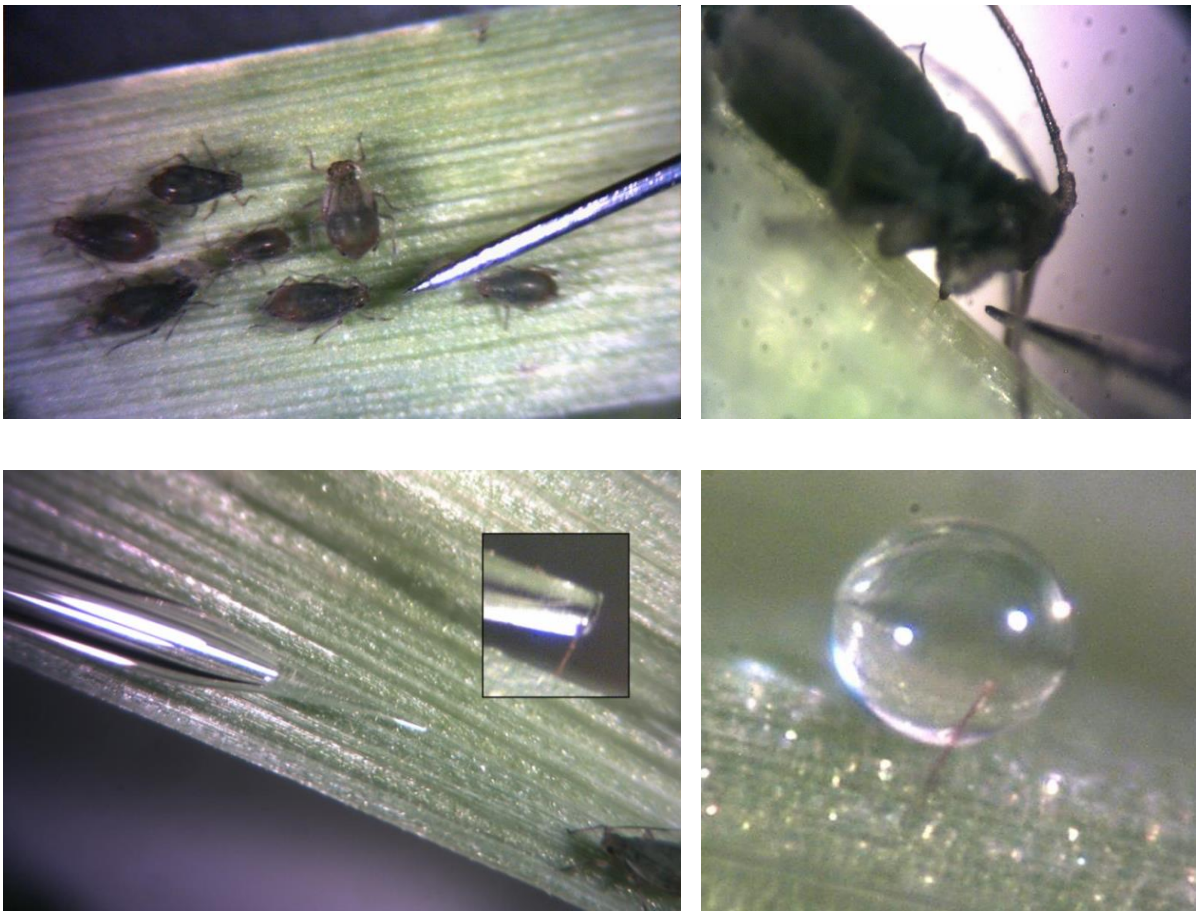


Figure 1-6 Aphid stylectomy. Showing (clockwise from top left): position of aphids on a barley leaf and sharpened tungsten needle; position of needle approaching stylets prior to cauterisation; phloem sap exuding from a cut stylets; collection of sap into a pulled glass microcapillary and (inset) close-up of position of collection capillary against stylets.

1.3 Phloem feeding insects (PFIs)

Despite being nutrient-rich relative to other plant tissues, the phloem has been exploited as a primary or sole food source by a relatively narrow range of animals, all of them insects (Douglas, 2006). All phloem-feeding insects (PFIs) are of the order Hemiptera. Phloem feeding has evolved multiple times among the Hemiptera, and is seen most commonly in the Sternorrhyncha (mealybugs, psyllids, whiteflies and aphids), the Auchenorrhyncha (most

planthoppers and many leafhoppers) and the phytophagous Heteroptera (lygaeids, pentatomids, and coreids)(Douglas, 2006).

A small number of other groups consume SE sap as a dietary supplement but do not rely on it for nutrition. These include animals outside of the insects, such as *Sphyrapicus* spp. (sapsuckers), which are a member of the woodpecker family and are able to drill into the phloem and extract sap (Daily *et al*., 1993). Sapsucker holes are exploited as a secondary food source by other animals such as hummingbirds, orange-crowned warblers, and chipmunks (Daily *et al*., 1993).

Aphids are the most studied phloem-feeders because they are an economically important pest of temperate and glasshouse crops (Blackman and Eastop, 2007). They can greatly reduce crop yield by removing large quantities of sugar-rich sap from their hosts and also by transmitting phytoviruses (Martin *et al*., 1997) and allowing growth of sooty moulds on their sugary excreta (Quisenberry and Ni, 2007). In order to develop crop protection strategies against aphids it is important to understand the interaction between aphids and plant hosts in its entirety, i.e. from whole-plant responses down to changes in metabolites, protein complement and gene expression. The relationship between susceptible plants and aphid pests is particularly interesting because an aphid population, or colony, can exist on a plant long-term, often with few or no external symptoms, as long as aphid numbers do not get too high. The aphids in this case must be supressing or avoiding plant defences to a sufficient degree to allow continual feeding. Whilst virus transmission requires only a single infected aphid (Kalleshwaraswamy and Kumar, 2008), yield loss due to nutrient withdrawal is dependent on the level of infestation. For example, the chlorophyll content, carbon dioxide assimilation and total soluble carbohydrate content of barley plants were all depressed by

relatively low levels of *S. graminum* infestation (more than 18 aphids per plant), whilst proline levels were elevated (Cabrera *et al*., 1994). In the same study, infestation levels of 100 insects per plant resulted in sugar concentrations five times lower than in control plants. Reduced leaf water potential was observed in infested plants in that study, suggesting that the observed metabolic changes may be linked to osmotic stress induced by sap removal. In the current study, different levels of *R. padi* infestation were imposed on barley plants to identify the scale of the effects on growth and SE sap exudation from cut stylets under various stress levels.

1.3.1 Aphid pests

Aphids are members of the super family Aphidoidea (Minks and Harewijn, 1988) and are a particular problem in northern temperate regions, although they can be found worldwide. They affect many agricultural and horticultural crops (Blackman and Eastop, 2007). Some of the most economically important aphid species, in terms of yield losses, are *Aphis fabae* (the black bean aphid), *Aphis gossypii* (the cotton aphid), *Diuraphis noxia* (the Russian wheat aphid), *Macrosiphum euphorbiae* (the potato aphid), *Myzus persicae* (the peach-potato aphid), *Rhopalosiphum padi* (the bird cherry-oat aphid) and *Sitobion avenae* (the grain aphid) (Blackman, 2007).

The current study focuses on the interaction of *R. padi* with host barley plants because individuals of this species are relatively large and have a sedentary nature, which makes them an easier subject for use in the stylectomy technique. *R. padi* have two major reproductive lineages (Figure 1-7)(Hulle *et al*., 1999). The first involves alternation between sexual and asexual reproduction (cyclical parthenogenesis)(Leather *et al*., 1989). These

aphids are heteroecious (colonise two host plant species): a sexual generation colonise the primary host, *Prunus padus* in autumn and lay eggs which overwinter on these trees; then, in spring, asexual (parthenogenetic) generations colonise cereals, including barley. The shorter autumnal days then trigger generation of sexual morphs, which move back to the primary hosts to lay eggs once again (Williams and Dixon, 2007). The phenomena of parthenogenesis, which allows female aphids to reproduce in the absence of males, doubles the intrinsic rate of population increase (r_m) and is the dominant method of reproduction when aphids are colonising new plants and expanding populations (Powell *et al*., 2006). Parthenogenesis is accompanied by viviparity, the birth of live young (Miura *et al*., 2003). This method of reproduction, as well as the phenomena of telescoping of generations, where a new nymph is already pregnant at birth, allows rapid parturition once a suitable host plant is located (Powell *et al*., 2006). Cyclical parthenogenesis has thereby enabled aphids to colonise less favourable climates as species can overwinter in the egg stage - eggs are able to withstand cooling to temperatures that would be fatal to live aphids (Strathdee *et al*., 1995) - then reproduce rapidly during the summer months (Minks and Harewijn, 1988). A second lineage, termed obligate parthenogenesis, involves asexual generations living permanently on cereals (monoecious). This approach leaves aphids susceptible to low winter temperatures but allows earlier colonisation of hosts in springtime (Carter *et al*., 2012).

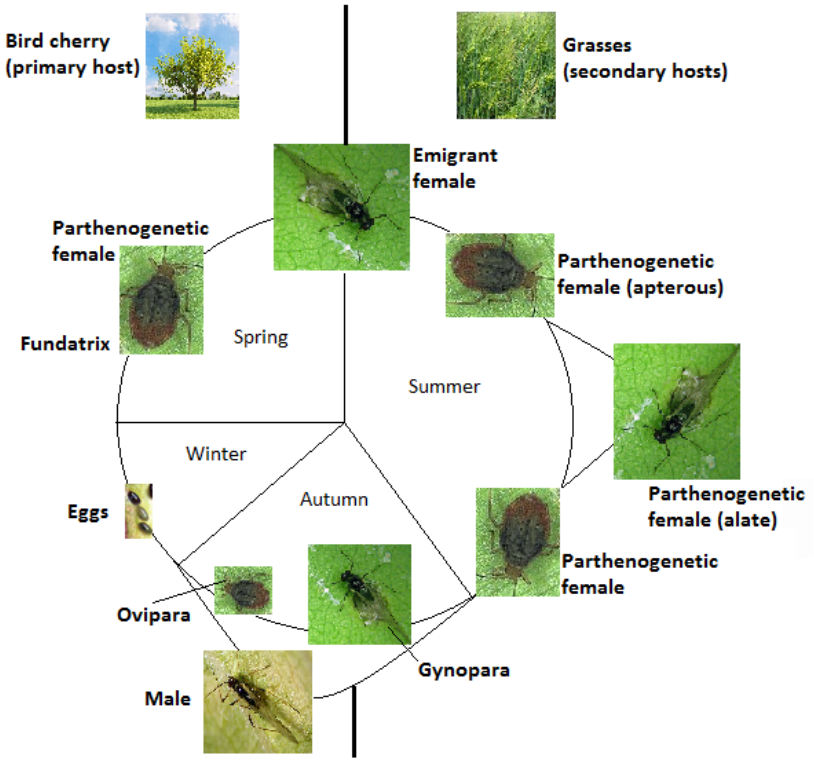


Figure 1-7 Life cycle of the heteroecious bird cherry-oat aphid, *Rhopalosiphum padi*. The holocyclic life cycle includes: in spring, a fundatrix, a wingless parthenogenetic female produces live offspring; emigration to the secondary host by a winged (alate) female; a period of parthenogenetic reproduction on the secondary host; gynopara, a migrant that flies back to the primary host to produce oviparae; ovipara, a wingless, sexual female that mates with a male and lays overwintering eggs. Diagram adapted from http://what-when-how.com/insects/sternorrhyncha-jumping-plant-lice-whiteflies-aphids-and-scale-insects/

R. padi also display a trait termed clonal alary polyphenism, whereby individuals may be alate (winged) or apterous (wingless)(Williams and Dixon, 2007). Production of alate clones is triggered by environmental factors experienced during development, such as host plant quality, crowding, temperature and abundance of natural enemies (Muller *et al*., 2001). This enables alate individuals to fly in search of new host plants when host quality declines or aphid density increases (Muller *et al*., 2001), whilst apterous aphids invest more resources in reproduction (Dixon *et al*., 1993).

1.3.1.1 Anatomy and feeding behaviour

Aphids vary in colour from black *A. fabae,* to pink *A. pisum,* to yellow or green-yellow *D. noxia,* and olive green *R. padi* (Blackman, 2007). The general aphid anatomy consists of the head, thorax and abdomen, a pair of antennae, compound eyes and six jointed legs and a pair of siphunculae, from which pheromones are secreted (Figure 1-8).

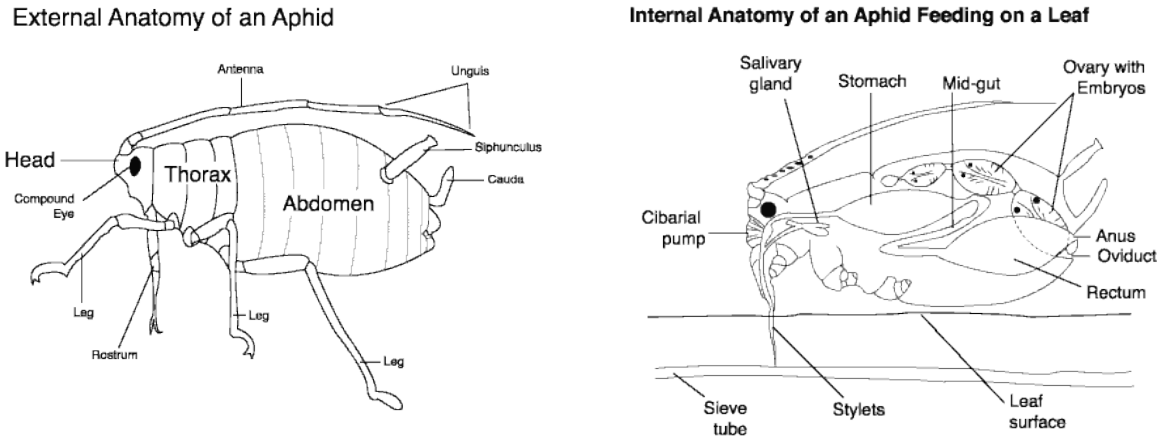


Figure 1-8 The external and internal aphid anatomy. Image from The University of Arizona, Centre for Insect Science Education Outreach (http://insected.arizona.edu/gg/resource/internal.html).

Aphids have evolved a furtive method of feeding whereby their mouthparts have become elongated such that the mandibles and maxillae form long stylets (Figure 1-9), designed to pierce tissues to remove nutrients required for growth (Minks and Harewijn, 1988). The stylets are composed of two outer mandibles and two inner maxillae, together forming a food canal and a salivary canal.

After landing on a potential host, aphids begin to probe the epidermis with their stylets (Powell and Hardie, 2000). The initial stylet insertions last only briefly, generally less than a minute, and seem to provide sufficient information for host acceptance or rejection and induction of flight (Tosh *et al*., 2003a, Powell and Hardie, 2000). Parturition often occurs before the stylets locate the phloem (Tosh *et al*., 2003b); in these cases it can be assumed that the necessary signals for host acceptance are perceived prior to this point and do not come from the SE contents (Tosh *et al*., 2002).

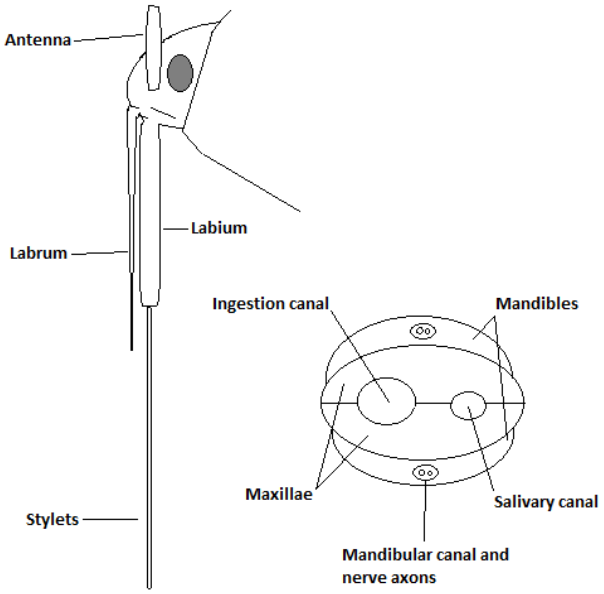


Figure 1-9 Lateral view and cross-section of aphid stylets. Shows labrum, labium and stylets on side view and mandibular and maxillary stylets, salivary and ingestion canals and mandibular canal and nerve axons on cross section. Diagram created from references within section 1.3.1.1.

In order to study aphid feeding behaviours and the movement of the stylets within plant tissues, a visualisation technique has been developed called the Electronic Penetration Graph (EPG)(Tjallingii and Esch, 1993). The EPG method uses a feeding aphid as the final link in a circuit and records voltage changes induced by the various feeding behaviours. It has been shown from analysis of waveforms that distinctive patterns are discernible during different feeding behaviours such as pathway through the mesophyll, cellular punctures, salivation (E1), phloem feeding (E2) and xylem ingestion. EPG in conjunction with microscopy has shown that the stylets are inserted between two epidermal cells and follow an apoplasmic route between the primary and secondary cell wall layers (Tjallingii and Esch,

1993). Cells along this pathway are punctured and sampled by the aphid for navigational cues. These intracellular probes involve puncturing the plasmalemma, injection of a small quantity of watery saliva (discussed below) into the cytosol and withdrawal and ingestion of a small quantity of cytosol/saliva mixture (Prado and Tjallingii, 1994). Cell punctures are brief and become more numerous as the stylets approach the phloem, supporting the theory that the aphid is assessing the internal chemistry of the punctured cells in order to locate the phloem (Martin *et al*., 1997, Tjallingii and Esch, 1993). Intracellular sampling in non-host plants will lead to rejection and stylet withdrawal (Powell *et al*., 2006). It is also thought that apoplasmic factors such as pH and sucrose gradients may play a role in stylet orientation while the stylets are in the extracellular space (Hewer *et al*., 2010).

1.3.2 Saliva

During transit of the stylets through the plant tissue, gelling saliva is secreted and forms a sheath around the stylets, which lubricates and supports the stylet pathway. As well as facilitating movement of the stylets within the plant tissue, the gel sheath is thought to stabilize coaption of the mandibles and maxillae (Pollard, 1973). The gelling saliva also seals cells that are punctured along the pathway so that the aphid can sample their contents. This probably limits plant defence responses due to calcium influx (Will *et al*., 2013). Gelling saliva is primarily composed of proteins (including phenoloxidases, peroxidases, pectinases and β-glucosidases), phospholipids and conjugated carbohydrates (Cherqui and Tjallingii, 2000).

A second type of saliva, termed watery saliva, is secreted into the plant immediately following stylet penetration (Moreno *et al*., 2011). Injection of saliva will continue periodically before and during sustained aphid feeding (Prado and Tjallingii, 1994), as well as

during cell punctures along the pathway to the SE (Martin *et al*., 1997). Watery saliva is a complex mixture of enzymes and other components. Its composition appears to vary amongst different aphid species, and even within the same species, according to diet (Carolan *et al*., 2009, Will *et al*., 2009). It had been hypothesised that aphid host range depends on watery saliva composition (Will *et al*., 2009). This is supported by observed alterations in the survival rates of *A. pisum* on its host *Vicia fabae* following RNA-i suppression of a single salivary protein (Mutti *et al*., 2006). RNAi silencing of the *C002* trancript, abundant in the aphid salivary gland, began to show lethal effects within 2-3 days. EPG studies on the *C002* knockdown mutant suggests that the gene product has a role in location or recognition of the SE and its removal precludes SE sap ingestion by affected individuals, thus reducing aphid longevity (Mutti *et al*., 2008).

1.4 The phloem as a food source

Although SE sap is generally free of toxins and feeding deterrents (Douglas, 2006), it does pose several problems for PFIs.

1.4.1 Limitations due to nitrogen content

Nitrogen is available in SE sap in the form of amino acids (AAs) but is limiting as a nutrient for aphids (Douglas, 2003). AAs can be separated into two categories based on their source. Eleven out of the 20 AAs required to make protein are the non-essential AAs, which animals are able to synthesise within their own bodies. The remaining nine AAs are referred to as the essential AAs as animals are unable to synthesise them and they are therefore required from a food source. The ratio of essential AAs: non-essential AAs in plant phloem sap is roughly 1:4–1:20, compared to a ratio of around 1:1 in animal protein (Douglas, 2003). The essential

AAs are all present in SE sap, but they represent just 8.2% of the total AA amount. The concentration of all of the essential AAs except histidine has been shown to be proportionately lower in phloem sap than in aphid protein (Douglas, 2006). The essential amino acid content of phloem sap therefore appears to be insufficient to support the growth rate of aphids. The single amino acid deletion technique has been used to demonstrate the requirement of AAs and the variability of this requirement amongst aphid species (Wilkinson and Douglas, 2003).

Aphids rely upon an endosymbiont, *Buchnera aphidicola,* to supply the essential AAs that are lacking in the aphid diet (Houk and Griffiths, 1980). *Buchnera* is an obligately intracellular microbe, which has the capacity to synthesise essential AAs from sucrose and aspartate (Douglas, 1998). The bacteria are located in specialised cells known as bacteriocytes in the aphid body cavity, or haemocoel (Macdonald *et al*., 2012). Aphid eggs or embryos are inoculated with *Buchnera* during development (Wilkinson *et al*., 2003) and the offspring will therefore be equipped with the facility to generate essential AAs.

This insight has been acquired through several approaches. Firstly, *Buchnera* have been eliminated from aphids using antibiotics in order to investigate the nutritional dependence of aphids on their symbionts (Wilkinson, 1998). Secondly, aphids will feed on artificial diets, ingested through a membrane (Mittler and Dadd, 1962). This allows manipulation of the diet for experimental purposes. For example, diets deficient in one or more essential AA can be used to investigate aphid capacity to access essential AAs via their symbionts (Sasaki *et al*., 1991). This approach can also be used to investigate diet-derived cues for host plant acceptance by allowing aphids to feed on host-specific artificial diets (Nam and Hardie, 2014). Thirdly, the genome of *Buchnera* has been sequenced (Shigenobu *et al*., 2000).

Integration of knowledge of the genome sequences of *Buchnera* and *A. pisum* (Richards *et al*., 2010) with pre-genomic observations has led to insight on areas such as lateral gene transfer, symbiotic metabolism and regulatory control of symbiont gene expression (Shigenobu and Wilson, 2011a). Crucially, it was shown that, despite losing many metabolic capabilities, including synthesis of most non-essential amino acids, *Buchnera* have retained genes coding for most enzymes in the biosynthetic pathways for essential amino acids.

1.4.2 Limitations due to high sugar content

The high sugar concentrations in SE sap mentioned above present aphids with a food source with an osmotic pressure 2–5 times greater than the osmotic pressure of their body fluids (Douglas, 2003). As aphids must ingest large quantities of nutrient-poor sap in order to obtain sufficient quantities of nutrients, aphids need to overcome the high osmotic pressure in order to prevent transfer of water from the bodily fluids to the gut contents down the osmotic gradient. Evidence as to how this is achieved comes from the aphid excreta, honeydew. When aphids are reared on chemically-defined diets with sucrose as the sole sugar, the dominant honeydew sugars are oligosaccharides comprising mostly glucose moieties (Ashford *et al*., 2000). This honeydew is isosmotic with the haemolymph, not the ingested fluid. It has been suggested that because the osmotic pressure exerted by solutes is determined by their molality and not their weight, the transformation of SE sap sucrose to oligosaccharides would reduce the osmotic pressure of the gut contents (Douglas, 2006).

1.5 Plant defences against phytophagous insects

Plant defence responses can be broadly categorised into two groups: antixenotic, where certain characteristics of a plant make it less attractive to an aphid, for example if the structure of the plant renders the insect mouthparts unsuitable for locating the SE; and antibiotic, where the plant produces toxins which alter aphid physiology, and consequently have a negative impact on aphid performance (Smith and Chuang, 2014). Defences may also differ spatially, with responses close to the feeding site differing from the systemic responses observed in distal tissues (Ferry *et al*., 2011).

Some plant defences are expressed whether or not a pest is present (constitutively), and may be physical (e.g. trichomes) or chemical (Thompson and Goggin, 2006). A subset of secondary metabolites also have a role in chemical defence, for example saponins and benzoxazinoids (Osbourn *et al*., 2003). Other defences are induced upon attack and can be very specific to the particular bioaggressor (War *et al*., 2012).

Initial events (see Figure 1-10 for timescale of defence responses) during an inducible response include changes in plasma transmembrane potential and accumulation of calcium and reactive oxygen species (ROS), both locally and systemically (Mittler and Blumwald, 2015). The changes to the normal levels of these molecules are detected and phytohormone-dependent pathways are triggered (Maffei *et al*., 2007b). It is at this point that the defence responses that are generated begin to differ, according to the particular aggressor (Baxter *et al*., 2014). Grazing insects, for example, which remove entire pieces of tissue and badly damage plants elicit wound responses, including ethylene- (ET) and jasmonate- (JA) dependent defence pathways (Maffei *et al*., 2007a). A similar reaction can be seen in response to necrotrophic pathogens (Thomma *et al*., 2001a, Thomma *et al*.,

2001b). Biotrophic pathogens trigger a salicylate- (SA) dependent response (Thomma *et al*., 2001a, Thomma *et al*., 2001b). The plant response to aphids in compatible interactions is interesting as the activation of the SA-dependent pathway appears to repress the JA response (Cipollini *et al*., 2004, Traw *et al*., 2003).

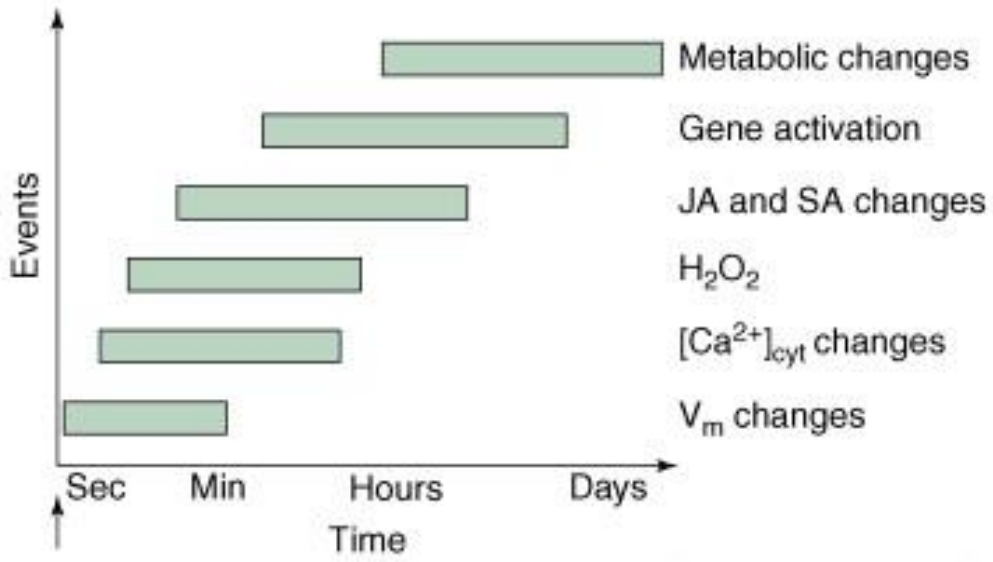


Figure 1-10 Representation of the timescale of the events of plant defence responses. Shows changes in plasma membrane potential (V_m), variations of cytosolic Ca^{2+} concentrations, hydrogen peroxide generation (H2O2), changes in jasmonic acid (JA) and salicylic acid (SA) signalling, gene expression changes and metabolic changes. From Maffei *et al* 2007.

ROS are a group of free radicals, reactive molecules, and ions that are derived from O_2. At low/moderate concentration, ROS act as secondary messengers in intracellular signalling cascades that mediate various responses in plant cells (Hancock *et al*., 2002, Yan *et al*., 2007). During stress events, levels of ROS increase within plant tissues. Elevated levels of ROS are toxic to both an attacking insect and to the host plant. The over-production of ROS under these conditions generates an oxidative burst, termed a hypersensitive response (HR), which is thought to restrict biotrophic pathogen growth (Pumplin and Voinnet, 2013). HRs are both local and systemic (Divol et al., 2005a).

In the case of race-specific innate resistance, an avirulence gene product in the pest is recognised by a specific resistance gene (*R* gene) in the plant (DeYoung and Innes, 2006). This may lead to the rapid generation of antibiotic compounds such as pathogenesis-related (PR) proteins (Kaloshian, 2004) and ultimately results in an incompatible interaction (Flor, 1971). Very few plant *R* genes have been identified that confer resistance to aphids. The best studied is the *Mi-1* gene from tomato which confers resistance to the potato aphid *M. euphorbiae* as well as to several root nematode species (*Meloidogyne* spp.)(Rossi *et al*., 1998). Another is the *Vat* gene, which confers resistance to *A. gossypii* in melon (*Cucumis melo* L.). Both Mi-1 and Vat both belong to the NBS-LRR class of R proteins, that is, they have a central nucleotide binding site (NBS) and a C-terminal Leu-rich repeat (LRR) domain. Mi-1 is an ATP-binding protein with ATPase activity (Tameling *et al*., 2002).

Another response to mechanical damage by piercing insects is sieve tube occlusion. The main objective of this is to prevent loss of SE sap from wounds. The most rapid response involves protein plugging of sieve plate pores (Furch *et al*., 2007, Pelissier *et al*., 2008, Knoblauch *et al*., 2012). A second type of response, one that is much slower to occur, is callose deposition. Callose is a β-1,3 glucan polymer that builds up around the sieve plate pores following wounding, and constricts damaged SEs, eventually occluding them. It is thought that damage to the SE, specifically to the cell wall and ER, causes an influx of Ca^{2+} ions (Will and van Bel, 2006, Furch *et al*., 2009). R-gene-mediated aphid resistance is often characterised by reduced sap ingestion following phloem location, suggesting that resistance might involve these phloem-sealing mechanisms (Caillaud and Niemeyer, 1996, Klingler *et al*., 1998, Kaloshian *et al*., 2000).

The result of these defence responses may be inhibitory or facilitatory to subsequent pests, depending on the host and pest species (Messina *et al*., 2002), and host plant genotype (Sauge *et al*., 2006). The effects can be seen as increased/decreased fecundity (Messina *et al*., 2002), altered host selection preferences (Sauge *et al*., 2002), and modified feeding behaviour (Prado and Tjallingii, 1997). Beyond aphid interspecies relationships, broader interguild facilitation effects have been observed. Research looking at the effects of *B. brassicae* pre-infestation on the leaf-chewing caterpillars of the Large Cabbage White butterfly, *Pieris brassicae*, at varying degrees of temporal separation, showed that the caterpillars developed faster and reached a larger body mass on plants previously infested with aphids (Soler *et al*., 2012). The aphids were found to attenuate the JA response of the plant and thus facilitate growth and development of the caterpillar. In the same study, the parasitoids of *P. brassicae* and *B. brassicae*, the parasitic wasps *Cotesia glomerata* and *Diaeretiella rapae* respectively, also performed better under multiple-infestation scenarios than in single-herbivore situations. Beyond that, even a cross-kingdom effect has been observed (Lee *et al*., 2012), with aphid feeding facilitating beneficial bacterial populations to help the plant cope with subsequent pathogen attacks. Results of such studies indicate that there is much cross-over between plant responses to various attackers and that the response to attack by one species will have wide ranging implications for other species.

1.6 Aphid reprogramming of plant physiology

1.6.1 Reallocation of nutrients

Studies have shown that aphids induce changes in nutrient allocation in host plants and these alterations may benefit the insects, improving the nutritional value of SE sap as a food source (Wilson *et al*., 2011, Petersen and Sandstrom, 2001). Several genes involved in nitrate and sugar remobilisation, including glutamine synthase, were induced in celery by *M. persicae* feeding (Divol et al., 2005a). Induction of glutamate synthase was also reported in *Nicotiana attenuata* infested by *Myzus nicotianae* (Voelckel *et al*., 2004). It is possible that aphids are inducing catabolism of leaf proteins and exploit the increased translocation (Dorschner *et al*., 1987, Riedell, 1989). Nitrogen reallocation has been observed in the hosts of *S. graminum* and *D. noxia* (Sandstrom *et al*., 2000, Telang *et al*., 1999). Various carbon assimilation-related genes were upregulated in celery during attack by *M. persicae* (Divol et al., 2005a), possibly as a response to assimilate loss from the plant during aphid feeding (Girousse *et al*., 2005).

1.6.2 Reversing early defence responses

Genes encoding proteins involved in ROS detoxification and inhibition of H_2O_2-generated cell death were found to be upregulated in *Arabidopsis* during infestation by *Brevicoryne brassicae*, the cabbage aphid (Kusnierczyk *et al*., 2008). Meanwhile, proteins involved in the synthesis of ROS, such as superoxide dismutase and polygalacturonase, were downregulated. HRs are rarely seen in compatible plant-aphid associations and it has been

hypothesised that aphid salivary secretions prevent ROS production (Giordanengo *et al*., 2010, Miles, 1999).

When an SE is pierced with a microcapillary, sieve plate occlusion occurs immediately (Knoblauch *et al*., 2001). When aphids feed on a plant however, penetration of the stylets may or may not lead to callose deposition, depending on the aphid species (Saheed *et al*., 2007). By using an aniline blue stain to label callose *in planta*, it has been shown that *D. noxia* induces callose-mediated occlusion of PD both between phloem parenchyma elements and between the CC and SE. *R. padi*, by contrast, did not induce callose formation until 14 days after infestation began (Saheed *et al*., 2007). This may explain the signs of stress and damage, including chlorosis and leaf necrosis, induced by *D. noxia*, which are absent during sustained *R. padi* feeding.

The volume of a 400 μm stylet, for example that of *A. fabae,* is calculated to be 95.03μm^3. This volume is 419 times smaller than that of a single SE so loss of pressure will be negligible when the stylets are inserted. Hence, stimulation of mechanosensitive Ca^{2+} channels will be unlikely (Will and van Bel, 2006). When forisome plugging of sieve tubes in the major veins of *V. fabae* was experimentally induced, feeding behaviour of the aphid *Megoura viciae* switched from phloem sap ingestion (E2) to salivation (E1)(Will *et al*., 2007). This suggests that watery saliva is being injected into the plant in an attempt to overcome SE occlusion, possibly by limiting calcium dependent signalling (Will *et al*., 2007). It has been suggested that phloem-specific proteins and aphid salivary proteins are likely to compete for free calcium (Will *et al*., 2007) and the ability of salivary proteins to bind Ca^{2+} may thus play a crucial role in enabling a compatible interaction between aphids and their hosts. Furthermore, transcripts of calcium-binding proteins were upregulated in *Arabidopsis* after

six hours of *B. brassicae* feeding (Kusnierczyk *et al*., 2008). Transcript abundance of eight glucan synthase-like genes was not found to differ between *R. padi* infested and uninfested barley plants (Saheed *et al*., 2009). Further to this, 5 β-1,3-glucanases, capable of degrading callose (a β-1,3-glucan) were upregulated in the same study by a callose-inducing aphid, *D. noxia*, but not by *R. padi,* which does not induce callose deposition. This indicates that the signalling involved in the induction of callose deposition is complex and is perhaps regulated post-translationally (Saheed *et al*., 2009). Watery saliva isolated from *M. viciae* was also shown to reverse forisome dispersion and was found to contain proteins with calcium-binding properties (Will *et al*., 2007, Will *et al*., 2009). This effect has not been demonstrated *in vivo*, however (Medina-Ortega and Walker, 2013), although salivation does prevent blocking of the aphid stylets during feeding (Tjallingii, 2006, Tjallingii and Esch, 1993).

1.6.3 Cell wall remodelling

The cell wall has also been shown to be a target for aphid-induced transcriptional reprogramming. Several genes involved in cell wall metabolism and remodelling have shown altered regulation in response to infestation. For example, protein kinases with cell-wall related activities, pectin acetyl esterase, cellulose synthase and expansin were all upregulated during infestation of *Arabidopsis* and *Apium graveolens* by the aphids *B. brassicae* and *M. persicae* respectively (Divol et al., 2005a, Kusnierczyk et al., 2008). Transcripts of pectin esterase inhibitor, which regulates pectin esterases, were under-expressed in those studies, potentially exposing the plant cell walls to enzyme-mediated breakdown (Lionetti *et al*., 2012).

One of the most important structural components of the cell wall is xyloglucan. Xyloglucans link cellulose microfibrils within the cell wall matrix (Campbell and Braam, 1999). They are metabolised by enzymes known as xyloglucan endotransglycosylase/hydrolases (XTHs)(Divol *et al*., 2007). Physical damage of the plant surface and vasculature during *M. persicae* stylet probing has been shown to induce over-expression of these enzymes in *B. vulgaris* (Dimmer *et al*., 2004). The importance of maintaining cell wall integrity has been highlighted in a study using *Arabidopsis* plants lacking the *XTH33* gene. This line was used in a choice-test experiment and *M. persicae* showed a preference for the mutant rather than the wild-type plants (Divol *et al*., 2007). This indicates that this gene, and the regulation it provides in cell wall metabolism, affects the interaction between the plant and aphid pests. It is likely that inhibiting the gene leads to weakening of the cell wall, which enables aphids to feed more easily.

1.6.4 Hormone signalling

Aphids induce transcription of phytohormones involved in disease responses, including ET, ABA, giberellic acid (GA), SA and JA, within their hosts (Thompson and Goggin, 2006b). SA- and JA- signalling in plant-aphid interactions is complex and varies between species pairings. For example, the SA-dependent pathway was strongly upregulated by *S. graminum* (Zhu-Salzman *et al*., 2004), *Macrosiphum euphorbiae* (de Ilarduya *et al*., 2003) and *M. persicae* (Moran *et al*., 2002, Moran and Thompson, 2001), whereas expression of JA-dependent genes was reduced. Overexpression of SA in tomato and *Arabidopsis* plants proved deleterious to *M. euphorbiae* (Cooper *et al*., 2004) and *M. persicae* (Moran and Thompson, 2001) respectively, whilst the SA-insensitive *npr1 Arabidopsis* mutant reduced *M. persicae* and *B. brassicae* feeding (Mewis *et al*., 2006). *B. brassicae* showed improved performance on

the JA-insensitive *coi1* mutant but constitutive expression of JA- and ET-signalling pathways in *cev1 Arabidopsis* mutants reduced aphid population growth (Mewis *et al*., 2006, Ellis *et al*., 2002b). This suggests that JA-mediated defence is an important aspect of the plant response to this aphid.

Aphids alter the balance of the JA and SA pathways, inhibiting JA-related genes and inducing those involved in the SA response (Singh *et al*., 2007, Zhu-Salzman *et al*., 2004, Moran and Thompson, 2001). Induction of JA-dependent gene expression pathways has been shown to alter the performance of *S. graminum* on sorghum (Zhu-Salzman *et al*., 2004) and *M. euphorbiae on* tomato (Cooper *et al*., 2004) and potato (Brunissen *et al*., 2010). Promoting an ineffective defence response would potentially be beneficial to the insects (Walling, 2008). Overall, it appears that the role of SA, JA and ET, and the interaction of these signalling pathways, is complex and varies among plant-aphid pairs.

1.7 Aphid pest control

1.7.1 Chemical pesticides

Traditionally, chemical pesticides have been used to control aphid pests. For example, the neonicotinoid Imidacloprid, which is a systemic neurotoxic insecticide (Matsuda *et al*., 2001) that is selective for insect receptors over those in vertebrates (Tomizawa and Casida, 2003). Chemical insecticides have several drawbacks, however, including: insect resistance; poisoning of animals further up the food chain, including humans; and indiscriminate insecticide action leading to reduced biodiversity and reduction in natural enemy populations (Hasken and Poehling, 1995, Jansen *et al*., 2010).

1.7.2 Biological control

The importance of natural enemies in controlling various aphid species has been established (Symondson *et al*., 2002). For example, in field experiments, aphid populations (including *S. avenae*, *Metopolophium dirhodum* and *R. padi*) were 18% higher at reduced densities of ground-dwelling predators (spiders and ground beetles), 70% higher when flying predators and parasitoids (such as parasitoid wasps) were removed, and 172% higher on the removal of both groups (Schmidt *et al*., 2003). This type of reduction of natural enemies is be observed following broad-spectrum insecticide application and highlights the problems with such control methods (Jansen *et al*., 2010).

Another aspect of biological control is applying or encouraging the growth of entomopathogens (Pell *et al*., 2010). Bacterial, viral, fungal and nematode entomopathogens have been successfully employed to control many insect species, however fungal pathogens have proved most useful against aphids (Stacey *et al*., 2003). Targeting pests with highly specific entomopathogens is an approach that preserves natural enemies and biodiversity and harbours no risks for human consumption (Lacey *et al*., 2001).

1.7.3 Genetic modification and transgenics

The current rapid expansion in genetic understanding is being applied to agriculture in two main ways: modifying traits within a species and transferring genes between different species (transgenics). Genetic modification can occur by natural breeding approaches or by artificial gene transfer, the latter being widely known as genetic engineering (Shelton *et al*., 2002).

Advances in genomic technology and bioinformatics has enabled discovery of new genes and regulatory sequences related to pest resistance. Genome-wide tools including molecular markers, high throughput genotyping strategies, and genetic maps are now available to breeders. It is now quicker and easier to screen mutant germplasm collections for allelic variants in specific genes. This has led to development of novel breeding strategies, including marker assisted backcross selection, 'breeding by design' and genomic selection. Aphid resistance genes have been bred into crop species. For example *D. noxia* resistance in barley and wheat cultivars, soybean aphid (*Aphis glycines*) resistance in soybean and greenbug (*Schizaphis graminum)* resistance in sorghum (Smith and Chuang, 2014).

One particular success of transgenics has been the insertion of genes from *Bacillus thuringiensis* (Bt), an aerobic, motile, gram-positive, endospore-forming bacterium, which has insecticidal properties (Lacey *et al*., 2001), into various crop plants. During sporulation, the bacteria produce crystals that are digested in the insect gut and release endotoxins, which attack the insect gut, leading to pore formation, disruption of membrane transport, and cell lysis leading ultimately to insect death (Gill *et al*., 1992). The endotoxins naturally produced by the bacteria are then expressed in the plant (Shelton *et al*., 2002). A long-term study in Arizona, USA has shown that the abundance of *Pectinophora gossypiella*, the pink bollworm, a major agricultural pest, has been suppressed by wide-spread Bt cotton planting (Carriere *et al*., 2003). The study concluded that Bt offered much longer-term pest suppression than insecticide sprays, highlighting the potential of transgenic crops for pest control. Similarly, suppression of *Ostrinia nubilalis,* the European corn borer, has been observed across parts of the USA where Bt maize is common (Hutchison *et al*., 2010). This study estimated that adopting this approach led to financial savings of approximately $3.2

billion over 14 years, which extended even to non-Bt maize growers due to the reduced regional pest populations. The decrease in pesticide use as a result of increased Bt utilisation (Cattaneo *et al*., 2006) has resulted in increased generalist predator numbers, which help to suppress aphid populations in both Bt crops and non-Bt crops in adjacent fields (Lu *et al*., 2012). As with chemical pesticides, however, some resistance has developed towards Bt in certain insect species, for example, the diamondback moth *Plutella xylostella* in the USA and Asia (Tabashnik *et al*., 2013, Tabashnik, 1994). Also, many important insect pests, including most hemipterans, are not amenable to Bt protection (Gordon and Waterhouse, 2007).

1.7.4 RNA silencing

Interference RNA, or RNAi, allows cells to break down foreign (for example viral) double-stranded RNA (dsRNA) (Fire *et al*., 1998). Dicer RNase III-type enzymes digest cytoplasmic dsRNAs into small interfering RNAs (siRNA) duplexes composed of 21–23 nucleotides (Mao and Zeng, 2014). These RNA fragments then bind to complementary RNA and, by doing so, tag that RNA for destruction by other cellular proteins. Even if an introduced RNA molecule is not viral, it will have the same effect, only now it will destroy transcripts of its own gene, thus providing a mechanism for sequence-specific post-transcriptional gene silencing (Fire *et al*., 1998). Insects have been particularly amenable to RNAi, and will take it up from their diet via cells in the midgut and the signal will spread throughout the insect body (Sapountzis *et al*., 2014). Plants can be induced to synthesise dsRNA which affects only insects with a particular target gene sequence. In this way, RNAi technology can be very specific, allowing targeting of a pest whilst leaving its natural enemies unaffected (Kupferschmidt, 2013). For example, a cytochrome P450 gene (*CYP6AE14*) has been identified in *Helicoverpa armigera*, the cotton bollworm, which permits this herbivore to tolerate high concentrations of the,

otherwise toxic, cotton-derived phenol, gossypol (Mao *et al*., 2007). When larvae were fed plant material expressing double-stranded RNA (dsRNA) specific to *CYP6AE14*, and thus silence the gene, larval growth was retarded. In the same study, a glutathione-S-transferase gene (*GST1*) was silenced the mid-gut of *H. armigera* feeding on *GST1* dsRNA–expressing *Arabidopsis* plants, and local GST activity decreased. GSTs are involved in the detoxification of plant allelochemicals (Gordon and Waterhouse, 2007).

The effect of plant-mediated RNAi on control of the aphid *M. persicae* has also been investigated. The gap gene *hunchback* (*hb*) was targeted as it is of crucial importance in insect axial patterning and knockdown of *hb* is deforming and lethal to the next generation (Schroder, 2003). The gene was cloned from the insect, plant RNAi vector was constructed, and transgenic tobacco expressing *Mphb* dsRNA was developed (Mao and Zeng, 2014). When neonate aphids were allowed to feed continuously on homozygous transgenic plants, reduced *Mphb* mRNA levels were observed in the aphids and insect reproduction was inhibited. Results of such studies indicate that knockdown of the target gene in insect pests can be achieved successfully by plant-mediated RNAi. Several candidate genes for RNAi in *S. avenae* have been identified by gene expression analyses of the alimentary canals of grain aphids before and after feeding on wheat plants (Zhang *et al*., 2013). Plant genes can also be targeted by RNAi in order to protect against attack. For example, an RNAi construct corresponding to a putative susceptibility gene in wheat (*TaS3*) was co-transformed with a GUS reporter construct into leaf epidermal cells (Li *et al*., 2013). This showed that powdery mildew was able to infest leaf epidermal cells and establish a mature haustorium and secondary hyphae in cells expressing only GUS, but in cotransformed cells the interaction was incompatible and fungal growth was arrested at the appressorial stage. The results of

these studies suggest that RNA silencing may hold much potential for pest and pathogen control in the future but identification of aphid effectors and plant susceptibility genes is vital in developing this approach.

1.8 Genomic resources

Development of the above approaches and maintaining successful control of aphid pests and combatting resistance requires ever more advanced technologies. Developments in the field of functional genomics are expediting the identification of genes, proteins and metabolites involved in the interaction between plants and aphids. The techniques available to researchers interested in the transcriptomics, proteomics and metabolomics of the plant-aphid interaction are outlined briefly below.

1.8.1 Transcriptomics

1.8.1.1 Microarray

The transcriptome is the total mRNA in a cell or organism and, as such, represents the genes being actively expressed (or transcribed) at a particular time (Horgan and Kenny, 2011). Development of the microarray chip has provided the means to study thousands of expressed genes in a single experiment (Alberts *et al*., 2005). It is a high-throughput method of studying differences in RNA populations between different treatment groups. Microarrays take advantage of the fundamental property of single stranded DNA/RNA to bind to its complementary strand. Microarrays, or Gene Chips, consist of thousands of individual spots, each of which represents a unique known DNA sequence. This is then used to probe the

target sequence, i.e. the collected sample, and if a single stranded RNA/DNA molecule complementary to the known sequence is present in the extract it will bind to the sequence that in turn is bound to the surface. By labelling the population of DNA/RNA molecules with florescent markers it is possible to visualise this binding pattern, using feature-extraction software, to determine whether a complementary strand is present in an extract.

There are two main microarray approaches: cDNA microarrays and oligonucleotide expression arrays (Gibson, 2002). cDNA array probes are pre-synthesised and deposited onto a glass surface, whilst oligonucleotide array probes are synthesised *in situ* on the chip. Whole genome oligonucleotide chips are commercially available for barley.

Microarrays also vary in the mode of substrate labelling utilised. Again there are two types: one-colour and two-colour microarrays (Patterson et al., 2006). For one-colour microarrays, arrays are performed with a single sample and the intensity measured for each gene. Intensities can be compared between chips showing differences in gene expression of different samples. Two-colour arrays rely on competitive hybridisation; two samples are labelled with different molecules, for example Cy dyes that fluoresce at different wavelengths, giving a green or a red signal. Following hybridisation, a scan of the chip gives a ratio of green to red fluorescence showing transcript abundance.

Using microarray technology in transcriptomic studies has several drawbacks such as: high background noise levels, caused when the scanning laser is reflected by an uneven surface, or, alternatively, by salts or debris remaining following hybridisation (http://array.mc.vanderbilt.edu/analysis/normal.fgsr); cross-hybridisation, usually caused by oligo fragments with a run of 10–16 nucleotides complementary to a probe (Wu *et al*., 2005); and a small dynamic range of around 200 fold. When conducting microarray

experiments it is important to record a core set of information about the procedure including material used, sample preparation, experimental design and array design description. This set of information is referred to as the 'minimum information about a microarray experiment' (MIAME)(Brazma *et al*., 2001). It is vital that this information is publicly available due to the importance of experimental design in determining the outcome of microarray experiments. For example, De Vos *et al.* (2005) and (Couldridge *et al*., 2005) both examined the transcriptomic response of *M. persicae* feeding on *Arabidopsis* using Affymetrix GeneChips but the studies returned very different results. De Vos *et al*. reported upregulation of 832 genes and downregulation of 1349 genes, whilst Couldridge *et al*. found that only two genes showed altered expression (one up- and one downregulated) two hours after infestation and only two were downregulated and twenty three were upregulated after 36 hours. The discrepancy could be due to the number of replicates used in each study: De Vos *et al*. employed only one biological replicate, whilst Couldridge *et al*. used 6, providing a higher statistical stringency. The variation in results in two studies testing the same hypothesis highlights the importance of transparency of experimental design and analysis methods.

1.8.1.2 RNA-seq

Early attempts at sequencing used a chain-termination capillary sequencing method: 'Sanger sequencing' (Sanger *et al*., 1977). In this method, DNA strands are separated and chain-terminating, fluorescently labelled dideoxynucleotriphosphates (ddNTPS) are incorporated randomly during complementary strand synthesis. Strands of varying lengths are synthesised and these are then separated by capillary electrophoresis and detected by fluorescence. The deduced sequences can be aligned to cover the whole genome.

More recently, next-generation sequencing (NGS) has become widely used, popular due to the highly parallel nature of the technique (Pareek *et al*., 2011). NGS has reduced the cost of genome sequencing and many genomes have now been fully sequenced (Pagani *et al*., 2012). Beyond genome sequencing, NGS has also facilitated transcriptomic profiling, enabling the quantification of transcripts and transcription factors (Soon *et al*., 2013). The sequencing of RNA (RNA-seq) is done in 3 main ways: sequencing by synthesis (Illumina), pyrosequencing (454 Life Sciences/Roche Diagnostics) and sequencing by ligation (SOLiD).

In preparation for Illumina sequencing (http://www.illumina.com/), mRNA is reverse transcribed and the resulting cDNA purified. The cDNA is then fragmented and tagged with adaptors by transposons in a process known as tagmentation (Marioni et al., 2008). Other regions, including primer binding sites, oligo binding sites, and sample identification indices (so multiple samples can be run simultaneously) are also added. The cDNA is amplified and sequenced on a glass slide. DNA polymerase synthesises complementary strands, which are clonally amplified before sequencing begins. A primer attaches and fluorescently labelled nucleotides compete for the binding site. After the complementary nucleotide binds, the cDNAs are excited by a light source and the fluorescent signal emitted is characteristic of each of the four bases. This process is performed repeatedly and the number of cycles determines the read length. Hundreds of millions of sequences are read simultaneously and these sequences are aligned back to a reference genome. Illumina sequencing has been used to investigate plant responses to pathogens (Lloyd et al., 2014) and insects (Nabity et al., 2013).

Applying pyrosequencing technology to RNA sequencing also requires reverse transcription of the RNA to cDNA (Bai et al., 2010). DNA polymerase then elongates the complimentary

strand, and as each nucleotide is added, pyrophosphate is released. ATP sulfurylase then converts pyrophosphate to ATP. ATP stimulates oxygenation of luciferin by luciferase. This generates a light signal recorded as a pyrogram peak. Nucleotides are added to the reaction in a predefined order so the pyrograms can be read as a sequence. These reactions take place while the cDNA fragments are bound to glass beads. A similar approach to this is employed by Ion Torrent sequencing (Life Technologies), but here it is the hydrogen atom released during the addition of a nucleotide that is measured via a change in pH.

SOLiD sequencing, which has proved useful in plant stress studies (Rahman et al., 2014), also uses glass beads as a support for template cDNA. Rather than adding nucleotides in turn, however, short oligos are added by the enzyme DNA ligase (www.lifetechnologies.com). These oligos are attached to fluorescent dyes which allow identification of the base at the end position. Ligation of further oligos allows every n^{th} base of the strand to be sequenced. Sequencing is then re-run one base along until the entire strand is sequenced.

These sequencing technologies all have an accuracy of around 99% and generate between 1 million (Pyrosequencing) and 3 billion (sequencing by ligation) reads per run. The time taken for each run varies by approach from 24 hours for a pyrosequencing run, to 1 to 10 days for Illumina sequencing and 1 to 2 weeks for SOLiD sequencing.

1.8.2 Proteomics

The proteome is defined as the set of all expressed proteins in a cell, tissue or organism. Much work has been done to elucidate the proteome of aphids (Francis *et al*., 2006, Nguyen *et al*., 2007) and their saliva (Sharma *et al*., 2014, Vandermoten *et al*., 2014, Nicholson *et al*., 2012) but less on the plant proteome following aphid attack. Ferry *et al*. (2011) isolated the

proteins from leaves of wheat seedlings, following feeding by *S. avenae,* using 2-D electrophoresis. The protein spots were analysed using matrix-assisted laser desorption ionization (MALDI)(Karas and Hillenkamp, 1988) time of flight mass spectrometry (MALDI-TOF MS). This technique allows separation of biological molecules based on mass, and thus provides an accurate identification technique for proteins derived from biological samples (Mann *et al*., 2001). A second approach is ionizing fragmented peptides and spraying them into a 'tandem mass spectrometer' which isolates the peptides in the mixture allowing identification (Pandey and Mann, 2000). This approach has been used to investigate the proteomic response of tomato to *M. euphorbiae* (Coppola *et al*., 2013). Proteomic analysis of phloem exudates (Walz *et al*., 2004, Giavalisco *et al*., 2006, Barnes *et al*., 2004), stylectomy-derived SE sap (Gaupels *et al*., 2008a) and aphid honeydew (Sabri *et al*., 2013) has also been achieved. The results of proteomic studies of SE sap and of the proteomic response to aphid infestation are discussed in chapters 4 and 5, respectively.

1.8.3 Metabolomics

The term metabolite includes a diverse range of small (<1500 Da) molecules including lipids, amino acids, organic acids, vitamins, thiols and carbohydrates. Metabolites represent the functional phenotype of a sample cell, tissue or organism; they are biomarkers for various processes or conditions. The diversity of these structures renders global analysis of metabolites a challenge, however (Zhang *et al*., 2012a). Modern approaches to metabolite analysis include: gas chromatography coupled to mass spectrometry (GC-MS)(Garcia and Barbas, 2011); high-performance liquid chromatography mass spectrometry (HPLC-MS)(Wilson *et al*., 2005); ultra-performance liquid chromatography mass spectrometry (UPLC-MS)(Plumb *et al*., 2006); capillary electrophoresis mass spectrometry (CE-MS)(Soga *et*

al., 2003); and nuclear magnetic resonance (NMR) spectroscopy (Pan and Raftery, 2007). Such approaches have been applied to studying the plant-pest relationship. For example, the metabolomic response of tomato plants (Mirnezhad *et al*., 2010) and *Senecio* spp. (Leiss *et al*., 2009) to cell-content-feeding thrips (*Frankliniella occidentalis*); and the response of *Arabidopsis* (Arany *et al*., 2008) and *Brassica rapa* (Widarto *et al*., 2006) to the tissue-chewing caterpillars *Plutella xylostella* and *Spodoptora exigua*. Kusnierczyk *et al*. (2008) used HPLC-MS to investigate the interaction between the aphid *B. brassicae* on *Arabidopsis* at several time points following infestation. This is an example of how metabolomics can be utilised to track the course of a plant-herbivore relationship to better understand the defence responses upregulated in the host.

1.9 Experimental species, agricultural significance and project objectives

1.9.1 Plant study system

Barley was domesticated around 10,000 years ago and, among the cereals, currently ranks fourth after maize (*Zea mays*), rice (*Oryza sativa*), and wheat (*Triticum aestivum*) in terms of total production (Schulte *et al*., 2009). Grain is predominantly used in animal feed and in the production of malt for use in the brewing and distilling industries. Barley cultivation occupies around 50 million hectares worldwide, resulting in the production of 133 million tonnes annually at a gross production value of around 17 billion US$ (2012 figures obtained from the website of the Food and Agriculture Organisation of the United Nations). It is widely cultivated in all temperate regions from the Arctic Circle to the tropics. Wild barley species are adapted to some of the most diverse and extreme ecosystems around the globe, which offers unexploited potential for the future adaptation of barley through the use of

germplasm resources. Germplasm collections containing geographically diverse elite varieties, landraces and wild accessions are accessible (Schulte *et al*., 2009).

As a diploid, inbreeding crop, barley has traditionally been considered a model for plant genetic research. Due to its simpler genome, it can be considered a good genomic model for hexaploid bread wheat, for example. The barley genome is one of the largest in cereal crops and, at 5.1 gigabases, is nearly twice the size of the human genome (Mayer *et al*., 2012). It has seven chromosomes containing around 32,000 genes. A wide range of genomic information is available for barley including: more than 460,000 public ESTs; several well-utilised genetic mapping populations; collections of aneuploids (plants with extra or missing chromosomes) and translocation lines (containing transfer DNA); several thousand single nucleotide polymorphism (SNP) markers for high throughput genotyping; genetic maps featuring nearly 5000 genes; and several bacterial artificial chromosome (BAC) libraries. These resources have made possible a series of studies in the fields of structural and functional genomics taking barley research to the forefront of plant genomics. Extensive mutant collections containing all of the morphological and developmental variation observed in the species have been generated (Caldwell *et al*., 2004). Until recently, the barrier to the exploitation of these resources was the absence of a reference genome sequence. Attempts to improve this situation have been fruitful, as summarised in a publication in *Nature* by the International Barley Sequencing Consortium (Mayer *et al*., 2012). The accessibility of this genetic information, and the availability of whole genome barley GeneChips (Agilent Technologies, Affymetrix, etc.), make barley an important study species.

1.9.2 Insect study system

As well as the lifecycle adaptations discussed above, which make *R. padi* an interesting study species, there were several practical considerations that made it useful in the current study. Firstly, *R. padi* are relatively large and have a sedentary nature, which makes them amenable to stylectomy. Secondly, *R. padi* prefer the mature leaves and leaf sheath of cereal plants (Gianoli, 1999), which makes these aphids useful for stylectomy as the leaf is easy to access. Aphids that induce leaf curling, e.g. *D. noxia* (Messina *et al*., 2002), make stylectomy more challenging. Finally, *R. padi* show a preference for barley as a host over other cereals (Leather *et al*., 1989), making this plant-pest pair a good example of a susceptible interaction and should make the effects of induced defences more obvious.

1.9.3 Project aims and hypotheses

An initial aim of this study was to characterise the effects of *R. padi* infestation on barley morphology and physiology. To achieve this, plant growth and inorganic ion content were measured in infested and uninfested plants, and SE osmotic pressure, sap exudation rate and exudation duration were measured from sap exuding from cut stylets. It was hypothesised that infestation would negatively impact on plant growth due to the withdrawal of photosynthate. SE sap exudation rate from cut stylets was expected to alter due to infestation, either decreasing due to induction of plant defence responses, such as callose-mediated occlusion of the sieve tubes, or increasing due to the effect of aphid salivary effectors inhibiting these defences.

A second aim was to use the EPG technique to investigate the effects of pre-infestation on the feeding behaviour of individual aphids subsequently attacking the host plant. A

facilitation or inhibitory effect of pre-infestation would provide evidence that transcriptomic and physiological responses taking place within the plant during infestation have an impact on the pest and would indicate whether these were plant defence responses, or if the aphids were facilitating their own feeding by modifying plant behaviours.

A third aim was to develop a technique for acquiring sufficient quantities of SE sap RNA to perform microarray analysis. Pure SE sap from aphid stylectomy can only be collected in minute volumes and the RNA concentrations within it are vanishingly small (Doering-Saad *et al*., 2002). It was aimed to quantify RNA in SE sap using *R. communis* sap as a proxy due to the larger volumes available from the exudation method. It was expected that an RNA amplification protocol would then be required to obtain sufficient RNA amounts for array hybridisation. If successful, such gene expression studies would provide a better understanding of the phloem.

Finally, this study aimed to investigate gene expression in infested and uninfested barley plants using whole tissue samples and microarray. Looking at local (infested first leaves) and systemic (uninfested second leaves of infested plants) effects using this approach, was designed to obtain information about whole plant integration of responses, for example reallocation of nutrients and long-distance signalling networks. Although the actual phloem signal is dilute in such samples, it would be possible to speculate over which responses may be phloem-localised or facilitated by a phloem-mediated signal. This whole tissue study aimed to provide a broader picture of the plant response to aphid pests and was a much more time-effective method of assessing plant defence responses. As this is a susceptible interaction (Ni and Quisenberry 2006), it was hypothesised that alterations to the plant transcriptome would occur that have beneficial effects for the aphid pests. This would allow

identification of candidate susceptibility genes. It was also hypothesised that a range of defence responses would take place during the interaction, particularly systemically as a result of systemically induced resistance. One or more of these genes could have potential as an RNAi target gene or a transgene. Ultimately, future work based on these genes could lead to the development of crops with enhanced resistance to aphids, thus improving crop yields to feed expanding populations. In the case of barley, improved yields would have a significant impact on pastoral agriculture.

2 *Rhopalosiphum padi* alter the growth, inorganic ion content and sieve element sap properties of barley

2.1 Summary

Removal of nutrients, cellular damage and injection of elicitors in saliva as a result of aphid feeding induces both defences and facilitation effects in plants. Identifying the whole-plant changes in response to aphid feeding can indicate the effects of infestation on plant development, composition and physiology. This study aimed to investigate the growth responses, inorganic ion fluctuations and alterations in phloem functioning of barley in response to infestation by the aphid *R. padi*. The phloem is an important site of interaction between aphids and host plants and is the site of injection of watery saliva during feeding. It is therefore hypothesised that aphid infestation will induce changes in sieve element functioning. To test this hypothesis, phloem sap osmotic pressure, rate of exudation of sap from severed stylets and duration of sap exudation was measured on heavily infested and lightly infested plants. It was found that infestation induces stunting and alters ion allocation both locally and systemically. Exudation rate did not differ significantly but the duration of exudation was extended on heavily infested plants, suggesting that aphids were inhibiting callose-mediated sealing of sieve tubes.

2.2 Introduction

Aphid infestation can alter plant development, morphology and resource allocation, and induce local and systemic symptoms (Goggin, 2007). Changes in the host plant, and especially in the SE, are likely to affect aphid performance. The relationship between *R. padi* and barley is a particularly intriguing one, as no visible symptoms occur in infested tissues, unlike those observed during the Russian wheat aphid *D. noxia* feeding, for example (Ni and Quisenberry, 2006). It is therefore important to understand the subtle changes induced by aphid feeding, both as a result of withdrawal of nutrients and due to injection of effector molecules in aphid saliva (Elzinga and Jander, 2013).

2.2.1 Changes to growth and development

Mallott and Davy (1978) showed that *R. padi* feeding on spring barley plants reduced growth but increased leaf area. This study infested plants with 3 or 4 aphids and allowed uncontrolled population growth. Aphid infestation levels reached over 1500 after 5 weeks, so this represents an extremely high level of infestation, likely to be higher than experienced in the field. In the current study, it was aimed to assess the growth of plants following a relatively short period of light to moderate infestation (5 to 30 aphids) to determine whether the stunting effects are consistent.

Infestation with *R. padi* has also been shown to reduce grain production of four wheat cultivars compared to uninfested plants (Savaris *et al*., 2013) and reduced yield of spring wheat by 789 kg ha^{-1} (Riedell *et al*., 2007). Field experiments have corroborated these

results. For example, a two year long field study looking at the effect of *R. padi* on spring wheat showed that plants had fewer, shorter tillers and less shoot dry weight when infested with aphids at the 2-3 leaf stage. Kernel weight was also reduced by 8% on infested plants (Riedell *et al*., 2003).

2.2.2 Changes to physiological processes

Photosynthetic capacity was not reduced in barley by *R. padi* feeding compared to control plants, according to carbon dioxide exchange studies (Mallott and Davy, 1978). Ni *et al.* (2002) found that feeding by *R. padi* did not alter chlorophyll a or b levels, or levels of carotenoids in wheat, unlike *D. noxia,* which reduced chlorophyll and carotenoid levels. Franzen *et al.* (2008) showed that, despite the lack of visible symptoms, *R. padi* does have an impact on the gas-exchange and chlorophyll fluorescence of its host plants. In the same study, *D. noxia* reduced the photosynthetic capacity of wheat plants more rapidly than *R. padi*. In general, neither *R. padi* nor *D. noxia* significantly affected chlorophyll content or chlorophyll fluorescence parameters (non-variable minimal fluorescence, maximal fluorescence, and variable fluorescence). Both aphid species significantly impacted on photochemical and non-photochemical quenching coefficients. That group suggested that aphid feeding may alter the thylakoid membrane pH gradient by influencing the photoprotective xanthophyll cycle. Feeding by both aphid species resulted in an increase in electron transport rate, but at different time periods. These studies show that aphid infestation can alter the fundamental functioning of host plants.

2.2.3 Mineral nutrient changes

During *R. padi* infestation, the levels of the mineral nutrients nitrogen, potassium, magnesium and manganese, as well as chlorophyll, in wheat leaves have been found to be significantly correlated with agronomic traits important in cereal plant responses to stress caused by aphid-feeding damage, such as leaf area and grain yield (Riedell *et al*., 2007).

Nitrogen-deficient barley plants have previously shown reduced growth and development and *R. padi* feeding on these plants was reduced four-fold compared to nitrogen-fertilized plants (Helenius, 1990). Addition of nitrogen was also found to increase the concentration of AAs in barley seedling phloem sap, which improved the growth rates of *R. padi* nymphs on these plants (Weibull, 1987). Similarly, Ponder *et al*. (2000) recorded a correlation between low nitrogen, reduced growth of barley seedlings and reduced *R. padi* intrinsic rate of increase (r_m). Conversely, Salas *et al*. (1990) found that fertilization with nitrate reduced the growth rate of *S. graminum)* colonies on barley seedlings. This was thought to be due to a resulting increase in production of secondary metabolites such as the indole alkaloid gramine, however, indicating that nitrate fertilization levels can alter plant resistance to pests. The nitrogen status of plants has also been shown to influence leaf chlorophyll levels, which can alter leaf colour (Montemurro *et al*., 2006). Colour is an important cue used by aphids in host location and for landing (Doring *et al*., 2009) so alteration due to changes in nitrogen levels may affect aphid host colonisation. Once aphids have located a host, nitrogen deficiency has been shown to hinder feeding. EPG studies showed *R. padi* aphids took longer to locate the SE and showed extended salivation into the SE on nitrogen-deficient barley plants (Ponder *et al*., 2001). Reduced feeding performance is likely to be a contributing

factor in the reduction in aphid r_m discussed above. As well as providing useful information on the role of nitrogen nutrition on plant-aphid interactions, results of such studies have important implications for agricultural fertilisation practices (Garratt *et al.*, 2010).

Potassium is important in providing turgor within plant cells. Hyperosmotic stress is known to significantly enhance net uptake of potassium and several other inorganic ions into plant cells. Shabala and Lew (2002) used a mannitol/sorbitol treatment to induce hyperosmotic stress. Turgor recovery started within 2-10 min of the treatment and was accompanied by an increased uptake of K^+, Cl^-, and Na^+ by *Arabidopsis* epidermal root cells. It is also responsible for maintaining membrane potential and transmitting electric signals via membrane depolarisation (Fromm and Hedrich, 2007). Interestingly, potassium-deficient soya bean plants (*Glycine max)* have been shown to host higher densities of soybean aphids (*Aphis glycines*)than those receiving adequate potassium (Walter and DiFonzo, 2007). This was suggested to be because potassium affects nitrogen use within the plant, leading to higher AA concentrations in phloem sap and resulting in earlier reproduction and higher fecundity of aphids feeding on these hosts. The interaction between potassium and nitrogen is an interesting factor to consider in aphid nutrition and the current study aimed to investigate both nutrients in infested and uninfested barley following *R. padi* infestation.

Calcium is another important mineral in plants and has a key role in defence signalling. Several defence pathways are triggered by an influx of Ca^{2+} ions from the apoplast. For example, Furch *et al.* (2009) studied the effect of remote stimuli on Ca^{2+} levels and on SE flow. They found that propagation of electropotential waves in response to leaf tip burning leads to transiently high levels of Ca^{2+} secreted through plasma membrane and ER Ca^{2+} channels, and ultimately triggers forisome dispersion and occlusion of sieve plates. Aphids

induce transient increases in cytosolic Ca^{2+} levels, which activate calmodulin and other calcium-sensing proteins that subsequently promote downstream signalling events, including protein phosphorylation and transcriptional responses (van Bel *et al*., 2014).

The studies outlined above highlight the importance of balanced mineral nutrition within plants under attack by aphids. Little is known, however, about the effects imposed by aphid infestation on nutrient levels. The current study therefore aimed to assess the inorganic ion content of infested verses uninfested plants following three days of the respective treatments, to identify any unbalance caused by aphid feeding. This was the same stage in the interaction that facilitation experiments (Chapter 3) and microarray experiments (Chapter 5) were performed and it was hoped that the results could be integrated to provide a holistic view of plant-aphid interaction and the effects on both the plant and aphid pests.

2.2.4 SE-specific changes

The phloem is an important site of interaction between aphids and their host plants. It is likely, therefore, that many of the aphid-induced changes will occur in the phloem tissues, or will be triggered by phloem-mobile signals. Due to its transport role, the phloem is likely to be the site of changes in nutrient allocation and of long-distance signalling that leads to systemic defence responses, both of which have been observed during the plant-aphid interaction (Girousse *et al*., 2005, Turnbull and Lopez-Cobollo, 2013).

Phloem sap has been shown to contain a wide range of solutes, including sugars, amino acids, secondary metabolites and RNA, consistent with its transport role (Will and van Bel,

2006). SE sap contains a range of inorganic ions including potassium, sodium, magnesium, calcium and ammonium cations and chloride, phosphate, nitrate and sulphate anions (Dinant *et al.*, 2010b). Potassium is found in highest concentrations (299mM in wheat)(Hayashi and Chino, 1986) and, alongside sugars and amino acids, is one of the primary osmotic components of SE sap. One important role for K^+ is stimulating sugar loading into the SE sap in source leaves (Lebaudy *et al.*, 2007). A mutant *Arabidopsis* showing loss-of-function of an AKT2/3 type potassium transporter contained only half the phloem sucrose of the wild type (Deeken *et al.*, 2002). The presence of voltage-gated ion channels in the phloem has been confirmed and it has been suggested that action potentials initiate the inhibition of phloem translocation during electric- and cold-shock events (Fromm and Bauer, 1994). Phosphate and other anions, such as bicarbonate and malate, are involved in the charge balance and are thought to play a role in the control of phloem sap pH. Magnesium, sodium and chloride are found at lower levels in the sap. SE sap is moderately alkaline, usually with a pH around 7.5 (Hafke *et al.*, 2005).

Stylectomy has been used to collect sap from barley to quantify AAs using high-performance liquid chromatography (HPLC)(Ponder *et al.*, 2000). They found the levels of non-essential, but not essential, AAs to be reduced under low nitrogen conditions. Stylectomy-derived samples from *Medicago sativa* (alfalfa) were collected to measure the sugar and AA content of SE sap from different genotypes (Girousse and Bournoville, 1994). The two genotypes induced differential effects on *A. pisum* population performance (weight, fecundity, survival). No difference was found in AA or sugar concentrations or the AA/sugar ratio, making it unlikely that either of these substances is responsible for the observed differences in aphid performance. The two genotypes did display different stylet exudation rates,

however, with the susceptible genotype exuding 2.8 times as much sap over the same time period and exuding for 2.3 times longer on average. This suggests that aphid feeding triggered a reduction in sap flow on the resistant plants, which would explain the reduced performance on these plants. A difficulty when using stylectomy-derived sap is the low quantities obtained by this method. A previous study reported phloem sap exuding from cut stylets at a rate of 60-120 nl $hour^{-1}$ (Doering-Saad *et al*., 2002), so the possibilities for downstream analysis of sap collected via this method is limited. In the current study, therefore, SE sap was collected from *R. communis* and used to optimise the techniques for very small samples. That was possible because *R. communis* exudes from excisions in the stem (Milburn, 1970), providing far greater quantities of sap than stylectomy.

As well as nutrient-stress, other stressors can affect aphid performance on host plants. Drought stress (-0.5MPa), for example, was shown to have a negative impact on performance (r_m) of *R. padi*, although this effect was inconsistent and depended on plant species and the level of drought imposed (Hale *et al*., 2003). In the same study, the osmotic pressure of phloem sap was measured using freezing point-depression picolitre osmometry (Tomos *et al*., 1994). This approach exploits the fact that increasing the concentration of a solution will lower the vapour pressure, elevate the boiling point and lower the freezing point of that solution. Because the osmolarity of a solution is determined by the number of particles contained within it, rather than their size or charge, this number can be calculated by measuring the freezing point of the solution. That is, one mole of any substance, dissolved in 1kg of water will decrease the freezing point by 1.858°C. Hale *et al*. (2003) found that the osmotic pressure of SE sap, and the concentration of AAs within it, were significantly higher in drought-stressed plants, although sap ingestion rates were reduced.

The quality of the sap was therefore suggested to be improved as a food-source for aphids by drought stress but the osmoregulation required to overcome the high osmotic pressure of the sap, i.e. the assimilation and transformation of sugars, is costly for aphids so the sap ingestion rate was reduced. In the current study, osmotic pressure was measured to determine whether aphid infestation itself affects osmotic pressure and, further to this, how any changes impact on the feeding behaviours of subsequent aphid populations (Chapter 3).

Overall, the results of the studies cited above suggest that the interplay between SE contents; properties of the sap such as osmotic pressure and exudation rate; and aphid performance, is variable and dependent on plant-pest pair. The current study therefore aimed to combine information on these factors during *R. padi* infestation of barley plants.

2.2.5 Aims and hypotheses

The current study aimed to look for alterations in barley physiology due to infestation by *R. padi*, by investigating changes in plant growth, inorganic ion content and properties of the sieve element:

1. Plant growth was measured to investigate carbon deposition rates in infested and uninfested plants. It was hypothesised, from previous research (Mallott and Davy, 1978), that *R. padi* infestation would reduce plant growth rate, but the extent of the effect, and its timescale, at various infestation levels was yet to be elucidated, and was a key area for investigation in this study.
2. Inorganic ion content was measured to determine how aphid feeding alters the concentration of these micronutrients. It was hypothesised that aphids would alter

ion content of barley, by removing nitrogen-containing AAs, imposing osmotic stress, and triggering calcium-mediated defence responses.

3. SE exudation rates and durations were measured in heavily and lightly infested plants using aphid stylectomy. The osmotic pressure of stylet sap was recorded using picolitre freezing-point depression osmometry. This was aimed at determining the effect of aphid feeding on plant water relations, which we hypothesised would be altered due to the sink imposed upon the plant by aphid feeding (Girousse *et al*., 2003).

2.3 Materials and methods

2.3.1 Plant material

'Morex' *Hordeum vulgare* L. (barley) seeds were surface sterilised in a 25% sodium hypochlorite solution for 7 minutes, followed by an ethanol wash and two rinses in water. Plants were grown individually in 6 cm pots containing 6 parts peat-based compost (Humax multipurpose) to 1 part Silvaperl under a 16:8 hour light:dark regime in 70% humidity. Plants were infested at 10 days and harvested at 13 days, at growth stages 11 and 12 respectively (Zadoks *et al*., 1974), unless stated otherwise. *R. communis* plants used for collection of larger volumes of SE sap for method development were grown in 10cm pots in the same compost, under the same conditions. Sap was collected from plants over 6 weeks old.

2.3.2 Aphid culture and infestation conditions

Rhopalosiphum padi L. (Hemiptera: Aphididae) were cultured from a clone kept at the University of Birmingham on *H. vulgare* plants, also under a 16:8 hour light:dark regime in 70% humidity. Culture plants were knocked to dislodge aphids and adult aphids were selected randomly for experimentation and were transferred using a soft paintbrush.

The first leaf of each plant was enclosed in a cage constructed of a plastic centrifuge tube with a slit cut into the lid for the leaf to protrude through and the other end removed and replaced with fine mesh for ventilation (Figure 2-1). The inside of the lid was also covered with mesh with a slit cut into it, to prevent aphids escaping. If, at any stage, aphids were found anywhere on the plant other than in the experimental region, that plant was discarded. The cages were carefully positioned so that the leaves were able to grow into them during normal development. By caging the aphids in this way, they were able to colonise approximately 6 cm of the leaf (on both adaxial and abaxial surfaces) by the end of the experiment. In the infested treatment, the aphids were placed inside the tube then the leaf was carefully inserted. The aphids found their own way to the leaf within an hour. All observed effects were therefore stimulated by the aphids, rather than touching of the leaf with a paintbrush for example.

The chambers were taped to a vertical stake, which was anchored in the soil of the pot, thereby keeping the leaf in a natural growing position and avoiding damage during the experiment. Control plants were set up in the same manner but no aphids were added. Infested and uninfested plants were randomly assigned to plant trays and were stored inside insect-proof tents. All infestations were left under standard growth conditions for 72 hours unless otherwise stated and no aphids were added or removed during this time.

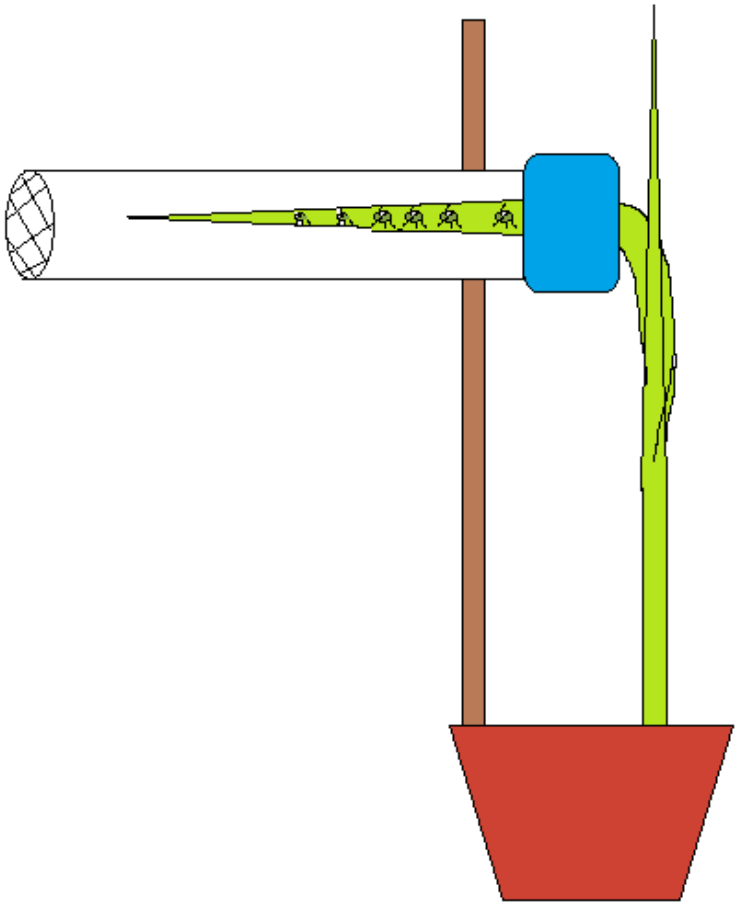


Figure 2-1 Diagram of aphid chamber set-up. First leaf of young barley plant protrudes through a slit in the lid of the chamber. The end of the tube is covered with fine mesh for ventilation. The chamber is supported by a stake which is seated in the soil of the plant pot.

2.3.3 Plant growth measurements

Plants were randomly assigned to one of five treatment groups: infested with either 0, 5, 10, 20 or 30 adult aphids. 22 plants were infested in each condition. Plants were measured immediately prior to infestation (day 0) then on days 2, 3, 6, 12 and 13 without removing the aphid cages. Measurements were taken from the soil to the tip of the first leaf. Change in height was calculated from the collected data, to account for different plant starting heights.

2.3.4 SE sap collection and analysis

Plants were infested for 72 hours with either 5 or 30 aphids, to represent lightly and heavily infested plants respectively. Stylectomy was performed on feeding aphids using a high-frequency microcautery device (Unwin, 1978). Phloem sap was collected into an oil-filled microcapillary (Pritchard, 1996) and stored at -70°C. During collection, the microcapillary could briefly be removed from the stylet, allowing a droplet of sap to form. This was measured with a calibrated eyepiece graticule and the change in volume of the droplet over time was calculated to give the exudation rate. Stylectomy was always performed between 9 am and 12 pm as sap composition is known to be affected by the diurnal cycle (Smith and Milburn, 1980, Gattolin *et al*., 2008).

The osmotic pressure of the sap was measured using a freezing point depression picolitre osmometer (Tomos *et al*., 1994). The apparatus consisted of a cooling system (cold water pump and ice bucket) a temperature-sensitive resistor (a thermistor) and a potential-difference measurement device graduated in temperature. Three stylectomy-acquired sap samples were taken from each of five plants infested with five aphids and from five plants infested with 30 aphids and the mean sap osmotic pressure, calculated from the respective freezing points was obtained for each condition.

SE sap was collected from *R. communis* using a clean razor blade to make an incision in the bark of a plant. 100µl of the exuding sap was collected with a pulled glass microcapillary and used immediately in downstream analysis.

2.3.5 Inorganic ion content

30 barley plants were randomly assigned to two groups and infested with either 0 or 30 adult aphids. Aphid cages were removed from the plants after 72 hours and aphids were gently brushed off using a soft paintbrush. Control leaves were treated similarly. The first and second leaves were excised using a sharp razor blade and transferred to separate 0.5 ml microcentrifuge tubes. The tissue was flash-frozen in liquid nitrogen and macerated using a miniature pestle. Holes were pierced in each end of the closed tube, which was subsequently placed inside a 1.5 ml microcentrifuge tube. The sample was then centrifuged for 10 minutes in order to expel cell lysate from the smaller to the larger tube and 25 µl of this sap was taken and used for ion analysis. The sample was initially diluted 1:20 with deionised water, then diluted by 1:20 again immediately prior to analysis.

Samples were tested for a range of ions. The cations measured were: calcium, sodium, ammonium, potassium and magnesium. The anions were: phosphate, sulphate, chloride and nitrate. Cation and anion standards were made up at 0.1 ppm, 0.5 ppm, 1.0 ppm, 5.0 ppm, 10.0 ppm, 25.0 ppm, 50.0 ppm, 100.0 ppm and 150.0 ppm. The standards and samples were loaded into autosampling cartridges seated inside an autosampler (Dionex® AS40 automated sampler) and run on a Dionex® DX500 ion chromatograph (Dionex Corporation, California, USA). Sample information was programmed into Peaknet® version 5.11 software, which was then used to compare the sample ion content with the known concentrations in the standards. The inorganic ion content was compared between treatment conditions and between leaves.

R. communis SE sap samples were also tested for cations. Either 1 µl, 1.025 µl or 1.25 µl of sap was expelled from the collection microcapillary into a Dionex vial. These volumes represented the maximum amounts reasonably collected by stylectomy for such analysis and aimed at assessing this analysis method (i.e. HPLC) for these small sample sizes. The volume was made up to 500 µl with deionised water. These were run with the same standards as above.

2.3.6 Statistical analysis

Statistical analyses were performed using SPSS version 19. The Anderson Darling test for normality (Anderson and Darling, 1952) was performed initially. This test was thought to have the most power and be most appropriate for the sample sizes used in this study (Henderson, 2006). If data followed a normal distribution and could be assumed to have equal variances according to Levene's Test for Equality of Variances, their means were compared using the t-test or a one-way ANOVA followed by post-hoc analysis with Tukey's test. If data were found to deviate from a normal distribution, the Mann Whitney U test for non-parametric data was used for analysis.

2.4 Results

2.4.1 Plant growth

The data for plant height increase followed a normal distribution (Anderson Darling test $p > 0.05$) and the variances were statistically similar according to Levene's test for equality of variances. A one-way analysis of variance (ANOVA) showed that the means were significantly

different ($p < 0.00$) at each time point measured (days 2, 3, 6, 12 and 13). Tukey's multiple comparison test showed that treatment with 20 and 30 aphids had significantly reduced plant growth by day 3 ($p < 0.05$); 5 and 10 aphids did not ($p > 0.05$). By day 3, uninfested plants had grown 45 ± 4 mm, while those infested with 5, 10, 20 and 30 aphids had grown 41 ± 7 mm, 31 ± 4 mm, 18 ± 5 mm and 18 ± 2 mm, respectively (Figure 2-2). By day 6, uninfested plants had grown 177 ± 2 mm since infestation. Plants infested with 5 and 10 aphids showed a significant reduction in height increase (142 ± 7 mm and 129 ± 6 mm, respectively; $p < 0.05$) compared to uninfested controls. The height increase of plants infested with 20 aphids was significantly less still (55 ± 11 mm; $p < 0.05$). No data are available for plants infested with 30 aphids as, by day 6, the primary leaves were displaying wilting and chlorosis. On day 12, plants infested with 5 and 10 aphids showed significantly less growth (198 ± 24 mm and 218 ± 33 mm) than uninfested plants (311 ± 7 mm; $p < 0.05$). Infestation with 20 aphids reduced plant height increase further still (62 ± 19 mm; $p < 0.05$). On day 13, plants infested with 5 aphids (263 ± 12 mm) did not show significantly reduced growth compared to controls (301 ± 9 mm; $p < 0.05$) but those infested with 10 or 20 aphids were significantly different from controls and from each other (185 ± 10 mm and 73 ± 15 mm; $p < 0.05$).

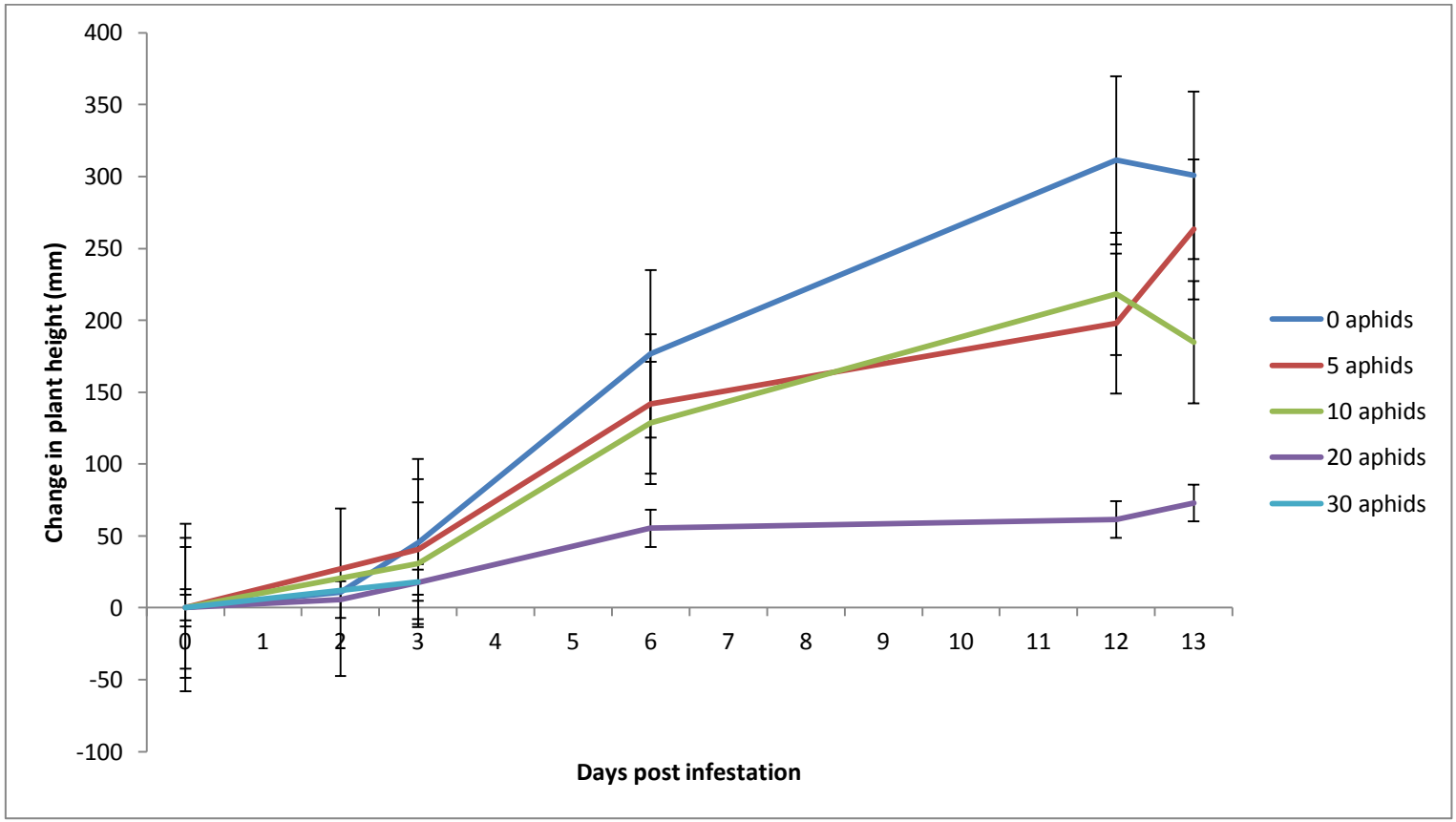


Figure 2-2 Mean height increase of *Hordeum vulgare* plants from the date of infestation (day 0) with *Rhopalosiphum padi* aphids, to 13 days post infestation. Plants were infested 10 days post sowing with either 0, 5, 10, 20 or 30 aphids. N=22 for all treatment conditions. Error bars show ± standard error.

2.4.2 Phloem sap exudation rate, exudation duration and osmotic pressure

The mean duration of sap exudation from a cut stylet was significantly greater (Mann Whitney U, $p < 0.05$) from heavily infested plants than lightly infested (246 ± 42 minutes and 114 ± 27 minutes, respectively)(Figure 2-3). The mean exudation rates on plants infested with 5 and 30 aphids were 0.6 ± 0.12 nl min^{-1} and 0.9 ± 0.22 nl min^{-1}, respectively (Figure 2-4). These values were not significantly different (Mann Whitney U; $p > 0.05$). Sap collected from exuding stylets on heavily infested barley plants was found to have an osmotic pressure of 2.49 MPa.

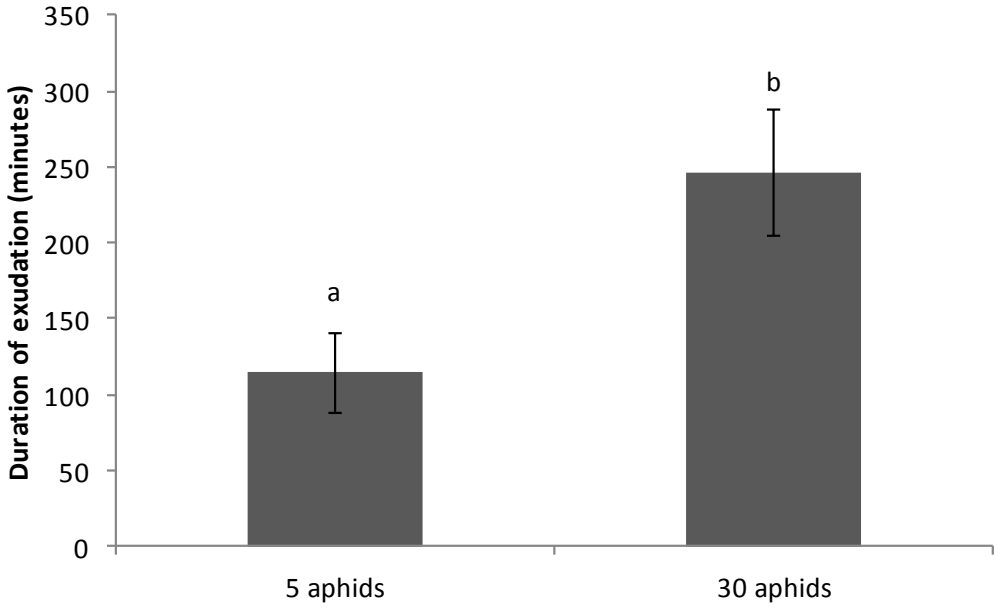


Figure 2-3 Mean duration of SE sap exudation from severed stylets on lightly infested (5 aphids, n=17) and heavily infested (30 aphids; N=23) *H. vulgare* plants. Error bars show ± standard error. Means with different letters are significantly different (Mann Whitney U, $p < 0.05$).

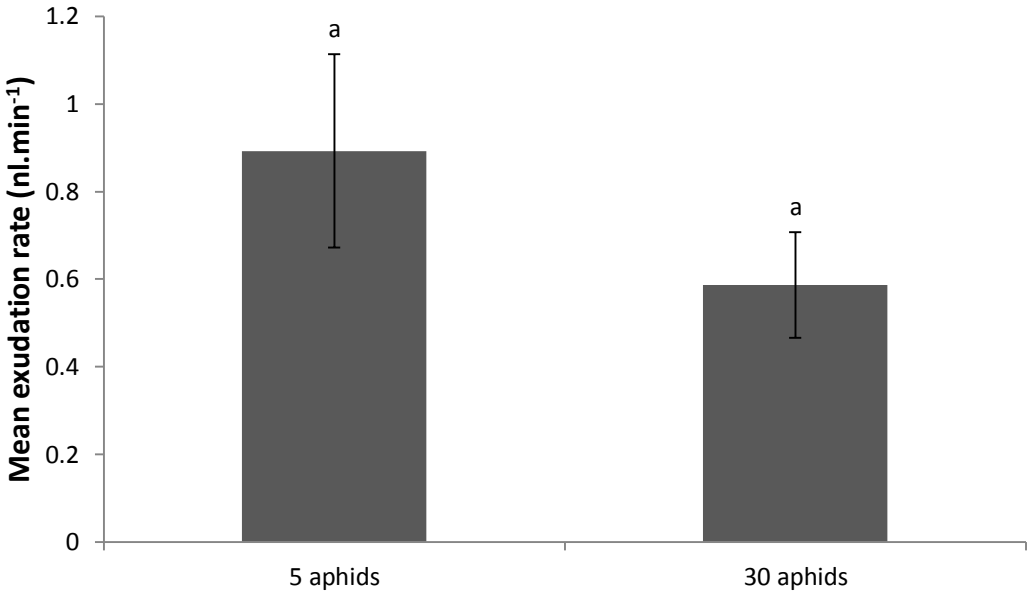


Figure 2-4 Mean exudation rate of stylets from *Hordeum vulgare* plants infested with 5 aphids (N=15) or 30 aphids (N=23). Error bars show ± standard error. Means with different letters are significantly different (Mann Whitney U, p < 0.05).

2.4.3 Inorganic ion content of infested and uninfested leaves

Infestation did not induce a significant change (t-test, $p > 0.05$) in concentration of any of the four anions in local tissues (Figure 2-5). Calcium levels were significantly higher (Mann Whitney U, $p < 0.05$) in infested leaves compared to controls (295.9 ± 34 ppm and 206.7 ± 11 ppm, respectively). None of the other cations were significantly different in these samples (Mann Whitney U, $p > 0.05$).

No systemic effect of infestation was observed for ammonium, potassium, sodium, chloride or phosphate (Mann Whitney U, $p>0.05$), but nitrate, sulphate, calcium and magnesium levels were significantly altered in second leaves by infestation (Mann Whitney U,

$p<0.05$)(Figure 2-6). Nitrate levels were significantly lower in infested plants than controls, falling from 8017.7 ±278 ppm to 7116.1 ± 328 ppm due to infestation. Sulphate levels rose from 559.0 ± 91 ppm in uninfested plants to 876.4 ± 95 ppm in aphid-treated plants. Calcium was 159.3 ± 16 ppm on average in uninfested plants but measured at 232.92 ± 14 ppm in infested plants and magnesium rose from 396.41 ± 10 ppm in control plants to 484.39 ± 22 ppm in infested plants.

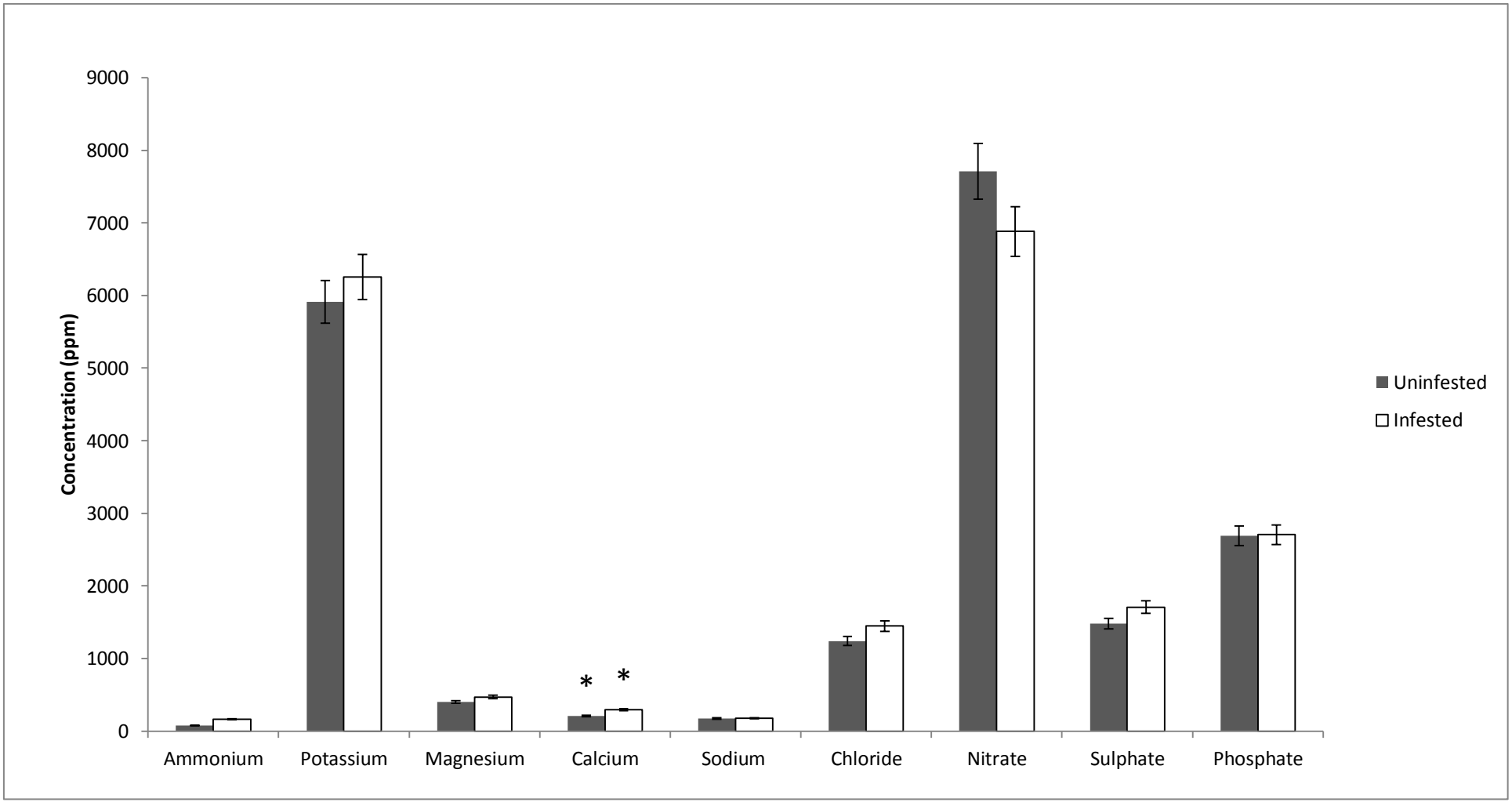


Figure 2-5 Mean concentration (ppm) of 9 inorganic ions in *Rhopalosiphum padi*-infested and uninfested primary leaves of 13 day old barley plants, infested for 72 hours from day 10. N=15 for each treatment. Error bars show ± standard error. Asterisks denote significant differences.

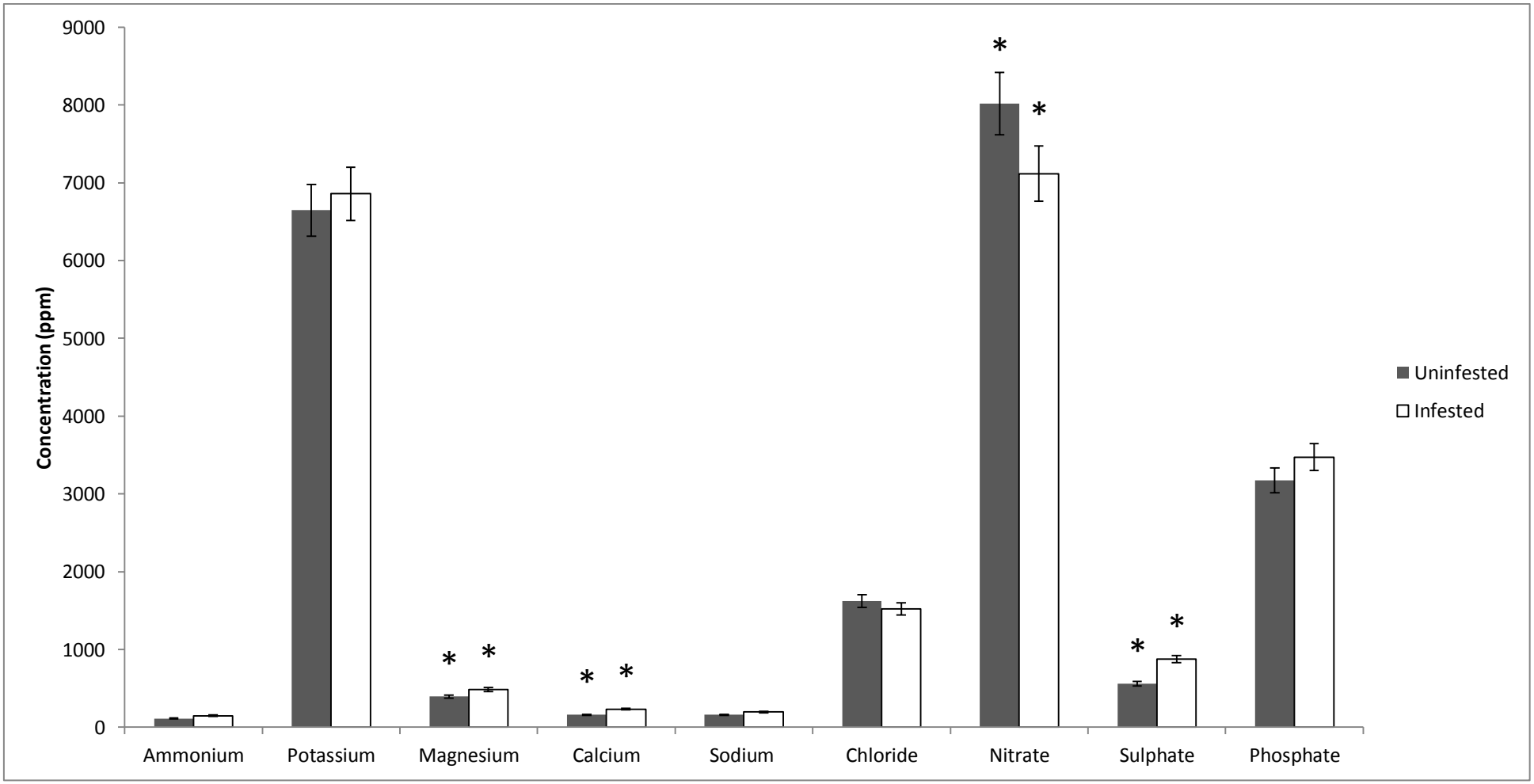


Figure 2-6 Mean concentration of 9 inorganic ions in the uninfested second leaves of *Rhopalosiphum padi*-infested 13 day old barley plants and the uninfested second leaves of uninfested control plants. N=15 for each treatment. Error bars show ± standard error. Asterisks denote significant differences.

Comparing ion levels in the first and second leaves of the same plants showed similar results for uninfested (Table 2-1) and infested (Table 2-2) plants. Potassium levels were significantly higher in the second leaves ($p < 0.01$) whilst calcium levels were significantly lower ($p < 0.01$). Ammonium, sodium and magnesium levels were not significantly different between first and second leaves of the same plants ($p > 0.05$). Significantly higher levels of chloride ($p < 0.01$) and phosphate ($p < 0.01$) were found in the second leaves of uninfested plants whilst significantly lower levels of sulphate ($p < 0.01$) were measured. Nitrate levels were not significantly different ($p > 0.05$).

Table 2-1 Mean ± S.E. ion concentration in the first and second leaves of the same, uninfested plants. Table shows mean ion concentration for each leaf, number of repeats and significance values. a= Wilcoxon signed ranks test; b= paired samples t test. Asterisks denote significant differences.

Ion	Mean		N	Significance (p value)
	Leaf 1	Leaf 2		
Ammonium	78 ±16	113 ± 10	15	.088[a]
Potassium	5912 ± 184	6648 ± 88	15	.001[a] *
Magnesium	402 ± 12	396 ± 10	15	.667[b]
Calcium	207 ± 11	159 ± 16	15	.001[b] *
Sodium	172 ± 22	160 ± 31	15	.820[a]
Chloride	1239 ± 69	1623 ± 111	15	.001[a] *
Nitrate	7708 ± 414	8018 ± 278	15	.429[b]
Sulphate	1480 ± 189	559 ± 91	15	.000[b] *
Phosphate	2690 ± 189	3176 ± 85	15	.009[b] *

In infested plants, potassium levels were significantly higher ($p < 0.05$) in leaf 2 whilst calcium was significantly lower ($p < 0.05$). The other cations were not significantly different between the two leaves. Sulphate levels were lower in the second leaves ($p < 0.01$) and phosphate levels were higher ($p < 0.01$). Chloride and nitrate levels were not significantly different ($p > 0.05$).

Table 2-2 Mean ± S.E. ion concentration of the first, infested leaves and the second, uninfested leaves of the same plants. Table shows mean ion concentration for each leaf, number of repeats and significance values. a= Wilcoxon signed ranks test; b= paired samples t-test. Asterisks denote significant differences.

Ion	Mean		N	Significance (p value)
	Leaf 1	Leaf 2		
Ammonium	163 ± 42	149 ± 23	15	0.701[a]
Potassium	6253 ± 247	6859 ± 232	15	0.046[b] *
Magnesium	470 ± 30	484 ± 21	15	0.281[a]
Calcium	296 ± 33	233 ± 14	15	0.013[a] *
Sodium	176 ± 40	195 ± 35	15	0.733[a]
Chloride	1448 ± 108	1521 ± 64	15	0.393[b]
Nitrate	6880 ± 242	7116 ± 328	15	0.438[b]
Sulphate	1707 ± 146	876 ± 94	15	0.001[a] *
Phosphate	2705 ± 158	3473 ± 159	15	0.001[a] *

2.4.3.1 Ricinus communis *phloem exudate ion concentration*

The sodium concentration of *R. communis* sap decreased from 129.5 (± 41) ppm with a 1 µl sap input in a 500 µl sample volume, to 89.6 (± 29) ppm with 1.025 µl sap input and further to 59.7 (± 23) ppm with 1.25 µl sap (Figure 2-7). Ammonium levels rose from 83.5 (± 26) ppm to 108.1 (± 35) ppm and then to 146.0 (± 57) ppm in the three sample concentrations, respectively. Magnesium levels were relatively constant, ranging from 51.3 (± 17) ppm to 74.3 (± 29) ppm. Potassium concentrations were given as 1141.4 (± 361) ppm 1191.4 (± 386) ppm and 1865.4 (± 737) ppm in the three input concentrations, respectively (Figure 2-8).

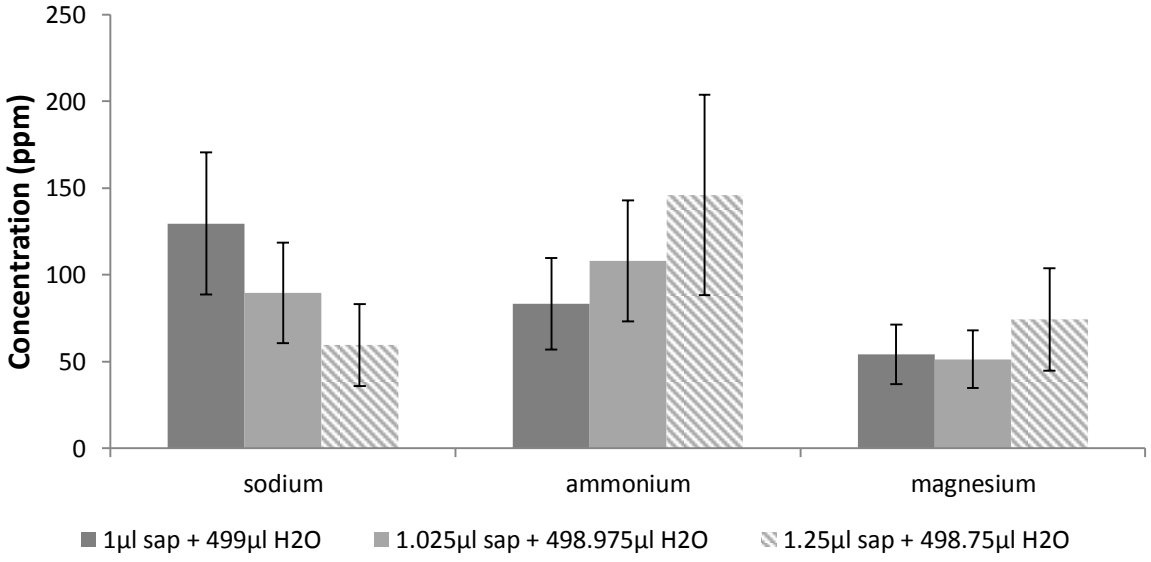


Figure 2-7 Mean concentration, ± standard error, of sodium, ammonium and magnesium ions in *R. communis* sap, collected by exudation, at 3 different input concentrations: 1:499µl, 1.025:498.975µl and 1.25:498.75µl. N=10 for each input sap concentration.

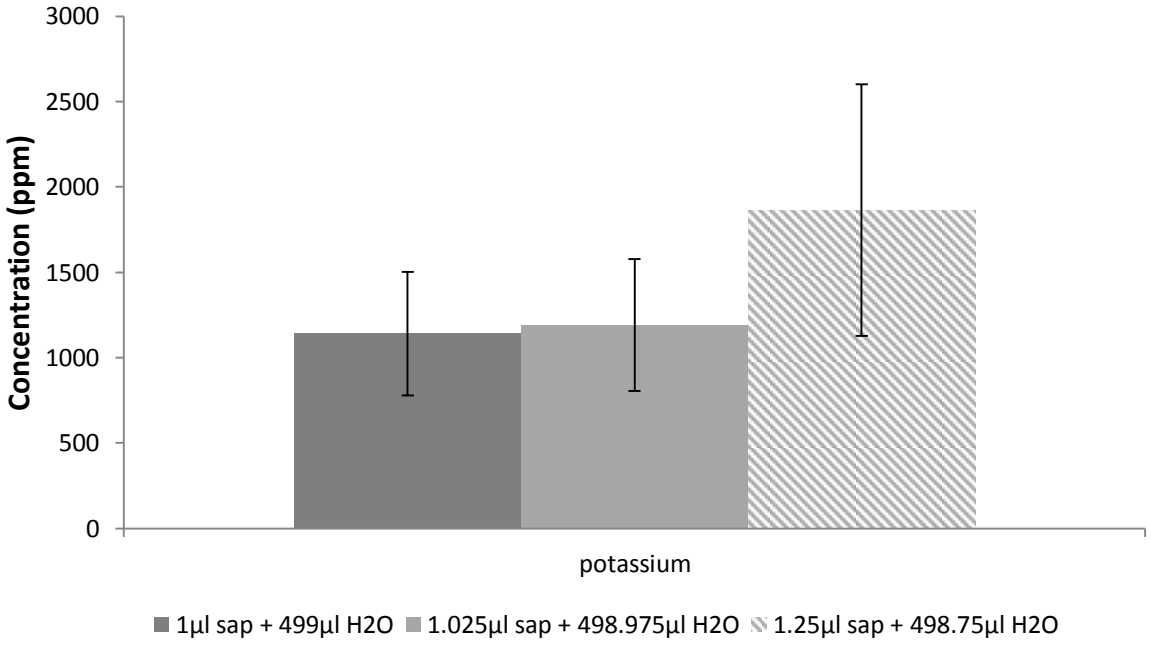


Figure 2-8 Mean concentration, ± standard error, of potassium ions in *R. communis* sap, collected by exudation, at 3 different input concentrations: 1:499µl, 1.025:498.975µl and 1.25:498.75µl. N=10 for each input concentration.

2.5 Discussion

2.5.1 Growth rate of infested and uninfested plants

These results show that aphid feeding has a significant effect on barley plants. Aphid infestation inhibits plant growth and the higher the infestation levels, the greater the resultant stunting. The effect on height in heavily infested plants (20 or 30 aphids) could be observed by 3 days post infestation and the disparity increased as time went on. This indicated that a period of 3 days infestation should be sufficient to characterise the effect of aphid infestation in future experiments such as gene expression and ion content investigations.

Aphids have previously been found to inhibit plant growth at very high infestation levels (over 1500 aphids on 5 week old plants)(Mallott and Davy, 1978). Our study showed that even moderate infestation levels (20-30 aphids) induce stunting. Plant growth can be hindered by two processes: some aphids induce phytotoxicoses by injecting toxic substances into the plant in their saliva (Giordanengo *et al*., 2010); other species inflict less direct damage and restricted growth is likely to result from removal of nutrients in these cases. For example, a correlation has been found between removal of labelled carbon by pea aphids and reduction in stem elongation rate (SER) of *Medicago sativa* (alfalfa)(Girousse *et al*., 2003). The infestation level, the position of feeding aphids on the plant, and the duration of infestation have also been shown to be important factors in determining the C and N fluxes within the host plant. Plants that were heavily infested in the stem growing zone showed reduced nitrogen deposition in the apical zone, for example (Girousse *et al*., 2005).

2.5.2 SE sap exudation rate, exudation duration and osmotic pressure

The exudation rates recorded in this study (0.6 and 0.9 nl min^{-1} on lightly and heavily infested plants, respectively) correspond well with those reported previously. Doering-Saad *et al*. (2002) reported exudation rates of 1-2 nl min^{-1} and Gaupels *et al*. (2008a) recorded exudation rates of 0.9 nl min^{-1}, both on barley. Heavy infestation did not significantly alter the exudation rate in the current study. The mean length of sap exudation, however, was significantly greater in plants heavily infested with aphids. This suggests that aphids were inhibiting callose-mediated sealing of the severed stylet and the sieve tubes in its immediate vicinity, thus allowing sap to flow freely for longer. This is consistent with previous studies

that showed limited callose induction by *R. padi* feeding (Saheed *et al*., 2009). The observed results are likely to be due to increased volumes of saliva being injected into the SE and overcoming plant sealing defences (Will *et al*., 2007). Therefore, a large number of aphids do not significantly improve the rate of SE sap flow, which presumably remains restricted by the physical properties of the SE (van Bel and Hafke, 2005) but the effect of increased volumes of saliva lasts longer in the plant, preventing callose-mediated sealing for longer. This is in accordance with a study showing that pre-infestation of broad beans (*Vicia faba*) led to prolonged honeydew excretion but did not affect the excretion rate (Prado and Tjallingii, 1997).

Previously measured SE osmotic pressure values have varied from ~0.8 MPa in *Arabidopsis* sap obtained by aphid stylectomy (Pritchard *et al*., 2010, Newbury *et al*., 2006) and *Opuntia ficus*, Indica sap obtained from stylectomy of cochineal insects (Wang and Nobel, 1995) to ~2.0-2.5 MPa in species such as *R. communis* (Smith and Milburn, 1980) and *H. vulgare* (Gould *et al*., 2005). Turgor pressure values measured in individual sieve tubes using aphid stylectomy range from ~0.8 MPa in *Salix babylonica* (Wright and Fisher, 1980) to ~1.2 MPa in *S. oleraceus* (Gould *et al*., 2004, Gould *et al*., 2005). The results of the current study are in line with this; sap collected from barley by stylectomy had an osmotic pressure of 2.49 MPa. These plants were infested with 30 aphids. It is not possible to provide an uninfested control measurement as plants must be infested with aphids in order to collect sap. It would be useful, however, to measure the osmotic pressure in lightly infested plants as a proxy for uninfested plants and as a comparator for heavily infested plants.

2.5.3 Nutrition

Nitrogen is found in the atmosphere, soil, and oceans in inorganic forms and is fixed by plants and microorganisms. Host plants that do not form microbial nitrogen-fixing associations absorb nitrogen from the soil through their roots in the form of either nitrate ions or ammonium ions and metabolise it via the glutamine synthetase-glutamate synthase (GS-GOGAT) pathway (Figure 2-9)(Stitt *et al*., 2002). Animals depend on this organic nitrogen supply, obtaining it in the form of protein and amino acids. Phloem sap is deficient in essential amino acids (Douglas, 1993) and therefore provides an unbalanced food source for aphids, requiring them to rely on a bacterial endosymbiont *Buchnera aphidicola* (Shigenobu and Wilson, 2011b). Generally nitrogen moves from the host plant to the feeding aphid but it has been shown that some nitrogen, in the form of aphid-derived proteins, is transferred back to the plant during injection of watery saliva (Wilson *et al*., 2011, Will *et al*., 2007, Mutti *et al*., 2006). This suggests that the nitrogen acquisition mechanisms employed by aphids, and the host-parasite interaction during feeding, are complex.

Data collected in this study show no change in nitrate concentration in the first leaves, which included the infested region (Figure 2-6). This may be because the plant is maintaining levels well in response to feeding by this particular parasite. There may be increased nitrate uptake in response to depleted nitrogen levels in the SE, either due to aphid feeding or stimulation by a component of the aphid saliva. A decrease was seen in the nitrate concentration of second leaves (Figure 2-7), consistent with a systemic shift in nitrogen allocation. Interestingly, there were no significant differences in ammonium levels in either the first or second leaves indicating that the turnover of ammonium remained the same. Ammonium is an important component of amino acid biosynthesis and is therefore important in aphid

nitrogen assimilation (Figure 2-9)(Wilson *et al*., 2011). Previous studies have reported changes in nitrate levels due to aphid feeding, with elevated levels at the site of infestation and reduced concentrations distant from this region, signalling a change from sink-to-source in growing zones and immature leaves (Girousse *et al*., 2005). A study looking at the effect of aphid feeding on soybean reported a 75% increase in nitrate post-infestation (Riedell *et al*., 2009). That was attributed to reduced nitrogen fixation in aphid-infested plants.

There are several steps along the nitrogen assimilation pathway that can be altered by aphids. A gene expression study on sorghum found a nitrite reductase gene (*NiR*) with a role in nitrogen-assimilation, to be significantly induced by aphid infestation (Zhu-Salzman *et al*., 2004). It was postulated by the authors that depletion of the nitrogen metabolites asparagine and glutamine due to aphid feeding could have led to the observed induction of *NiR*. This study highlights a complex interplay between aphid feeding and nitrogen fixation/reallocation. Similarly, glutamine synthase genes have been found to be upregulated due to infestation (Divol et al., 2005a). The aphid *M. nicotianae* induced up-regulation of three nitrogen uptake and metabolism genes (a nitrate transporter, a ferroxin-dependent glutamate synthase and a glutamine synthetase) in *N. attenuate* (Voelckel *et al*., 2004*)*. Treatment with salicylic acid upregulated the same nitrate transporter (Baldwin and Heidel, 2004). It would be interesting to try to localise the effect on nitrate concentration within the plant tissues to give a better insight into the plant-aphid interaction. As aphids feed exclusively from the phloem, it could be assumed that the effect would be greatest in the cells of the CC-SE complex. However, in their role as a sink whilst parasitizing a host (Girousse *et al*., 2003), it could be that nitrate transport is altered on a greater scale.

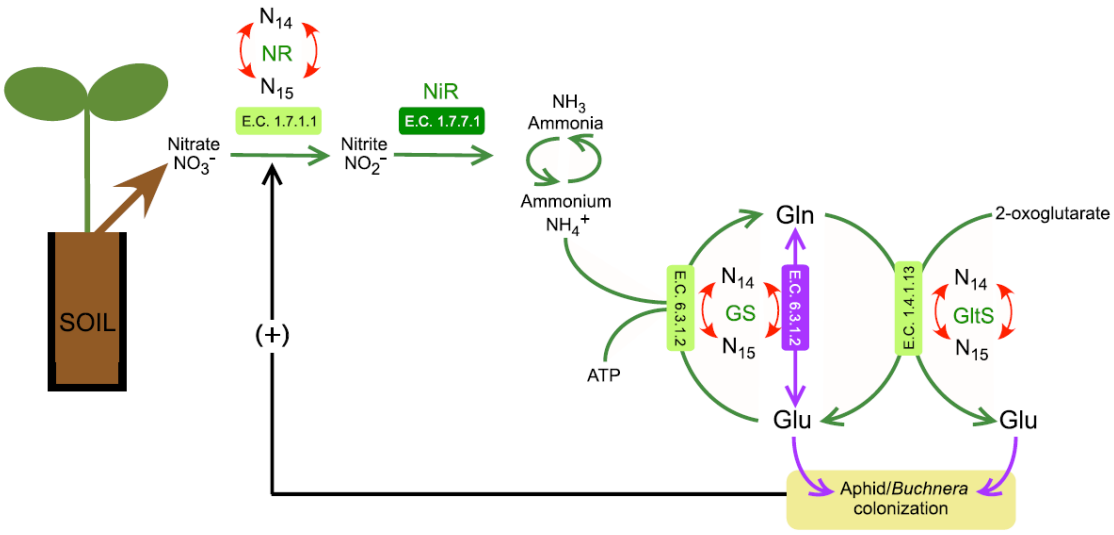


Figure 2-9 Nitrogen assimilation pathway (Wilson *et al.*, 2011). Shows the uptake of nitrate (NO_3^-) from the soil and its conversion to nitrite (NO_2^-) by the enzyme nitrate reductase (NR). Nitrite (NiR) reductase then facilitates the conversion of nitrite to ammonia. Ammonia is converted to ammonium and feeds into the GS/GOGAT cycle of AA biosynthesis. Glutamine synthetase (GS) converts glutamate and ammonium to glutamine. Glutamate synthase then converts glutamine + 2-oxoglutarate to glutamate. Green arrows and boxes show plant metabolism and purple arrows and boxes illustrate metabolism of aphid pests.

The data in this study show that nitrate levels are relatively constant throughout the plant; they are not significantly different between the first and second leaves of the same plant, in both infested and uninfested conditions.

The data showed a significant increase in calcium levels in infested samples compared to uninfested samples in both first and second leaf samples. This shows that aphids may be inducing calcium-mediated defence responses. This is not consistent with the theory that aphids inhibit calcium-mediated induction of defence pathways by salivation (Will *et al.*, 2007). These data highlight the importance of understanding the phloem-specific response as the interpretation of the results will differ according to whether there is a SE-localised Ca^{2+} increase or a rise outside of the phloem tissues. Radioactively labelled calcium could be

used to visualise Ca^{2+} distribution within the plant tissues. None of the other cations were significantly different in these samples.

In the second leaf samples, magnesium levels were significantly higher ($p<0.01$) in the aphid-treated plants. Magnesium has been shown to have an important role in preventing damage from several stressors. Mengutay *et al*. (2013) showed that magnesium deficiency increases the susceptibility of wheat and maize plants to heat stress. They found that levels of the antioxidative enzymes superoxide dismutase, glutathione reductase and ascorbate peroxidase were elevated in Mg-deficient plants subjected to heat, indicating that heat stress probably increases oxidative cellular damage caused by ROS in these plants. Mg deficiency has also been shown to increase susceptibility to photooxidative damage in bean (*Phaseolus vulgaris* L.) leaves (Cakmak and Marschner, 1992). Chlorosis and O^{2}- and H_20_2-scavenging enzyme levels were increased when Mg-deficient leaves were exposed to high light intensities. When aphids feed on host plants, no ROS increase is generally observed. It has been suggested that aphid salivary enzymes scavenge ROS (Rao *et al*., 2013) in order to allow a compatible relationship. Furthermore, *R. padi* does not induce chlorosis in the leaves from which it feeds, unlike other aphid species (Saheed *et al*., 2007). Cakmak *et al*. (1994) showed that another cause of chlorosis in Mg-deficient plants is impaired phloem loading. By varying the Mg supply to bean plants, they showed this micronutrient has a key role in loading and export of photosynthates, especially sucrose, from the source leaves. In Mg-deficient plants the rate of sucrose export was decreased by 80-90% compared to controls. In the same study, sucrose levels were elevated in whole leaf samples but reduced in SE sap samples collected by EDTA-facilitated exudation. That showed that sucrose was not being loaded into the phloem but was instead accumulating in the leaf tissues. Inhibition of root

growth and high shoot/root dry weight ratios were key symptoms of Mg deficiency in that study. A reduction in the phloem export of amino acids due to Mg deficiency was also observed. As aphids obtain most of their nitrogen from amino acids in phloem sap, a reduction of AA loading into the SE would have an impact on aphid performance on Mg deficient plants. Maintaining an adequate Mg level is therefore highly advantageous to aphids, and this is what the results of this study show is happening. Whether or not this is also due to a component of aphid saliva is speculation and further investigation would be needed to demonstrate this.

Interestingly, potassium levels were not altered significantly by aphid feeding. As mentioned above, potassium is one of the main osmotic components within the plant and deviation from normal levels would be likely to have severe consequences throughout the plant. It appears from the data that a mechanism is in place to control levels of this anion. This is consistent with measurements of SE sap osmotic pressure, which were around 2.5MPa in heavily infested plants, similar to measurements of uninfested barley plants in other studies (Gould *et al.*, 2005). Another implication of stable potassium levels is that sugar uploading to the SE is likely to be maintained as potassium has a key role in the regulation of this process (Deeken *et al.*, 2002). Potassium concentration was shown to be affected by the diurnal cycle in *R. communis*, where there was reciprocity between potassium and sucrose concentrations (Smith and Milburn, 1980) but, as the infestation and harvesting of the experimental plants in this study were always performed at the same time of day, this was unlikely to have affected the results. Again, potassium levels could vary in different regions of the leaf as only the overall measurement was taken.

2.5.4 Evaluation and future directions

The aphids were asexually reproducing adults so aphid numbers increased on the experimental plants. The biology and feeding behaviour of the aphids may therefore have obscured the direct response to infestation, but the results are more representative of a natural population increase in the field. Nymphs have a lower nutritional requirement than adults due to their reduced size. Also, the first nymphs deposited on the day of infestation would not have reached maturity by the end of the experiment; the average time to maturity is 10 days, depending on plant conditions such as leaf quality leaf quality (Leather and Dixon, 1981).

It has been suggested that exudation rate measurements from cut stylets are affected by evaporation (Pritchard, 1996). Collection of sap under water-saturated mineral oil, a common approach to stylectomy collection to reduce evaporation, also affects volume calculations due to the curvature of the surface of the oil (Palmer *et al*., 2013). Palmer *et al*. (2013) took a sequence of photographs of the sap collection period to reduce the air-exposure time of sap droplets to only 5 seconds. Measurements from these photos were compared with measurements taken from sap droplets collected under oil and a linear correction factor was generated which allowed correction for evaporation in sample volumes measured in air. A similar approach may have been more accurate in this study, however our method was sufficient for its purpose, i.e. to give a comparison between heavily and lightly infested plants to determine the effect of aphid feeding on sieve tube occlusion. Where exudation levels have been used to inform other chapters, this has been for preliminary calculations and a mean exudation rate has been used, rather than the

maximum value. These values are therefore likely to be generally representative of sap exudation levels, if a correction for evaporation had been applied.

When using *R. communis* SE sap to measure cation concentration in small quantity samples, representative of stylet sap, different values for the same ion were returned, depending on the input volume. For example, inputting 1 µl and 1.025 µl in 500 µl aliquots returned readings for potassium of 1141 ppm and 1191 ppm, respectively, whilst inputting 1.25 µl in 500 µl gave a value of 1865 ppm (Figure 2-7 & Figure 2-8). Therefore this method is clearly inappropriate for analysing small sap samples due to the limited resolution of the apparatus at such low ion levels, and no stylet samples were run on the ion chromatograph. One approach to overcoming this problem would be to collect greater phloem sap samples than were acquired in this study and concentrate them by vacuum or freeze-drying to improve SE sap ion measurements. It may not subsequently be possible to collect equivalent quantities of *H. vulgare* sap by stylectomy, however. Ion analysis of cellular and vacuolar contents microsampled from cells of the upper epidermis of wheat seedling leaves has previously been achieved (Bazzanella *et al*., 1998). These samples, in the picolitre range, were directly injected, using a micropump and modified microsyringe, into a custom built capillary electrophoresis system to identify cations and anions within them. This approach may have proved more successful with the *R. communis* SE samples in the current study and would an avenue for further SE sap ion analysis of stylet-collected samples.

Previous studies have shown a reduction in photosynthetic capacity, and a subsequent reduction in growth, of host plants following aphid infestation (Simpson *et al*., 2012). It would be interesting to investigate physiological traits such as maximum photosynthetic rate, gaseous exchange, leaf fluorescence, and leaf chlorophyll content during *R. padi*

infestation of barley, to determine whether these factors are affected by infestation and whether they are linked to the reduced growth observed in this study.

As well as providing important insights into the physical changes caused by aphid infestation, the results of this work offered useful information for further chapters of this thesis. Most importantly, it allowed identification of an appropriate level of infestation for array studies, where the impact of aphid infestation, rather than widespread tissue damage, could be recorded. Specifically, it was identified in the growth analysis that 3 days infestation with 30 aphids induced a reduction in growth but no other visible symptoms, whereas infesting for longer than this period led to wilting and chlorosis in the infested leaves. Bostock *et al*. (2005) noted that unnaturally high inoculum levels used in plant-pathogen relationship investigations can have a profound effect on the outcome. Our data demonstrate that this is likely to also be true of plant-pest relationships.

Plants are not uniform, and comparing ion concentration in first and second leaves of the same plant was aimed at showing how ions are partitioned throughout the plant in normal, uninfested conditions. Comparing this with data from infested plants was aimed at showing how infestation alters the balance of ions within the plant and revealing whether the second leaves of infested plants were at a different stage in the sink-to-source transition to those from uninfested plants. The data show that, although the levels of the various ions were altered by aphid feeding, the balance of those ions within the plant remained roughly the same. The only ion showing a change was chloride, which showed higher levels in the second than the first leaves of uninfested plants but no difference between first and second leaf concentrations in infested plants. Overall, these results suggests that the sink/source status of the second leaves of infested plants was the same and the systemic effects recorded in

Chapter 5 can be attributed with relative confidence to aphid infestation, rather than developmental delay.

3 *R. padi* have an inhibitory effect on the feeding behaviour of subsequent conspecifics on barley

3.1 Summary

Chapters 2, 4 and 5 of this thesis investigate the morphological, physiological and transcriptomic changes induced in barley by *R. padi* infestation. Plant responses to aphid infestation may alter the suitability of that plant as a host for subsequent populations. The aim of this chapter was to investigate the effects of pre-infestation on aphid feeding behaviour. The Electrical Penetration Graph (EPG) was used to monitor feeding behaviours on pre-infested and uninfested plants. The results indicate that *R. padi* pre-infestation inhibits feeding performance, reducing the duration of phloem sap ingestion and increasing the proportion of time spent in pathway through the leaf. The duration of aphid salivation and xylem sap ingestion were not significantly altered by pre-infestation.

3.2 Introduction

Aphids obtain their full nutritional intake from their hosts and inject saliva, containing effector molecules, into the plant at all stages of feeding (Prado and Tjallingii, 1994). The physiological and transcriptional responses to aphid feeding have been demonstrated in a number of host-pest pairs (Goggin, 2007, Thompson and Goggin, 2006a). It is reasonable to assume that induced changes will affect aphids subsequently colonising pre-infested hosts. This may take the form of an induced defence response, reducing aphid performance, or a facilitatory effect, suppressing plant defence responses and improving aphid performance.

Such effects have been observed in certain plant-attacker combinations. For example, *Macrosiphum euphorbiae*, the potato aphid, was significantly more attracted to control potato plants than to those pre-infested with conspecific individuals, and spent less time ingesting phloem sap when feeding on pre-infested plants (Brunissen *et al*., 2009). The performance of the aphids was not affected by previous conspecific infestation, however. In the same study, aphid settlement and colony growth rates were greatly enhanced by previous infestation by *Myzus persicae*, even though their feeding behaviour was not altered. A similar effect was observed during *M. persicae* feeding on a resistant peach cultivar (Sauge *et al*., 2002). Significantly fewer aphids settled on pre-infested than on uninfested plants, and phloem sap ingestion was 4-fold shorter on pre-infested than on uninfested plants. Conversely, in a susceptible cultivar, pre-infestation led to slightly enhanced larviposition, less sieve element salivation and more continuous sap ingestion. A study looking at the effects of pre-infestation of wheat on the performance of *R. padi* (Messina *et al*., 2002) showed a negative effect of pre-infestation. Aphid treatment reduced subsequent colony growth by 50% and individuals preferred leaves from control plants to those from *R. padi*-infested plants. In the same study, alates of *R. padi* within flight cages settled more often and showed a higher fecundity on control plants than on previously infested plants.

Due to the variety of responses in those pre-infestations, it has been widely acknowledged that the presence of a pre-infestation effect, and the form it takes, is host-parasite specific (Messina *et al*., 2002). It has been shown that these effects can occur within a very short space of time, even if the damage is of short duration, and aphids will shift their host preferences soon after biotic or abiotic damage (Couty *et al*., 2007).

The current study was designed to provide an insight into the feeding behaviours of *R. padi* on a susceptible barley plant and the changes in feeding patterns resulting from the physiological and transcriptomic changes identified in Chapters 2 and 5, respectively. Specifically, it was hoped to determine whether pre-infestation of barley plants facilitated or hindered the feeding of subsequent colonising individuals.

3.2.1 Electrical penetration graph (EPG)

An important indicator of a facilitatory or inhibitory effect is altered feeding behaviour. To investigate the feeding behaviours of phloem- and xylem-feeding insects, the electrical penetration graph (EPG) technique is often used (Tjallingii and Esch, 1993). EPG allows real-time visualisation of aphid feeding behaviour. Looking at feeding behaviours is also a useful method of determining where defence- or facilitation-related effects are taking place. For example, if aphids take longer to locate the SE, the effect can be assumed to be occurring in the epidermis or mesophyll; more cell punctures indicate problems navigating through tissues *en route* to the SE; shorter periods of ingestion from the SE (E2) show a rejection of SE, and the effect is in the SE sap itself (Prado and Tjallingii, 1994).

EPG has been used to investigate the effects of pre-infestation by aphids on subsequent populations. For example, the yellow sugarcane aphid, *Sipha flava,* displayed improved feeding abilities on pre-infested *Sorghum halepense* (Johnson grass) leaves (Gonzales *et al*., 2002). Aphids spent more time in penetration activities in infested leaves and the phloem was located more rapidly. A similar facilitation effect was observed during *A. fabae* feeding on pre-infested leaves of its host plant *V. faba* showing longer and more continuous sap ingestion and less salivation into sieve elements before sap ingestion (Prado and Tjallingii,

1997). This suggests a role of phloem factors in the facilitation effect. There were also fewer non-probing periods before the first phloem phase on previously infested leaves compared to controls, suggesting that aphids are picking up cues from the mesophyll and non-vascular tissues more effectively on pre-infested plants. In the same study, excretion of honeydew continued for longer periods on previously colonised plants, in line with the prolonged SE sap ingestion observed on EPGs although the excretion rate did not change. Unlike *A. fabae*, *R. padi* did not show responses to previous colonisation.

The effect of previous herbivory has been found to alter according to plant genotype, particularly with regard to the resistance status of the plant (Sauge *et al*., 2006). *M. persicae*, for example, were found to increase the length of time taken to reach the phloem and to reduce the length of sustained phloem feeding by 4-fold in a resistant peach cultivar (Sauge *et al*., 2002). On a susceptible cultivar, however, previous infestation improved the feeding performance of aphids leading to greater host plant acceptance with less salivation and more sustained SE sap ingestion. Plants resistant to herbivore feeding are generally divided into two categories based on their resistance mechanisms. Plants exhibiting avoidance resistance affect herbivore recognition or preference for the plant, deterring potential attackers before they reach the phloem, whereas those employing antibiosis resistance increase the mortality or reduce the longevity and fecundity of an insect, often in a phloem-mediated response (Painter, 1958).

EPG has previously been coupled with other techniques such as honeydew clocks and video monitoring, in order to link feeding behaviours to other factors, such as excretion rate and larvipositioning. For example, Nam and Hardie (2012) coupled EPG with video recording to investigate probing and larviposition behaviours and were able to show that all gynoparae

and 55% of winged virginoparae in the study initiated larviposition before phloem contact, indicating that phloem contact may not be a pre-requisite for those aphid forms to initiate reproduction. These types of studies make EPG a useful tool to investigate aphid feeding, host selection and facilitation.

3.2.2 Aim and hypotheses

In summary, the aim of this study was to use EPG to monitor aphid feeding on pre-infested barley plants and compare this to data from uninfected controls. It was hypothesised that aphid infestation, specifically the injection of aphid saliva into the plant, would induce a facilitation effect and aphids would either locate the phloem more rapidly, or would ingest sap for longer, or both, on pre-infested plants, depending on whether the induced responses were phloem-mediated or not.

The EPG experiments were run at the same time-points and under the same, carefully controlled, conditions as the microarrays in Chapter 5 and so provided an opportunity to map the gene expression data onto aphid behaviours on the plant, thus translating the molecular data collected into real-world applications. For example, increased phloem ingestion on a pre-infested plant could be explained by simultaneous upregulation of phloem-specific susceptibility genes and these genes would be candidates for further investigation.

3.3 Materials and Methods

3.3.1 Plant growth conditions and infestation

H. vulgare plants were grown as described in Chapter 2. Plants were infested with 30 adult aphids on day 10. A second set of plants were fitted with cages but no aphids. After 72 hours, the cages were removed from both sets of plants and the aphids gently brushed off with a soft paintbrush. An apterous adult experimental aphid, which had been removed from the parent culture and starved for 1 hour, was then applied to the previously infested region, or the equivalent position on the uninfested plants. These aphids were wired into a circuit using a fine 18.5 µm diameter gold wire and conductive silver paint.

3.3.2 Electrical penetration graph

Aphid feeding behaviour was recorded and classified using a DC EPG system (from EPG Systems, Wageningen Agricultural University, Wageningen, The Netherlands). The equipment (including an electrical resistor, a voltage source and a Giga 8- DC EPG amplifier) was set up as per Prado and Tjallingii (1997). An aphid was attached to a fine gold wire with conductive silver paint and acted as an electrode; a second electrode, constructed of thicker copper wire, was placed in the soil of the experimental plant (**Error! Reference source not found.**). The EPG was located within a faraday cage covered in silver foil to minimise interference and was run at a temperature of 24 ± 3°C and 60 ± 10% humidity. 8 channels were run concurrently for each repeat, 4 pre-infested and 4 control plants. Each recording lasted for 8 hours. All the signals were recorded and analysed using STYLET+ software.

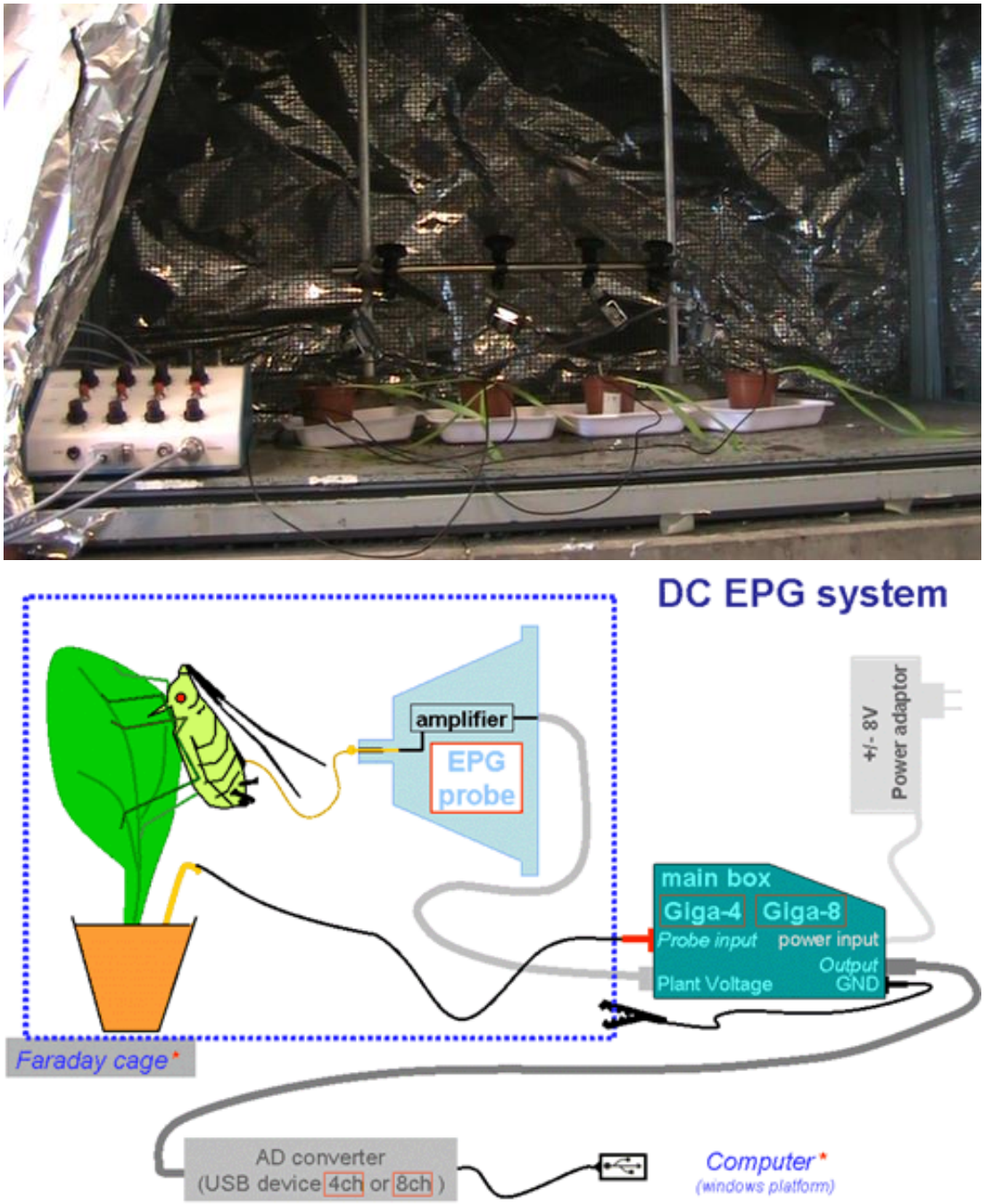


Figure 3-1 Electrical Penetration Graph equipment. Top: A 4 channel Electrical Penetration Graph system. Recording the feeding behaviour of four aphids feeding on young barley plants inside a Faraday cage surrounded by silver foil. Bottom: The set-up comprises of an electrical resistor, a voltage source and a Giga 4- or 8- DC EPG amplifier with a 109 Ω input resistance. An aphid is wired up with gold wire and silver paint and acts as one of two electrodes; a second electrode, constructed of copper wire, is placed in the growing medium of the experimental plant. http://www.epgsystems.eu/systems.htm

EPG waveforms have been previously characterised (Tjallingii and Esch, 1993). Typical traces displaying the main feeding behaviours are shown below (Figure 3-2 to 3-3). This study concentrated on 5 main behaviours: non-probing (NP; out of the plant); pathway (C; including cell penetrations); salivation in the SE (E1); SE sap removal (E2); and xylem feeding (G). A further pattern, described as 'derailed stylet mechanics' was also recorded, as was the number and average length of potential drops (pds), which represent cell penetrations. However, the latter two waveforms were classed as pathway behaviours during analysis. It was thought that focusing on the main, easily identifiable behaviours, and in particular grouping all pathway behaviours, would lend more power to the analysis.

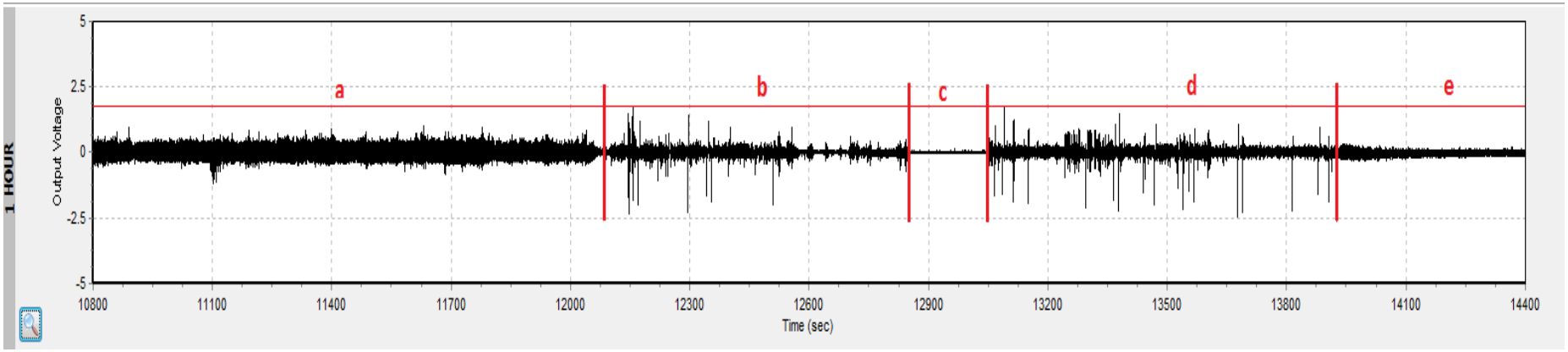
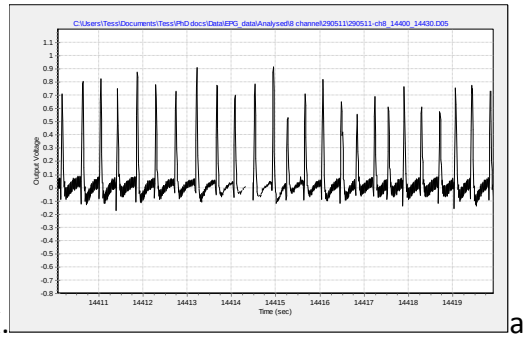
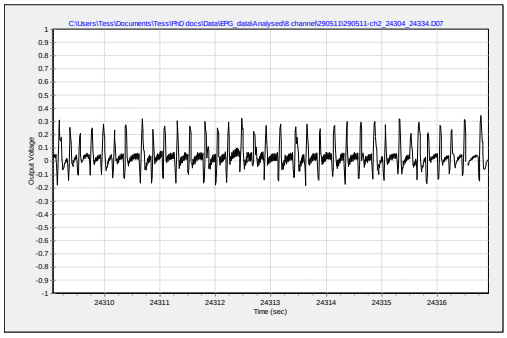
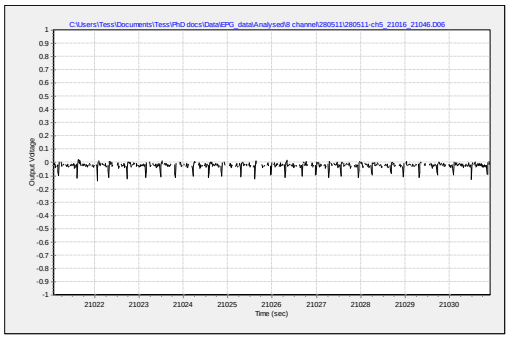
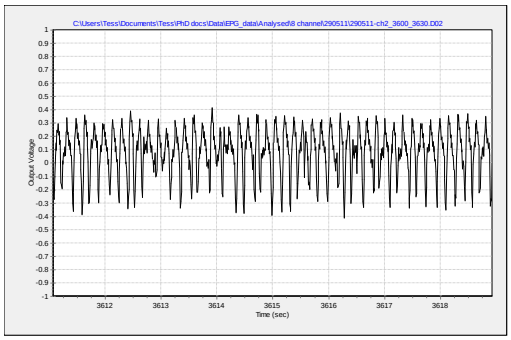


Figure 3-2 An electrical penetration graph trace. Shows a one hour recording including a stretch of xylem feeding (a; 10800-12100 seconds); pathway, including numerous cell penetrations (b and d; 12100-12850 and 13050-13900 seconds); a stretch of non-probing (c; 12850-13050 seconds); and phloem feeding (e; 13900-14400 seconds).

a.I.  a.II 

b  c 

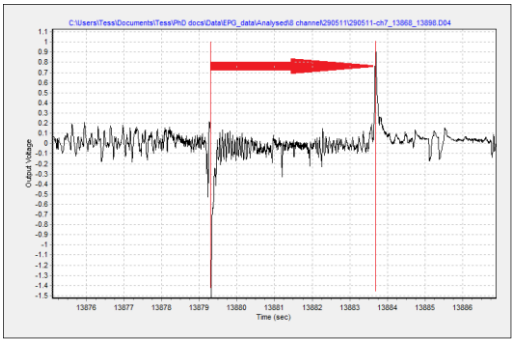
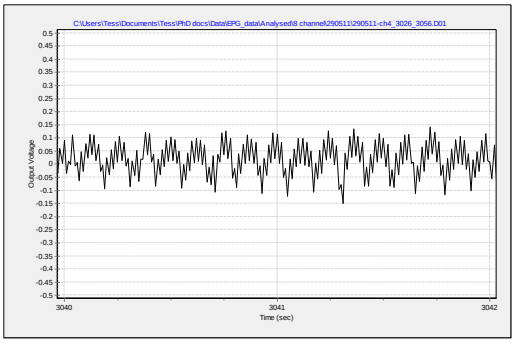
d  e 

Figure 3-3 Electrical penetration graph traces. a) I and II. Two examples of E1 salivation of varying amplitudes. E1 is characterised by regular peaks at a frequency of approximately 3-4 per second. b) E2 SE sap ingestion. Characterised by regular troughs (2-3 per second). c) Xylem ingestion. Large amplitude waveform, simple wave pattern. d) Characteristic wave pattern of a cell penetration, showing a potential drop. Potential drops were measured from trough to peak as indicated. e) Stylets derailed. An irregular pattern, thought to be indicative of difficulty feeding (Tjallingii, 1988).

3.4 Results

The data for duration of potential drops (pd) followed a normal distribution (Anderson Darling $p > 0.05$), so a t-test for independent samples was used. The rest of the data were not normally distributed (Anderson Darling $p < 0.00$) so the Mann Whitney U test was used for analysis.

There were no significant differences between the lengths of time spent not probing (np) and in E1 and xylem phases in pre-infested and control plants (Table 3-1). There were significant differences, however, in time spent performing pathway behaviours and time spent in E2. Aphids on pre-infested plants spent less time feeding from the phloem and more time performing pathway activities over the 8-hour period.

There were no significant differences in the number of occurrences of the various feeding events. The total number of pds did not increase with the increase in pathway time in infested plants, meaning that the pd frequency decreased in these samples (0.27 pds per minute in pre-infested plants compared to 0.35 per minute in controls). The time taken to reach E2 was not significantly different between pre-infested and uninfested plants. This was measured from the start of recording, rather than from the first penetration as it was assumed that, following an hour starvation period, the aphids would have been seeking a suitable food source and any non-probing time between the start of recording and SE location could be indicative of antixenotic effects of pre-infestation.

Table 3-1 Mean duration in minutes (± SE) and number of occurrences of the feeding behaviours of bird cherry-oat aphid (*R. padi*) over an 8-h period on uninfested and pre-infested barley plants. Asterisks denote significant results.

EPG parameter	Mean uninfested N=12	Mean pre-infested N=11	P value
Duration of waveforms (minutes)			
Non-probing	289.67 (±57.81)	326.07 (±61.64)	0.474 [a]
Pathway	861.42 (±143.39)	1454.77 (±164.42)	0.026 [a] *
E1	185.53 (±59.03)	136.79 (±44.10)	0.821 [a]
E2	1548.70 (±177.86)	908.25 (±156.74)	0.023 [a] *
Xylem	527.69 (±119.61)	774.12 (±141.85)	0.232 [a]
pd (seconds)	3.97 (±0.12)	3.96 (±0.16)	0.971 [b]
Occurrences of waveforms			
Non-probing	1.04 (±0.19)	1.63 (±0.29)	0.174 [a]
E1	0.48 (±0.14)	0.60 (±0.16)	0.834 [a]
E2	0.59 (±0.08)	0.49 (±0.10)	0.063 [a]
Xylem	0.31 (±0.06)	0.37 (±0.06)	0.916 [a]
pd	43.9 (±15.5)	55.9 (±17.7)	0.619 [b]
Time to E2	13837.18 (±3370.51)	16059.73 (±3716.57)	0.970 [a]

[a]Independent samples Mann Whitney U test
[b]Independent t-test

The data were plotted per hour to give a picture of aphid feeding behaviour over the 8-hour recording period (Figure 3-4 & Figure 3-5). Non-probing (NP) time was similar across all 8 hours for pre-infested and uninfested plants. NP initially constituted around 19% of time spent on both uninfested and pre-infested plants. The proportion of time spent not probing declined in both conditions and both ended at around 7% at the end of recording. The proportion of time spent in pathway phase decreased throughout the traces from 46% for both pre-infested and non-infested plants. This decrease was much more extreme for uninfested plants, which had reached a mean of 26% by the fourth hour and 8.7% by the final hour. Aphids on the pre-infested plants spent an average of 40-45% in pathway until hour 5 after which it gradually decreased to 30.6% in the final hour. The proportion of time spent salivating in the SE followed a similar pattern in both conditions, increasing between hours 1 and 3 then decreasing sharply in the fourth hour. It then rises again in hours 5 and 6 and falls again towards the end of the trace. E2 duration increased throughout both sets of traces. In uninfested plants the proportion of E2 feeding began at only 7.8%, increasing rapidly until it had reached 50.1% in the fourth hour, then increasing more slowly reaching 69.5% by hour 8. Aphid-treated plants showed a much slower, gradual increase, finishing at 39.8% in hour 8. Xylem duration remained relatively constant in both treatment conditions, decreasing slightly from 20% in the first hour to 11.9% in the 8th hour in uninfested plants and fluctuating in infested plants.

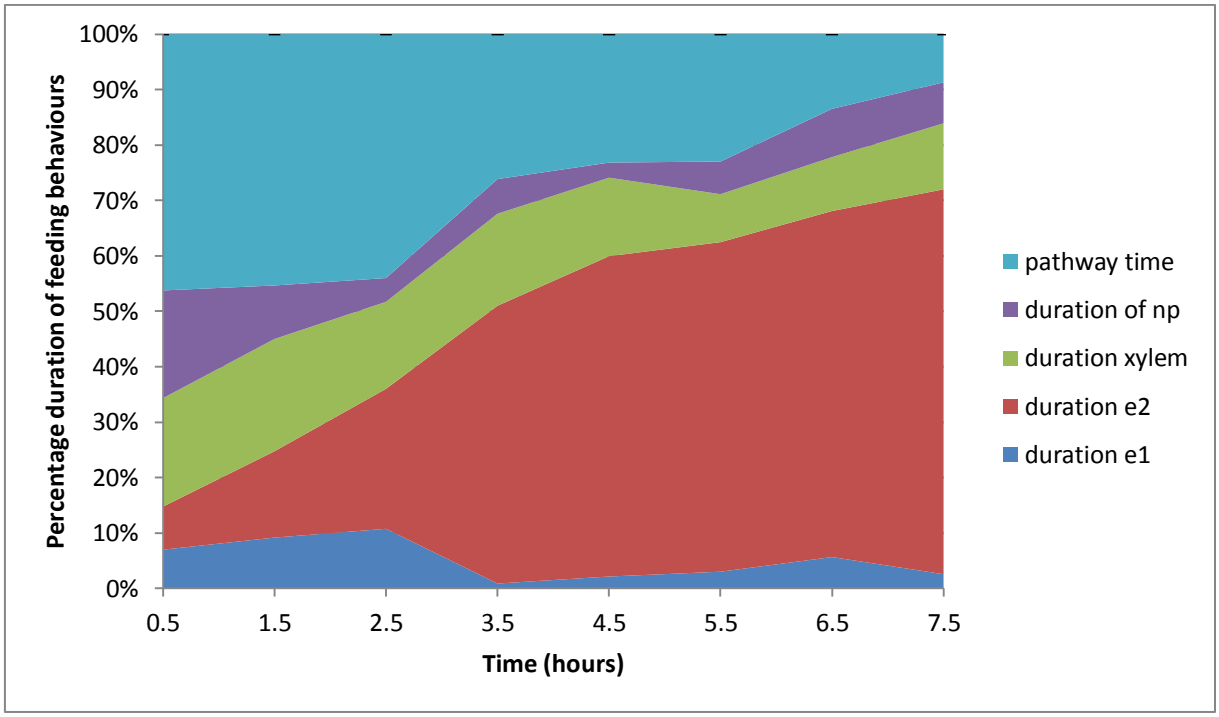


Figure 3-4 Mean percentage duration of five EPG waveform patterns: Non probing, pathway, E1 salivation, E2 feeding and xylem ingestion in uninfested plants. The graph shows the changes in percentage duration over 8 hours.

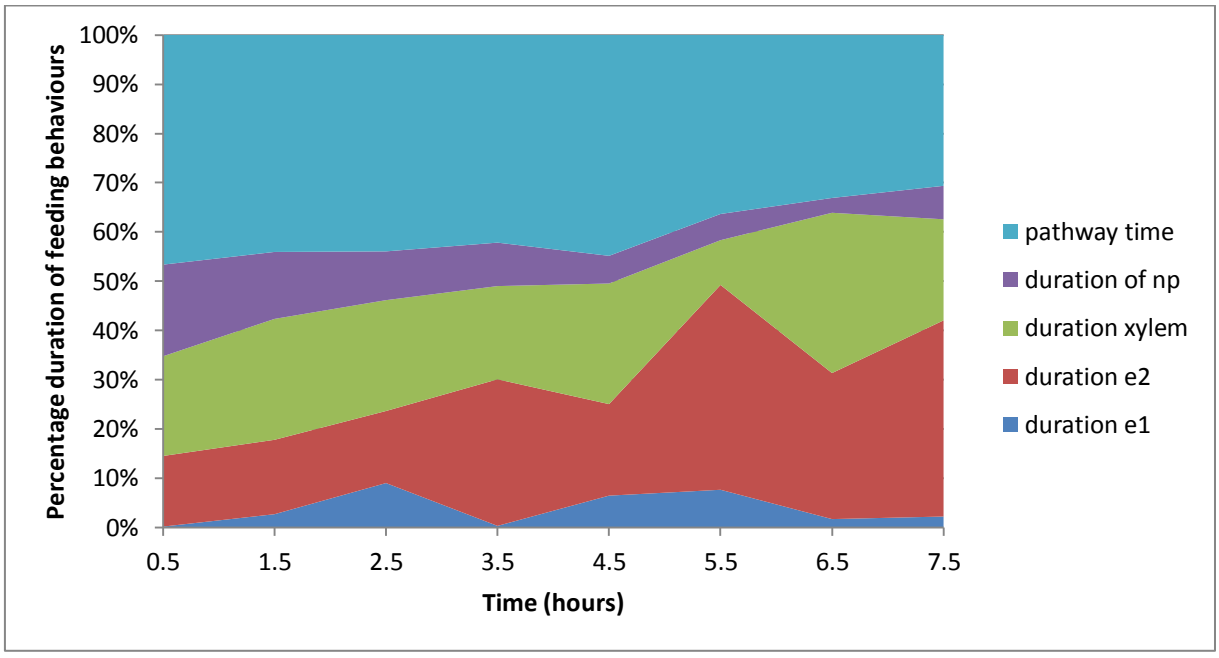


Figure 3-5 Mean percentage duration of five EPG waveform patterns: Non probing, pathway, E1 salivation, E2 feeding and xylem ingestion in pre-infested plants. The graph shows the changes in percentage duration over 8 hours.

3.5 Discussion

The time taken for aphids to commence phloem feeding (E2) was not significantly different between pre-infested and uninfested plants. This would seem to suggest that pre-infestation has not induced changes in the plant that alter the ability of an aphid to locate the phloem. The duration of E2 was significantly less when the aphid was feeding on a pre-infested plant. It appears that aphid-induced changes caused the experimental insects to reject the SE sap once located and revert to probing behaviours, the duration of which was significantly increased in pre-infested plants. Interestingly, there was no significant difference in salivation (E1) duration or number between the two conditions, so the aphids were not making an attempt to overcome any feeding problems by increasing salivation. Previous studies have reported increased salivation by *R. padi* prior to sustained sap ingestion on nitrogen-deficient barley (Ponder *et al*., 2001) and by *M. persicae* on mutant *Arabidopsis* plants which had reduced levels of amino acids (Hunt *et al*., 2010) suggesting salivation is an important part of improving plant nutritional quality for aphids. It is also known that aphid saliva contains proteins designed to overcome plant defences such as forisome- and callose-sealing of sieve tubes (Will *et al*., 2009, Will *et al*., 2007) and altered salivation has been observed in EPG studies of pre-infested plants (Prado and Tjallingii, 1997). *R. padi* have been shown to induce only limited callose deposition when feeding on barley (Saheed *et al*., 2008), making this result even more surprising. Furthermore, the pd frequency decreased during the stylet pathway phase in pre-infested plants, despite the duration of pathway increasing. Pd represent puncturing of cells along the stylet pathway; as the stylet breaches the plasma membrane, a potential drop is induced. These brief probes allow sampling/olfaction of cell contents for cues to assist in location of the SE. A reduction in such

probing and sampling of cellular contents suggests that greater efforts are not being made to reach the phloem in pre-infested plants, despite reduced feeding durations.

One previous study has reported no modification of *R. padi* probing behaviour after pre-infestation of *Triticum aestivum* (Prado and Tjallingii, 1997) but the majority have shown a negative effect of aphid infestation, even if it is temporally separated and, in all cases, *R. padi* feeding led to a heterospecific inhibition of aphid performance. For example, one group found that conspecific competition hindered *R. padi* feeding performance (Chongrattanameteekul *et al*., 1991b) and reduced fecundity and lifespan on wheat (Chongrattanameteekul *et al*., 1991a). In the latter study, the performance of *S. avenae* was also inhibited by *R.padi* infestation, whereas *R. padi* were not hindered by competition from *S. avenae*.

A negative effect of pre-infestation of winter wheat has even been reported where the secondary infestation was subject to a lag time, allowing the plant to recover to a certain degree (Messina *et al*., 2002). These authors carried out choice tests and population growth studies, whereby wheat plants were pre-infested for 5 days with either *R. padi* or *D. noxia* and then the aphids were removed. The plants were allowed to recover for 2 days then were re-infested with one or the other of these species. The results showed that *R. padi* avoided whole plants and excised leaves that had been previously infested by *R. padi* colonies. They were not deterred, however, by previous *D. noxia* infestation. *D. noxia* were not deterred by pre-infestation by either species. The authors speculated that specificity of elicitation may be due to differing salivary chemicals between the two species, which may generate different profiles of plant allelochemicals. In the current study, aphids had only one choice of host and, due to tethering, were obliged to feed on the pre-infested plant. The results of the

Messina study, combined with the reduced duration of sap ingestion and increased pathway time observed in the current study, suggest that pre-infested plants are not ideal hosts for *R. padi*; the aphids will feed out of necessity but not as efficiently as on uninfested plants. In fact, it would be useful to investigate the intrinsic rate of natural increase (r_m)(Wyatt and White, 1977) to record *R. padi* reproductive success on pre-infested barley.

Taken together, the results from these studies show clearly that *R. padi* have a negative impact on the feeding behaviour and performance of conspecifics and heterospecifics when sharing a host. The alteration in feeding behaviour tends to appear as a reduction in E2, suggesting that the change is phloem-mediated. The effects of pre-infestation in wheat remain after the removal of the primary colony and will affect secondary outbreaks for at least two days post infestation (Messina *et al*., 2002). It would be interesting to extend the delay between primary and secondary infestations of *R. padi* on barley in this manner, to investigate how long the effect remains. The longevity of the aphid-induced effects also has implications for systemic responses to aphid infestation and it would be useful to perform EPGs on *R. padi* feeding on an uninfested part of an infested plant. A previous study investigating the role of a systemic signal in aphid resistance/facilitation effects found that *M. persicae* feeding was enhanced on previously infested plants whereas feeding was reduced on uninfested areas of infested plants (Cherqui *et al*., 2007). That shows that there was a systemic defence response, even in the presence of a localised facilitation effect. The effect at the primary site of infestation differs between *R. padi* and *M. persicae* so it is difficult to extrapolate the results for systemic effects, but they are consistent with the expected effect for *R. padi*, as a defence-inducing species.

Chapter 5 of this study looks at the transcriptomic responses to aphid infestation. By identifying differentially expressed genes and characterising the barley response to *R. padi* attack, we will gain insight into the causes of the inhibitory effect of pre-infestation on aphid feeding behaviours reported here. Chapter 5 also aims to compare the transcriptomes of leaf tissue local to and systemic from the site of infestation. It is thought that the systemic effect is likely to be mediated by phloem-localised signals, so that will give us some insight into the role of the phloem in the plant-aphid interaction. Comparing this data with the feeding behaviour recorded in response to pre-infestation, reported above, will allow conclusions to be drawn as to the role of the phloem in aphid feeding ability. It would be useful to examine the sap of infested and uninfested barley plants more closely to determine whether *R. padi* induce a greater range of defensive compounds in the phloem of host plants, thus explaining the reduced sap ingestion duration in this study. It would also be interesting to contrast this with feeding behaviour on a resistant variety to determine whether the defence response is qualitatively or merely quantitatively different. Due to time constraints, it was not possible to use EPG to record the feeding behaviours of aphids on leaves distant to the site of infestation. This would be an important next step in investigating the systemic responses to pre-infestation and would provide behavioural information to integrate with the transcriptomics data from second leaves in Chapter 5.

4 Method development: towards determining the transcriptome of phloem sap

4.1 Summary

The phloem is a site of prolonged interaction between aphids and their host plants. Studying the transcriptome of the phloem helps to build a better understanding of its role as a long-distance communication network, and can reveal the gene expression changes induced by aphid attack. RNA quantities within the sieve element (SE) are vanishingly small, however, and this poses a challenge for gene expression studies. The aim of this study was to develop a method of obtaining sufficient RNA from SE sap for whole genome microarray analysis.

The stylectomy and EDTA-facilitated exudation techniques were employed to sample SE sap. Various attempted approaches showed that the collected samples are too small, and the RNA levels within them too low, to provide sufficient RNA quantities for microarray via these methods. The method development leading to this conclusion is described in this chapter. Subsequent developments in genomic techniques provide possibilities for further study and these are discussed here also.

4.2 Introduction

4.2.1 Functional genomics of the phloem

As functional genomic technologies have improved, a wealth of data has been provided on ever more specific tissue types and their responses to various biotic and abiotic factors. This has included investigations of the transcriptome, proteome and metabolome of samples from single cell-types (Asano *et al*., 2002, Casson *et al*., 2005, Gaupels *et al*., 2008a). Sampling from the plant phloem provides a unique challenge, due to the inaccessible, interconnected and responsive nature of the SE. For example, SE defences include a callose-mediated occlusion response which hinders sampling (King and Zeevaart, 1974, Furch *et al*., 2007).

In some species, phloem-enriched samples can be collected relatively easily. For example, phloem tissues can be stripped from the surrounding tissues in celery, allowing investigation of the transcriptome of these cells (Divol et al., 2005a). Analysis of expressed sequence tags (ESTs) generated in this way has shown that aphid feeding induces an upregulation of genes belonging to various functional categories, including: carbon and nitrogen metabolism; cell wall modification; photosynthesis; vitamin biosynthesis; and water transport. Similarly, Anstead *et al*. (2013) removed phloem fibres from an epidermal peel of *Brassica oleracea* (broccoli) plants and isolated the soluble and membrane-bound proteins within the tissues. They found approximately 400 proteins in the phloem-enriched samples including some involved in defence responses such as: myrosinase-binding like protein and myrosinase-associated protein, with roles in glucosinolate production; β-1,3-glucanases, which have roles in stress responses; glucose-6-phosphate dehydrogenase 6 (G6PD6), which is involved

in ROS detoxification. As well as defence-related proteins, some with structural (actin, profillin, tubulin) and transport (S-adenosylmethionine synthase) roles were identified.

SE sap exudation from incisions in the bark of species with reduced sealing mechanisms, such as *R. communis* (Milburn, 1970) has been collected for gene expression and proteomics studies. For example, functional analysis of a complementary DNA (cDNA) library constructed from *R. communis* sap showed roles including transport, interaction with the environment, DNA/RNA binding, protein turnover and ribosomal function, (Doering-Saad *et al*., 2006). Analysis of the closest *Arabidopsis thaliana* (L.) homologues showed a two-fold increase in expression in SE sap of genes involved in cell, tissue and organ localisation, protein synthesis, organ differentiation, and cell fate, compared with the whole *Arabidopsis* genome. In another study, 18 proteins were identified in exuding *R. communis* sap (Barnes *et al*., 2004). These fitted into 4 broad categories: sugar metabolism and redox regulation; structure and defence; long-term SE functioning; and molecular chaperones. A number of identified proteins were of unknown function. 1- and 2-dimentional polyacrylamide gel electrophoresis (PAGE) was used to separate proteins from *Brassica napus* sap obtained by the exudation technique (Giavalisco *et al*., 2006). Quadrupole time of flight mass spectrometry analysis of the 140 isolated proteins showed that chaperones, heat shock proteins, protease inhibitors, antioxidative proteins, metal-binding proteins, components of the myrosinase system, lectins, profillins and glycine-rich RNA-binding proteins were included in the phloem-localised proteome. Using the sap exudation method, it was shown that a high proportion of SE mRNAs in *Cucumis melo* (melon) are associated with biotic stimuli, stress responses and metal-ion binding (Wolf *et al*., 2007).

EDTA-facilitated exudation has been employed to study sugars and AAs in the phloem (Caputo and Barneix, 1999, Hijaz and Killiny, 2014) but not RNA. EDTA-facilitated exudation is generally considered unsuitable for proteomics of the phloem as it introduces artefacts which make data interpretation difficult (Gaupels *et al*., 2008c). This may be also a problem for gene expression analysis and this study aims to assess the quality and quantity of RNA samples collected by this method.

Stylectomy sap has been analysed but has yet to provide substantial transcriptomic data. Sasaki *et al.* (1998) collected sap from rice using laser stylectomy on brown planthoppers (BPH). This technique allowed collection of 1 µl of sap and RT-PCR was performed using primers for 3 specific mRNAs: thioredoxin; actin; and oryzacystatin-I. All three transcripts were located and presumed to have been translocated into the SE from the CC. Similarly, Doering-Saad *et al.* (2002) used RT-PCR to amplify specific mRNA known to have phloem-related functions. Transcripts encoding the H^+/sucrose symporter SUT1, a putative aquaporin and the H^+/ATPase PPA1 were all detected in SE sap. One study modified the stylectomy technique to collect large quantities of sap (Gaupels *et al*., 2008a). Aphids were placed on a plant inside a silicone ring which had been glued to the leaf. The stylets were cut and the ring was flooded with paraffin oil. Exuding sap could be aspirated from the oil into glass microcapillaries. Using this approach, sap was collected from up to 30 plants and 600 *R. padi* aphids in parallel and an average of 10µl of SE sap could be obtained within 6 hours of sampling. The group used cDNA amplified fragment length polymorphism (AFLP) to identify RNA from the sap. A subset of the total cDNA pool was selectively amplified using a PCR-based approach and nonspecific primers and then sequenced. They found transcripts related to metabolism, signalling, and pathogen defence. Proteomic analysis of SE sap sampled in

the same manner showed proteins with roles in stress responses and transport, as well as many unknown proteins. Collecting multiple samples in parallel under oil was an approach attempted in the current study.

Stylectomy using BPH provided sap for proteomic analysis of rice (Aki *et al*., 2008). 5µl of sap was collected and analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). 107 proteins were identified in phloem sap, 50% of which had previously been reported in the SE sap of other species, collected by exudation techniques (Giavalisco *et al*., 2006, Walz *et al*., 2004, Barnes *et al*., 2004). As in previous studies, the proteins could broadly be categorised as metabolism-related, defence-related, response to stress, cell wall modifying and structural components. Palmer *et al*. (2014) used gas chromatography–mass spectrometry (GC-MS) to analyse the metabolome of wheat phloem sap collected by stylectomy. They found 79 metabolites, mainly sugars and AAs, and 26 of unknown identity.

Overall, there is a lack of transcriptomic data for SE sap, particularly whole genome analysis such as that which would be provided by microarray studies. A method of collecting RNA samples of sufficient quality and quantity for microarray would therefore be of great benefit to this field of study.

4.2.2 Collecting RNA from the sieve element

Whole tissue samples are the most common type of sample used for gene expression studies due to the ease of sample collection and preparation. RNA isolation kits designed for whole leaf samples have been available commercially for some time, allowing fast and simple sample processing from tissue harvesting to pure RNA sample (http://www.lifetechnologies.com/ and http://www.qiagen.com/).

Much of the interaction between aphids and their host plants is in the vasculature, however, which makes up only a small proportion of the leaf, perhaps as little as 0.4% of the total volume (Sjolund, 1997). Whole leaf transcriptional assays could therefore miss low-copy-number transcripts that are expressed within sieve elements. Also, although advances in high-throughput genomic approaches have made transcriptomic studies faster, they remain sensitive to changes in experimental conditions and to different sampling techniques. When designing a transcriptomic experiment it is therefore important to consider which tissue type to sample and which technique to use in sampling it. In this study the stylectomy technique was identified as providing the purest SE samples and it was hoped to compare it with sap acquired by EDTA-facilitated exudation to identify any contamination or artefacts introduced by this method.

Although the transcriptomes of ever-decreasing sample sizes have been discovered (Asano *et al*., 2002, Casson *et al*., 2005, Deeken *et al*., 2008), whole-genome microarray and RNA sequencing have not been performed on plant tissues with RNA concentrations as low as SE sap, which is orders of magnitude lower than that of the cytoplasm in surrounding parenchyma cells (Doering-Saad *et al*., 2002). There are two main reasons for this. Firstly, the SE lacks transcriptional and translational machinery. No mRNA originates from the SE and mRNA is not targeted to the SE for translation into proteins. This means that any transcripts in the SE must have moved there from neighbouring cells, that is, the CCs (van Bel and Knoblauch, 2000). Secondly, mRNA transport into the SE from the CC has been shown to be selective (Lee *et al*., 2003). For these two reasons the estimate of RNA in the SE must be greatly reduced from that of other plant cells, which is between 150-760 fg mRNA, depending on cell type (Dresselhaus *et al*., 1994). Furthermore, when amplifying such low

RNA amounts, it is likely that there will be a bias against low copy number transcripts (Casson *et al*., 2005) and a 3' bias (Frohman *et al*., 1988) so stringent quality controls, are vital. Attempting to elucidate the transcriptome of SE sap is therefore a huge challenge.

Barley SE sap flowed for up to 10 hours from severed *R. padi* stylets in the current study, although the average duration of exudation was only around 2 hours on lightly infested plants and 4 hours on heavily infested plants (see Chapter 2). Exudation rates were measured at 0.6 nl min^{-1} and 0.9 nl min^{-1} on lightly and heavily infested plants, respectively. Stylet-acquired SE sap is therefore difficult to collect in large quantities. Sap collected from *R. communis* via the exudation method was therefore used to aid method development and to provide an estimate of the RNA concentration of SE sap in this study, as it could be collected in much larger quantities. Similarly, when developing RNA amplification protocols, diluted whole leaf RNA was used for method development to expedite the process.

4.2.3 Microarray and small samples

The methods employed in the studies mentioned above lack the potential or the sensitivity to identify the full transcriptomic profile of SE sap acquired by stylectomy. As this was the aim of the current study, an alternative approach was required. Microarray was identified as a technique that will detect a wide range of transcripts, and full-genome microarrays are available for many species, including barley. In the past, a limitation to the application of microarray technology was the large amount of RNA required per hybridisation; 50−200 µg total RNA or 2−5 µg poly(A) mRNA were required per target, per array to achieve adequate fluorescence on an array (Duggan *et al*., 1999). For mRNA present at very low transcript

levels per cell it was likely that hybridisation levels would fall below the lower limit of fluorescence detection, and may have easily been rendered undetectable by assay noise.

In order to analyse gene expression in single cell types, various approaches have been used to overcome this problem. One area of improvement has focussed on increasing hybridisation efficiency. This has included reducing the physical array area by creating an 8 x 60k array; 8 arrays, each with 60,000 spots, containing different oligonucleotide probes, on a single slide. These require only a 40 µl volume of sample, containing target RNA, for hybridisation, compared to the hundreds of microlitres required for the other array formats (Table 4-1; figures obtained from Agilent Low Input QuickAmp labelling kit manual). With a low sample volume, each target complementary RNA (cRNA) strand will have a greater chance of coming into contact with, and hybridising to, the appropriate probe. Only 600 ng of input RNA is required for an 8 x 60k array. Improving mixing procedures to increase hybridisation of low quantity samples is another aspect of this approach (Duggan *et al*., 1999).

Table 4-1 RNA input amounts and hybridisation volumes required for the various Agilent microarray formats.

	Agilent microarray format			
	1x244k	2x105k	4x44k	8x60k
RNA input amount (µg)	5	3.75	1.65	0.6
Volume to hybridise (µl)	490	240	100	40

Despite these improvements in array technology, the low concentrations of RNA in phloem sap meant that it was necessary to include an RNA amplification step to provide sufficient RNA for input into an array. It was calculated from RNA labelling for whole leaf array that the QuickAmp Low Input Labeling kit (Agilent Technologies, Palo Alto, CA, USA) gave rise to a 10 to 40 fold sample amplification from input RNA to resulting cRNA. 600 ng cRNA is required for a one-colour microarray using the 8 x 60k platform. The aim of this study was therefore to reach a starting threshold of 60 ng input RNA, ideally within a 1.5 µl sample volume, i.e. 40 ng μl^{-1}, which is the quantity required for input into the labelling kit. Whilst based on existing methodologies and commercially available protocols and techniques, the small sample volumes in this study, and the small quantities of RNA within them, meant that the methods used invariably required modification both through intuitive alterations and through trial-and-error approaches. The various stages of method development have been outlined to give a full account of the attempts made to achieve the objectives of the study.

The two most common RNA amplification techniques are polymerase chain reaction (PCR)-based amplification and linear T7 RNA polymerase mediated amplification. PCR (Saiki *et al*., 1985) is a widely used technique for amplifying DNA and can be used to study low copy number transcripts from extremely small tissue samples such as microdissected chromosomes (Ludecke *et al*., 1989). PCR requires the synthesis of primers for both the 5' and 3' ends, however, and this sequence information is not usually available for all messages in a cell population. The PCR method has been modified to improve the range of cDNAs that can be cloned. These modifications include homopolymeric tailing of the 3' terminus (Frohman *et al*., 1988) and synthesising highly degenerate oligonucleotide primers (Gould *et al*., 1989). However, the widely used *Thermus aquaticus* (Taq) DNA polymerase has relatively

low fidelity (Saiki *et al*., 1985, Saiki *et al*., 1988), and each misincorporation event will be retained and propagated through subsequent cycles of the amplification. Thus, the number of errors is cumulative and directly related to the number of rounds of PCR performed. The Taq polymerase also has difficulty transcribing sequences longer than 3 kilobases (kb), which results in a bias towards smaller cDNAs with subsequent rounds of amplification (Vangelder *et al*., 1990). Inherent limitations to the PCR amplification of limited amounts of complex template have been documented (Karrer *et al*., 1995).

The T7 RNA polymerase mediated RNA amplification procedure was first published in 1990 (Vangelder *et al*.), where it was used to generate antisense RNA (aRNA) from cerebellar tissue sections of rats. T7 RNA polymerase is used to transcribe multiple copies of target aRNA. Luo *et al.* (1999) showed that RNA from 1000 rat ganglion cells could be amplified sufficiently for microarray analysis after 3 rounds of T7 RNA polymerase amplification. The technique has subsequently been adapted successfully for use with plant-derived samples (Nakazono *et al*., 2003, Asano *et al*., 2002, Casson *et al*., 2005, Deeken *et al*., 2008). The QuickAmp Low Input Labeling kit used in this study is based on T7 polymerase amplification.

Amplifying RNA has inherent problems, both methodological and biological. The first is maintaining the population distribution of transcripts; different transcripts may be amplified to different degrees due to variations in size or structure. Another is the phenomenon of transcript truncation, whereby sequence is lost during amplification. This can particularly affect downstream analysis where transcripts are probed for by an oligonucleotide sequence. When probes consist of sequence from close to the 5' end, for example, 5' truncation during amplification will reduce or eliminate signal from these transcripts. These problems must be taken into consideration when designing an RNA amplification/microarray

experiment and appropriate quality control procedures must be in place to ensure that biases inherent in amplification are reduced or accommodated satisfactorily.

4.2.4 Aims of the study

The primary aim of this study was to develop a method of isolating and amplifying stylectomy-derived SE sap RNA to provide sufficient quantities for microarray analysis and thereby obtain the transcriptome of pure phloem sap. A second aim was to compare the transcriptomes of SE sap obtained by stylectomy and EDTA-facilitated exudation, to assess the efficacy of the latter technique in representing pure phloem sap. Characterising the transcriptome of the phloem in response to aphid feeding would provide candidate genes for further investigation into this relationship, with a view to developing novel defence strategies against aphid pests.

4.3 Materials and method development

4.3.1 Plant Material and aphid infestation

H. vulgare plants for stylectomy and EDTA-facilitated exudation were grown individually in 6cm pots containing 6 parts peat based compost (Humax multipurpose) to 1 part Silvaperl under a 16:8 hour light:dark regime in 70% humidity. *R. communis* plants for preliminary experiments were grown in 10cm pots in the same compost, under the same conditions.

Barley plants used for EDTA collection were not infested with aphids. Plants used for stylectomy were heavily infested after 10 days and stylectomy performed before the plants were 20 days old. *R. communis* plants were used after 20 days.

4.3.2 Quality Assessment of RNA/cRNA

4.3.2.1 Quantifying collected and amplified RNA

RNA was quantified at various stages of the process, including following RNA isolation, following one round of RNA amplification and following two rounds of amplification. Two quantification methods were used in order to maximise confidence in the results. Firstly, samples were run on the Bioanalyzer 2100 (Agilent Technologies) following the RNA 6000 Nano LabChip reagent kit guide using the Eukaryote Total RNA Nano assay for whole-leaf samples or the RNA 6000 Pico LabChip using the Eukaryote Total RNA Pico assay for sap samples. The electrophoresis-based system gives information on RNA concentration and on the number of fragments of various lengths. The Bioanalyzer produces an electropherogram for each sample and these traces show distinct peaks representing RNA bands including ribosomal subunits. As the Bioanalyzer does not require samples to be labelled, this was used at all stages, including following initial RNA isolation. A second device used to quantify RNA was the NanoDrop Spectrophotometer. The NanoDrop requires minimal sample (1-2 µl) and gives details of RNA concentration and dye-labelling efficiency. The NanoDrop was therefore used after labelling/amplification.

4.3.2.2 Assessing the quality of the RNA

For the assessment of RNA quality, the Agilent 2100 Expert Software automatically provides a RNA Integrity Number (RIN). RIN provides a quantitative value for RNA integrity that facilitates the standardization of quality interpretation. The RIN is calculated from the electrophoretic trace and will flag up the presence of degradation products. Only starting RNA samples that have a high RIN (>8) are used for downstream analysis to ensure that a specific, easily degraded fraction of the RNA will not be lost and all of the expressed species will be represented. The NanoDrop Spectrophotometer also provides information on the RNA quality (T042 - Technical Bulletin). Nucleic acids and proteins absorb at 260 nm and 280 nm, respectively. The NanoDrop calculates a 260/280 ratio to assesses sample purity, based on the absorbance properties of a DNA or RNA sample. Readings for RNA samples should be above 2.0 to be considered pure enough for downstream use. A lower reading indicates contamination by protein, phenol, etc., which also absorbs at 280. A second ratio, the 260/230, also measures the purity of nucleic acid samples. The required range for this is 2.0-2.2. A lower reading, again, indicates a sample containing contaminants absorbing at 230 nm, such as carbohydrate, which should be discarded.

4.3.3 Sap collection and RNA isolation

4.3.3.1 EDTA-facilitated exudation

The EDTA-facilitated exudation technique has been well documented (King and Zeevaart, 1974). Five barley plant stems were severed with an RNase-free razor blade just above the soil level, and immediately re-cut. They were immersed in RNase-free 3mM EDTA solution (Ambion®-Life Technologies) for one minute then transferred to centrifuge tubes containing

1.5ml aliquots of the same solution and left to exude for several hours. RNA was isolated from the collection solution using the Stratagene low input RNA isolation kit. Nanodrop analysis showed that the quality and quantity of the resulting RNA were insufficient. RNA yield was low (2.6-3.1 ng μl^{-1}) and the 260/280 and 260/230 ratios never exceeded 1.57 and 0.2 respectively. Further to this, much of the collection solution was taken up by the plant during transpiration so a method of limiting this was required to maximise sample collection.

Subsequently, the duration of sap collection was reduced so that RNA within samples was not exposed to room temperature for extended periods, which would be likely to lead to RNA degradation. Cut stems were left exuding for only half an hour before being transferred to a fresh collection set-up. Samples were stored at -20°C. In order to reduce the rate of transpiration within the experimental plants, the collection-chamber was saturated with water-vapour. The resulting rates of transpiration of samples following collection in this way appeared to be reduced. However, there was a possibility of contamination of samples from condensation inside the chamber. Samples collected in this way were still not of sufficient quantity for use in microarray (1.5-6.6ng μl^{-1}). Consequently, it was considered necessary to pool samples in order to concentrate the RNA. Samples were pooled immediately prior to RNA isolation. It was convenient to pool sap collected from 4 plants into one RNA sample.

A further precaution taken was to seat collection tubes within a cool box during collection. This was aimed at reducing degradation by RNases and this strategy was augmented by the addition of RNase inhibitors to the collection solution. The use of a cool box was, however, incompatible with a humid environment and condensation within the collection area and inside the collection tubes led to contamination and reduced RNA quality and yield. The samples were therefore collected in darkness, as an alternative method of reducing

transpiration, and in a cool box with RNase inhibitors to reduce RNA degradation. This setup gave RNA yields of 6.7-12.3 ng μl^{-1} and 260/230 ratios of 0.33 and 0.44, which were sufficient to collect the minimum amount of RNA required for an 8 x 60k microarray. The 260/280 ratios were low (1.89 and 1.70), however, but this may have been due to the very low RNA concentration. The spectral profiles were improved compared to those of previous samples.

In order to test the suitability of these samples for array, they were labelled with the QuickAmp Labeling Kit (Agilent Technologies). 10 µl of each sample was dried down to 3.5 µl giving samples of 67 ng and 123 ng of RNA in 3.5 µl aliquots respectively. The labelled samples were cleaned up using the RNeasy Plant Mini Kit (QIAGEN). Briefly, the sample volume was adjusted to 100 µl with RNase-free water and 350 µl Buffer RLT added. This buffer contains β-mercaptoethanol to eliminate RNases within the sample, and guanidine thiocyanate, a chaotropic salt, to denature the RNA and promote its binding to the silica membrane of the spin column. 250 µl of 98% ethanol was then added to bring the pH below 7.5, as required for efficient binding, and the sample was transferred to a spin column and centrifuged for 15 seconds at 10,000 rpm. The low salt Buffer RPE (500 µl) was then added to wash the column and centrifuged for 15 seconds at 10,000 rpm. This wash step was repeated. The spin column was then seated in a fresh collection tube and 30 µl RNase-free water was added directly to the spin-column membrane and centrifuged for 1 minute at 10,000 rpm to elute the RNA. The RNA was analysed on the NanoDrop spectrophotometer. The sample yields were 0.510 µg and 0.831 µg and the specific activities were 5.88 and 21.66, respectively. The latter indicated good labelling efficiency and the corresponding yield was well over the 600 ng threshold. These readings indicated that the threshold for analysis of this type of sample would probably be somewhere between 67 ng and 123 ng RNA input.

4.3.3.2 **Ricinus communis** ***exudation as a proxy for stylectomy***

R. communis was used to allow approximate quantification of RNA concentration within phloem sap due to the larger quantities available from exudation from excisions in the bark of the plant. Sap exuding from a wound in the stem inflicted with a sharp razor blade was collected in glass capillaries, which had been baked at 200°C for 8 hours to remove RNases, and expelled into an RNase-free microcentrifuge tube. The sap was aliquotted into two lots each of 2 µl, 4 µl, 10 µl and 20 µl volumes and the first buffer of the RNeasy plant mini kit (QIAGEN) added. RNA was then isolated by following the kit protocol and was eluted in RNase-free water. It was then quantified using an Agilent Bioanalyzer 2100. Due to the small quantities of RNA involved, the Absolutely RNA nanoprep kit (Agilent Technologies) was trialled to attempt to achieve a higher yield.

Nanodrop readings for RNA isolated from *R. communis* sap showed low RNA yields (2.0-4.3 ng/µl; Figure 4-1), and the 260/230 ratios were also low (0.07-0.14). Samples isolated using the Stratagene Absolutely RNA nanoprep kit had higher yields (3.2-27.8 ng/µl) but the 260/230 ratios were still poor (maximum 0.17). The 260/280 ratios were good (~2), however.

Taking the highest yield measurement of 5 ng μl^{-1}, 120 µl sap would be required to achieve the 600 ng RNA required for array. At the highest exudation rate measured for stylectomy on barley (0.9 nl min^{-1}; Figure 2-4), over 2000 hours of collection would be required.

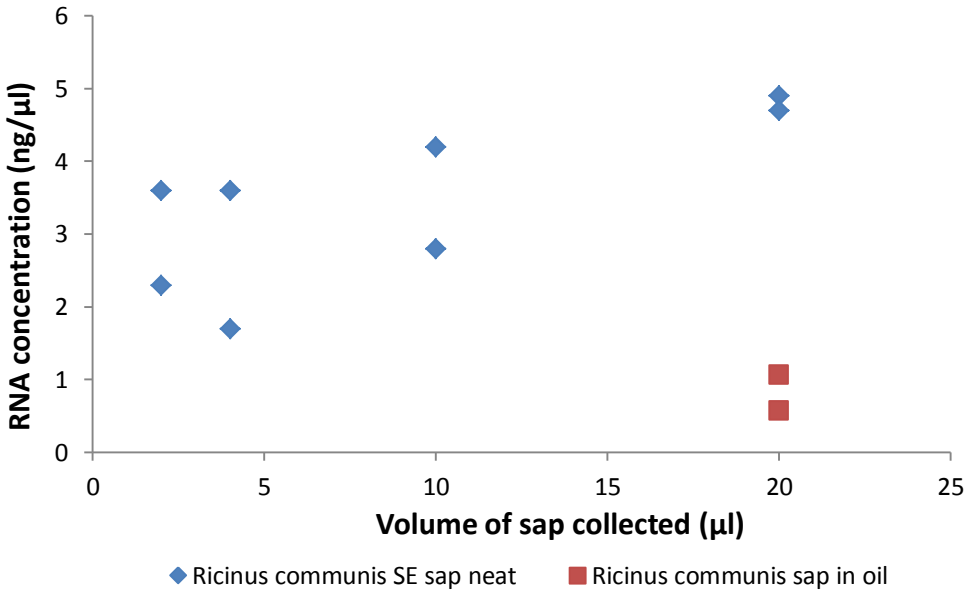


Figure 4-1 Concentration of RNA in neat *Ricinus communis* phloem exudate samples (blue) and *R. communis* sap samples collected into oil-backfilled microcapillaries (red) as measured on an Agilent 2100 Bioanalyzer. Data show a similar RNA concentration for all input volumes but a variation in measurements at low levels. Sap collected in oil showed low RNA concentration.

R. communis was also used to assess collection of stylet samples under oil, as per Gaupels *et al*. (2008a). 20 µl volumes of *R. communis* sap were collected into RNase-free glass microcapillaries that had been backfilled with paraffin oil to replicate the procedure that would be used to collect sap exuding from cut stylets under oil. Equivalent volumes of *R. communis* sap were collected into clean RNase-free microcapillaries. The samples were then expelled into RNase-free microcentrifuge tubes containing aliquots of the first buffer of the RNeasy Plant Mini Kit (QIAGEN) and the kit protocol followed to completion.

The RNA yield of oil-collected samples was unexpectedly low (Figure 4-1) and both the Bioanalyzer and Nanodrop reported poor quality RNA in comparison to pure sap samples. It was concluded that oil interferes with the RNA clean-up protocol. From these data, it will be impossible to collect sufficient SE sap for analysis without an amplification step.

4.3.4 RNA amplification

4.3.4.1 One round of RNA amplification- whole leaf samples

The Quick Amp Low Input RNA Labeling Kit (Agilent Technologies) labels RNA by first synthesising cDNA using the sample RNA as a template, then transcribing to complementary RNA, which is used as a probe during array hybridisation. Throughout this process the amount of RNA can be amplified as much as 100 times. This amplification was exploited to attempt to achieve sufficient RNA from small starting samples for microarray analysis.

Isolated whole-leaf RNA was diluted to concentrations suitable for the Quick Amp Kit. Two whole leaf RNA samples of concentration 905 ng μl^{-1} (RIN 8.1) and 1071 ng μl^{-1} (RIN 8.8), respectively (see Figure 4-2) were diluted to starting amounts of 200 ng (amplification kit upper recommended limit), 100 ng, 10 ng (lower recommended limit), 5 ng, 3 ng, 1.45 ng, 0.75 ng and 0.4 ng. The latter 5 samples were below the recommended lower threshold of the kit to assess the suitability of this kit for amplification of the low concentrations of RNA in phloem sap and to determine whether a second round of amplification would be required to amplify the RNA to levels suitable for microarray analysis. These diluted samples were processed as per the kit instructions and the labelled, amplified cRNA product was eluted in 30µl nuclease-free water and stored at -70°C until required. The RNeasy plant mini kit (QIAGEN) was used for RNA clean-up at all stages (as outlined in 4.3.3.1 above).

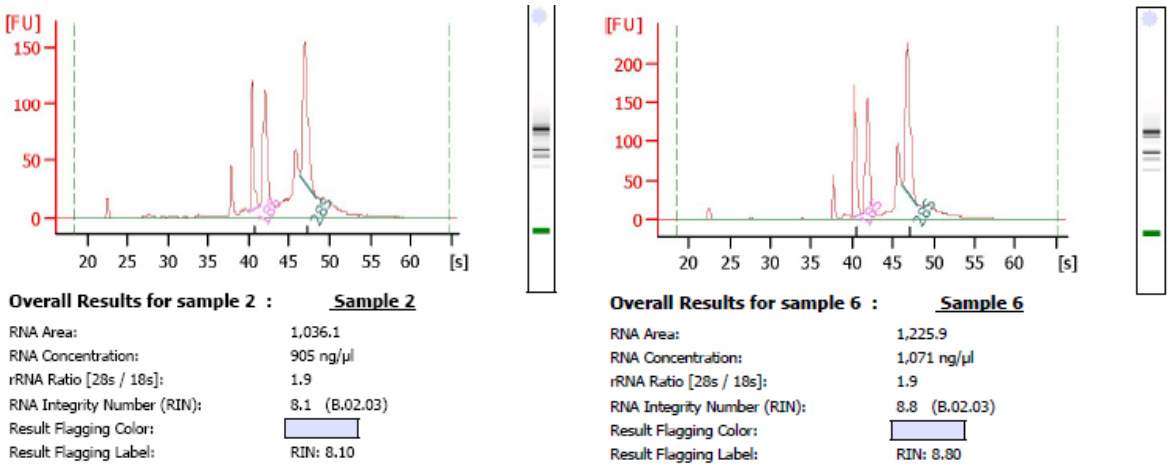
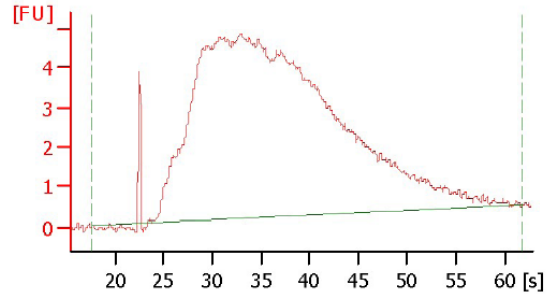


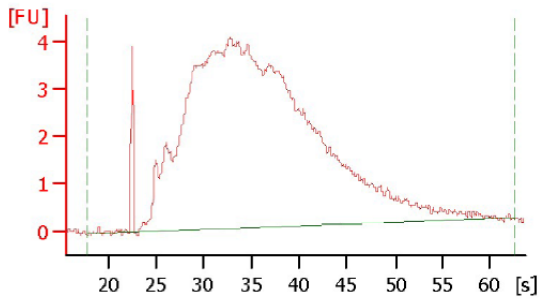
Figure 4-2 Bioanalyzer electropherograms of starting RNA samples prior to dilution. Traces show fluorescence on the y axis and time, in seconds, along the x axis. A marker peak can be seen at 23 seconds and distinct peaks representing sample ribosomal RNA bands appear at various intervals. The large and small cytosolic ribosomal subunits (labelled 18S and 28S) are used in calculation of RNA integrity numbers (RIN). The samples have high RIN numbers (8.1 and 8.8 respectively).

The Bioanalyzer electropherograms from the amplified RNA showed a broad smear of fragments between 25 seconds and 50 seconds (Figure 4-3). The RIN numbers were low (2.1-2.4) but this was expected due to the low RNA starting concentrations. The quality of these samples was good with the traces characteristic of RNA samples following Quick Amp labelling.



Overall Results for sample 3 : **Sample 3**

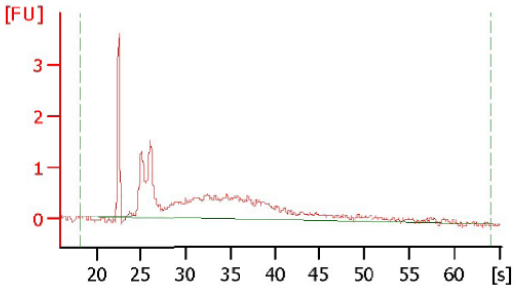
RNA Area:	224.0
RNA Concentration:	171 ng/µl
rRNA Ratio [28s / 18s]:	0.0
RNA Integrity Number (RIN):	N/A (B.02.03)



Overall Results for sample 6 : **Sample 6**

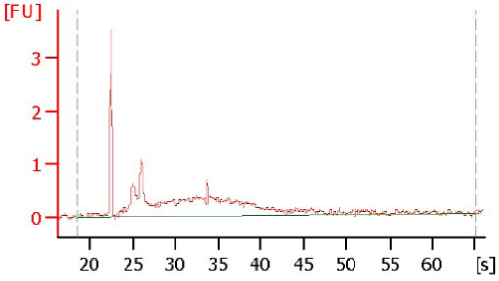
RNA Area:	177.4
RNA Concentration:	135 ng/µl
rRNA Ratio [28s / 18s]:	0.0
RNA Integrity Number (RIN):	2.1 (B.02.03)

Figure 4-3 Bioanalyzer electropherograms of labelled, amplified cRNA from different starting amounts. Starting concentrations of 200ng (Sample 3 above), 100ng (Sample 6 above), 10ng (Sample 9 below) and 5ng (Sample 12 below) are shown. The output RNA concentrations and RNA integrity numbers are shown.



Overall Results for sample 9 : **Sample 9**

RNA Area:	26.5
RNA Concentration:	20 ng/µl
rRNA Ratio [28s / 18s]:	0.0
RNA Integrity Number (RIN):	2.4 (B.02.03)



Overall Results for sample 12 : **Sample 12**

RNA Area:	18.4
RNA Concentration:	14 ng/µl
rRNA Ratio [28s / 18s]:	0.0
RNA Integrity Number (RIN):	2.3 (B.02.03)

The Bioanalyzer measurements showed levels of amplification in the range of 25-84 fold (Table 4-2). These results suggest that a starting amount of at least 10 ng of total RNA would be required to provide sufficient cRNA for microarray hybridisation (600 ng required for 8 x 60k array) following only one round of amplification. This is in agreement with Agilent Technologies literature (http://www.agilent.co.uk/about/newsroom/presrel/2010/19may-ca10031.html).

Table 4-2 RNA yield and fold increase following amplification according to Bioanalyzer measurements.

Bioanalyzer	Starting RNA amount (ng)			
	200	100	10	5
cRNA yield (ng)	5130	4050	600	420
Fold increase	25.7	40.5	60	84

NanoDrop measurements indicated a high level of amplification in all samples although fold increase in RNA concentration was inversely related to starting amount (Table 4-3). According to these results the four highest starting concentrations (200 ng, 100 ng, 10 ng and 5 ng RNA) provided sufficient output to reach the 600 ng threshold for array. Sample labelling efficiency was good and all samples showed sufficient labelling to be used for hybridisation (i.e. had a specific activity > 9) except the samples of 5 ng and 3 ng starting amounts.

Table 4-3 Shows RNA yield, fold increase and specific activity following one-round amplification according to NanoDrop measurements.

NanoDrop	Starting RNA amount (ng)							
	200	100	10	5	3	1.45	0.75	0.4
cRNA yield (ng)	7476	5289	948	651	474	336	330	297
Fold increase	37.4	52.9	94.8	130.2	158	232	440	742.5
Specific activity (pmolCy3/µg cRNA)	26.89	30.09	9.5	4.6	6.33	35.7	9.09	20.20

The NanoDrop readings indicate that sufficient RNA for microarray hybridisation could be obtained from 5 ng starting amount following one round of amplification. With an exudation rate of 0.9 nl min^{-1} (heavily infested plants; Figure 2-4), 18.5 hours worth of collection would be required to collect 5 nl sap or 37 hours to collect 10 nl. Using the lower sap exudation rate of 0.6 nl min^{-1}, from lightly infested plants, these figures rise to 28 hours and 56 hours collection for 5 nl and 10 nl, respectively. These collection durations appear more feasible; however there are two main problems with them. Firstly, the RNA sample is required to be in a 1.5 µl volume for input into the Low Input RNA Labeling kit. This would require the samples to be dried down, from the 30 µl RNA isolation kit output, prior to labelling and this prolonged exposure to ambient temperatures would lead to RNA degradation. Secondly, lightly infested plants only exuded for an average of two hours from cut stylets. It is desirable to collect sap at the same time each day, to control for diurnal fluctuations (Gattolin *et al*., 2008) so sap collection could take up to 18 days on heavily infested plants and 28 days on lightly infested plants. As the aim of this study was to develop a usable method for these types of studies, this length of collection is not ideal.

4.3.4.2 Two rounds of RNA amplification

In order to acquire larger quantities of cRNA for array, a double amplification protocol was attempted, again based on the amplifying nature of the Low Input Labeling kit. The modified protocol aimed to convert the sample RNA to cDNA, transcribe to RNA and use this as a template for another round of cDNA synthesis, followed by conversion back to complementary RNA. In theory this would amplify the sample concentration massively and would produce sufficient RNA for array from very low inputs.

Two sample types were used. Firstly a whole leaf RNA sample (concentration 308 ng/µl; RIN 9.4) was used to make two diluted samples containing 750 pg RNA each. This is the lower detection limit of the Agilent 2100 Bioanalyzer RNA 6000 Pico assay, which was used to quantify the RNA (see below). Using such low RNA concentrations simulated samples that were likely to be obtained from phloem sap. Secondly, RNA from two stylectomy sap samples was isolated (also adjusted to 750 pg starting amount) and amplified alongside the whole-leaf samples. The samples were processed using a novel protocol, based around the Quick Amp Low Input Labeling Kit with modifications as outlined below.

Isolated RNA samples were dried to 3.5 µl under vacuum in a rotary desiccator. A volume of 1.8 µl diluted T7 polymerase primer was added to the samples to give a total volume of 5.3 µl. The primer and template were then denatured by incubating the reaction at 65°C in a heat block for 10 minutes. Meanwhile, a cDNA Master Mix was prepared as outlined in the Quick Amp Low Input Labeling Kit protocol. A volume of 4.7 µl of the Master Mix was added to each reaction and incubated for 2 hours in a 40°C circulating water bath and for 15 minutes at 70°C on a heating block to denature before placing on ice for 5 minutes. A Transcription Master Mix was then added to the first strand cDNA reaction mixture, 5.76µl per sample, made up as per the kit instructions. This Master Mix contained the reagents necessary for the transcription of RNA from the cDNA template, including nucleotide triphosphates and T7 polymerase. The reaction was incubated overnight at 40°C in a circulating water bath. The amplified RNA was purified using a QIAGEN RNeasy mini kit, following the RNA clean-up protocol. Samples were then dried to 10.5 µl.

Random hexamers (1 µl per reaction mixture) were then added for incorporation into first-strand cDNA in preparation for second strand synthesis. The reaction was heat denatured by

incubating for 10 minutes at 60°C and placed on ice. A second batch of cDNA Master Mix was prepared and 8.5 µl added to each sample. The samples were then incubated for 2 hours at 40°C and denatured at 70°C for 10 minutes before being placed on ice. The samples were RNase treated by adding 1 µl RNase in order to remove the RNA and ensure that the next stage of cDNA synthesis used only the first-strand cDNA as a template. The newly synthesised first-strand cDNA was recovered on a clean-up column (QIAquick PCR purification kit, QIAGEN) following the protocol (reproduced below) with several modifications. Five volumes of buffer PB were added to 1 volume of cDNA reaction and mixed by pipetting. The solution was transferred to a QIAquick spin column seated in a 2ml collection tube and centrifuged for 60 seconds at approximately 13,000 rpm (>10,000xg). The flow-through was discarded and the spin column re-seated in the collection tube. The column was then washed with 400 µl Buffer PE and centrifuged for 60 seconds at 13,000rpm. The flow-through was discarded and the wash step was repeated. The flow-through was discarded again and the spin column was transferred to a fresh collection tube. The isolated cDNA was then eluted by adding 30 µl of Buffer EB directly to the centre of the column, incubating at room temperature for 1 minute then centrifuging for 60 seconds. The elution step was repeated with a further 30 µl buffer into the same collection tube to give a final eluate volume of approximately 60 µl. This was then dried under vacuum in a rotary desiccator to a volume of 20 µl. T7 promoter primer was added for incorporation into the second-strand cDNA the reaction was incubated for 10 minutes at 65°C to denature and placed on ice. cDNA Master Mix was added and the reaction was incubated at 40°C for 2 hours and denatured at 70°C for 10 minutes then placed on ice. The sample was then dried to 20 µl and 60 µl Transcription Master Mix was added and the reaction was incubated

overnight in a circulating water bath at 40°C. The labelled, amplified cRNA was then purified using the QIAGEN plant mini kit RNA clean-up protocol, as before, and stored at -70°C.

The NanoDrop and Bioanalyzer readings for the resulting samples are not in concordance. The Nanodrop showed RNA yields of between 30 µg and 80 µg, vastly exceeding expectations (see Table 4-4). Specific activity was good with all samples around or exceeding the threshold of 9.

Table 4-4 RNA yield, fold increase and specific activity following two-round amplification according to NanoDrop measurements. Starting samples are 2 x whole leaf diluted (WLD 1 & 2) and 2 x stylectomy-derived samples (S1 & S2).

NanoDrop	**Sample type**			
	WLD 1	WLD 2	S1	S2
Starting RNA amount (pg)	750	750	750	750
cRNA yield (µg)	79	65.2	62.5	30.3
Fold increase (approx.)	10^5	10^5	10^5	10^4
Specific activity (pmolCy3/µg cRNA)	8.8	10.5	13.6	18.81

The Bioanalyzer electropherograms, however, showed a smear, low in concentration, from 25 seconds onwards suggesting that the sample contained many labelled small fragments (Figure 4-4). RNA yield was measured at around 8 µg, considerably lower than the NanoDrop measurements of around 80 µg.

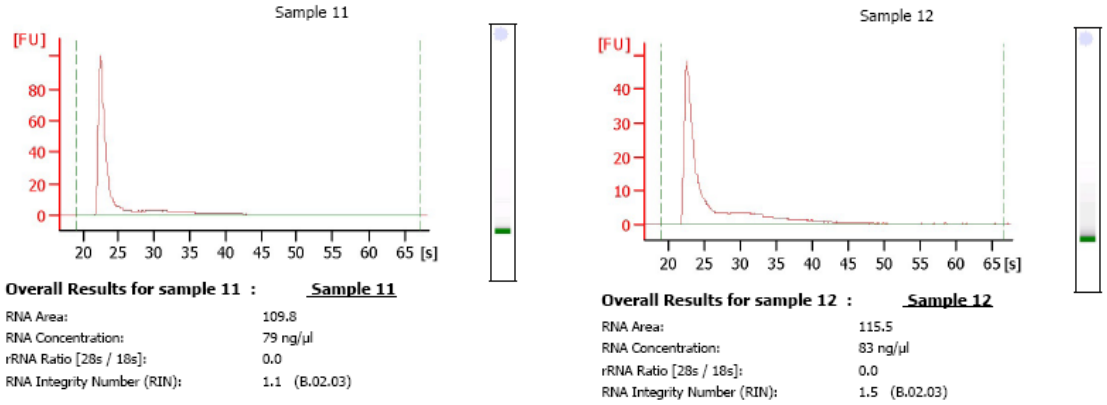


Figure 4-4 Bioanalyzer traces of samples obtained by two rounds of amplification. Sample 11 represents an amplified whole-leaf sample and sample 12 a stylectomy sap sample. RIN numbers are 1.1 and 1.5 respectively.

The discrepancy between the Nanodrop and Bioanalyzer readings may simply be due to an insufficient sensitivity of the equipment at such low RNA concentrations, but nevertheless reduces the reliability of the data and confidence in the success of the amplification protocol. After consultation with experts at Agilent, these samples were not considered to be of good enough quality to take forward to array as the uncertainty regarding what the RNA fragments actually were would have made analysis ambiguous. It was agreed that the problem may have been the use of random hexamers in second-strand cDNA synthesis and that specific promoters may have been more effective. Time restrictions made it impossible to attempt this during the current study but it could be an avenue for further investigation in future studies.

4.4 Discussion

4.4.1 Obstacles to studying small samples

The results of preliminary studies using *R. communis* sap show that RNA levels in the SE sap are too low to allow use of small sap samples such as those obtained by stylectomy without an amplification step. It was also demonstrated that collection under oil inhibits RNA extraction using a commercial kit, making adapted stylectomy techniques such as that developed by Gaupels *et al.* (2008a) unavailable for microarray studies. It is therefore concluded from the *R. communis* sap work that it is not possible to analyse the transcriptome of SE sap by array without some form of RNA amplification step.

The results of the one-round amplification suggest that sufficient RNA quantities for microarray analysis using the 8 x 60k format (i.e. >600 ng) could be achieved with a starting amount as low as 5 ng using this approach. The fold increase shown from the Bioanalyzer measurements, however, is lower than predicted by the kit (i.e. around a 100-fold increase). This may be because of the sample type. Phloem sap contains high sugar quantities and sugars are known to interfere with the amplification procedure (Low Input Labeling Kit protocol, Agilent Technologies). The increases seen in the NanoDrop readings are closer to what we would have expected from the kit guidelines, however, so it may be due to the sensitivity of the instruments rather than the samples.

The Quick Amp labelling kit selects mRNA. Traces for these samples are therefore expected to show a mass of RNA fragments of varying lengths, rather than the distinct peaks, which represent ribosomal RNA subunits, observed on those of freshly isolated RNA. The traces for the amplified cRNA samples show this characteristic, suggesting that the amplification

procedure was successful and the samples reaching the 600ng threshold could be used confidently for microarray.

It is recommended that only samples with a specific activity of 9 or higher are used for hybridisation to ensure an adequate level of dye incorporation; a lower dye incorporation than this may not be sufficient to give a broad enough spectrum of intensities to pick out subtleties in expression differences (Agilent Quick Amp labelling kit Manual). That is, if a saturated spot does not fluoresce brightly then it will be harder to differentiate between it and less well represented spots. The amplified cRNA samples in this study were all around or exceeding the minimum labelling requirement with the exception of the samples of RNA input 5ng and 3ng. The low specific activity of the latter samples could be due to contamination of the sample by reagents used in the amplification procedure. In a sample of low RNA concentration, this contamination would be more apparent than in a sample with a high RNA concentration where the signal from the labelled RNA would exceed that of any contaminants. This sample could be used, taking into consideration the issue with sample visualisation mentioned above, however it is likely that this value could be improved by ensuring no carry-over of solute from one stage to the next during amplification.

Whilst it is possible to collect sap for the periods of time required for single amplification (between 18 and 56 hours), the unpredictability of the stylectomy method renders this approach impractical for repeated experimentation. A double amplification method would reduce the necessary collection times significantly, and would make the stylectomy technique more efficient for use by investigators looking at gene expression in small samples.

The low yield shown in the Bioanalyzer trace from the two-round amplification, and the contrast between this and the NanoDrop measurements, raises doubts as to what exactly was labelled. The broadness of the lower marker peak in these samples (which appeared to be normal in other samples run on the same Nano chip) could indicate that a large number of small fragments have been amplified. This might not be a problem if the small fragments were indeed amplified cRNA and the sample was suitable in its current state, however it might be necessary to do a shearing, i.e. fragmenting of the RNA in preparation for analysis, to reduce the size of the larger strands before hybridisation. This may shorten the smaller fragments further, leading to a problem with non-specific binding. If the samples do indeed contain amplified cRNA, then it is unlikely that it is of sufficient quality to use for microarray. The low RIN numbers generated by the Bioanalyzer suggests that the samples have been degraded and the amplified product does not accurately represent the starting RNA sample. It is a possibility that a by-product of one of the amplification steps was not removed completely. For example, if an insufficient concentration of RNases was added prior to second-strand cDNA synthesis, RNA would have remained and become a template for another round of reverse transcription, leading to further first-strand cDNA synthesis. It must also be considered that it may be the type of substrate being investigated that is causing the difficulties in RNA amplification. SE sap is rich in sugars, for example, and this may interfere with the RNA isolation or amplification protocol. In fact, carbohydrate carryover is cited as a possible cause of low 260/230 ratios following labelling with the Quick Amp labelling kit (T042 Technical bulletin, ThermoScientific).

Several studies have used laser capture microdissection (LCM) to collect samples for transcriptome analysis and included an amplification step to improve RNA yield.

Amplification of RNA from single cell types obtained by LCM has successfully generated sufficient RNA for microarray. For example, Asano *et al*. (2002) extracted Total RNA from approximately 150 phloem cells obtained by LCM and constructed a cDNA library following T7 RNA polymerase amplification. Nakazono *et al*. (2003) isolated large numbers of epidermal cells and vascular tissues from maize coleoptiles by LCM and amplified the RNA from them with T7 RNA polymerase. The technique was developed further in 2005 when RNA was extracted and amplified from only 20 *Arabidopsis* embryonic cells to generate cDNA for microarray (Casson *et al*., 2005). The group found that 3 rounds of amplification using a commercial amplification kit (MessageAmp, Ambion) produced approximately 5-10 µg of amplified RNA, sufficient for use with the Affymetrix GeneChip. Deeken *et al*. (2008) collected samples of *Arabidopsis* phloem parenchyma cells and companion cell–sieve element complexes by LMPC. They also isolated mobile transcripts in sieve elements from leaf phloem exudates collected by EDTA-facilitated exudation. The group found that several commercial amplification kits did not allow linear amplification of the RNA-derived cDNA samples. They eventually achieved sufficient yields of RNA for microarray by modifying the SMART mRNA amplification protocol (BD/Clontech). Karrer *et al*. (1995) also faced problems with the T7 RNA polymerase amplification method when it was found to fail, for unknown reasons, when used to make complex cDNA libraries from samples collected by microsampling from tomato guard cells. So, although success has ultimately been achieved in some studies, the method development has not been without problems.

Furthermore, it was generally conceded in previous studies that amplifying RNA populations, whilst allowing the large-scale identification of strongly differentially expressed genes, is less useful for reliably characterising the expression of genes encoding very low abundance

transcripts (Casson *et al*., 2005). These effects were ameliorated to some extent with careful experimental design, for example performing more technical repeats and adjusting filtering thresholds during analysis, as well as confirming the results with RT-PCR and comparing the outcome with the results of other studies, but some uncertainty remains. These groups were also using as many as 150-10,000 whole cells, which would be likely to contain far more RNA than the equivalent volumes of SE sap. The SE is anucleate and lacks any transcriptional/translational machinery so any RNA contained within it comes selectively through PD from the associated companion cells (Lee *et al*., 2003). Therefore the bias against low copy number transcripts and the 3' bias seen in these amplified samples are likely to be even more problematic in phloem sap samples.

Whilst the results of the EDTA-facilitated exudation approach were encouraging, there would be little point in running these samples on an array in the absence of a stylet-derived sample for comparison. In this case it would be impossible to judge the purity of the sap obtained by this method or to assess how accurately it represents the transcriptome of pure phloem sap.

From the results of this study it is concluded that it should be possible to amplify sufficient RNA from SE sap but further method development is required. The low RNA levels and high sugar concentrations of SE sap are likely to have been responsible for the limited success achieved in the amplification procedures. These problems are unique to phloem sap and make the approaches described for LCM-derived samples, for example, unsuitable for this sample type. Future protocol modifications should involve further purification of the sap and utilisation of an alternative primer for second-round amplification. If double-amplification of

RNA from SE sap is achieved, a wide range of robust quality control and result confirming methods should be applied.

4.4.2 Advances in studying small samples

Since this study was completed, improvements in commercial RNA amplification kits have reduced the amount of input RNA required. For example The Illumina® TotalPrep™ RNA Amplification Kit (Life Technologies), which employs T7 RNA polymerase mediated amplification, generates biotinylated, amplified RNA suitable for analysis on Illumina arrays from as little as 50 ng total RNA. This kit has improved on previous T7 mediated amplification by using an alternative reverse transcriptase (RT), ArrayScript™ RT. This is an engineered RT which produces higher cDNA yields than natural enzymes. Further to this, a new *in vitro* transcription (IVT) technology, MEGAscript®, generates hundreds of aRNA copies of each target mRNA. The Ovation PicoSL WTA System (NuGEN Technologies Inc.) is another kit designed for small samples. It requires an input of 500pg total RNA and the cDNA produced is suitable for use with a variety of array platforms. As well as advances in transcriptomics, other functional genomics techniques have been developed for small samples. For example, Palmer *et al*. (Palmer *et al*., 2014)developed a mass spectrometry-based metabolomics technique, which allowed them to identify metabolites from only 19.5 nl of SE sap.

Table 4-5 RNA inputs required for old and new technologies.

Stage	Method	RNA input	RNA output
RNA isolation	QIAGEN plant mini kit	100mg plant tissue	25-65ug
	Stratagene Absolutely RNA	1 cell	<0.01ng
	nanoprep kit	10 cells	<0.01ng
		100 cells	1ng
		10,000 cells	100ng
	QIAGEN micro kit	1 cell – $5x10^5$ cells	<45ug
	QIAGEN REPLI-g Single Cell kit	2–1000 cells	<40ug
RNA preparation for array or sequencing	Agilent Technologies Low Input Quick Amp Labelling kit	10 ng	
	Illumina® TotalPrep™ RNA Amplification Kit	50ng	
	Clontech SMARTer Universal Low Input RNA Kit	2-10ng	
	RNA sequencing (ultra low input Clontech SMARTer kit)	10pg	
Microarray	2^{nd} generation	5ug	
	1x244k	3.75ug	
	2x105k	1.65ug	
	4x44k		
	3^{rd} generation	600ng	
	8x60k		

4.4.3 New transcriptomics technology

Advances in RNA sequencing (RNA-seq) have made this method more widely available to researchers investigating the transcriptomes of small samples. The principles behind RNA-seq have been discussed in Chapter 1, but various efforts have also been made to develop the technique for very small scale studies (Adiconis *et al*., 2013). For example, RNase H (also known as selective depletion of abundant RNA, or SDRNA), Ribo-Zero (Epicentre), duplex-specific nuclease with light normalization (DSN-lite), the Ovation RNA-seq system ('NuGEN') and switching mechanism at the 5' end of the RNA template (SMART; Clontech). The merits of each approach have been assessed and it was concluded that RNase H was most effective with degraded RNA, whilst SMART and NuGEN were advantageous for low quantity samples (Adiconis *et al*., 2013). The SMARTer Ultra Low Input RNA Kit for Sequencing (Clontech), for example, allows sufficient quantities of cDNA to be synthesised from RNA collected from single cells for transcriptome analysis by RNA-seq. Full length cDNA libraries for sequencing can be generated from as little as 10pg of total RNA, or a single cell.

Studies on very small samples collected by microsampling or LCM are frequently utilizing RNA-seq for transcript identification. For example, transcripts from 90-100 individually sampled cancer cells have been successfully sequenced (Navin *et al*., 2011, Hou *et al*., 2012). More recently, the approach has been applied to plant studies. For example, Honaas *et al* (2013) used LCM to sample cells from the host-parasite junction between *Triphysaria versicolor* and its hosts *Zea Mays* and *Medicago truncatula* to investigate the interaction between parasitic plants and their hosts. RNA was collected and pooled from interface cells of approximately 110 regions of interest covering an area of approximately 6 million μm^2 in total and two rounds of linear T7 amplification performed using the Message Amp™ II aRNA

kit (Ambion). Around 50-100ug of amplified RNA (aRNA) was generated from around 100ng of starting sample RNA by this approach. An Illumina sequencing library was created from this aRNA and this was sequenced accordingly. Triphysaria genes overexpressed at the host-parasite junction included those encoding pathogenesis-related proteins and cell wall modifying enzymes. Host transcription factors were also upregulated at this site. SOLiD and Illumina sequencing have been used to study gene expression in various developmental stages of *Arabidopsis*, including the meiocyte (Chen *et al.*, 2010, Yang *et al.*, 2011), megaspore (Schmid *et al.*, 2012) pollen and seedling (Loraine *et al.*, 2013). In the former studies, cells were obtained by micromanipulation. In the latter study, Arabidopsis embryo sac cells were collected by LCM and approximately 300-1000pg RNA was isolated from 450 cells. Sequencing libraries were created and sequenced using the SOLiD platform. This highlights the usefulness of RNA-seq for small-sample studies. Currently, RNA-seq produces large amounts of data so the costs of analysis are high. It is likely that, as analysis software and annotation databases improve, RNA-seq will become the standard method in small sample gene expression studies in the future.

5 *R. padi* induce gene expression changes both locally and systemically in barley

5.1 Summary

Given the difficulties of sampling and analysing very small quantities of RNA, whole tissue studies can be used as a way of collecting sufficient sample to study the broader effects of a treatment. It was the aim of the current study to provide plant transcriptomic information on the interaction of *R. padi* and barley in leaf tissue, both locally and distally from the site of infestation. Two microarray studies were carried out to investigate changes in gene expression in infested first leaves of young barley plants and in uninfested second leaves of infested plants. More genes were upregulated systemically than locally and the expression pattern was characteristic of a local facilitation and a systemic defence response. The differentially regulated genes have roles in defence responses, nutrient partitioning, cell wall modification and metabolic activities.

5.2 Introduction

5.2.1 How aphids alter the plant transcriptome, proteome and metabolome

It has been established that plants undergo many changes in response to aphid attack (Thompson and Goggin, 2006a). This study seeks to identify induced defences that reduce the severity of aphid infestation or improve tolerance to aphid pests, and possible susceptibility genes that facilitate aphid colonisation, in a compatible host-pest interaction.

Advances in functional genomics techniques have allowed identification of plant traits with roles in broad-spectrum induced resistance, such as cell wall modification, proteins or secondary metabolites with antixenotic or antibiotic properties, and plant volatiles that attract the natural enemies of PFIs (Thompson and Goggin, 2006a). Microarray, for example, has proven to be an important technique in gene expression studies and has been used to provide information on plant-insect interactions. Early microarray studies used small defence-gene biased cDNA arrays, and therefore only studied genes already implicated in the plant-aphid interaction (Moran *et al*., 2002, Voelckel *et al*., 2004, Zhu-Salzman *et al*., 2004). Development of whole-genome arrays has provided a more comprehensive view of the plant response and a wealth of data are now available in online resources (e.g. National Centre for Biotechnology Information: Gene Expression Omnibus).

De Vos *et al*. (2005) used Affymetrix GeneChips to investigate the response of *Arabidopsis* to *M. persicae.* They identified 832 upregulated genes and 1349 downregulated genes after 72 hours infestation. They contrasted this with gene expression changes induced by a range of herbivores and pathogens with different modes of attack, including a bacterium (*Pseudomonas syringae* pv. *tomato*), a fungus (*Alternaria brassicicola*), leaf-chewing caterpillars (*Pieris rapae*) and cell-content-feeding thrips (*Frankliniella occidentalis*). Although aphids caused the fewest symptoms in their host plants, they induced the largest number of differentially expressed genes. A differential proteomic response to different attackers was also recorded (Duceppe *et al*., 2012). Using two-dimensional gel electrophoresis (2-DE), this study identified 31 proteins that were differentially induced in potato plants (*Solanum tuberosum*) by one of three stress treatments: mechanical wounding, Colorado potato beetle (*Leptinotarsa decemlineata*) herbivory and *M. euphorbiae*

aphid feeding. None of the proteins was differentially regulated by all three stress treatments, and most of them were up- or downregulated by only one treatment, illustrating how specific the plant response is to specific attackers.

Couldridge *et al*. (2007) used Affymetrix microarrays to look at the interaction between *Arabidopsis* and *M. persicae* at two hours and 36 hours post-infestation. After two hours, one gene was upregulated and one downregulated. After 36 hours 23 genes were upregulated and two downregulated. Functional categorisation of these genes showed important categories to be: response to oxidative stress; pathway defence; cell wall modification; calcium signalling; and regulation of transcription. Kusnierczyk *et al*. (2008) performed a more extensive time series-based microarray study of *Arabidopsis* subjected to attack by *B. brassicae*. Transcriptome changes were studied at 6, 12, 24 and 48 h after infestation using full-genome oligonucleotide microarrays. Genes involved in ROS metabolism, calcium signalling, and SA and JA signalling were all upregulated from 6 hours onwards. Metabolome changes were also investigated using high-performance liquid chromatography–mass spectrometry (HPLC-MS) and showed a decrease in the level of aliphatic glucosinolates 48 h after attack.

Coppola *et al.* (2013) investigated the transcriptomic and proteomic relationship between *Solanum lycopersicum* (tomato) and *Macrosiphum euphorbiae* (the potato aphid). Microarrays were performed on plant material after 24, 48 and 96 hours of infestation. The study showed that 148, 637 and 34 genes were differentially expressed at these timepoints, respectively. Functional analysis of the transcripts showed a high level of oxidative stress in this interaction and modification of the expression of various cell wall related genes. An elevated level of ROS detoxification proteins and a reduction in photosynthetic proteins

were the key findings of the proteomic analysis. Interestingly, the group found a limited match between transcripts and proteins and speculated that this may be due to translational regulation and post-translational processing.

Ferry *et al.* (2011) performed proteomic analysis of the interaction of the cereal aphid *S. avaenae* and wheat. Using 2D gel electrophoresis and mass spectrometry, the group isolated 500 proteins from leaves of wheat seedlings. In local tissues, 35 proteins were differentially regulated after 24 hours, whilst 40 were differentially regulated after 8 days infestation. In systemic tissues, 32 were differentially regulated after 24 hours, whilst 40 were differentially regulated after 8 days of infestation. The identity of the proteins was determined by peptide mass fingerprinting. This showed that the majority of aphid-induced proteins were related to metabolic processes and photosynthesis, while a smaller proportion were involved in signalling, stress responses and detoxification of ROS. The responses were spatially as well as temporally regulated.

5.2.2 Gene expression analysis

At the time of experimentation, microarray was the most cost-effective and subject-appropriate method of carrying out expression studies. Emerging technologies such as high-throughput sequencing may, and perhaps subsequently have, overtaken microarray in this domain. What microarray does, however, is give a wide overview of the molecular interactions between a host plant and herbivorous insect, and large quantities of data suitable for identifying candidate genes for future study. One-colour arrays were considered to be preferable in this study as they allow comparison between all samples, not just between populations that are co-hybridised.

5.2.3 Aims and hypotheses

This study aimed to simultaneously characterise the gene expression profiles of local and systemic barley leaves during *R. padi* feeding in order to provide information on the integration of responses by the host plant and to implicate genes involved in long-distance trafficking, mediated by the phloem. This data, when supplemented with that from EPG experiments, will give a picture of what behaviours aphids are exhibiting and the concomitant host gene expression adjustments. It was hypothesised that genes with roles in defence responses, cell wall modification and primary metabolism would be differentially regulated, both from the studies discussed above and from the results of Chapter 2 of the current study, which showed growth reduction, nutrient reallocation and altered SE sap exudation duration following infestation. It was also hypothesised that the systemic response would differ from the local one. Specifically, this may involve a systemic induction of defence-related genes.

5.3 Materials and Methods

5.3.1 Plant Material and aphid infestation

The plant and aphid growth conditions and the infestation techniques were as outlined in Chapter 2. For arrays investigating local responses, the first leaves were infested at 10 days post sowing and harvested on day 13 (Table 5-1). For systemic arrays, the first leaves were infested on day 10 and the second leaves harvested on day 13.

Table 5-1 Experimental design. Shows the infestation status of the first and second leaves of *Hordeum vulgare* plants used for arrays investigating the local and systemic effects of infestation. Both local and systemic arrays had an infested condition and an uninfested condition. The leaves of the plants that were harvested and processed for array are highlighted in red. Each of the four treatments had four replicates, making a total of 16 arrays.

	Leaf 1	Leaf 2
Local arrays		
Infested condition	**Infested (30 aphids)**	Uninfested
Uninfested condition	**Uninfested**	Uninfested
Systemic arrays		
Infested condition	Infested (30 aphids)	**Uninfested**
Uninfested condition	Uninfested	**Uninfested**

5.3.2 Whole leaf tissue harvesting and RNA isolation

Four plants were selected at random from each of the control and treatment conditions. If aphids were observed on the plant anywhere other than the desired infestation site, the plant was discarded and another was randomly selected. Insect cages were removed from the plants and aphids were brushed from infested plants using a soft paintbrush. A leaf was removed using a sterile, RNase-free blade and was immediately flash-frozen in liquid nitrogen. No more than 100mg of leaf material could be processed using the preferred RNA isolation kit so, after preliminary measurements, this amount was sampled by eye to reduce sample degradation during weighing. Samples were processed immediately with no storage time. The flash-frozen leaf tissue was ground to a fine powder under liquid nitrogen in a pestle and mortar. This was transferred to a chilled micro centrifuge tube and the liquid nitrogen was allowed to evaporate without the sample defrosting. 450 µl of the first buffer

from the QIAGEN RNeasy Plant Mini Kit was added to the tube and the kit protocol was followed to completion without modification. The resulting RNA was eluted in 30 µl and was transferred to -20°C for storage.

5.3.3 RNA labelling

All samples were labelled using the Low Input Quick Amp Labeling Kit for one-colour microarrays (Agilent Technologies). All of the following steps, from labelling to feature extraction, can be found in detail in the Low Input Quick Amp Labeling Protocol v.6.5, May 2010 (available from www.agilent.com/chem/dnamanuals-protocols). This kit generates fluorescent complementary RNA (cRNA) with a sample input RNA range between 25 ng and 200 ng of total RNA or a minimum of 5 ng of poly A+ RNA for one-colour processing. The method uses T7 RNA polymerase, which simultaneously amplifies target material and incorporates cyanine 3- (Cy3) labelled cytidine triphosphate (CTP). Amplification is typically at least a 100-fold from total RNA to cRNA with the use of this kit. It was necessary to dilute the RNA samples down to a suitable starting concentration prior to labelling. All samples went into the labelling procedure with 200 ng RNA in a 1.5 µl volume.

A spike-in kit was used for control purposes and was added at this stage (Agilent One Color RNA Spike-In Kit; Figure 5-1). Spike-in probes are known probes replicated across the microarray that are hybridised with known quantities of a target spike-in cocktail which is added to the sample prior to array. They are used to perform a quality check of the microarray experiment.

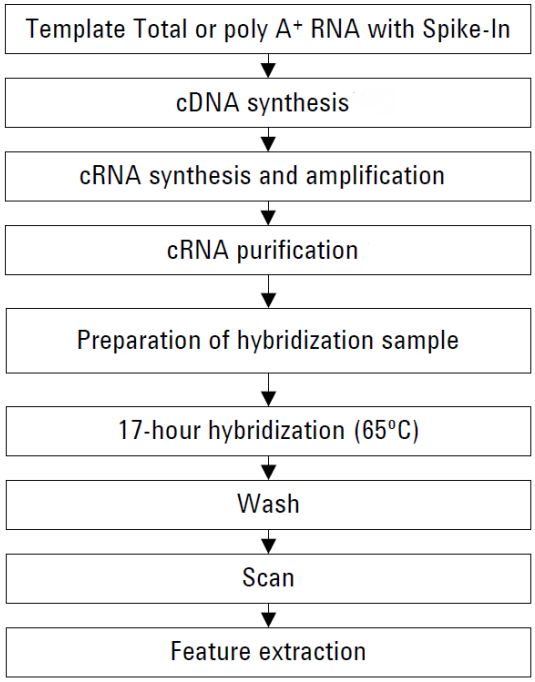


Figure 5-1 Workflow for sample preparation and array processing. From 'Low Input Quick Amp Labeling Protocol v.6.5, May 2010' (available from www.agilent.com/chem/dnamanuals-protocols)

5.3.4 Quality Assessment of RNA/cRNA

It was necessary to assess the quality of template RNA and labelled cRNA before proceeding with amplification and hybridisation, respectively. This was achieved using the NanoDrop UV-VIS Spectrophotometer and the Agilent 2100 Bioanalyzer, as outlined in Chapter 4. Only RNA samples with a RIN of 8, or greater, were taken forward to labelling in order to eliminate experimental bias due to poor RNA quality, in accordance with Agilent recommendations.

5.3.5 Microarray

5.3.5.1 Hybridisation, washing, feature extraction

The Cy3 labelled, linearly amplified cRNA was fragmented and a hybridisation buffer was added. The 8 samples were loaded onto the array immediately and the hybridisation assembly, consisting of an Agilent SureHyb chamber, gasket slide and clamp, was prepared. The assembled slide chamber was transferred to the rotator rack in a hybridisation oven set to 65°C for 17 hours. The slide was then washed and scanned and the data extracted using the Agilent Feature Extraction software. Following feature extraction, a QC report was generated for each array. QC reports include statistical results to enable evaluation of the reproducibility and reliability of microarray data.

5.3.5.2 Array design

A custom *H. vulgare* oligonucleotide microarray chip was designed to an 8 x 60k format using existing Agilent probesets. The sequences databases that were used to design the barley array are:

- RefSeq (Release 31), Sep 2008
- UniGene (Build 52), Feb 2008
- TIGR Plant Transcript Assemblies (Release 2), Jul 2008
- TIGR Gene Indices (Release 10), Jun 2006

Two chips were required, with a total of 16 arrays. The first held 4x infested first leaf sample arrays and 4x uninfested first leaf sample arrays. The second held 4x second leaf samples from infested plants and 4x second leaf samples from uninfested plants. Sample position on the chip was randomised, in case of damage to one area of the chip. This microarray had 10

spike-in sequences with 30 replicates each. The quality control (QC) Report uses these replicated probes to evaluate reproducibility of both the signals and the log ratios.

5.3.6 Analysis

Various programs are available for data analysis in a microarray experiment. Genespring (Agilent Technologies) is a user-friendly dedicated microarray analysis software program designed for use in conjunction with specific chips, e.g. Affymetrix, Agilent etc. Another statistical package is *R* (www.r-project.org). *R* requires programming scripts to be written, but does thereby allow the user more freedom for data manipulation. Due to the simple experimental design, Genespring was deemed most suitable for the purposes of this experiment.

5.3.6.1 Quality control

In Genespring, several quality control criteria are available, including Principal Component Analysis (PCA), Agilent Quality Control Metrics and Sample Correlation Matrix. Microarray experiments can generate high volumes of data, from many samples simultaneously. Such high-dimensionality can make it difficult to capture an overview of the data. PCA is an eigenvector-based analysis that reduces the dimensionality of multivariate data to allow visualisation and exploration of the data. It does this by identifying new variables which represent the variations in the samples (Ringner, 2008). Data are transformed linearly and plotted on a matrix as co-ordinates. The first component represents the greatest variance, the second component the second largest, unrelated variance, and so on. Using this approach, the data can be plotted using only a few components, making it clearer to discern patterns such as groupings. PCA allows comparison between the expression profiles of samples based on the assumption that samples representing the same experimental

condition should be more similar to each other than to samples representing different experimental conditions. Thus, they should group closer together in a PCA plot. Deviation from this assumption could be due to poor quality samples in the dataset or true biological variation within the populations under study.

The sample correlation matrix shows the correlation coefficients calculated using Spearman's correlation coefficient for all pair-wise comparisons of the samples in the experiment. It is similar to the PCA in the assumption that samples representing the same experimental condition should be more similar to each other than to samples representing a different treatment. It compares the intensity across all arrays and would identify any that are outliers. The minimal value of this coefficient gives a good idea of the dataset homogeneity: low coefficients indicate important differences between array intensities.

5.3.6.2 Normalisation and filtering

Data was normalised in order to allow comparisons across arrays. Normalisation removes variation due to factors such as dying efficiency by comparing identical spots/markers present on each array. The term 'normalised signal intensity value' in a microarray experiment refers to the value generated after log transformation and normalization (percentile shift, scale, normalize to control genes or quantile) and baseline transformation. This study used quantile normalisation.

In large data sets such as those generated in microarray studies, it is advantageous to filter the data to reduce the number of data points to increase the power of statistical tests performed on it, including importantly the multiple testing corrections (discussed below).

There are several methods of filtering the data. The data can be assigned ‘flag values’ which denote the quality of the entities. The terms Detected, Not Detected and Compromised are used and entities are filtered based on this. The resultant flag value of any probe is decided by the following logic shown. For each probe with multiple flags, the order of importance is:

Compromised>Not Detected>Detected.

If there is even one `Compromised', then the resultant flag is `Compromised'. If there is no Compromised, but 'Not Detected' and `Detected', then `Not Detected' is assigned. If there are only `Detected' then only the resultant flag is assigned as `Detected'. If there are equal numbers of `Not Detected' and `Detected', then `Not Detected' is assigned. Each probe is ultimately assigned one flag. A second method of filtering is by using the probe signal intensity values. By allowing only those probes between 20-100% through the filter, those with very low signal values, biased heavily by background, were removed. Similarly, by reducing maximum expression, those probes that have reached saturation can be removed. However, as the data contained no Compromised flags, this was deemed unnecessary. A third method is filtering by error. Here, entities are filtered based on the deviation of signal intensities within a condition, i.e. genes with low variance across arrays (ignoring treatment) are removed. Expression for equally expressed genes should not differ greatly between treatment groups, hence leading to small overall variance. The stringency can be controlled by changing the coefficient of variation, for example, <20% is more stringent than <50%.

5.3.6.3 Significance analysis

A variety of statistical tests can be used in the analysis of microarray data, including t-tests, ANOVA and Wilcoxon’s test. In this study, the moderated t-test, a modification of the unpaired t-test, was selected for analysis. The higher the calculated t-statistic for a gene, the

greater the confidence with which this gene can be declared as being differentially expressed. The modified t-test is designed to ameliorate two conditions that prove problematic for the unpaired t-test. They are: 1. Two conditions with a small difference between the means and a very low variance within each condition, which may lead to a false positive result with the unpaired t-test; 2. Two conditions with a large difference between their means and very high variance within each condition, which might result in false negatives.

In a very large dataset, such as that in a microarray experiment, there is a problem with false-positive results. If data is analysed with a p-value cut-off of 0.05, then for every 100 significant genes, 5 will be detected due to chance. In an array of 60,000 probes, this can severely reduce the validity of the data. The GeneSpring software includes 5 methods for correcting this, the first three correct for family-wise error and the remaining correct for false discovery. The two most common approaches are the Bonferroni method and the Benjamini-Hochberg method (Benjamini and Hochberg, 1995). The Bonferroni method corrects for family-wise error. It is a single step procedure, where each p-value is corrected independently. The p-value of each gene is multiplied by the number of genes in the gene list. If the corrected p-value is still below the error rate, the gene will be significant. For example, if testing 1000 genes, the highest accepted individual p-value is 0.00005. This method is therefore very stringent and will lead to fewer false positives. The trade-off for this is that the rate of false negatives will be higher. Less stringent than Bonferroni, the Benjamini-Hochberg method assumes independence of p-values across genes. This procedure makes use of the ordered p-value, multiplying each p-value by the total number of genes tested, divided by its rank. So, if the second largest p-value in a group of 1000 is

0.06, the FDR p-value is 0.06 x (1000/999) or 0.06006. This test is therefore more stringent the lower the p-value.

Following statistical analysis, the significant probes were filtered by fold change (FC) to leave only those most overexpressed for further analysis. FC analysis is used to identify genes where expression ratios between treatment and control are over a certain threshold. Stringency can be controlled by moving the FC cut-off but a FC threshold of 2 is generally considered acceptable in microarray studies.

5.3.6.4 Gene ontology analysis

Information on gene function was gathered from several online resources, including the Universal Protein Resource (Uniprot), the Plant Expression Database (PLEXdb; a gene expression resource for plants and plant pathogens), and the National Center for Biotechnology Information (NCBI) GenBank. The majority of microarray data has associated annotations, this includes information on the function of the genes (whether known or inferred) in the form of gene ontology (GO) divided into three categories: biological processes, cellular component and molecular function. By searching the gene list for enriched ontologies it is often possible to identify clusters of related genes. Web-based ontology enrichment tools such as GO-EAST (Zheng and Wang, 2008), can be used to search for enriched ontological categories compared with the GeneChip as a whole.

5.4 Results

5.4.1 Local (first leaf) transcriptome responses to infestation

5.4.1.1 Quality Control

Following feature extraction, a QC report was generated, summarising the signal strength and distribution of data probes and of spike-in probes. These QC reports confirmed that the arrays were all of sufficiently high quality to use for downstream analysis.

PCA of infested and uninfested first leaf samples showed that the samples from the two treatment conditions do form distinct clusters (Figure 5-2), although one sample in each condition did lie outside of the main group. The lowest correlation between these outliers and the other samples was 0.87 and 0.77 in the uninfested and infested conditions, respectively (Figure 5-3), indicating that there is a high degree of correlation between samples. The variation within treatments was therefore assumed to be due to natural biological variation and all samples were retained. The correlation across all samples was high (0.71) so normalising all arrays in the same experiment was considered appropriate.

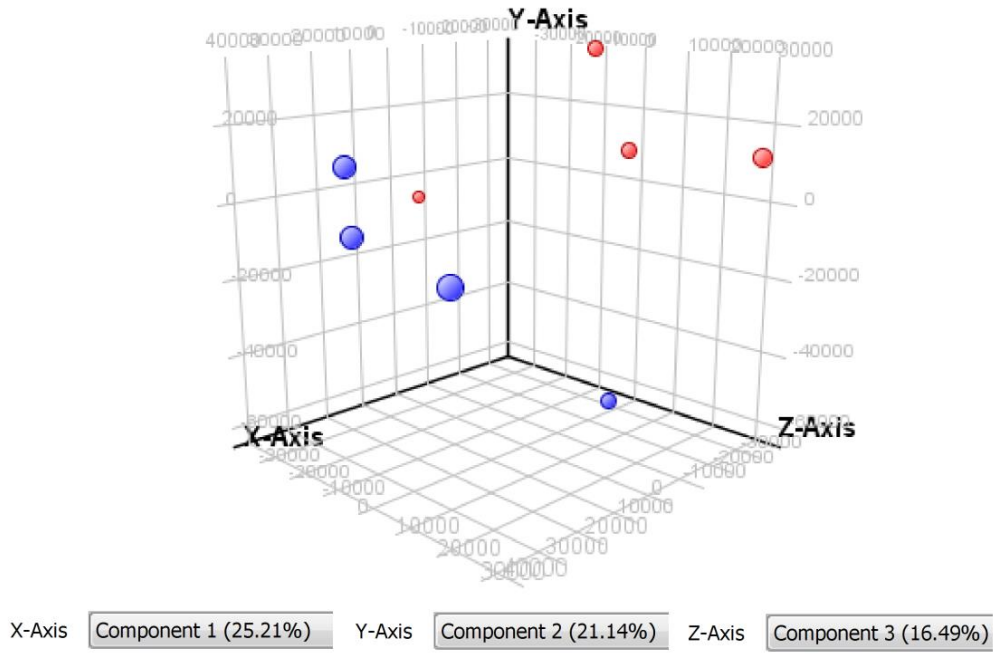


Figure 5-2 PCA plot of normalised samples. Red markers represent samples from aphid infested barley plants; blue markers represent uninfested sample arrays. The third dimension is represented by the size of the data points, i.e. the larger the marker, the further it would stand from the page.

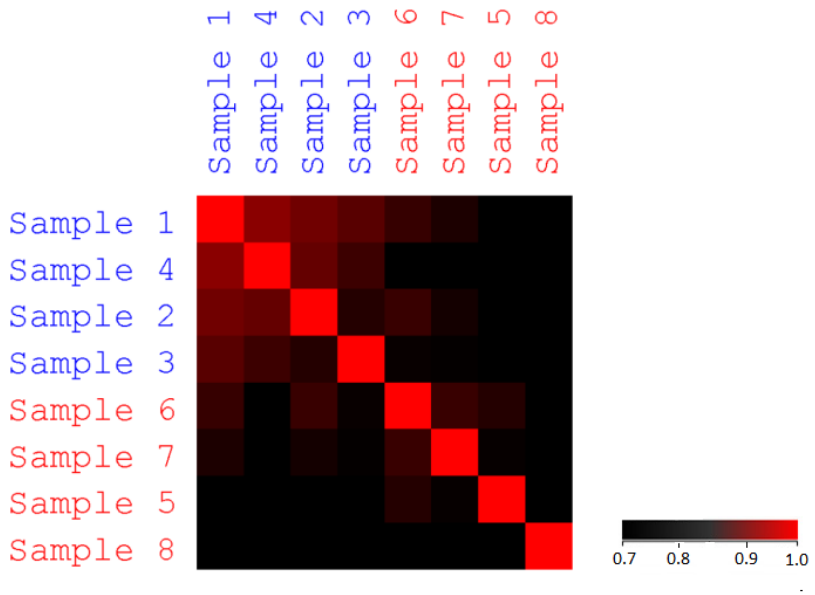


Figure 5-3 Correlation coefficient plot of first leaf arrays. Depicts the value of Spearman correlation coefficient for each pair of arrays in the dataset. Samples 1-4 are uninfested; samples 5-8 are infested. Key shows possible Z-scores ranging from black (normalized expression of 0.7) to red (normalized expression value of 1.0)

5.4.1.2 Data

Data from the arrays were normalised and a box plot of the sample distributions was generated (Figure 5-4). The samples all show similar medians and interquartile ranges following normalisation, indicating that the arrays are of suitable quality and that normalisation was successful. A profile plot showing those probes which have passed the filter conditions was also generated (Figure 5-5). This plot displays the direction of regulation of each transcript, those depicted in red were strongly upregulated in the infested condition, blue were strongly downregulated by infestation, and yellow were moderately up- or down-regulated.

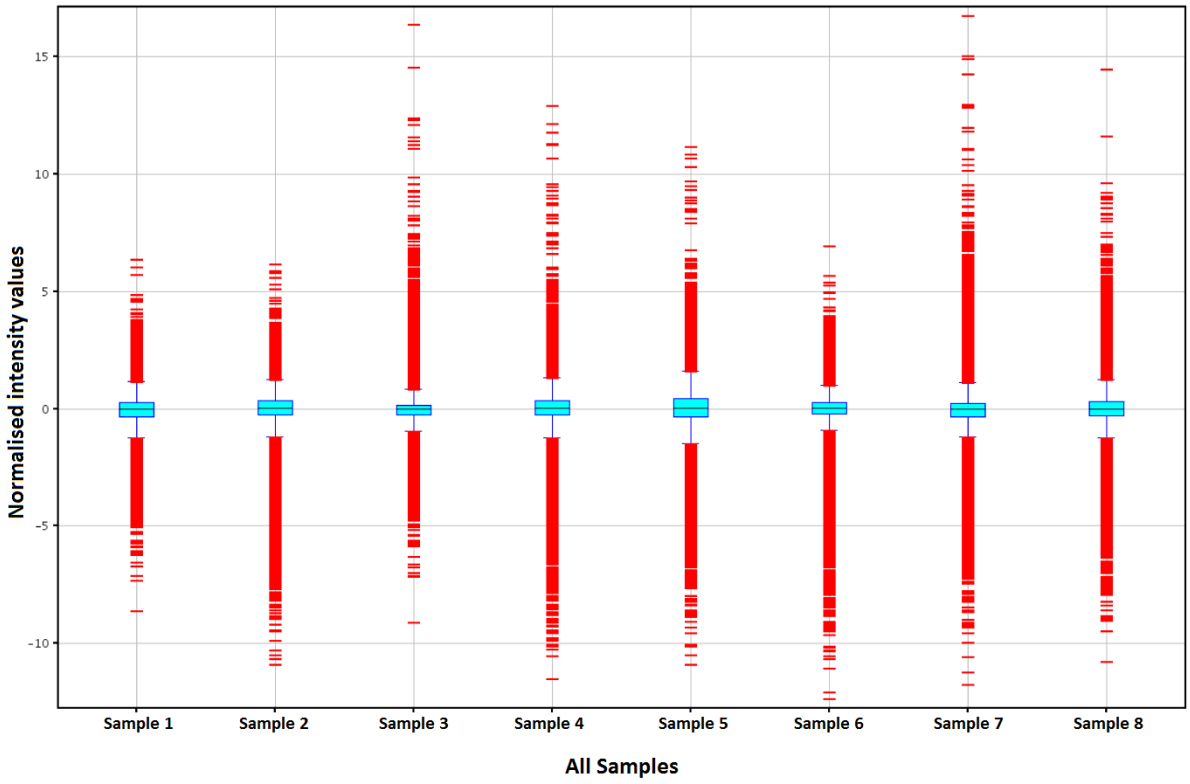


Figure 5-4 Box plot of the normalised intensity values for all 8 arrays. Shows the distribution of the data, the median value and the inter-quartile range for four uninfested sample arrays (Samples 1-4) and four aphid infested sample arrays (Sample 5-8), following quantile normalisation.

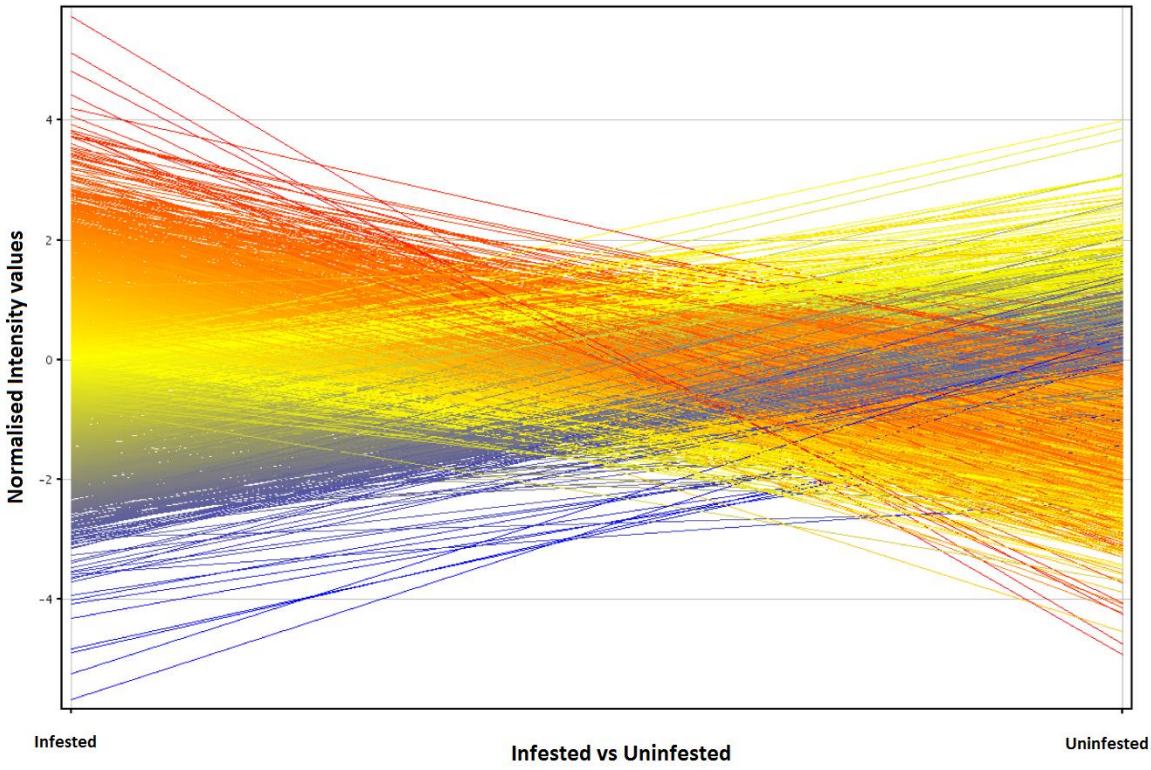


Figure 5-5 Profile plot showing normalised intensity values of all infested and uninfested samples. For each probe, the relationship between expression in infested and uninfested plants is depicted. Red lines indicate upregulation in the infested plants, blue lines indicate probes which were downregulated by infestation. Yellow probes showed only moderate up- or down-regulation.

982 genes were differentially regulated ($p < 0.05$) more than 2-fold due to aphid infestation when the Benjamini Hochberg false discovery rate correction was applied. 690 genes were upregulated ($p < 0.05$) with a FC greater than 2 and 292 genes were downregulated ($p < 0.05$) more than 2-fold.

When the Bonferroni false discovery rate correction was used, only 100 genes were found to be upregulated ($p < 0.05$) and 7 downregulated ($p < 0.05$) more than 2-fold. Similarly, when the data were filtered by error <20 rather than error <50, fewer genes were found to be altered (114; 94 upregulated and 20 downregulated).

Using the more stringent filtering and correction methods (i.e. error < 20 and Bonferroni respectively) is likely to lead to some type II errors (false negatives). The less stringent approach (i.e. error <50 and Benjamini Hochberg FDR correction) was used in this study because, although type I errors (false positives) may be generated, the aim of the study was to identify candidate genes for further study and so it was deemed more detrimental to discount true positive results than to include true negatives. This approach will ultimately provide a wider spectrum of genes for physiological/functional analysis and any false positives can then be eliminated.

5.4.1.3 Functional analysis of differentially expressed genes

A small proportion (2%) of the most highly overexpressed genes appeared to be of non-plant origin (Figure 5-6). They were assumed to be contaminants from the sample processing procedure so were discounted. The vast majority (557 upregulated genes and 255 downregulated genes) of the differentially regulated genes were predicted protein-coding sequences based on previous studies, but no further information exists for them beyond this, either in the Agilent annotation file for the array or by the Basic Local Alignment Search Tool (BLAST).

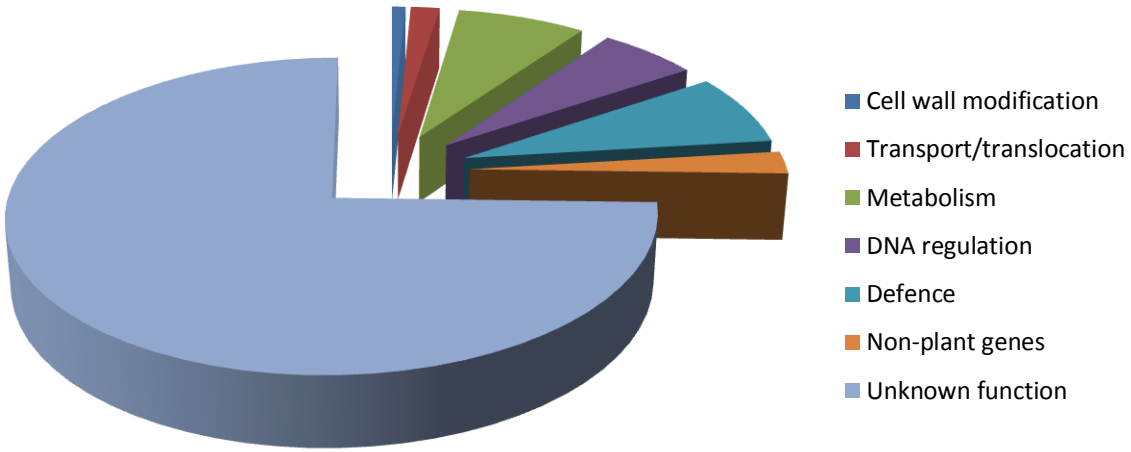


Figure 5-6 Functional grouping of the 982 barley genes significantly altered by *R. padi* infestation. Shows that the majority of differentially regulated transcripts are of unknown function, a small proportion are of non-plant origin and the rest fit into the categories cell wall metabolism, transport, metabolism, DNA regulation and defence.

Analysis showed that 48 of the upregulated genes are directly related to, or have previously been implicated in, plant defence responses. Some are associated with response to biotic stress, some abiotic and others a more general response. They include genes with roles in ROS detoxification, *R*-gene mediated defence, callose digestion, JA signalling and transcriptional regulation of defence genes. Amongst the downregulated genes, seven are implicated in defence responses. In contrast to the upregulated genes, those which were downregulated have roles in response to abiotic stress (dehydration and salinity). Genes with a role in various metabolic activities accounted for 74 differentially expressed genes (52 up and 22 down), whilst 18 DNA regulatory genes were upregulated and none downregulated. Eight genes with transport/translocation roles were upregulated and five

downregulated. Finally, ten genes related to cell wall modification were differentially expressed (seven upregulated and three downregulated).

Table 5-2 Defence genes differentially regulated locally following 72 hours of *Rhopalosiphum padi* feeding on 13 day old barley plants. Table shows Agilent probe name; role of gene product in the plant according to annotation and/or BLAST search; absolute fold change; and gene annotation. P < 0.05 (moderated t-test); Benjamini-Hochberg false discovery rate correction applied. A positive fold change indicates upregulation in infested plants; a negative fold change indicates downregulation in infested plants.

ProbeName	Role	FC (abs)	Annotation
A_13_P199664	Detection	3	Resistance-related receptor-like kinase - Triticum aestivum
A_13_P182464	Detection	11	Avr9/Cf-9 rapidly elicited protein-like - Oryza sativa
A_13_P184229	Calcium binding	4	EF hand family protein, expressed - Oryza sativa
A_13_P318042	JA response	8	Hordeum vulgare mRNA for allene oxide cyclase (aoc gene)
A_13_P480235	JA response	8	Allene oxide synthase - Hordeum vulgare (Barley)
A_13_P128410	JA response	4	Jasmonate induced protein - Hordeum vulgare (Barley)
A_13_P538169	JA response	4	CI2E - Hordeum vulgare (Barley)
A_13_P338482	JA response	8	Proteinase inhibitor Rgpi9 - Triticum aestivum (Wheat)
A_13_P416215	JA response	5	Subitilisin-chymotrypsin inhibitor - Oryza sativa
A_13_P044211	JA response	25	Hordeum vulgare partial mRNA for putative cytochrome P450
A_13_P169955	JA response	2	Sterol C-22 desaturase-like - Oryza sativa
A_13_P204584	JA response	2	Wound and phytochrome signalling involved receptor like kinase
A_13_P538399	ROS	7	Hordeum vulgare mRNA for predicted peroxidase
A_13_P499769	ROS	12	Peroxidase precursor - Hordeum vulgare (Barley)
A_13_P179944	ROS	4	Pox1 protein - Triticum aestivum (Wheat)
A_13_P143440	ROS	7	Glycolate oxidase - Zantedeschia aethiopica (White calla lily)
A_13_P128055	PCD regulator	3	Hordeum vulgare hypersensitive-induced reaction protein 3
A_13_P169465	PCD regulator	3	Leaf senescence related protein-like - Oryza sativa
A_13_P465923	PCD regulator	2	Protein spotted leaf 11 - Oryza sativa subsp. indica (Rice)
A_13_P523629	Heat shock	15	DnaJ-related protein ZMDJ1 - Zea mays (Maize)
A_13_P567774	Heat shock	3	Heat-shock protein precursor - Secale cereale (Rye)

A_13_P116850	Heat shock	6	Hordeum vulgare subsp. vulgare mRNA for predicted protein
A_13_P102415	Heat shock	3	Heat stress transcription factor Spl7 - Oryza sativa
A_13_P569894	PR gene	9	Protein WIR1A - Triticum aestivum (Wheat)
A_13_P162600	PR gene	11	WIR1 protein - Triticum aestivum (Wheat)
A_13_P127900	PR gene	19	PR-1a pathogenesis related protein (Hv-1a) precursor
A_13_P522469	PR gene	24	Pathogenesis-related 1a - Triticum monococcum
A_13_P094360	PR gene	17	Pathogenesis-related protein 1 precursor - Hordeum vulgare
A_13_P262922	PR gene	39	Pathogenesis-related protein 4 - Triticum monococcum
A_13_P489725	PR gene	25	Hordeum vulgare pathogenesis-related protein 10 (PR-10)
A_13_P438076	PR gene	20	Pathogenesis-related protein precursor - Hordeum vulgare
A_13_P136575	PR gene	17	H. vulgare,intracellular pathogenesis-related protein PR-107
A_13_P171025	PR gene	6	Thaumatin family protein, expressed - Oryza sativa
A_13_P113435	PR gene	93	Thaumatin-like protein TLP7 - Hordeum vulgare
A_13_P477528	R-gene defence	30	Hordeum vulgare subsp. vulgare mRNA for predicted protein
A_13_P138520	R-gene defence	5	Pathogen-related protein - Hordeum vulgare (Barley)
A_13_P152445	R-gene defence	6	Ice recrystallization inhibition protein 1 precursor
A_13_P186194	R-gene defence	4	LRR binding protein - Oryza sativa subsp. japonica (Rice)
A_13_P226329	R-gene defence	2	NBS-LRR disease resistance protein-like - Oryza sativa
A_13_P022816	R-gene defence	42	Hordeum vulgare subsp. vulgare cDNA clone: Bln2
A_13_P099510	Callose	49	Hordeum vulgare beta-1,3-glucanase 2a mRNA
A_13_P281559	Callose	10	Hordeum vulgare mRNA for beta-1,3-glucanase
A_13_P152495	Callose	3	Beta-glucanase-like protein - Oryza sativa
A_13_P095890	Transcription	3	WRKY DNA binding domain containing protein
A_13_P052611	Transcription	7	WRKY transcription factor - Triticum aestivum
A_13_P551009	Transcription	7	WRKY transcription factor 47 - Oryza sativa
A_13_P084966	Abiotic stress	17	Hordeum vulgare subsp. vulgare germin-like protein 1a (GER1a)

A_13_P577839	Abiotic stress	10	Cadmium tolerance factor - Triticum aestivum (Wheat)
A_13_P067791	Epicuticular wax	-4	H. vulgare putative very long chain fatty acid condensing enzyme
A_13_P597329	Epicuticular wax	-2	H. vulgare subsp. vulgare mRNA for long-chain fatty acid
A_13_P552529	MAPK	-6	Mitogen activated protein kinase 20-4 - Oryza sativa
A_13_P483075	ROS metabolism	-3	Class III peroxidase 62 precursor - Oryza sativa
A_13_P003103	NBS-LRR	-3	Hordeum vulgare NBS-LRR disease resistance protein (rga S-112)
A_13_P207034	Abiotic stress	-4	Dehydrin ERD14 - Arabidopsis thaliana (Mouse-ear cress)
A_13_P149440	Abiotic stress	-7	ERD4 protein - Arabidopsis thaliana (Mouse-ear cress)

Table 5-3 Genes with annotations linked to metabolism that were differentially regulated locally after 72 hours of *Rhopalosiphum padi* feeding on 13 day old barley plants. Table shows Agilent probe name; absolute fold change; and gene annotation. P < 0.05. Benjamini-Hochberg false discovery correction applied. A positive fold change indicates upregulation in infested plants; a negative fold change indicates downregulation in infested plants.

ProbeName	FC (abs)	Description
A_13_P027791	30	Barley alpha-amylase type B isozyme mRNA
A_13_P184039	11	Wall-associated kinase 1 - Triticum aestivum (Wheat)
A_13_P064931	9	Soluble acid invertase - Hordeum vulgare (Barley)
A_13_P024726	5	UDP-D-glucuronate decarboxylase - Hordeum vulgare (Barley)
A_13_P112910	3	Reversibly glycosylated polypeptide - Triticum aestivum (Wheat)
A_13_P541682	2	Xylose isomerase - Hordeum vulgare (Barley)
A_13_P000241	2	Hordeum vulgare mRNA for SnRK1-interacting protein 1
A_13_P183464	8	Transferase family protein, expressed - Oryza sativa subsp. japonica (Rice)
A_13_P137050	16	Alternative oxidase - Oryza sativa (Rice)
A_13_P232814	6	Hordeum vulgare mRNA for putative S-adenosylhomocystein hydrolase 2 (ahh2)
A_13_P520064	3	Malic enzyme - Oryza sativa subsp. indica (Rice), partial (11%)
A_13_P135890	3	Phosphoenolpyruvate/phosphate translocator - Oryza sativa (Rice)
A_13_P553174	31	Carboxyl-terminal peptidase-like - Oryza sativa subsp. japonica (Rice)
A_13_P189504	70	U-box domain containing protein, expressed - Oryza sativa subsp. japonica
A_13_P464513	8	Polyubiquitin containing 7 ubiquitin monomers - Oryza sativa subsp. indica
A_13_P157355	6	Putative subtilisin-like proteinase - Oryza sativa subsp. japonica (Rice)
A_13_P006746	18	Hordeum vulgare Sec61 alpha subunit mRNA
A_13_P186299	25	Protein kinase domain containing protein, expressed - Oryza sativa
A_13_P192794	12	Protein kinase APK1B, chloroplast precursor - Arabidopsis thaliana
A_13_P168145	9	S-domain receptor-like protein kinase precursor - Zea mays (Maize)
A_13_P539674	7	S-locus receptor kinase - Brassica oleracea (Wild cabbage)
A_13_P208964	6	Protein kinase domain containing protein, expressed - Oryza sativa
A_13_P199679	2	Fructokinase-like protein - Arabidopsis thaliana (Mouse-ear cress)
A_13_P146245	2	Protein kinase-like protein - Oryza sativa subsp. indica (Rice)

A_13_P155325	3	CR4 - Oryza sativa (Rice)
A_13_P464593	3	Holocarboxylase synthetase - Triticum aestivum (Wheat)
A_13_P179154	7	T1N24.22 protein - Arabidopsis thaliana (Mouse-ear cress)
A_13_P160825	3	Lipase class 3-like - Oryza sativa subsp. japonica (Rice)
A_13_P212564	3	Glutamate dehydrogenase 2 - Oryza sativa subsp. japonica (Rice)
A_13_P168310	12	Tryptophan decarboxylase - Hordeum vulgare (Barley)
A_13_P122135	5	Serine hydroxymethyltransferase - Triticum monococcum (Einkorn wheat)
A_13_P549244	4	Tryptophan synthase alpha chain - Arabidopsis thaliana (Mouse-ear cress)
A_13_P511064	4	Indole-3-glycerol phosphate synthase, chloroplast precursor - Arabidopsis
A_13_P053796	4	Hordeum vulgare mRNA for methionine synthase 2 enzyme (ms2 gene)
A_13_P246191	3	3-phosphoshikimate 1-carboxyvinyltransferase - Lolium multiflorum
A_13_P026666	16	KI domain interacting kinase 1 - Zea mays (Maize)
A_13_P415440	4	ABA-responsive protein-like - Oryza sativa subsp. japonica (Rice)
A_13_P551594	4	ZmRR2 protein - Zea mays (Maize)
A_13_P548664	3	Dual-specificity protein-like phosphatase 3 - Zea mays (Maize)
A_13_P206969	10	Integral membrane protein DUF6 containing protein - Oryza sativa
A_13_P495980	6	Hydrolase, alpha/beta fold family protein, expressed - Oryza sativa
A_13_P120715	3	Peptidyl-prolyl cis-trans isomerase - Triticum aestivum (Wheat)
A_13_P572464	2	Phenazine biosynthesis family protein - Oryza sativa subsp. japonica (Rice)
A_13_P130625	2	MAPEG family protein, expressed - Oryza sativa subsp. japonica (Rice)
A_13_P129635	2	High molecular mass early light-inducible protein HV58, Hordeum vulgare
A_13_P141195	6	Glyoxalase II - Brassica juncea (Leaf mustard) (Indian mustard)
A_13_P082621	3	Glucose-6-phosphate 1-dehydrogenase - Triticum aestivum (Wheat)
A_13_P052356	4	Transducin / WD-40 repeat protein-like - Oryza sativa subsp. japonica (Rice)
A_13_P187574	18	10-deacetylbaccatin III-10-O-acetyl transferase-like - Oryza sativa
A_13_P550529	4	Chalcone-flavanone isomerase family protein, expressed - Oryza sativa
A_13_P139265	15	Flavanone 3-hydroxylase - Triticum aestivum (Wheat)
A_13_P030866	4	Acid phosphatase-like - Oryza sativa subsp. japonica (Rice)

A_13_P526319	-5	Hordeum vulgare partial CA8 gene for P-type ATPase
A_13_P499299	-2	ATP synthase subunit beta - Triticum aestivum (Wheat)
A_13_P027771	-10	Hordeum vulgare cytokinin dehydrogenase 2 mRNA
A_13_P372642	-7	Type-III chlorophyll a/b-binding polypeptide of the light-harvesting complex-ii
A_13_P001881	-5	Barley mRNA for NADPH-protochlorophyllide oxidoreductase
A_13_P068411	-10	Hordeum vulgare mRNA for predicted Glucose 6 phosphate dehydrogenase
A_13_P235214	-3	Hordeum vulgare myo-inositol 1-phosphate synthase mRNA
A_13_P078951	-5	Alkaline alpha-galactosidase - Oryza sativa subsp. japonica (Rice)
A_13_P499179	-4	At1g56600/F25P12_16 - Arabidopsis thaliana (Mouse-ear cress)
A_13_P555239	-8	Beta transducin-like protein HET-E2C-like - Oryza sativa subsp. japonica (Rice)
A_13_P160670	-2	Cycloartenol synthase - Avena strigosa (black oat)
A_13_P440366	-3	Far-red impaired response protein-like - Oryza sativa subsp. japonica (Rice)
A_13_P000831	-2	Ferredoxin-sulfite reductase precursor - Zea mays (Maize)
A_13_P062606	-5	Hydrolase, alpha/beta fold family protein, expressed - Oryza sativa
A_13_P297382	-3	Light-harvesting complex I - Hordeum vulgare (Barley)
A_13_P136355	-5	Sterol desaturase family protein, expressed - Oryza sativa
A_13_P447773	-6	Ubiquitin carrier protein - Oryza sativa subsp. japonica (Rice)
A_13_P160950	-2	Zinc finger, C3HC4 type family protein, expressed - Oryza sativa
A_13_P439546	-7	Zinc finger, ZZ-type; Zinc finger, C2H2-type - Medicago truncatula (Barrel medic)
A_13_P635812	-6	Hordeum vulgare subsp. vulgare cDNA clone: FLbaf44a22, mRNA sequence
A_13_P357047	-2	Serine/threonine-protein kinase Nek4 - Oryza sativa subsp. japonica (Rice)
A_13_P350722	-5	Hordeum vulgare subsp. vulgare mRNA for predicted protein clone: NIASHv1051G03

Table 5-4 DNA regulatory genes that were differentially regulated locally after 72 hours of Rhopalosiphum padi feeding on 13 day old barley plants. Table shows Agilent probe name; absolute fold change; and gene annotation. P < 0.05 (moderated t-test). Benjamini- Hochberg false discovery correction applied. A positive fold change indicates upregulation in infested plants; a negative fold change indicates downregulation in infested plants

ProbeName	FC (abs)	Description
A_13_P445968	4	Basic helix-loop-helix protein-like - Oryza sativa subsp. japonica (Rice)
A_13_P146940	21	Two component, sigma54 specific, transcriptional regulator, Fis family
A_13_P357267	34	ZIM motif family protein, expressed - Oryza sativa subsp. japonica (Rice)
A_13_P392745	2	Myb4 transcription factor - Hordeum vulgare (Barley)
A_13_P128210	14	Aleurone ribonuclease - Hordeum vulgare (Barley)
A_13_P550939	6	Pentatricopeptide (PPR) repeat-containing protein-like - Oryza sativa
A_13_P139635	2	Eukaryotic translation initiation factor 5A-4 - Solanum tuberosum (Potato)
A_13_P428546	2	60S ribosomal protein L27a-3 - Arabidopsis thaliana (Mouse-ear cress)
A_13_P165415	3	Ribosomal large subunit pseudouridine synthase C-like - Oryza sativa
A_13_P480080	3	Hordeum vulgare mRNA for putative cytosine-5 DNA methyltransferase (met1 gene)
A_13_P194039	5	Mre11A - Zea mays (Maize)
A_13_P580979	3	ATP-dependent DNA helicase RecG - marine gamma proteobacterium
A_13_P127115	3	Nucleosome chromatin assembly protein - Oryza sativa subsp. indica (Rice)
A_13_P117980	3	Histone Protein H2A.6 - Triticum aestivum (Wheat)
A_13_P428351	8	Histone H2A - Zea mays (Maize)
A_13_P116390	3	Histone H2B.2 - Triticum aestivum (Wheat)
A_13_P347287	7	Histone H2B.3 - Triticum aestivum (Wheat)
A_13_P116525	5	Histone H2B.5 - Triticum aestivum (Wheat)

Table 5-5 Transport/transfer-related genes that were differentially regulated locally after 72 hours of Rhopalosiphum padi feeding on 13 day old barley plants. Table shows Agilent probe name; absolute fold change; and gene annotation. P < 0.05 (moderated t-test). Benjamini- Hochberg false discovery correction applied. A positive fold change indicates upregulation in infested plants; a negative fold change indicates downregulation in infested plants

ProbeName	FC (abs)	Description
A_13_P000401	10	Hordeum vulgare subsp. vulgare putative high-affinity potassium transporter (HAK1)
A_13_P118810	3	Actin-3 - Oryza sativa subsp. indica (Rice)
A_13_P414110	2	GTP-binding protein Rab6 - Oryza sativa subsp. japonica (Rice)
A_13_P035231	3	Iron transporter Fe2 - Oryza sativa subsp. japonica (Rice)
A_13_P349247	2	Non-specific lipid-transfer protein - Oryza sativa subsp. japonica (Rice)
A_13_P553589	3	Phragmoplast-associated kinesin-related protein 1 - Oryza sativa subsp. japonica
A_13_P145920	4	Secretory carrier-associated membrane protein 2 - Oryza sativa subsp. japonica
A_13_P184134	4	Transporter-related - Arabidopsis thaliana
A_13_P184854	-2	Hordeum vulgare subsp. vulgare mRNA for predicted protein kinase AKT-2
A_13_P144995	-3	Amino acid transporter-like - Oryza sativa subsp. japonica (Rice)
A_13_P020086	-13	Hordeum vulgare HvMATE mRNA for aluminum activated citrate transporter
A_13_P305392	-10	Hordeum vulgare HvPIP1;4 mRNA for aquaporin
A_13_P189149	-2	Myosin heavy chain-like - Oryza sativa subsp. japonica (Rice)

Table 5-6 Genes with annotations linked to cell wall modification that were differentially regulated locally after 72 hours of *Rhopalosiphum padi* feeding on 13 day old barley plants. Table shows Agilent probe name; absolute fold change; and gene annotation. P < 0.05 (moderated t-test). Benjamini-Hochberg false discovery correction applied. A positive fold change indicates upregulation in infested plants; a negative fold change indicates downregulation in infested plants

ProbeName	FC (abs)	Description
A_13_P461208	23	Xylanase inhibitor 801OS - Triticum aestivum (Wheat)
A_13_P144840	4	Polygalacturonase-like protein - Fragaria ananassa (Strawberry)
A_13_P303952	4	N-acetylmuramoyl-L-alanine amidase
A_13_P083386	3	Hordeum vulgare subsp. vulgare mRNA for UDP-D-xylose epimerase 2
A_13_P116875	2	Caffeoyl-CoA O-methyltransferase - Phyllostachys edulis
A_13_P230504	3	4-coumarate--CoA ligase 4CL1 - Lolium perenne (Perennial ryegrass)
A_13_P499494	4	4-coumarate--CoA ligase 4CL3 - Lolium perenne (Perennial ryegrass)
A_13_P012726	-4	Hordeum vulgare subsp. vulgare Cellulose synthase (CesA1)
A_13_P017091	-4	Hordeum vulgare subsp. vulgare Cellulose synthase (CesA3)
A_13_P388159	-5	Hordeum vulgare subsp. vulgare cinnamoyl CoA reductase (CCR)

5.4.2 Systemic (second leaf) transcriptome responses to infestation

5.4.2.1 Quality Control

PCA shows an outlier in the uninfested condition (Figure 5-7). The correlation between the samples in this condition was higher than 0.93, however, so it is assumed that the variation was due to natural variability in the biological population and the sample was retained. The lowest correlation coefficient between arrays is 0.85 (Figure 5-8), indicating a high degree of correlation between arrays so normalising of all arrays in the same experiment is appropriate.

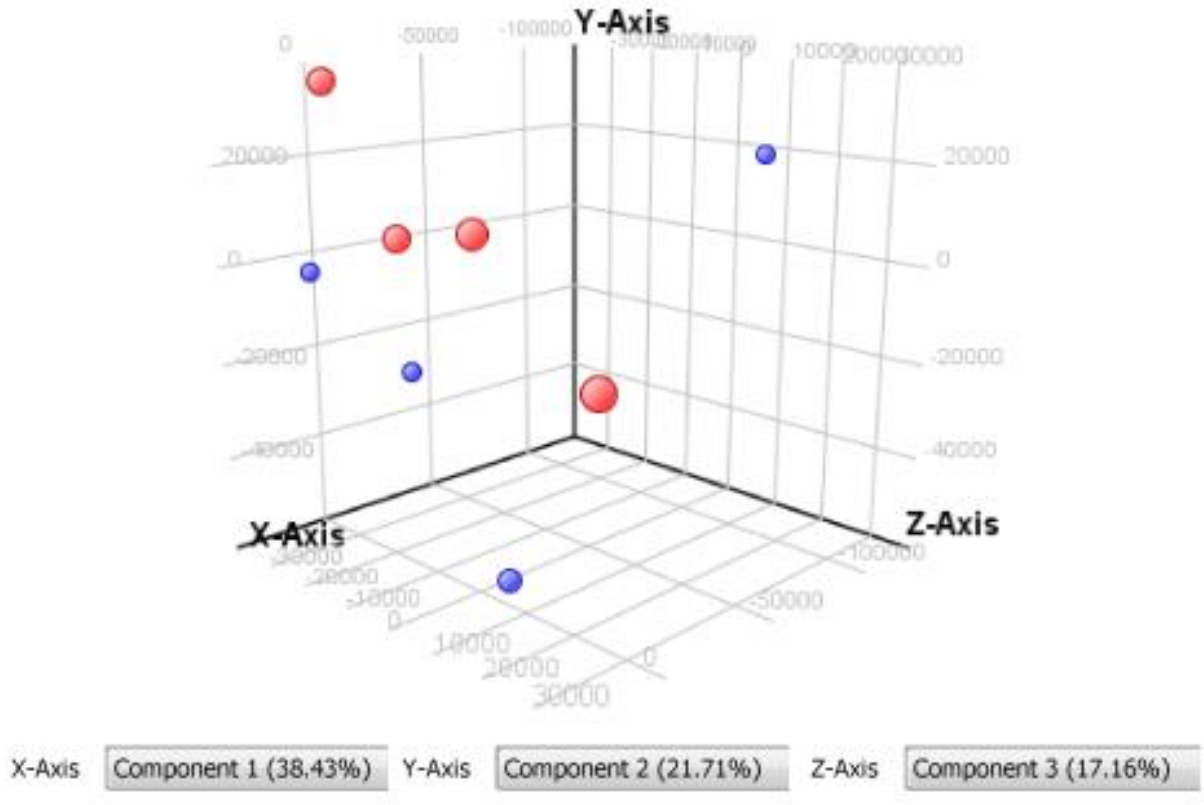


Figure 5-7 PCA plot of normalised arrays. Red markers represent samples from aphid infested barley plants; blue markers represent uninfested sample arrays. The third dimension is represented by the size of the data points, i.e. the larger the marker, the further it would stand from the page.

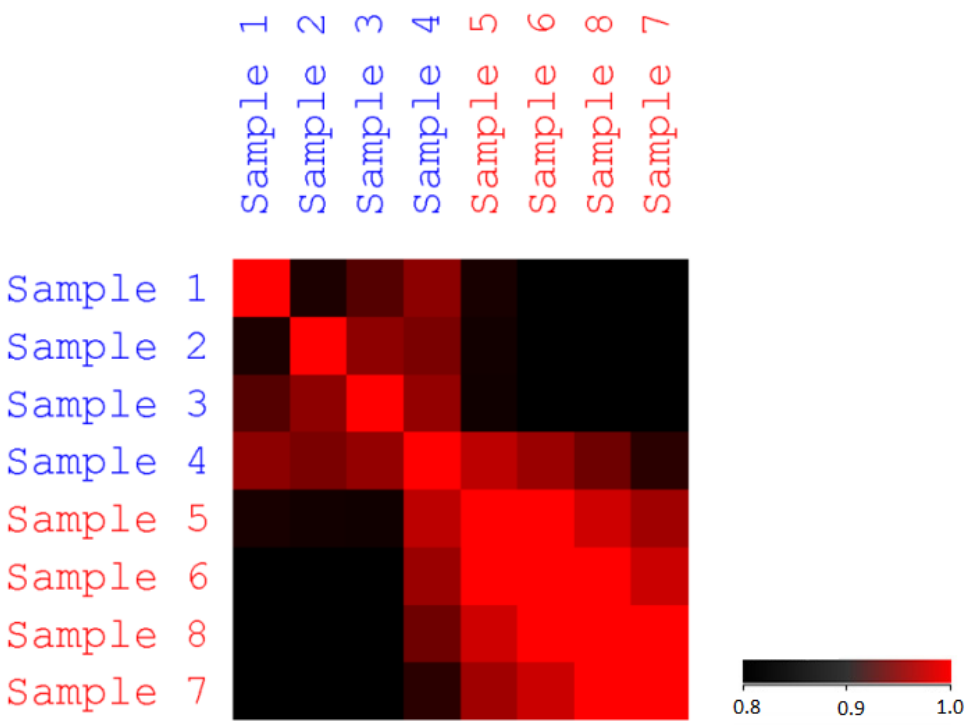


Figure 5-8 Correlation coefficient heatmap for second leaf arrays. Depicts the value of Spearman correlation coefficient for each pair of arrays in the dataset. Samples 1-4 are uninfested; samples 5-8 are infested. Key shows possible Z-scores ranging from black (normalized expression of 0.8) to red (normalized expression value of 1.0)

5.4.2.2 Data

A box plot of the normalised samples (Figure 5-9) shows that all samples have similar medians and interquartile ranges indicating that normalisation was successful. A profile plot of the normalised intensity values of each sample was generated (Figure 5-10). The profile plot shows the direction of regulation of those probes which have passed the filter conditions. Transcripts depicted in red were strongly upregulated in the infested condition,

blue were strongly downregulated by infestation, and yellow were moderately up- or down-regulated.

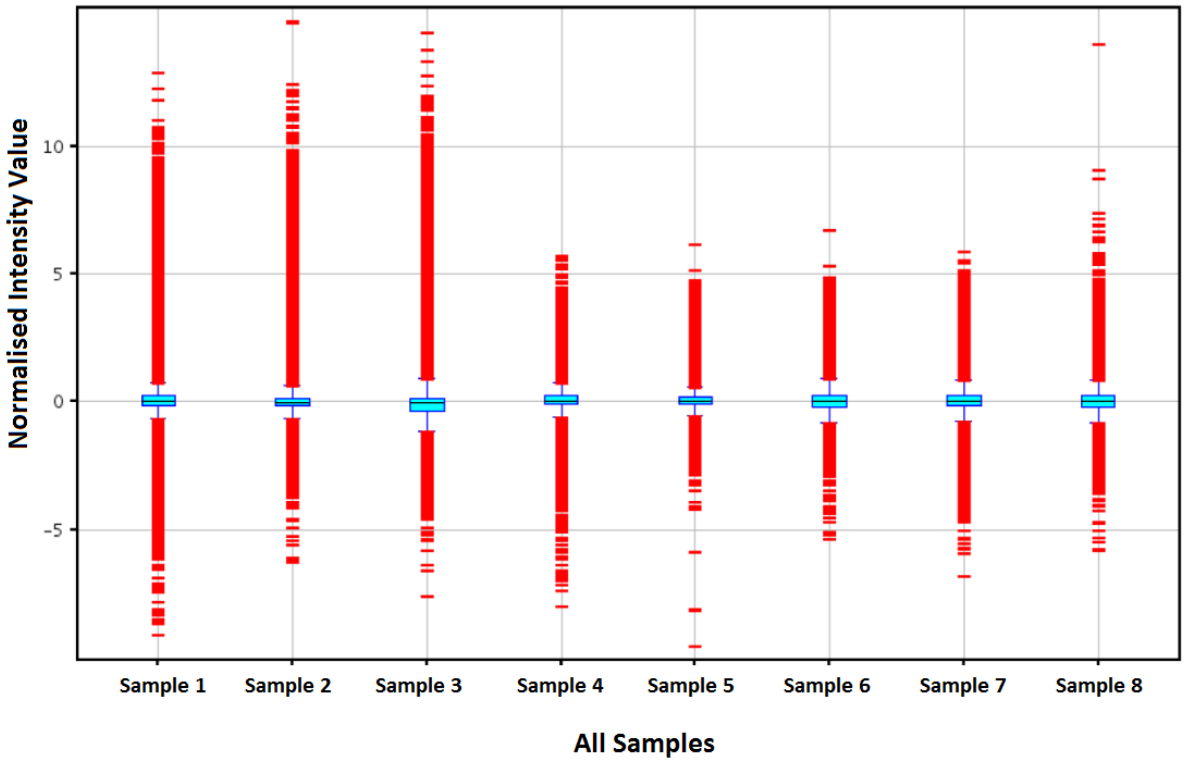


Figure 5-9 Normalised intensity box plot for each array. Shows the distribution of the data, the median value and the inter-quartile range for four uninfested sample arrays (Samples 1-4) and four aphid infested sample arrays (Samples 5-8), following quantile normalisation.

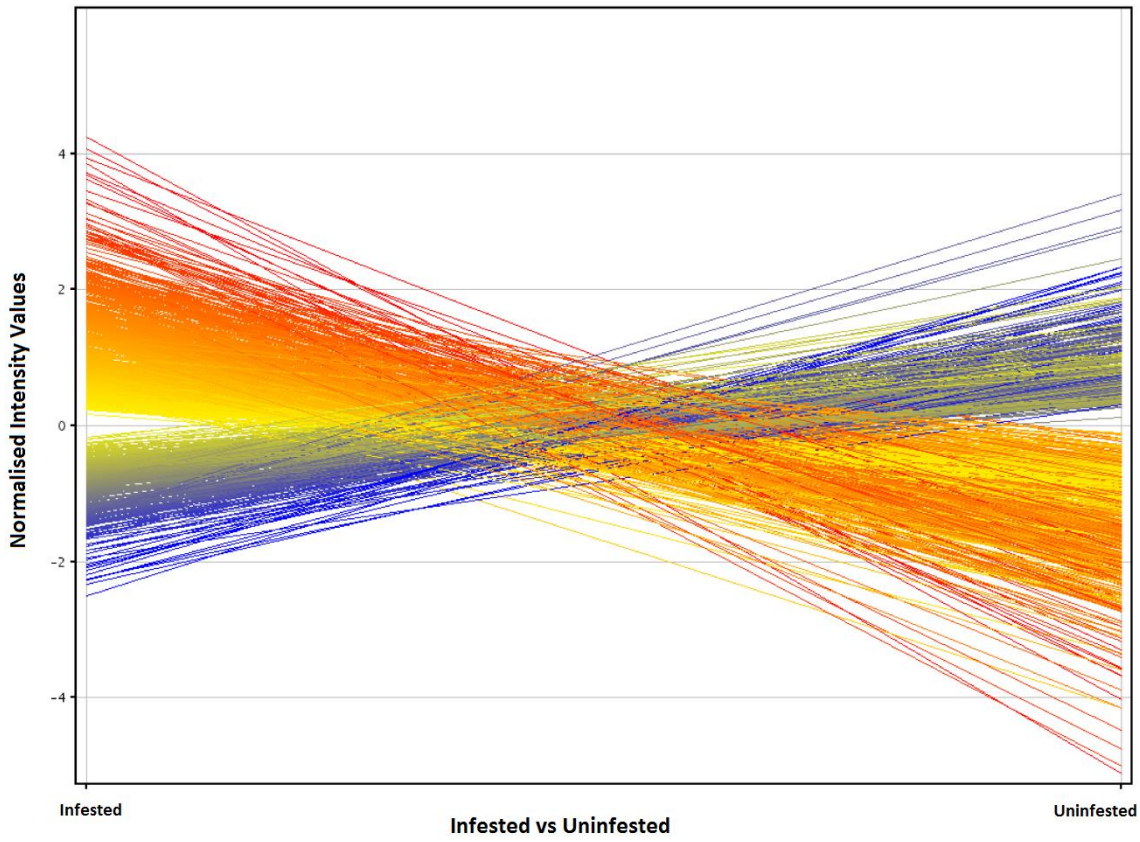


Figure 5-10 Profile plot of normalised intensity values for significant genes. For each probe, the relationship between expression in infested and uninfested plants is depicted. Red lines indicate upregulation in the infested plants, blue lines indicate probes which were downregulated by infestation. Yellow probes were only moderately up- or down-regulated.

1747 genes were differentially regulated ($p < 0.05$) more than 2-fold due to aphid infestation when the Benjamini Hochberg false discovery rate correction was applied. 1183 genes were upregulated ($p < 0.05$) with a FC greater than 2 and 564 genes were downregulated ($p < 0.05$) more than 2-fold. When the Bonferroni false discovery rate correction was used, 201 genes were found to be upregulated ($p < 0.05$) and 70 downregulated ($p < 0.05$) more than 2-fold.

5.4.2.3 Functional analysis of differentially expressed genes

Around 1% of the differentially regulated genes was thought to be of non-plant origin and was discounted (Figure 5-11). 1457 (962 upregulated and 495 downregulated) genes have been annotated as protein-coding sequence in previous studies, but limited information exists beyond this and the gene function is unknown.

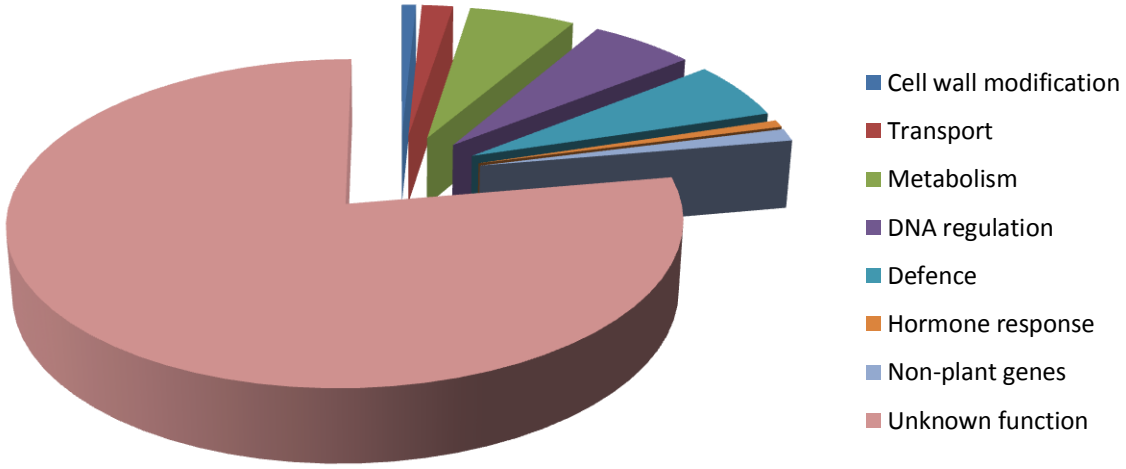


Figure 5-11 Functional grouping of the 1747 barley genes significantly altered systemically by *R. padi* infestation. Shows that the majority of differentially regulated transcripts are of unknown function, a small proportion are of non-plant origin and the rest fit into the categories cell wall metabolism, transport, metabolism, DNA regulation and defence.

Analysis showed that 61 of the upregulated genes are directly related to, or have previously been implicated in, plant defence responses. They include genes with roles in ROS metabolism, hormone responses, R-mediated defence, epicuticular wax biosynthesis, heat shock and Ca^{2+}/calmodulin signalling. 13 transcripts upregulated that code for cold- or

drought-induced proteins. Amongst the downregulated genes, 20 are implicated in defence responses, including several WRKY transcription factors (named for the most highly conserved amino acid sequence found within the family), a cytochrome p450, several heat shock proteins and an ethylene-responsive factor. Genes with roles in various metabolic activities accounted for 108 differentially expressed genes (90 up and 18 down), whilst 46 DNA regulatory genes showed altered expression (25 up and 21 down). 22 genes with transport/translocation roles were upregulated and 6 downregulated. Nine transcripts with a role in general hormone signalling were upregulated, and three downregulated. Finally, 15 genes related to cell wall modification were differentially expressed (14 upregulated and one downregulated).

Table 5-7 Defence-related genes differentially regulated systemically following 72 hours of *Rhopalosiphum padi* feeding on 13 day old Hordeum vulgare plants. Table shows Agilent probe names; role of the gene product in the plant, according to annotations or BLAST search; absolute fold change; and gene annotation. P < 0.05; Benjamini-Hochberg false discovery correction applied. A positive FC indicates that infestation causes upregulation; a negative FC indicates that infestation causes downregulation.

ProbeName	Role	FC (abs)	Annotation
A_13_P028446	Epicuticular wax	7	H. vulgare long chain fatty acid condensing enzyme CUT1;3
A_13_P067791	Epicuticular wax	9	H. vulgare long chain fatty acid condensing enzyme CUT1;1
A_13_P212754	Signalling	2	LRR transmembrane protein kinase 1 – Z. mays (Maize)
A_13_P569404	Signalling	6	LRR family protein /protein kinase family protein- like O. sativa
A_13_P186269	Signalling	3	LRR family protein, expressed - Oryza sativa
A_13_P094175	Signalling	4	Calmodulin-like protein - Oryza sativa
A_13_P500534	Signalling	2	Calcium-dependent protein kinase 2 - Triticum aestivum
A_13_P138630	Signalling	2	MLA10 - Hordeum vulgare (Barley), complete
A_13_P137185	Signalling	2	MAP KINASE - Avena sativa (Oat)
A_13_P549524	Signalling	4	Chitin elicitor-binding protein precursor – O. sativa
A_13_P318042	JA response	8	Hordeum vulgare mRNA for allene oxide cyclase (aoc gene)
A_13_P136120	JA response	2	Cytochrome P450 reductase - Triticum aestivum
A_13_P226219	JA response	3	Cytochrome P450 - Triticum aestivum (Wheat)
A_13_P128410	JA response	8	Jasmonate induced protein - Hordeum vulgare
A_13_P115355	SA response	2	Zinc finger protein-like - Oryza sativa
A_13_P037146	SA response	7	VQ motif family protein, expressed - Oryza sativa
A_13_P146220	ABA induced	14	Protein HVA22 - Hordeum vulgare (Barley)
A_13_P235924	ROS metabolism	2	Glutathione peroxidase-like protein GPX54Hv
A_13_P000231	ROS metabolism	2	Catalase isozyme 1 - Hordeum vulgare (Barley)
A_13_P592754	ROS metabolism	4	H. vulgare subsp. vulgare mRNA for peroxidase (prx7)
A_13_P483075	ROS metabolism	3	Class III peroxidase 62 precursor - Oryza sativa
A_13_P114715	ROS metabolism	5	Peroxidase precursor - Hordeum vulgare
A_13_P556809	ROS metabolism	10	Peroxidase family protein, expressed - Oryza sativa
A_13_P576864	ROS metabolism	11	Plasma membrane-bound peroxidase 1 - Zea mays

A_13_P358072	ROS metabolism	10	Metallothionein-like protein - Hordeum vulgare
A_13_P157355	PCD regulation	9	Putative subtilisin-like proteinase - Oryza sativa
A_13_P132695	PCD regulation	3	Autophagy-related protein 3 - Oryza sativa subsp.
A_13_P171025	PR protein	4	Thaumatin family protein, expressed - Oryza sativa
A_13_P203744	R defence	2	NBS-LRR class RGA - Aegilops tauschii (Tausch's goatgrass)
A_13_P065586	R defence	3	NB-ARC domain containing protein, expressed - Oryza sativa
A_13_P537164	R defence	3	H. vulgare NBS-LRR disease resistance protein (rga S-9217)
A_13_P013931	R defence	2	Non-TIR-NBS-LRR type resistance protein - S. officinarum
A_13_P171580	Heat shock	2	DnaK protein, expressed - Oryza sativa
A_13_P267027	Abiotic stress	49	Dehydrin - Triticum turgidum subsp. durum
A_13_P582609	Abiotic stress	18	Cold acclimation protein WCOR615 - Triticum aestivum
A_13_P429681	Abiotic stress	9	Cold acclimation protein WCOR413 - Triticum aestivum
A_13_P088786	Abiotic stress	11	H. vulgare clone BLTI-5 low temperature induced protein mRNA
A_13_P076016	Abiotic stress	15	H. vulgare subsp. vulgare cultivar Morex HvCBF7 (CBF7) mRNA
A_13_P137345	Abiotic stress	31	Low temperature-induced protein lt101.1 – H. vulgare
A_13_P117005	Abiotic stress	4	Paf93 protein - Hordeum vulgare (Barley)
A_13_P344907	Abiotic stress	4	Oleosin low molecular weight isoform-Linum usitatissimum
A_13_P583369	Abiotic stress	9	Late-embryogenesis abundant protein - Euphorbia esula
A_13_P149445	Abiotic stress	7	Dehydrin ERD4 protein - Arabidopsis thaliana
A_13_P149235	Abiotic stress	2	Early response to drought 3 - Pinus pinaster (Maritime pine)
A_13_P125685	Abiotic stress	3	Ramosa 3-like protein - Phalaris canariensis
A_13_P120000	Abiotic stress	5	Protein BLT4 precursor - Hordeum vulgare (Barley)
A_13_P232879	Abiotic stress	19	At1g23250.1 - Putative calsosin Arabidopsis thaliana
A_13_P500249	Abiotic stress	2	Hypoxia-responsive protein/zinc finger (C3HC4-type RING finger)
A_13_P564009	Abiotic stress	3	Hypoxia-responsive family protein-like - Oryza sativa
A_13_P225109	Universal stress	3	Universal stress protein family protein, expressed - Oryza sativa
A_13_P120585	Basal resistance	8	Germin-like protein 4c - Hordeum vulgare var. distichum
A_13_P463623	Deters feeding	10	Hordeum vulgare Agl2 mRNA for alpha-glucosidase
A_13_P025166	Phenylpropanoid biosynthesis	4	Dirigent-like protein, expressed - Oryza sativa

A_13_P025516	Phenylpropanoid biosynthesis	3	O-methyltransferase ZRP4 - Zea mays (Maize)
A_13_P486455	Phenylpropanoid biosynthesis	3	O-methyltransferase family protein, expressed – O. sativa
A_13_P127930	Phenylpropanoid biosynthesis	4	O-methyltransferase - Secale cereale (Rye)
A_13_P139755	Phenylpropanoid biosynthesis	2	Cinnamoyl CoA reductase - Hordeum vulgare (Barley)
A_13_P136025	Phenylpropanoid biosynthesis	3	Caffeoyl-CoA O-methyltransferase - Mesembryanthemum crystallinum
A_13_P287564	Detoxification	2	Glutathione-S-transferase 28e45 - Triticum aestivum
A_13_P126970	Detoxification	4	Glutathione S-transferase - Oryza sativa
A_13_P278514	Detoxification	3	Glutathione S-transferase - Arabidopsis thaliana
A_13_P091700	WRKY	-13	WRKY19-b transcription factor - Triticum aestivum (Wheat
A_13_P151415	WRKY	-8	WRKY transcription factor - Triticum aestivum (Wheat)
A_13_P440286	Calcium binding	-6	EF hand family protein, expressed - Oryza sativa
A_13_P136530	JA response	-2	P450 - Lolium rigidum (Annual ryegrass)
A_13_P024536	ET response	-5	Ethylene-responsive factor - Thinopyrum intermedium
A_13_P145165	SA response	-4	Zinc finger protein family-like - Oryza sativa
A_13_P165035	SA response	-4	Zinc finger protein 1 - Triticum aestivum (Wheat)
A_13_P166570	SA response	-2	Zinc finger POZ domain protein-like - Oryza sativa
A_13_P579354	Signalling	-6	Leucine Rich Repeat family protein, expressed - Oryza sativa
A_13_P029371	Signalling	-2	H. vulgare NBS-LRR disease resistance protein (rga S-9201)
A_13_P099965	R defence	-4	NB-ARC domain containing protein, expressed - Oryza sativa
A_13_P235539	Heat shock	-2	DnaJ protein - Oryza sativa subsp. indica (Rice)
A_13_P567774	Heat shock	-3	Heat-shock protein precursor - Secale cereale (Rye)
A_13_P170665	Heat shock	-2	Heat stress transcription factor A-4a - Arabidopsis thaliana
A_13_P198754	Heat shock	-4	Heat shock protein 70 - Arabidopsis thaliana
A_13_P257837	Heat shock	-2	Hordeum vulgare cytosolic heat shock protein 90 (HSP90) mRNA
A_13_P171765	Stress response	-3	GroEL protein; chaperonin, 60 kDa - Arabidopsis thaliana
A_13_P119830	Stress response	-2	Stress responsive protein - Triticum aestivum (Wheat)
A_13_P547204	Detoxification	-4	Heavy metal-associated domain containing protein, Oryza sativa
A_13_P207034	Abiotic stress	-6	Dehydrin ERD14 - Arabidopsis thaliana (Mouse-ear cress)

Table 5-8 Genes with roles in metabolism that are differentially regulated systemically following 72 hours of Rhopalosiphum padi feeding on 13 day old Hordeum vulgare plants. Table shows Agilent probe names; role of the gene product in the plant, according to annotations or BLAST search; absolute fold change; and gene annotation. P < 0.05; Benjamini-Hochberg false discovery correction applied. A positive FC indicates that infestation causes upregulation; a negative FC indicates that infestation causes downregulation.

ProbeName	FC(abs)	Description
A_13_P199094	4	Ubiquitin-like - Oryza sativa
A_13_P461413	5	Ubiquitin carrier protein - Oryza sativa
A_13_P130805	2	Ubiquitin carrier protein - Arabidopsis thaliana
A_13_P118410	2	Ubiquitin - Arabidopsis thaliana
A_13_P121370	3	SKP1/ASK1-like protein - Triticum aestivum
A_13_P153210	4	Wpk4 protein kinase - Triticum aestivum
A_13_P203639	22	Serine/threonine-protein kinase SAPK4 - Oryza sativa
A_13_P156365	6	Serine carboxypeptidase family protein, expressed - Oryza sativa
A_13_P136585	3	Sec61beta family protein, expressed - Oryza sativa
A_13_P104035	3	RING zinc finger protein - Chlamydomonas reinhardtii
A_13_P168605	3	Receptor-like protein kinase-like protein - Oryza sativa
A_13_P559909	5	Receptor-like kinase - Sorghum bicolor (Sorghum)
A_13_P037891	3	Protein phosphatase-like - Oryza sativa subsp. japonica (Rice)
A_13_P134265	2	ESTs AU065232 - Oryza sativa subsp. japonica (Rice)
A_13_P557159	8	Elongation factor - Triticum aestivum (Wheat)
A_13_P203964	2	At1g79720/F19K16_30 - Arabidopsis thaliana
A_13_P236249	3	H. vulgare subsp. vulgare mRNA for papain-like cysteine proteinase (pap-7)
A_13_P132960	27	H. vulgare subsp. vulgare mRNA papain-like cysteine proteinase (pap-22)
A_13_P077351	121	Hordeum vulgare subsp. vulgare mRNA for farnesylated protein 1
A_13_P120715	2	Peptidyl-prolyl cis-trans isomerase - Triticum aestivum (Wheat)
A_13_P139245	3	Kelch repeat-containing F-box-like - Oryza sativa
A_13_P484620	2	Sucrose:sucrose 1-fructosytransferase - Triticum aestivum (Wheat)

A_13_P112910	13	Reversibly glycosylated polypeptide - Triticum aestivum (Wheat)
A_13_P585839	6	Predicted glycosyl transferase - Arabidopsis thaliana
A_13_P135430	3	High pI alpha-glucosidase - Hordeum vulgare (Barley)
A_13_P510964	6	Glycosyltransferase - Triticum aestivum (Wheat)
A_13_P466613	3	Fructokinase-1 - Zea mays (Maize), partial (30%) [TC247468]
A_13_P393050	8	H. vulgare partial mRNA for beta3-glucuronyltransferase (pglcat4 gene)
A_13_P075526	3	Hordeum vulgare alpha-glucosidase (AGL1) mRNA
A_13_P507694	2	Fructose-bisphosphate aldolase - Oryza sativa subsp. indica (Rice)
A_13_P272709	3	Phosphoglycerate kinase, chloroplast precursor - Triticum aestivum
A_13_P140540	13	Phosphoenolpyruvate carboxykinase - Zoysia japonica
A_13_P426021	5	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic – H. vulgare
A_13_P145370	3	Aldose reductase - Zea mays (Maize)
A_13_P428116	3	Malate dehydrogenase - Triticum aestivum (Wheat)
A_13_P473978	2	Cytochrome c oxidase subunit Vb - Oryza sativa
A_13_P271329	4	Oxygen-evolving enhancer protein 2, chloroplast precursor – T. aestivum
A_13_P121000	2	ATP/ADP carrier protein - Triticum turgidum (Poulard wheat)
A_13_P514559	3	ATP synthetase alpha chain-like - Oryza sativa subsp. japonica (Rice)
A_13_P242605	3	Photosystem II reaction centre psb28 protein - Oryza sativa
A_13_P117660	2	Photosystem I reaction centre subunit VI, chloroplast precursor – barley
A_13_P235694	4	Indole-3-glycerol phosphate synthase, chloroplast precursor - Arabidopsis
A_13_P212564	3	Glutamate dehydrogenase 2 - Oryza sativa subsp. japonica (Rice)
A_13_P141610	4	Chloroplast ornithine carbamoyltransferase - Oryza sativa
A_13_P483015	2	Betaine aldehyde dehydrogenase - Hordeum brevisubulatum
A_13_P128330	2	Aspartate aminotransferase - Oryza sativa subsp. japonica (Rice)
A_13_P155355	2	Asparaginase - Hordeum vulgare (Barley)
A_13_P113735	6	Epoxide hydrolase - Ananas comosus (Pineapple)
A_13_P166330	2	Enoyl-CoA hydratase - Arabidopsis thaliana (Mouse-ear cress)
A_13_P128030	3	Acyl-CoA oxidase - Glycine max (Soybean)
A_13_P010331	12	Hordeum vulgare putative aldehyde decarbonylase enzyme CER1;1 mRNA

A_13_P113775	6	Ferritin - Triticum aestivum (Wheat)
A_13_P047206	16	Hordeum vulgare subsp. vulgare GA 2-oxidase 4 (GA2ox4) mRNA
A_13_P125120	8	Selenium binding protein - Oryza sativa subsp. indica (Rice)
A_13_P134925	2	Soluble inorganic pyrophosphatase - Hordeum vulgare var. distichum
A_13_P144705	4	Similarity to amine oxidase - Arabidopsis thaliana (Mouse-ear cress)
A_13_P128610	3	Ripening-related protein-like - Oryza sativa subsp. japonica (Rice)
A_13_P233514	22	Oxidase-like protein - Arabidopsis thaliana (Mouse-ear cress)
A_13_P028871	2	Hydrolase, alpha/beta fold protein-like - Oryza sativa
A_13_P558499	2	Growth-regulating factor 3 - Oryza sativa subsp. japonica (Rice)
A_13_P555954	9	Glycine rich protein - Cenchrus ciliaris (Buffelgrass)
A_13_P234299	3	Glucosyltransferase like protein - Arabidopsis thaliana
A_13_P411685	9	Flavin containing polyamine oxidase precursor - Hordeum vulgare
A_13_P152785	19	Epsin-1 - Schizosaccharomyces pombe (Fission yeast)
A_13_P434826	7	Embryonic abundant protein-like - Oryza sativa
A_13_P468563	2	Dihydrolipoyl dehydrogenase - Oryza sativa
A_13_P117280	2	Dihydrolipoamide S-acetyltransferase - Zea mays (Maize)
A_13_P565084	2	Cytidylyltransferase family protein, expressed - Oryza sativa
A_13_P550529	60	Chalcone-flavanone isomerase family protein, expressed – O. sativa
A_13_P076536	2	Biogenesis protein - Hordeum vulgare var. distichum
A_13_P151440	3	AT5g64130/MHJ24_11 - Arabidopsis thaliana (Mouse-ear cress)
A_13_P186259	2	At5g49350 - Arabidopsis thaliana (Mouse-ear cress)
A_13_P125355	3	At4g31290 - Arabidopsis thaliana (Mouse-ear cress)
A_13_P164215	3	At1g14020 - Arabidopsis thaliana (Mouse-ear cress)
A_13_P555549	22	Arabinogalactan peptide 16 precursor – A. thaliana (Mouse-ear cress)
A_13_P149015	13	Anthranilate N-benzoyltransferase-like protein - Arabidopsis thaliana
A_13_P124635	12	Annexin p35 - Zea mays (Maize)
A_13_P549614	3	Aldehyde dehydrogenase - Saccharum officinarum (Sugarcane)
A_13_P212529	7	Adenosine 5'-phosphosulfate reductase 1 - Zea mays (Maize)
A_13_P181154	5	AAA ATPase, central region (50.1 kD)-like protein - Oryza sativa

A_13_P212014	4	3-methyl-2-oxobutanoate hydroxy-methyl-transferase - Arabidopsis
A_13_P096400	9	Hordeum vulgare partial mRNA for putative acid phosphatase (bci-3 gene)
A_13_P029011	3	Hordeum vulgare partial CA9 gene for P-type ATPase
A_13_P526319	2	Hordeum vulgare partial CA8 gene for P-type ATPase
A_13_P503454	28	Hordeum vulgare leucoanthocyanidin dioxygenase-like protein mRNA
A_13_P000371	5	Hordeum vulgare subsp. vulgare mRNA for predicted protein, CA1 gene
A_13_P069671	2	Hordeum vulgare subsp. vulgare General regulatory factor (GRF1)
A_13_P258797	7	Hordeum vulgare subsp. vulgare Plasma membrane protein (PM19)
A_13_P134940	3	Uncharacterized protein At5g53050.2 - Arabidopsis thaliana
A_13_P216754	3	Translationally-controlled tumor protein homolog - Hordeum vulgare
A_13_P199154	-3	Xyppx repeat family - Stigmatella aurantiaca DW4/3-1
A_13_P251237	-2	Triacylglycerol Lipase - Triticum aestivum (Wheat)
A_13_P140235	-3	Thioredoxin domain 2; Thioredoxin fold - Medicago truncatula (Barrel medic)
A_13_P165460	-3	Streptomyces cyclase/dehydrase family protein, expressed - Oryza sativa
A_13_P248093	-3	Serine/threonine kinase - Sorghum bicolor (Sorghum) (Sorghum vulgare)
A_13_P583034	-7	Selenium-binding protein-like - Oryza sativa subsp. japonica (Rice)
A_13_P567594	-4	No apical meristem (NAM)-like protein - Solanum tuberosum (Potato)
A_13_P147035	-2	Cyclin-T1-4 - Oryza sativa subsp. japonica (Rice)
A_13_P329217	-2	Cyclin-T1-3 - Oryza sativa subsp. japonica (Rice)
A_13_P566364	-2	Ankyrin repeat family protein / methyltransferase-related - Musa acuminata (Banana)
A_13_P355872	-3	Hordeum vulgare subsp. vulgare mRNA for predicted protein, AS2
A_13_P546099	-7	Hordeum vulgare subsp. vulgare germin-like protein 1a (GER1a) mRNA
A_13_P199689	-2	Nuclear pore protein 84/107 containing protein, expressed - Oryza sativa
A_13_P234334	-22	Hordeum vulgare subsp. vulgare mRNA for legumain (leg-5 gene)
A_13_P131270	-9	Cysteine proteinase precursor - Hordeum vulgare (Barley)
A_13_P000116	-2	Signal recognition particle 54 kDa protein 3 - Hordeum vulgare (Barley)
A_13_P170075	-9	F-box protein family, AtFBX13-like protein - Oryza sativa
A_13_P555159	-8	Alpha-amylase /trypsin inhibitor - Oryza sativa

Table 5-9 Genes with roles in DNA regulation that are differentially regulated systemically following 72 hours of Rhopalosiphum padi feeding on 13 day old Hordeum vulgare plants. Table shows Agilent probe names; role of the gene product in the plant, according to annotations or BLAST search; absolute fold change; and gene annotation. P < 0.05; Benjamini-Hochberg false discovery correction applied. A positive FC indicates that infestation causes upregulation; a negative FC indicates that infestation causes downregulation.

ProbeName	FC (Abs)	Annotation
A_13_P129835	3	ZIM motif family protein, expressed - Oryza sativa
A_13_P152705	3	Myb-related protein MYBAS2 - Oryza sativa
A_13_P156985	3	Myb-related protein - Triticum aestivum (Wheat)
A_13_P445968	2	Basic helix-loop-helix protein-like - Oryza sativa
A_13_P044541	29	Hordeum vulgare subsp. vulgare mRNA for predicted protein, KN6
A_13_P548469	2	BHLH protein family-like - Oryza sativa subsp. japonica (Rice)
A_13_P135775	3	HMG-I/Y protein HMGa - Triticum aestivum (Wheat)
A_13_P025451	6	Hordeum vulgare HvTFL1 mRNA for homologous protein to TFL1
A_13_P226664	5	Von Willebrand factor type A domain containing protein- Oryza sativa
A_13_P224719	5	CONSTANS-like protein CO8 - Hordeum vulgare var. distichum
A_13_P029031	2	CONSTANS-like protein CO7 - Hordeum vulgare var. distichum
A_13_P179884	5	Similarity to Myb-related transcription factor - Arabidopsis thaliana
A_13_P448793	17	Ribonuclease - Hordeum vulgare (Barley)
A_13_P118035	19	Protein H2A.6 - Triticum aestivum (Wheat)
A_13_P075481	7	MIKC-type MADS-box transcription factor WM29B - Triticum aestivum
A_13_P563289	3	BLE1 protein - Oryza sativa (Rice)
A_13_P582269	4	60S ribosomal protein L18A-like - Oryza sativa
A_13_P212139	4	High mobility group box protein 2 - Oryza sativa
A_13_P117440	9	Histone H3 - Oryza sativa subsp. japonica (Rice)
A_13_P442678	5	Histone H2B.5 - Triticum aestivum (Wheat)
A_13_P459393	16	Histone H2B.4 - Triticum aestivum (Wheat)
A_13_P116405	2	Histone H2B.2 - Zea mays (Maize)
A_13_P119730	56	Histone H2A.2.1 - Triticum aestivum (Wheat)

A_13_P509454	11	Histone H2A.1 - Triticum aestivum (Wheat)
A_13_P133220	15	Probable histone H2AXb - Oryza sativa subsp. indica (Rice)
A_13_P450638	-3	SMC5 protein - Oryza sativa (Rice)
A_13_P132765	-5	Root abundant factor - Hordeum vulgare (Barley)
A_13_P517134	-5	Hordeum vulgare root abundant factor (RAF) mRNA
A_13_P209789	-2	RNA recognition motif (RRM)-containing protein-like - Oryza sativa
A_13_P220159	-2	Nucleosome/chromatin assembly factor A - Zea mays (Maize)
A_13_P132870	-2	Nucleosome assembly protein 1-like protein 1 - Oryza sativa (Rice)
A_13_P127185	-2	Nucleolin - Arabidopsis thaliana (Mouse-ear cress)
A_13_P225044	-3	Mitochondrial ribosomal protein L11 - Triticum aestivum (Wheat)
A_13_P180249	-4	MIKC-type MADS-box transcription factor WM27B - Triticum aestivum
A_13_P234499	-2	H/ACA ribonucleoprotein complex subunit 2 - Oryza sativa
A_13_P562064	-5	DNA-directed RNA polymerase subunit beta - Hordeum vulgare (Barley)
A_13_P473883	-3	DNA-directed RNA polymerase - Hordeum vulgare (Barley)
A_13_P413845	-2	Ribosomal protein L18a-like - Oryza sativa subsp. japonica (Rice)
A_13_P398496	-3	Chloroplast 50S ribosomal protein L16 - Hordeum vulgare (Barley)
A_13_P397560	-2	Chloroplast 30S ribosomal protein S8 - Hordeum vulgare (Barley)
A_13_P246830	-4	Chloroplast 30S ribosomal protein S12 B – P. trichocarpa (Western poplar)
A_13_P226889	-2	60S ribosomal protein L37a - Oryza sativa subsp. japonica (Rice)
A_13_P574299	-4	60S ribosomal protein L36 - Triticum aestivum (Wheat)
A_13_P275814	-2	60S ribosomal protein L30 - Zea mays (Maize)
A_13_P093320	-2	40S ribosomal protein S24 - Oryza sativa subsp. japonica (Rice)
A_13_P012111	-2	H. vulgare DNA-directed RNA polymerase, chloroplast precursor (rpoT)

Table 5-10 Genes with roles in transport that are differentially regulated systemically following 72 hours of *Rhopalosiphum padi* feeding on 13 day old *Hordeum vulgare* plants. Table shows Agilent probe names; role of the gene product in the plant, according to annotations or BLAST search; absolute fold change; and gene annotation. P < 0.05; Benjamini-Hochberg false discovery correction applied. A positive FC indicates that infestation causes upregulation; a negative FC indicates that infestation causes downregulation.

ProbeName	FC (abs)	Description
A_13_P293457	2	Transporter-related-like - Oryza sativa subsp. japonica (Rice)
A_13_P150685	3	P-type ATPase - Hordeum vulgare (Barley)
A_13_P141220	3	Pleiotropic drug resistance protein 4 - Oryza sativa
A_13_P538434	3	Phosphatidylinositol 3,5-kinase-like - Oryza sativa
A_13_P349247	3	Non-specific lipid-transfer protein - Oryza sativa
A_13_P120515	76	Nonspecific lipid transfer protein - Hordeum vulgare (Barley)
A_13_P168075	4	Microtubule-associated protein-like - Oryza sativa
A_13_P396050	4	Major Facilitator Superfamily protein, expressed - Oryza sativa
A_13_P559074	2	Integral membrane protein-like - Oryza sativa
A_13_P116970	4	Hydrophobic protein OSR8 - Oryza sativa
A_13_P421490	4	Hydrophobic protein LTI6B - Oryza sativa
A_13_P174435	36	Cortical cell-delineating protein - Oryza sativa
A_13_P320487	3	Hordeum vulgare subsp. vulgare mRNA for predicted protein, CAX
A_13_P156055	3	Cation/H+ exchanger - Hordeum vulgare (Barley)
A_13_P447038	12	Cation diffusion facilitator 9 - Stylosanthes hamata (Caribbean stylo)
A_13_P543777	4	Potassium transporter 10 - Oryza sativa subsp. japonica (Rice)
A_13_P439461	3	Protein transport protein SEC61 subunit gamma - Oryza sativa
A_13_P120270	19	Amino acid selective channel protein - Hordeum vulgare (Barley)
A_13_P402980	5	Hordeum vulgare boron transporter (Bot1) mRNA
A_13_P166400	13	Boron transporter 2 - Triticum aestivum (Wheat)
A_13_P484825	2	Alpha tubulin-5A - Triticum aestivum (Wheat)
A_13_P561749	3	C3H2C3 RING-finger protein-like - Oryza sativa
A_13_P574759	-3	Na(+) dependent transporter-like - Oryza sativa

A_13_P173895	-7	At5g50300 - Oryza sativa subsp. japonica (Rice)
A_13_P224714	-2	Ammonium transporter - Triticum aestivum (Wheat)
A_13_P189089	-2	Amino acid ABC transporter protein, permease protein - Rhizobium loti
A_13_P407846	-25	Alanine aminotransferase 2 - Hordeum vulgare (Barley)
A_13_P000146	-2	Hordeum vulgare HvPIP2;1 mRNA for PIP aquaporin

Table 5-11 Genes with a role in general hormonal responses that are differentially regulated systemically following 72 hours of *Rhopalosiphum padi* feeding on 13 day old Hordeum vulgare plants. Table shows Agilent probe names; role of the gene product in the plant, according to annotations or BLAST search; absolute fold change; and gene annotation. P < 0.05; Benjamini-Hochberg false discovery correction applied. A positive FC indicates that infestation causes upregulation; a negative FC indicates that infestation causes downregulation.

ProbeName	FC (abs)	Description
A_13_P190909	3	Probable gibberellin receptor GID1L1 - Arabidopsis thaliana
A_13_P133360	4	FERONIA receptor-like kinase - Poncirus trifoliata (Hardy orange)
A_13_P520979	3	FERONIA receptor-like kinase - Arabidopsis lyrata (Lyre-leaved cress)
A_13_P090361	2	Auxin responsive factor 10 - Oryza sativa subsp. indica (Rice)
A_13_P206744	2	Auxin response factor 6 - Oryza sativa subsp. indica (Rice)
A_13_P149915	3	Auxin response factor 17 - Oryza sativa subsp. indica (Rice)
A_13_P089271	15	ACC synthase - Triticum aestivum (Wheat)
A_13_P415440	7	ABA-responsive protein-like - Oryza sativa subsp. japonica (Rice)
A_13_P000481	6	ABA-responsive protein - Hordeum vulgare (Barley)
A_13_P183884	-2	Probable auxin efflux carrier component 1c - Oryza sativa
A_13_P176495	-4	Auxin-responsive protein-like - Oryza sativa subsp. japonica (Rice)
A_13_P297012	-8	9-cis-epoxycarotenoid dioxygenase 2 - Oryza sativa subsp. japonica

Table 5-12 Genes with roles in cell wall modification that are differentially regulated systemically following 72 hours of Rhopalosiphum padi feeding on 13 day old Hordeum vulgare plants. Table shows Agilent probe names; role of the gene product in the plant, according to annotations or BLAST search; absolute fold change; and gene annotation. P < 0.05; Benjamini-Hochberg false discovery correction applied. A positive FC indicates that infestation causes upregulation; a negative FC indicates that infestation causes downregulation.

ProbeName	FC (Abs)	Annotation
A_13_P180304	5	Polygalacturonase PG1-like - Oryza sativa
A_13_P515404	2	Hordeum vulgare subsp. vulgare mRNA for predicted protein, UGE2
A_13_P083386	2	Hordeum vulgare subsp. vulgare mRNA for predicted protein, UXE2
A_13_P518134	3	Hordeum vulgare UDP-D-glucuronate decarboxylase (UXS2)
A_13_P077911	22	Xyloglucan endotransglycosylase - Hordeum vulgare (Barley)
A_13_P396415	7	Hordeum vulgare beta-D-xylosidase mRNA
A_13_P499494	2	4-coumarate--CoA ligase 4CL3 - Lolium perenne
A_13_P133180	5	4-coumarate--CoA ligase 4CL2 - Lolium perenne
A_13_P230504	13	4-coumarate--CoA ligase 4CL1 - Lolium perenne
A_13_P162805	4	Latex cyanogenic beta glucosidase - Hevea brasiliensis
A_13_P567834	5	Hordeum vulgare cellulose synthase-like CslF10 (CslF10)
A_13_P463923	3	Cellulose synthase - Oryza sativa subsp. indica (Rice)
A_13_P011191	3	Hordeum vulgare putative cellulose synthase catalytic subunit (CesA6)
A_13_P563894	2	CSLC7 - Oryza sativa (Rice)
A_13_P570999	-4	Alpha-expansin EXPA2 - Triticum aestivum (Wheat)

5.5 Local vs systemic responses

More genes were differentially regulated systemically than locally (1747 and 982, respectively). The proportion of genes up- and down-regulated in each sample type was similar, however (Figure 5-12). Upregulated genes comprised 70% of the local response and 68% of the systemic response. Downregulated genes comprised 30% and 32% of the local and systemic responses, respectively.

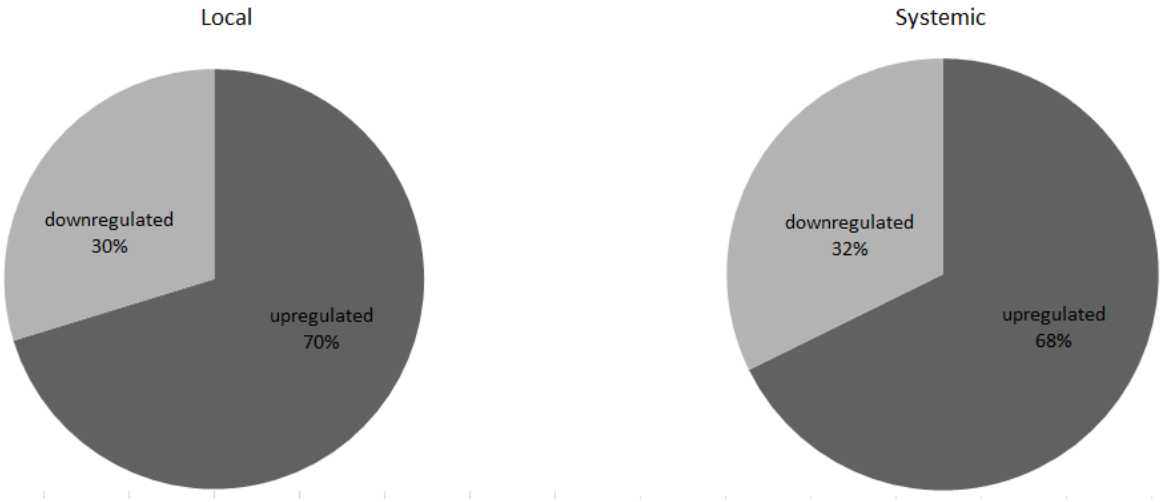


Figure 5-12 Percentage of genes upregulated and downregulated in local (infested first leaf) and systemic (uninfested second leaf of infested plant) barley tissues following 72 hours *R. padi* infestation.

A Venn diagram of the annotated transcripts that showed altered expression in local or systemic tissues shows very little overlap between the responses (Figure 5-13). Only 19 genes were altered in both conditions. Of these, four were differentially expressed in the different leaves (i.e. upregulated locally and downregulated systemically, or vice versa)(Table 5-13). One transcript was downregulated both locally and systemically and the

rest were upregulated in both conditions. The majority of the overlapping genes are defence- (8 transcripts) or metabolism- (6 transcripts) related (Table 5-12).

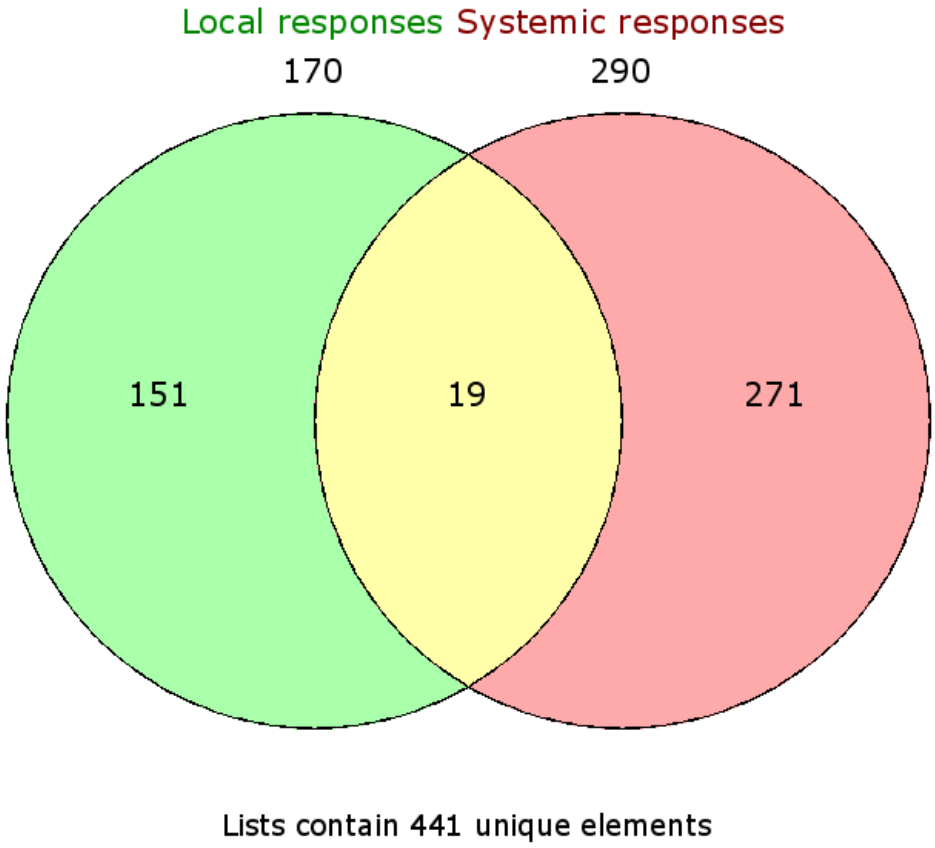


Figure 5-13 Venn diagram showing the numbers of barley genes with known functions that were differentially regulated locally (151), systemically (271) or both (19) by *R. padi* infestation. Created at http://www.bioinformatics.lu/venn.php

Table 5-13 Transcripts differentially expressed in both local and systemic barley tissues following 72 hours *R. padi* infestation. Transcripts for which the directions of regulation differ (i.e. upregulated in one sample and downregulated in the other) between local and systemic tissues are highlighted in red. Transcripts which were upregulated in both local and systemic tissues are in black and both downregulated are in blue.

Probe name	Functional category	Role	Annotation
A_13_P067791	Defence	Epicuticular wax	Long chain fatty acid condensing enzyme CUT1;1
A_13_P318042	Defence	JA response	Allene oxide cyclase (aoc gene)
A_13_P128410	Defence	JA response	Jasmonate induced protein - Hordeum vulgare
A_13_P157355	Defence	PCD regulation	Putative subtilisin-like proteinase - Oryza sativa
A_13_P171025	Defence	PR protein	Thaumatin family protein, expressed
A_13_P207034	Defence	PR protein	Dehydrin ERD14 - Arabidopsis thaliana
A_13_P483075	Defence	PR protein	Class III peroxidase 62 precursor - Oryza sativa
A_13_P567774	Defence	HS protein	Heat-shock protein precursor - Secale cereale (Rye)
A_13_P083386	Cell wall		Hordeum vulgare UDP-D-xylose epimerase 2
A_13_P230504	Cell wall		4-coumarate--CoA ligase 4CL1 - Lolium perenne
A_13_P499494	Cell wall		4-coumarate--CoA ligase 4CL3 - Lolium perenne
A_13_P212564	Metabolism		Glutamate dehydrogenase 2 - Oryza sativa
A_13_P112910	Metabolism		Reversibly glycosylated polypeptide
A_13_P120715	Metabolism		Peptidyl-prolyl cis-trans isomerase
A_13_P415440	Metabolism		ABA-responsive protein-like
A_13_P550529	Metabolism		Chalcone-flavanone isomerase family protein
A_13_P526319	Metabolism		Partial CA8 gene for P-type ATPase
A_13_P445968	Dna regulation		Basic helix-loop-helix protein-like
A_13_P349247	Transport		Non-specific lipid-transfer protein

5.6 Discussion

5.6.1 Defence

Genes from several functional groups were differentially regulated in aphid-infested tissues. Naturally, one of the most interesting groups is defence-related genes. The responses are spatially separated, with some responses affecting the epidermis, some the pathway cells and cell wall and some, systemic responses, presumably in the vascular tissues.

5.6.1.1 Aphid-host physical interaction

Two transcripts encoding a long chain fatty acid (A_13_P597329) and a long chain fatty acid condensing enzyme (A_13_P067791) were downregulated locally to the site of infestation (Table 5-2). The systemic response, however, included upregulation of two long chain fatty acid condensing enzymes (A_13_P028446 and A_13_P067791; Table 5-7). These enzymes are required for cuticular wax biosynthesis in *Arabidopsis* (Millar *et al*., 1999). When aphids initially encounter a potential host, they are exposed to the chemical and physical properties of the epidermis, and any waxy layers coating it, first. It is unsurprising, therefore, that

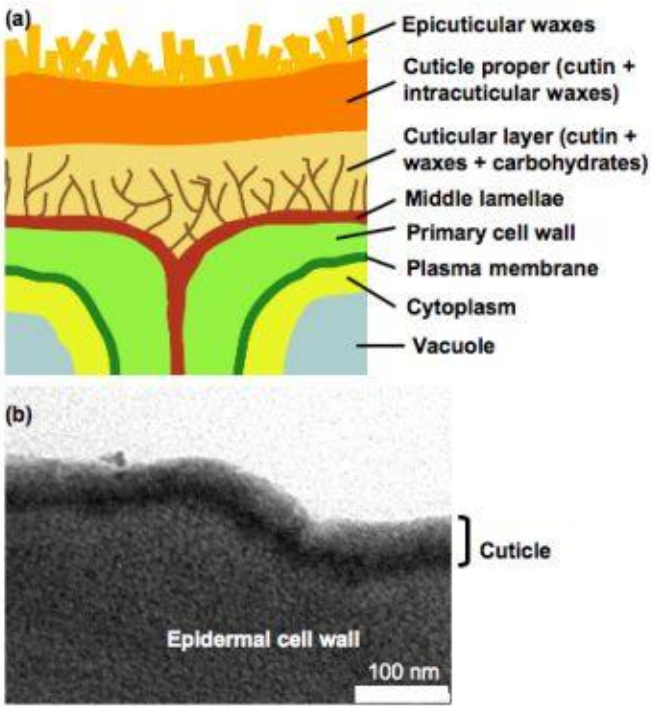


Figure 5-14 Structure of the plant cuticle. a) Diagrammatic representation of outer leaf layers, including epicutic ular wax layer, cuticular layer, middle lamella, cell wall, plasma membrane, cytoplasm and vacuole. b) TEM image of epidermal cell wall and cuticle. Image from ACOS lipid library: http://lipidlibrary.aocs.org/index.html

the outermost wax layer is thought to play an important role in providing chemical cues involved in the primary step of the plant-insect interaction, affecting oviposition, movement and feeding (Eigenbrode and Espelie, 1995, Powell *et al*., 1999) and leading to host acceptance or rejection (Muller and Riederer, 2005). The amount of cuticular wax has been found to have a negative correlation with the level of *R. padi* infestation on barley plants (Tsumuki *et al*., 1989). Scanning electron micrographs of the surface of *S. halepense* leaves showed less epicuticular wax in *S. flava* infested leaves compared to non-infested leaves (Gonzales *et al*., 2002). If aphids were able to downregulate this gene then it could confer an advantage. The observation that these enzymes are downregulated in local tissues but upregulated in systemic tissues suggests that aphids are able to alter the host plant to their advantage at the site of infestation, but elsewhere in the plant a defence response (i.e. greater production of epicuticular wax) is occurring. The results of EPG experiments in this study (see Chapter 3) looking at the effect of pre-infestation on the feeding behaviour of subsequent aphid populations showed that pre-infestation did not alter the probing behaviours of aphids and the time taken to locate the phloem was not significantly different. It appears likely from these data that changes in epicuticular wax on infested plants does not affect the feeding success of aphids but may still hinder performance by restricting movement or larvipositioning on the leaf surface. These are areas for further investigation using observation and r_m experiments. It would also be useful to investigate the effects of epicuticular wax on aphid feeding more closely by comparing EPGs on uninfested plants and aphid infested plants with those on plants with the wax removed using ethyl ether (Ni *et al*., 1998). This would remove other variables associated with aphid feeding, e.g. upregulation of chemical defence responses, to allow assessment of the effects of leaf surface wax only. The results of this experiment would indicate what proportion of the response to pre-infestation

is due to the downregulation of transcripts encoding epicuticular waxes. Another important avenue for investigation, in light of these results, would be to record aphid feeding behaviour on the uninfested second leaves of pre-infested plants. This would give an indication as to the role of upregulated wax production on aphid feeding performance.

5.6.1.2 Hormone responses

Several phytohormone signalling pathways appeared to be active in infested plants. The pathway involving jasmonic acid (JA) was triggered both locally and systemically (Table 5-13). Transcripts encoding an allene oxide cyclase (AOC; A_13_P318042) and an allene oxide synthase (AOS; A_13_P480235) were upregulated in infested (first leaf) tissues. Both gene products catalyse steps in octadecanoid pathway of the biosynthesis of jasmonic acid (Figure 5-15)(Wasternack, 2007). A jasmonate-induced protein (A_13_P128410) was also upregulated, as well as a cytochrome p450 (A_13_P044211). Cytochrome p450s are involved in jasmonate signalling (AOS is a cytochrome p450 enzyme)(Devoto and Turner, 2003). Systemically, four JA-related genes were upregulated: an allene oxide cyclase (AOC; A_13_P318042); a cytochrome p450 (A_13_P226219); a cytochrome p450 reductase (A_13_P136120); and a jasmonate-induced protein (A_13_P128410). One cytochrome p450 (A_13_P136530) was down regulated due to infestation. Interestingly, defensin, which is often used as an indicator of a JA response (Moran and Thompson, 2001), does not appear to be upregulated either locally or systemically.

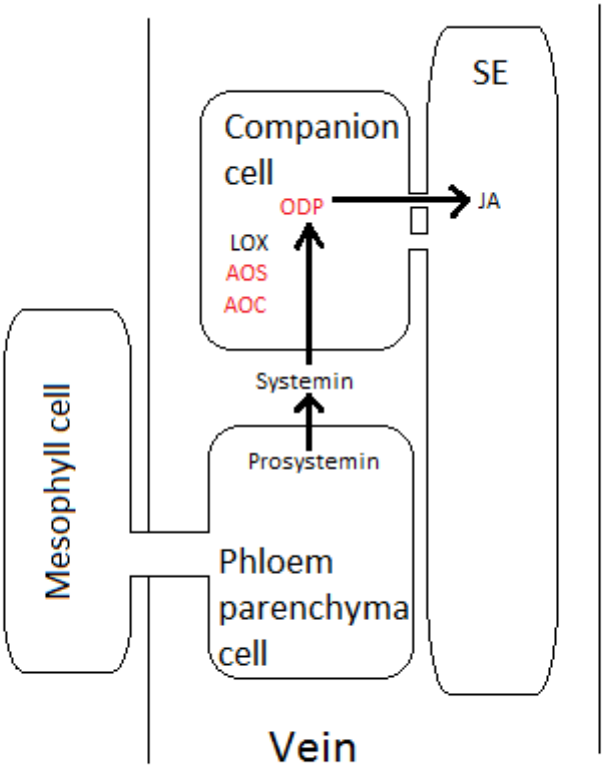


Figure 5-15 Location of the components of the jasmonate signalling pathway in the plant vasculature. Shows the steps of the octadecanoid pathway (ODP), including lipoxygenase (LOX), allene oxide synthase (AOS) and allene oxide cyclase (AOC). Also systemin and its precursor prosystemin, and movement from the phloem parenchyma to the companion cell. Direction of movement is depicted by black arrows. Enzymes and pathways upregulated in the current study are highlighted in red. Diagram created from references within section 5.6.1.2 of the text.

Jasmonate-mediated responses have been shown to have a negative effect on herbivores (Thaler, 1999, Omer *et al*., 2000, Omer *et al*., 2001, Ellis *et al*., 2002b, Cooper *et al*., 2004, Zhu-Salzman *et al*., 2004, Singh *et al*., 2007, Brunissen *et al*., 2010). It has been demonstrated that exogenous JA application can induce systemic defences in a susceptible tomato cultivar that reduce population growth of *M. euphorbiae*, the potato aphid (Cooper *et al*., 2004). JA signalling pathways are known to be triggered by the phloem-mobile

defence signal systemin (Howe, 2004a), induced by chewing insects on solanaceous species (Stratmann and Ryan, 1997). Moran and Thompson, (2001) found that a rapid local defence response involving the JA and SA pathways was induced in Arabidopsis infested with the aphid *M. persicae*. In the same study, a systemic induction of defence genes associated with wounding and pathogen attacks was not observed. This is contrary to the results of the current study, however, although, as discussed at length in Chapter 3, defence responses can differ greatly among different host-parasite pairs.

There was also evidence of a SA-facilitated response in local tissues (Table 5-2). The SA-dependent signalling pathway regulates the expression of a range of defence genes, including the *pathogenesis-related protein* (*PR*) genes (Zarate *et al*., 2007). A number of *PR* genes were differentially expressed in both local and systemic tissues (Table 5-13). Locally upregulated PR genes include: PR1a (A_13_P522469); a PR1a precursor (A_13_P127900); PR1 precursor (A_13_P094360); PR4 (A_13_P262922); PR10 (A_13_P489725); and two thaumatin family proteins (and A_13_P113435). Only one of these (the thaumatin family protein coding transcript A_13_P171025) was also upregulated systemically, showing a severely limited systemic SA response. This is consistent with the results of a previous study looking at the effect of *M. persicae* infestation on Arabidopsis (Moran and Thompson, 2001). It was shown that upregulation of *PR-1* and a 1,3-beta-glucosyltransferase (*BGL2*) – both associated with the SA-dependent response pathway – took place locally, but not systemically in the uninfested apical leaves of infested rosettes within 48 to 96 hours of infestation. Two wheat-induced resistance 1 (WIR1) genes (A_13_P569894 and A_13_P162600) were also upregulated locally to the infestation site. WIR1 encodes for a small glycine- and proline-rich PR protein, the upregulation of which has been found to be

triggered by pathogen attack by *Blumeria graminis*, the barley powdery mildew fungus (Douchkov *et al*., 2011). Its exact function is unknown but it appears to be specific to grass species. This is a significant defence response and would likely be metabolically costly to the plant (Heil and Baldwin, 2002).

Overall, these responses were ineffective, however, as this is a compatible host-parasite pairing. This may be due to the interaction between SA and JA signalling. SA signalling has been shown to be upregulated by *M. persicae* on Arabidopsis, for example, but does not improve resistance to this pest (Louis and Shah, 2013). In fact, it was suggested in that study that SA antagonises the JA signalling pathway, facilitating aphid infestation.

5.6.1.3 R-mediated defence

A suite of genes was upregulated with known roles in *R* gene-mediated defence. This is also known as effector-triggered immunity (ETI) as it relies upon recognition of effector molecules, which have been secreted into the plant by a pest or pathogen, by the product of *R* genes (Figure 5-16)(DeYoung and Innes, 2006). The largest class of *R* genes encodes proteins with a nucleotide-binding site (NBS) and a Leucine-rich repeat (LRR) region (Dangl and Jones, 2001). In the current study, an NBS-LRR containing protein (A_13_P226329) and a LRR-binding protein (A_13_P186194) were upregulated locally after aphid infestation and one NBS-LRR containing protein was downregulated 3 fold. Systemically, an NBS-LRR class resistance gene analogue (A_13_P203744), an NBS-LRR disease resistance protein (rga S-9217; A_13_P537164) and a non-TIR-NBS-LRR type resistance protein (A_13_P013931) were upregulated. An LRR family protein (A_13_P579354) and an NBS-LRR disease resistance protein homologue (A_13_P029371) were downregulated six and two fold respectively. One

NB-ARC domain-containing R protein (A_13_P065586) was overexpressed systemically and another (A_13_P099965) was downregulated. NB-ARC is a signalling motif found in plant resistance gene products and it contains a functioning ATPase domain and a nucleotide binding region (van der Biezen and Jones, 1998, Van Ooijen *et al*., 2008).

The NBS-LRR domain architecture is consistent with a role in pathogen recognition and defence response signalling (Ameline-Torregrosa *et al*., 2008). Both Mi-1 and Vat, which confer resistance to aphids on tomato and melon, respectively, are members of the NBS-LRR protein family. The *Arabidopsis* genome contains 149 predicted NBS-LRR genes, at least eleven of which have been identified as functionally active disease resistance genes (Madsen *et al*., 2003). Silencing one family of NBS-LRR-encoding genes in lettuce resulted in reduced resistance to the oomycete *Bremia lactucae,* which carries the avirulence gene, Avr3 and causes downy mildew (Wroblewski *et al*., 2007). The simultaneous up- and downregulation of members of the NBS-LRR gene family in this study suggests that the regulation of the defence response is complex but it is likely that the downregulation of an *R*-gene would increase susceptibility of a host plant to aphid pests. This makes these differentially regulated transcripts obvious candidates for further investigation. It would be useful to ascertain initially whether the downregulation of these genes is contributing to susceptibility. This could be achieved by knocking out the genes individually, possibly using *Arabidopsis* mutants deficient in homologues of these genes, and monitoring aphid performance using EPGs and r_m experiments.

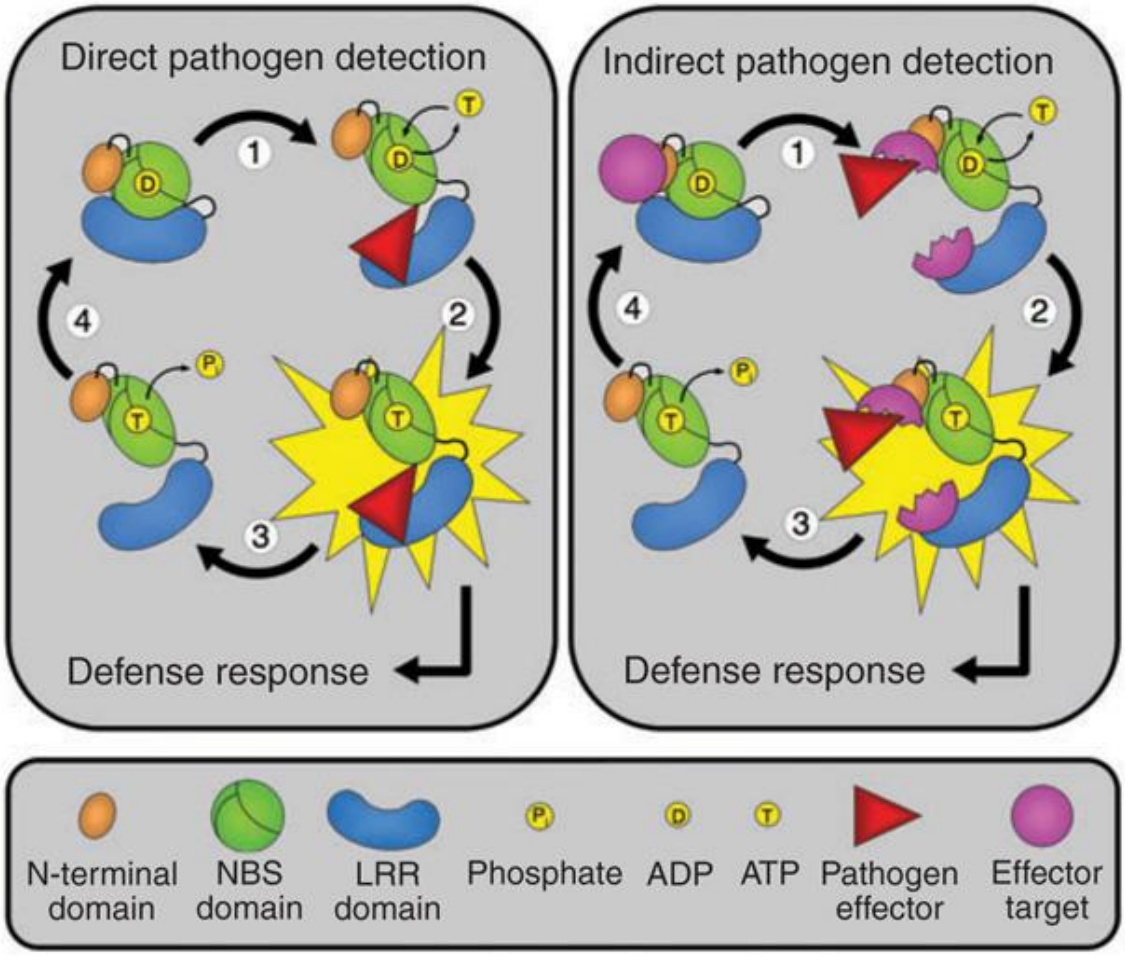


Figure 5-16 Simplified diagram of NBS-LRR signalling in pathogen recognition. Shows both direct activation (left) by a pathogen effector molecule, and indirect activation (right) via an effector target. (1) A pathogen effector directly binds to the NBS-LRR protein (left), or interacts with effector target proteins (right) altering the NBS-LRR protein structure; (2) binding sites are thereby created for downstream signalling molecules; (3) The effector molecule dissociates; (4) NBS-LRR protein returns to the inactive state. Diagram taken from DeYoung and Innes (2006) with permission.

Interestingly, a much higher proportion of the systemically upregulated genes were related to abiotic stress responses, particularly to low temperature and drought. These included genes coding for: two dehydrins (A_13_P267027 and A_13_P149445); two cold-acclimation proteins (A_13_P582609 and A_13_P429681); a low-temperature-induced protein (A_13_P137345); a late embryogenesis abundant protein (A_13_P583369); an early

response to drought protein (A_13_P149235); and two hypoxia-responsive family proteins (A_13_P500249 and A_13_P564009). One dehydrin-coding transcript (A_13_P207034) was downregulated both locally and systemically by aphid feeding. The protein products of these genes have been shown to have protective functions such as membrane protection, cryoprotection of enzymes, and protection from reactive oxygen species (Graether and Boddington, 2014). It could be that the systemic response observed in the current study is due to osmotic stress imposed by aphids, which remove fluids from both the phloem and xylem (Pompon *et al*., 2011). Previous studies have shown an overlap in the transcriptomic responses to biotic and abiotic stressors (Sham *et al*., 2014). It is clear from the data presented in the current study that the barley response to *R. padi* feeding involves both differential activation and repression of defence-related genes.

5.6.1.4 Callose plugging of sieve plate pores

Damage to a SE, for example leaf-tip burning, leads to sieve plate occlusion with callose (Furch *et al*., 2007). When aphids feed on a plant, however, the deposition of callose is species dependent. For example, it has been shown that *D. noxia* induces deposition of callose-mediated occlusion of the PD connecting phloem parenchyma cells and also between the CC and SE. *R. padi* does not induce callose deposition until after 14 days of infestation (Saheed *et al*., 2007). The lack of callose in recently infested plants was confirmed in this study by staining a 13 day old barley leaf, which had been heavily infested with *R. padi* for 72 hours with the callose-binding aniline blue stain (Figure 5-17). An absence of fluorescence at the sieve plates indicated that no callose-mediated occlusion was taking place.

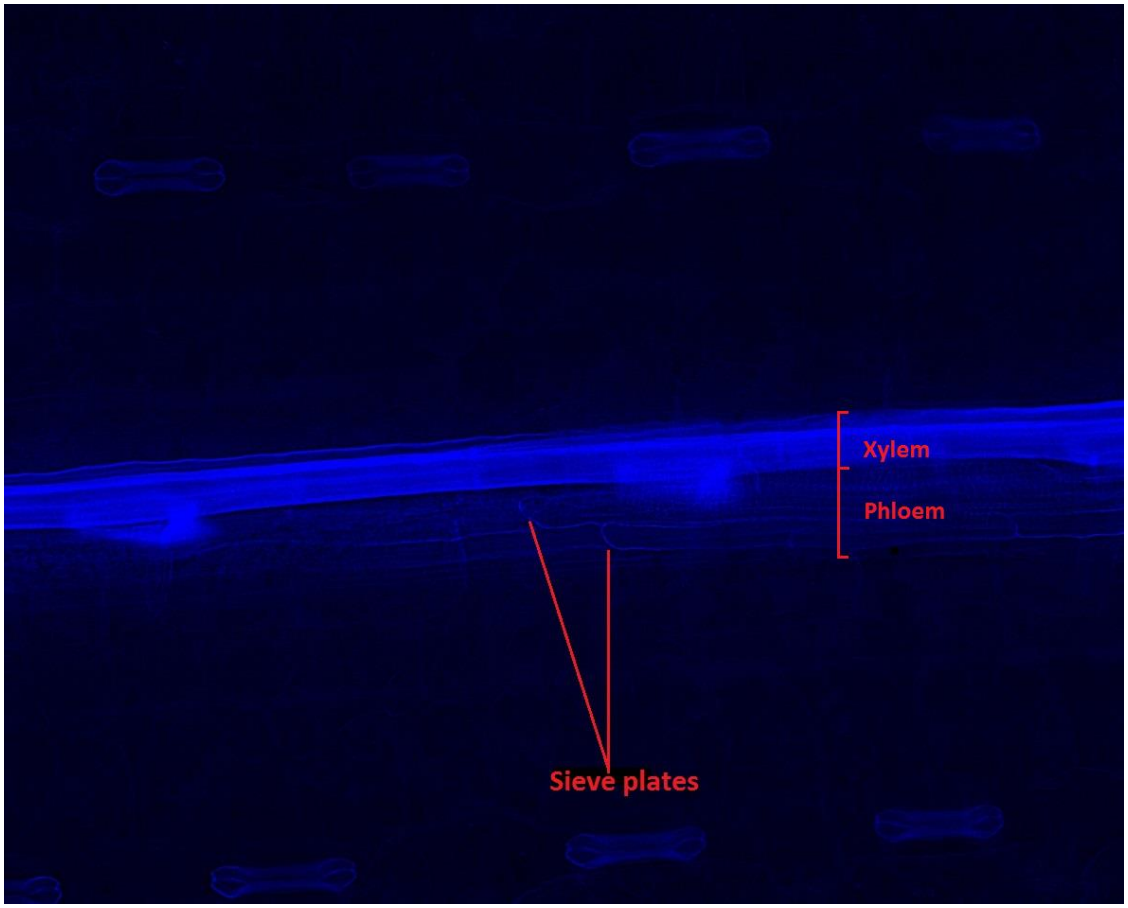


Figure 5-17 Fluorescence microscope image of an aniline blue stained 13 day old barley leaf that had been heavily infested for 3 days with *R. padi*. Image shows the lightly stained cell walls of the phloem sieve elements, including the sieve plates, which all contain the β-1,3 glucan callose. There is no intense fluorescence expected from areas rich in callose, however, indicating that no aphid-induced plugging of the sieve plates has occurred. The xylem elements can be identified by the high fluorescence associated with secondary wall thickenings.

The difference in *R. padi*- and *D. noxia*-induced responses has been attributed to differences in the watery salivary secretions of the two aphid species (Saheed *et al*., 2007). Two β-1,3 glucanases (A_13_P099510 and A_13_P281559) and a β-glucanase-like protein (A_13_P152495) were upregulated locally in the current study. These enzymes break down callose. This suggests that some effort was being made to overcome callose-mediated defences/occlusion of pores. Interestingly no callose synthase genes were upregulated. This suggests that *R. padi* are both preventing callose synthesis and upregulating β-glucanases in

a prophylactic manner. β-1,3 glucanase is a good indicator of SA-mediated defence signalling and has been seen in response to aphid infestation (Moran and Thompson, 2001, Uknes *et al*., 1992, Rogers and Ausubel, 1997). No similar gene expression changes were observed in second leaves, indicating the absence of a systemic callose-mediated defence response.

5.6.2 Cell wall modification

A UDP-D-xylose epimerase 2 (UXE; A_13_P083386) was upregulated both locally and systemically in this study. UXE has been found to be coordinated with the incorporation of pentose sugars into cell walls in barley leaves, roots, and developing endosperm (Zhang *et al*., 2010b)(Figure 5-18). A UDP-D-glucose epimerase 2 (UGE; A_13_P515404) and a UDP-glucuronate decarboxylase (UXS; A_13_P518134) were also overexpressed systemically due to aphid feeding. UGE acts as an activated sugar donor for the biosynthesis of cell wall polysaccharides such as cellulose, xyloglucans, (1,3;1,4)-β-D-glucan and pectins, together with other biologically significant compounds including glycoproteins and glycolipids (Zhang *et al*., 2006). It has been found to be upregulated in rice in response to drought stress (Nguyen *et al*., 2004). UXS catalyses the formation of UDP-d-Xylose from UDP-d-glucuronate (Zhang *et al*., 2005). UXE meanwhile catalyses the reversible interconversion of UDP-xylose and UDP-arabinogalactan (Zhang *et al*., 2010b). Upregulation of these transcripts appears to be a significant indication of the triggering of sugar nucleotide interconversion pathways and hence modification of cell wall biosynthesis, particularly in systemic tissues.

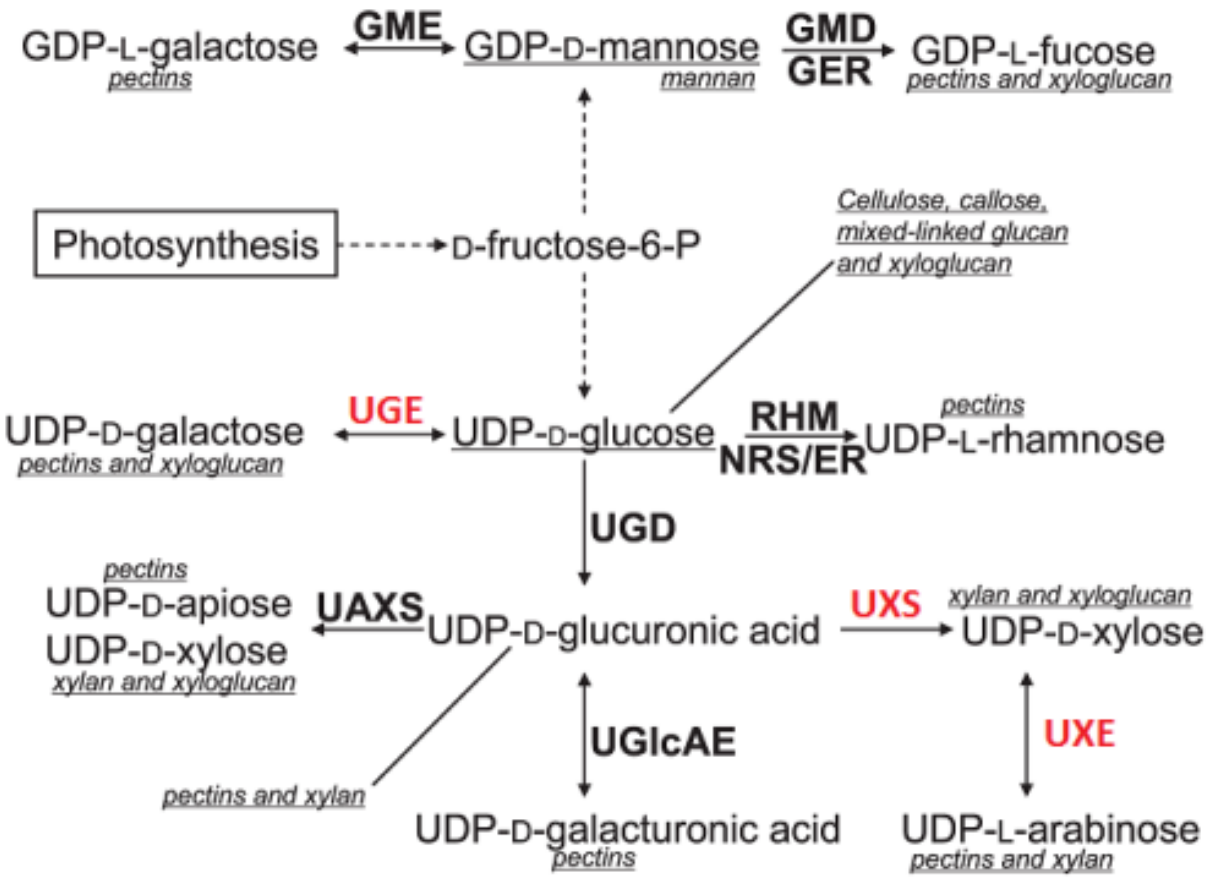


Figure 5-18 A schematic showing the interconversion of NDP-sugars which form cell wall polysaccharides. Polysaccharides are shown (italic and underlined) as are the interconversion enzymes (bold). Enzymes for which transcripts were upregulated in the current study are highlighted in red. GME (GDP-D-mannose 3,5-epimerase);GMD (GDP-D-mannose-4,6-dehydratase); GER (GDP-4-keto-6-deoxy-D-mannose-3,5-epimerase-4-reductase); UGE (UDP-D-glucose 4-epimerase); RHM (UDP-L-rhamnose synthase); NRS/ER (nucleotide-rhamnose synthase/epimerase-reductase); UGD (UDP-D-glucose dehydrogenase); UAXS (UDP-D-apiose/UDP-D-xylose synthase); UXS (UDP-D-xylose synthase); and UGlcAE (UDP-D-glucuronic acid 4-epimerase); UXE (UDP-D-xylose 4-epimerase). Diagram modified from Yin *et al*. (2011).

Two cellulose synthase genes (*CesA1* and *CesA3;* A_13_P012726 and A_13_P017091 respectively) were downregulated locally upon infestation. Cellulose is an important component of cell walls (Figure 5-19 a & b) and downregulation would be likely to facilitate aphid puncturing of cells along the probing pathway (Richmond and Somerville, 2000). Interestingly, two cellulose synthase genes (A_13_P463923 and A_13_P011191) and two

cellulose synthase-like genes (A_13_P567834 and A_13_P563894) were upregulated systemically, indicating that reorganisation of the cell wall is taking place in the second leaves of infested plants. The hemicellulose xyloglucan (XG) is responsible for linking adjacent cellulose microfibrils in the primary cell wall and can be severed and assembled by the enzyme xyloglucan endotransglycosylase (XET). XET is therefore a central regulatory factor in cell expansion (Darley *et al*., 2001). An XET (A_13_P077911) was upregulated systemically in the current study and possibly has a role in strengthening the cell wall as a defence against aphid feeding. The only systemically downregulated gene with any putative cell wall-related function was an alpha-expansin (*EXPA2;* A_13_P570999). This gene is annotated as having a plant cell wall organization role and is often involved in processes where cell wall loosening is observed, such as fruit ripening (Cosgrove, 2000). Expansins respond to auxin-, GA-, ET-, cytokinin- and brassinosteroid-induced cell wall changes/cell enlargement (McQueen-Mason and Rochange, 1999, Cho and Cosgrove, 2002, Cho and Kende, 1997). Loosening of the cell wall in this manner would be advantageous to aphids, so downregulation of this gene may represent an important systemic plant defence response, with the result being a strengthened cell wall.

Studies into aphid stylet pathways have shown that cells are punctured along the pathway to the SE and breaks in the cell wall are subsequently filled with aphid gelling saliva (Tjallingii and Esch, 1993). It therefore seems likely that a response involving rebuilding the cell wall following damage, or reinforcing the cell wall as an induced defence response is occurring in infested plants in this study.

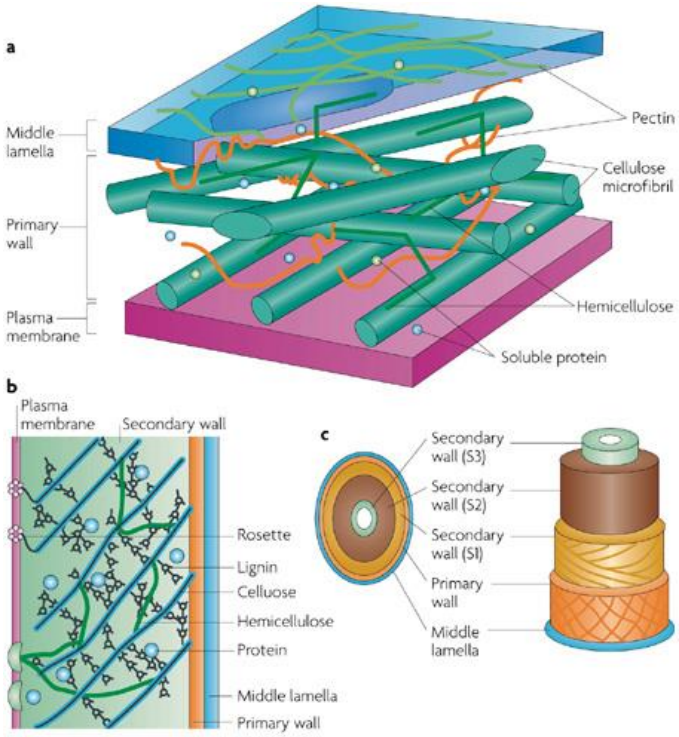


Figure 5-19 Diagram of plant cell wall structure. (a) Primary wall containing cellulose microfibrils, hemicellulose, pectin, lignin and soluble proteins. (b) Secondary wall structure, including lignin and cellulose synthase enzymes in the form of rosette complexes. (c) Shows the S1, S2 and S3 layers of the secondary cell wall, which is where lignification takes place. Reproduced from Sticklen (2008) with permission.

A Cinnamoyl CoA reductase (CCR; A_13_P388159) gene was downregulated in infested tissues. CCRs play an important role in the lignin biosynthesis pathway and are particularly important in lignification around vasculature (Tamasloukht *et al*., 2011). In CCR deficient tobacco, deleterious effects on plant development were observed, including stunting and collapsed xylem vessels (Piquemal *et al*., 1998). *Arabidopsis* plants with a downregulated CCR gene (*AtCCR1*) showed a 50% decrease in lignin content (Goujon *et al*., 2003). In CCR downregulated poplar, transcript and metabolite profiling suggested that, in addition to altered lignification, CCR deficiency resulted in a decrease in hemicellulose and pectin biosynthesis (Leple *et al*., 2007). A reduction of lignin, particularly around the vasculature,

and decreased levels of hemicellulose and pectin in the cell walls would confer a significant advantage to aphids when penetrating cells in the search for the SE. Three 4-coumarate-CoA ligase enzymes (A_13_P499494, A_13_P133180 and A_13_P230504) and a β-glucosidase (A_13_P162805) were upregulated systemically. These enzymes are known to have an important role in cell wall lignification (Cairns and Esen, 2010). Lignin deposition in the secondary cell wall (Figure 5-19c) is important in reinforcing plant cell walls, facilitating water transport, providing compressive strength to conducting tissues, and acting as a mechanical barrier to pathogens (Boudet, 2007). These results show a local reduction and a systemic increase in lignification.

5.6.3 Metabolism

In photosynthesising C_4-plants, pyruvate is produced by the malic enzyme (ME) in bundle sheath cells and then returned to mesophyll cells for conversion to phosphoenolpyruvate (PEP) by the chloroplast-localised pyruvate-phosphate dikinase (Christin and Osborne, 2014)(Figure 5-20). There follows an efflux of PEP, regulated by the PEP/phosphate translocator (PPT), from the stroma to the cytosol for primary carbon fixation via PEP carboxylase (PEPCase)(Wang *et al.*, 2014). Both a malic enzyme (A_13_P520064) and a PPT (A_13_P135890) were overexpressed locally in this study (Table 5-3). This suggests that the plant is upregulating this cycle, perhaps to accommodate for carbohydrate losses due to aphid feeding. This response was not observed in systemic tissues.

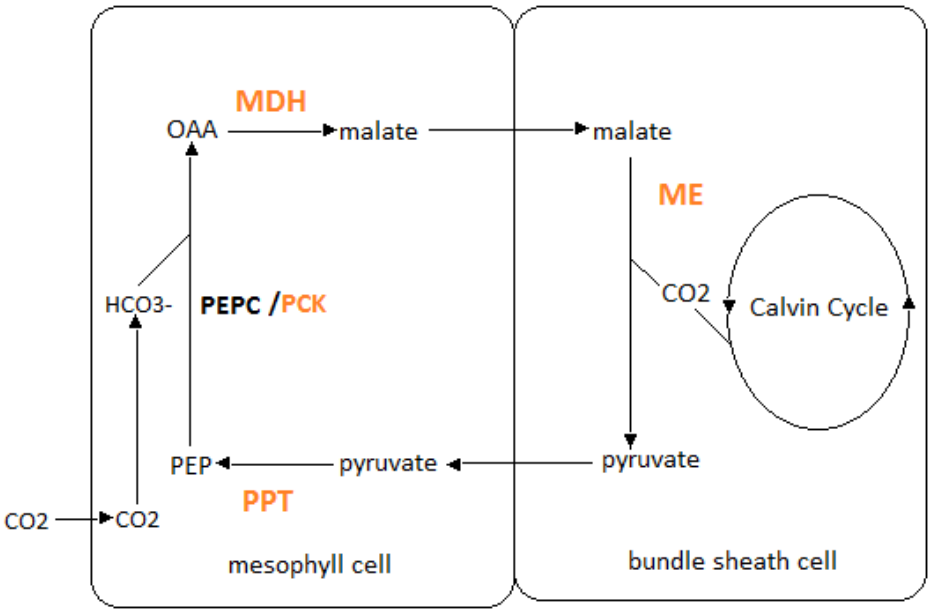


Figure 5-20 Simplified diagram of C_4 metabolism. Showing the enzymes involved in each step of the cycle (bold). Enzymes upregulated in the current study are highlighted in red. OAA, oxaloacetate; MDH, malate dehydrogenase; ME, malic enzyme; PPT, phosphoenolpyruvate phosphate translocator; PEPC, phosphoenolpyruvate carboxylase; PCK, phosphoenolpyruvate carboxykinase. Modified from Peterhansel (2011).

A ubiquitin carrier protein (A_13_P447773) was downregulated 6 fold in response to aphid attack. This is in agreement with a previous study, which showed a downregulation of ubiquitin-carrier protein transcripts in tobacco (*Nicotiana attenuate*) following feeding by *Myzus nicotianae* aphids (Voelckel *et al*., 2004). Two ubiquitin carrier protein transcripts (A_13_P461413 and A_13_P130805), a ubiquitin (A_13_P118410) and a ubiquitin-like (A_13_P199094) protein transcript were upregulated systemically in response to aphid feeding in the current study. Ubiquitin tags proteins for destruction and has been implicated in the control of numerous processes, such as cell-cycle progression, signal transduction,

transcriptional regulation and receptor down-regulation (Hershko and Ciechanover, 1998). The type of post-translational modification modulated by the ubiquitin-proteasome system (UPS) has been shown to be a target for pest and pathogen effector proteins (Banfield, 2015). The local downregulation of ubiquitin carrier proteins and the systemic upregulation of ubiquitination suggests that effector proteins in the aphid saliva may be disrupting host regulatory protein activity at the point of attack, but is not having a long-distance effect. In fact the host plant appears to be upregulating this activity in tissues distant from the site of aphid infestation. There are currently no insect effector proteins that have been shown to directly interfere with the host UPS, so it would be of interest to identify the trigger for the differential regulation of the UPS-related genes in this study.

5.6.4 Transport

Several genes involved in inter- and intra-cellular mobilisation of ions and molecules were upregulated locally in this study. They include HAK1 (A_13_P000401; Table 5-5), a high affinity potassium transporter which has previously been isolated in barley roots (Santa-Maria *et al*., 1997) and leaves (Doering-Saad *et al*., 2002) and is involved in transport of both K^+ and Na^+ (Waters *et al*., 2013). Interestingly, Doering-Saad *et al.* (2002) could not amplify the HAK1 transcript from phloem sap obtained by stylectomy, suggesting that it is not present in the SE, or is present at undetectable levels. An AKT2 transcript (A_13_P184854) was downregulated locally due to infestation. AKT2 is a K^+ channel responsible for at least 50% of the K^+ permeability in the mesophyll cells of leaves (Dennison *et al*., 2001). The other 50% is transported by AKT1. AKT2 has been shown to be upregulated by ABA, and the suggestion of a role in response to drought has been made (Lacombe *et al*., 2000). A K^+ transporter (A_13_P543777) was also upregulated systemically (Table 5-10). In the

investigation into the effects of infestation on inorganic ion content in this study (see Chapter 2), K^+ and Na^+ levels were not altered locally or systemically by aphid treatment (Figure 2-5 and Figure 2-6). The simultaneous upregulation and downregulation of K^+ transport-related transcripts following infestation, despite constant K^+ levels in the leaf, suggests that an alteration in potassium flux is occurring in specific cell types. Two other cation transporters were upregulated systemically: a cation/H^+ exchanger (A_13_P156055); and a cation diffusion facilitator (A_13_P447038). The results of the ion study in Chapter 2 show that calcium and magnesium levels were both elevated in the second leaves of infested and uninfested plants. These findings may be linked. Overall, further work is required to localise the differentially regulated transporters, and/or ions, in order to confirm the speculations above.

5.6.5 Transcripts of unknown function

A large proportion of the transcripts implicated in the *R. padi*-barley interaction in this study are of unknown function. Whilst this impedes conclusion formation, functional assessment of these genes offers the greatest possibility for discovery of novel targets for biotechnological manipulation in genetic crop defence approaches against aphid pests, such as transgenics and RNA silencing. Due to the high throughput and highly parallel nature of gene expression studies, particularly with the advent of microarray and RNA-seq, genomic information available to the researcher is increasing exponentially. As efforts are combined, and gene annotation is improving, much useful information is becoming available on gene function.

A method of identifying the function of unknown genes is to silence them and observe the effects on phenotype and plant-aphid interaction. Collections of mutant germplasm are limited for most cereal crops due to the large or complex genomes (for example hexaploid wheat). One approach to overcoming this is to use *Arabidopsis* knockout mutants lacking homologues of the previously uncharacterised genes. This approach has already provided much information on the plant-aphid interaction (Louis and Shah, 2013). A second approach is to employ a gene-silencing strategy such as virus-induced gene silencing. This approach has successfully been used to knockdown putative resistance genes (a WRKY53 transcription factor and a phenylalanine ammonia-lyase) and demonstrate reduced transcript abundance, phenotype changes and improved aphid performance on these plants (van Eck *et al*., 2010). Similarly, this approach has been used to target putative susceptibility genes, such as (*1,3;1,4*)-β-*glucanase*, which are highly expressed in susceptible plants, and showed that aphid reproductive performance, and plant symptom development, was reduced in the knockdown plants (Anderson *et al*., 2014). This is a useful way of identifying susceptibility factors to exploit as targets for resistance breeding.

5.6.6 Evaluation and future research directions

In order to confirm the results presented here, it would be desirable to perform RT-PCR on some key genes. One important thing to bear in mind with transcriptomics experiments is that, just because there are transcripts present, it does not mean there are active proteins; protein synthesis can be regulated translationally (Ingolia *et al*., 2009). It is also difficult to accurately predict the protein product from an mRNA sequence because of recoding signals associated with ribosomal frameshifting, hopping, termination codon suppression, and the

incorporation of the unusual amino acids selenocysteine and pyrrolysine (Namy *et al*., 2004). For example, initiation of translation may begin somewhere other than at the standard initiation codon (AUG). The protein products of transcripts translated in this fashion will have altered amino terminal domains which may have a crucial role for the biological function of the gene product (Touriol *et al*., 2003). So microarray or RNA sequencing is merely the first step in understanding gene expression patterns. Even the presence of proteins does not mean that there are successful pathways in place. The unknown quantity in genomics experiments means that any gene product or pathway could be disrupted by an unknown gene product. Until we have the full and accurate annotation of the barley genome and characterisation of every signalling network and interference pattern, it will be impossible to know for sure what will be the effect of upregulation of a certain gene or, conversely, the transcriptional cause of a physiological phenomenon.

As whole-leaf samples were used in this study, it would be useful to localise the transcripts within the plant tissue to gather more information about their function. It is possible to locate mRNA within tissue sections by *in situ* hybridisation using labelled single stranded RNA fragments (Engler *et al*., 2001). Alternatively, *in situ* PCR can be used to amplify specific mRNA within plant sections, simultaneously incorporating labelled nucleotides, which can be detected by immunohistochemistry (Athman *et al*., 2014).

As discussed in Chapter 4, RNA-seq is becoming a more popular approach in gene expression studies. RNA-seq has been applied widely to plant and insect research. For example, it has been used to investigate gene expression changes in response to abiotic stress such as drought (Bowman *et al*., 2013), NaCl (Villarino *et al*., 2014) and nutrient deficiency (Wang *et al*., 2013). Bouvaine *et al.* (2012) used the Illumina platform to investigate the effects of diet

sterol content on *Acrythosiphon pisum* aphids feeding on *V. faba* plants and on chemically defined diets. This method provides a more compreshensive approach to transcript detection than microarray and offers potential for gene expression studies of the future.

6 General discussion

Using a range of physiological and functional genomic techniques it was possible to characterise responses in barley to feeding by *R. padi*. As this is a susceptible interaction (Ni and Quisenberry, 2006), it was hypothesised that alterations to the plant physiology and transcriptome would occur that would have beneficial results for the aphid pests. It was also hypothesised that a range of defence responses would be upregulated during the interaction, due to cellular damage caused by the aphid stylets (Hewer *et al*., 2011) and due to elicitors within aphid saliva (De Vos and Jander, 2009). It was possible to investigate the plant responses in great detail using whole genome microarray studies, and also to observe the effects of these changes at the whole plant level, and further, to effects on aphid populations subsequently colonising a pre-infested host.

It was further hypothesised that, as in previous studies (Divol et al., 2005b), a systemic response would occur in uninfested second leaves of infested plants. By comparing the transcriptomes of infested and uninfested leaves it was possible to investigate the differences in response in these tissues. The ion content of local and systemic tissues was recorded but, due to time constraints, it was not possible to record the feeding behaviours of aphids on leaves distant to the site of infestation.

6.1 Impact of *R. padi* infestation on barley growth, physiology and transcriptome

This study has shown that *R. padi* infestation causes stunting of barley plants. The reduction in growth may be a trade-off with the substantial defence response observed in the transcriptomic data; energy put into changing gene expression and synthesising defence

compounds may have been put towards biomass increase under normal, non-infested conditions (Herms and Mattson, 1992). *N. attenuata* plants with a silenced jasmonic acid signalling cascade were shown to exhibit enhanced auxiliary branch growth, in comparison to wild-type plants, following defoliation by leaf-chewing caterpillars (*Manduca sexta* larvae)(Zavala and Baldwin, 2006). Addition of methyl jasmonate (MeJa) to the silenced plants suppressed regrowth, thus restoring the wild-type phenotype. This suggests that these signalling pathways, and their associated defence responses, involve a trade-off with growth. The results of the study presented in this thesis support and augment these findings by showing that *R. padi* infestation induces upregulation of several components of the JA signalling pathway and a range of antibiosis-related proteins (e.g. PR1, PR4, WIR1; Tables 5-2 and 5-6), and reduces the growth rate of infested barley plants (Figure 2-2). It would be useful to confirm a causal relationship between these two effects in barley by silencing JA signalling and looking for improved growth under infestation conditions. Nevertheless, the reduced growth observed following infestation highlights why these aphids are such a serious pest of cereal crops as yield losses under high infestation levels are likely to be great. In order to quantify the yield loss due to aphid infestation, it would be interesting to measure biomass (e.g. fresh weight/dry weight ratios) in older plants and look at seed production. The microarray data obtained in this study provide some possibilities for explaining the loss of biomass deposition but further work would be required to confirm the speculation.

Further to this, an *Arabidopsis* mutant (*cev1*) displaying constitutive expression of jasmonate and ethylene signalling pathways possessed a stunted phenotype (Ellis *et al*., 2002a). That study showed that the *cev1* phenotype was caused by a mutation in the cellulose synthase

gene *CeSA3*. It was suggested that chemical or genetic disruption of cellulose synthesis may alter cell wall synthesis and release oligosaccharides and cell wall components. These compounds may then act as effectors, triggering stress signalling including JA synthesis. This would explain the enhanced resistance to pathogens in these mutants (Ellis and Turner, 2001). The data presented within this thesis are in line with this: the cellulose synthase gene *CeSA3* (A_13_P017091; Table 5-6) was downregulated four-fold in local tissues following infestation. Our results support the theory that the cell wall can mediate JA defence responses (Ellis *et al*., 2002a) and extend this effect to include responses to insect pests.

EPG showed that *R. padi* pre-infestation reduces the feeding duration (E2) of subsequent aphids but does not stimulate an increase in E1 salivation or cell puncture rate. There was, therefore, no evidence of increased efforts to feed. Without doing performance studies such as intrinsic rate of increase (r_m) and mean relative growth rate (Dahlin and Ninkovic, 2013) it is difficult to know whether the reduced E2 duration actually had a negative effect on aphids. Hale *et al*. (2003) found that SE sap was improved as an aphid food source, in terms of increased osmotic pressure and elevated AA content, under drought stress, however sap ingestion rates were reduced on these plants. The authors suggested that, although aphids are able to osmoregulate when faced with concentrated sap (Wilkinson *et al*., 1997), it is metabolically costly for them to do so (Hendrix *et al*., 1992) and a solution to this may be to reduce sap ingestion rates when feeding on sap with high osmotic pressure. It is a possibility that, in the current study, barley SE sap was becoming more concentrated in pre-infested plants due to reallocation of photosynthate so aphids reduced imbibition duration. It would be useful to measure the osmotic pressure on uninfested barley plants as a comparator for that recorded for heavily infested plants in this study, to determine whether this explanation

is likely. Interestingly, transcripts encoding aquaporins were downregulated both locally (A_13_P305392; Table 5-5) and systemically (A_13_P000146; Table 5-10). This suggests that changes in plant water relations are taking place, but without localising the aquaporins it is difficult to draw conclusions as to what the effect of this would be. This would be an avenue for further investigation.

Comparing the local vs systemic effects on gene expression, some overlap in responses could be seen but a much greater discordance in differentially regulated transcripts was recorded. Of the annotated genes, 19 were differentially regulated both locally (infested first leaves) and systemically (uninfested second leaves of infested plants), 151 were differentially regulated only locally, and 271 showed altered expression only in a systemic manner. Functional annotation of these genes showed a general local improvement for aphids but a systemic enhancement of defences. Local downregulation of epicuticular wax biosynthesis genes and genes associated with ubiquitination and responses to abiotic stress, and upregulation of transcripts encoding callose-degrading enzymes, are all responses likely to facilitate aphid feeding. Meanwhile, systemically, epicuticular wax related transcripts, abiotic stress-related transcripts and ubiquitination genes were upregulated, and no callose-related transcripts were differentially regulated. This suggests that a protective response is taking place in systemic tissues. In contrast, the SA-mediated response appeared weaker in systemic tissues, with none of the locally upregulated pathogenesis related proteins being upregulated in second leaves. This could be explained by a 'decoy' hypothesis which suggests that SA antagonises the JA signalling pathway, facilitating aphid infestation (Walling, 2008). SA signalling has been shown to be upregulated by *M. persicae* on *Arabidopsis*, for example, but does not improve resistance to this pest (Louis and Shah,

2013). The differential SA-related gene expression observed in our study would support a local disabling, and a systemic induction, of defences.

This pattern of localised suppression and systemic induction of defences has been observed previously in behavioural studies. For example, Dugravot *et al*. (2007) used EPG to investigate the local and systemic effect of *M. persicae* pre-infestation on potato. They found that aphid food acceptance improved on previously infested leaves (local response), but was reduced on uninfested leaves of infested plants (systemic response). This indicated that a systemic induction of resistance was taking place, even in the presence of a localised facilitation effect. Similarly, Prado and Tjallingii (1997) found no local resistance in *B. oleracea* to the aphid *B. brassicae* on pre-infested plants using EPG. There was no reduction of phloem feeding and no increase in probing behaviours. Systemically, however, the total duration of non-probing time increased and E1 (salivation) and E2 (phloem sap ingestion) were both delayed and reduced, indicating an upregulation of defence responses. The EPG results indicated that the resistance factors were phloem located. In the same study, choice tests using *B. oleracea/B. brassicae*, *C. annuum*/*M. persicae* and *T. aestivum/R. padi* host/pest pairs showed that aphids chose control leaves (uninfested plants) over the uninfested leaves of infested plants. EPG and choice test experiments are both approaches that could be employed with the barley/*R. padi* plant/pest study system in order to confirm the induction of systemic defence responses reported here, and their effects on aphid behaviour.

6.2 Evaluation of approaches

Overall, more genes were differentially regulated systemically (1747) than locally (982). One concern is that a proportion of the systemically regulated genes may be implicated due to delayed growth in infested plants. Both the infested and uninfested plants were in growth stage 11 at infestation and growth stage 12 upon harvesting (Zadoks *et al*., 1974), but the switch could have taken place at different times over the three day period. The differential regulation of more metabolism-related genes and phytohormones would seem to support this, although studies linking upregulation of defence pathways with stunting (see above) would seem to refute it. It would be desirable to confirm whether the plants are stunted or merely developmentally delayed by performing longer-term growth studies of barley plants to determine whether infested and uninfested plants reach subsequent growth stages simultaneously and produce seeds simultaneously, etc. If not, and development is delayed, then a proportion of the differentially regulated genes could be due to differences in growth/development processes rather than solely response to aphids.

This study investigated the effects of aphid infestation in isolation. In the field, these plants would have faced other biotic and abiotic stressors, which may have altered the plant response. For example, Dahlin and Ninkovic (2013) showed that aphid population development was negatively affected by weed-crop interactions in field studies. Johnson *et al*. (2014) showed that the resistance status of *Medicago sativa* (alfalfa) to *A. pisum* altered according to CO_2 levels. Genotypes displaying low levels of resistance showed reduced aphid colonisation and reproduction rates under elevated CO_2 conditions, whilst a highly resistant variety experienced higher pest colonisation levels. Studies of this nature highlight the importance of understanding the genome of crop species and the various responses to both

biotic and abiotic factors, so that changes in resistance levels in response to altered environmental conditions can be pre-empted by crop breeders.

6.3 Role of aphid salivary effectors

Aphid saliva contains effector molecules which either trigger plant defence responses or enhance susceptibility and are important factors determining resistance level of a plant to aphid attackers (Elzinga and Jander, 2013). Improved functional genomics techniques have also been applied to aphid pests and particularly their saliva. This has provided much insight into the role of aphid saliva in the host-pest relationship, which is briefly outlined below, but many effectors remain to be identified. RNAi silencing of the *C002* trancript, abundant in the aphid salivary gland, began to show lethal effects in *A. pisum* within 2-3 days (Mutti *et al*., 2006). EPG studies on the *C002* knockdown mutant suggests that the gene product has a role in location or recognition of the SE and its removal precludes SE sap ingestion by affected individuals, thus reducing aphid longevity (Mutti *et al*., 2008). *M. persicae* reproduction was elevated on *Arabidopsis* plants transgenically expressing the *M. persicae* salivary effectors PIntO1 and PIntO2 (Progeny Increase to Overexpression 1 and 2 respectively)(Pitino and Hogenhout, 2012). This effect was not observed when the transgenic plants were expressing the *A. pisum* ortholog of these genes, however. In the same study, RNAi silencing of *PIntO2* and *C002* in *M. persicae* feeding on *Arabidopsis* and *Nicotiana benthamiana* reduced the fecundity of these aphids. RNAi knockdown of the *Rack-1* gene, which is predominantly expressed in the aphid gut, also reduced the performance of *M. persicae* on *Arabidopsis* and *N. benthamiana* (Pitino *et al*., 2011). *C002*, *PIntO1*, and *PIntO2* transcripts have been found to be abundant in the salivary glands (Bos *et al*., 2010)

and C002 and PIntO1 have been identified in proteomics studies of *M. persicae* and *A. pisum* saliva (Carolan *et al*., 2009, Harmel *et al*., 2008). Generation of a salivary gland transcriptome by RNA sequencing identified two further aphid effectors that alter the fecundity of aphid pests. Reproduction of the potato aphid (*Macrosiphum euphorbiae*) increased on *N. benthamiana* upon transient expression in the host of Me10 and Me23 proteins, and also when Me10 was expressed in tomato (Atamian *et al*., 2012). The function of Me10 is unknown but Me23 is thought to encode a glutathione peroxidase with a role in reducing the effect of the oxidative burst triggered by aphid feeding (Lamb and Dixon, 1997).

Some effector proteins have been shown to have a negative effect on aphid performance. For example, Mp10 and Mp42 proteins from *M. persicae* saliva were found to decrease aphid fecundity on *N. benthamiana*, possibly by eliciting plant defence responses. Mp10 was found to induce chlorosis and cell death in *N. benthamiana,* supporting this theory (Bos *et al*., 2010). The Russian Wheat aphid (*D. noxia*) has been shown to cause a range of symptoms in host plants, including chlorosis, stunting, and leaf curling; *R. padi*, in contrast do not cause visible symptoms (Saheed *et al*., 2007). This difference is intriguing given their similar leaf penetration behaviours. It has been postulated that differences in aphid salivary components may be responsible for the differential response profiles. It has been shown that *D. noxia* and *R. padi* have similar salivary hydrolases, but different oxido-reductases (catalase and peroxidase)(Ni *et al*., 2000). The group suggested that it could be these variations in salivary components that are behind the presence and absence of leaf chlorosis in *D. noxia* and *R. padi* infested plants, respectively (Ni and Quisenberry, 2003).

A number of calcium-binding proteins have also been identified during proteomic studies of aphid saliva (Carolan *et al*., 2009, Harmel *et al*., 2008). A calcium-binding protein (NcSP84)

was isolated from salivary gland extracts and from the saliva of Green rice leafhoppers (*Nephotettix cincticeps*) and was also detected in the SE sap of rice plants fed upon by leafhoppers (Hattori *et al*., 2012). This study shows the pathway of the protein from its production in the aphid salivary gland into the plant SE, via the saliva. The calcium-binding nature of the protein suggests a role in suppression of calcium-mediated defence responses such as sieve-tube occlusion (van Bel *et al*., 2014).

2D-PAGE has also been used to analyse the proteins in honeydew of the aphid *A. pisum* (Sabri *et al*., 2013). The results identified a number of proteins originating from the endosymbiotic bacteria *Buchnera*, as well as several with purported roles in the plant-aphid interaction, including a chaperonin, GroEL and Dnak chaperones.

The results of these studies support the hypothesis that these are aphid-derived effector proteins, which induce an effect in the host plant in a species-specific manner. As more information becomes available on salivary effector molecules and plant defence and susceptibility genes, there will be greater scope for breeding and biotechnological approaches that directly target a specific gene.

6.4 The future of the field

6.4.1 Small sample studies

One of the main aims of this study was to develop a protocol for performing whole genome microarray of barley phloem sap. Although this was not completed successfully, advances in the field of RNA-sequencing have made transcriptomic studies of small samples viable (Navin

et al., 2011, Honaas *et al*., 2013, Loraine *et al*., 2013)(see Chapter 4 for a full discussion). RNA-seq has also been applied to the plant-aphid relationship. For example, the transcriptomic response of Chrysanthemum (*Chrysanthemum morifolium)* to the aphid *Macrosiphoniella sanbourni* was investigated using this method and implicated genes with roles in ROS generation, phytohormone signalling, cell wall modification and photosynthesis, as being central to the defence response (Xia *et al*., 2014). Applying RNA-seq to studying SE sap will generate much information on the plant phloem and enable a clearer understanding of plant-aphid interaction. If the current study were to be performed today, it is likely that an approach employing RNA-seq would prove more fruitful than a microarray-based method.

As well as transcriptomic studies, metabolomics has been targeted at small samples such as SE sap. The metabolome of wheat phloem sap collected by stylectomy has now been identified using gas chromatography–mass spectrometry (GC-MS)(Palmer *et al*., 2014). This approach would be available to the plant-host study system investigated in the current study and would provide useful complementary data to a stylectomy-derived sap transcriptome.

6.4.2 Approaches to crop protection

6.4.2.1 Breeding

The importance of identifying novel resistance genes for crop breeding has been highlighted by the development of virulence, or resistance-breaking, in 17 aphid species (Jimoh *et al*., 2011). Breeding crops for high levels of antibiosis resistance often contributes to the development of aphid virulence and it has been suggested that a more balanced approach

would be advantageous, employing aphid-tolerant cultivars and better integrating them with biological, chemical and cultural practices (Smith and Chuang, 2014). Large RNA-seq or microarray datasets, like the one in the current study, offer many possibilities for identification of genes involved in more moderate antibiosis resistance or tolerance.

6.4.2.2 RNA silencing and transgenics

New control strategies such as RNAi have been successful against lepidopteran pests/tissue chewing insects but there were concerns that phloem-feeders would not ingest sufficient quantities of dsRNA to have an effect. Being able to identify key SE/aphid interaction genes is key to developing this approach. Plant-mediated RNA silencing has recently been successfully employed as a method to engineer plant resistance towards aphids. Guo *et al*. (2014) targetted nine *M. persicae* genes for silencing and created viruses expressing hairpin RNA or micro RNA. The genes included a V-type proton ATPase subunit E-like (V-ATPaseE), a tubulin folding cofactor D (TBCD) and an acetylcholinesterase 2 coding gene (MpAChE2). The vectors were transformed into tobacco plants (*N. tabacum cv. Xanti*) and insect challenge assays performed. Most of the transgenic plants showed enhanced aphid resistance. In that study, target genes were identified by studying sequence information in GenBank but further studies identifying genes involved in the aphid-plant interaction, particularly looking at phloem-specific samples, will be beneficial.

RNA silencing approaches have also been applied to the control of aphid-vectored plant viruses. For example, transgenic *Vigna unguiculata* (cowpea or black eye pea) lines were generated with enhanced resistance to the Cowpea aphid-borne mosaic virus (CABMV)(Cruz and Aragão, 2014). This virus represents one of the greatest causes of yield loss for this crop. The use of RNAi will advance the development of transgenic pest resistant crops but there

remains a need to identify target genes for plant-mediated silencing, both aphid effectors and plant susceptibility genes.

As well as gene silencing, overexpression of genes to enhance resistance has been achieved. Chen *et al*. (2013) overexpressed a gene they termed *Increased Resistance to Myzus persicae 1 (IRM1)*, which they identified from an *Arabidopsis* mutant collection as being a resistance gene. A dominant gain-of-function phenotype is generated in overexpression mutants by inserting a 35S enhancer upstream of the natural promoter. Investigating aphid feeding behaviour on these plants using EPG showed that the aphid stylets encounter resistance factors, perhaps of a mechanical nature *en route* to the phloem, although the function of the gene is unknown.

As genomic resources improve, other approaches become available to the search for resistance and susceptibility genes. For example, genome-wide association studies, which look for quantitative trait loci controlling a particular trait, such as resistance, are made possible with a large quantity of genomic information. Debibakas *et al* (2014) were able to compare genomic information of *Saccharum* spp. (sugarcane) cultivars of known phenotype with regards to resistance against the Sugarcane yellow leaf virus (SCYLV), which is transmitted by aphids. This allowed them to identify genomic regions controlling resistance to the virus. The results of such studies provide much relevant information, including candidate genes for involvement in the aphid-virus-plant relationship, which may be important targets in crop breeding. This could be applied to *R. padi*-barley.

The advantages of understanding the phloem and transfer of substances (i.e. saliva and SE sap) between an aphid and its host has been demonstrated by efforts to improve uptake of

Bt toxins and other intrahaemocoelic toxins by aphids. Transgenic approaches utilising Bt toxins have not been highly effective against hemipteran pests due to limited binding to the gut epithelium of these insects (Li *et al*., 2011a). Attempts have been made to enhance Bt toxicity to hemipterans, by modifying the Cry Bt toxins for improved binding to the insect gut (Chougule and Bonning, 2012). For example, Cry1Ac was fused to the nontoxic ricin B-chain (RB), which is a galactose/N-acetyl galactosamine binding lectin (Mehlo *et al*., 2005). N-acetyl galactosamine residues are thought to be important Bt toxin-binding receptors and the RB lectin binds these residues with high affinity (Frigerio and Roberts, 1998). The normally resistant hemipteran pest, the leafhopper *Cicadulina mbila,* was rendered susceptible to the modified toxin (Mehlo *et al*., 2005). Similarly, fusing the snowdrop lectin, *Galanthus nivalis* agglutinin (GNA) with neurotoxic venom collected from the spider *Segestria florentia* is deleterious to both lepidopteran and hemipteran pests (Down *et al*., 2006). Both *M. persicae* and *N. lugens* were among the hemipteran pests susceptible to this toxin fusion in that study.

As well as lectins, other delivery molecules have been investigated to improve transport of toxins from the gut of hemipteran pests in transgenic resistance. Persistently-transmitted plant viruses, for example, by their nature, invade and remain within the haemocoel of the vector (Tamborindeguy *et al*., 2010). This transcytosis of the virus molecules has made them of particular interest for engineering delivery of toxins into the insect haemocoel. An insecticidal toxin derived from a spider was fused to the coat protein of the aphid-transmitted plant virus, Pea enation mosaic virus (PEMV)(Bonning *et al*., 2014). The toxin is ineffective against aphids when delivered orally in its natural form, but is highly effective when injected into the haemocoel (Pal *et al*., 2013). When fused to the viral coat protein,

the toxin was taken up from the aphid diet and delivered into the haemocoel, leading to increased mortality of *A. pisum*, *R. padi*, *A. glycines* and *M. persicae* (Bonning *et al*., 2014). Such delivery molecules should ideally be phloem-translocated to maximise transmission to aphid vectors. This is why viruses and phloem-specific lectins make good targets (Stoger *et al*., 1999). Development of these approaches depends on identifying suitable target genes and on demonstrating their practical function across broad areas of crop production. Until such a time as this has been achieved, conventional breeding of aphid resistance will dominate the field.

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Websites and other resources:

The University of Arizona, Centre for Insect Science Education Outreach: http://insected.arizona.edu/.html

The Arabidopsis Information Resource (TAIR): http://www.arabidopsis.org

Universal Protein Resource (Uniprot): http://www.uniprot.org/

The Plant Expression Database (PLEXdb) : http://www.plexdb.org/

National Center for Biotechnology Information (NCBI) GenBank:

http://www.ncbi.nlm.nih.gov/

T042 Technical bulletin, ThermoScientific. Assessment of Nucleic Acid Purity:

http://www.nanodrop.com/Library/T042-NanoDrop-Spectrophotometers-Nucleic-Acid-Purity-Ratios.pdf

Agilent Technologies. Low Input Labeling Kit protocol:

http://www.chem.agilent.com/library/usermanuals/Public/G4140-90040_GeneExpression_OneColor_6.7.pdf

Rhopalosiphum padi life cycle adapted from:

http://what-when-how.com/insects/sternorrhyncha-jumping-plant-lice-whiteflies-aphids-and-scale-insects/

Venn diagram software:

http://www.bioinformatics.lu/venn.php

NCBI Gene Expression Omnibus

http://www.ncbi.nlm.nih.gov/geo/