Local and systemic responses to *Myzus persicae* in *Arabidopsis*. A role for redox components.

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The candidate confirms that the work submitted is his own, except where work which has formed part of jointly-authored publications has been included. The contribution of the candidate and the other authors to this work has been explicitly indicated below. The candidate confirms that appropriate credit has been given within the thesis where reference has been made to the work of others.

Chapters 5 and 7 of the thesis are based on work of jointly-authored publications.

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Abstract

The mechanisms that enable plants to perceive and respond to aphids remain poorly characterised, particularly with respect to systemic signalling pathways. The aim of the studies reported in this thesis was to characterise the local and systemic signalling pathways induced by *Myzus persicae* infestation of *Arabidopsis thaliana* with translational aspects to potato (*Solanum tuberosum*). Particular emphasis was placed on the role of redox signalling pathways, which were studied in wild type *A. thaliana* (Col0) and in mutants that were deficient either in the major low molecular weight antioxidant ascorbic acid *vitamin C defective 2* (*vtc2*) or in the abscisic acid (ABA) Insensitive-4 (ABI4) transcription factor or both components. Transcriptome analysis and metabolite profiling of leaves infested by *M. persicae* and on leaves from the same rosettes that were remote from the site of aphid attack revealed that the plant responses to aphids involved rapid local and systemic transcriptome re-programming in the absence of marked changes in the metabolite profiles. Moreover, the transcriptome re-programming observed in infested leaves was different from the systemic response. The aphid-induced transcriptome signature of the infested leaves bore strong hallmarks of redox-signalling, salicylic acid (SA) signalling and ABA signalling, while that of the systemic leaves revealed a transcript profile where redox signalling was present but SA signalling was decreased. The *vtc2* mutant showed decreased aphid fecundity, while aphid numbers were increased on the *abi4* mutants. The differences in resistance to aphids between these genotypes are linked to alterations in ABA-dependent jasmonate-signalling pathways. When potato leaves were enriched in asorbate aphid fecundity was increased. These findings demonstrate the central role of redox signalling pathways in plant responses to aphids. Further characterisation of genes crucial for maintenance of redox homeostasis following aphid attack will inevitably facilitate development of aphid-resistant crops through GM technologies and marker-assisted selection.

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Abbreviations

ABA	abscisic acid
ABI4	ABA INSENSITIVE 4
APX	ascorbate peroxidase
BHT	benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester
BIT	1,2-benzisothiazol-3(2H)-one1,1-dioxide
Bq	becquerel
CAT	catalase
CK	cytokinin
Col0	Columbia 0
DHA	dehydroascorbate
DHAR	dehydroascorbate reductase
DPI	diphenylene iodonium
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ET	ethylene
eV	electron volt
GA	gibberellic acid
GC-MS	gas chromatography – mass spectrometry
GDP	duanosine-5'-diphosphate
GPX	glutathione peroxidases
GR	glutathione reductase
GS	glucosinolate
GSH	reduced glutathione
GSSG	glutathione disulphide
hpi	hours post infestation
HPLC	high-performance liquid chromatography
HR	hypersensitive response
JA	jasmonic acid
JAZ	JA ZIM-domain
kD	kiloDalton

L-GalL	L-galactono-1,4-lactone
MAMP	microbe-associated molecular pattern
MAPK	mitogen-activated protein kinase
MDA	monodehydroascorbate
MDAR	monodehydroascorbate reductase
MPA	methaphosphoric acid
MS	Murashige and Skoog
MSTFA	N-methyl-N-(trimethylsilyl)-trifluoroacetamide
NADPH	nicotinamide adenine dinucleotide phosphate
NBS-LRR	nucleotide-binding site and leucine-rich region
NPR-1	NONEXPRESSER OF PR GENES 1
ODD	oxoacid-dependant dioxygenases
PCR	polymerase chain reaction
PR	pathogenesis-related
PVPP	polyvinylpyrrolidone
R	resistance
SA	salicylic acid
SAR	systemic acquired resistance
SD	standard deviation
SE	standard error
ROS	reactive oxygen species
SOD	superoxide dismutase
TCA	tricarboxylic acid
TCEP	tris(2-carboxyethyl)phosphine hydrochloride
TMSi	trimethylsilyl
VTC	vitamin C

Chapter 1. Introduction

1. Introduction

Aphids are major agricultural pests that cause extensive damage to both crop and garden plants. Aphid feeding deprives plants of essential photo-assimilates. More importantly, aphids are the vectors for over 100 plant disease-causing viruses (Blackman and Eastop, 2000; Ng and Perry, 2004). For example, the bird cherry-oat aphid (*Rhopalosiphum padi* L.) invades cereal fields across Europe, causing extensive damage. Aphid-induced damage routinely results in decreases in crop yield of up to a 15% (Leather et al., 1989).

The majority of aphid species exploit a small number of host plants. The biochemical and molecular factors that restrict the colonization of non-host plants are poorly understood. However, it appears that aphids are able to differentiate between hosts and non-hosts using a range of markers/cues in the epidermal or mesophyll tissues that are sensed during brief probing periods prior to colonization (Powell et al., 2006).

1.1 The green peach aphid (*Myzus persicae*)

Unlike many aphid species, the green peach aphid *Myzus persicae* (Sulzer) has been designated as a “generalist” feeder because it can colonise numerous plant species (Fig. 1.1). It is a global agricultural pest that feeds on more than 30 plant families and transmits over 100 viruses (van Emden et al., 1969). *M. persicae* is also considered to be the major insect pest of potato worldwide largely because it is the predominant vector of potato viruses such as leafroll virus, potato virus A and potato virus Y.

Figure 1.1 Fourth instar nymphs of the green peach aphid (*Myzus persicae*) feeding on *Arabidopsis thaliana* Col0. Scale bar equals 0.5 cm.

In addition to potato, *M. persicae* infests a range of vegetable crops including cabbage, oil seed rape and other *Brassica*s. The detrimental effects of infestation are the result of the high aphid reproduction rates. Massive increases in population are possible because of clonal reproduction resulting in genetically identical individuals (Loxdale and Lushai, 2003). Sexual reproduction is limited to one generation per year. Significant variations in performance and reproduction success on different hosts have been found in different *M. persicae* clonal lineages (Weber, 1985; Vorburger et al., 2003). For example, the *M. persicae* populations found in Scotland comprise of numerous insecticide-sensitive and insecticide-resistant clones with variable reproduction rates on different host plants (Fenton et al., 2010). Host specialization and the emergence of insecticide-resistant super-clones appear to be ongoing processes that will inevitably increase problems for agriculture (Kasprowicz et al., 2008).

The model plant species *Arabidopsis thaliana,* which is closely related to *Brassica* crops, is also a host for *M. persicae.* Indeed, this aphid can be a major problem for researchers worldwide who grow *A. thaliana* for research purposes. Arabidopsis is a plant of choice for many studies in plant biology because of the availability of genome information and mutants and the ease of genetic manipulation. It has been widely used to study the plant-insect interactions and to dissect defence signalling pathways (de Vos et al., 2007).

1.2 Aphid feeding

Aphids feed exclusively on phloem sap using their anatomically adapted mouthparts called stylets to probe between the plant cells and reach the sieve elements. During the probing activities, aphids secrete rapidly-gelling sheath saliva, which is deposited between the cells along the stylet track (Tjallingii and Hogen Esch, 1993). Saliva secretion is important because it prevents the direct contact of the chitinous stylet with the plant tissues. The

saliva contains metabolites such as phospholipids and conjugated carbohydrates and proteins such as phenoloxidases, peroxidises, pectinases, and β-glucosidases that have the potential to elicit plant defence responses (Miles, 1999). The probing stylet can also occasionally puncture epidermal and mesophyll cells on its way to the phloem (Martín et al., 1997; Fig. 1.2). These puncture points, which are considered to serve as positional cues, are accompanied by the secretion of a specific type of saliva called “watery saliva” and ingestion of cell contents (Tjallingii, 2006). Watery saliva is also secreted at the feeding site, once it becomes established after the stylet tip punctures a sieve element (Prado and Tjallingii, 1994; Fig. 1.2). The secretion of watery saliva appears to prevent the wound defence responses of the sieve element that would otherwise block or repair any tissue damage (Will et al., 2009). Such mechanisms allow the aphid to feed for long periods from a single sieve element (Will and van Bel, 2006). Sustained sap ingestion is essential for optimal aphid growth and reproduction. In contrast to chewing insects, which macerate leaves and other plant tissues causing extensive wounding, the mode of aphid feeding allows minimal interaction of the stylet with the plant tissue and hence does not cause significant damage.

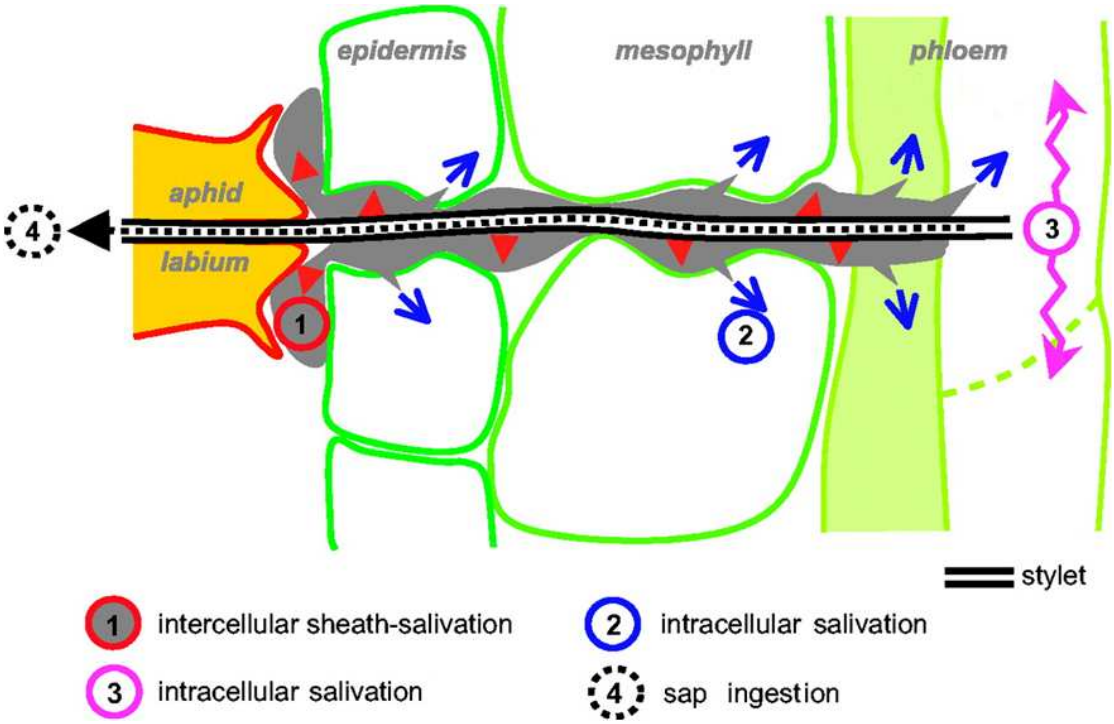

Figure 1.2 A schematic diagram illustrating different types of saliva secretion along the stylet track during the aphid probing and feeding process (Tjallingii, 2006). The grey area depicts the deposited sheath saliva that limits the contact of the stylet with the plant tissue. Red arrowheads (1) illustrate sheath saliva secretion points. Intracellular watery salivation (2) during brief punctures of epidermal and mesophyll cells is illustrated with open blue arrowheads. Purple arrows (3) indicate a secretion of watery saliva in the phloem flow after the stylet has punctured the sieve element.

1.3 Plant perception of aphid attack

Although aphid feeding does not cause extensive damage, plant tissues appear to perceive aphid probing in a manner that induces significant transcriptome changes leading to the elicitation of rapid defence responses (de Vos et al., 2005). However, little is known concerning the mechanisms that enable plants to perceive aphids. Similarly, the nature of the elicitors involved in the induction of plant defence responses is poorly understood. Potential elicitors include metabolites and proteins synthesized by the aphids and also compounds produced by the endosymbiotic bacteria that reside in the aphid and are introduced into the plant tissues with the saliva (Urbanska et al., 1998; Miles, 1999; Forslund et al., 2000). In addition, stylet probing activities and cell wall punctures may activate mechanoreceptors in the plant cells, resulting in wound-like responses. The intimate contacts between the chitinous stylet and the plant tissues, together with the prolonged probing activity could be compared to similar processes occurring during the intercellular growth of the hyphae of fungal pathogens (Hamel and Beaudoin, 2010; Perfect and Green, 2001). Chitin, found in fungal cell walls, is a potent elicitor of plant innate immune responses that induces microbe-associated molecular patterns (MAMP) signalling (Wan et al., 2008). MAMPs are important in the detection of potential pathogens (Boller and Felix, 2009). Thus, it is possible that some aspects of aphid feeding are perceived by similar mechanisms to those used to detect fungal pathogens and so similar programmed responses are involved. However, it should be noted that stylet penetration is accompanied by deposition of significant amounts of gelling saliva that is specifically proteinaceous and envelopes the stylet. Moreover, the gelling saliva remains along the stylet track, even after the stylet has been withdrawn thus minimizing the contact between the stylet and the plant tissues.

While the specific composition of aphid saliva varies between species (Harmel et al., 2008; Carolan et al., 2009), all aphids produce both the “gelling” and “watery”, types of saliva during the feeding process. While the functions of the different components of the aphid saliva are largely unknown, the watery saliva appears to contain factors that can trigger programmed cell death responses in the host cells. For example, a proteinaceous elicitor with a molecular mass of between 3 and 10 kD that is present in the saliva of *M. persicae* induces defence responses in Arabidopsis (de Vos and Jander, 2009). Moreover, the ectopic expression of another *M. persicae* salivary gland effector protein (Mp10) in *Nicotiana benthamiana* caused the induction of chlorosis and local cell death (Bos et al., 2010). Aphid reproduction was also reduced in the transgenic lines (Bos et al., 2010). Other elicitors of plant origin, such as oligogalacturonides, may contribute to aphid perception and trigger defence responses. Oligogalacturonides are released from plant cell walls by the action of the enzymes released by the stylet sheath and they have been implicated in the induction of defence signalling cascades (Heil, 2009).

1.4 Defence mechanisms against aphids

Plants exploit a plethora of constitutive and inducible defence mechanisms to restrict aphid feeding. These processes can alter the physiology of the aphids, resulting in reduced growth rate and survival. The constitutive defence mechanisms include traits associated with general morphology, such as wax layers, leaf thickness and the number of trichomes, which can prevent or deter aphids from settling on a plant. Plant proteins such as lectins and protease inhibitors also influence aphid fecundity (Dutta et al., 2005; Rahbe et al., 2003).

Secondary metabolites such as glucosinolates (GS) contribute to the constitutive and inducible plant defences. In the absence of insect feeding, GS and the enzymes that catalyze their hydrolysis (β-thioglucosidases or myrosinases) are spatially separated between

different tissue types. However, the enzymes and substrates are brought together when mechanical damage disrupts cellular structure (Thangstad et al., 2004). The products of GS hydrolysis have a deterrent effect on insect herbivores (Kliebenstein et al., 2005). A number of studies have shown that GS modulate resistance to aphids (Levy et al., 2005; Mewis et al., 2005). For example, aphid resistance was impaired on mutants deficient in a cytochrome P_{450} called CYP81F2, which converts indole-3-yl-methyl GS to 4-hydroxy-indole-3-yl methyl GS (Pfalz et al., 2009).

Aphid feeding triggers the release of a range of volatile compounds. Some of them play important roles in plant defence against aphids such as negatively effecting aphid reproduction (Hildebrand et al., 1993, Hardie et al., 1994). Repression of a hydroperoxide lyase that is involved in the production of volatile C6 aldehydes in transgenic potato plants resulted in increased aphid fecundity (Vancanneyt et al., 2001). The release of volatiles from plants can attract aphid predators and serve as an indirect defence against aphid infestation. For example, *M. persicae* feeding on *Solanum tuberosum* triggers the emission of a range of volatile organic compounds (Gosset et al., 2009). There is little evidence that the synthesis of volatile compounds incurs a significant metabolic cost. Rather, the available data suggest that volatile production has a low cost in terms of energy and metabolites and has little effect on plant growth and development (Fritzsche et al., 2001). Plants with constitutively high levels of volatile emissions did not show impaired growth (Lücker et al., 2001; Ohara et al., 2003).

Other forms of aphid resistance depend upon plant resistance (R) genes. These are able to recognize avirulence gene products in the pest, a process that triggers an incompatible interaction in the host, characterized by a hypersensitive response (HR) that leads to programmed cell death. The majority of the R genes encode proteins with a nucleotide-

binding site and leucine-rich region (NBS-LRR) motif. The tomato NBS-LRR gene called *Mi-1.2* confers resistance to certain biotypes of the potato aphid, *Macrosiphum euphorbiae.* It triggers an incompatible interaction in infested tomato leaves that is characterized by a rapid, local defence response that blocks or dramatically reduces aphid performance (Martin et al., 2003; Rossi et al., 1998). Similarly, the *Vat* gene encoding a cytoplasmic protein with a NBS-LRR domain, mediates resistance to the aphid *Aphis gossypii* Glover in melon. This resistance response involves the formation of microscopic lesions together with deposition of callose and lignin in the apoplast of infested leaves (Villada et al., 2009). The signalling mechanisms that are triggered in R-gene-mediated resistance responses remain poorly understood, but may include changes in calcium fluxes, the generation of reactive oxygen species (ROS), localized HR and accumulation of pathogenesis-related (PR) proteins (Smith and Boyko, 2007). R-gene-mediated resistance mechanisms result in reduced sap ingestion, suggesting that the deterrent compounds accumulate in the phloem or that the phloem is blocked in the response leading to enhanced aphid resistance. Phloem blocking mechanisms involve the deposition of callose in the sieve plates, together with the production of phloem proteins that "clog" the system. Such mechanisms have been found in both compatible (*Megoura viciae – Vicia Faba*) and incompatible (*Aphis gossypii - Cucumis melo*) interactions (Will et al., 2007; Villada et al., 2009). Sieve elements are sealed immediately following damage (Furch et al., 2007) by calcium-mediated mechanisms that involve dispersal of phloem proteins, or in the case of legumes, protein bodies termed forisomes (Knoblauch et al., 2001).

1.5 Sieve element occlusion by forisomes

Protein occlusions serve to limit the flow of phloem sap through the damaged sites and are crucial for preventing excessive loss of nutrients. The reaction of forisomes to wounding

have been extensively studied in *Vicia faba* due to the conspicuous appearance of these crystalloid proteins as spindle-like bodies under electron and confocal laser scanning microscopy (Peters et al., 2007; Behnke, 1991; Knoblauch and Van Bel, 1998). Moreover, they are easily extracted and manipulated *in vitro* which makes them a useful model system (Knoblauch et al., 2001). Even under slight injury the forisomes detach from the plasma membrane and aggregate on the sieve plates forming an extensive mesh-like structure occluding the sieve plates (Ehlers et al., 2000). The mechanical damage inflicted by the aphid stylet fails to provoke forisome dispersion and a subsequent plugging reaction in the *Megoura viciae – Vicia Faba* interaction (Will and van Bel, 2006). In contrast, an impalement by a microcapillary with a comparable tip diameter results in sieve element occlusion suggesting that aphids overcome the defence reaction of the phloem to mechanical damage and interfere with the clogging mechanism in order to secure sustained sap ingestion (Will et al., 2007). The dispersal of forsimes is mediated by increases of the calcium levels and redox perturbations (Knoblauch et al., 2001). Isolated forisomes from *V. faba* change their conformation after application of 1 mM $CaCl_2$ from a contracted to a dispersed state (Will et al., 2007). Only the forisomes in the later conformation are able to occlude the sieve tubes. In contrast, chelating agents such as EDTA sequestering Ca^{2+} has been shown to reverse the dispersal of forisomes both *in vitro* and *in vivo* (Will et al., 2007). In a similar manner aphid saliva injected in the sieve elements appears to provoke forisome dispersion and secure continuous flow of phloem sap. This effect is attributed exclusively to the calcium-chelating proteins present in the watery saliva because protease K treatment of saliva collected from *Megoura viciae* abolishes its effect on forisome conformation (Will et al., 2007).

1.6 Hormones involved in plant defence responses

While a range of constitutive and inducible defence mechanisms that restrict aphid feeding have been described, little is known about the molecular controls that underpin these systems. Over the last 10 years considerable evidence has accumulated to suggest that plant hormones play a central role in the plant response to aphid attack. Plants synthesize a plethora of hormones, including abscisic acid (ABA), auxin, cytokinins (CKs), gibberellins (GAs), salycilic acid (SA), jasmonates (JAs) and ethylene (ET). Hormone signaling pathways regulate plant growth and development, and they play crucial roles in responses to biotic and abiotic stimuli. The role of SA, JA and ET has been extensively studied in plants challenged with pathogens and herbivory. They are also important players in the plant–aphid interaction. Aphid feeding does not result in detectable increases in the tissue levels of SA, JA or ET upon *M. persicae* attack in Arabidopsis. However, hormone accumulation might be transient or occur in discrete locations, making detection difficult (de Vos et al., 2005).

1.6.1 SA signalling pathways

SA plays a key role in plant defence responses. It is required for the induction of effective defences against biotrophic and hemi-biotrophic pathogens. Pathogen-challenged plants accumulate SA and transcripts encoding pathogenesis related (PR) proteins. The SA-dependent pathways lead to the establishment of systemic acquired resistance (SAR) that facilitates enhanced resistance to a broad range of pathogens. Mutations that lead to either reduced SA production or perception enhance susceptibility to pathogens (Loake and Grant, 2007). The stealthy system of aphid feeding, together with the intimate interaction of the stylet with the intercellular spaces induce gene expression patterns that are characteristic of SA defence signalling (Moran and Thompson, 2001).

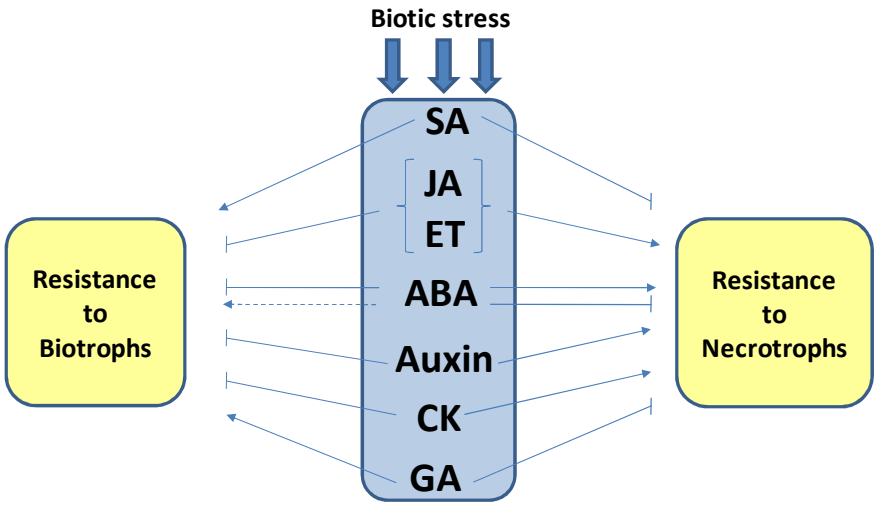

Figure 1.3 A simplified scheme outlining the role of different hormones in biotic stress responses. The levels of different hormones are altered upon biotic stress. Changes in hormone contents trigger down-stream defence responses. Alterations leading to enhanced resistance are indicated by arrows while blocked lines indicate repression of the defence response or negative interaction (Bari and Jones, 2009).

However, the evidence to show that aphid fitness is altered in plants with impaired SA defence signalling is not conclusive. Transgenic plants (*NahG*) over-expressing the bacterial salicylate hydroxylase that converts SA to catechol fail to accumulate PR proteins after pathogen attack. The decreased SA abundance in the *NahG* plants decreased the reproduction of *M. persicae* and *Brevicoryne brassicae* relative to controls (Mewis et al., 2005). However, a similar study by Pegadaraju et al. (2005) found no differences in *M. persicae* feeding between the *NahG* plants and controls. Mutants lacking the non-expressor of PR-1 (NPR-1) protein are compromised in SA defence signaling. NPR-1 is a crucial component of the SA signalling pathway. In the absence of the SA signal, NPR-1 is localized in the cytosol, where it forms oligomeric structures involving redox-sensitive disulphide bridges (Tada et al., 2008). The expression of SA responsive genes requires monomerization and movement to the nucleus. Experiments involving aphid performance on *npr1* mutants have failed to produce consistent results. Some authors have reported negative effects on *M. persicae* and *B. brassicae* while others have found no effect (Mewis et al., 2006; Moran and Thompson, 2001; Pegadaraju, et al., 2005). Aphid reproduction was not affected by feeding on mutants lacking isochorismate synthase 1, which is involved in SA biosynthesis (Pegadaraju et al., 2005). The SA responsive gene *PAD4* encodes a nucleo-cytoplasmic protein that has sequence similarities to lipases (Feys et al., 2001). While transgenic Arabidopsis plants over-expressing *PAD4* showed enhanced resistance to *M. persicae* (Pegadaraju et al., 2007), *pad4* mutants were more susceptible to aphid feeding (de Vos et al., 2007). Analysis of aphid feeding behavior in these lines showed that PAD4 is involved phloem-based defence mechanisms. *M. persicae* spent more time actively feeding from the sieve elements of the *pad4* mutants than wild-type plants. Similarly less time was spent in feeding on transgenic plants in which *PAD4* was ectopically expressed.

In plant-pathogen interactions, PAD4 functions require interaction with another protein, EDS1 (Pegadaraju et al., 2007). Aphid performance was not affected on *eds1* mutants (Pedagaraju et al., 2005) and PAD4 appears to be uncoupled from EDS1 during aphid feeding. This finding suggests that plants employ somewhat different mechanisms in responses to aphids from those employed for fungal pathogens.

1.6.2 JA/ET-dependent defence pathways

JA and ET act synergistically in plant defences against necrotrophic pathogens and herbivorous insects. Chewing insects macerate the tissue and release oral secretions in the wounded areas that activate the JA/ET dependent defence pathways. JA and ET signalling pathways are also induced in response to necrotrophic pathogens. In contrast, SA defence pathways are predominately activated in response to biotrophic pathogens. Extensive cross talk between the JA, SA and ET signalling pathways allows specificity in plant defence strategies with respect to the type of attacker. Antagonistic relationships between the SA and JA/ET defence pathways have been reported but synergistic interactions have also been found. The attackers have also evolved mechanisms that suppress inducible plant defences or modulate plant signalling networks and so they can manipulate plant defences to increase invasion or feeding. For example, aphids can activate SA-signalling pathways that tend to suppress effective JA/ET-dependent resistance (Kempema et al., 2007). Resistance to *M. persicae* in Arabidopsis appears to be jasmonate-dependent. Jasmonate-treated plants support significantly less aphids than untreated controls (Ellis et al., 2002). The *cev1* mutant that constitutively expresses JA and ET responsive genes displays enhanced resistance to aphids (Ellis et al., 2002). In contrast, aphid reproduction was enhanced in the JA-insensitive *coi1* mutant compared to the wild type (Mewis et al., 2006). *COI1* encodes an F-box protein, which physically interacts with JA ZIM-domain (JAZ) transcriptional

repressors of the JA response (Chini et al., 2007). Isoleucine JA conjugates promote interactions between JAZ proteins and the SCF^{coi1} ubiquitin ligase, leading to JAZ degradation via the 26S proteasome leaving transcription factors that were previously inactivated by the JAZ repressors free to stimulate JA dependent gene expression.

1.6.3 ABA-mediated plant responses

1.6.3.1 Effects of ABA on whole plant physiology

Essentially, ABA is a stress hormone that mediates plant responses to adverse environmental conditions and enhances plant survival under stresses, such as drought, salinity and low temperature that can severely affect plant growth and development (Shinozaki et al., 2003). During the onset of drought, for example, ABA accumulates rapidly in the root tissues and is being transported aboveground via the xylem elements of the vasculature (Jiang and Hartung, 2008; Sauter et al., 2001). In the shoot ABA exerts it role mainly by affecting the activities of the stomata and the shoot apical meristem (Zhang and Davies, 1991; Davies et al., 2005). The accumulation of the imported ABA in the shoots is not a prerequisite for its action. For example, in phosphorous deficient plants ABA is rapidly degraded following its action on the stomata (Jeschke et al., 1997). The vital role of the roots in ABA synthesis has been shown in transgenic plant unable to accumulate wild type levels. Plants deficient in ABA synthesis fail to close their stomata and as a result could not limit the transpirational stream resulting in increased sensitivity to drought (Borel et al., 2001). The grafting of roots from these plants onto plants able to synthesis ABA was not sufficient to restore the normal stomatal behavior. Whereas the response of the guard cells to ABA is rapid and characterized by high sensitivity, the effect of increased ABA levels on the shoot meristems require more time ultimately resulting in impaired growth and development (Dodd and Davies, 1994). Additional long-term changes

in the shoot include synthesis of waxes, thorn and hair formation (Trewavas and Jones, 1991). Apart from mediating acclimation to harsh environmental conditions, ABA actively regulates numerous developmental processes. The ABA seed content is crucial for the dormancy release and seed maturation (Finch-Savage and Leubner-Metzger, 2006). Moreover, exogenous ABA application inhibits root growth by arresting the mitotic cell activity (Robertson et al., 1990). Taken together these findings pinpoint the role of ABA as a negative regulator of plant growth.

1.6.3.2 ABA as an essential component of plant immunity

Hormonal pathways governing plant responses to biotic and abiotic stresses are interconnected in a complex network of synergistic and antagonistic interactions. ABA is emerging as an important component of plant immunity. For example, ABA deficiency in tomato mutants enhanced susceptibility to the lepidopteran insect *Spodoptera exigua* (Thaler and Bostock, 2004). ABA negatively regulates SA-mediated signal transduction pathways and so modulates plant immunity. The application of ABA or the imposition of drought stress increased the susceptibility of Arabidopsis to the avirulent pathogen *Pseudomonas syringae* pv. *tomato* (Mohr and Cahill, 2003). Similarly, resistance to the rice blast fungus *Magnaporthe grisea* was suppressed following ABA application or low temperature treatment. The inhibition of ABA biosynthesis abolished the low temperature-mediated increase in susceptibility to *M. grisea*, suggesting that the accumulation of ABA plays an important role in modulating the outcome of the infection (Koga et al., 2004). The imposition of salt stress can also suppress SA-mediated signal transduction and alter disease susceptibility. Pretreatment with NaCl compromised SA accumulation and abolished resistance to *P. syringae* induced by two biologically active SA analogues, BHT (benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester) and BIT (1,2-benzisothiazol-

3(2H)-one1,1-dioxide: Yasuda et al., 2008). BHT and BIT act up-stream and down-stream of SA biosynthesis respectively in the induction of SAR in Arabidopsis. Pre-treatment with ABA suppressed SA accumulation and abolished SAR-dependent limitations on growth in *P. syringae*. Interestingly, the induction of SAR involved the NPR1-dependent suppression of genes involved in ABA biosynthesis and signalling (Yasuda et al., 2008).

1.6.3.3 ABA signalling via ABA INSENSITIVE 4 (ABI4)

ABI4, a nuclear localized APETALA2-type transcription factor, was originally characterized as an important factor in ABA signalling (Finkelstein et al., 1998). ABI4 is involved in the transduction pathways regulating the expression of nuclear genes in response to signals originating from organelles such as the chloroplasts, in the process called retrograde signalling. A role of ABI4 in plastid-derived retrograde signalling has been described (Koussevitzky et al., 2007). In addition, ABI4 has been implicated in the regulation of the expression of *ALTERNATIVE OXIDASE1a*, a marker for the mitochondrial retrograde response (Giraud et al., 2009) suggesting a link between mitochondrial and chloroplast retrograde signals. Roles for ABI4 in sugar and nitrogen signalling pathways, plant development and defence have also been demonstrated (Yang et al., 2011; Bossi et al., 2009; Kaliff et al., 2009; Signora et al., 2001).

1.7 Effects of aphid feeding on plant growth

Aphids negatively influence plant growth (Cuperus et al., 1982). Aphid-dependent limitations on growth can sometimes be accompanied by visible symptoms (phytotoxicoses) but also show no other visible phenotypic effects (Mittler and Sylvester, 1961). Growth reductions observed with phytotoxicoses are usually associated with the toxic effects of injected saliva that accompanies the uptake of photoassimilates, whereas the

absence of visible symptoms is believed to result only from the removal of photoassimilates (Miles, 1989). It is generally accepted that aphids act as external "sinks" for photoassimilates while feeding passively on the phloem sap. The amount of assimilates taken by the aphids can be calculated indirectly based on an estimation of honeydew secretion. Aphids (*Drepanosiphum platanoides*) feeding on sycamore trees caused a reduction in leaf size, which was greater than would be expected from the negative relationship between leaf area and the quantity of removed nutrients (Dixon, 1971). Barlow et al. (1977) showed that pea aphid infestation (0.46 pea aphid per cm^2) on young pea plants removed photoassimilates at a rate comparable to that of primary production, leading to a decrease in surface area of 54% after 10 days. If the infestation is of sufficient density, the newly formed sink can effectively compete for resources with existing plant sinks and so influence the partitioning of assimilates between different plant organs. Several studies have demonstrated that assimilate partitioning between shoots and roots is altered by aphid feeding (Wu and Thrower, 1981; Veen, 1985). Girousse et al. (2005) studied the effect of pea aphid infestation on stem elongation rates in alfalfa. This study showed that only a part of the reduction in the stem elongation rate could be explained by the assimilate withdrawal. Moreover, aphids were able to re-program plant growth by affecting apical sink strength and triggering systemic signalling pathways.

1.8 Effects of aphid feeding on plant metabolism

It has been suggested that aphids can alter plant metabolism for their own benefit. For example, increases in the levels of essential amino acids were observed in stylets and leaf exudates following *Diuraphis noxia* feeding on a susceptible wheat variety but not on a resistant one (Telang et al., 1999). Similar changes were observed with *Schizaphis graminum* but not with *R. padi* (Sandström et al., 2000). Heavy aphid infestations can cause

the conversion of plant apical sinks to source tissues. This in turn can impact on nitrogen metabolism (Girousse et al., 2005). For example, *M. persicae* feeding on potato induced increased glutamate dehydrogenase and glutamine synthetase activities in the vicinity of the feeding sites. The induction of glutamine synthetase in response to aphid feeding has been observed in local and systemic leaves (Giordanengo et al., 2010). The abundance of transcripts encoding glutamine synthetase was increased in the phloem of celery infested by *M. persicae* (Divol et al., 2005). Glutamine synthetase plays a key role in primary nitrogen assimilation and in nitrogen remobilization, whereas glutamate dehydrogenase is important in the recycling of ammonium released during protein catabolism (Tercé-Laforgue et al., 2004). Infestation with *Myzus nicotianae* enhanced the expression of a gene encoding glutamate synthase in *Nicotiana attenuata* (Voelckel et al., 2004). However, aphid reproduction rates were not changed by feeding on Arabidopsis mutants deficient in the amino acid transporter ATN1, which have very high phloem amino acid contents relative to the wild type plants (Hunt et al., 2006). Similarly, the lower phloem amino acid content resulting from a mutation in the amino acid permease *AAP6* had no significant effects on aphid feeding or reproduction (Hunt et al., 2010). The influence of phloem sugar content on aphid feeding behaviour were illustrated by studies on transgenic potato plants with antisense expression of the sucrose-H^+ symporter, *StSUT1* (Pescod et al., 2007). The lower sucrose content of the phloem in the StSUT1 antisense plants had no effect on the feeding behaviour or performance of either *M. persicae* or *Aulacorthum solani*. In contrast, *M. euphorbiae* was not able to locate the sieve tubes of the plants with low *StSUT1* expression and these aphids withdrew their stylets prematurely (Pescod et al., 2007).

1.9 *Buchnera aphidicola:* the primary aphid symbiont

The high concentrations of sugars and the low (insufficient) amounts of essential amino acids in the phloem sap (Douglas, 1993) make this an unbalanced diet. Aphids harbour the obligate endosymbiotic coccoid γ-proteobacterium *Buchnera aphidicola*, which can supplement essential amino acids intake (Douglas, 1998). A unique feature of *Buchnera* is their large cell size, which ranges from 2 to 4 μm between aphid species (Mira and Moran, 2002). A special type of hypertrophied cells (mycetocytes) in which a significant amount (approximately 5% of the aphid's biomass) of *Buchnera* resides, accommodates the endosymbionts in the hemocoel of the aphid (McLean and Houk 1973; Whitehead and Douglas, 1993; Buchner, 1965; Douglas, 1998). In a single *Acyrthosiphon pisum* adult for example can be found between 60 and 100 mycetocytes (Wilkinson and Douglas, 1998). The mycetocytes expand as the proliferation rates of the bacteria increase but this is rarely accompanied with division (Baumann and Baumann, 1994). *Buchnera* are transmitted from one generation to the next so that the bacterial population in the offspring originates from a single maternal mycetocyte (Mira and Moran, 2002).

As a result of the strictly symbiotic life style the genome of *Buchnera* has undergone a significant reduction due to degradation and vertical transmission and is significantly smaller than the genome of other free living γ-proteobacteria (Moran, 2003). For example, it only represents around 15% of the *Escherichia coli* genome reflecting the dependence of *Buchnera* on the aphid host (Blattner et al., 1997). Many genes essential for free living life such as defence related genes and genes involved in the synthesis on non-essential amino acids and biosynthesis of cell wall are absent from the genome of *Buchnera* (Pérez-Brocal et al., 2006; Shigenobu et al., 2000). However, genes important for the replication, transcription and translation machineries have been preserved together with sequences

encoding enzymes synthesizing essential amino acids (Pérez-Brocal et al., 2006; Tamames et al., 2007). These essential amino acids are crucial for aphid success as in the absence of *Buchnera* both nymph development and fecundity are significantly impaired (Ishikawa, 1998; Douglas, 1996). *Buchera* readily release the synthesized amino acids to the aphid host in exchange for nutrients and growth environment provided by the insect (Birkle et al., 2002).

In contrast to the high proportion of essential amino acids found in aphid proteins (approximately 50%), on average only one tenth of the amino acids present in the phloem flow cannot be synthesized by the aphids (Sandström and Pettersson, 1994). With the exception of methionine, all other essential amino acids synthesized by *Buchnera* exceed aphids' dietary requirement and allow them to feed on phloem sap (Gündüz and Douglas, 2009). Methione shortage could be overcome by the availability of 5-methylmethionine in the digested sieve element content, which can be utilized by the aphid (Gündüz and Douglas, 2009).

1.10 Osmoregulation in aphids

Aphids feed exclusively on phloem sap with sugars, potassium and amino acids being the main osmotic component. The high osmotic pressure of the phloem sap requires a sophisticated mechanism for osmoregulation within the insect body that prevents desiccation (Douglas, 2006). Its high sugar content, sometimes exceeding 1 M, results in an osmotic pressure up to 5 fold higher than the osmotic potential of the aphid body fluids (Rhodes et al., 1997; Kehr et al., 1998). The resulting osmotic gradient is sufficient to dehydrate aphids as a consequence of water loss from body fluids in the gut (Ashford et al., 2000). Aphids overcome this obstacle by actively metabolising the main osmotic component of the phloem content (sucrose) by converting it first to glucose and fructose

and subsequently to glucose-based oligosaccharides thus reducing the overall molarity of the ingested sap (Wilkinson et al., 1997; Walters and Mullin, 1998). The enzymatic activities of the aphid midgut are crucial for maintaining a physiological osmotic potential and avoidance of dehydration. Sucrose hydrolysis is mediated by sucrase that possesses an α-glucosidase activity (Price et al., 2007). Subsequently, part of the hexoses (mainly fructose) is absorbed through the gut wall and the glucose is used to synthesize oligosaccharides via a transglucosidase activity (Ashford et al., 2000). The resulting oligosaccharides are excreted with the honeydew which has an osmotic pressure comparable to that of the aphid haemolymph, clearly showing that dietary sucrose present in the phloem sap undergoes a comprehensive metabolic rearrangement in the gut (Douglas, 2006). Furthermore, there is a positive correlation between the dietary sucrose concentration and the amount of oligosaccharides found in the honeydew.

An alternative mechanism of osmoregulation is the active sucking of xylem sap that possesses significantly lower osmotic pressure than phloem content, which is mainly reported following periods of starvation (Powell and Hardie, 2002; Ramirez and Niemeyer, 2000). The ingestion of xylem sap is sometimes referred as ‘drinking’ and described as a rehydration mechanism (Pettersson et al., 2007). The significance of rehydration in starved aphids has been shown with *R. padi* feeding on wheat treated with a sublethal dose of neonicotinoid thiamethoxam (Daniels et al., 2009). The systemic movement of this insecticide occurs via the xylem and aphid feeding on treated plants showed impaired xylem sap uptake. As a result *R. padi* exhibited reduced body water content, growth and reproduction.

1.11 Oxidative signalling

Reactive oxygen species are generated continuously in plant metabolism and they are produced by numerous biological processes. The transfer of energy or electrons to ground state molecular oxygen during metabolism can lead to the production of much more reactive oxygen forms such as singlet oxygen, superoxide, hydrogen peroxide and the hydroxyl radical (Fig. 1.4; Cadenas, 1989). The high reactivity of ROS means that they are not generally compatible with the efficient operation of cellular functions as they rapidly react with cellular components. However, their high reactivity also makes them powerful signalling molecules. For many years the concept of 'oxidative stress', considered only aspects related to the potential harm caused by the production and accumulation of reactive oxygen species such as the irreversible oxidation of proteins, lipids and DNA. However, this concept has largely been superseded by the concept of oxidative signalling in which ROS play multifaceted roles in the regulation of a diverse range of processes including growth, development and defence (Mittler et al., 2011; Monshausen et al., 2007). The different ROS forms are considered to participate in the oxidative modifications of proteins and other components that act as signaling molecules to influence gene expression patterns in specific ways in response to biotic and abiotic stresses.

1.11.1 Hydrogen peroxide

Hydrogen peroxide is the least reactive of the ROS forms. It is generated in multiple cellular compartments such as chloroplast, peroxisome, and plasma membrane (Apel and Hirt, 2004). Membranes can restrict the movement of these molecules and hence they can be compartmentalized in vesicles, which can be transported in a targeted manner (Leshem et al., 2006). Hydrogen peroxide is considered to be involved in long-distance stress signalling cascades. Although the role of hydrogen peroxide as a mobile signal remains controversial, ROS might be continuously generated along signalling pathways.

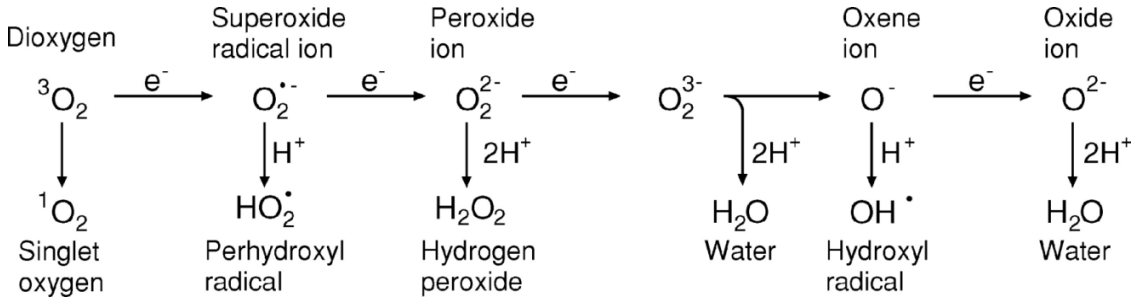

Figure 1.4 A scheme depicting the generation of different ROS forms by energy transfer or reduction of ground state triplet oxygen (Apel and Hirt, 2004).

Alternatively, a mobile signal might be induced by hydrogen peroxide at the point of stress perception and this signal may then lead to the generation of hydrogen peroxide at the point of arrival. Hormones such as ABA induce hydrogen peroxide production in response to abiotic stresses. ABA is an attractive candidate for the mobile signal that can travel long distances during plant stress responses (Zhang et al., 2007). ABA is translocated via the xylem from the roots to the shoots and its movement is not restricted by apoplastic barriers (Jiang and Hartung, 2008).

1.11.2 Singlet oxygen

By virtue of its high reactivity, singlet oxygen was only considered for many years as a highly toxic molecule with limited diffusion. However, it is now well established that singlet oxygen is involved in signalling and it is considered that it might diffuse significant distances from the site of its production and even move between cellular compartments (Kim et al., 2008; Fischer et al., 2007). The most significant source of singlet oxygen production in plants is the photosystem (PS) II reaction centre of the photosynthetic electron transport chain in the chloroplasts. Singlet oxygen is predominantly produced by excited triplet-state chlorophyll molecules in PSII that can transfer energy to O_2 modifying its electron configuration to produce highly reactive singlet oxygen. Global transcriptome analysis of the conditional fluorescent (*flu*) mutant that accumulates singlet oxygen in the light, revealed a distinct signalling fingerprint (Laloi et al., 2006). The specificity of singlet oxygen signalling is difficult to establish unequivocally because it can be rapidly converted to other ROS forms such as hydrogen peroxide following reaction with ascorbate (Kramarenko et al., 2006).

1.11.3 Superoxide and hydroxyl radicals

Superoxide molecules rapidly undergo a dismutation reaction in aqueous solution, making the diffusion of superoxide over significant distances unlikely except within a lipid environment. Moreover, various forms of the enzyme superoxide dismutase (SOD) are present in most cell compartments. In the presence of transition metal ions superoxide radicals can reduce hydrogen peroxide to form highly reactive hydroxyl radicals. Hydroxyl radicals are highly reactive and will react immediately with multiple cell components in the vicinity of the site of production. While hydroxyl radicals essentially cannot move large distances, the limited diffusion of the hydroxyl radical has been implicated in oxidative signalling. Moreover, hydroxyl radical generation is important in cell wall metabolism (Müller et al., 2009). The high reactivity of the hydroxyl radical and its lack of diffusion ability may be overcome by highly targeted and timed production in order for signalling events to take place.

1.12 Control of cellular redox homeostasis

Cellular redox homeostasis is central to the regulation of plant metabolism, and the control of development, growth, and defence responses against biotic and abiotic stresses (Foyer and Noctor, 2011). The extent of ROS accumulation is controlled by the plant antioxidant network, which involves the concerted action of low molecular antioxidants and antioxidant enzymes. The antioxidant network limits the accumulation of ROS and facilitates transient and spatial variations of ROS signalling (Foyer and Noctor, 2009; Chaouch et al., 2010).

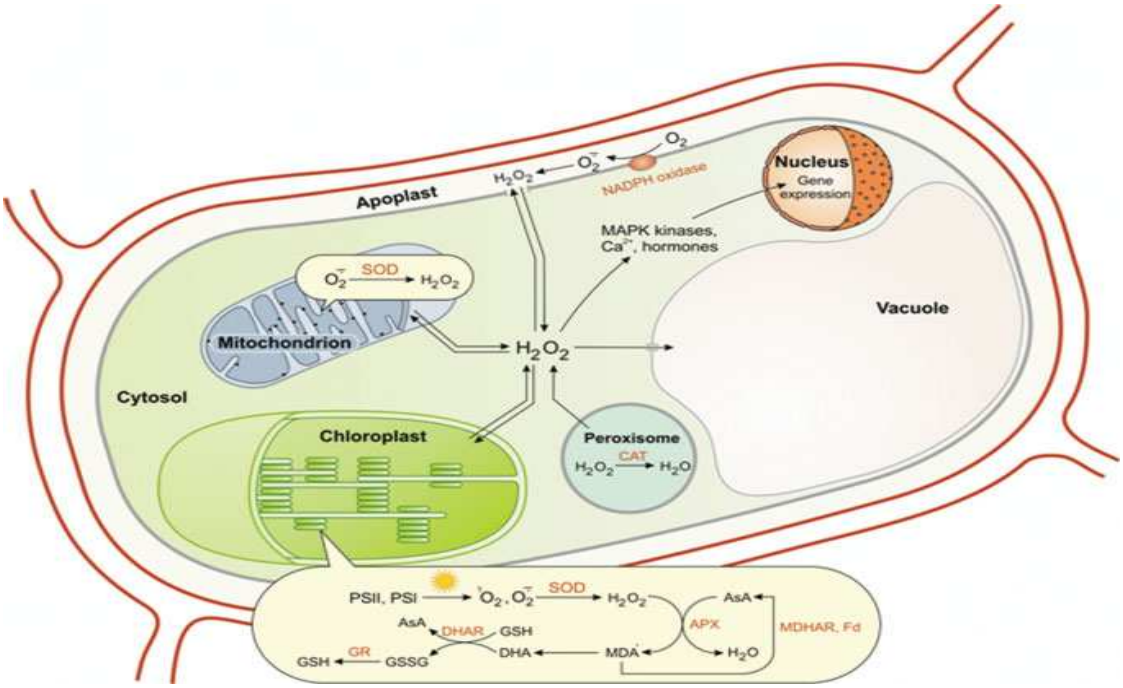

Figure 1.5 A scheme illustrating major sources of ROS generation and enzymatic systems maintaining ROS homeostasis in the plant cell (Genchev et al., 2006). The primary sources of ROS generation in the photosynthetic plant cells are Photosystem II (PSII) and Photosystem I (PSI). In mitochondria superoxide anion ($O^{-\cdot}_2$) is generated by the activity of the mitochondrial electron transport chain. Superoxide dismutases (SOD) convert the superoxide produced in both organelles to hydrogen peroxide (H_2O_2), which is subsequently neutralized by ascorbate peroxidases (APX) activity. APX requires ascorbate (AsA) which is oxidized to monodehydroascorbate (MDA) and then recycled in the ascorbate-glutathione cycle in a series of enzymatic reactions requiring monodehydroascorbate reductase (MDHAR), reduced ferredoxin (Fd), dehydroascorbate reductase (DHAR), glutathione reductase (GR) and reduced (GSH) and oxidized glutathione (GSSG). Substantial amounts of H_2O_2 are generated during photorespiration and fatty acid oxidation in the peroxisomes and detoxified by catalases (CAT). An important source of superoxide anion is the NADPH oxidase activity in the apoplast.

Signal strength depends on the rates of ROS production, removal or compartmentation and the presence of ROS receptors, or receptors for molecules that are modified as a result of interaction with ROS. Many components of the elaborate plant antioxidant system such as thioredoxins, ascorbate, glutathione and peroxiredoxins also participate in redox signal transduction. The participation of these molecular antioxidants in redox signal transduction pathways facilitates a large repertoire of specific signalling pathways, allowing transient, directional and spatial variations (Foyer and Noctor, 2009; Chaouch et al., 2010).

Glutathione and ascorbic acid are the major water soluble antioxidants of plant cells. They accumulate to milimolar concentrations in plant tissues. In the removal of ROS, reduced glutathione (GSH) is oxidized to glutathione disulphide (GSSG), and ascorbate is oxidized firstly to monodehydroascorbate (MDA) and then dehydroascorbate (DHA). The ascorbate and glutathione pools are interconnected through the ascorbate-glutathione cycle (Foyer and Noctor, 2011). The antioxidants are maintained largely in their reduced forms in the ascorbate-glutathione cycle in plant cells by the enzymes glutathione reductase (GR), monodehydroascorbate reductase (MDAR), and dehydroascorbate reductase (DHAR). The ascorbate-glutathione cycle functions alongside catalases (CAT), superoxide dismutases (SOD), ascorbate peroxidases (APX), and thiol (glutathione) peroxidases (GPX) to control cellular hydrogen peroxide levels. APX uses ascorbate to detoxify hydrogen peroxide generating MDA and DHA. Ascorbic acid is subsequently regenerated in the ascorbate-glutathione cycle.

The levels of GSH and ascorbate in plant cells are responsive to environmental stimuli and metabolic cues (Foyer and Noctor, 2005). Situations that lead to severe oxidative stress often have a more profound impact on the cellular GSH/GSSG ratio than on the redox status of the ascorbate pool. Glutathione has been suggested to be a key transmitter of

intracellular ROS signals (Mhamdi et al., 2010). Low GSH/GSSG ratios can cause significant redox modifications to target proteins, whereas no similar mechanisms are known at present that link changes in ascorbate-DHA ratios directly to modifications in signalling proteins (Palmieri et al., 2010; Holtgrefe et al., 2008; Foyer and Noctor, 2011). However, changes in ascorbate levels can have a profound effect on hormone signalling pathways (Kerchev et al., 2011; Pavet et al., 2005).

1.13 Interactions between ROS and hormone signalling pathways

Evidence for links between plant hormones, ROS and redox signalling has been found in numerous plant responses to environmental stimuli as well as in the control of growth and development (Mittler et al., 2011; Tognetti et al., 2011; Hu et al., 2007). Redox-modulated signalling events are now considered to be an integral part of the signalling pathways triggered by hormones such as auxin, ethylene, ABA, SA and JA (Fig. 1.6). For example, ROS signals are amplified by SA in a positive feedback manner (Shirasu et al., 1997; Straus et al., 2010). Hydrogen peroxide and SA are thought to act in a co-operative manner to modulate photoperiod signalling. When Arabidopsis mutants that are deficient in the major leaf form of catalase (*cat2*) were grown under short day conditions the leaves had elevated levels of glutathione and defence genes (Queval et al., 2007). Conversely, under long day growth conditions, hydrogen peroxide signalling led to cell death in a strictly SA dependant manner (Queval et al., 2007). Components of the ethylene and SA signalling pathways such as EDS1 and PAD4 that are important in plant pathogen responses are linked to ROS signalling (Mateo et al., 2004). Singlet oxygen induces the peroxidation of polyunsaturated fatty acids present in the chloroplast membranes to produce signalling molecules termed phytoprostanes (Thoma et al., 2003).

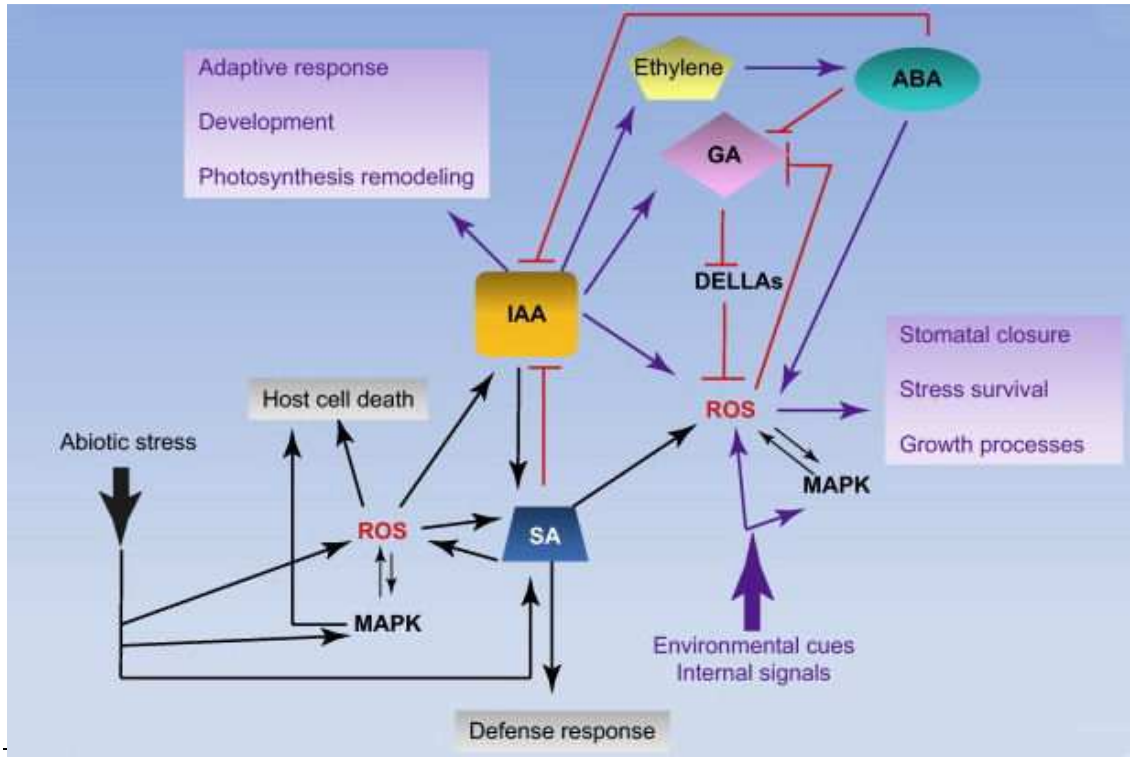

Figure 1.6 Interactions between ROS and hormone signalling pathways in the regulation of plant growth and defence. With black arrows are depicted mechanisms triggered by abiotic stresses, whereas purple lines represent activation initiated by environmental cues and internal signals. Repression is shown with red arrows. Plant hormones and signaling components are abbreviated as follows: ABA, abscisic acid; GA, gibberellins; IAA, auxins; MAPK, mitogen-activated protein kinase; ROS, reactive oxygen species; SA, salicylic acid (Mittler et al., 2011).

They are distinct from the oxidation products arising from the enzymatic oxidation of lipids, such as JA and together with them are collectively known as oxylipins (Blée, 2002). JA is an oxylipin that is synthesized in the chloroplast from linolenic and linoleic acid by the enzyme lipoxygenase (Feussner and Wasternack, 2002). When the *flu* mutant is exposed to light, 13-hydroxyoctadecatrienoic acid, an intermediate in JA synthesis, is accumulated in the leaves and this may be an intermediate in the signal transduction process (Przybyla et al., 2008).

1.14 Modulation of redox homeostasis during plant–aphid interactions

The hypersensitive response (HR) is an important part of plant responses to pathogens. HR induces programmed cell death that serves to limit the spread of the pathogen. HR involves an apoplastic oxidative burst that is characterized by ROS production in the apoplast by plasma membrane-bound NADPH oxidases and cell wall peroxidases (Torres et al., 2002; Chen and Schopfer, 1999). HR-like responses that are characterised by small lesions in the immediate vicinity of stylet penetration have been also observed in plant-aphid interactions (Villada et al., 2009; Lyth, 1985; Belefant-Miller et al., 1994). The growth rates of *M. persicae* populations are approximately 4 times faster on the Arabidopsis *respiratory burst oxidase homolog D* (*RbohD*) mutants that lack a functional NADPH oxidase than on wild-type plants (Miller et al., 2009). Such observations suggest a key role for NADPH oxidase-dependent ROS production in orchestrating the plant defence response to aphids. The production of ROS during the aphid-induced oxidative burst could be involved in the induction of localised programmed cell death and also in the initiation of signalling events leading to expression of defence genes in the cells surrounding the lesions. ROS production can involve different sites that are physically and temporally localized across the cellular compartments. In some concepts, the ROS signals are considered to be amplified in

signalling waves (Mittler et al., 2011). Superoxide and hydrogen peroxide produced on the plasma membrane and in the apoplast may move into the cytosol and alter cellular redox homeostasis. The impact of enhanced ROS production on cellular redox homeostasis is modulated by the antioxidative system. Enhanced cellular oxidation will cause oxidative modifications in plasma membrane proteins and ion-channels that might play an important role in signalling (Demidchik et al., 2010). Moreover, the transient redox gradient across the plasma membrane can also potentiate secondary events such as calcium release (Mori and Schroeder, 2004).

Evidence from microarray studies also suggests that ROS play important roles in the initial stages of plant–aphid interactions (Kuśnierczyk et al., 2008; Gutsche et al., 2009b). Numerous transcripts encoding enzymes involved in ROS production and detoxification are up-regulated early in the plant response to aphid infestation (de Vos et al., 2005; Couldridge et al., 2007). For example, a study on the transcriptomic response of Arabidopsis leaves to infestation by the cabbage aphid (*B. brassicae*) revealed that ROS responsive genes were among the most strongly represented class (Kusnierczyk et al., 2008). These included genes encoding hydrogen peroxide-generating copper amine oxidase and NADPH oxidase (Kusnierczyk et al., 2008). Such data suggest the presence of redox signalling events that result from alterations in cellular redox status induced by aphids. The induction of ROS responsive genes occurred in the absence of hydrogen peroxide accumulation in the infested leaves (Kusnierczyk et al., 2008). This may suggest that signalling occurs through increased flux through ROS metabolizing pathways rather than hydrogen peroxide accumulation. The production of H_2O_2 might also be transient and restricted to the cells surrounding stylet penetration site. However, rapid H_2O_2 accumulation has been observed in a resistant wheat line following infestation by the

Russian wheat aphid. H_2O_2 accumulation was observed within 3 h of the onset of aphid attack in the resistant but not the susceptible wheat line (Moloi and van der Westhuizen, 2006). Hydrogen peroxide accumulation in the resistant cultivar was accompanied by an increase in NADPH oxidase activity suggesting a link between these parameters (Moloi and van der Westhuizen, 2006). The view that ROS play an important role in activating plant defences to aphids is also supported by studies involving the inhibition of NADPH oxidase activity by diphenylene iodonium (DPI), which abolished ROS accumulation and the activation of defence genes following aphid attack (Moloi and van der Westhuizen, 2006). Xantine oxidase (XO), which is a key enzyme in purine degradation that produces superoxide, has also been implicated in aphid-triggered ROS accumulation. Pre-treatment of wheat with the XO inhibitor allopurinol substantially inhibited the aphid-induced accumulation of hydrogen peroxide, chitinase and peroxidase activities in resistant lines (Berner and Van der Westhuizen, 2010).

1.15 Ascorbic acid

1.15.1 Ascorbic acid biosynthesis in plants

The isolation and characterisation of Arabidopsis mutants impaired in ascorbate synthesis and accumulation was instrumental in the identification of the pathway of ascorbate biosynthesis (Kotchoni et al., 2009; Colville and Smirnoff, 2008). The major pathway for ascorbate biosynthesis in Arabidopsis leaves is the GDP-mannose pathway (Hancock and Viola, 2005) although other different pathways occur in different plant organs (Fig. 1.7). The GDP-mannose pathway also delivers intermediates for the synthesis of cell wall polysaccharides and glycoproteins containing D-mannose, L-fucose and L-galactose (Smirnoff, 2000). The importance of the GDP-mannose pathway for ascorbate biosynthesis in Arabidopsis leaves has been demonstrated in mutants that lack GDP-L-galactose

phosphorylase (VTC2 and VTC5), which are embryo lethal. These mutants grow only if supplemented with ascorbate (Dowdle et al., 2007). The low levels of ascorbic acid in the *vtc* (*vitamin C*) defective mutants results in slow shoot and root growth (Velojovic-Jovanovic et al., 2001). They accumulate only 10-25% of wild-type ascorbic acid, constitutively express PR genes and have elevated resistance against bacterial pathogens (Pavet et al., 2005; Mukherjee et al., 2010). The leaves of the *vtc* mutants have higher levels of ABA and they show altered patterns of gene expression particularly of transcripts encoding ABA signalling components (Kerchev et al., 2011; Pastori et al., 2003; Kiddle et al., 2003). Other steps of the GDP-mannose pathway have also been identified. The *vtc1* mutant harbours a point mutation in GDP-Man pyrophosphorylase and has about 30% of the leaf ascorbate found in Col0 (Conklin et al., 1999). The *vtc4* mutants have a defect in L-Gal-1-phosphate phosphatase (Laing et al., 2004) and have about half the leaf ascorbic acid of Col0 (Conklin et al., 2006).

1.15.2 Vitamin C Defective 2 (VTC2)

The *vtc2* mutation affects the ascorbate biosynthetic enzyme GDP-L-galactose phosphorylase/L-Galactose guanylyltransferase (Linster and Clarke, 2008). Four different alleles of the *VTC2* gene have been described (Linster and Clarke, 2008). The *vtc2-1* allele has a G to A change and is an intron-splice mutant. The *vtc2-1* mRNA is spliced one base pair later introducing a premature stop codon, shortening the protein product with 1/3 of the wild-type length. The transcript abundance of *vtc2-1* is significantly reduced to about 20% that of the wild type, most probably as a result of nonsense-mediated mRNA decay (Muller-Moule et al., 2008). The enzyme violaxanthin deepoxidase, which plays an important role for photoprotection by the xanthopyll cycle, requires ascorbate as a cofactor. The *vtc2* mutants are more susceptible to high light than the wild type plants because of

impaired accumulation of the xanthophyll pigments, zeaxantin and anthocyanin that are involved in thermal energy dissipation (Giacomelli et al., 2006).

1.15.3 Physiological roles of ascorbic acid in plants

Ascorbic acid is the major water soluble antioxidant in plants. However, its function goes far beyond its antioxidant activities and ROS detoxification (Foyer and Noctor; 2011; Olmos et al., 2006; Gilbert et al., 2009). The ascorbate pool is predominantly reduced in leaves under stress-free conditions. Oxidation of the ascorbate pool leading to decreases in the ascorbate-DHA ratio reflects an enhanced oxidative load. The ascorbate-DHA ratio is often considered to be a redox-status indicator. Current evidence suggests that the apoplast is the site of ascorbate degradation (Green and Fry, 2005). The apoplast contains the enzyme ascorbate oxidase (AO), which oxidizes ascorbate to DHA. The apoplastic ascorbate-DHA ratio is lower than that of the cytosol (Foyer and Noctor, 2011). The apoplastic ascorbate pool is important in the control of cell growth and expansion (Kärkönen and Fry, 2006).

The abundance of ascorbate in leaves and other tissues is also an important regulator of many pathways because ascorbate is an enzyme cofactor. In addition to violaxanthin deepoxidase, ascorbate is a cofactor for the 2-oxoacid-dependant dioxygenase (2ODD) enzymes that are involved in the biosynthesis of hormones such as ABA, GA, and ethylene (Arrigoni and Tullio, 2002; Mirica and Klinman, 2008). The pathways of anthocyanin, flavonoid and glucosinolate biosynthesis also require ascorbate (Turnbull et al., 2004).

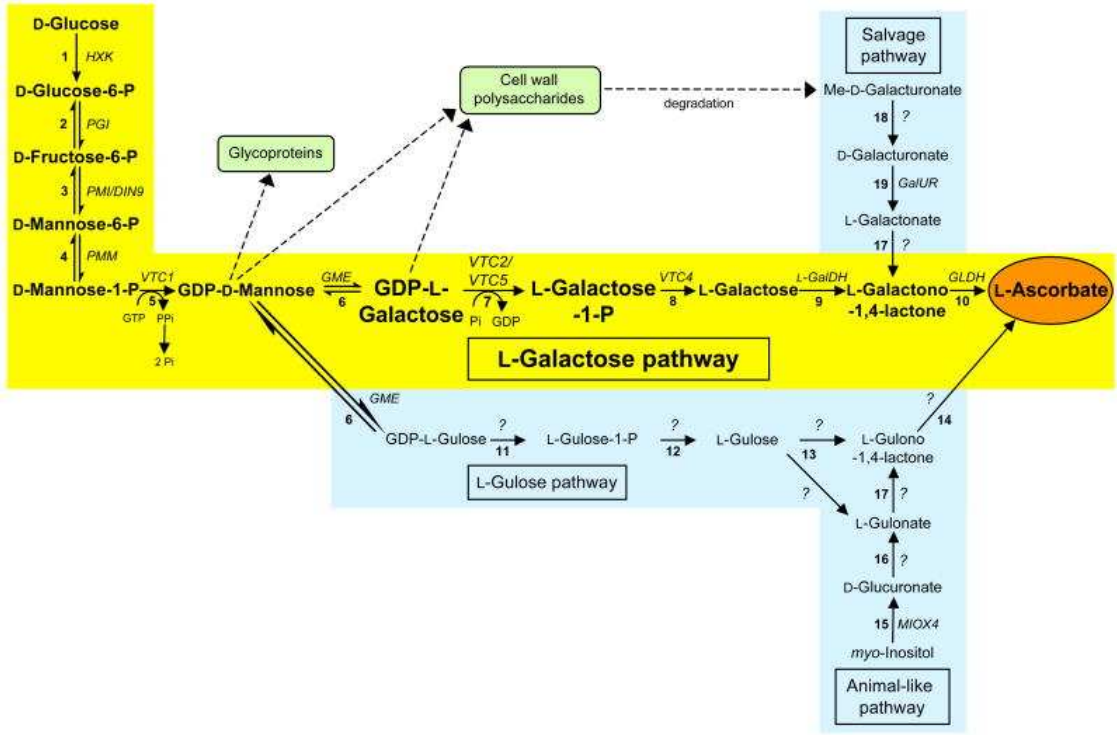

Figure 1.7 Biosynthesis of L-ascorbate in higher plants. The major L-galactose pathway is shown in yellow while alternative routes are shown in blue (Linster and Clarke, 2008). Genes encoding enzymes experimentally proven to catalyze reactions from the pathway are shown in italics. Putative reactions mediated by unknown enzymes are designated with question marks. Enzymes catalyzing the reactions are as follows: HXK, hexokinase; PGI, phosphoglucose isomerase; PMI, phosphomannose isomerase; PMM, phosphomannomutase, VTC1, GDP-D-mannose pyrophosphorylase; GME, GDP-D-mannose 3′,5′-epimerase, VTC2/VTC5, GDP-L-galactose phosphorylase, VTC4, L-galactose-1-P phosphatase, L-GalDH, L-galactose dehydrogenase; GLDH, L-galactono-1,4-lactone dehydrogenase, 11, nucleotide pyrophosphatase or sugar-1-P guanylyltransferase; 12, sugar phosphatase; 13, sugar dehydrogenase; MIOX4, L-gulono-1,4-lactone dehydrogenase/oxidase; 15, *myo*-inositol oxygenase; 16, uronate reductase; 17, aldonolactonase; 18, methylesterase; GaIUR, D-galacturonate reductase.

1.15.4 Ascorbic acid biosynthesis in insects

The role of ascorbic acid in insect physiology has not received great attention and the majority of studies in this regard have explored ascorbate function as a dietary antioxidant. Much uncertainty remains concerning the ability of insects to synthesize ascorbic acid. The silk moth (*Bombyx mori*) can convert mannose to ascorbic acid (Lipke and Fraenkel, 1956). The activities of ascorbate synthesizing enzymes that can convert hexoses to ascorbate were found in the housefly (*Musca domestica*) (Briggs, 1960). However, a more comprehensive study failed to detect ascorbate synthesis activities in nine insect species, including the silk moth (Gupta et al., 1972). Numerous studies on the optimal ascorbate concentrations required for artificial diets for insects further support the view that insects are unable to synthesize this compound. The absence of dietary ascorbic acid limited insect growth and development, and led to severe deficiency symptoms (Chippendale et al., 1965; Chang and Kurashima, 1999). *M. persicae* requires dietary ascorbate for development and it cannot utilize the biosynthetic precursor L-gulono-1,4-lactone (Mittler et al., 1970; Dadd et al., 1967). However, aphids reared on ascorbic acid enhanced diets show detrimental effects such as lower weights and a higher mortality (Mittler et al., 1970; Dadd et al., 1967). Analogies with mammalian studies suggest that these adverse effects are caused by the presence of free iron, which is also an important component of the artificial diets. In such circumstances ascorbic acid can act as a pro-oxidant.

The optimal ascorbate content for *M. persicae* artificial diets is considered to be 0.2% (Goggin et al., 2010). While this amount exceeds typical amounts found in whole plant extracts and phloem exudates (Smirnoff, 1996; Hancock et al., 2008), the high requirement implies that ascorbate is a limiting factor in the diet. However this interpretation must be viewed with caution because ascorbate degrades rapidly and the input amounts may not

reflect the actual ascorbic acid available to the insects (Navon, 1978). The antioxidant action of ascorbate may prevent the oxidation of other compounds present in the diet, such as linolenic acid and increase its stability (Fraenkel and Blewett, 1946).

1.15.5 Role of ascorbic acid in plant-insect interactions

The presence of ascorbate in the midgut of certain herbivores may limit the toxicity of pro-oxidants found in the insect diet, such as tannins and phenols. The tolerance of the white-marked tussock moth (*Orgyia leucostigma*) to tannic acid oxidation and generation of peroxide has been related to higher concentrations of ascorbic acid in the midgut lumen (Barbehenn et al., 2001). Similarly, the presence of enzyme activities involved in recycling of ascorbate has been demonstrated in the midgut of *Helicoverpa zea* (Felton and Summers, 1995).

The effects of insect feeding on leaf ascorbate contents and the redox status of the ascorbate pool have not been studied extensively (Bi and Felton, 1995; Bi et al., 1997). The levels of ascorbic acid in *Medicago sativa* leaves were decreased following infestation by the spotted alfalfa aphid (Jiang, 1996). Aphid feeding also alters activities of enzymes involved in ascorbate metabolism such as MDHAR, DHAR, APX and AO and it also influences the expression of genes encoding these enzymes. For example, the Russian wheat aphid increased APX activity in a resistant wheat cultivar (Moloi and van der Westhuizen, 2008). The abundance of AO transcripts was enhanced following *M. persicae* infestation of celery (Divol et al., 2005).

Artificially enhancing leaf ascorbate levels can modify insect resistance. Infiltrating alfalfa stems with exogenous ascorbic acid limited the reproduction of the spotted alfalfa aphid

(*Therioaphis trifolii maculata*) and the blue-green aphid (*Acyrthosiphon kondoi*) (Miles and Oertli, 1993).

1.16 Systemic signalling in plant defence

Communication between herbivory-challenged organs and the rest of the plant is crucial for controlling the intensity of infestation by restricting the movement of pests. Organs subjected to herbivory transmit signals to other parts of the plant that have not been colonized by the insects. This systemic signalling process results in the activation of plant defence responses or the priming of such responses for rapid activation. A mobile signal or signals that rapidly transmit information about the presence of insects to the intact distant leaves is required for this role. In order to fulfill their role as long-distance signals these compounds have to be able to induce a defense response and accumulate at the site of the attack. Furthermore, they have to be transported to the systemic tissues. The vasculature provides an ideal conduit for translocation of mobile substances and their distribution to systemic tissues. Alternatively, the systemic induced resistance can result from translocation of defense compounds from the site of attack to the non-affected tissues. Experimental evidence support the role of both mechanisms in mounting an effective resistance in distant plant parts (Heil and Ton, 2008).

1.16.1 Long-distance signaling leading to SAR

Historically, triggering of systemic resistance was demonstrated for the first time in tobacco plants inoculated with tobacco mosaic virus (Ross, 1961). A localized hypersensitive response in this plant-pathogen system resulted in enhanced resistance when upper leaves of previously infested plant were inoculated (Ross, 1961). SAR was not detected before 48 hours following the initial inoculation reflecting the time needed for synthesis and

translocation of the mobile signal (Ross, 1961). Significant efforts have been devoted to the elucidation of the actual signals and the mechanisms involved in triggering systemic defence. For many years SA was considered to be a long-distance signal triggering a systemic resistance to pathogens due to the observations that SA accumulates in the leaves of numerous plant species including Arabidopsis and tobacco subjected to immediate infestation and exogenous application of SA enhances pathogen resistance (Cameron et al., 1999; Malamy, 1990; Durrant and Dong, 2004). Moreover, SA was detected in exudates from infested leaves and radio-labeled SA was transported throughout the plant following pathogen attack (Molders et al, 1996; Metraux et al., 1990). However, the role of SA as a mobile signal was questioned when it was shown that SAR can be induced even if the infected cucumber leaves are removed before SA accumulation (Vernooij et al., 1994). Methyl SA (MeSA) was identified as the actual mobile signal, and it was shown that SA perception and accumulation are only important in the systemic tissues (Park et al., 2007; Shulaev et al., 1997). A series of grafting experiments with tobacco plants in which the *SA-binding protein 2* (*SABP2*) and *SA methyltransferase 1* (SAMT1) genes were silenced unequivocally showed that MeSA is the mobile signal in triggering SAR following infection with tobacco mosaic virus (Park et al., 2007). The MeSA esterase activity of SABP2 was crucial for signal perception and development of SAR in the non-infected tissues. Moreover, SA methyltransferase activity converts SA to MeSA and when the *SAMT1* gene was silenced, inoculated leaves failed to export a signal required for SAR (Park et al., 2007).

However, the role of MeSA in other plant species remains debated and in Arabidopsis, for example, an important role for other long-distance signals in SAR activation has been shown (Truman et al., 2007; Jung et al., 2009). The nine-carbon dicarboxylic acid, azelaic

acid, accumulates in the vascular sap of Arabidopsis following *P. syringae* infection and primes plants to accumulate SA (Jung et al., 2009).

1.16.2 Long-distance signals following insect attack

Herbivore-damaged plant tissues can induce defense responses in distant, non-attacked plant parts, which resemble those stimulated by wounding (Major and Constabel, 2006). Reports on the insect induced proteinase inhibitors in tomato showed that their synthesis is triggered in both damaged and undamaged leaves (Green and Ryan, 1972). A mobile 18-amino-acid peptide, systemin, was shown to accumulate in the damaged sites upon attack by chewing insects (Narvaez-Vasquez et al., 1995). However, grafting experiments revealed that despite its systemic mobility, an intact perception of systemin is required only in the damaged leaf for a systemic response (Lee and Howe, 2003). Moreover, jasmonates appear to be the actual long-distance messengers and systemin is required only to induce jasmonates biosynthesis locally (Schilmiller and Howe, 2005; Wasternack et al., 2006). Systemic signaling depended on both JA synthesis at the site of wounding and JA perception in the distant tissues, in grafting experiments involving tomato plants impaired in JA biosynthesis and perception (Wang et al., 2008*)*. Reciprocal grafting of the JA-deficient *suppressor of prosystemin* (*spr2*) mutant and the jasmonate-insensitive (*jai1*) mutant showed that *spr2* can recognize the signal, whereas *jai1* plants were not responsive to the distant signal but were unable to produce it (Li et al., 2002). JA and its derivatives were proposed to act as a phloem-mobile signal (Truman et al., 2007). Moreover, radioactively labeled JA in tobacco was shown to move from leaves to roots exclusively via the phloem (Zhang and Baldwin, 1997).

Hydraulic and electrical signals were also suggested to be initiated following insect perception (Malone et al., 1994; Zimmermann et al., 2009). Xylem and phloem represent

ideal ‘conductors’ through which electrical signals may propagate (Rhodes et al., 1996). Electrical signals have been shown to induce the systemic expression of defense related genes such as those encoding proteinase inhibitors in tomato (Stankovic and Davies, 1997).

1.16.3 Airborne signaling

Volatile compounds produced in herbivory-damaged leaves can serve as airborne signals triggering defence responses in distant plant parts and in neighboring plants (Heil and Bueno, 2007). Within the plant this enables a rapid expression of resistance even in leaves that lack a vascular connection with the attacked leaf (Mutikainen et al., 1996). For example, air contact between the branches of mechanically damaged sagebrush was required to mount systemic induced resistance (Karban et al., 2006). Extrafloral nectar secretion, an induced defense mechanism that attracts predators, was induced systemically in lima bean exposed to volatile organic compounds released by beetle-damaged leaves (Heil and Bueno, 2007). The highly volatile and potent inducers of defense MeJA and MeSA, derivatives of JA and SA respectively, are ideal candidates for signaling compounds transmitted with the airflow (Farmer and Ryan, 1990; Shulaev et al., 1997). *Zea mays* plants exposed to an herbivory-induced emission of volatiles accumulated JA. Moreover, plants that had previously been exposed to airborne signals exhibited significantly higher levels of JA production following subsequent herbivory attack (Engelberth et al., 2004). C6-compounds, which are rapidly released upon tissue damage, have also been associated with airborne signalling (Engelberth et al., 2004). For example, lima bean leaves attacked by the spider mite (*Tetranychus urticae*) emitted a distinct blend of terpenoid compounds and six-carbon aldehydes, alcohols, and esters that induced the expression of defence-related genes (Arimura et al., 2000). The gaseous hormone ET has also been shown to modulate volatile emission in *Zea mays* (Ruther and Kleier, 2005). Mechanically damaged

leaves emit (Z)-3-hexenol that triggers the release of volatile compounds in intact leaves. The volatile emission of leaves exposed to (Z)-3-hexenol was increased in the presence of ET implying a synergistic mode of action (Ruther and Kleier, 2005).

1.16.4 Systemic signalling following aphid attack

The majority of the studies on the systemic signalling pathways induced by insect attack to date have concerned plant responses to chewing insects. By contrast, the systemic signalling that follows aphid attack remains largely unexplored. Voelckel et al. (2004) exploited the feeding preferences of *M. nicotianae* on *N. attenuata* to study the systemic effects of aphid infestation. Using a custom made cDNA array containing 240 defence-related N. attenuata genes, these authors found that M. nicotiannae preferentially settles on sink leaves and that aphid movements are limited. A small number of genes, such as a trypsin inhibitor and an 18S rRNA were differentially regulated in both local and systemic tissues. Transcripts induced by *M. nicotiannae* only in the attacked leaves included lipoxygenase and glutamate synthase, whereas triosephosphate isomerase and alpha-dioxygenase were differentially expressed only in systemic leaves (Voelckel et al., 2004). Phloem-based resistance was observed in distant, non-attacked leaves of potato plants infested with *M. persicae* (Dugravot et al., 2007). Divol et al. (2005) also reported transcriptome changes in the phloem tissue of celery infested with *M. persicae*.

1.17 Hypothesis

Minimizing the damage caused by aphid feeding is crucial to obtaining higher yields. Furthermore, controlling the spread of viruses is of agricultural importance. Currently available transgenic approaches to control herbivores are predominantly orientated towards chewing insects (http://www.isaaa.org/kc/). The lack of knowledge of plant defence

responses against aphids further limits the development of biotechnological methods for aphid resistance. Currently, the use of pesticides is the predominant method of choice for aphid control. However, the highly variable resistance to insecticides together with the acquisition of resistance to the most commonly used insecticides makes aphid control an expensive and cumbersome process (Kasprowicz et al., 2008). Furthermore, current legislation anticipates a reduction in the inputs of pesticides despite the increased demand for better food quality because of the need to minimize the impact of agriculture on the environment. Conventional methods of breeding for aphid resistance are promising but the efficiency of this process could be greatly improved by a better knowledge of the exact molecular mechanisms that underpin aphid resistance. Methods of integrated pest management are also currently being developed (Peusens et al., 2006). Pest management strategies include the use of natural aphid predators such as lady birds and parasitoids. However, the usage of such bio-control methods is limited to small scale farms and it is unlikely to have much impact on agriculture at a global scale.

Our understanding of the molecular basis of plant–aphid interactions has significantly advanced in recent years because of the application of genomic technologies that have provided new details concerning the signal transduction pathways that underpin plant perception of aphid infestation and defence mechanisms. Despite these advances, much remains uncertain regarding the role and order of specific signalling events, the interplay of signalling networks and the defence systems that determine the outcome of aphid attack. In particular, little information is available concerning the long-distance systemic signalling cascades that trigger appropriate defences in tissues that are remote from the site of aphid feeding. Moreover, although the local responses at the sites of aphid feeding have been shown to include effects on redox homeostasis, the precise role of redox signalling that is

triggered after aphid perception is largely uncharacterized. Thus, an increase in our current understanding of the role of the cellular redox signalling hub in plant–aphid interactions will clarify the importance of such mechanisms in the early events that orchestrate plant defence.

Numerous signals that influence plant growth, development and stress responses converge at the level of cellular redox homeostasis. Moreover, long-distance signalling cascades are considered to involve redox components. It is highly likely therefore that aphid feeding on local leaves is sensed, at least in part, by redox mechanisms and that long distance signalling pathways also includes redox components. Reductive and oxidative signalling events are likely to interact directly with the plant hormonal network and involve elements of the JA, SA and ethylene signalling pathways.

1.18 Aims of the project

The overall aim of the research described in this thesis was to elucidate the role of redox processes involving ascorbate in plant–aphid interactions, with particular reference to the characterization of the role of ascorbate in aphid–induced systemic signalling pathways. The nature of the signalling events that follow aphid infestation was therefore investigated using the aphid *M. persicae*, which is an important pest worldwide, on the model plant, *A. thaliana* and in the crop species potato (*S. tuberosum*). *A. thaliana* mutants that are deficient in either ascorbate biosynthesis or that lack the ABI4 transcription factor, or are defective in both components, were used to elucidate the role of ascorbate and associated ABA signalling pathways in aphid perception and responses.

The specific objectives of the project were:

1) To characterize the local and systemic responses of *A. thaliana* leaves to *M. persicae* infestation using transcriptomic and metabolomic approaches coupled to biochemical analyses.
2) To determine the effects of altered redox buffering capacity in the *vitamin C* (*vtc*) mutants that have decreased leaf levels of ascorbate biosynthesis and associated altered ABA signalling pathways in the *abi4* mutants on the local and systemic responses in *A. thaliana*.
3) To characterize the role of redox processes in plant–aphid interactions in a system of agricultural importance, *M. persicae* feeding on *S. tuberosum.*

Chapter 2. Materials and Methods

2.1 Plant material and growth conditions

2.1.1 Arabidopsis thaliana

Seeds for wild-type *Arabidopsis thaliana* accession Columbia 0 (Col0), *vtc1-1* (Conklin et al., 1999), *vtc2-1* (Jander et al., 2002), *vtc4-1* (Conklin et al., 2006), *abi4-102* (Laby et al., 2000), and *abi4vtc2* double mutants were used in the following study. The double mutant *abi4vtc2* was made by Till Pellny (Rothamsted Research, UK) and characterized at the molecular level by Perdo Diaz Vivancos in an earlier study at the University of Leeds (Kerchev et al., 2011).

Plants were grown in trays in a potting growing medium (William Sinclair Horticulture Ltd, UK) in controlled environment cabinets under a light intensity of 250 μmol $m^{-2}s^{-1}$, with a 10h photoperiod, a day/night temperature of 22^{o}C and a relative humidity of 70%. Physiological measurements such as photosynthesis were performed on whole rosettes or individual attached leaves as described in detail later. For molecular and metabolic measurements, whole rosettes or leaves were harvested at the time-points indicated on the tables and figures. Metabolism was arrested immediately in liquid nitrogen and samples were stored at -80^{o}C until analysis.

2.1.2 Solanum tuberosum

Potato plants (*Solanum tuberosum* L. cv. Desiree) were grown from tubers in glasshouses with a 16h photoperiod in 11 cm diameter pots in a mixture containing 85% (v/v) Irish moss peat (bedding grade), 7% (v/v) Pavoir sand, 7% (v/v) Perlite, 0.2% (w/v) limestone (magnesium), 0.2% (w/v) limestone (calcium), 0.1% (w/v) Sincrostart base fertiliser

(William Sinclair Horticulture Ltd, UK), 0.1% (w/v) Celcote wetting agent (LBS Horticulture, UK) and 0.15% (w/v) Osmocote mini controlled release fertiliser (Scotts, UK). Leaves were harvested at the time-points indicated on the tables and figures. Metabolism was arrested immediately in liquid nitrogen and samples were stored at -80^{o}C until analysis.

2.2 Reagents

Unless otherwise stated, the reagents used in this work were of HPLC grade obtained from Sigma-Aldrich (Dorset, UK) and VWR International (Lutterworth, UK).

2.3 Aphid material and culture conditions

Two multilocus genotypes of the green peach aphid (*Myzus persicae* Sulzer) that had been collected in Scotland in the years 2002-2004 and assigned genotypes G and E by using four microsatellite loci and three insecticide resistance mechanisms (Kasprowicz et al., 2008) were used in these studies. Aphids were maintained in an insectiary on mature potato plants in transparent Perspex® cages with a 16h photoperiod and day/night temperature of 18^{o}C.

2.4 Aphid infestation

Only apterous (wingless) aphids were used in the following experiments. Unless otherwise indicated, *M. persicae* clone G was used in these studies.

2.4.1 Arabidopsis thaliana

One mature fully expanded rosette leaf per plant was enclosed in a mesh (mesh size 200 µm) covered clip cage (2.5 cm internal diameter) and 60 aphids were confined to it. For molecular and metabolic measurements leaf material was collected as described in detail later at the time-points indicated on the tables and figures. Metabolism was arrested immediately in liquid nitrogen and samples were stored at -80^{o}C until analysis.

2.4.2 Solanum tuberosum

For molecular and metabolic measurements the third fully expanded leaf of seven-week old potato plants was used for infestation. 60 wingless aphids were transferred to the leaves with a fine paint brush and confined to the abaxial leaf surface in a 2.5 cm diameter clip cage. Aphid-free cages were used in control experiments. To minimize diurnal differences in metabolites levels and gene expression, all samples were collected at the same time point (~18:00 h). To achieve this all cages were clipped at the same time in the beginning of the experiment and aphids were transferred to the corresponding cages subsequently. Infested leaf area and leaf area confined in empty cages from 5 independent replicates was harvested at the time-points indicated on the tables and figures. Metabolism was arrested immediately in liquid nitrogen and samples were stored at -80^{o}C until analysis.

2.5 Aphid reproductive performance

2.5.1 Arabidopsis thaliana

At least 15 plants per genotype were analysed in these studies. Aphid fecundity was estimated by the method described by Fenton et al. (2009). Briefly, a single one-day-old nymph was placed in the centre of each 3 week-old rosette. To retain aphids, each plant was covered with clear Perspex® cages (4 cm internal diameter) capped with a thin mesh (mesh size 200 µm). Plants were then grown under the conditions described in section 2.3. After 15 days the rosettes were cut and each was carefully transferred to a paper bag. The bags were stored at -20^{o}C for later counting of the aphid numbers.

2.5.2 Aphid pre-infestation studies in A. thaliana

In these experiments, ten plants per genotype were used to estimate nymph survival both on the local and systemic leaves on previously infested plants and ten plants were used as controls. Control plants were treated as below but had only empty cages in the first step of

the study. In step 1, sixty aphids were confined on a mature leaf of a 4 week-old *A. thaliana* plant with a clip cage (internal diameter 2.5 cm, as illustrated in Fig. 2.1). In step 2, the aphids were carefully removed after 24 h with a fine paint brush. In step 3, 10 one-day-old nymphs were applied to either the leaf that had been previously infested or a systemic (uninfested) leaf on the same plant. For controls at step 3, aphids were placed in the cage that had not previously contained aphids. In step 4, aphid numbers were recorded after 3 days.

2.5.3 Aphid infestation studies in potato with different leaf ascorbic acid contents

In these experiments, a fully expanded potato leaf was placed into a cylindrical Perspex® container, which was open at one end and had a 10 mm diameter hole at the other end through which the leaf petiole was passed. The container was inside a second container so that the leaf petiole was submerged in either water (control ascorbate) or 50 mM L-galactono-1,4-lactone (L-GalL; high ascorbate). A lid with a 5cm hole covered with a fine mesh was placed over the open end of each cylindrical Perspex® container. On day one of the experiment, a single 1 day-old nymph was placed onto each leaf. The containers were then incubated in the conditions described in section 2.3. After 15 days the total number of individuals present in each colony was recorded. The aphids were weighed, frozen in liquid nitrogen and stored at -80^{o}C prior to determination of ascorbic acid contents by using the method described in section 2.9.1. Ten independent replicates were performed for each treatment.

2.6 RNA extraction and cDNA synthesis

RNA was isolated from homogenized Arabidopsis rosettes or individual leaves or mature fully expanded potato leaves (~50 mg) using the Qiagen® RNeasy Plant Mini kit (Qiagen) according to the manufacturer's protocol, including a DNA digestion step, and quantified

with Nanodrop ND-1000 (Thermo). 1 µg total RNA was reverse-transcribed using Ready-To-Go You-Prime First-Strand Beads (GE Healthcare) or QuantiTect Reverse Transcription Kit (Qiagen) according to the appropriate manufacturer's protocol.

2.7 Quantitative Real-Time RT-PCR (qRT-PCR)

The cDNA equivalent of 10 to 20 ng of total RNA was used in a 25 µL PCR reaction on an ABI 7700 real-time PCR system (Applied Biosystems). The reaction mixture comprised of 1 µL cDNA, 12.5 µL QuantiTect SYBR Green PCR Kit (Qiagen), 7 µL H_2O and 2.25 µL of each forward (Fwd) and reverse (Rev) primer (10 µM). In all experiments, three biological replicates of each sample type were used and reactions were performed on 96 well plates in three technical replicates. Controls that had not been reverse transcribed were used to confirm the absence of genomic DNA. The formation of primer dimers was investigated for each pair of primers with water controls and dissociation curves. Real-time cycler conditions were as follows: (1) incubation at 95^{o}C for 15 min; (2) 40 cycles of amplification consisting of 95^{o}C for 15 s, 55-60^{o}C (depending on the melting temperature of the primer pair) for 30 s and 72^{o}C for 30 s.

Three reference genes were used to normalize the qRT-PCR data for Arabidopsis experiments (based on Czechowski et al., 2005): *At2g28390* (*SAND family protein*), *At4g34270* (*TIP41-like family protein*) and *At3g18780* (*Actin-2*). The stable expression of these transcripts in the experimental conditions was further confirmed by the microarray data. Relative expression values for transcripts from potato were calculated using *elongation factor 1-α* (*AB061263*) as a reference gene (based on Nicot et al., 2005).

All primers were designed using the NCBI Primer–Blast tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) and are listed in Tables 2.1 and 2.2. The amplification efficiency (E) of each primer pair was estimated with the LinReg software

(Ramakers et al., 2003). The relative gene expression was calculated using the Livak method ($2^{-\Delta\Delta C_T}$) when the amplification efficiencies of the target and reference genes were similar (Livak and Schmittgen, 2001). When the amplification efficiencies of the target and reference genes differed the relative expression ratio was calculated according to the Pfaffl method (Pfaffl, 2001).

Primer	Sequence	Target accession
EF1-α Fwd	5’-ATTGGAAACGGATATGCTCCA-3’	AB061263
EF1-α Rev	5’-TCCTTACCTGAACGCCTGTCA-3’	
CAT2 Fwd	5’-GCAGCTCCCAGTTAATGCTC-3’	AY500290
CAT2 Rev	5’-CAAACCTCGAGGGCAAATAA-3’	
FeSOD Fwd	5’-AATCTATGAAGCCCAACGGA-3’	EU545469
FeSOD Rev	5’-GCTGCAGCTGCCTTAAATTC-3’	
CuZnSOD Fwd	5’-CTCCTGAAGATGAGGTGCGT-3’	AF354748
CuZnSOD Rev	5’-GAGAGGAATCTGCTTGTCGG-3’	
PR-1 Fwd	5’-TATCTTGCGGTTCACAACGA-3’	AJ250136
PR-1 Rev	5’-CACCAGTTCTTGAGTTGGCA-3’	
JAZ-1 Fwd	5’-CCATGAATCTTTTCCCTCAAG-3’	EF591123
JAZ-1 Rev	5’-TTTGTGCCTTTTCTGGTTGA-3’	

Table 2.1 Primers used for qRT-PCR in *Solanum tuberosum*. Primer sequences were designed for the reference gene *AB061263* and the five genes of interest using *S. tuberosum* sequences obtained from the NCBI database. Primers were designed using the NCBI Primer–Blast tool so that the target amplicon size is approximately 100 bp.

Table 2.2 Primers used for qRT-PCR in *Arabidopsis thaliana*. Primer sequences were designed for the reference genes (*At2g28390*, *At4g34270*, and *At3g18780*) and the genes of interest using *A. thaliana* sequences obtained from the TAIR database. Primers were designed using the NCBI Primer–Blast tool so that the target amplicon size is approximately 100 bp.

Primer	Sequence	Target accession
PR1 Fwd	5'-AAGAGGCAACTGCAGACTCA-3'	At2g14610.1
PR1 Rev	5'-TCTCGCTAACCCACATGTTC-3'	
GA20ox1 Fwd	5'-CCGGTGAGAGTGTTGGCTACGC-3'	At4g25420.1
GA20ox1 Rev	5'-GAGCGGCTCATGTCGTCGCA-3'	
GA20ox2 Fwd	5'-AGAAGCTTGCACCAAACACGGC-3'	At5g51810.1
GA20ox2 Rev	5'-TTTGCCGGCGAGAGGCATGT-3'	
GA20ox3 Fwd	5'-TGGCCCGACCACGAGAAACCT-3'	At5g07200.1
GA20ox3 Rev	5'-AGCCTCCGATGCCAAGCACG-3'	
GA3ox1 Fwd	5'-CACGGCGTGCCTTTGGGACT-3'	At1g15550.1
GA3ox1 Rev	5'-GACGCCGTAGCCGGACACAC-3'	
GA3ox2 Fwd	5'-CTGGGTTACCGCGCCACCTG-3'	At1g80340.1
GA3ox2 Rev	5'-CCCTGGCTCGGTGAAGCACG-3'	
GA2ox2 Fwd	5'-CCCCGTCGTCAACCTAGCCG-3'	At1g30040.1
GA2ox2 Rev	5'-TCGGGTCGGACTCCGTGGTT-3'	
GA2ox6 Fwd	5'-CGGTCCGGCGAGTCCGTTC-3'	At1g02400.1
GA2ox6 Rev	5'-ACAGCTGTCGGATTTGCGTGGA-3'	
SAND family protein Fwd	5'-CGAATTTAGCGGTGGCGGCG-3'	At2g28390.1
SAND family protein Rev	5'-CCCGCCACACCTTCACGCAA-3'	

TIP41-like family protein Fwd	5’-TCCGGGGCTGAGCTTCTTCC-3’	At4g34270.1
TIP41-like family protein Rev	5’-GAGAATCGTGCCGCGGAGGG-3’	
Actin-2 Fwd	5’- GGCTCCTCTTAACCCAAAGG-3’	At3g18780.1
Actin-2 Rev	5’-GAGAGAACAGCTTGGATGGC-3’	
VSP2 Fwd	5’-ACTCCCGGAGGCCTTGCATCT-3’	At5g24770.1
VSP2 Rev	5’-AGGTCACGCCAGCAGCTTCG-3’	
ANNAT4 Fwd	5’-TGCACACGCTCTGCTGAGGA-3’	At2g38750.1
ANNAT4 Rev	5’-TTGCGCTGAGGACCGTGGAC-3’	
ATSERAT3;2 Fwd	5’-TGGCCGACCCGGTTTTGCTG-3’	At4g35640.1
ATSERAT3;2 Rev	5’-ACCGGCTCCTCTTCTGCCTCA-3’	
ERD9 Fwd	5’- GGCAAGCAGCGACGTGAAGC -3’	At1g10370.1
ERD9 Rev	5’- GGAGGAACTCGTAGGGGACAGAC -3’	
P5CS1 Fwd	5’-GCGTTTCCTCAGCCGCCGAT-3’	At2g39800.1
P5CS1 Rev	5’-CGCGTGTTCGTTGGGTGGTG-3’	
alliinase family protein Fwd	5’-TGGTCCTCTCGTTGGCCATCAACT-3’	At4g24670.1
alliinase family protein Rev	5’-GTGGTGGATACCACGGGCCAT-3’	
CYCP2;1 Fwd	5’-TCGGCAACGGAAGCAGCCAC-3’	At3g21870.1
CYCP2;1 Rev	5’-ACGCCTCCAAGCTCTTCCCA-3’	
MYB51 Fwd	5’-GTGCGGACACCGTGTTGCAAAG-3’	At1g18570.1
MYB51 Rev	5’-CCATCCACCTTCACCGTGGCG-3’	

2.8 Whole transcriptome profiling

2.8.1 Global transcriptome reprogramming of local and systemic leaves of Arabidopsis Col0 upon aphid attack

The experimental procedures related to the microarray processing described in the following section were performed by Jenny Morris at the James Hutton Institute, Dundee. Data extraction, quality control analysis and initial statistical analysis were carried out by Pete Hedley at the James Hutton Institute, Dundee.

To study the global transcriptome effect of aphid feeding on infested (local) and uninfested (systemic) leaves 6-week old *A. thaliana* Col0 plants were used. One mature fully expanded rosette leaf per plant was enclosed in a mesh covered clip cage. At the start of the experiment all plants to be used were caged and 60 adult wingless aphids were confined to 4 cages (Figure 2.1). Following 24 h, 60 adult aphids were placed within each of a further 4 clip cages and after a further 18 h, 60 adult aphids were added to each of a further 4 clip cages. 6h after the final addition of aphids, all clip cages were removed and aphids were quickly brushed from leaves using a fine paint brush. Both the caged leaf and a second mature fully expanded rosette leaf immediately opposite the caged leaf were removed with a scalpel, transferred to 1.5 ml eppendorf tubes and rapidly frozen in liquid N_2. Controls for the caged and non-caged leaves were collected from plants that had empty cages on. Leaves were then stored at -80^{o}C until further processing.

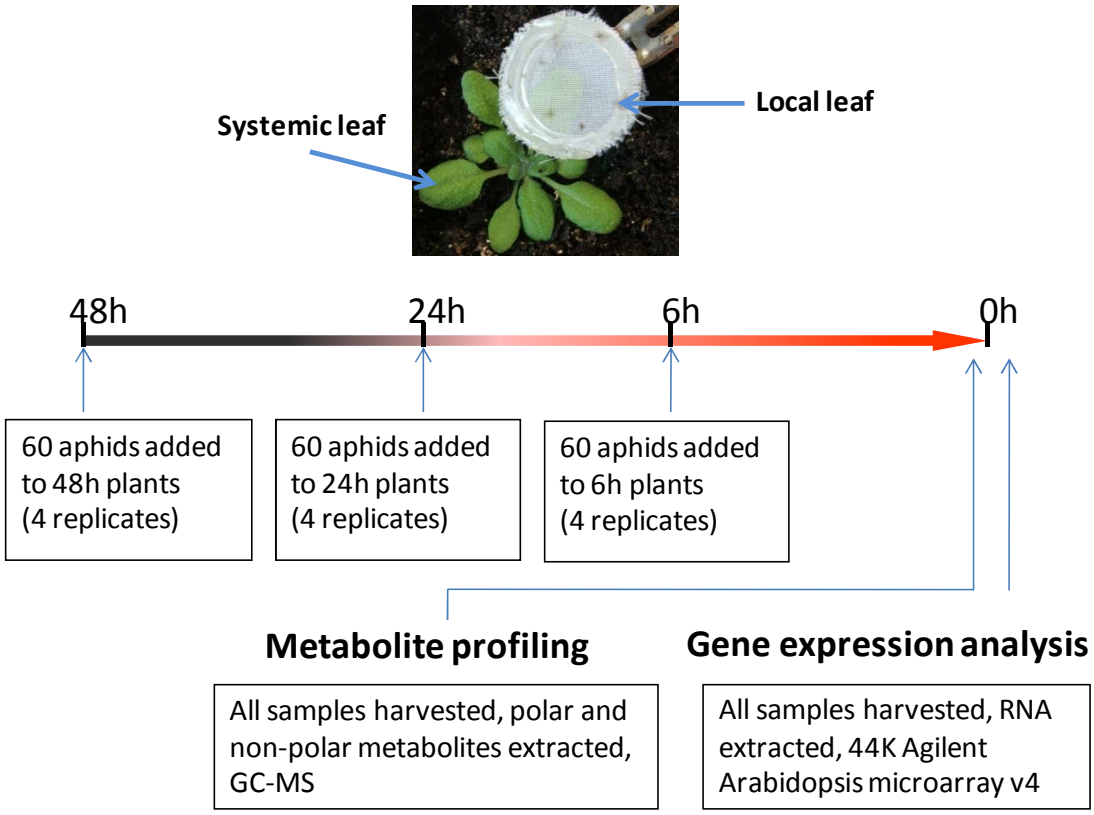

Figure 2.1 Experimental design used for time course microarray analysis and metabolite profiling of Arabidopsis following aphid attack. Fully expanded mature Arabidopsis rosette leaves were caged at the beginning of the experiment. Sixty wingless aphids were confined to 4 plants and following 24 h, further 60 adult aphids were placed within each of a further 4 clip cages. Six hours before the termination of the experiment another 4 plants were infested with 60 aphids. Both caged (local) and non-caged (systemic) leaves from the same rosette were collected. Plants that had empty cages on were used as references. Samples were snap frozen on liquid nitrogen and used for metabolite profiling and gene expression analysis.

Leaf material was ground under liquid N_2 with a sterile micropestle directly in the collection tube and RNA extracted as described above. The quality of RNA was assessed using Agilent 2100 Bioanalyzer. Agilent's Quick Amp Labeling kit (version 5.7) was used to amplify and label target RNA according to the manufacturer's instructions. Levels of cDNA synthesis and dye incorporation were determined using spectrophotometry.

Sixteen two-colour Agilent V4 arrays (Arabidopsis V4 Gene Expression Microarray (4x44K) containing 43,803 probes, Agilent Technologies) were used to compare infested and non-infested leaves of four biological replicates at four time points (48, 24, 6 and 0h). The hybridization strategy incorporated balanced dye-swaps to minimize any residual dye effects (Table 2.3).

Hybridization and washing of the slides were performed according to the manufacturer's protocols (Agilent Two-Color Microarray-Based Gene Expression Analysis, version 5.5). Briefly, aliquots (20 µl) of the labelled samples were combined with 5 µl 10x blocking agent (Agilent 5188-5242) and denatured at 98^{o}C for 3 min followed by cooling at room temperature. The hybridization step was conducted at 65^{o}C for 17 h after addition of 2x GE hybridization buffer HI-RPM (25 µl). Wash 1 buffer (Agilent, 5188-5327) was used to disassemble the slides, followed by washing in Wash 1 buffer (1 min), then Wash 2 buffer (Agilent, 5188-5327) for 1 min, and then final drying by centrifugation. Agilent G2505B scanner was used to scan the hybridized slides at resolution of 5 µm at 532 nm (Cy3) and 633 nm (Cy5).

Slide	Array	Cy3	Cy5
1	1	L0	S0
1	2	L6	S6
1	3	L24	S24
1	4	L48	S48
2	1	S48	L48
2	2	S0	L0
2	3	S6	L6
2	4	S24	L24
3	1	L24	S24
3	2	L48	S48
3	3	L0	S0
3	4	L6	S6
4	1	S6	L6
4	2	S24	L24
4	3	S48	L48
4	4	S0	L0

Table 2.3 Hybridization strategy used for the time course microarray analysis of local and systemic leaves of Arabidopsis Col0 plants infested with *M. persicae*. Four slides each containing 4 two-colour Agilent V4 arrays were used to compare infested (local) and non-infested (systemic) leaves of four biological replicates at four time points (48, 24, 6 and 0h). The combinations between letters (L and S) and numbers (48, 24, 6, and 0) represent samples from caged (L) or systemic (S) leaves collected from plants infested for 48, 24, 6 or 0 h respectively. Two out of the four biological replicates were labeled with Cy3 dye and the other two with Cy5 dye prior to hybridization.

Data were extracted with Agilent Feature Extraction (v.9.5.3) software and normalised using the Lowess algorithm to minimize differences in dye incorporation efficiency. Each channel of the Lowess normalised data were imported into GeneSpring (v.7.3, Agilent Technologies) software as if it were single-channel arrays. Subsequently a minimum of 5.0 was set to intensity values, data from each array was normalized to the 50th percentile of all measurements on the array and the signal from each probe was normalised to the median of its values across the entire dataset. Unreliable measurements flagged as absent by the Feature Extraction software were discarded if not present in at least 3 out of 32 samples. Volcano plots were used to indentify statistically significant differentially expressed genes based on cut-offs of fold change ≥2x and Student's t-test p-value ≤0.05.

2.8.2 Microarray analysis of abi4, vtc2 and abi4vtc2 double mutants

Whole rosettes of 6 week old Arabidopsis plants were homogenized under liquid nitrogen using a mortar and pestle. RNA was extracted from aliquots (50–100 mg) using Qiagen® RNeasy Plant Mini kit according to the manufacturer's protocol. Between 3 and 6 biological replicates were used per genotype. Gene expression profiles were analyzed using commercial oligonucleotide microarrays, Genechip Arabidopsis ATH1 Genome Arrays (Affymetrix, USA) at the NASC's (Nottingham Arabidopsis Stock Centre) International Affymetrix Service. The scanned Arabidopsis ATH1 Genome Arrays were analyzed using GeneSpring GX 11.00 (Agilent Technologies) software. Raw intensity values were normalized using the Robust Multichip Average (RMA) summarization algorithm. All genotypes were analyzed against the wild type (Col0) as a control condition and p-values calculated by asymptotic unpaired t-test subjected to multiple testing correction (Benjamini-Hochberg False Discovery Rate). To identify genes with statistically significant

differential expression between the studied mutants and the wild type a cut-off of $\log_2$ expression ratio ±1 and p-value ≤0.05 was adopted.

2.8.3 Microarray analysis of local and systemic responses of Col0, abi4, vtc2 and abi4vtc2 upon aphid attack

The experimental procedures related to the microarray processing described in the following section were performed by Jenny Morris at the James Hutton Institute, Dundee. Data extraction, quality control analysis and initial statistical analysis were carried out by Pete Hedley at the James Hutton Institute, Dundee.

Four-week-old Arabidopsis wild type, *abi4*, *vtc2* and *abi4vtc2* double mutants were used for infestation. One mature fully expanded rosette leaf per plant was enclosed in a mesh covered clip cage. At the start of the experiment all plants to be used were caged and 60 adult wingless aphids were placed within each of 4 cages per genotype. 6h after the addition of aphids, all clip cages were removed and aphids were quickly brushed from leaves using a fine paint brush. Both the caged leaf and a second mature fully expanded rosette leaf immediately opposite the caged leaf were removed with a scalpel, transferred to 1.5 ml eppendorf tubes and rapidly frozen in liquid N_2. Controls for the caged and non-caged leaves were collected from plants that had empty cages on. Leaves were then stored at -80^{o}C until further processing. RNA was extracted, quantified and labeled as described in section 2.8.1.

In total 32 two-colour Agilent V4 arrays (Arabidopsis V4 Gene Expression Microarray (4x44K) containing 43,803 probes, Agilent Technologies) were used to compare infested and non-infested leaves of four biological replicates of Col0, *abi4*, *vtc2* and *abi4vtc2* double mutants infested with aphids for 6 hours. The hybridization strategy incorporated dye-swaps to minimize any residual dye effects (Table 2.4).

Hybridization and washing of the slides were performed as described in section 2.8.1. The slides were scanned as described above. Data were extracted with Agilent Feature Extraction (v.9.5.3) software and normalised using the Lowess algorithm to minimize differences in dye incorporation efficiency. Normalised datasets for each array from the Feature Extraction software were imported into GeneSpring (v.7.3, Agilent Technologies) software for further analysis. Data were transformed to account for dye-swaps and unreliable measurements flagged as absent by the Feature Extraction software were discarded if not present in at least 3 out of 32 samples. Volcano plots were used to identify statistically significant differentially expressed genes based on cut-offs of fold change $\geq$2x and Student's t-test p-value $\leq$0.05.

Table 2.4 Hybridization strategy used for the microarray analysis of local and systemic responses of Col0, *abi4*, *vtc2* and *abi4vtc2* following *M. persicae* attack. Eight slides each containing 4 two-colour Agilent V4 arrays were used to compare infested (local) and non-infested (systemic) leaves at 6 hpi in four biological replicates. Non-infested caged plants were used as controls. L and S represent local and systemic leaves, respectively. Two out of the four biological replicates were labeled with Cy3 dye and the other two with Cy5 dye prior to hybridization.

Slide	Array	Cy3		Cy5	
		Genotype	Treatment	Genotype	Treatment
1	1	Col0 (L)	Control	Col0 (L)	Infested
1	2	*abi4* (L)	Control	*abi4* (L)	Infested
1	3	*abi4vtc2* (L)	Control	*abi4vtc2* (L)	Infested
1	4	*vtc2* (L)	Control	*vtc2* (L)	Infested
2	1	Col0 (S)	Control	Col0 (S)	Infested
2	2	*abi4* (S)	Control	*abi4* (S)	Infested
2	3	*abi4vtc2* (S)	Control	*abi4vtc2* (S)	Infested
2	4	*vtc2* (S)	Control	*vtc2* (S)	Infested
3	1	*vtc2* (L)	Infested	*vtc2* (L)	Control
3	2	Col0 (L)	Infested	Col0 (L)	Control
3	3	*abi4* (L)	Infested	*abi4* (L)	Control
3	4	*abi4vtc2* (L)	Infested	*abi4vtc2* (L)	Control
4	1	*vtc2* (S)	Infested	*vtc2* (S)	Control
4	2	Col0 (S)	Infested	Col0 (S)	Control
4	3	*abi4* (S)	Infested	*abi4* (S)	Control
4	4	*abi4vtc2* (S)	Infested	*abi4vtc2* (S)	Control
5	1	*abi4vtc2* (L)	Control	*abi4vtc2* (L)	Infested
5	2	*vtc2* (L)	Control	*vtc2* (L)	Infested
5	3	Col0 (L)	Control	Col0 (L)	Infested
5	4	*abi4* (L)	Control	*abi4* (L)	Infested
6	1	*abi4vtc2* (S)	Control	*abi4vtc2* (S)	Infested
6	2	*vtc2*(S)	Control	*vtc2* (S)	Infested
6	3	Col0 (S)	Control	Col0 (S)	Infested
6	4	*abi4* (S)	Control	*abi4* (S)	Infested
7	1	*abi4* (L)	Infested	*abi4* (L)	Control
7	2	*abi4vtc2* (L)	Infested	*abi4vtc2* (L)	Control
7	3	*vtc2* (L)	Infested	*vtc2* (L)	Control
7	4	Col0 (L)	Infested	Col0 (L)	Control

8	1	*abi4* (S)	Infested	*abi4* (S)	Control
8	2	*abi4vtc2* (S)	Infested	*abi4vtc2* (S)	Control
8	3	*vtc2* (S)	Infested	*vtc2* (S)	Control
8	4	Col0 (S)	Infested	Col0 (S)	Control

2.8.4 Transcriptional reprogramming induced by two M. persicae clones on wild type Arabidopsis

The experimental procedures related to the microarray processing described in the following section were performed by Jenny Morris at the James Hutton Institute, Dundee. Data extraction, quality control analysis and initial statistical analysis were carried out by Pete Hedley at the James Hutton Institute, Dundee.

One mature fully expanded Arabidopsis Col0 (4-week old) rosette leaf per plant was enclosed in a mesh covered clip cage containing 60 aphids. Leaves were infested with two different *M. persicae* clonal lineages (G and E) and four biological replicates for each aphid clone were used. Six hours after the addition of aphids, all clip cages were removed and aphids were quickly brushed from leaves using a fine paint brush. Leaves were flash frozen in liquid N_2 and stored at -80^{o}C until further processing. RNA was extracted, quantified and labeled as described in section 2.8.1.

Four two-colour Agilent V4 arrays (Arabidopsis V4 Gene Expression Microarray (4x44K) containing 43,803 probes, Agilent Technologies) were used to compare infested leaves of four biological replicates of Col0 leaves infested with *M. persicae* clones G and E for 6 hours. The hybridization strategy incorporated dye-swaps to minimize any residual dye effects (Table 2.5).

Slide	Array	Cy3	Cy5
1	1	1G	1E
1	2	2E	2G
1	3	3G	3E
1	4	4E	4G

Table 2.5 Hybridization strategy used for the microarray analysis of the transcriptional reprogramming induced by *M. persicae* clones G and E on Arabidopsis Col0. One slide containing 4 two-colour Agilent V4 arrays was used to compare fully expanded Arabidopsis Col0 leaves infested with two different *M. persicae* clones. Samples representing four biological replicates from leaves infested with clone E were hybridized against clone G infested samples. Two of the four biological replicates for each aphid clone were dyed with Cy3 and the other two with Cy5.

Slides were processed as described in section 2.8.1. Data were extracted with Agilent Feature Extraction (v.9.5.3) software and normalised using the Lowess algorithm to minimize differences in dye incorporation efficiency. Normalised datasets for each array from the Feature Extraction software were imported into GeneSpring (v.7.3, Agilent Technologies) software for further analysis. Data were transformed to account for dye-swaps and unreliable measurements flagged as absent by the Feature Extraction software were discarded if not present in at least 3 out of 4 samples. Volcano plots were used to identify statistically significant differentially expressed genes in Arabidopsis Col0 as a result of the feeding of the two aphid clones (≥2x fold change genotype E/G at Student's t-test p-value ≤0.05).

2.9 Metabolite analysis and enzyme activities

2.9.1 Determination of ascorbic acid contents in potato leaves upon aphid attack

The following method was used to quantify the changes in the ascorbate pool of potato leaves following aphid infestation. Leaf material was collected as described in section 2.4.2 and extracted in ice-cold 5% metaphosphoric acid (MPA) at a ratio of 1ml/100 mg FW using pestle and mortar. The homogenate was centrifuged at 14,000g for 10 min at 4^{o}C. Total ascrobate was quantified in samples to which tris(2-carboxyethyl)phosphine hydrochloride (TCEP) was added to a final concentration of 5 mM to allow reduction of dehydroascorbic acid to ascorbic acid (Walker et al., 2006). Reduced ascorbate was estimated in the samples without any additional treatments. Ascorbic acid was quantified by cation interaction HPLC using a 300 x 7.8 mm IC-Sep ICE-Coregel 64H column (Transgenomic Inc., USA) with 8 mM H_2SO_4 as mobile phase pumped at 0.6 ml/min. Dionex UVD 340U diode array detector was used to quantify ascorbate by determination of the area under the peak at 245 nm.

2.9.2 Determination of honeydew ascorbic acid content

In these studies, aphids were cultured for 3 days on control and high ascorbate potato leaves, as described in section 2.5.3. In each case, ten aphids were then transferred from these leaves to 2 cm discs of either control or high ascorbate leaves that had no prior infestation. The discs were placed on 1% agar gel, one disc per well in six well plates. Each plate was closed with a lid lined with foil (to collect the honeydew) and the plate was then inverted and cultured for 24h under the conditions described in section 2.3. The foil containing the honeydew was then washed with 5% (w/v) MPA containing 5 mM TCEP and the washings stored at -80^{o}C prior to ascorbic acid quantification by HPLC as described above (section 2.9.1). In a similar series of experiments, the foil was replaced with filter paper dipped in 0.25% ninhydrin (dissolved in acetone) and extensively dried. Five aphids were allowed to settle on each disc for 3 h after which the number of spots of honeydew produced over a period of 4 hours was determined. Data are presented as the average number of honeydew spots produced per aphid per hour.

2.9.3 Determination of ascorbic acid content of rosette leaves of abi4, vtc2, and abi4vtc2 double mutant plants

Whole Arabidopsis rosettes were ground to a fine powder in a mortar using liquid nitrogen. Aliquots were homogenized in ice cold 1 M $HClO_4$ at a ratio of 1 ml 50 mg FW^{-1} and centrifuged at 4^{o}C for 10 min (14,000g). An aliquot of the supernatant was neutralized with 5M K_2CO_3. The resulting $KClO_4$ pellet was removed after centrifugation and the supernatant was used for ascorbic acid analysis in a microplate-adapted enzymatic assay (Queval and Noctor, 2007). The reduced ascorbate was quantified after addition of 20 µl neutralized supernatant to a well containing 100 µl 0.2 M phosphate buffer (pH 5.6) and 75µl H_2O. The absorbance was recorded at 265 nm and 5 µl ascorbate oxidase (AO) were

added to specifically degrade the ascorbate present in the sample. The decrease in absorbance, monitored at 265 nm, was recorded for 5 min untill stable values were reached. The amount of reduced ascorbate was calculated after subtraction of the final value from the initial measurment using a molar extinction coefficient of 14 $mM^{-1}cm^{-1}$. Aliquots of AO were prepared daily in 0.2 M phosphate buffer pH 5.6 (40U/ml) and discarded after the end of the experiment. Total ascorbic acid was measured after reducing dehydroascorbate present in 100 µl neutralized extract in a reaction mixture containing 10 µL 25mM DDT and 140 µl 0.12 M phosphate buffer (pH 7.5) for 30 min at room temperature. After incubation the levels of total ascorbic acid were quantified as described above.

2.9.4 Total protein quantification

Total protein contents were quantified in the pellets obtained from the samples for ascorbate analysis processed as described above (section 2.9.3). The pellet was resuspended in 0.2 M phosphate buffer (pH 7.0) and 20 µl aliquots were mixed with Bradford reagent to a final volume of 200 µl in microplate wells. Absorption was read at 595 nm after 5 min incubation and protein content estimated against a standard curve. Bovine Serum Albumin (BSA) was used as a standard.

2.9.5 Determination of glutathione

GSH and GSSG were quantified using the GR-dependent reduction of 5,5'-dithiobis(2-nitro-benzoic acid) (DTNB, Ellman's reagent) in a recycling assay monitored at 412 nm in method adapted for microplate reader by Queval and Noctor (2007). Samples were ground with a pestle and mortar in 1% trichloroacetic acid (TCA) at a ratio of 1ml 100 mg FW^{-1}. The homogenate was centrifuged at 14,000g for 10 min at 4°C and subsequently neutralized with 0.2 M NaH_2PO_4 (pH 5.6) and 0.2 M NaOH to a final pH between 5 and 6. To specifically measure GSSG, 0.2 ml of the neutralized extract were incubated with 5 µl 2-

vynylpiridine (VPD) for 30 min at room temperature and centrifuged before analysis. Total glutathione (GSH plus GSSG) was measured without pre-treatment of the extract. To calculate the amount of reduced glutathione GSSG values were multiplied by 2 before subtraction from the total glutathione values. The absolute amounts of GSH and GSSG were quantified using a standard curve constructed using standards run concurrently on the same plate. The increase in absorbance was measured for 300 s and corrected for the non-specific reduction of DTNB using a blank sample.

2.9.6 Chlorophyll analysis

Whole Arabidopsis rosettes were ground to a fine powder in a mortar using liquid nitrogen. Aliquots were homogenized in 80% acetone and centrifuged at 4^{o}C for 10 min (14,000g). The absorbance values at 663 and 645 nm were used to calculate the amount total chlorophyll according to the following equation:

$$\text{Total Chlorophyll (mg/l)} = (8.02 \times A_{663}) + (20.2 \times A_{645})$$

2.9.7 Hydrogen peroxide

The levels of hydrogen peroxide were quantified using commercially available Amplex Red Hydrogen Peroxide/Peroxidase Assay kit (Invitrogen Ltd., Paisley, UK). Leaf material was collected as described in section 2.5.1 and extracted in cold 5% $HClO_4$ at a ratio of 1 ml 10 mg FW^{-1} using a pestle and mortar. The homogenate was centrifuged at 14,000g for 10 min at 4^{o}C. The SN was neutralized with 5M K_2CO_3 and used for determination of hydrogen peroxide according to the manufacturer's recommended protocol with a fluorescence microplate reader using excitation at 530 nm and fluorescence detection at 590 nm. The absolute amounts of hydrogen peroxide were quantified using a standard curve as standards were run concurrently on the same plate.

2.9.8 Total polyphenols

Leaf samples collected as described above (section 2.4.2) were ground with a pestle and mortar in 1% trichloracetic acid (TCA) at a ratio of 1 ml 100 mg FW^{-1}. The homogenate was centrifuged at 14,000g for 10 min at 4°C and subsequently neutralized with 0.2 M NaH_2PO_4 (pH 5.6) and 0.2 M NaOH to a final pH of 6.0. The reaction mixture consisted of 100 µl SN, 690 µl potassium phosphate buffer (pH 8.0), 100 µl 4-aminophenazone, 100 µl 20 mM H_2O_2 and 10 µl 100 U/ml horse radish peroxidise. After mixing vigorously the samples were incubated for 5 min and absorbance was read at 500 nm. The amount of polyphenols was quantified by reference to a standard curve constructed using catechin and presented as µg catechin equivalents/g FW.

2.9.9 Non-specific peroxidise activity

Leaf samples were collected as described in section 2.4.2 and extracted in cold potassium phosphate buffer (pH 6.7) containing 1 mM EDTA, 2% (w/v) PVPP and 1 mM phenylmethylsulfonyl fluoride (PMSF) at a ratio of 1 ml 100 mg FW^{-1}. The homogenate was centrifuged at 14,000g for 10 min at 4°C. Enzyme activity was quantified in a reaction mixture containing 1.1 ml potassium phosphate buffer (pH 6.7), 10 µl SN and 360 µl 1% guaiacol. The reaction was started by addition of 20 µl H_2O_2 and the increase in absorbance at 470 nm was monitored. The enzyme activity was expressed as guaiacol dehydrogenated product min^{-1} g FW^{-1} using an extinction coefficient of 26.6 mM^{-1} cm^{-1}.

2.9.10 Ascorbate-glutathione recycling enzymes

Dehydroascorbate reductase (DHAR) and glutathione reductase (GR) activities were quantified using a microplate method described by Murshed et al. (2008). Enzyme activities were extracted from samples collected as in 2.4.2 in 50 mM MES buffer (pH 6.0) with 40 mM KCl, 2 mM $CaCl_2$ and 1 mM ascorbate at a ratio of 1 ml 250 mg FW^{-1}. The

homogenate was centrifuged at 14,000g for 10 min at 4^{o}C, and the enzyme activities were quantified as follows. For the DHAR reaction 10 µl SN was added to individual wells containing 185 µl reaction buffer (50 mM Hepes (pH 7.0), 0.1 mM EDTA and 2.5 mM GSH) and the reaction was started by the addition of 5 µl DHA (8 mM). The increase of OD was measured for 5 minutes at 265 nm and the enzyme activity calculated using an extinction coefficient of 14 mM^{-1} cm^{-1}. The GR activity was quantified in a reaction mixture containing 10 µl SN and 185 µl reaction buffer (50 mM Hepes (pH 7.0), 0.1 mM EDTA and 0.25 mM NADPH). The oxidation of NAPDH was started by the addition of 5 µl GSSG (20 mM) and monitored at 340 nm for 5 minutes. The enzyme activity was calculated using an extinction coefficient of 6.22 mM^{-1} cm^{-1}.

2.9.11 Analysis of gibberellins

Freeze-dried Arabidopsis rosettes (Col0, *abi4*, *vtc2* and *abi4vtc2*) were ground to powder using a ball mill. Aliquots of approximately 500 mg were resuspended in 80% aqueous methanol (100 ml) containing a mixture of ^{2}H- and ^{3}H-labeled gibberellin standards ([833 Bq / 5 ng of each: {1,2-3H2}-GA_1, {1,2-3H2}-GA_4], and deuterated GAs [5 ng of: GA_1, GA_3, GA_4, GA_8, GA_9, GA_{12}, GA_{15}, GA_{19}, GA_{20}, GA_{24}, GA_{25} GA_{29}, GA_{34}, GA_{44}, GA_{53}]). The methanol extracts were then stirred overnight at 4 ^{o}C, filtered through filter paper and evaporated on a rotary evaporator. After adjusting the pH to 8.0 with KOH, the samples were loaded onto a QAE Sephadex A-25 (Pharmacia) anion exchange column that had been pre-equilibrated with sodium formate (0.5 M) and then washed with formic acid (0.2 M) and water (pH 8.0). The column was washed with water and eluted with 0.2 M formic acid through a C_{18} Sep-Pak cartridge (primed with CH_3OH and water). The C_{18} Sep-Pak column was eluted with 80% aqueous CH_3OH, taken to dryness *in vacuo* and then redissolved in MeOH and methylated with ethereal diazomethane. The methylated mixture was dissolved

in ethyl acetate and partitioned with water. The ethyl acetate phase was applied to an aminopropyl ion exchange cartridge [Bond Elut (100 mg)] and the run through taken to dryness. The samples were dissolved in 50 µL CH_3OH, injected onto an analytical C_{18} reversed phase HPLC column (4.9 mm i.d x 250 mm) and eluted with a linear gradient of increasing CH_3OH in 2 mM acetic acid (19% CH_3OH to 100% CH_3OH over 40 min) at a flow rate of 1ml/min. 1 ml fractions were collected. HPLC fractions containing the tritiated gibberellin standards were pooled together and taken to dryness in *vacuo*. The samples were converted to TMSi ethers by heating with N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA) (10 µL) at 90^{o}C for 30 min and quantified by GC-MS.

2.9.12 Targeted metabolomics

Frozen local and systemic leaves collected from 4-week old Arabidopsis plants (Col0, *abi4*, *vtc2* and *abi4vtc2*) infested with aphids as described in section 2.8.1 were freeze-dried and homogenized. Approximately 10–20 mg leaf powder was extracted in 3 ml CH_3OH. Ribitol (100 µl, 2mg/ml) and methyl nondecanoate (100 µl, 0.2 mg/ml), dissolved in water and methanol respectively, were added as internal standards. The extraction was performed at 30^{o}C under vigorous shaking. After 30 min water (0.75 ml) was added to the mixture, followed by chloroform (6 ml) 30 min later. At the end of the extraction procedure 1.5 ml water was added, the tubes were manually shaken, and centrifuged for 10 min. The resulting upper (polar) and lower (organic) fractions were isolated and subjected to derivatization and further analysis.

2.9.12.1 Derivatization of the polar fraction

An aliquot (500 µl) of the polar fraction was taken to dryness under reduced pressure in a centrifugal evaporator. Methoxylamine hydrochloride (80 µl, 20 mg/ml) dissolved in anhydrous pyridine was added to oximate the carbonyl functional groups for 4 hours at

50°C. Silylation was performed with 80 µl MSTFA at 37°C for 30 min. 40 µl of the reaction mixture was diluted with pyridine (1:1) in an auto sampler vial containing 50 µl evaporated retention standard mixture (undecane, tridecane, hexadecane, eicosane, tetracosane, triacontane, tetratriacontane, and octatriacontane dissolved in isoxane at 0.2 mg/ml).

2.9.12.2 Derivatization of the non-polar fraction

The non-polar fraction (4 ml) was taken to dryness under reduced pressure in a centrifugal evaporator. Sulphuric acid (1 % (v/v), 2 ml) dissolved in methanol was added and the mixture was incubated overnight at 50°C to allow trans-esterification. NaCl (5 ml, 5% (w/v)) and chloroform (3 ml) were added and the mixture was manually shaken. The upper-phase was discarded and $KHCO_3$ (3ml, 2% (w/v)) was added. The mixture was briefly centrifuged and the lower chloroform layer was collected and run through an anhydrous sodium sulphate column. After evaporating to dryness, chloroform (40 µl) and pyridine (10 µl) were added and a silylation step was carried out with MSTFA at 37°C for 30 min. 40 µl of the reaction mixture was diluted with pyridine (1:1) in an auto sampler vial containing 50 µl evaporated retention standard mixture (undecane, tridecane, hexadecane, eicosane, tetracosane, triacontane, tetratriacontane, and octatriacontane dissolved in isoxane at 0.2 mg/ml).

2.9.12.3 Sample analysis

Samples were analysed on a DSQ II Single Quadrupole GC-MS system (Thermo). 1 µl of the sample was injected with a split ratio of 40:1 into a programmable temperature vaporising injector under the following conditions: injection temperature of 132°C for 1 min, transfer rate 14.5°C/s, transfer temperature 320°C for 1 min, clean rate 14.5°C/s and clean temperature 400°C for 2 min. Analytes were chromatographed on a DB5-MSTM

column (15 m x 0.25 mm x 0.25 µm; J&W, Folsom, USA) using helium at 1.5 ml/min in constant flow mode as mobile phase. The temperature gradient was 100^{o}C for 2.1 min, then 25^{o}C/min to 320^{o}C, and isothermal for 3.5 min. The interface temperature was 250^{o}C. Mass data were acquired at 70 eV electron impact ionization conditions over a 35 – 900 a.m.u mass range at 6 scans per sec with a source temperature 200^{o}C and a solvent delay of 1.3 min. Acquisition rates were set to give approximately ten data points across a chromatographic peak. XcaliburTM v1.4 and XcaliburTM v2.0.7 software packages were used to acquire and analyze the data, respectively. A processing method developed at SCRI was used to assign identities to the peaks. It uses the retention times and masses of known standards and the Genesis algorithm (part of the XcaliburTM package) for peak integration. The expected retention time for each peak was adjusted using the retention times of the retention standards. The integrated area of the annotated peaks was normalized against the integrated area of the respective internal standards, ribitol and nonadecanoic acid for the polar and non-polar fractions, respectively. The peak area ratios were normalized on a dry weight basis.

2.10 Sugar signalling

Seeds of Col0, *vtc2*, *abi4* and *abi4vtc2* were germinated on 0.8% agar containing full strength Murashige and Skoog (MS) medium alone or MS medium supplemented with 6% (w/v) glucose. The seedlings were grown for up to 10 days in controlled environment chambers under a light intensity of 30 µmol m^{-2} s^{-1}, with a 12h photoperiod.

2.11 Physiological measurements

2.11.1 Photosynthetic gas exchange

Mature Arabidopsis leaves were enclosed in an open-circuit infrared gas exchange system that allowed simultaneously measurement of steady-state photosynthesis in six leaves on

different plants. CO_2 exchange were measured with an Infrared Gas Analyser (model ADC 225 Mark 3, The Analytical development Co Ltd, Hoddesdon, UK) set at 500 μmol m^{-2} s^{-1} photosynthetically active radiation (PAR), 40–50% relative humidity in the leaf chamber, leaf chamber CO_2 and O_2 concentrations maintained respectively at 350 ± 10 μmol mol^{-1} and 210 μmol mol^{-1}. The temperature of the leaf chambers was set at 20 ± 0.5^{o}C and calculations of CO_2 assimilation rate were performed as described previously (von Caemmerer and Farquhar, 1981).

2.11.2 Microscopy

Potato leaves were prepared following 48 h of infestation by decolouring in boiling 70% ethanol. To obtain surface images, decolourised leaves were mounted on microscope slides and viewed under a 5X objective. Alternatively, leaves were embedded in 8% agarose and 100 μm transverse sections were prepared using a vibrotome prior to mounting on slides and viewing under a 20X objective.

Chapter 3. Transcriptional changes in local and systemic leaves of wild type *A. thaliana* upon *M. persicae* attack

3.1 Introduction

3.1.1 Use of microarrays to study plant-aphids interactions

Transcriptome reprogramming after aphid attack has been studied extensively in numerous plant–aphid combinations in the recent years using microarray technologies (Thompson and Goggin, 2007). The majority of these studies have focused on compatible interactions with the aim of identifying conserved defence signalling pathways that are induced in plants as a result of the perception of aphids. This information is considered to be a pre-requisite for the development of new strategies for the limitation of the detrimental effects of aphids in an agricultural context. It is assumed that genes conferring aphid resistance can be identified from comparisons of changes in the leaf transcriptome profiles of resistant and susceptible cultivars in response to aphid infestation (Delp et al., 2009). However, the aphid-induced changes in gene expression patterns are proving to be complex and multifaceted. The plant response to aphids has considerable overlap with other more general plant defence responses and the identification of specific pathways has proved elusive to date even in near-isogenic lines. The genes which expression is responsive to aphid feeding cover a wide range of functional categories, including metabolism, transport, protein and carbohydrate degradation, signal transduction, and plant defence (De Vos et al., 2005). For example, more than 2000 genes were differentially expressed in Arabidopsis leaves after 72 hours of *M. persicae* infestation (De Vos et al., 2005). The sample preparation procedure in which RNA from four biological replicates was pooled together and only one sample was used for hybridization questions the statistical adequacy of this

experiment (De Vos et al., 2005). There are large discrepancies in the results obtained in this study and the findings of Couldridge et al. (2007) who found that only 25 transcripts were enhanced in abundance and 2 were decreased in the infested plants 36 h after the onset of the infestation. Performed with six biological replicates for each time point and accounting for the false discovery rate (Benjamini and Hochberg) this study is the most statistically stringent microarray evaluation of transcriptional changes following aphid attack so far and reflects the need for an adequate biological replication (Couldridge et al., 2007). Moreover, at least five biological replicates are required to obtain stable results when analyzing microarray data (Pavlidis et al., 2003). Nevertheless, nine common genes were identified between the two studies (Couldridge et al., 2007). In another study, aphid-induced transcriptome changes were monitored 6, 12, 24, and 48 h after the onset of infestation of *A. thaliana* with the cabbage aphid, *B. brassicae* (Kusnierczyk et al., 2008). Four biological replicates each consisting of 24 plants on average were used for hybridization (Kusnierczyk et al., 2008). Genes were considered statistically significant if having P values (calculated using the Iimma approach; Smyth, 2004) and false discovery rate (Benjamini and Hochberg) lower than 0.05. In this study, 196 genes were significantly up- or down-regulated 6 h post infestation and the numbers of transcripts that were altered in abundance had increased by almost 2.5 times at the last time point (Kusnierczyk et al., 2008). A comparison of aphid (*M. persicae*)-induced transcriptome changes in three different Arabidopsis ecotypes (Wassilewskija (Ws), Cape Verde Islands (Cvi), and Landsberg erecta (Ler)) revealed that considerable genotypic variation exists in the aphid response, the transcriptome changes being greatest in Cvi and lowest in Ler (Kusnierczyk et al., 2007).

3.1.2 Global transcriptome changes induced by aphid feeding

The transcriptome studies reported in the literature show that large numbers of genes can be differentially regulated in response to aphid attack. However, because the different reports have failed to identify common patterns of aphid-induced gene expression when studying the same plant–aphid interaction, it is difficult to determine the significance that each plays in the plant defence against aphids. Moreover, the transcriptomes of different Arabidopsis ecotypes were found to differ after attack from the same aphid species. Many of the variations in the results obtained in different studies may be explained, at least in part, by variations in factors such as the aphid strains, the host plants on which the insects were reared, environmental conditions during the course of the experiments, the density of infestation and the statistical criteria used in the processing of the data. Nevertheless, some conclusions can be drawn from the literature data, based on the functional classifications of differentially regulated genes. Genes encoding calcium-binding proteins and proteins involved in the detoxification of ROS were induced in all experiments, indicating that aphid infestation triggers oxidative and calcium signaling events. Moreover, the hormone-mediated signaling network is activated by aphid attack suggesting that the perception of aphids leads to adjustments in the cellular hormonal balance. The hormonal response is considered to play a universal role in plant–aphid interactions (Moran and Thompson, 2001). In addition, transcripts encoding components involved in cell wall modifications are generally observed following aphid attack. This response is likely to be a consequence of the physical damage caused by the intercellular penetration of the aphid stylet, which may provoke strengthening of the cell wall against further attack.

The effect of aphid feeding on directly attacked leaves have been studied extensively (Kuśnierczyk et al., 2008; Pegadaraju et al., 2005; Zhu-Salzman et al., 2004). However,

the systemic reprogramming of plant metabolism and defences is likely to be of pivotal importance in priming non-infested tissues and preparing them for subsequent aphid attack. Moreover, the coordinated response of infested and non-infested leaves must be tightly spatio-temporally regulated to ensure timely activation of defense mechanisms while maintaining adequate sink-source relationships and utilization of nutrients between the infested leaves and the rest of the rosette. The following experiments were performed firstly to explore the effects of aphid infestation on transcriptome patterns at different time points (6, 24 and 48 hours) after the onset of attack in leaves directly subjected to aphids (local) and in distant (systemic) aphid-free leaves. The second aim of the experiments was to compare the transcriptome responses of local leaves to two different aphid clones that exhibit a significant degree of reproductive variation on certain hosts (Fenton et al., 2010). For this analysis, two *M. persicae* clonal lineages (G and E) were selected as they had been shown to reproduce at different rates on host plants such as oil seed rape and potato (Fenton et al., 2010). Finally, the effects of the transcriptome changes on nymph survival rates were assessed by introducing nymphs to leaves that had previously been infested for 24h.

3.2 Results

3.2.1 Local and systemic transcriptome changes in response to aphid attack

The responses of the Arabidopsis leaf transcriptome to aphid feeding was studied using clonal lineage G. Transcriptome patterns were monitored in leaves directly subjected to attack (local) and aphid-free (systemic) leaves of the same rosette for up to 48h after the onset of infestation. Aphid feeding rapidly elicited a transcriptome response both in the local leaves and in systemic leaves far removed from the site of aphid attack (Table 3.1). Marked changes in specific transcripts were observed in both local infested leaves and in systemic leaves at the earliest time-point measured i.e. 6 h post-infestation (hpi).

At this time-point, a total of 134 transcripts were differentially regulated (2 fold-change cut off and p-value ≤0.05) in the local infested leaves (Table 3.1).

Moreover, most of the changes in transcripts observed at this time point involved an increase rather than a decrease. Only 14 transcripts were decreased in abundance at 6 hpi in the local tissue relative to controls compared to 125 genes whose expression was increased following aphid attack. Thereafter, there was a large increase in the number of transcripts with altered expression in the local leaves (Table 3.1) with 1551 transcripts changed in abundance at 24h. Of these 485 transcripts were decreased in abundance relative to controls while 1066 genes were increased. However, at the last time point measured (48 hpi) the number of transcripts with altered abundance had slightly decreased once more (Table 3.1). Of the 1108 transcripts that showed altered abundance in the local leaves at 48 hpi, 292 were decreased in abundance while 816 were increased. These changes in total numbers of transcripts suggest that the leaf response to aphids is a dynamic process with suites of genes induced and repressed. Marked changes in specific transcripts were also observed in the systemic leaves at the earliest time-point measured i.e. 6 hpi.

However, unlike the situation in local leaves, where most transcripts were increased in abundance, similar numbers were increased and decreased in the systemic leaves (Table 3.1). Moreover, in the systemic leaves, the increase in the number of transcripts that occurred between 6 and 24 hpi was much smaller than that observed in the local leaves (Table 3.1). However, as with 6 hpi, approximately equal numbers of transcripts were increased and decreased at 24h (Table 3.1). At the last time point measured (48 hpi) the number of transcripts with altered abundance had slightly decreased in the systemic leaves (Table 3.1). In this case, of the 137 transcripts that showed altered abundance in the systemic leaves at 48 hpi, most were increased in abundance (Table 3.1).

Time point	Leaf type	Number of genes		
		Up-regulated	Down-regulated	Total
6 h	Local	120	14	134
6 h	Systemic	91	78	169
24 h	Local	1066	485	1551
24 h	Systemic	144	97	241
48 h	Local	816	292	1108
48 h	Systemic	114	23	137

Table 3.1 Number of genes differentially regulated at 6, 24 and 48 hpi in the local (infested) and systemic (uninfested) leaves of *A. thaliana* Col0 during *M. persicae* infestation. Fully expanded mature rosette leaves were infested with 60 apterous aphids confined in a mesh covered clip cage. Both the caged leaf and a second mature fully expanded rosette leaf immediately opposite the caged leaf were used to extract RNA. Sixteen two-colour Agilent V4 arrays were used to compare infested and non-infested leaves of four biological replicates for each time point. Non-infested caged plants were used as controls. Raw data were normalised using the Lowess algorithm and each channel imported in GeneSpring v.7.3 as a single-channel array. Data from each array was normalized to the 50^{th} percentile of all measurements on the array and the signal from each probe was normalised to the median of its values across the entire dataset. Statistically significant differentially expressed genes were identified on volcano plots with a cut-off of fold change ≥ 2 and Student's t-test p-value ≤ 0.05.

The transcripts that were changed in abundance in the local leaves (Appendix 1-3) and systemic leaves (Appendix 4-6) at 6, 24 and 48 hpi were analyzed and used to explore the characteristics plant response to aphids. The kinetics of global changes in transcript patterns in local and systemic leaves are shown in Fig. 3.1.

A large number of the transcripts that were increased 6 hpi were also increased at 24hpi and 48 hpi in the local leaves (Fig. 3.2 A). In total, about 67% of the induced transcripts at 24 hpi were also increased at 48 hpi. In contrast, only 24% of the transcripts that were decreased in abundance at 24 hpi were also decreased at 48 hpi (Fig. 3.2 B). In the systemic leaves, very little overlap in the transcripts that were changed in abundance was observed at 6 hpi, 24 hpi and 48 hpi time points (Fig. 3.2 C, D). Only 3 transcripts (*miR408*, *SERINE ACETYLTRANSFERASE 3;2* and *At5g03670*; similar to unknown protein) were increased in the systemic leaves at the 6 hpi and 24 hpi (Fig. 3.2 C). Moreover, only 2 transcripts (*At2g23270*; similar to unknown protein and *Monooxygenase 2* (*MO2*), partial (58%) were decreased at the 6 hpi, 24 hpi and 48 hpi in the systemic leaves (Fig. 3.2 C). Twenty six transcripts that were increased in the systemic leaves at 24 hpi were also increased at 48 hpi (Fig. 3.2 C).

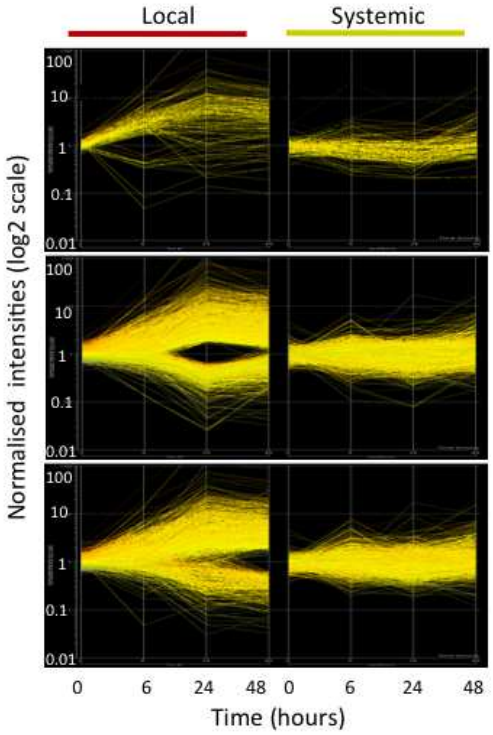

Figure 3.1 A comparison of expression intensities of differentially regulated transcripts in the local (infested) and systemic (uninfested) leaves of Arabidopsis wild type (Col0) upon *M. persicae* attack at 6, 24 and 48 hpi. 60 aphids were confined to mature rosette leaves in clip cages. Both the caged leaf and a second mature fully expanded rosette leaf immediately opposite the caged leaf were used to extract RNA. Four biological replicates for each time point were collected. Agilent V4 arrays were used to compare infested and non-infested leaves of four biological replicates for each time point. Volcano plots were used to identify significant differentially expressed genes with a cut-off of fold change ≥2 and Student's t-test p-value ≤0.05.

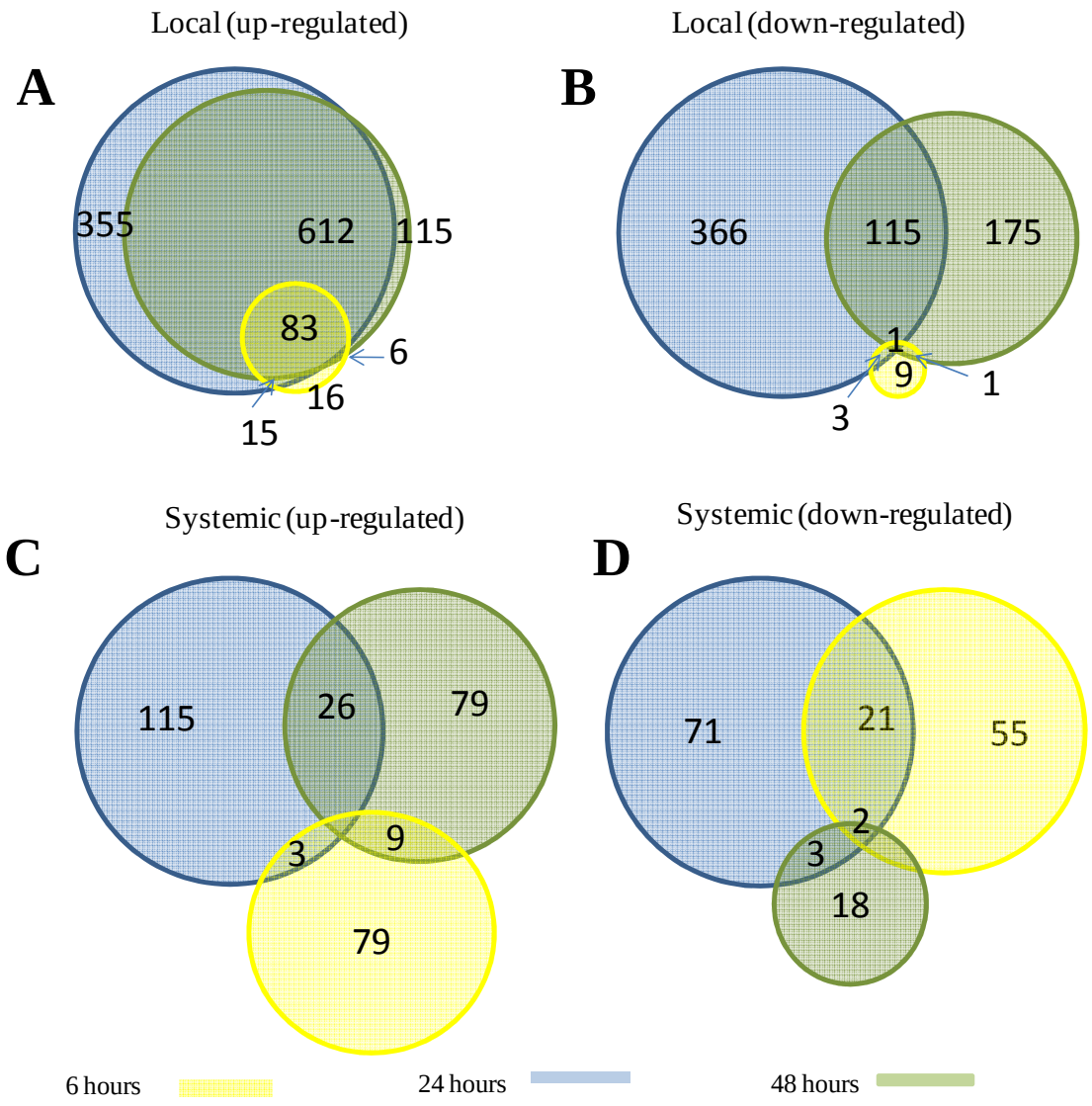

Figure 3.2 Venn diagrams showing common and unique genes differentially regulated following aphid attack at 6, 24 and 48 hpi in the local and systemic tissue of Arabidopsis Col0. Agilent V4 arrays were used to compare infested and non-infested leaves of four biological replicates for each time point. Statistically significant differentially expressed genes were identified with a cut-off of fold change ≥2 and Student's t-test p-value ≤0.05. A) Common and unique up-regulated genes in the local leaves. B) Common and unique down-regulated genes in the local leaves. C) Common and unique up-regulated genes in the systemic leaves. D) Common and unique down-regulated genes in the systemic leaves.

Similarly, 21 of the transcripts that were decreased in the systemic leaves 24 hpi were also decreased at 48 hpi (Fig. 3.2 D). These data show that there is a rapid response in the systemic leaves as well as the local leaves, suggesting a rapid transcriptional reprogramming in leaves that are remote from the site of infestation. The transcripts which showed the greatest fold changes in response to aphid attack in the local leaves provide evidence of the nature of plant response. For example, the transcript which showed the greatest increase in the local tissues at 6 hpi encodes *REDOX RESPONSIVE TRANSCRIPTION FACTOR 1* (Fig 3.3 A).

This transcript was accumulated to levels about 17 times higher at the site of infestation than those measured in the aphid-free leaves. Other transcripts encoding proteins involved in plant defense responses were also greatly increased early in the response to aphid infestation (*ATRRD*3, *ATHSFA*, *WRKY62*, *LHT7*, *CNGC19* and *ATERF13*). In marked contrast, the transcripts that were highly induced at this time point in systemic leaves do not appear to suggest such a marked increase in defense related processes (Fig 3.3 B). The transcript whose abundance was most decreased in the local leaves at 6 hpi encodes a putative peroxidase (Fig 3.3 A). The transcript whose abundance was most decreased in the systemic leaves at this time point encodes a wound induced protein (Fig 3.3 B).

Only one transcript (*S-adenosylmethionine-dependent methyltransferase*; *At1g15125*) was regulated in a similar manner in the local and systemic leaves at 24 hpi (Fig. 3.4). Moreover, only two transcripts (*UDP-Glycosyltransferase superfamily protein*; *At2g36780* and *alpha/beta-Hydrolases superfamily protein*; *At3g03990*) were increased in both local and systemic leaves at 48 hpi (Fig. 3.4).

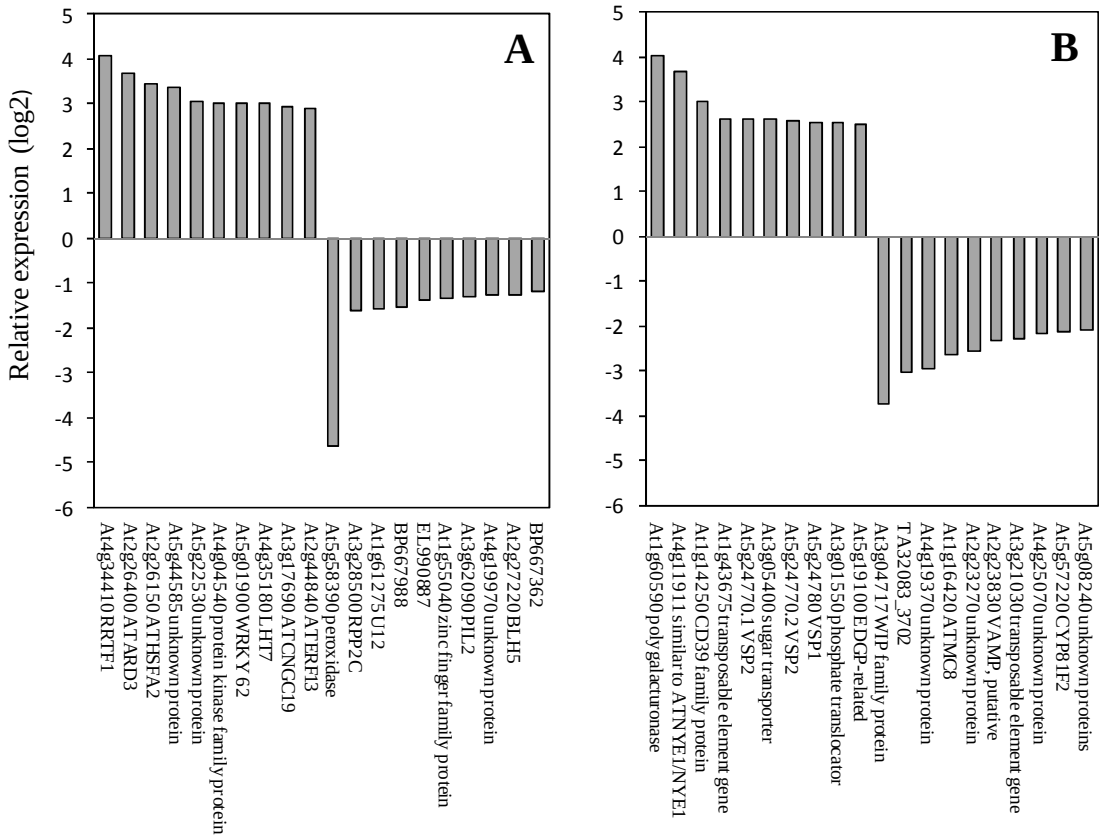

Figure 3.3 Top ten most induced or repressed transcripts upon *M. persicae* attack at 6 hpi in the local (A) and systemic (B) leaves of 6 week-old Arabidopsis Col0. Mature rosette leaves were infested with sixty wingless aphids. Both the infested (local) leaf and a second fully expanded rosette (systemic) leaf immediately opposite the local leaf were harvested. Agilent V4 arrays were used to compare infested and non-infested leaves of four biological replicates. Statistically significant gene expression changes in the local and systemic leaves relative to non-infested caged controls were identified with a cut-off of fold change ≥2 and Student's t-test p-value ≤0.05.

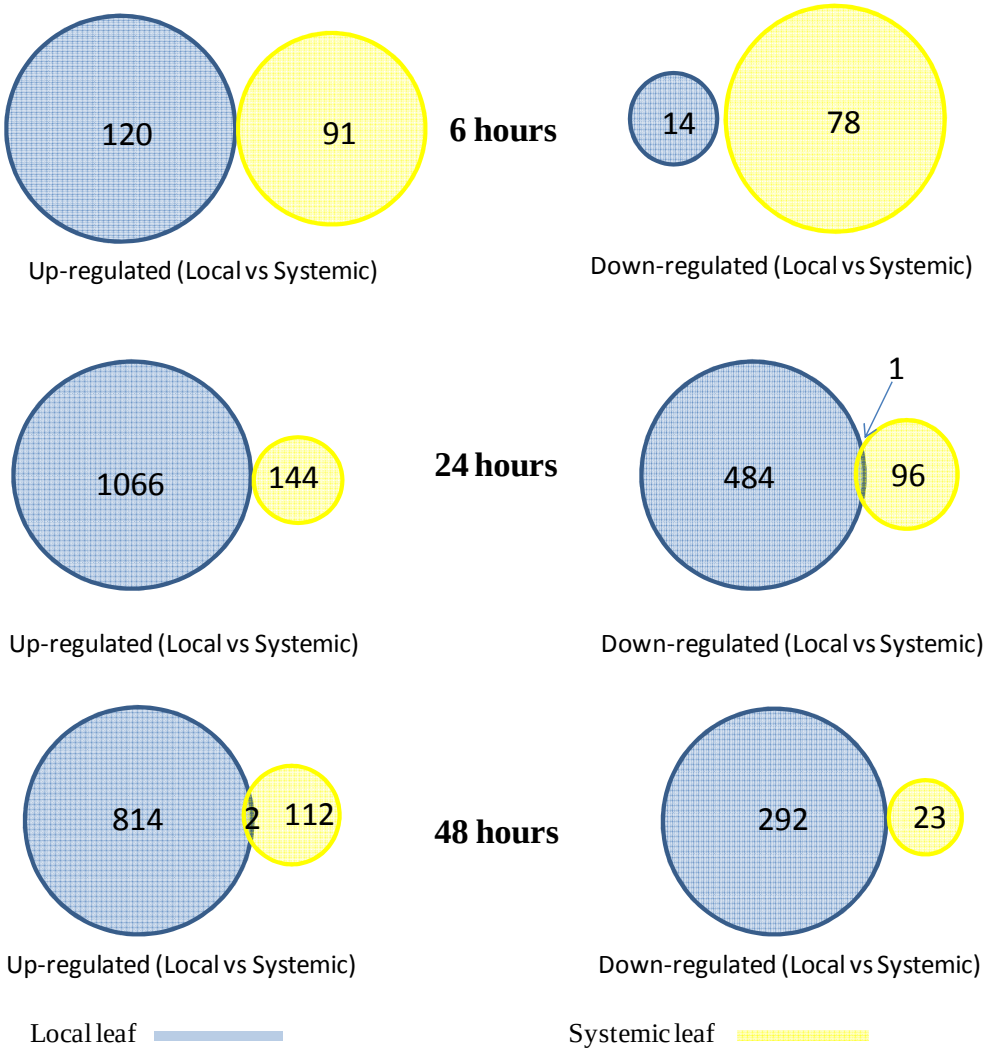

Figure 3.4 Venn diagrams showing the numbers of common and unique transcripts that were differentially expressed in local and systemic leaves of Arabidopsis Col0 plants infested with 60 wingless *M. persicae* for 6, 24 and 48h. Both the infested (local) leaf and a second fully expanded rosette leaf immediately opposite the local leaf were harvested. Agilent V4 arrays were used to compare infested and non-infested leaves of four biological replicates. Statistically significant gene expression changes in the local and systemic leaves relative to non-infested caged controls were identified with a cut-off of fold change ≥2 and Student's t-test p-value ≤0.05.

3.2.2 Functional classification of suites of transcripts modified in response to aphid infestation

For simplicity, the functions of the transcripts described above that were differentially modified in the local and systemic tissues were determined using the Classification SuperViewer tool (http://bar.utoronto.ca) in conjunction with functional categories defined by the MapMan database (http://mapman.gabipd.org). This analysis provides a very simple approach to the classification of genes that normalizes them to the number of genes present in the whole genome as follows:

Normed Frequency : $(Number\_in\_Class_{input\_set}/Number\_Classified_{input\_set})/$

$(Number\_in\_Class_{reference\_set\ ()}/Number\_Classified_{reference\_set})$.

Hence, this analysis does represent the absolute number of genes falling into each functional category but rather represents enrichment in specific functional categories.

Considering only transcripts that were increased in abundance, this analysis revealed that the most represented functional classes in the local leaves 6 hpi were related to stress, hormone metabolism and nucleotide classes (Fig. 3.5 A). However, stress was the only category that was represented at all three time points in the local leaves (Fig. 3.5 A). In contrast, the most represented functional classes in the systemic leaves varied according to the time points of analysis but the stress category was conspicuously absent from all three time points (Fig. 3.5 A).

Considering transcripts that were decreased in abundance, this analysis revealed that there was no pronounced functional class in the local leaves at 6 hpi (Fig 3.5 B). However, classes related to metabolism such as sulfur assimilation and polyamine biosynthesis were represented at the later time-points (Fig 3.5 B). In contrast, amino acid metabolism was represented in the systemic leaves at the two earlier time points (Fig 3.5 B).

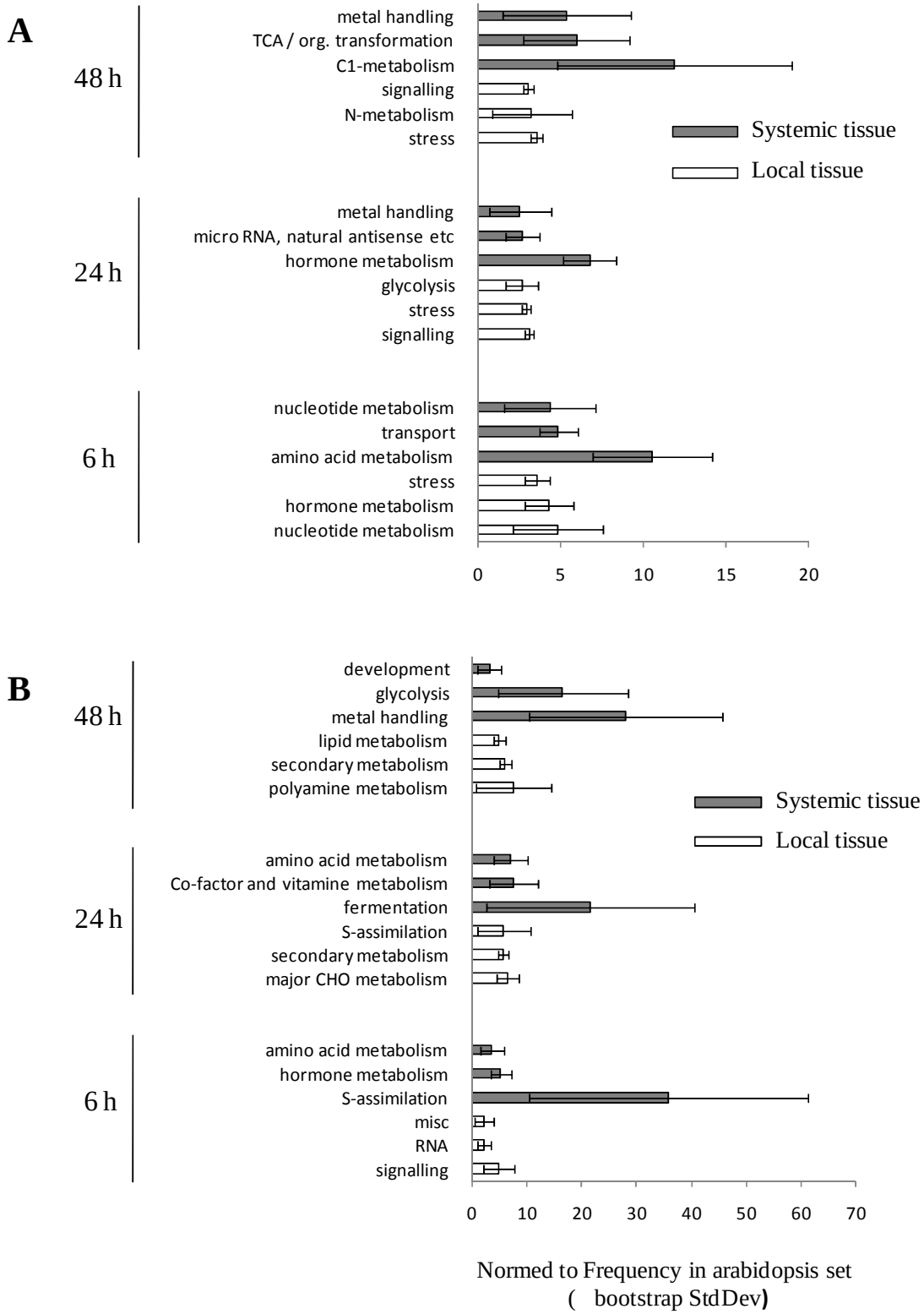

Figure 3.5 Classification of the three most represented functional categories based on the MapMan database in the local and systemic leaves of infested wild type (Col0) plants at 6, 24 and 48 hpi. A) Functional classification of the genes up-regulated in the local and systemic leaves. B) Functional classification of the genes down-regulated in the local and systemic leaves.

3.2.3 Validation of microarray data by qRT-PCR

The following experiments were performed in order to provide further evidence concerning the responses of the systemic leaves to aphid attack. Eight genes were selected from the microarray analysis described above based on their transcriptional patterns, being either repressed (*MYB51*) or induced (*VSP2*, *SERAT3;2*, *CYCP2;1*, *ANNAT4*, *P5CS1*, *ERD9*, and *At4g24670*) by *M. persicae* attack at 6 hpi. Transcript abundance was measured by qRT-PCR and compared with that determined in the microarray analysis (Fig. 3.6). The values obtained by qRT-PCR analysis were strongly consistent with the values obtained from the microarray analysis at 6 hpi (Fig. 3.6) with a Pearson correlation coefficient $r = 0.868$ ($P=0.005$).

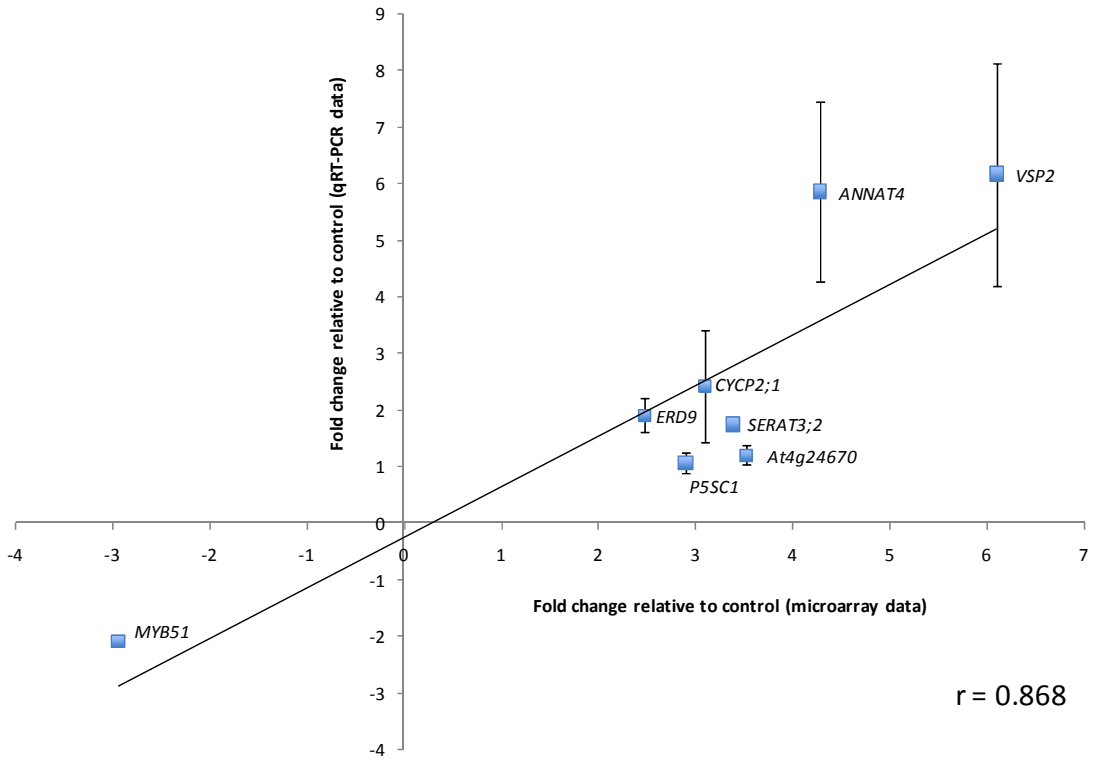

Figure 3.6 Correlation of transcript abundance determined by quantitative RT-PCR (qRT-PCR) results and microarray analysis for eight selected genes in systemic leaves of Arabidopsis Col0 plants infested with *M. persicae* for 6 hours. qRT-PCR results represent the average expression values of three biological replicates of *M. persicae*-infested plants relative to aphid-free control plants calculated using the Livak method ($2^{-\Delta\Delta C_T}$). Volcano plots were used to identify significant differentially expressed genes in the microarray analysis with a cut-off of fold change ≥2 and Student's t-test p-value ≤0.05. Pearson correlation coefficient (r) indicating the significance of the correlation is shown on the graph (P = 0.005).

3.2.4 Evidence in support of transcriptional reprogramming in infested tissues

3.2.4.1 ROS associated transcripts

Aphid feeding rapidly altered the expression of a number of genes that can be classified as involved in the maintenance of redox homeostasis in the infested (local) leaves (Table 3.2). As mentioned previously, the transcript that was most increased in abundance at 6 hpi encodes the *REDOX RESPONSIVE TRANSCRIPTION FACTOR 1* (*At4g34410*). This transcription factor, which is considered to be central to the control of cellular redox networks (Khandelwal et al., 2008), was induced more than 15 fold in local leaves relative to the non-infested control leaves. In addition, genes encoding inhibitors of hydrogen peroxide-induced cell death (BAP1 and BAP2) and their interacting partners BON1 and BON3 (Yang et al., 2007) were highly induced in a transient manner in response to *M. persicae* attack (Table 3.2). BAP1 is a membrane-associated protein that binds phospholipids in a calcium-dependent manner (Yang et al., 2006). Loss of BAP1 function confers enhanced SA-mediated pathogen resistance (Yang et al., 2007).

A transcript encoding a 2OG-Fe (II) oxygenase family protein (*At3g13610*) whose gene product is involved in hydrogen peroxide-mediated programmed cell death (Gechev et al., 2005), was increased by aphid feeding at 24 hpi, the point where *BAP1*, *BAP2* and *BON1* transcripts were highest. Disruption of the gene encoding 2OG-Fe(II) oxygenase significantly reduced hydrogen peroxide-induced cell death (Genchev et al., 2005). Transcripts encoding HEAT SHOCK PROTEIN 101, which is responsive to hydrogen peroxide (Nishizawa et al., 2006), accumulated in the aphid-challenged leaves at 24 hpi. *GLUTATHIONE PEROXIDASE 4* mRNA levels which are increased in response to oxidative stress (Rouhier and Jacqout, 2005) were increased at 24 hpi and remained at the high level at 48 hpi in the infested tissues. Interestingly, several genes encoding proteins of the Nudix (nucleoside diphosphates linked to some moiety X) hydrolase

family (NUDT4, NUDT5, NUDT6, NUDT7, NUDT13, NUDT17 and NUDT21) were increased in response to aphid infestation. Nudix hydrolases are important in nucleoside metabolism and homeostasis, especially because the uncontrolled build-up of derivatives can be toxic (Kraszewska, 2008). It is interesting to note that *AtNUDT7* transcripts, which encode a hydrolase that protects DNA against oxidative damage by maintaining NAD^+ levels (Ishikawa, et al. 2009), were significantly increased at 6 hpi (Table 3.2). Taken together, these data demonstrate that redox signalling and associated processes are induced early in the response to aphids and that the accumulation of transcripts encoding these proteins is maximal at the 24 h time-point.

In addition, to the above changes in redox-regulated transcripts, the expression of genes associated with antioxidant defence processes was modified in response to aphid attack but only at the 24 and 48h time points (Table 3.2). For example, transcripts encoding a member of the monodehydroascorbate reductase family, which is responsible for ascorbic acid regeneration, were greatly increased at 24 hpi. Moreover, transcripts for a putative L-ascorbate oxidase (*At4g39830*), which is an apolastic enzyme involved in cell wall metabolism, were increased at 24 and 48 hpi. Aphid feeding induced the expression of three types of thioredoxins (*H-type 5*, *Y1* and *H2*). Thioredoxins are involved in reversible thiol-disuphide exchange reactions (Gelhaye et al., 2005), as are protein disulphide isomerases (PDI). Transcripts encoding two PDI-like proteins (*PDI-LIKE 1-3* and *PDI-LIKE 1-1*), were increased at 24 hpi (Table 3.2). Moreover, three genes encoding glutathione S-transferases (*GSTs 5*, *8* and *11*) had higher expression at 24 hpi (Table 3.3), as did several glutaredoxins.

Table 3.2 Genes involved in maintenance of redox homeostasis or responsive to ROS which expression was altered in the local leaves of *A. thaliana* Col0 at 6, 24 and 48 h upon *M. persicae* attack. Sixty aphids were confined to a clip cage attached to a mature rosette leaf and gene expression analysed with Agilent V4 arrays relative to non-infested caged controls. Statistically significant differentially expressed genes were identified on volcano plots with a cut-off of fold change ≥2 and Student's t-test p-value ≤0.05. Genes were attributed to this category based on either literature evidence or Gene Ontology (GO). GO classification was made according to Biological Process (oxidation-reduction process or response to oxidative stress) as found at The Arabidopsis Information Resource (TAIR; www.arabidopsis.org). na (not applicable) indicates that the corresponding transcript did not fulfil the adopted statistical criteria for differential expression. All ratios are expressed on a linear scale.

Accession	Description	Fold change		
		6h	24h	48h
At4g34410	RRTF1; REDOX RESPONSIVE TRANSCRIPTION FACTOR 1	16.8	40.9	na
At2g26150	ATHSFA2; heat shock transcription factor A2	11.0	11.9	na
At2g45760	BAP2; BON1-associated protein 2	6.1	33.1	12.5
At4g12720	NUDT7; Nudix hydrolase 7	3.2	6.3	4.0
At3g13610	2OG-Fe(II) oxygenase family protein	na	69.2	na
At1g19250	FMO1; FLAVIN-DEPENDENT MONOOXYGENASE 1	na	39.8	na
At2g04450	NUDT6; Nudix hydrolase 6	na	32.3	16.4
At5g26700	germin-like	na	17.2	9.2
At3g09940	MDHAR;MONODEHYDROASCORBATE REDUCTASE	na	16.0	na
At1g03850	glutaredoxin family protein	na	12.6	16.5
At5g39100	GLP6; GERMIN-LIKE PROTEIN 6	na	11.7	na
At3g61190	BAP1; BON1-associated protein 1	na	11.5	6.9
At1g08860	BON3; BONZAI 3	na	10.8	7.3
At4g39830	putative L-ascorbate oxidase	na	9.6	8.1
At3g28580	AAA-type ATPase family protein	na	7.3	6.0
At2g04430	NUDT5; Nudix hydrolase 5	na	5.9	4.2
At1g18300	NUDT4; Nudix hydrolase 4	na	5.7	5.7
At1g73540	NUDT21; Nudix hydrolase 21	na	4.9	na
At3g54960	ATPDIL1-3; PDI-LIKE 1-3	na	3.8	2.5

At1g05260	RCI3; RARE COLD INDUCIBLE GENE 3; peroxidase	na	3.7	na
At1g72060	serine-type endopeptidase inhibitor	na	3.7	3.9
At5g39150	germin-like	na	3.5	na
At5g61900	BON1; BONZAI 1	na	3.5	2.4
At1g45145	ATTRX5; thioredoxin H-type 5	na	3.4	na
At2g48150	GPX4; GLUTATHIONE PEROXIDASE 4	na	3.2	3.2
At3g26690	NUDT13; Nudix hydrolase 13	na	3.1	na
At2g01670	NUDT17; Nudix hydrolase 17	na	2.5	2.4
At1g21750	ATPDIL1-1; PDI-LIKE 1-1	na	2.9	na
At4g12400	putative stress-inducible protein	na	2.9	na
At5g39950	ATTRX2; ARABIDOPSIS THIOREDOXIN H2	na	2.6	2.1
At1g76760	ATY1; ARABIDOPSIS THIOREDOXIN Y1	na	2.1	na
At1g74310	ATHSP101; HEAT SHOCK PROTEIN 101	na	2.1	na
At2g47730	ATGSTF8; GLUTATHIONE S-TRANSFERASE 8	na	2.0	na
At4g14630	GLP9; GERMIN-LIKE PROTEIN 9	na	na	6.3
At5g58390	peroxidase, putative	0.1	na	0.1
At3g03190	ATGSTF11; GLUTATHIONE S-TRANSFERASE F11	na	0.2	na
At1g02940	ATGSTF5; Glutathione S-transferase (class phi) 5	na	0.3	na
At5g60540	PDX2; PYRIDOXINE BIOSYNTHESIS 2	na	0.5	na
At5g01410	PDX1; PYRIDOXINE BIOSYNTHESIS 1.3	na	0.5	na
At5g58530	glutaredoxin family protein	na	na	0.5

Taken together, these data suggest that there is not only a strong antioxidant response to aphid attack in the local leaves but also pronounced re-adjustment of related thiol signalling pathways involving interacting thioredoxin and glutathione utilising enzymes.

3.2.4.2 Effect of aphid feeding on the expression levels of genes encoding proteins related to calcium homeostasis

Redox signalling and calcium signalling have been linked in many studies, particularly at the level of ROS production by NADPH oxidases that are activated by calcium-dependent protein kinases (CDPKs) (Kobayashi et al., 2007; Sagi and Fluhr, 2001). CDPKs are Ca^{2+}-binding sensory proteins that contain both protein kinase and calmodulin-like domains. CDPKs are activated during pathogen responses and most probably are important calcium sensors in inducible defence responses. CDPK antagonists block ROS formation (Kobayashi et al., 2007). A number of transcripts encoding proteins associated with calcium homeostasis were increased as early as 6 hpi, in the infested leaves suggesting a rapid change in cellular calcium homeostasis and the activation of defense responses through calcium-dependent pathways (Table 3.3).

Calcium is stored in multiple compartments, including the apoplast, vacuole, endoplasmic reticulum, chloroplast and mitochondria (McAinsh and Pittman, 2009). Cytosolic Ca^{2+} levels are generally considered to be in the 100–200 nM range (McAinsh and Pittman, 2009). Cytosolic Ca^{2+} concentrations are affected by Ca^{2+} efflux, which is mediated by H^+/Ca^{2+}-antiporters and Ca^{2+}-ATPases (Dodd et al., 2010). A range of environmental and metabolic stimuli influence the activities of these differentially localized Ca^{2+} pumps leading to a variations in the characteristic calcium wave produced by plant cells (Dodd et al., 2010). In this study, four genes encoding auto-inhibited Ca^{2+}-ATPases (*ACA1*, *ACA2*, *ACA10* and *ACA13*) were up-regulated following aphid attack in the local leaves (Table 3.3). Transcripts encoding ACA2,

which is localized in the endoplasmic reticulum (Harper et al., 1998) and ACA1 and ACA10, which are found in the inner chloroplast membrane and the plasma membrane, respectively (George et al., 2008; Huang et al., 1993) were increased at 24 hpi. Only, *ACA13* transcripts were significantly increased at 6hpi (Table 3.3).

Increases in cytosolic calcium are thought to be sensed via Ca^{2+}-binding proteins, which usually contain Ca^{2+}-binding EF-hand motif(s) (De Falco et al., 2009), in which each of the helix-loop-helix motifs binds a single Ca^{2+} ion. Three major classes of EF-hand sensors (calmodulins, CDPKs and calcineurin B-like proteins) have been characterized in plants, based on their structural similarities and the number of EF-hand motifs (Luan et al., 2002). Transcripts encoding members of all three classes were differentially regulated in the local leaves in response to *M. persicae* attack (Table 3.3). With the exception of *CBL10* and *NPGR1*, transcripts encoding EF-hand sensors were increased early in the response to infestation (Table 3.3). In addition, transcripts encoding calmodulin-binding proteins were differentially regulated in response to infestation. Calmodulin is a small acidic protein found in all eukaryotes with four EF-hand motifs, which regulates numerous enzymes and undergoes conformational changes upon binding of Ca^{2+} (Chin and Means, 2000).

Table 3.3 Genes related to calcium homeostasis which expression was altered in the local leaves of *A. thaliana* Col0 at 6, 24 and 48 h upon *M. persicae* attack. Sixty aphids were confined to a clip cage attached to a mature rosette leaf and gene expression analysed with Agilent V4 arrays relative to non-infested caged controls. Statistically significant differentially expressed genes were identified on volcano plots with a cut-off of fold change ≥2 and Student's t-test p-value ≤0.05. Genes were attributed to this category based on either literature evidence or Gene Ontology (GO). GO classification was made according to Biological Process (calcium ion transmembrane transport or calcium ion transport) or Molecular Function (calcium ion binding) as found at The Arabidopsis Information Resource (TAIR; www.arabidopsis.org). na (not applicable) indicates that the corresponding transcript did not fulfil the adopted statistical criteria for differential expression. All ratios are expressed on a linear scale.

Accession	Description	Fold change		
		6h	24h	48h
At5g39670	calcium-binding EF hand family protein	4.5	11.5	7.6
At2g41100	TCH3 (TOUCH 3)	4.1	6.4	5.4
At4g27280	calcium-binding EF hand family protein	2.9	5.9	8.2
At3g29000	calcium-binding EF hand family protein	2.9	na	na
At3g22910	calcium-transporting ATPase, plasma membrane-type, putative / Ca^{2+}-ATPase, putative (ACA13)	2.8	13.8	5.3
At1g73805	calmodulin binding	2.4	6.8	5.8
At1g76040	CPK29 (calcium-dependent protein kinase 29); calmodulin-dependent protein kinase/ kinase	2.1	7.7	4.6
At3g01830	calmodulin-related protein, putative	na	21.5	12.5
At5g44460	calcium-binding protein, putative	na	19.9	17.4
At5g42380	CML37/CML39; calcium ion binding	na	13.3	11.6
At3g47480	calcium-binding EF hand family protein	na	11.4	8.1
At4g04695	CPK31 (calcium-dependent protein kinase 31); calmodulin-dependent protein kinase/ kinase	na	4.8	3.6
At1g66400	calmodulin-related protein, putative	na	4.2	na
At4g29900	ACA10 (autoinhibited Ca^{2+} -ATPase 10); calcium-transporting ATPase/ calmodulin binding	na	3.4	na
At5g66210	CPK28 (calcium-dependent protein kinase 28)	na	2.7	2.9
At3g56800	CAM3 (CALMODULIN 3); calcium ion binding	na	2.5	2.6

At4g37640	ACA2 (CALCIUM ATPASE 2); calmodulin binding	na	2.5	na
At1g27770	ACA1 (autoinhibited Ca^{2+} -ATPase 1); calcium-transporting ATPase/ calmodulin binding	na	2.4	2.2
At1g18890	ATCDPK1; calmodulin-dependent protein kinase/ kinase/ protein kinase	na	2.3	2.1
At3g25600	calmodulin, putative	na	2.2	3.0
At4g01010	ATCNGC13 (cyclic nucleotide gated channel 13); calmodulin binding / cyclic nucleotide binding / ion channel	na	2.2	na
At2g26190	calmodulin-binding family protein	na	2.2	2.4
At4g21940	CPK15 (calcium-dependent protein kinase 15); calmodulin-dependent protein kinase/ kinase	na	2.0	na
At4g33000	CBL10 (CALCINEURIN B-LIKE10); calcium ion binding	na	0.4	na
At1g27460	NPGR1 (NO POLLEN GERMINATION RELATED 1); calmodulin binding	na	0.3	na

3.2.4.3 Effect of aphid feeding on the expression levels of genes encoding mitogen activated protein kinases (MAPKs).

Mitogen activated protein kinase (MAPK) cascades act as universal signal transduction networks and they are also key players in the reactive oxygen species (ROS)-mediated and Ca^{2+}-regulated signalling cascades that connect many diverse signalling pathways. The redox signalling hub involves not only oxidants and antioxidants but also interacting hormone, calcium and MAPK cascades, which in some cases serve to regulate ROS production (Takahashi et al., 2011). MAPKs catalyse the phosphorylation of proteins on specific serine and threonine residues, to regulate intra- and extracellular signalling networks (Rodriguez et al., 2010).

The expression of several MAPKs was induced by aphid feeding in the local leaves at all time points (Table 3.4). Of these, two MAPKinases (*MKK1* and *MKK2*) are known to form part of the MEKK1-MKK1/MKK2-MPK4 pathway, which has been implicated in biotic and abiotic stress responses and is a negative regulator of cell death by influencing SA signalling and redox pathways (Gao et al., 2008; Qiu et al., 2008; Pitzschke et al., 2009). Like the *BAP1* and *BAP2* transcripts and their interacting partners *BON1* and *BON3*, which are inhibitors of hydrogen peroxide-induced cell death (Yang et al., 2007), *MKK1* and *MKK2* transcripts were enhanced at 24 hpi. Double mutants (*mkk1mkk2*) lacking MKK1 and MKK2 die at seedling stage (Gao et al., 2008), the seedling lethality phenotype being accompanied by accumulation of high levels of hydrogen peroxide, spontaneous cell death, constitutive expression of PR genes, and enhanced pathogen resistance. Conversely, over-expression of *MKK2* enhanced resistance to the biotrophic pathogen *P. syringae*, but decreased sensitivity to the fungal necrotroph *Alternaria brassicola* (Brader et al., 2007). These findings are consistent with the view that *MKK2* is a negative regulator of ROS-mediated cell death pathways.

Accession	Description	Fold change		
		6h	24h	48h
At4g36950	MAPKKK21; ATP binding / protein kinase	3.8	7.5	na
At1g01560	ATMPK11 (Arabidopsis thaliana MAP kinase 11); MAP kinase/ kinase	na	7.1	na
At4g26070	MEK1 (mitogen-activated protein kinase kinase 1); MAP kinase kinase/ kinase	na	2.6	2.3
At4g29810	ATMKK2 (MAP KINASE KINASE 2); MAP kinase kinase/ kinase	na	2.6	na
At3g21220	ATMKK5 (MITOGEN-ACTIVATED PROTEIN KINASE KINASE 5)	na	2.4	na
At4g08470	MAPKKK10 (Mitogen-activated protein kinase kinase kinase 10)	na	2.2	2.1
At2g32510	MAPKKK17 (Mitogen-activated protein kinase kinase kinase 17)	na	na	0.5

Table 3.4 Genes encoding MAPKs which expression was altered in the local leaves of *A. thaliana* Col0 at 6, 24 and 48 h upon *M. persicae* attack. Sixty aphids were confined to a clip cage attached to a mature rosette leaf and gene expression analysed with Agilent V4 arrays relative to non-infested caged controls. Statistically significant differentially expressed genes were identified on volcano plots with a cut-off of fold change ≥2 and Student's t-test p-value ≤0.05. Genes were attributed to this category based on either literature evidence or Gene Ontology (GO). GO classification was made according to Molecular Function (MAP kinase kinase activity or MAP kinase activity) as found at The Arabidopsis Information Resource (TAIR; www.arabidopsis.org). na (not applicable) indicates that the corresponding transcript did not fulfil the adopted statistical criteria for differential expression. All ratios are expressed on a linear scale.

Its induction in response to abiotic stresses enhances plant defenses while limiting genetically programmed cell suicide events. The enhanced resistance to *P. syringae* observed in the *MKK2* over-expressing lines was accompanied smaller pathogen-induced increases in JA and SA, indicating that MKK2 is involved in regulating these hormone signalling pathways (Brader et al., 2007). Together with MPK6, MKK1 forms part of a phosphorelay system that modulates an ABA-dependent signalling cascades that regulate hydrogen peroxide production (Xing et al., 2008).

3.2.4.4 Genes related to ET biosynthesis and signalling

In addition to the changes in the redox-regulated signalling pathways described above, changes in transcripts encoding components involved in hormone metabolism and signalling were also markedly changed in the leaves infested by aphids. For example, *ATARD3* transcripts (*At2g26400*) encoding acireductone dioxygenase 3, an enzyme from the methionine cycle, were enhanced at all time points (Table 3.5). The *ATARD3* transcripts were the most highly expressed at the later time-points. Acireductone dioxygenase 3 is required for the recycling of methionine from methylthioadenosine, which is released as a side product during the first committed step in ethylene synthesis (Bürstenbinder et al., 2007). Methylthioadenosine is also formed as a by-product of polyamine biosynthesis (Bürstenbinder et al., 2007). The expression of a gene (*At1g03400*) encoding a protein with sequence similarity to tomato 1-amino-cyclopropane-1-carboxylic acid oxidase, which is also involved in ethylene synthesis (Trentmann and Kende, 1995) was induced at 24 and 48 hpi. Other evidence supports the hypothesis that there is a rapid response in ethylene synthesis and signalling. Firstly, transcripts encoding several members of the ETHYLENE-RESPONSIVE FACTOR (ERF)/APETALA2 (AP2) transcription factor family were up-regulated, particularly the later time-points (Table 3.5).

Table 3.5 Transcripts encoding components of ET biosynthesis and signalling pathways which expression was altered in the local leaves of *A. thaliana* Col0 at 6, 24 and 48 h upon *M. persicae* attack. Sixty aphids were confined to a clip cage attached to a mature rosette leaf and gene expression analysed with Agilent V4 arrays relative to non-infested caged controls. Statistically significant differentially expressed genes were identified on volcano plots with a cut-off of fold change ≥2 and Student's t-test p-value ≤0.05. Genes were attributed to this category based on either literature evidence or Gene Ontology (GO). GO classification was made according to Biological Process (ethylene mediated signalling pathway) as found at The Arabidopsis Information Resource (TAIR; www.arabidopsis.org). na (not applicable) indicates that the corresponding transcript did not fulfil the adopted statistical criteria for differential expression. All ratios are expressed on a linear scale.

Accession	Description	Fold change		
		6h	24h	48h
At2g26400	ARD/ATARD3 (ACIREDUCTONE DIOXYGENASE)	12.6	346.0	189.4
At4g34410	AP2 domain-containing transcription factor, putative	16.8	40.9	39.9
At2g44840	ATERF13/EREBP (ETHYLENE-RESPONSIVE ELEMENT BINDING FACTOR 13); transcription factor	7.5	10.3	8.1
At1g33760	AP2 domain-containing transcription factor, putative	3.4	11.3	na
At5g51190	AP2 domain-containing transcription factor, putative	3.2	4.6	6.4
At2g22200	AP2 domain-containing transcription factor	3.1	na	na
At4g17490	ATERF6 (ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 6); DNA binding / transcription factor	2.4	na	na
At1g19210	AP2 domain-containing transcription factor, putative	na	17.1	na
At5g21960	AP2 domain-containing transcription factor, putative	na	13.7	na
At5g61600	ethylene-responsive element-binding family protein	na	2.9	5.0
At1g03400	2-oxoglutarate-dependent dioxygenase, putative	na	2.9	2.3
At1g77640	AP2 domain-containing transcription factor, putative	na	2.7	na
At1g06160	ORA59 (OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF 59)	na	0.2	na

At1g12010	1-aminocyclopropane-1-carboxylate oxidase, putative / ACC oxidase, putative	na	0.2	na

ERF/AP2 transcription factors fulfil important roles in plant responses to environmental stress as well as in the control of the hormonal regulation of growth and development (Gutterson and Reuber, 2004).

Other transcripts indicate an interaction between JA and ET signalling pathways in the infested leaves. For example, *OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF 59* (*ORA59*), which is involved in the cross talk between the JA and ET signalling pathways during plant responses to pathogens, was down-regulated at 24 hpi. The expression of several JA and ET-induced defence genes is dependent on ORA59 (Pré et al., 2008). Plants over-expressing *ORA59* were more resistant to the fungus *Botrytis cinerea* (Pre et al., 2008). Silencing of *ORA59* makes plants more susceptible to fungal attack (Pre et al., 2008). Decreases in *ORA59* at the 24 hpi time point may indicate that JA responses are decreased in favour ET-induced defences.

3.2.4.5 Salicylic acid associated transcripts

Other evidence in support of rapid responses in hormone signalling pathways following infestation is provided by the large numbers of transcripts associated with SA-mediated signal transduction that are increased in response to *M. persicae* attack (Table 3.6). For example, *WRKY62, 38, 18* and *70* transcripts, and *PAD4, EDS5, EDS1* and NIM1 mRNAs were all increased at 6 hpi (Table 3.6). The expression of *WRKY62* is induced by SA and by JA in a NPR1-dependent manner (Mao et al., 2007). *WRKY62* is considered to be a negative regulator of basal defenses against pathogens, *wrky62* mutants showing enhanced disease resistance and *PR1* gene expression (Mao et al., 2007). Large increases in even greater numbers of transcripts involved in SA

biosynthesis and SA-mediated signal transduction were observed at 24 and 48 hpi (Table 3.6). For example, *ISOCHORISMATE SYNTHASE I* (*ICS1*), which encodes and enzyme involved in SA biosynthesis, was induced at 24 and 48 hpi. Isochorismate synthase is required for pathogen-induced SA biosynthesis (Garcion et al., 2008). Plants overexpressing *EDS5* accumulate SA and show a heightened pathogen resistance (Nawrath et al., 2002).

SA-mediated responses are linked to calcium signalling via AtSR1/CAMTA3 encoding a Ca^{2+}/calmodulin binding transcription factor (Du et al., 2009). AtSR1/CAMTA3 acts as a negative regulator of SA biosynthesis. Mutants that lack AtSR1/CAMTA3 display higher SA levels and greater pathogens resistance (Du et al., 2009). In the preset study (Table 3.3), the infested leaves accumulated transcripts encoding a calmodulin binding protein 60 g (CBP60G), which is also induced during pathogen attack (Wang et al., 2011). Mutants lacking a functional CBP60G protein have higher SA levels and are more susceptible to bacterial infection (Wang et al., 2011).

A gene encoding *OCS-ELEMENT BINDING FACTOR 5* (*OBF5*), which belongs to the basic leucine zipper transcription factor family, was induced in the local tissue at 24 hpi. OBF5 interacts with NPR1 to activate expression of SA-induced genes (Kim and Delaney, 2002). Transcripts encoding pathogenesis related proteins (*PR1*, *PR2* and *PR5*), which are widely used as molecular markers of SA-defence pathways (Yoshimoto et al., 2009), were increased at 24 and 48 hpi. Moreover, transcripts encoding two NPR1-like proteins (*NPR3* and *NPR4*) were also slightly increased at these time points. The Arabidopsis *npr3npr4* double mutant has elevated basal levels of *PR1* and displays enhanced resistance against bacterial pathogens (Zhang et al., 2006).

Table 3.6 Salicylic acid associated transcripts which expression was altered in the local leaves of *A. thaliana* Col0 at 6, 24 and 48 h upon *M. persicae* attack. Sixty aphids were confined to a clip cage attached to a mature rosette leaf and gene expression analysed with Agilent V4 arrays relative to non-infested caged controls. Statistically significant differentially expressed genes were identified on volcano plots with a cut-off of fold change ≥2 and Student's t-test p-value ≤0.05. Genes were attributed to this category based on either literature evidence or Gene Ontology (GO). GO classification was made according to Biological Process (salicylic acid mediated signaling pathway, salicylic acid biosynthetic process or systemic acquired resistance) as found at The Arabidopsis Information Resource (TAIR; www.arabidopsis.org). na (not applicable) indicates that the corresponding transcript did not fulfil the adopted statistical criteria for differential expression. All ratios are expressed on a linear scale.

Accession	Description	Fold change		
		6h	24h	48h
At5g01900	WRKY62 (WRKY DNA-binding protein 62); transcription factor	8.1	na	18.2
At1g02450	NIM1-INTERACTING 1; protein binding	6.5	28.8	12.9
At5g22570	WRKY38 (WRKY DNA-binding protein 38); transcription factor	4.4	14.5	9.6
At4g31800	WRKY18 (WRKY DNA-binding protein 18); transcription factor	4.0	9.3	6.9
At3g52430	PAD4 (PHYTOALEXIN DEFICIENT 4); triacylglycerol lipase	3.6	10.0	7.6
At3g56400	WRKY70 (WRKY DNA-binding protein 70); transcription factor	3.5	7.9	6.3
At4g39030	EDS5 (ENHANCED DISEASE SUSCEPTIBILITY 5); antiporter/ transporter	3.4	na	4.8
At5g26920	CAM-BINDING PROTEIN 60-LIKE G	2.5	6.1	4.5
At3g48090	EDS1 (ENHANCED DISEASE SUSCEPTIBILITY 1); signal transducer/ triacylglycerol lipase	2.3	5.1	3.9
At2g13810	ALD1 (AGD2-LIKE DEFENSE RESPONSE PROTEIN1); transaminase	na	47.0	na
At5g13320	PBS3 (AVRPPHB SUSCEPTIBLE 3)	na	14.2	na
At2g14610	PR1 (PATHOGENESIS-RELATED GENE 1)	na	12.0	17.3
At1g74710	ICS1 (ISOCHORISMATE SYNTHASEI); isochorismate synthase	na	10.4	6.6
At5g54610	ANK (ANKYRIN); protein binding	na	8.3	6.5
At1g75040	PR5 (PATHOGENESIS-RELATED GENE 5)	na	5.1	8.1

At3g57260	BGL2 (PATHOGENESIS-RELATED PROTEIN 2)	na	5.1	5.7
At2g25000	WRKY60 (WRKY DNA-BINDING PROTEIN 60); transcription factor	na	3.6	2.7
At5g45110	NPR3 (NPR1-LIKE PROTEIN 3); protein binding	na	3.5	3.0
At4g14400	ACD6 (ACCELERATED CELL DEATH 6); protein binding	na	3.2	3.4
At2g30250	WRKY25 (WRKY DNA-binding protein 25); transcription factor	na	2.7	na
At3g11280	myb family transcription factor	na	2.8	2.5
At4g23170	EP1; protein kinase	na	2.6	2.2
At2g37710	RLK (RECEPTOR LECTIN KINASE); kinase	na	2.5	2.2
At2g40750	WRKY54 (WRKY DNA-binding protein 54); transcription factor	na	2.4	2.4
At5g06960	OBF5 (OCS-ELEMENT BINDING FACTOR 5); DNA binding / transcription factor	na	2.2	na
At4g19660	NPR4 (NPR1-LIKE PROTEIN 4); protein binding	na	2.0	2.3
At3g50930	BCS1(CYTOCHROME BC1 SYNTHESIS)	na	na	6.2
At3g25882	NIM1-INTERACTING 2	na	na	4.5

3.2.4.6 Expression profiles of genes related to JA biosynthesis and signalling

The aphid-induced changes in transcriptome patterns provide some evidence in support of altered JA biosynthesis pathways in the infested leaves (Table 3.7). For example, transcript encoding ALLENE OXIDE CYCLASE 3, one of the key enzymes in JA synthesis pathway (Schaller et al., 2008), were increased relative to controls at all time points measured. Similarly, the expression of *lipoxygenase 5* (*LOX5*) and *12-oxophytodienoate reductase* (*At1g09400*), which have also been implicated in JA synthesis (Stenzel et al., 2003) was enhanced at 24 and 48 hpi. However, transcripts encoding a fatty acid desaturase (*FAD8*) were decreased 24 hpi. The omega-3 trienoic fatty acids (TFAs) are considered to serve as precursors for JA synthesis (Mène-Saffrané et al., 2009), the triple fatty acid desaturase mutant (*fad3fad7fad8*) being JA deficient (Mène-Saffrané et al., 2009).

Defensins are low-molecular cysteine-rich antifungal proteins, which are responsive to ET and JA. Defensin expression is often used as a marker for activation of JA/ET signalling events (Brown et al., 2003). In this study, the expression of five genes from the plant defensin family (*PDF1.2*, *PDF1.2b*, *PDF1.2c*, *PDF1.3*, and *PDF2.2*) was decreased but only at 24 hpi (Table 3.7). Moreover, transcripts encoding vegetative storage proteins (*VSP1* and *VSP2*) that are used as markers of the JA/ET transduction pathway were also decreased at 48 hpi. VSP1 and VSP2 are considered to have anti-insect properties that result in retarded insect development (Liu et al., 2005).

Accession	Description	Fold change		
		6h	24h	48h
At3g25780	AOC3 (ALLENE OXIDE CYCLASE 3)	3.2	5.4	4.0
At2g27690	CYP94C1 (cytochrome P450, family 94, subfamily C, polypeptide 1); oxygen binding	2.9	5.6	4.9
At2g24850	TAT3 (TYROSINE AMINOTRANSFERASE 3); transaminase	na	30.1	na
At3g22400	LOX5; lipoxygenase	na	6.3	5.0
At1g09400	12-oxophytodienoate reductase, putative	na	2.6	na
At5g05580	FAD8 (FATTY ACID DESATURASE 8); omega-3 fatty acid desaturase	na	0.3	na
At2g02100	LCR69/PDF2.2 (Low-molecular-weight cysteine-rich 69); protease inhibitor	na	0.2	na
At5g44420	PDF1.2 (Low-molecular-weight cysteine-rich 77)	na	0.05	na
At2g26010	PDF1.3 (plant defensin 1.3)	na	0.04	na
At5g44430	PDF1.2c (plant defensin 1.2c)	na	0.03	na
At2g26020	PDF1.2b (plant defensin 1.2b)	na	0.02	na
At5g24780	VSP1 (VEGETATIVE STORAGE PROTEIN 1)	na	na	0.1
At5g24770	VSP2 (VEGETATIVE STORAGE PROTEIN 2)	na	na	0.1

Table 3.7 Genes related to JA biosynthesis and signalling which expression was altered in the local leaves of *A. thaliana* Col0 at 6, 24 and 48 h upon *M. persicae* attack. Sixty aphids were confined to a clip cage attached to a mature rosette leaf and gene expression analysed with Agilent V4 arrays relative to non-infested caged controls. Statistically significant differentially expressed genes were identified on volcano plots with a cut-off of fold change ≥2 and Student's t-test p-value ≤0.05. Genes were attributed to this category based on either literature evidence or Gene Ontology (GO). GO classification was made according to Biological Process (jasmonic acid biosynthetic process or response to jasmonic acid stimulus) as found at The Arabidopsis Information Resource (TAIR; www.arabidopsis.org). na (not applicable) indicates that the corresponding transcript did not fulfil the adopted statistical criteria for differential expression. All ratios are expressed on a linear scale.

3.2.4.7 Expression profiles of genes involved in ABA-mediated signaling

Taken together, the data shown in Table 3.7 suggest that JA synthesis is stimulated at the site of infestation, but there is only limited evidence of local activation of JA-signalling responses. However, transcripts encoding a member of the Dehydration Responsive Element Binding (DREB) transcription factor family (*ORA47*) were increased early in the local response to aphids (Table 3.8).

Plants over-expressing *ORA47* are insensitive to ABA and showed decreased tolerance to dehydration in comparison to wild type plants (Hsing-Yu et al., 2010). Crucially, the *ORA47* transcription factor has been linked to fine tuning of JA signalling pathways, for example by activation of lipoxygenases (Pauwels and Goossens, 2008). Similarly, transcripts encoding ABI FIVE BINDING PROTEIN 3 (AFP3), a protein involved in JA metabolism (Pauwels et al., 2010), are enhanced at 24 hpi supporting the arguments in favour of fine tuning of JA signalling pathways in the local response to infestation. In addition, the altered *ORA47* and *AFP3* expression pattern is part of a more general response of drought responsive genes to aphid infestation particularly at 24 and 48 hpi (Table 3.8). Moreover, *NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 5* (*NCED5*), one of the six genes encoding the ABA-biosynthetic enzyme 9-cis-epoxycarotinoid dioxygenase, was up-regulated in the local leaves at the last two time points of the experiment. Transcripts encoding other ABA and drought responsive transcription factors including *ARABIDOPSIS ZINC-FINGER PROTEIN 2* (*AZF2*), *ABA-OVERLY SENSITIVE 1* (*ABO1*), *DREB2A, ACT Domain Repeat 8, MYB15* and *WRKY63* were also enhanced infested leaves (Table 3.8).

Table 3.8 Genes implicated in ABA-mediated signalling pathways which expression was altered in the local leaves of *A. thaliana* Col0 at 6, 24 and 48 h upon *M. persicae* attack. Sixty aphids were confined to a clip cage attached to a mature rosette leaf and gene expression analysed with Agilent V4 arrays relative to non-infested caged controls. Statistically significant differentially expressed genes were identified on volcano plots with a cut-off of fold change ≥2 and Student's t-test p-value ≤0.05. Genes were attributed to this category based on either literature evidence or Gene Ontology (GO). GO classification was made according to Biological Process (abscisic acid biosynthetic process or response to abscisic acid stimulus) as found at The Arabidopsis Information Resource (TAIR; www.arabidopsis.org). na (not applicable) indicates that the corresponding transcript did not fulfil the adopted statistical criteria for differential expression. All ratios are expressed on a linear scale.

Accession	Description	Fold change		
		6h	24h	48h
At5g67450	AZF1 (ARABIDOPSIS ZINC-FINGER PROTEIN 1); nucleic acid binding / transcription factor/ zinc ion	5.8	15.0	13.0
At1g74930	ORA47; DNA binding / transcription factor	4.9	6.2	na
At5g43650	basic helix-loop-helix (bHLH) family protein	na	36.1	24.5
At1g66600	WRKY63 (WRKY DNA-binding protein 63); transcription factor	na	11.9	11.1
At3g23250	AtMYB15/AtY19/MYB15 (myb domain protein 15); DNA binding / transcription factor	na	6.9	6.9
At1g30100	NCED5 (NINE-CIS-EPOXYCAROTENOID DIOXYGENASE 5)	na	6.0	2.5
At3g19580	AZF2 (ARABIDOPSIS ZINC-FINGER PROTEIN 2); nucleic acid binding / transcription factor/ zinc ion binding	na	4.4	na
At5g13680	ABO1/ELO2 (ABA-OVERLY SENSITIVE 1); transcription elongation regulator	na	3.2	na
At1g12420	ACR8 (ACT Domain Repeat 8)	na	2.8	2.9
At3g29575	AFP3 (ABI FIVE BINDING PROTEIN 3)	na	2.8	na
At2g40180	ATHPP2C5; protein serine/threonine phosphatase	na	2.8	na
At4g34390	XLG2 (EXTRA-LARGE GTP-BINDING PROTEIN 2); signal transducer	na	2.5	2.1
At5g05410	DREB2A (DRE-BINDING PROTEIN 2A); DNA binding / transcription activator/ transcription factor	na	2.4	2.6
At4g09570	CPK4 (calcium-dependent protein kinase 4); calmodulin-dependent protein kinase/ kinase	na	2.1	na

At1g15080	ATPAP2 (PHOSPHATIDIC ACID PHOSPHATASE 2); phosphatidate phosphatase	na	0.5	na
At5g35750	AHK2 (ARABIDOPSIS HISTIDINE KINASE 2)	na	0.5	na
At3g26520	TIP2 (TONOPLAST INTRINSIC PROTEIN 2); water channel	na	0.4	na
At5g25610	RD22 (RESPONSIVE TO DESSICATION 22)	na	0.4	0.3
At4g25480	DREB1A (DEHYDRATION RESPONSE ELEMENT B1A); DNA binding / transcription activator/ transcription factor	na	0.4	0.3

3.2.4.8 Effect of aphid feeding on senescence-related genes in the leaves directly subjected to aphid attack

Aphid feeding has been shown to cause premature senescence in Arabidopsis leaves (Pegadaraju et al., 2005). In support of this view, the expression of more than 10 genes encoding various peptidases, proteases and senescence-associated proteins was enhnaced at 24 and 48 hpi (but not at 6 hpi) in the infested leaves (Table 3.9). For example, *SENESCENCE-ASSOCIATED GENE 101* (*SAG101*) transcripts, which are expressed in leaves undergoing senescence (He and Gan, 2002) were enhanced as a result of *M. persicae* infestation, as was the expression of *FLG22-INDUCED RECEPTOR-LIKE KINASE 1* (*FRK1*) (Table 3.9). FRK1, which is a receptor-like protein kinase that is involved in early defence signalling pathways, is strongly up-regulated during leaf senescence (Robatzek and Somssich, 2002). Transcripts encoding WRKY53, a key regulator of leaf senescence, accumulated in the infested leaves at 24 and 48 hpi. WRKY53 functions in a complex transcriptional network governing senescence (Miao and Zentgraf, 2010). Changes in hydrogen peroxide levels have been implicated in WRKY53 function (Miao and Zentgraf, 2010).

SENESCENCE-ASSOCIATED E3 UBIQUITIN LIGASE 1 (*SAUL1*) transcripts were also increased as a result of infestation. This protein is considered to prevent premature leaf senescence by targeting ABSCISIC ALDEHYDE OXIDASE 3 (a key enzyme in ABA biosynthesis) for degradation. The *saul1* mutants accumulate ABA and exhibit premature senescence, together with increased expression of senescence genes (Raab et al., 2009).

Table 3.9 Genes related to senescence which expression was altered in the local leaves of *A. thaliana* Col0 at 6, 24 and 48 h upon *M. persicae* attack. Sixty aphids were confined to a clip cage attached to a mature rosette leaf and gene expression analysed with Agilent V4 arrays relative to non-infested caged controls. Statistically significant differentially expressed genes were identified on volcano plots with a cut-off of fold change ≥2 and Student's t-test p-value ≤0.05. Genes were attributed to this category based on either literature evidence or Gene Ontology (GO). GO classification was made according to Biological Process (aging or apoptotic process) as found at The Arabidopsis Information Resource (TAIR; www.arabidopsis.org). na (not applicable) indicates that the corresponding transcript did not fulfil the adopted statistical criteria for differential expression. All ratios are expressed on a linear scale.

Accession	Description	Fold change		
		6h	24h	48h
At3g12230	SCPL14 (serine carboxypeptidase-like 14)	na	27.8	13.1
At4g23810	WRKY53 (WRKY DNA-binding protein 53)	na	8.4	7.0
At2g19190	FLG22-INDUCED RECEPTOR-LIKE KINASE 1	na	7.1	na
At5g10760	aspartyl protease family protein	na	6.6	8.6
At3g51330	aspartyl protease family protein	na	6.2	4.2
At3g45860	CYSTEINE-RICH RLK 4	na	5.2	7.1
At2g13790	BRI1- ASSOCIATED KINASE 7 (BAK7)	na	4.2	3.2
At5g11870	similar to SAG18 (SENESCENCE ASSOCIATED GENE 18)	na	4.1	2.6
At1g49050	aspartyl protease family protein	na	2.9	2.4
At1g21750	PROTEIN DISULFIDE ISOMERASE 5	na	2.9	na
At5g10380	SAUL1 (SENESCENCE-ASSOCIATED E3 UBIQUITIN LIGASE 1)	na	2.8	3.9
At3g52400	SYP122 (SYNTAXIN OF PLANTS 122)	na	2.7	3.0
At3g12220	SCPL16 (serine carboxypeptidase-like 16)	na	2.6	2.3
At2g33530	SCPL46 (serine carboxypeptidase-like 46)	na	2.5	2.3
At2g44180	MAP2A (METHIONINE AMINOPEPTIDASE 2A); methionyl aminopeptidase	na	2.5	2.2
At5g14930	SENESCENCE-ASSOCIATED GENE 101 (SAG101)	na	2.3	2.3
AT3g11820	SYP121 (SYNTAXIN OF PLANTS 121)	na	2.3	na
At5g37540	aspartyl protease family protein	na	2.3	na
At5g47120	ARABIDOPSIS BAX INHIBITOR 1	na	2.2	na
At4g39795	senescence-associated protein-related	na	0.2	na

At5g19740	peptidase M28 family protein	na	0.3	na
At2g23010	SCPL9; serine carboxypeptidase	na	0.3	0.4
At2g22980	SCPL13; serine carboxypeptidase	na	0.3	0.5
At2g22920	SCPL12; serine carboxypeptidase	na	0.4	na
At1g74940	senescence-associated protein-related	na	0.4	na
At1g78020	senescence-associated protein-related	na	0.4	na
At1g79720	aspartyl protease family protein	na	0.4	na
At4g15450	senescence/dehydration-associated protein-related	na	0.5	na
At2g47940	DEGP2 (DEGP PROTEASE 2)	na	0.5	na

Interestingly, *SYNTAXIN OF PLANTS 121* (*SYP121*) and *SYNTAXIN OF PLANTS 122* (*SYP122*) transcripts, which encode members of the Soluble NSF (*N*-ethylmaleimide–sensitive factor Attachment Protein Receptors (SNARE) superfamily of proteins, were enhanced at 24 hpi. The SYP121 and SYP122 proteins are considered to act as negative regulators of programmed cell death, through SA and JA/ET signalling pathways (Zhang et al., 2007).

3.2.4.9 Expression patterns of genes involved in glucosinolate biosynthesis and signalling in infested leaves

Glucosinolates are considered to be important components of pathways that activate MAMP-mediated defence responses such as callose accumulation (Clay et al., 2009). In addition, they fulfil important roles as herbivore deterrents (Kliebenstein et al., 2005). Indole glucosinolates (and their breakdown products) are considered to be important plant signalling molecules (Clay et al., 2009). They have now been implicated in plant innate immunity, with hormones such as ET involved in the induction of glucosinolates accumulation (Clay et al., 2009). Callose deposition is suppressed by defence hormones such as ABA and JA, which negatively affect ET signalling and indole glusosinolates breakdown (Clay et al., 2009). In the present study, a number of transcripts encoding proteins involved in glucosinolate biosynthesis and metabolism were altered in response to aphid infestation (Table 3.10). For example, *HIGH INDOLIC GLUCOSINOLATE 1* (*HIG1* also known as *MYB51*), which binds specifically to the promoters of the indole glucosinolate biosynthetic genes, was significantly increased by aphid feeding at 24 and 48 hpi. Overexpression of *MYB51* in transgenic plants leads to the accumulation of indole glucosinolates, and conversely, low levels of glucosinolates are found in *hig1-1* mutants (Gigolashvili et al., 2007). Moreover, *MYB51* overexpressing plants were less attractive to the generalist lepidopteran herbivore, *Spodoptera exigua* (Gigolashvili et al., 2007).

Accession	Description	Fold change		
		6h	24h	48h
At1g18570	HIG1 (HIGH INDOLIC GLUCOSINOLATE 1)	na	2.8	2.9
At4g13770	CYP83A1 (CYTOCHROME P450 83A1)	na	0.4	na
At1g18590	SULFOTRANSFERASE 17	na	0.4	na
At1g74090	SOT18 (DESULFO-GLUCOSINOLATE SULFOTRANSFERASE 18)	na	0.4	na
At5g23010	MAM1 (2-isopropylmalate synthase 3)	na	0.4	na
At2g43100	isopropylmalate isomerase 2	na	0.4	na
At5g14200	IMD1 (ISOPROPYLMALATE DEHYDROGENASE 1)	na	0.4	na
At3g19710	BCAT4 (BRANCHED-CHAIN AMINOTRANSFERASE4)	na	0.3	na
At1g16410	CYP79F1 (SUPERSHOOT 1)	na	0.3	na
At1g65860	FMO GS-OX1 (FLAVIN-MONOOXYGENASE GLUCOSINOLATE S-OXYGENASE 1)	na	0.3	na
At4g03060	AOP2 (ALKENYL HYDROXALKYL PRODUCING 2)	na	0.2	na
At4g12030	BAT5 (BILE ACID TRANSPORTER 5	na	0.3	na
At5g60890	ATR1 (ALTERED TRYPTOPHAN REGULATION 1)	na	0.3	na
At1g52030	MBP2 (MYROSINASE-BINDING PROTEIN 2)	na	0.3	0.2

Table 3.10 Genes related to glucosinolate biosynthesis which expression was altered in the local leaves of *A. thaliana* Col0 at 6, 24 and 48 h upon *M. persicae* attack. Sixty aphids were confined to a clip cage attached to a mature rosette leaf and gene expression analysed with Agilent V4 arrays relative to non-infested caged controls. Statistically significant differentially expressed genes were identified on volcano plots with a cut-off of fold change ≥2 and Student's t-test p-value ≤0.05. Genes were attributed to this category based on either literature evidence or Gene Ontology (GO). GO classification was made according to Biological Process (indole glucosinolate biosynthetic process or glucosinolate biosynthetic process) as found at The Arabidopsis Information Resource (TAIR; www.arabidopsis.org). na (not applicable) indicates that the corresponding transcript did not fulfil the adopted statistical criteria for differential expression. All ratios are expressed on a linear scale.

MYB51-dependent biosynthesis of certain indole glucosinolates is required for the activation of MAMP-mediated defence responses such as callose accumulation (Clay et al., 2009).

Transcripts encoding ALTERED TRYPTOPHAN REGULATION 1 (ATR1), which is a major regulator of indole glucosinolate and tryptophan biosynthesis, were decreased at 24h in the infested leaves. *ATR1* encodes a Myb transcription factor, which modulates the expression of the *anthranilate synthase alpha subunit 1* (*ASA1*) gene (Bender and Flink, 1998). Mutations in *ATR1* result in reduced levels of indole glucosinolates and impaired expression of indole glucosinolate synthesis genes (Celenza et al., 2005). Anthranilate synthase catalyzes the rate-limiting step of tryptophan synthesis (Li and Last, 1996). *ASA1* is considered to have a role in JA-induced auxin biosynthesis (Sun et al., 2009).

Several genes associated with aliphatic glucosinolate biosynthesis were repressed in the infested leaves at 24 hpi (Table 3.10). These include CYTOCHROME P450 83A1 (CYP83A1), which catalyzes the conversion of aliphatic oximes derived from chain-elongated homologs of methionine, during the synthesis of aliphatic glucosinolates (Naur et al., 2003). Desulfoglucosinolate sulfotransferases, sulfotransferase 17 and desulfoglucosinolate sulfotransferase 18 are involved in the final step of glucosinolate biosynthesis (Klein and Papenbrock, 2009). Isopropylmalate isomerise 2 is involved in the chain elongation during the biosynthesis of methionine-derived glucosinolates (Gigolashvili et al., 2009). *ISOPROPYLMALATE DEHYDROGENASE 1* (*IMD1*) is involved in both methionine chain-elongation of glucosinolates and leucine biosynthesis (He et al., 2009). It is of interest to note that IMD1 activity is subject to redox regulation (He et al., 2009). Other transcripts such as those encoding a methionine-oxo-acid transaminase (BCAT4), a cytochrome P450 (CYP79F1) and FLAVIN-MONOOXYGENASE GLUCOSINOLATE S-OXYGENASE that are involved in the biosynthesis of aliphatic

glucosinolates (Schuster et al., 2006; Chen et al., 2003; Hansen et al., 2007) were also repressed at 24 hpi.

A gene encoding MYROSINASE-BINDING PROTEIN 2, which may play a role in the myrosinase-glucosinolate system (Capella et al., 2001), was also repressed at 24 and 48 hpi.

3.2.4.10 Expression profile of cell wall associated transcripts

The structure of the plant cell wall is an important factor that might be used to limit infestation because of alterations in properties that can prevent or impede aphid stylet penetration. Stylet penetration is accompanied by mechanical damage arising from brief punctures of the cell wall. In general, the plant response to mechanical damage is to induce processes that strengthen or repair the cell wall structure. However, stimulation of any processes that loosen the cell wall will be beneficial to the aphids. In this study, the expression pattern of a number genes encoding components involved in cell wall metabolism and remodelling (Table 3.11), may suggest that cell wall modification is central to plant defences against aphids.

For example, transcripts (*TOUCH 4*) encoding a xyloglucan endotransglycosylase/hydrolase (XTH) where increased at 6 hpi, and remained high at 24 and 48 hpi (Table 3.11). In addition, transcripts encoding two cell wall-associated protein kinases, *WAK1* and *WAK6,* were increased at 24 and 48 hpi, and a third kinase *WAK2* was induced at 48 hpi (Table 3.11).

Several transcripts encoding proteins involved in cell wall loosening such as expansin-like B1 (Li et al., 2002) and *EXTENSIN 3* (Hall and Cannon, 2002) were also increased in infested leaves relative to controls at the later time points, as were transcripts encoding proteins showing similarity to cellulose synthase components *(cellulose synthase-like A11* and *cellulose synthase-like A10*).

Table 3.11 Cell wall associated transcripts which expression was altered in the local leaves of *A. thaliana* Col0 at 6, 24 and 48 h upon *M. persicae* attack. Sixty aphids were confined to a clip cage attached to a mature rosette leaf and gene expression analysed with Agilent V4 arrays relative to non-infested caged controls. Statistically significant differentially expressed genes were identified on volcano plots with a cut-off of fold change ≥2 and Student's t-test p-value ≤0.05. Genes were attributed to this category based on either literature evidence or Gene Ontology (GO). GO classification was made according to Biological Process (plant-type cell wall biogenesis) or Molecular Function (cellulose synthase activity) as found at The Arabidopsis Information Resource (TAIR; www.arabidopsis.org). na (not applicable) indicates that the corresponding transcript did not fulfil the adopted statistical criteria for differential expression. All ratios are expressed on a linear scale.

Accession	Description	Fold change		
		6h	24h	48h
At5g57560	TCH4 (TOUCH 4); hydrolase, acting on glycosyl bonds / xyloglucan:xyloglucosyl transferase	2.9	6.6	12.2
At5g46960	invertase/pectin methylesterase inhibitor family protein	na	22.9	12.7
At5g57550	XTR3 (XYLOGLUCAN ENDOTRANSGLYCOSYLASE 3)	na	14.2	na
At1g02360	chitinase, putative	na	6.4	4.3
At4g01700	chitinase, putative	na	6.3	4.9
At2g43570	chitinase, putative	na	6.1	4.8
At1g21250	WAK1 (CELL WALL-ASSOCIATED KINASE); kinase	na	4.4	3.4
At2g26440	pectinesterase family protein	na	3.7	3.4
At1g72680	CINNAMYL ALCOHOL DEHYDROGENASE 1	na	2.9	2.1
At3g47380	invertase/pectin methylesterase inhibitor family protein	na	2.5	3.4
At1g16110	WAKL6 (WALL ASSOCIATED KINASE-LIKE 6); kinase	na	2.1	2.3
At2g33160	glycoside hydrolase family 28 protein / polygalacturonase (pectinase) family protein	na	2.1	na
At5g07130	LAC13 (laccase 13); copper ion binding / oxidoreductase	na	2.1	na
At2g33160	glycoside hydrolase family 28 protein / polygalacturonase (pectinase) family protein	na	2.1	na
At1g78570	RHM1/ROL1 (RHAMNOSE BIOSYNTHESIS1); UDP-L-rhamnose synthase/ UDP-glucose 4,6-dehydratase/ catalytic	na	2.1	na

At1g21310	ATEXT3 (EXTENSIN 3); structural constituent of cell wall	na	na	3.4
At1g21270	WAK2 (wall-associated kinase 2); protein serine/threonine kinase	na	na	2.2
At5g16190	ATCSLA11 (Cellulose synthase-like A11); transferase, transferring glycosyl groups	na	0.5	na
At5g64640	pectinesterase family protein	na	0.4	0.4
At5g64640	pectinesterase family protein	na	0.4	0.4
At3g54920	PMR6 (POWDERY MILDEW RESISTANT 6); lyase/ pectate lyase	na	0.4	na
At5g58600	PMR5 (POWDERY MILDEW RESISTANT 5)	na	0.4	na
At5g62350	invertase/pectin methylesterase inhibitor family protein / DC 1.2 homolog (FL5-2I22)	na	0.3	na
At3g61490	glycoside hydrolase family 28 protein / polygalacturonase (pectinase) family protein	na	na	0.5
At2g47670	invertase/pectin methylesterase inhibitor family protein	na	na	0.4
At1g24070	ATCSLA10 (Cellulose synthase-like A10); transferase, transferring glycosyl groups	na	na	0.1

In addition, several genes encoding invertase/pectin methylesterase inhibitor family proteins showed a differential expression at 24 and 48 hpi (Table 3.11) as did *CINNAMYL ALCOHOL DEHYDROGENASE 1* and *LACCASE 13* (Raes et al., 2003; Gavnholt and Larsen, 2002) suggesting that lignin synthesis is modified in response to aphid infestation.

3.2.5 Evidence in support of transcriptional reprogramming in systemic tissues

3.2.5.1 ROS associated transcripts

The affects of aphid feeding on the expression of genes that can be classified as involved in the maintenance of redox homeostasis in the infested (local) leaves was described in section *3.2.4.1.* The transcriptome pattern observed in systemic leaves shows a response in transcripts that encode components of redox metabolism and signaling (Table 3.12) that is distinct from that observed in the infested leaves (Table 3.2). For example, *REDOX RESPONSIVE TRANSCRIPTION FACTOR 1* (*At4g34410*), was highly induced late in the response (at 48hpi) in the systemic leaves compared to early induction in infested leaves (at 6hpi). Similarly, transcripts encoding *HEAT SHOCK TRANSCRIPTION FACTOR A2* (*ATHSFA2*), which accumulated in the infested tissue from at 6 hpi onwards, only increased in the systemic leaves at 48 hpi. The defence-related genes that are under the control of ATHSFA2 serve to alleviate ROS-induced cell death (Zhang et al., 2009). Other gene products that are involved in regulation of H_2O_2-induced programmed cell death are also modified in the systemic leaves. For example, transcripts encoding METACASPASE 8 (ATMC8) were strongly repressed at 6 and 24 hpi in the leaves distant from the site of aphid attack (Table 3.12). Transgenic plants overexpressing *ATMC8* are more sensitive to H_2O_2-induced cell death than the wild type (He et al., 2008). *OXIDATIVE SIGNAL-INDUCIBLE1* (*OXI1*) transcripts, which encodes a redox sensitive serine/threonine kinase, were decreased at 24 hpi.

Accession	Description	Fold change		
		6 h	24 h	48 h
At2g29450	ARABIDOPSIS THALIANA GLUTATHIONE S-TRANSFERASE TAU 5	2.4	na	2.1
At5g51890	Peroxidase 66	na	2.1	na
At4g34410	REDOX RESPONSIVE TRANSCRIPTION FACTOR 1	na	na	19.9
At4g37925	NAD(P)H:PLASTOQUINONE DEHYDROGENASE COMPLEX	na	na	3.8
At2g26150	HEAT SHOCK TRANSCRIPTION FACTOR A2	na	na	3.5
AT1G02940	Arabidopsis thaliana Glutathione S-transferase (class phi) 5	na	na	3.5
At5g26700	germin-like protein, putative	0.4	na	na
At1g80840	WRKY DNA-BINDING PROTEIN 40	0.3	0.3	na
At1g16420	METACASPASE 8	0.2	0.1	na
At3g25250	OXIDATIVE SIGNAL-INDUCIBLE1	na	0.5	na
At4g02520	Arabidopsis thaliana Glutathione S-transferase (class phi) 2	na	0.3	0.3
At1g08830	COPPER/ZINC SUPEROXIDE DISMUTASE 1	na	0.3	na
At5g20230	BLUE-COPPER-BINDING PROTEIN	na	0.3	na
At2g28190	COPPER/ZINC SUPEROXIDE DISMUTASE 2	na	0.3	na

Table 3.12 Genes involved in maintenance of redox homeostasis or responsive to ROS which expression was altered in the systemic leaves of *A. thaliana* Col0 at 6, 24 and 48 h upon *M. persicae* attack. Sixty aphids were confined to a clip cage attached to a mature rosette leaf and a second mature fully expanded rosette leaf immediately opposite the caged leaf was used to analyse gene expression with Agilent V4 arrays relative to non-infested caged controls. Statistically significant differentially expressed genes were identified on volcano plots with a cut-off of fold change ≥2 and Student's t-test p-value ≤0.05. Genes were attributed to this category based on either literature evidence or Gene Ontology (GO). GO classification was made according to Biological Process (oxidation-reduction process or response to oxidative stress) as found at The Arabidopsis Information Resource (TAIR; www.arabidopsis.org). na (not applicable) indicates that the corresponding transcript did not fulfil the adopted statistical criteria for differential expression. All ratios are expressed on a linear scale.

OXI1 positively regulates H_2O_2-mediated resistance to *P. syringae* and *Peronospora parasitica* (Petersen et al., 2008). In such responses, the expression of *OXI1* is increased by hydrogen peroxide produced by the activation of NADPH oxidases (Petersen et al., 2008).

Genes encoding two antioxidant enzymes (*CSD1* and *CSD2*), were decreased at 24 hpi. These genes encode the cytosolic and chloroplastic Cu/Zn superoxide dismutase isoforms respectively. These superoxide dismutases are subject to postranscriptional regulation by miR398, whose expression of is down-regulated by oxidative stress resulting in the accumulation of *CSD1* and *CSD2* transcripts (Sunkar et al., 2006).

3.2.5.2 Transcripts associated with calcium homeostasis

Like the infested leaves (Table 3.3), a number of transcripts encoding proteins associated with calcium homeostasis were modified in the systemic leaves early in the plant response to aphids (Table 3.13). For example, *ANNEXIN ARABIDOPSIS 4* (*ANNAT4*), one of the four annexins identified in Arabidopsis, was rapidly increased (at 6 hpi) in the leaves distant from the site of attack (Table 3.13). Annexins are a multi-gene protein family binding to membranes in a calcium-dependent manner. They play important roles in plant stress responses particularly, osmotic stress (Huh et al., 2010). The expression of annexins is triggered in ABA-mediated stress responses and is linked to ROS- signalling events via Ca^{2+} transport pathways (Laohavisit et al., 2010). Transcripts encoding CALMODULIN-BINDING RECEPTOR-LIKE CYTOPLASMIC KINASE 1 (CRCK1) are also modified in the systemic leaves early in the plant response to aphids. The *CRCK1* mRNA accumulates in response to abscisic acid and hydrogen peroxide as well as during cold and salt stress (Yang et al., 2004).

Accession	Description	Fold change		
		6h	24h	48h
At2g38750	ANNAT4 (ANNEXIN ARABIDOPSIS 4)	4.3	na	na
At4g23060	IQD22 (IQ-domain 22); calmodulin binding	2.6	na	na
At2g30790	PSBP-2 (photosystem II subunit P-2); calcium ion binding	na	2.1	na
At5g58940	CRCK1 (CALMODULIN-BINDING RECEPTOR-LIKE CYTOPLASMIC KINASE 1)	0.5	na	na
At1g01340	ATCNGC10 (CYCLIC NUCLEOTIDE GATED CHANNEL 10)	0.4	0.3	na
At2g41410	calmodulin, putative	na	0.5	na
At3g57330	ACA11 (AUTOINHIBITED CA^{2+}-ATPASE 11); calcium-transporting ATPase/ calmodulin binding	na	0.4	na
At3g63380	calcium-transporting ATPase, plasma membrane-type, putative / Ca^{2+}-ATPase, putative (ACA12)	na	0.1	na

Table 3.13 Genes related to calcium homeostasis which expression was altered in the systemic leaves of *A. thaliana* Col0 at 6, 24 and 48 h upon *M. persicae* attack. Sixty aphids were confined to a clip cage attached to a mature rosette leaf and a second mature fully expanded rosette leaf immediately opposite the caged leaf was used to analyse gene expression with Agilent V4 arrays relative to non-infested caged controls. Statistically significant differentially expressed genes were identified on volcano plots with a cut-off of fold change ≥ 2 and Student's t-test p-value ≤ 0.05. Genes were attributed to this category based on either literature evidence or Gene Ontology (GO). GO classification was made according to Biological Process (calcium ion transmembrane transport or calcium ion transport) or Molecular Function (calcium ion binding) as found at The Arabidopsis Information Resource (TAIR; www.arabidopsis.org). na (not applicable) indicates that the corresponding transcript did not fulfil the adopted statistical criteria for differential expression. All ratios are expressed on a linear scale.

Other transcripts associated with calcium homeostasis were modified at 24 hpi (Table 3.13). For example, mRNAs encoding two Ca^{2+}-transporting ATPases, *ACA11* and *ACA12*, ACA11, which is localized to the vacuole membrane, were decreased at 24hpi, as were transcripts encoding *CYCLIC NUCLEOTIDE GATED CHANNEL 10* (*ATCNGC10*). Ca^{2+}-transporting ATPase have been shown to have a role in ABA-mediated stomatal closure (Lee et al., 2005) while the *ATCNGC10* encodes a calmodulin binding ion channel that is involved in long-distance transport of Na^+ and K^+ (Guo et al., 2008).

3.2.5.3 Effect of aphid feeding on the expression levels of genes encoding mitogen activated protein kinases (MAPKs).

In contrast to the sites of infestation where transcripts encoding MAPKs were generally increased in response to aphid attack (Table 3.4), the expression levels of three MAPKs were decreased in the systemic tissues (Table 3.14). For example, *MKK9* transcripts were decreased early (6 hpi) in the experiment, whereas *MKK4* and *MAPKKK20* were decreased only at 24 hpi.

Accession	Description	Fold change		
		6h	24h	48h
At1g73500	ATMKK9 (Arabidopsis thaliana MAP kinase kinase 9); kinase	0.5	na	na
At1g51660	ATMKK4 (MITOGEN-ACTIVATED PROTEIN KINASE KINASE 4); MAP kinase kinase/ kinase	na	0.5	na
At3g50310	MAPKKK20 (Mitogen-activated protein kinase kinase kinase 20); kinase	na	0.2	

Table 3.14 Genes encoding MAPKs which expression was altered in the systemic leaves of *A. thaliana* Col0 at 6, 24 and 48 h upon *M. persicae* attack. Sixty aphids were confined to a clip cage attached to a mature rosette leaf and a second mature fully expanded rosette leaf immediately opposite the caged leaf was used to analyse gene expression with Agilent V4 arrays relative to non-infested caged controls. Statistically significant differentially expressed genes were identified on volcano plots with a cut-off of fold change ≥2 and Student's t-test p-value ≤0.05. Genes were attributed to this category based on either literature evidence or Gene Ontology (GO). GO classification was made according to Molecular Function (MAP kinase kinase activity or MAP kinase activity) as found at The Arabidopsis Information Resource (TAIR; www.arabidopsis.org). na (not applicable) indicates that the corresponding transcript did not fulfil the adopted statistical criteria for differential expression. All ratios are expressed on a linear scale.

3.2.5.4 Genes related to ET biosynthesis and signalling

Transcripts encoding components involved in ET metabolism and signalling were markedly changed in the leaves infested by aphids (Table 3.5).

In contrast to the sites of infestation, where most ET-related transcripts were increased, ET-responsive transcripts tended to decrease in the response of the systemic leaves to aphid attack (Table 3.15). For example, *ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 1* and *5*, and *ETHYLENE RESPONSE FACTOR 2* mRNAs were decreased early (6hpi) in the response in the systemic leaves. Two other ET related transcripts: *ETHYLENE FORMING ENZYME* and ethylene-responsive transcription factor *ERF011*, were decreased at 24 hpi.

Accession	Description	Fold change		
		6h	24h	48h
At4g17500	ATERF-1 (ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR 1)	0.4	na	na
At5g47230	ERF5 (ETHYLENE RESPONSIVE ELEMENT BINDING	0.4	na	na
At5g47220	ATERF-2/ATERF2/ERF2 (ETHYLENE RESPONSE FACTOR 2)	0.3	na	na
At3g50260	Ethylene-responsive transcription factor ERF011	na	0.5	na
At1g05010	EFE (ETHYLENE FORMING ENZYME)	na	0.3	na

Table 3.15 Genes related to ET biosynthesis and signalling which expression was altered in the systemic leaves of *A. thaliana* Col0 at 6, 24 and 48 h upon *M. persicae* attack. Sixty aphids were confined to a clip cage attached to a mature rosette leaf and a second mature fully expanded rosette leaf immediately opposite the caged leaf was used to analyse gene expression with Agilent V4 arrays relative to non-infested caged controls. Statistically significant differentially expressed genes were identified on volcano plots with a cut-off of fold change ≥2 and Student's t-test p-value ≤0.05. Genes were attributed to this category based on either literature evidence or Gene Ontology (GO). GO classification was made according to Biological Process (ethylene mediated signalling pathway) as found at The Arabidopsis Information Resource (TAIR; www.arabidopsis.org). na (not applicable) indicates that the corresponding transcript did not fulfil the adopted statistical criteria for differential expression. All ratios are expressed on a linear scale.

3.2.5.5 Salicylic acid associated transcripts

In marked contrast to the situation in local leaves, where many transcripts implicated in SA-signalling pathways were rapidly increased in response to aphid attack (Table 3.6), no mRNAs associated with SA-linked processes where changed in the systemic leaves at the early time point (Table 3.16). Moreover, transcripts encoding *ICS1* and *NIMIN-2* were repressed at 24 hpi in the leaves distant from the site of aphid attack (Table 3.16). NIMIN-2 (NIM1-INTERACTING 2) interacts physically with the NPR1/NIM1 complex, which is a key component of the signal transduction cascade leading to systemic acquired resistance in Arabidopsis (Weigel et al., 2001). In contrast to the situation the local leaves where *ICS1* transcript s increased in abundance, *ICS1* mRNAs were decreased at 24 hpi in the systemic leaves. This may suggest that SA synthesis is increased in the infested leaves but not in the systemic leaves.

Accession	Description	Fold change		
		6h	24h	48h
At1g74710	ICS1 (ISOCHORISMATE SYNTHASEI)	na	0.4	na
At3g25882	NIMIN-2 (NIM1-INTERACTING 2)	na	0.3	na

Table 3.16 Salicylic acid associated transcripts which expression was altered in the systemic leaves of *A. thaliana* Col0 at 6, 24 and 48 h upon *M. persicae* attack. Sixty aphids were confined to a clip cage attached to a mature rosette leaf and a second mature fully expanded rosette leaf immediately opposite the caged leaf was used to analyse gene expression with Agilent V4 arrays relative to non-infested caged controls. Statistically significant differentially expressed genes were identified on volcano plots with a cut-off of fold change ≥2 and Student's t-test p-value ≤0.05. Genes were attributed to this category based on either literature evidence or Gene Ontology (GO). GO classification was made according to Biological Process (salicylic acid mediated signaling pathway, salicylic acid biosynthetic process or systemic acquired resistance) as found at The Arabidopsis Information Resource (TAIR; www.arabidopsis.org). na (not applicable) indicates that the corresponding transcript did not fulfil the adopted statistical criteria for differential expression. All ratios are expressed on a linear scale.

3.2.5.6 Expression profiles of genes related to JA biosynthesis and signalling

In contrast to the local leaves, where aphid feeding caused a pronounced decrease in the abundance of *VSP2* and *VSP1* transcripts (Table 3.7), *VSP2* and *VSP1* mRNAs were rapidly (6hpi) increased in the systemic tissue (Table 3.17). qRT-PCR analysis confirmed that *VSP2* and *VSP1* transcripts were increased at 6hpi (Fig. 3.6). Moreover, these mRNAS remained at high abundance at 24 and 48 hpi (Fig. 3.6). While *Lipoxygenase 4* (*LOX4*) transcripts were decrease in the systemic tissues at 24 hpi, *PEROXISOMAL 3-KETO-ACYL-COA THIOLASE 2* (*PKT2*) mRNAs were increased at 6 hpi. *PKT2* expression is triggered by JA and by mechanical damage but only in a systemic manner (Cruz Castillo et al., 2004). Thus, while the decrease in *LOX4* transcripts, which encode an enzyme involved in JA biosynthesis (Caldelari et al., 2011), may suggest that JA synthesis is decreased in the systemic leaves, JA synthesised in the infested leaves may trigger a systemic response, in leaves far removed from the site of aphid attack.

Accession	Description	Fold change		
		6h	24h	48h
At5g24770	VSP2 (VEGETATIVE STORAGE PROTEIN 2)	6.1	na	na
At5g24780	VSP1 (VEGETATIVE STORAGE PROTEIN 1)	5.8	na	na
At5g48880	KAT5/PKT1/PKT2 (PEROXISOMAL 3-KETO-ACYL-COA THIOLASE 1, PEROXISOMAL 3-KETO-ACYL-COA THIOLASE 2)	3.1	na	na
At3g15850	FAD5 (FATTY ACID DESATURASE 5)	na	na	3.5
At1g18020	12-oxophytodienoate reductase, putative	na	0.4	na
At1g72520	(LOX4) Lipoxygenase 4	na	0.3	na

Table 3.17 Genes related to JA biosynthesis and signalling which expression was altered in the systemic leaves of *A. thaliana* Col0 at 6, 24 and 48 h upon *M. persicae* attack. Sixty aphids were confined to a clip cage attached to a mature rosette leaf and a second mature fully expanded rosette leaf immediately opposite the caged leaf was used to analyse gene expression with Agilent V4 arrays relative to non-infested caged controls. Statistically significant differentially expressed genes were identified on volcano plots with a cut-off of fold change ≥2 and Student's t-test p-value ≤0.05. Genes were attributed to this category based on either literature evidence or Gene Ontology (GO). GO classification was made according to Biological Process (jasmonic acid biosynthetic process or response to jasmonic acid stimulus) as found at The Arabidopsis Information Resource (TAIR; www.arabidopsis.org). na (not applicable) indicates that the corresponding transcript did not fulfil the adopted statistical criteria for differential expression. All ratios are expressed on a linear scale.

3.2.5.7 Expression profiles of genes involved in ABA-mediated signaling

Transcripts encoding Dehydration Responsive Element Binding (DREB) transcription factors and other ABA responsive genes were increased in the infested leaves in response to aphids (Table 3.8). ABA responsive genes were also increased in the systemic leaves from the first time point of the experiment (Table 3.18).

For example, gene encoding a glutathione-S-transferase (*EARLY-RESPONSIVE TO DEHYDRATION 9*) was up-regulated in the systemic leaves at 6 hpi. ERD9 is considered to serve as a negative regulator of ABA signalling pathways that modulate drought and salt stress responses (Jui-Hung et al., 2010). Transcripts encoding two water channels, SMALL AND BASIC INTRINSIC PROTEIN1B (Ishikawa et al., 2005) and RESPONSIVE TO DESICCATION 28 (Javot et al., 2003), were also induced at 6hpi. Similarly, aphid feeding induced the expression of *delta1-pyrroline-5-carboxylate synthase* (*P5CS1*) and *PROLINE TRANSPORTER 3* (*ProT3*) in the systemic leaves (Table 3.18). *P5CS1* encodes the rate-limiting enzyme in proline biosynthesis (Székely et al., 2008). Other proteins involved in drought stress responses such as the dehydrin family proteins *XERO1* and *XERO2*, and the *ARABIDOPSIS ZINC-FINGER PROTEIN 2* were increased at 24 hpi and 48 hpi respectively. In contrast, transcripts encoding ALDEHYDE OXIDASE 4, an enzyme that is involved in ABA biosynthesis (Seo et al., 2004), were decreased at 48 hpi.

Table 3.18 Genes related to ABA-mediated signalling and drought response*s* which expression was altered in the systemic leaves of *A. thaliana* Col0 at 6, 24 and 48 h upon *M. persicae* attack. Sixty aphids were confined to a clip cage attached to a mature rosette leaf and a second mature fully expanded rosette leaf immediately opposite the caged leaf was used to analyse gene expression with Agilent V4 arrays relative to non-infested caged controls. Statistically significant differentially expressed genes were identified on volcano plots with a cut-off of fold change ≥2 and Student's t-test p-value ≤0.05. Genes were attributed to this category based on either literature evidence or Gene Ontology (GO). GO classification was made according to Biological Process (abscisic acid biosynthetic process or response to abscisic acid stimulus) as found at The Arabidopsis Information Resource (TAIR; www.arabidopsis.org). na (not applicable) indicates that the corresponding transcript did not fulfil the adopted statistical criteria for differential expression. All ratios are expressed on a linear scale.

Accession	Description	Fold change		
		6h	24h	48h
At2g39800	P5CS1 (DELTA1-PYRROLINE-5-CARBOXYLATE SYNTHASE 1)	2.9	na	na
At2g36590	ProT3 (PROLINE TRANSPORTER 3); amino acid transmembrane transporter	2.8	na	na
At1g10370	EARLY-RESPONSIVE TO DEHYDRATION 9	2.5	na	na
At5g18290	SIP1;2 (SMALL AND BASIC INTRINSIC PROTEIN1B)	2.7	na	na
At2g37180	RD28 (plasma membrane intrinsic protein 2;3); water channel	2.3	na	na
At3g50980	XERO1 (DEHYDRIN XERO 1)	na	3.1	na
At4g27410	RD26 (RESPONSIVE TO DESSICATION 26)	na	3.1	3.0
At5g66400	RAB18 (RESPONSIVE TO ABA 18)	na	2.8	na
At5g37500	GORK (GATED OUTWARDLY-RECTIFYING K^+ CHANNEL)	na	2.6	na
At3g50970	XERO2 (DEHYDRIN XERO 2)	na	2.2	na
At3g19580	AZF2 (ARABIDOPSIS ZINC-FINGER PROTEIN 2)	na	na	2.8
At1g27730	STZ (SALT TOLERANCE ZINC FINGER)	na	0.3	na

At2g40330	REGULATORY COMPONENTS OF ABA RECEPTOR 9	na	0.2	na
At1g04580	AAO4 (ALDEHYDE OXIDASE 4)	na	na	0.5

3.2.5.8 Expression patterns of genes involved in glucosinolate biosynthesis and signalling in systemic leaves

The expression profile in the infested leaves showed that a number of transcripts involved in glucosinolates biosynthesis were modified in response to aphid attack (Table 3.10). The expression profile of the systemic leaves also involved changes in transcripts involved in the synthesis of indole glucosinolates, which were decreased (Table 3.19). For example, a gene encoding a recently characterized cytochrome P450 monooxygenase (CYP81F2), which is involved in indole glucosinolate biosynthesis, was decreased in the leaves distant from the site of attack at 6 and 24 hpi. CYP81F2 has been shown to play an important role in plant responses to *M. persicae* (Pfalz et al., 2009). Transcripts encoding enzymes of the tryptophan biosynthetic pathway, such as indole-3-glycerol phosphate synthase (IGPS), tryptophan synthase (TSA1) and a putative anthranilate synthase beta subunit (*At1g25083*), were repressed in the systemic leaves at 24 hpi (Table 3.19). IGPS catalyzes the synthesis of a plastid-originated precursor of indole containing compounds, including tryptophan, auxin, phytoalexins, and glucosinolates (Li et al., 1995), whereas TSA1 converts indole-3-glycerolphosphate to indole in one of the final reactions of tryptophan biosynthesis (Radwanski and Last, 1995).

Accession	Description	Fold change		
		6h	24h	48h
At5g57220	CYP81F2 (cytochrome P450, family 81, subfamily F, polypeptide 2)	0.2	0.2	na
At2g04400	indole-3-glycerol phosphate synthase (IGPS)	na	0.4	na
At3g54640	TSA1 (TRYPTOPHAN SYNTHASE ALPHA CHAIN); tryptophan synthase	na	0.4	na
At1g25083	anthranilate synthase beta subunit, putative	na	0.4	na
At4g31500	CYP83B1 (CYTOCHROME P450 MONOOXYGENASE 83B1)	na	0.4	na

Table 3.19 Genes involved in the biosynthesis of glucosinolates which expression was altered in the systemic leaves of *A. thaliana* Col0 at 6, 24 and 48 h upon *M. persicae* attack. Sixty aphids were confined to a clip cage attached to a mature rosette leaf and a second mature fully expanded rosette leaf immediately opposite the caged leaf was used to analyse gene expression with Agilent V4 arrays relative to non-infested caged controls. Statistically significant differentially expressed genes were identified on volcano plots with a cut-off of fold change ≥2 and Student's t-test p-value ≤0.05. Genes were attributed to this category based on either literature evidence or Gene Ontology (GO). GO classification was made according to Biological Process (indole glucosinolate biosynthetic process or glucosinolate biosynthetic process) as found at The Arabidopsis Information Resource (TAIR; www.arabidopsis.org). na (not applicable) indicates that the corresponding transcript did not fulfil the adopted statistical criteria for differential expression. All ratios are expressed on a linear scale.

It is also worthy of note that *CYTOCHROME P450 MONOOXYGENASE 83B1* (*CYP83B1*) transcripts were decreased at 24 hpi in the systemic leaves because the CYP83B1 gene product is involved in the conversion of indole-3-acetaldoxime to indole glucosinolates. This step is a major branch point between several biosynthetic pathways, particularly indole glucosinolates, camalexin and auxin (Morant et al., 2010).

3.2.5.9 Expression profile of cell wall associated transcripts

Similar to the situation in the local leaves, there was a rapid systemic response in several transcripts encoding proteins involved in cell wall re-organisation and biosynthesis as a result of aphid attack (Table 3.20). Of these, mRNAs encoding pectinase (*At1g60590*) and pectin methylesterase inhibitor (*PME1*) were increased early in the systemic response. Pectin methylesterases (PMEs) catalyse the demethylesterification of cell wall polygalacturonans (Willats et al., 2001). Pectin de-esterification is considered to be an important cue for pectin degradation, a process that involves pectin methylesterase inhibitors. These proteins control pectin esterification by inhibition of endogenous PMEs (Giovanea et al., 2004). Pectinases are present in aphid watery saliva and their cell wall degrading action might be important in stylet penetration because they undermine cell wall integrity.

The abundance of transcripts encoding several other cell wall-related components including *extracellular dermal glycoprotein-related* (*At5g19100*) and *CELLULOSE SYNTHASE LIKE* were increased early (at 6 hpi) in the systemic response, while others such as *Cellulose synthase-like B4*, a putative *xyloglucanxyloglucosyl transferase* (*At2g36870*) and member of the expansin superfamily, *EXPANSIN-LIKE B1*, were increased at later time points (24 hpi). However, transcripts encoding EXTENSIN 3, which is a structural component of the cell wall (Hall and Cannon, 2002), were decreased at the same time point.

Accession	Description	Fold change		
		6h	24h	48h
At1g60590	polygalacturonase, putative / pectinase, putative	16.5	na	na
At5g19100	extracellular dermal glycoprotein-related / EDGP-related	5.7	na	na
At4g12390	PME1; pectinesterase inhibitor	2.1	na	2.8
At2g35650	ATCSLA07 (CELLULOSE SYNTHASE LIKE)	2.1	na	na
At2g36870	xyloglucan:xyloglucosyl transferase, putative	na	2.8	na
At4g17030	ATEXLB1 (ARABIDOPSIS THALIANA EXPANSIN-LIKE B1)	na	2.2	na
At2g32540	ATCSLB04 (Cellulose synthase-like B4)	na	2.1	na
At1g16090	WALL ASSOCIATED KINASE-LIKE 7	0.4	0.4	na
At1g21310	ATEXT3 (EXTENSIN 3); structural constituent of cell wall	na	0.4	na
At2g43050	ATPMEPCRD; pectinesterase	na	0.3	na

Table 3.20 Cell wall associated transcripts which expression was altered in the systemic leaves of *A. thaliana* Col0 at 6, 24 and 48 h upon *M. persicae* attack. Sixty aphids were confined to a clip cage attached to a mature rosette leaf and a second mature fully expanded rosette leaf immediately opposite the caged leaf was used to analyse gene expression with Agilent V4 arrays relative to non-infested caged controls. Statistically significant differentially expressed genes were identified on volcano plots with a cut-off of fold change ≥2 and Student's t-test p-value ≤0.05. Genes were attributed to this category based on either literature evidence or Gene Ontology (GO). GO classification was made according to Biological Process (plant-type cell wall biogenesis) or Molecular Function (cellulose synthase activity) as found at The Arabidopsis Information Resource (TAIR; www.arabidopsis.org). na (not applicable) indicates that the corresponding transcript did not fulfil the adopted statistical criteria for differential expression. All ratios are expressed on a linear scale.

3.2.6 Effect of priming on nymph survival rates on local and systemic leaves

Based on the above evidence of substantial transcriptome re-programming in both infested leaves and in systemic leaves within hours of the onset of aphid attack, it was pertinent to address the question of whether such changes were accompanied by a modification in aphid resistance. The following experiments were performed to determine whether the observed transcriptional reprogramming resulting from aphid infestation alters aphid survival and/or fecundity on previously infested leaves. For these studies, leaves were primed by placing 60 aphids on a single leaf for 24h, after which these aphids were removed (Fig. 3.7). Thereafter, the survival of one-day old nymphs on the previously infested leaves was compared to that on leaves on other rosettes that had never been challenged by aphids. However, pre-infestation did not significantly alter nymph mortality rates over the subsequent 3 day period. Similarly, nymph mortality rates were unchanged when nymphs were placed on the systemic leaves. Nymph mortality was identical in all conditions examined in this study, i.e. in unchallenged leaves, previously infested leaves and systemic leaves from previously infested rosettes according to the Kruskal-Wallis test ($P = 0.254$).

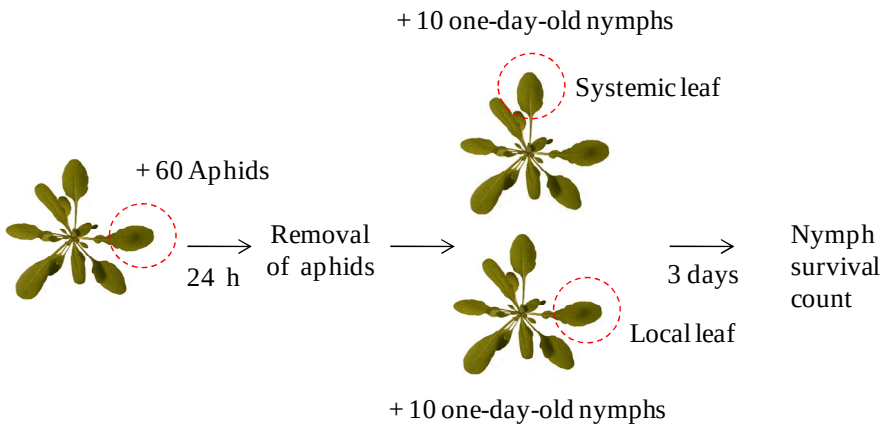

Figure 3.7 Experimental design used to study the local and systemic effect of priming on nymph survival rate. Sixty aphids were caged on mature 4-week old Arabidopsis leaves and allowed to feed for 24 h. After their removal, 10 one-day old nymphs were placed on previously infested (local) leaves or non-infested (systemic) leaves from the same rosette. Non-infested plants were used as controls. Nymph survival rate was counted after three days. The experiments were performed with 10 biological replicates.

3.2.7 Reproduction rates of two clonal lineages of *M. persicae* on Arabidopsis.

In a further series of experiments, the reproductive success of two *M. persicae* clonal lineages G and E (Fig. 3.8) was compared on Arabidopsis wild type (Col0). These experiments were performed by counting the number of aphids produced from a one-day-old nymph on each plant after 15 days. In this study, the average numbers of aphids per colony formed by clone G was significantly higher than that of clone E (Fig. 3.8).

Based on the above information, the transcript re-programming triggered in the local leaves by each of the *M. persicae* clones was compared at a single time-time. On the basis of the experiments discussed above, the 6h time-point was chosen for these studies. In these experiments, sixty aphids were confined on each single mature rosette leaf using a clip cage and allowed to feed for 6h, after which 55 transcripts were differentially expressed between the two aphid clones (Table 3.21). Of these, 27 transcripts were increased in abundance in the leaves infested with clone E relative to those infested with clonal lineage G. The largest effect was observed in mRNAs encoding an unknown protein (*At5g52900*), in which transcripts were 3.3 fold higher in the plants infested with clone E than in those infested with clone G. In total, 28 transcripts where decreased in expression in the plants attacked by clone E relative to those infested with *M. persicae* clone G (Table 3.21).

The transcripts that were altered in abundance in plants attacked by clone E relative to clone G cover a wide range of functional categories (Table 3.21). For example, *TERPENE SYNTHASE 10* mRNAs were 5 times higher in leaves infested with clone G than those challenged with clone E. Cell wall associated genes such as *expansin A1* and *Cellulose synthase-like G1*, and stress related transcripts including *GLUTATHIONE PEROXIDASE 7*, *DEHYDRATION RESPONSE ELEMENT B1A* and *METACASPASE 7* were differentially expressed within the range of 2-3 fold.

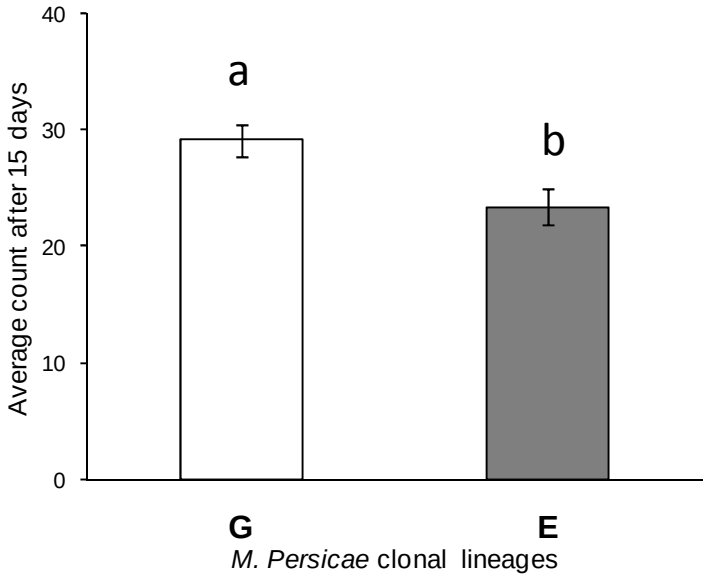

Figure 3.8 A comparison of the reproduction of two clonal lineages of *M. persicae* on *A. thaliana* Col0 plants. One-day-old nymphs were placed in the middle of 3-week old rosettes of Arabidopsis Col0 plants and the newly formed aphids were counted after 15 days. To retain aphids, each plant was covered with a meshed capped clear cage. The numbers represent means of 15 replicates ± SE. Different letters indicate a significant difference according to one-way ANOVA at the 5% level (P = 0.011).

Table 3.21 Differentially expressed genes between Arabidopsis Col0 leaves infested with *M. persicae* clonal lineages E and G for 6 hours. Sixty aphids were confined to a clip cage attached to a mature rosette leaf of 4-week old plants and gene expression analysed with Agilent V4 arrays. Leaves were infested with two different *M. persicae* clonal lineages (G and E) and four biological replicates for each aphid clone were collected. Volcano plots were used to identify statistically significant differentially expressed genes between the leaves infested with clones E and G (≥2x fold change genotype E/G at Student's t-test p-value ≤0.05). All ratios are expressed on a linear scale.

Accession	Description	Fold change difference (E relative to G)
At5g52900	unknown protein	3.3
At4g27450	Aluminium induced protein with YGL and LRDR motifs	3.2
At1g15125	S-adenosylmethionine-dependent methyltransferase	3.1
At1g03870	fasciclin-like arabinogalactan-protein 9	3.0
At1g49210	zinc finger (C3HC4-type RING finger) family protein	2.7
At5g19190	unknown protein	2.6
At1g69530	ARABIDOPSIS THALIANA EXPANSIN A1	2.5
At1g49200	zinc finger (C3HC4-type RING finger) family protein	2.5
At2g45180	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein	2.5
At1g49130	B-box type zinc finger protein with CCT domain	2.5
At1g22330	RNA-binding (RRM/RBD/RNP motifs) family protein	2.4
At3g06070	unknown protein	2.3
At2g18300	basic helix-loop-helix (bHLH) DNA-binding superfamily protein	2.3
At5g03120	unknown protein	2.3
At5g22920	CHY-type/CTCHY-type/RING-type Zinc finger protein	2.3
At2g36050	ovate family protein 15	2.3
At5g44020	HAD superfamily, subfamily IIIB acid phosphatase	2.2
At1g28330	DORMANCY-ASSOCIATED PROTEIN 1	2.2
At4g23820	Pectin lyase-like superfamily protein	2.2
At3g26760	NAD(P)-binding Rossmann-fold superfamily protein	2.1
At2g32100	ovate family protein 16	2.1

At5g44680	DNA glycosylase superfamily protein	2.1
At4g34760	SAUR-like auxin-responsive protein family	2.1
At5g51460	trehalose-phosphatase	2.1
At5g28770	BASIC LEUCINE ZIPPER 63	2.1
At3g07340	basic helix-loop-helix (bHLH) DNA-binding superfamily protein	2.0
At5g40450	Unknown protein	2.0
At2g39030	Acyl-CoA N-acyltransferases (NAT) superfamily protein	0.5
At2g25530	AFG1-like ATPase family protein	0.5
At3g11480	S-adenosylmethionine-dependent methyltransferase	0.5
At1g09240	NICOTIANAMINE SYNTHASE 3	0.5
At3g15990	SULFATE TRANSPORTER 3;4	0.5
At5g38120	4-coumarate--CoA ligase family protein / 4-coumaroyl-CoA synthase family protein	0.5
At1g66725	miRNA163	0.5
At2g37760	aldo/keto reductase family protein	0.5
At1g64910	UDP-Glycosyltransferase superfamily protein	0.5
At4g27570	UDP-Glycosyltransferase superfamily protein	0.5
At5g48880	PEROXISOMAL-3-KETO-ACYL-COA THIOLASE 1	0.5
At4g15490	UDP-glycosyltransferase/ sinapate 1-glucosyltransferase/ transferase	0.5
At1g12370	PHOTOLYASE 1	0.5
At3g55100	ATP-BINDING CASSETTE G17	0.5
At1g79310	METACASPASE 7	0.5
At4g09750	NAD(P)-binding Rossmann-fold superfamily protein	0.4
At4g24010	Cellulose synthase-like G1	0.4
At3g48350	Cysteine proteinases superfamily protein	0.4
At5g17050	UDP-GLUCOSYL TRANSFERASE 78D2	0.4
At4g25480	DEHYDRATION RESPONSE ELEMENT B1A	0.4
At4g31870	GLUTATHIONE PEROXIDASE 7	0.4
At2g23910	NAD(P)-binding Rossmann-fold superfamily protein	0.4
At1g57590	Pectinacetylesterase family protein	0.4
At2g30830	2-oxoglutarate-dependent dioxygenase, putative	0.4
At5g03210	Unknown protein	0.4
At3g55120	CHALCONE FLAVANONE ISOMERASE	0.3
At4g15210	BETA-AMYLASE 5	0.3
At2g24210	TERPENE SYNTHASE 10	0.2

3.3 Discussion

3.3.1 Effects of normalization and replication on gene expression microarray experiments

Raw microarray data are intrinsically variable and need to be normalized in order to obtain reliable gene expression data (Clarke and Zhu, 2006). Normalization acts to minimize experimental and systemic variations such as those originating in the array manufacturing process, the amount of initial RNA material, the hybridization conditions, and the quantification of the spot intensities (Quackenbush, 2002). A number of normalization techniques, taking into account various assumptions, are currently in use (Do and Choi, 2006). None of them is perfect and the appropriate choice of normalization is important for the correct analysis of the microarray data. The Lowess algorithm that eliminates the dye bias dependent on the overall spot intensity was used in this study (Yang et al., 2002). Lowess uses locally weighted linear regression to smooth the data and is one of the most widely applied normalisation methods. When choosing this approach it has to be kept in mind that it assumes that the biggest percentage of the genes are not differentially expressed (Do and Choi, 2005). Previous microarray results showed that only a small part of the transcriptome is responsive to aphid attack. For example, in one of the most stringent microarray experiments performed so far, aphid feeding affected only 0.15% of the genome (Couldridge et al., 2006). Therefore, it is accurate to utilize the lowess approach in studying genes expression altered by aphid feeding.

The value of the information obtained from microarray experiments can be increased through replication (Jeffery et al., 2006; Novak et al., 2002). However, replication is associated with high costs and sometimes limited by the availability of biological material. Despite the apparent need to define the minimum number of biological replicates in order to achieve a desired statistical power, the optimal level of replication

is still a matter of debate. It has been suggested that for most analysis, an optimal level of sensitivity and specificity is obtained between 8 and 15 replicates (Hwang et al., 2002; Zien et al., 2003) Moreover, fewer than 5 replicates lead to unstable results with poor quality (Pavlidis et al., 2003). The majority of the published studies in plant molecular biology rarely use more than four biological replicates. Similarly, in microarray experiments designed to study plant-aphid interactions the authors have typically used three or four replicates, and only one study have utilized six biological replications (Couldridge et al., 2007; Kusnierczyk et al., 2008; Delp et al., 2009). Nevertheless, even studies that have used hardly any replication reported results that have been subsequently corroborated (De Vos et al., 2005). It seems that even poorly replicated experiments can be useful as long as they can identify expression changes that are robust enough (Pavlidis et al., 2003). The experiments performed in this study used four biological replicates for each time point and identified transcriptional patterns reported by other studies. Given the current cost of the microarray experiments the four replicates represent a good trade-off between value and statistical power that can provide biologically meaningful results.

3.3.2 Global transcriptional patterns triggered upon aphid attack

The transcriptome profiles of the infested leaves provide information concerning the local signalling networks that operate in the plant response to aphids. Rapid responses to aphids have previously been observed at the feeding sites. The results presented in Chapter 3 supports this view and show that aphid attack leads to rapid transcriptome reprogramming in both infested and non-infested tissues. In addition to the marked responses of the infested leaves, aphid feeding caused a rapid response in the transcript profile of the systemic tissues suggesting that within hours of the onset of aphid probing, signals are transmitted from the site of infestation to all the leaves of the rosettes. The data presented here therefore provide evidence of the presence of rapid

systemic signalling pathways in *A. thaliana*. The gene expression patterns observed in distant leaves are quantitatively and qualitatively different from the transcriptome changes found in leaves directly subjected to aphid attack. The profile of transcripts that were modified in expression in the systemic leaves shows little overlap with that observed in the systemic leaves, suggesting that the signal that triggers the systemic responses is ether different in nature to that occurring at the site of infestation, or that it has a different context in the systemic leaves. Key questions that have to be addressed therefore concern the nature of the long distance signals and the mechanism by which they are perceived and function in tissues that are free from immediate stress. While the threat is not immediate, the systemic leaves face the possibility of becoming infested as aphids multiply and move from already infested leaves in search of new feeding sites. Presumably, the intensity as well as the nature of the signal, alerts the systemic leaves to the possible threat.

Similar to previous observation (de Vos et al., 2005; Kuśnierczyk et al., 2008), the studies described here provide strong evidence in support of rapid aphid-induced activation of calcium-dependent and kinase signalling cascades, that occurs in parallel with the activation of redox signalling pathways. In addition, the results presented here provide strong evidence for the activation of specific hormonal defence pathways, particularly those involving ET and SA that interface with calcium, MAPK, and ROS signalling in response to aphid infestation. The induction of transcripts encoding a large number of transcription factors supports the view that aphids trigger transcriptional programming at the sites of infestation.

3.3.3 Oxidative signalling events triggered upon aphid attack

Hydrogen peroxide is an important messenger molecule in plants that exerts a strong influence on global gene expression patterns (Neil et al., 2002; Desikan et al., 2001, Queval et al., 2009). However, ROS signalling is complex and remains poorly

understood (Mittler et al., 2011). The pathogen-induced oxidative burst that is part of the HR in incompatible plant-pathogen interactions generates hydrogen peroxide that is an important part of the resistance to invasion. Mutants with a reduced capacity to generate an oxidative burst display compromised resistance (Pogány et al., 2009). A hypersensitive response in the vicinity of stylet penetration has been shown in some plant-aphid interactions (Belefant-Miller et al., 1994; Lyth, 1985). Apart from its role in HR, ROS accumulation may be largely related to triggering down-stream signalling cascades. In addition, disruption of the leaf ROS homeostasis has been shown to adversely affect insects, causing oxidative damage in the midgut (Bi and Felton 1995). The induction of *BAP1* and *BAP2* and their interacting partners *BON1* and *BON3*, acting as inhibitors of hydrogen peroxide induced cell death, suggests that aphid feeding altered the levels of hydrogen peroxide in the infested leaves. Kusnierczyk et al. (2008) also observed accumulation of the transcripts encoding BAP and BON proteins in Arabidopsis following infestation with the cabbage aphid but failed to detect hydrogen peroxide accumulation using DAB staining. This result has to be interpreted with caution since hydrogen peroxide accumulation may be transient and subtle changes in its levels may be enough to trigger downstream responses. On the contrary, the levels of hydrogen peroxide have been shown to increase in wheat following infestation with the Russian wheat aphid (Moloi et al., 2006). Transcripts encoding the H_2O_2-metabolizing enzyme catalase were decreased in sorghum plants infested by the greenbug aphid (*Schizaphis graminum*), while transcripts encoding GSTs were increased (Zhu-Salzman et al., 2004). The data presented in Chapter 3 shows that the expression of several genes encoding GSTs was enhanced in the infested leaves. Of these, the expression of *GLUTATHIONE S-TRANSFERASE 8* is known to be regulated by SA and H_2O_2 (Foley at al., 2006). However, the expression of genes that might be involved in either ROS production such as those encoding germin-like proteins or ROS detoxification like

MONODEHYDROASCORBATE REDUCTASE was only induced at 24 hpi in the infested Arabidopsis leaves. This indicates that the balance between ROS generation and detoxification is maintained on transcriptional level at the later time points. Thus the induction of ROS-responsive genes within the first six hours upon attack might be the result of rapid activation of ROS generating enzymes.

The *respiratory burst oxidase homolog D* (*RbohD*) gene encodes an NADPH oxidase that is involved in ROS production in response to biotic and abiotic stresses. Lack of functional RbohD resulted in increased susceptibility to *M. persicae* (Miller et al., 2009). Transcripts encoding NADPH oxidases increased in Arabidopsis in response to infestation by the aphids *M. persicae* and *B. brassicae* (Kusnierczyk et al., 2007). Similarly, the expression of NADPH oxidases was increased in tomato following infestation by phloem-feeding whiteflies (Estrada-Hernández et al., 2009). Such observations suggest that NADPH oxidase-dependent ROS generation is important in the signalling cascades that are triggered following infestation by phloem feeders. However, the transcriptome re-programming observed in the present experiments did not reveal any changes in NADPH oxidase mRNAs. It may be that aphid-induced activation of existing NADPH oxidase enzymes was sufficient to trigger the redox signalling cascades observed in these experiments.

In this study, aphid feeding rapidly altered the expression of a large number of redox-regulated transcripts in the systemic leaves. Systemic induction of genes associated with oxidative stress was observed previously in phloem tissue from infested celery plants (Divol et al., 2005). In the systemic leaves, transcripts encoding ROS-metabolising enzymes such as superoxide dismutases were decreased only at 24 hpi but this change occurred before transcripts encoding REDOX RESPONSIVE TRANSCRIPTION FACTOR 1 were increased. Interestingly, *WRKY 40* and *METACASPASE 8*, which are also implicated in H_2O_2–mediated cell death, were repressed up to 24 hpi. These results

suggest that any increase in ROS in systemic tissues is related at least in part to a down-regulation of the antioxidant system that serves to induce redox-sensitive defence pathways.

3.3.4 Transcriptional evidence for perturbation of calcium homeostasis during *M. persicae - Arabidopsis* interaction

When a sieve element is punctured, the concentration of cytosolic Ca^{2+} rises and initiates a cascade of defense events, including plugging of the sieve plates by a calcium-mediated aggregation of phloem proteins and deposition of callose (Will and van Bel, 2006). This mechanism prevents the flow of phloem sap via the damaged sieve tube thus preventing the loss of vital nutritional resources. The occlusion of SE is highly unfavourable for the phloem feeders. Additionally, clogging of proteins might happen also inside the stylet's food canal compromising aphid feeding. Before ingesting phloem sap aphids inject watery saliva containing calcium-binding proteins (Will et al 2007). They prevent the increase in phloem calcium levels and subsequent protein clogging. The massive up-regulation of genes associated with calcium homeostasis in the local leaves already at 6 hours after the onset of the attack indicates that aphid feeding leads to a rapid disruption of calcium homeostasis. Moreover, calcium fluctuations can be associated with signalling events involving ROS and SA since these pathways are closely intertwined (Sagi and Fluhr 2001; Du et al., 2009). Transient fluxes of calcium ions are important messenger events in the transduction of developmental and environmental signals, including biotic and abiotic stresses.

In contrast to the local tissue where the majority of the genes involved in calcium homeostasis and signaling were upregulated, the picture in the non-infested leaves was not uniform and involved as well reduction. Nevertheless, aphid feeding altered the expression of calcium related genes in the systemic leaves already at 6 hpi indicating

that calcium-dependent signalling cascades also play important roles following aphid attack in the leaves distant from the site of attack.

3.3.5 Ethylene dependent pathways are an important component of the hormonal signalling network induced by aphid feeding

A burst of ET production has been observed in many plant-pathogen and plant-insect systems but the role of ET remains controversial (van Loon et al., 2006). While ET has been shown to activate defence responses (Diaz et al., 2002), it can also promote virulence (Weingart et al., 2001). The induction of *ATARD3* following aphid attack implies enhanced rates of ET biosynthesis. ET accumulation and induction of ET responsive genes has been shown in several plant-aphid combinations (Argandoña et al., 2001; Mantelin et al., 2009). For example, the Russian wheat aphid induced ET production in susceptible barley varieties (Miller et al., 1994). Transcripts encoding two ET-responsive transcription factors (ATERF13 and ATERF 6) and other ethylene-responsive components were rapidly increased in infested leaves, supporting the hypothesis that *M. persicae* induces ET accumulation in Arabidopsis leaves.

In contrast to the pronounced evidence for ET accumulation and activation of down-stream ET signalling pathways in the leaves directly subjected to aphid attack, the transcriptome profile of the systemic tissues was markedly different with a number of ET responsive transcription factors being repressed. This transcriptional pattern might reflect cross-talk differences between signalling components occurring in the two tissue types, in particular the interplay between ET accumulation and ROS. Experimental evidence support the view that ET and ROS are intricately linked. For example, wounding of winter squash has been shown to induce rapid accumulation of ROS and ethylene (Wang et al., 2002). The transcriptional activation of the ethylene producing enzyme 1-aminocyclopropane-1-carboxylate was inhibited following treatment with DPI prior wounding suggesting that ROS accumulation is required for enhanced rates of

ET synthesis (Wang et al., 2002). In contrast, ethylene synthesis was positioned upstream of ROS production during signalling events in the Arabidopsis guard cells. Ethylene accumulation was required for stomatal closure via hydrogen peroxide production dependent on NADPH oxidase AtrbohF in the guard cells (Desikan et al., 2006). Regardless of the actual position of ET and ROS in the signalling relays activated upon *M. persicae* attack in Arabidopsis the provided data support the view that the information about aphid feeding is at least partly mediated by increases of ET and ROS levels and their concerted action.

3.3.6 SA-dependent defence pathways in the attacked leaves

The results presented in Chapters 3 therefore provide strong evidence in support of a role for SA and SA-transduction pathways in the response to aphid attack since the infested leaves had hallmarks of a SA defence response. For example, the enhanced expression of SA marker genes such as *PR1*, *PR2* and *PR5*, SA-signalling pathway components such as *PAD4*, *EDS1* and *EDS5* and the gene encoding the SA synthesizing enzyme *ICS1*, provide compelling evidence for aphid triggered increases in SA synthesis and signalling. The results obtained in the present study are consistent with the findings of other studies published in the last decade that have implicated SA defence signalling pathways in plant responses to aphids. While de Vos et al. (2005) were unable to detect changes in leaf SA contents during *M. persicae* feeding on Arabidopsis, the evidence presented here would argue that such changes are the inevitable consequence of aphid feeding. For example, infestation of barley by the aphid *Schizaphis graminum* led to increases in the levels of both free and conjugated SA forms (Chaman et al., 2003). The increased abundance of *ISC1* transcripts in the infested tissues further implies SA accumulation since ISC1 is required for pathogen-induced SA biosynthesis. This hypothesis is further supported by the induction of genes

encoding PR proteins such as PR1, PR2 and PR5, which are commonly used as late markers for the SA pathway.

The expression of *PR1* transcripts in Arabidopsis leaves challenged with *M. persicae* is restricted to the site of stylet penetration as revealed by histological staining of β-glucuronidase reporter system (De Vos et al., 2005). In support to the highly localized induction of PR genes and related SA signalling components, the transcriptional pattern of the systemic tissue did not show hallmarks of a SA response. Moreover, the transcript abundance of *ICS1* and the SA signalling components *EDS5* and *NIMIIN-2* was decreased as opposed to the induction observed in the infested tissues. Taken together these observations suggest that the SA signalling pathways triggered upon aphid attack are tightly spatially regulated which leads to enhanced SA synthesis and signalling in the infested leaves and systemic repression of SA signalling components. The experimental design exploited in my studies designated as systemic tissue a mature rosette leaf situated opposite to the infested one. Such leaves in Arabidopsis are lacking a symplastic connection and signals initiated in the attacked leaf most probably do not reach the leaf from the opposite site of the rosette through the vasculature. Nevertheless, induction of *PR* genes following a localized pathogen challenge was observed throughout the Arabidopsis rosette suggesting that non-phloem based signalling mechanisms are involved in the induction of SAR (Kiefer and Slusarenko, 2003). The enhanced signalling through SA mediated pathways in aphid challenged leaves might trigger a systemic signal similar to that required for mounting a broad-spectrum disease resistance. However, the infested Arabidopsis plants did not show altered abundance of *PR* transcripts systemically at least up to 48 hpi implying the involvement of alternative long-distance signalling pathways that fail to induce expression of *PR* genes in the leaves distant from the site of attack. The question if more time is needed to activate SA

mediated gene expression in the remote plant parts upon aphid attack remains open and merits future research.

3.3.7 Spatial regulation of the SA/JA crosstalk in Arabidopsis during aphid attack

SA and JA response pathways interact antagonistically allowing the plant to fine tune its defence mechanisms depending on the type of attacker. Exogenous application of SA or its accumulation triggered upon biotrophic infection antagonizes the JA-dependent defences and confers enhanced susceptibility to herbivory and necrotrophic pathogens. For example, Arabidopsis plants challenged with the biotroph *Hyaloperonospora arabidopsidis* displayed suppressed JA-mediated responses that were induced following feeding by caterpillars of the small cabbage white butterflies *Pieris rapae* (Koornneef et al., 2008). Conversely, activation of JA mediated signalling can negatively affect SA-dependent defence. The phytotoxin corotine produced by *P. syringae* structurally resembles JA and upon delivery in the plant tissue is able to compromise the effective defence mediate via the SA defence pathway. Moreover, the transcription of JA-responsive genes, such as *PDF1.2* and *VSP2*, is suppressed by very low concentrations of exogenously applied SA (Koornneef et al., 2008).

The increased abundance of transcripts such as *ALLENE OXIDE CYCLASE 3* and *LIPOXYGENASE 5* in the infested leaves may suggest that the pathway of JA synthesis is enhanced in responses to aphid feeding. However, the repression of a number of marker genes for JA signalling such as *PDF1.2*, *PDF1.3, VSP1* and *VSP2*, would infer that JA signalling is repressed in the infested leaves. It is possible to speculate that the repression of JA signalling pathways results from the strong induction of SA-mediated signal transduction pathways and appears to be uncoupled from the activation of JA biosynthesis. Mechanical wounding results in accumulation of jasmonates and the fact that aphids inflict mechanical damage may trigger JA biosynthesis in the infested leaves. Moreover, a transient increase of the JA content in systemic Arabidopsis leaves

has been observed following localized mechanical damage. Interestingly, *M. persicae* attack increased transiently the abundance of the JA marker genes *VSP2* and *VSP1* systemically suggesting that a peak of JA accumulation may be triggered in the non-infested leaves. In line with this observation is the systemic repression of SA signalling components that may reflect the antagonistic cross talk between SA- and JA- dependent defence pathways. Further supporting this hypothesis is the systemic induction of *PEROXISOMAL 3-KETO-ACYL-COA THIOLASE 2* (*PKT2*). *PKT2* is responsive to JA and mechanical damage induces its expression only systemically (Cruz Castillo et al., 2004).

3.3.8 ABA-mediated signalling pathways triggered upon *M. persicae* attack

A number of drought responsive genes and transcripts implicated in ABA signalling were induced both locally and systemically upon aphid attack. Moreover, the increased abundance of transcripts encoding the ABA biosynthetic enzyme NCED5 suggests that ABA synthesis is stimulated at the site of infestation. Nevertheless, previous studies failed to show ABA accumulation during the interaction between *M. persicae* and Arabidopsis (De Vos et al., 2005).

Emerging evidence implicates ABA as a negative regulator of plant immunity by antagonistically affecting SA-mediated defence. Transcriptional evidence support the induction of ABA signalling pathways in the infested leaves in the presence of strong hallmarks of a SA defence response suggesting that both SA- and ABA-dependent pathways are simultaneously triggered upon aphid attack. Although the majority of the published studies reported a negative impact of ABA on SA signaling cascades, a positive regulation of ABA by SA has also been shown. Thus the pathogen-induced accumulation of ABA in the SA deficient mutant *sid2* was reduced in comparison to the wild type (de Torres-Zabala et al., 2009). Similarly, the transcription factor MYB96 involved in ABA mediated signalling positively regulates SA biosynthesis and pathogen

resistance (Seo and Park, 2010). The cross talk between SA and ABA was predominantly studied following exogenous ABA or abiotic stress pre-treatment. The events occurring upon aphid attack are most probably more complex and the timing and intensity of activation of both pathways should be taken into account.

The repression of ICS1 limited to the systemic tissue may reflect the negative impact of ABA on SA transduction pathways. ABA has been shown to down-regulate SA synthesis by transcriptional regulation of ICS1. The ABA deficient mutant *abscisic acid aldehyde oxydase* (*aao3*) accumulates higher levels of SA relative to the wild type and constitutively expresses ICS1 (de Torres-Zabala et al., 2009). Similarly, tomato plants deficient in ABA show higher constitutive expression of *PHENYLALANINE AMMONIA LYASE (PAL)* required for SA biosynthesis and display enhance resistance to *B. cinerea* (Audenaert et al., 2002).

3.3.9 Effect of aphid feeding on the expression patterns of cell wall associated genes

The integrity of the plant cell wall is an important factor governing the penetration of the stylet in the plant tissue. While strengthening of the cell wall will inevitably hamper aphid success, cell wall loosening could be beneficial for the aphids. A number of genes involved in cell wall metabolism and remodelling were differentially regulated upon aphid attack in the infested leaves implying that cell wall modification is a crucial component of the defence response to *M. persciae*. The majority of the cell wall related genes were responsive at the later time points positioning the rearrangement of the cell wall among the late events during aphid attack. *TOUCH4* encoding a xyloglucan endotransglycosylase/hydrolase (XTH) that modifies cell wall xyloglucans was the only cell wall associate genes at 6 hpi. Increased accumulation of XTHs was also observed following *M. persicae* and cabbage aphid attack on Beta Vulgaris and Arabidopsis, respectively (Dimmer et al., 2004; Kusnierczyck et al., 2008). Studying the phloem transcriptome response of celery to *M. persicae* attack, Vilaine et al. (2005) identified

XTH1 as an aphid-induced gene. Moreover, an Arabidopsis ortholog of XHT1 was found to affect aphid-settling behaviour suggesting that cell wall modifications are important in plant-aphid interactions (Divol et al., 2007).

Several transcripts encoding proteins involved in cell wall reorganisation and biosynthesis were affected rapidly after aphid feeding in the systemic tissue. Among them were genes encoding pectinase and pectin methylesterase inhibitor. Pectin methylesterases (PMEs) catalyse the demethylesterification of cell wall polygalacturonans (Willats et al., 2001). The de-esterification of pectin is an important cue for its degradation by plant enzymes (Brummell and Harpster, 2001). Involvement of pectin methylesterase inhibitors in controlling pectin esterification by inhibition of endogenous PMEs might be important factor determining cell wall integrity during stylet penetration. Pectinase activity is present in the aphid watery saliva and its cell wall degrading action might be modulated by the esterification status of the cell wall components.

3.3.10 Transcriptional evidence for an early senescence is limited to the leaves experiencing aphid attack

Activation of programmed cell death serves as a potent defence strategy against bacterial and fungal invasion. Similarly, *M. persicae* feeding on Arabidopsis has been shown to cause premature senescence dependent on *PAD4* (Pegadaraju et al. 2005, 2007). The synchronized induction of *PAD4* and other genes associated with senescence such as *WRKY53* and senescence markers at 24 and 48 hpi in the infested leaves indicates that the local tissue undergoes a premature senesce. *B. brassicae* attack on Arabidopsis induced the expression of *PAD4* and a number of senescence related genes implying that accelerated senescence is also a favourable defence strategy in this interaction (Kusnierczyk et al., 2008). Signalling pathways mediated by SA and JA may play a significant role in this process. Both hormones modulate the transcriptional

abundance of *WRKY53*, which governs the expression of senescence-associated genes in the initial stages of leaf senescence (Hinderhofer and Zentgraf, 2001; Miao et al., 2004).

Redistribution of nutrients from the infested leaves undergoing senescence to the aphid-free plant parts might limit their availability to the phloem feeders and thus impede aphid success. On the other hand, redirecting the flow of nutrients via the phloem may be beneficial for the phloem feeders. There was no conclusive evidence for premature senescence in the systemic leaves indicating that aphid feeding only affects the leaves directly subjected to attack.

3.3.11 Changes in the abundance of glucosinolate related transcripts upon *M. persicae* infestation

Aphid feeding has been shown to manipulate GS levels and provoke significant transcriptome reprogramming of genes related to GS biosynthesis. The performance of *M. persicae* and *B. brassicae* was negatively correlated with the total GS content in Arabidopsis mutants affected in phytohormone signalling (Mewis et al. 2005). Indole glucosinolates and their breakdown products were identified as the major deterrent GSs against aphids (Kim et al., 2008). Phloem sap contains GSs that are ingested by the aphids during feeding (Chen et al., 2001).

3.3.11.1 Transcripts related to indole glucosinolates biosynthesis

The mRNA levels of the transcription factor HIGH INDOLE GLUCOSINOLATE (HIG1) controlling indole GS synthesis were elevated throughout the experiment in the infested tissues as opposed to the systemic leaves where aphid feeding repressed its expression. *HIG1* was also induced in Arabidopsis challenged with *B. brassicae* and this correlated with increase of the levels of total indole GSs, mainly due to the accumulation of 1-methoxyindol-3-ylmethyl (1MI3M) GS in the infested plants (Kusnierczyk et al., 2008). In contrast, the expression of *ATR1*, a major regulator of indole glucosinolate and tryptophan biosynthesis, was repressed locally. Moreover, the

transcript abundance of several other genes involved in tryptophan biosynthesis was also decreased in the systemic leaves. The differential expression of genes implicated in indole glucosinolate biosynthesis in the whole rosette suggests that localized aphid feeding triggers systemic signalling events that eventually might alter GS synthesis in remote leaves thus modulating their defence mechanisms. The expression of *CYP81F2* was repressed early during the infestation in the non-infested leaves. Aphid performance on mutant plants with disrupted CYP81F2, converting indole-3-yl-methyl GS (I3M) to 4-hydroxy-indole-3-yl methyl GS (4OH-I3M), was impaired (Pfalz et al., 2009). This might suggest that aphids can manipulate the production of this deterrent compound in the systemic leaves and impede their resistance in future attack.

The opposite trends in indole glucosinolate biosynthesis (up-regulation of *HIG1* and repression of tryptophan biosynthetic genes) in the local leaves might be explained by the fact that aphids may manipulate only certain key points in the biosynthetic pathway. *HIG1* is situated downstream of tryptophan biosynthesis and is possible to speculate that aphids are able to limit indole glucosinolate biosynthesis by repressing genes early in the biosynthesis of tryptophan, which is used as a structural component of indole glucosinolates. The findings in this thesis are different from the observations of Kusnierczyk et al. (2007) and Moran and Thompson (2001) who showed that tryptophan biosynthetic genes were upregulated upon aphid attack. However, a significant number of GS biosynthetic genes were repressed in Arabidopsis following 72 h feeding of *M. persicae* (De Vos et al., 2005).

Wounding and MeJA treatment have been shown to increase the levels of indole GSs and induce the expression of genes involved in GS biosynthesis (Mikkelsen et al., 2000). For example, GUS fusions of CYP79B2 and CYP79B3 were strongly responsive following application of MeJA (Kusnierczyk et al. 2007). Transcriptional evidence support the hypothesis that JA synthesis is enhanced upon aphid attack which might

result in increased abundance of HIG1 transcripts in the infested leaves. Moreover, it follows that the repression of JA-dependent transduction pathways by SA signalling events might result in repression of genes involved in GS biosynthesis. Relevant findings were reported by Kliebenstein et al. (2002) who showed that Arabidopsis accumulated different GSs following treatment with both MeJA and SA, whereas their simultaneous application abolished these effects.

3.3.11.2 Transcripts related to aliphatic glucosinolates biosynthesis

In contrast to the differential regulation of genes involved in biosynthesis of indole GS in the infested tissues, aphid attack predominantly repressed genes implicated in aliphatic GS synthesis locally. Similarly, upon cabbage aphid attack on Arabidopsis the total aliphatic GS content declined and the transcript levels of genes associated with aliphatic GS biosynthesis were down regulated (Kusnierczyk et al., 2008). There is no conclusive evidence for the role of aliphatic glucosinolates in aphid resistance (Kim and Jander, 2007). Feeding of *M. persicae* and *B. brassicae* on Arabidopsis increased aliphatic GS levels without affecting indole GS content (Mewis et al., 2005). In another study conducted by the same authors *M. persicae* attack increased the levels of aliphatic GS which was accompanied with up-regulation of genes implicated in aliphatic glucosinolate biosynthesis (Mewis et al., 2006). The experimental variations might reflect the complexity of the signalling events orchestrating GS biosynthesis and the highly variable conditions between individual experiments such as growth conditions, time and density of aphid infestation, Arabidopsis ecotype and aphid strain.

3.3.12 Two different *M. persicae* clones elicit distinct transcriptional changes in Arabidopsis

Relatively small number of genes (56) showed different transcriptional levels between the plants infested with *M. persicae* clones E and G. However, the absolute number of

differentially regulated genes upon *M. persicae* (clone G) attack in a similar experiment described in Chapter 6 of this thesis is approximately 200. Thus a substantial number of the genes expected to respond during aphid feeding display different expression patterns between the leaves infested with *M. persicae* clones E and G. Based on these results it is possible to speculate that the aphid clonal lineage significantly influences the transcriptional profile of the infested tissue. Moreover, the difference could be even more pronounced at later time points.

Aphids deliver a plethora of potential effectors in the plant tissue with the injected saliva and these might modulate plant defence responses. The observed reproductive difference between the two *M. persicae* clones on Arabidopsis may be explained by intrinsic, genetic variations affecting the utilisation of nutrients or genetically predetermined growth rates and fitness. On the other hand, the genetic variation between these clones may result in a unique proteinaceous profile of the injected saliva leading to activation of specific defence signalling pathways and modulation of the timing and intensity of the defence responses. Most probably, the overall effect is a combination of both of them and other yet unknown factors. Nevertheless, since aphid saliva is the main elicitor of defence responses, a significant overlap between the transcriptional changes elicited upon attack from the two clones is expected provided that their salivary composition is identical. The different transcriptome profiles of Arabidopsis infested with *M. persicae* clones E and G suggests that the injected saliva is not identical. Furthermore, the genetic pressure to host specialization and the variable reproduction success on different host plants may result in quantitative and qualitative changes in aphid saliva.

3.3.13 Modulation of defence mechanisms in Arabidopsis by aphid pre-infestation

The rapid reprogramming of the systemic leaf transcriptome could modulate the resistance of the non-infested leaves in order to prepare them for a future aphid attack.

The experiments reported here suggest that this is not the case because nymph survival rates were not increased after prior aphid infestation. These observations agree with those of a previous study where Arabidopsis leaves were primed with saliva (de Vos et al., 2009). In this case, the reproduction of *M. persicae* on the systemic leaves of the same rosette was unchanged but the primed leaves were less attractive to *M. persicae* and supported lower aphid numbers (de Vos et al., 2009). More experiments are therefore required to determine how the transcript reprogramming in infested and systemic leaves influences aphid behaviour and aphid success.

Chapter 3: Summary

- Transcriptional changes induced by *M. persicae* in Arabidopsis Col0 were studied using Agilent V4 microarrays. Transcriptome patterns were monitored in leaves directly subjected to aphid attack and in aphid-free leaves from the same rosette at 6, 24 and 48 hpi.
- Rapid transcriptional changes were observed in both infested leaves and in leaves distant from the site of attack already at 6 hpi.
- Marked changes in transcripts involved in ROS and calcium homeostasis, hormonal (ET, ABA, SA, JA) and MAPK signalling were observed, together with altered expression of genes implicated in senescence, cell wall remodelling and glucosinolate biosynthesis.
- Priming did not significantly alter nymph mortality on previously infested Arabidopsis Col0 leaves. Similarly, nymph mortality was not affected when nymphs were placed on the systemic leaves.
- The reproductive success of two *M. persicae* clonal lineages G and E was compared on Arabidopsis Col0.

- The transcriptional response elicited by *M. persciae* clonal lineages G and E in Arabidopsis Col0 was compared at 6 hpi in directly infested leaves.

Chapter 4. Metabolome analysis and related changes in the transcript profiles of local and systemic leaves of wild type *A. thaliana* upon *M. persicae* attack

4.1 Introduction

4.1.1 Impact on insect herbivory on primary metabolic processes

Plants have developed constitutive and inducible defence strategies that prevent or minimize the effects of insect herbivory, and these include re-direction of a number of pathways associated with primary and secondary metabolism (Baldwin and Callahan, 1993; Herms and Mattson, 1992; Bolton, 2009). A key hypothesis in this regard concerns the possible fitness costs associated with the re-direction of metabolites and energy away from growth into secondary metabolic pathways (Frost et al., 2008; Schwachtje and Baldwin, 2008). The inducible nature of such responses has led to the suggestion that in the absence of herbivore pressure, the expression of tolerant or defensive phenotypes incurs fitness costs that reduce the plants capacity to compete in herbivore-free environments (Herms and Mattson, 1992). A number of mechanisms by which induced defences may impose fitness costs have been proposed. These include the diversion of limiting resources away from growth and development into defence (Mole, 1994), the autotoxicity of defence compounds (Kessler and Baldwin, 2002), and trade-offs between defences against different enemies in which mounting of defence against one enemy enhances susceptibility to another (Frost et al., 2008). Alternatively, cell division and expansion may be limited under conditions of stress where an unbalanced cellular redox state can impact the cell cycle (Hirt, 2000).

A range of inducible mechanisms have been observed following insect infestation that function to reduce the suitability of the plant as a host. These include the induction of

mechanisms that increase the physical barrier to insect feeding with the up-regulation of genes involved in cell wall remodelling (Thompson and Goggin, 2006; Goggin, 2007), the induction of anti-nutritive compounds including protease inhibitors, protein cross-linking polyphenol oxidases (Kessler and Baldwin, 2002) and other enzymes catalysing the degradation of essential amino acids such as threonine and arginine (Chen, 2008), and the induction of a range of toxic secondary metabolites (Kessler and Baldwin, 2002). Secondary metabolites can accumulate to significant levels within the tissues of plants undergoing herbivore attack, for example following induction up to 6% of the whole plant nitrogen pool of *Nicotiana attenuata* can be diverted into the toxic alkaloid nicotine (Baldwin et al., 1998). Such changes in secondary metabolite accumulation are usually associated with significant changes in primary metabolism following insect or pathogen attack (Frost et al., 2008; Schwachtje and Baldwin, 2008; Bolton, 2009). Several reasons for the up-regulation of primary metabolism have been postulated including the provision of energy and carbon skeletons required for the synthesis of secondary metabolites. It is suggested that such alterations in primary metabolism allow the plant to tolerate herbivory while minimizing impacts on fitness traits. Moreover, primary metabolites such as sugars are often signalling molecules and as such alterations in the levels of key primary metabolites might themselves have the potential to have a defensive mode of action (Paul et al., 2008; Fernandez et al., 2010; Hanson and Smeekens, 2009).

4.1.2 Role of photosynthesis in plant-insect interactions

Given that photosynthetic rates are often constrained by metabolic limitations and photosynthesis thus operates at well below the maximum values (Stitt, 1986), one prediction of an increased diversion of fixed carbon towards secondary metabolite synthesis following insect herbivory would be an enhancement of photosynthesis, providing that other factors such as CO_2 availability were not limiting. However,

although a number of compensatory changes have been observed for specific plant–insect interactions, in the majority of cases defence responses are associated with a reduction in photosynthesis (Bilgin et al., 2010). The classic plant response to biotic and abiotic stresses involves stomatal closure, decreased photosynthesis and enhanced respiration. In the case of insect infestation, decreased photosynthesis has been associated with physiological phenomena such as reduced leaf water potentials caused by severed vasculature (Nabity et al., 2009), enhanced water loss from wound sites (Aldea et al., 2005), stomatal closure (De Freitas Bueno et al., 2009) and higher non-photochemical quenching in photosystem II (Aldea et al., 2006). There is also accumulating evidence to suggest that reduced photosynthesis is a genetically programmed plant response. Gas exchange measurements coupled with fluorescence techniques have demonstrated that at least in certain plant–insect interactions repressed photosynthetic rates result not from impaired CO_2 availability or impairment of the light reaction but to reduced rates of CO_2 fixation within the dark reaction (Peterson et al., 1998; Macedo et al., 2005; Gutsche et al., 2009a). This suggests that limitation is associated with either ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) activity or the regeneration of ribulose bisphosphate, which is dependent on enzymes and metabolites of the photosynthetic carbon reduction cycle. Furthermore, several studies have demonstrated inhibition of photosynthesis following insect oviposition even where eggs are laid on the leaf surface in the absence of tissue damage (Schröder, et al., 2005; Velikova et al., 2010). This provides additional support to the hypothesis that down-regulation of photosynthesis is a plant-driven response to the perception of stress rather than a secondary physiological response to tissue damage.

4.1.2.1 Transcriptome and proteomic evidence for impact of herbivory attack on photosynthesis

The view for down-regulation of photosynthesis is further supported by evidence from transcriptomic studies, which have demonstrated significant changes in expression of photosynthesis-related genes following feeding by chewing (Hermsmeier et al., 2001; Hui et al., 2003; Voelckel and Baldwin, 2003; Ralph et al., 2006; Lawrence et al., 2008) and piercing/sucking insects (Zhu-Salzman et al., 2004; Yuan et al., 2005; Botha et al., 2006; Mozoruk et al., 2006; Park et al., 2006; Broekgaarden et al., 2008; Estrada-Hernández et al., 2009; Gutsche et al., 2009b). Furthermore, transcriptomic studies have demonstrated anticipatory regulation of photosynthesis-related genes following oviposition by adult butterflies (Little et al., 2007) or following exposure to herbivory induced leaf volatiles (Arimura et al., 2000).

Proteomic studies corroborate the evidence generated from transcriptomic studies and moreover, demonstrate that the observed changes in transcript levels are translated into changes in protein abundance. For example, caterpillar feeding led to differential accumulation of seven photosynthesis-related proteins in *N. attenuata* (Giri et al., 2006), while infestation of rice by the phloem feeding brown planthopper resulted in differential accumulation of five photosynthesis-related proteins (Wei et al., 2009).

The functional significance of insect-induced changes in photosynthetic gene expression has been tested by transgenic manipulation of gene expression. Transgenic *N. attenuata* plants with reduced transcript levels of either Rubisco through the introduction of an antisense construct (asRub) or Rubisco activase (RCA) through the introduction of an inverted repeat construct (irRCA) (Mitra and Baldwin, 2008) showed reductions in photosynthetic capacity and reduced growth performance. Following elicitation by treatment of wounds with regurgitant of *M. sexta* larvae both asRub plants and empty vector control plants accumulated trypsin proteinase inhibitors (TPIs), diterpene

glycosides (DTGs) and nicotine to similar levels. However, accumulation of TPIs and DTGs was impaired in the irRCA plants, which were also deficient in their capacity to accumulate key signalling jasmonic acid (JA) conjugates. Both the generalist *Spodoptera littoralis* and specialist *M. sexta* gained mass more rapidly when cultured on irRCA plants than when cultured on empty vector control plants whereas asRUB plants supported enhanced weight gain only for *M. sexta*. These data illustrate a link between the regulation of photosynthesis, hormonal signalling and defence against insect herbivores.

4.1.2.2 Compensatory changes in photosynthesis in plant–insect interactions

The hypothesis that insect-induced changes in photosynthesis are genetically programmed is supported by the observation that plant genotypes that are able to maintain photosynthesis under insect attack often exhibit greater resistance. Infestation of barley (Gutsche et al., 2009a) and wheat (Haile et al. 1999; Franzen et al. 2007) by the economically significant Russian wheat aphid (*Diurpahis noxia*) revealed that tolerant lines were able to maintain photosynthetic capacity more strongly during insect feeding or were able to recover photosynthetic rates more rapidly following removal of aphids. Crucially, the rate of ribulose bisphosphate turnover (as estimated from gas exchange measurements) was maintained in resistant but not in susceptible cultivars (Franzen et al., 2007; Gutsche et al., 2009a). Conversely, other investigators failed to detect reduced carboxylation efficiency in other susceptible wheat lines and therefore proposed that the major difference between tolerant and susceptible lines lay within the regulation of photochemistry and electron transport following aphid injury (Haile et al., 1999). A large number of genes encoding photosynthesis-related proteins were differentially expressed in both wheat (Botha et al., 2006) and barley (Gutsche et al. 2009b). This finding together with the observation that there were significant differences in photosynthetic gene expression between susceptible and tolerant barley

cultivars suggests that plant responses to insects are not merely physiologically based but rather that they are caused by the adoption of different genetic re-programming strategies between cultivars.

Similar compensatory photosynthetic mechanisms have been observed in tolerant but not susceptible cultivars of the turf grass species *Buchloë dactyloides* (Heng-Moss et al., 2006) and the forage crop pearl millet (Ni et al., 2009) following exposure to phloem-feeding chinch bugs (*Blissus occiduus*). In the *B. dactyloides–B. occiduus* interaction, susceptible lines accumulated carbohydrate in the leaves, exhibited reduced rates of CO_2 fixation, photosynthetic electron transport and the photochemical efficiencies of photosystem II (PSII) leading to the conclusion that insect attack led to end-product inhibition of photosynthesis with impaired electron flow through PSII. Crucially, these symptoms were not observed in resistant lines. Other experiments using $^{14}CO_2$ labelling have confirmed that cotton leaves export less fixed carbon following infestation by the phloem-feeding silverleaf whitefly and that soluble sugars accumulated in leaves, which further supports the hypothesis regarding the role of feedback inhibition of photosynthesis (Lin et al., 2000). Similar findings were observed in the *Pennisteum glaucum–B. leucopterus* interaction, where resistant millet varieties demonstrated enhanced CO_2 fixation under feeding injury whereas susceptible lines demonstrated a decline. Furthermore, higher CO_2 fixation rates were observed following infestation of resistant maize lines with the chewing lepidopteran pest *Spodoptera frugiperda* than were observed in the susceptible lines (Chen et al., 2009).

Taken together, the studies outlined earlier suggest that there is genetic reprogramming of plant development in response to insect herbivory and that this involves some degree of interaction between the pathways of primary metabolism, particularly photosynthesis and plant defence responses. The studies described in the previous chapter (Chapter 3)

examined the transcriptional reprogramming of wild type *A. thaliana* upon *M. persicae* attack. This chapter describes experiments that were performed to determine the responses of metabolites in local and systemic leaves to aphid attack, under the same experimental conditions as those used for the studies on transcriptional reprogramming. This analysis was undertaken to provide a better understanding of the responses of primary and secondary metabolism to aphid infestation in local and systemic leaves, and compliment the information obtained from the microarray analysis.

4.2 Results

A targeted metabolite profiling approach was used to explore the time course of changes in major leaf metabolites in infested leaves and in non-infested leaves, for up to 48 hours after the onset of the infestation. In these experiments, more than sixty compounds were unambiguously identified and quantified by GC-MS (Fig. 4.1; Appendix 7), including major amino acids, organic acids involved in the tricarboxylic acid (TCA) cycle, sugars, fatty acids, long-chain alcohols and various non-polar compounds. In the absence of aphids, the presence of the cage alone for 2 days on the local leaves caused some changes in the abundance of amino acids and sugars between the local and systemic leaves from the same rosette (Fig. 4.2, Appendix 7). These variations have been noted and taken into consideration in the following discussion.

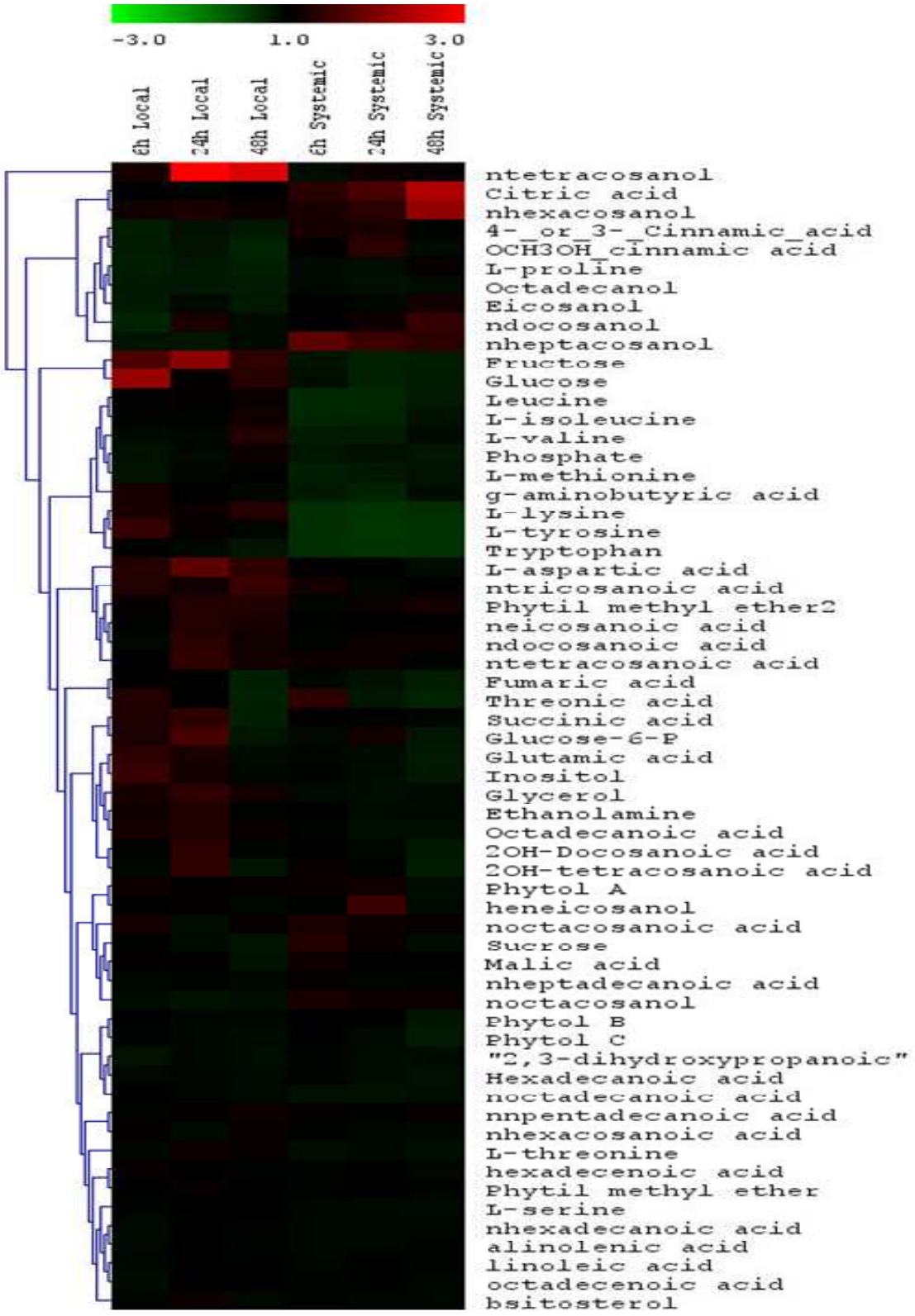

Figure 4.1 Hierarchical clustering analysis of metabolite profiles of local and systemic leaves of wild type Arabidopsis plants infested with *M. persicae* at 6, 24 and 48 hpi. The values represent ratios relative to control samples. The metabolites were clustered according to Euclidean distances.

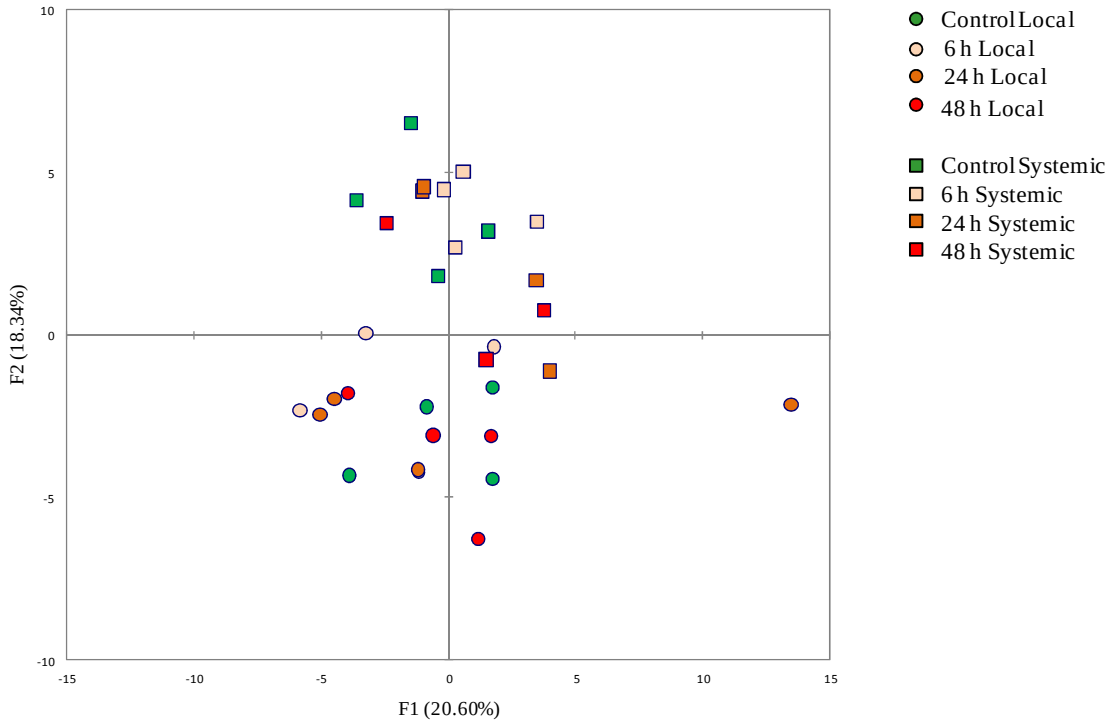

Figure 4.2 Principal component analysis of GC-MS profiles of Arabidopsis Col0 plants infested with *M. persicae* for 6, 24 and 48 h in four biological replicates. The plot represents the first (PC1) and second components (PC2) of the metabolite profiles of infested (local) and uninfested (systemic) leaves from *M. persicae*-challenged wild type Arabidopsis plants and corresponding controls. The first and second components together explain 38.94% of the total variation.

In the first 48 hpi, aphid feeding had little effect on the abundance of the metabolites that were measured in this study, in either the infested leaves or the systemic leaves (Fig. 4.2). The pool sizes of only a small number of metabolites were significantly different between the local and systemic leaves of infested plants and the corresponding aphid-free controls according to two-way ANOVA ($p \leq 0.05$). However, in these studies whole leaves were harvested and analyzed to provide global patterns of metabolite pools. Therefore, any localized changes in metabolites content in the cells in the vicinity of stylet penetration may fall below the level of detection. Similarly, these analyses may not detect changes in the amino acid content of the phloem sap, which could also be altered as a result of aphid feeding (Hunt et al., 2006; Hunt et al., 2010; Zhu et al., 2005).

4.2.1 Metabolite changes in leaves following aphid attack

Aphid feeding decreased the pools of 4- or 3-cinnamic acid in the local leaves (Appendix 7). The 4- or 3-cinnamic acid pool size was significantly lower (up to 54%) at 6 and 24 hpi but values at the 48 hpi were not significantly different. A significant decrease of the levels of OCH_3OH-cinnamic acid was observed in the systemic leaves at 48 hpi (Appendix 7).

No statistically significant changes in TCA cycle metabolite pools were observed in either the infested or systemic leaves (Fig. 4.2). However, significant decreases in the pools of the branched-chain amino acids (valine, leucine and isoleucine) were observed in the systemic leaves at 6 and 24 hpi, and also at 48 hpi for leucine. This finding is interesting as branched-chain amino acids are an essential part of the insect diet and their availability can have a direct effect on herbivore success.

Myristic acid is a saturated C:14 fatty acid that is an important in the post-translational modification of proteins such as protein kinases, GTP-binding proteins, thioredoxins and several NBS-LRR proteins (Boisson et al., 2003). It was calculated that about 1.7%

of the Arabidopsis proteome undergoes N-myrostoylation (Boisson et al., 2003). Myristate is added to N-terminal glycines of target proteins by the action of N-myristoyltransferase. Aphid feeding increased the myristic acid pool of the local leaves at 48 hpi (Fig. 4.3).

Moreover, n-octadecanoic (stearic) acid was significantly reduced in the systemic leaves at 6, 24 and 48 hpi (Fig. 4.4). The stearic acid pool is important for the synthesis of polyunsaturated fatty acids such as the 18:3 fatty acids that serve as jasmonate precursors (Gfeller et al., 2010).

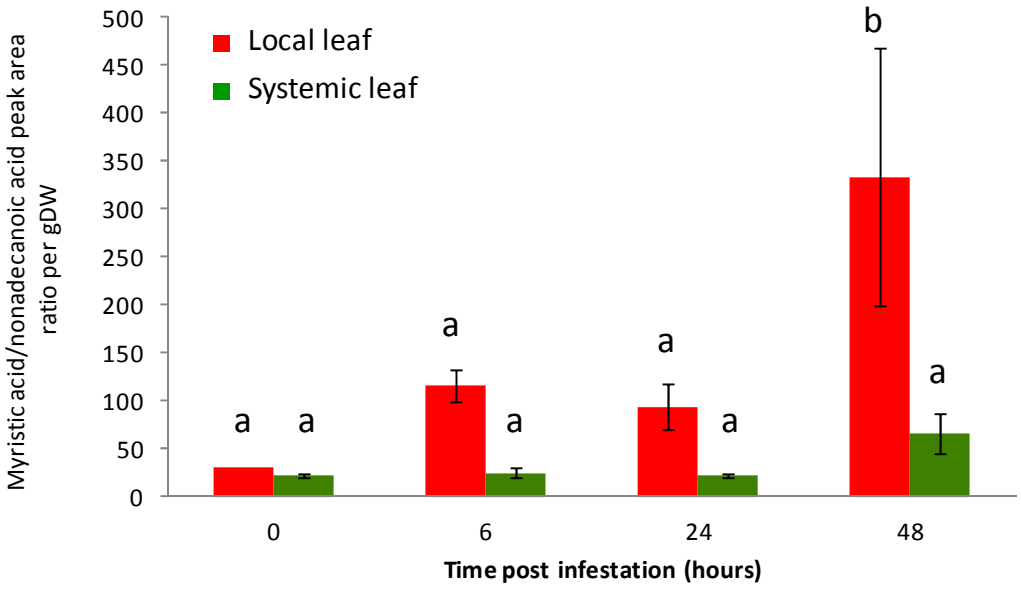

Figure 4.3 Effect of aphid feeding on the levels of myristic acid in local and systemic leaves of Arabidopsis Col0 upon *M. persicae* attack. Sixty aphids were confined to single mature leaves of 4-week-old plants in a clip cage and samples infested for 6, 24 and 48h were harvested. Non-infested caged plants were used as references. Four biological replicates for each time point were derivatized and analyzed by GC-MS. The integrated area of the myristic acid peaks was normalized against the integrated area of the internal standard nonadecanoic acid peaks and the ratio was normalized on a dry weight basis. Red bars represent myristic acid contents in caged (local) leaves and green bars represent non-caged (systemic) leaves. Significant differences were analyzed with two-way ANOVA with post hoc analysis by Student-Newman-Keuls' pairwise comparison. Means with different letters are significantly different at the 5% level.

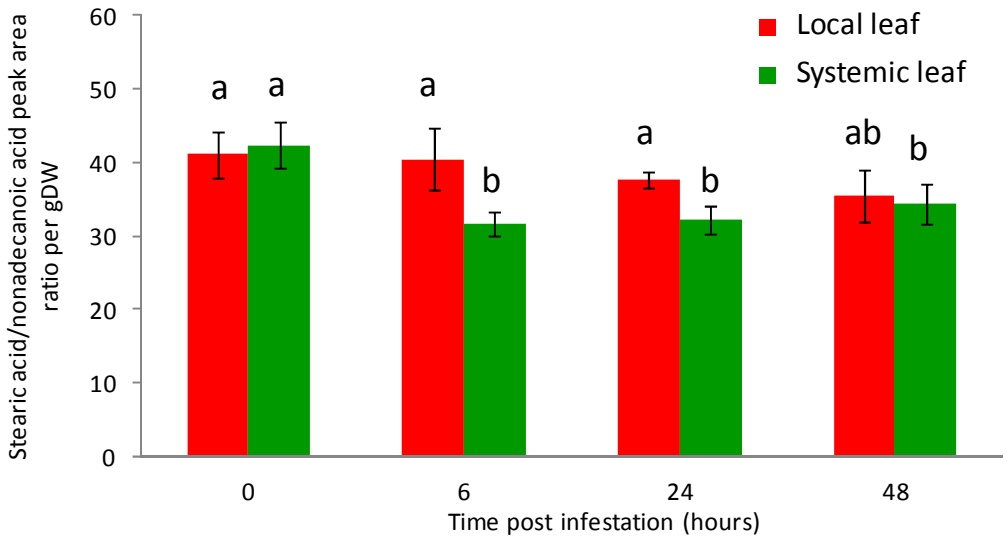

Figure 4.4 Effect of aphid feeding on the levels of stearic acid in local and systemic leaves of *Arabidopsis* Col0 upon *M. persicae* attack. Sixty aphids were confined to single mature leaves of 4-week-old plants in a clip cage and samples infested for 6, 24 and 48h were harvested. Non-infested caged plants were used as references. Four biological replicates for each time point were derivatized and analyzed by GC-MS. The integrated area of the stearic acid peaks was normalized against the integrated area of the internal standard nonadecanoic acid peaks and the ratio was normalized on a dry weight basis. Red bars represent stearic acid contents in caged (local) leaves and green bars represent non-caged (systemic) leaves. Significant differences were analyzed with two-way ANOVA with post hoc analysis by Student-Newman-Keuls' pairwise comparison. Means with different letters are significantly different at the 5% level.

4.2.2 Effects of aphid feeding on photosynthesis in the systemic leaves

Localized *M persicae* attack did not provoke significant changes in the photosynthesis rates of non-infested (systemic) leaves from the same rosette following 24 h of aphid feeding. No significant differences were observed between infested plants and aphid free controls according to one-way ANOVA ($p = 0.505$). Six biological replicates were used in the analysis and the photosynthesis rates were calculated based on leaf CO_2 gas exchange measured with an Infrared Gas Analyser.

This finding is consistent with the absence of any detectable changes in transcripts encoding photosynthetic proteins in the systemic leaves. The photosynthetic rates of the infested leaves could not be determined in this study due to technical limitations.

4.2.3 Effects of aphid feeding on transcripts encoding enzymes involved in metabolism in local and systemic leaves

4.2.3.1 Transcripts involved in phenylpropanoid metabolism in infested leaves

The metabolite profiling studies discussed above showed an increase in the 4- or 3-cinnamic acid pool at 6 and 24 hpi. Cinnamic acid is the first product of the general phenylpropanoid pathway producing precursors to a variety of important phenolic compounds including SA (Winkel-Shirley, 2001). It is synthesized by the enzyme phenylalanine ammonia-lyase (PAL) from phenylalanine, an important regulation point between primary and secondary metabolism (Schilmiller et al., 2009). The abundance of transcripts encoding several enzymes involved in flavonoid and anthocyanin biosynthesis was modified by aphid feeding. For example, *chalcone-flavanone isomerase family protein* (*At5g05270*), *ANTHOCYANIN 5-AROMATIC ACYLTRANSFERASE 1* and *PRODUCTION OF ANTHOCYANIN PIGMENT 1* were decreased in the local tissue at the last two time points of the experiment (Appendix 2-3). However, *CINNAMYL ALCOHOL DEHYDROGENASE 1*, and *LACCASE 13*, which

are involved in lignin biosynthesis (Raes et al., 2003) were increased in the infested leaves at 24 and 48 hpi (Appendix 2-3).

4.2.3.2 Transcripts involved in starch metabolism in infested leaves

Although the metabolite profiling data failed to detect any aphid-induced changes in carbon metabolites, a number of transcripts encoding enzymes involved in carbohydrate metabolism were differentially expressed in the local leaves (Table 4.1). In particular, transcripts encoding starch-modifying enzymes such as *ISOAMYLASE 1* and *ARABIDOPSIS THALIANA ISOAMYLASE 2*, were repressed at 24 hpi. These are required for the formation of the isoamylase-type debranching enzyme complex Iso1 which shows the major isoamylase activity in leaves (Wattebled et al., 2005). Mutations in both genes disrupt the normal starch structure and reduce the starch content. Similarly, aphid feeding decreased the expression of *BETA-AMYLASE 5* (*BMY5*) and *BETA-AMYLASE 3* (*BMY3*), which are involved in starch degradation (Lloyd et al., 2005), in local leaves at 24 hpi. *BMY5* is expressed in rosette leaves and is induced by sugars (Laby et al., 2001). *BRANCHING ENZYME 3* (*BE3*) was also repressed in the infested leaves at 24 hpi. Mutations in *BE3* affect the amylopectin structure (Dumez et al., 2006). *BMY3* is expressed preferentially in vascular tissues in source and sink organs and is required for leaf starch breakdown at night (Nga et al., 1999). The expression of genes involved in starch metabolism was repressed upon *M. persicae* attack in the infested leaves. These data would suggest that starch metabolism is modified as a result of aphid infestation. Changes in starch content would not be detected using the metabolite profiling approaches used in this study. However, sucrose, glucose and fructose levels can in some circumstances indicate changes in starch metabolism and turnover. An increase in transcripts encoding a CYTOSOLIC INVERTASE 1 (CINV1) were increased in the infested leaves at 24 hpi, suggesting enhanced starch turnover at the later stages of the infestation process. Disruption of

CINV1 reduces the activities of both neutral and acid invertases, shortens the roots and alters the sugar metabolism (Lou et al., 2007). Further analysis of starch metabolism in the local leaves is required to determine precisely how aphids modify leaf carbohydrates. For example, accumulation of starch was shown after yellow sugarcane aphid infestation on *Sorghum bicolor* (Gonzales et al., 2002). Other studies have suggested that starch plays a role in aphid resistance because mutants that are deficient in phosphoglucomutase (*pgm1*) and have depleted starch are able to support higher aphid populations (Singh et al., 2011). Similarly, the inclusion of starch in artificial diets acts as a deterrent against aphid feeding (Campbell et al., 1986).

4.2.3.3 Transcripts involved in carbohydrate metabolism and transport in infested leaves

While the present study failed to show any changes in the sucrose pool of local or infested leaves as a result of aphid feeding, the expression of genes encoding sugar transporters was altered, particularly in the infested leaves (Table 4.1). For example, *SUGAR TRANSPORTER 4* (*STP4*) that encodes a monosaccharide hydrogen ion symporter, which under normal conditions catalyzes monosaccharide import into sink tissues, was induced at 48 hpi. *STP4* is rapidly induced by wounding, chitin treatment and fungal attack in the vicinity of the affected area (Truernit et al., 1996). Moreover, its transcript levels have been shown to increase during *M. persicae* feeding on Arabidopsis (Moran and Thompson, 2001). Similarly, *SUCROSE-PROTON SYMPORTER 1* (*SUC1*) was also up-regulated at 48 hpi. SUC1 plays an important role in sugar signaling and *in vitro* experiments showed that ABA-dependent transcription factors bind to the promoter region of SUC1 (Hoth et al., 2010). It is induced in stress responses such as nematode attack (Hammes et al., 2005). *SUC1* belongs to the sucrose transporter gene family in Arabidopsis (Sivitz et al., 2008). Sucrose transporters mediate the predominant way for delivery of sucrose into the phloem flow of

Arabidopsis (Truernit, 2001). Sucrose released from the mesophyll cells accumulates in the apoplastic space and is actively taken up by sucrose transporters that act like hydrogen ion-symporters located in the sieve element-companion cell complexes (Chen et al., 2012). Sucrose uptake is driven by a transmembrane proton gradient that results in pumping of sucrose across the plasma membrane into the phloem thus contributing to the pressure gradient between source and sink tissues (Ayre et al., 2011). The major role in phloem loading can be attributed to SUC2. Mutant plants harboring a disrupted SUC2 display a severely stunted phenotype and accumulate high levels of sugar, starch and anthocyanin implying the crucial role of SUC2 for normal carbohydrate partitioning (Srivastava et al., 2008; Srivastava et al., 2009).

These observations are consistent with the absence of any detectable changes in leaf sucrose at the early time-points of the experiments. It is likely that changes in leaf sucrose will follow the above changes in sucrose transporters and therefore will only be detectable after 48 hpi.

Transcripts encoding sucrose-phosphatase 3 (SPP3) were increased by aphid feeding in the infested leaves at 24 and 48 hpi (Table 4.1). Sucrose-phosphatase catalyzes the last reaction in the sucrose biosynthesis pathway (Lunn, 2003). Similarly, the abundance of *GLUCOSE-6-PHOSPHATE DEHYDROGENASE 6* transcript, which plays a role in the pentose-phosphate pathway, was increased in the infested leaves at 24 hpi (Table 4.1). Taken together, these findings may reflect enhanced sucrose production and metabolism in infested leaves.

Table 4.1 Genes related to carbohydrate metabolism and transport which expression was altered in the local leaves of *A. thaliana* Col0 at 6, 24 and 48 h upon *M. persicae* attack. Sixty aphids were confined to a clip cage attached to a mature rosette leaf and gene expression analysed with Agilent V4 arrays relative to non-infested caged controls. Statistically significant differentially expressed genes were identified on volcano plots with a cut-off of fold change ≥2 and Student's t-test p-value ≤0.05. Genes were attributed to this category based on either literature evidence or Gene Ontology (GO). GO classification was made according to Biological Process (carbohydrate metabolic process, transmembrane carbohydrate transport or carbohydrate biosynthetic process) as found at The Arabidopsis Information Resource (TAIR; www.arabidopsis.org).

Accession	Description	Fold change		
		6h	24h	48h
At5g11920	ATCWINV6 (6-&1-FRUCTAN EXOHYDROLASE); hydrolase, hydrolyzing O-glycosyl compounds / inulinase/ levanase	na	6.3	8.9
At4g16600	glycogenin glucosyltransferase (glycogenin)-related	na	4.7	na
At3g13560	glycosyl hydrolase family 17 protein	na	4.3	4.5
At3g47050	glycosyl hydrolase family 3 protein	na	3.9	3.5
At4g22590	trehalose-6-phosphate phosphatase, putative	na	3.4	na
At4g34480	glycosyl hydrolase family 17 protein	na	3.2	2.7
At3g47040	glycosyl hydrolase family 3 protein	na	3.0	3.2
At3g54270	SPP3 (sucrose-phosphatase 3)	na	2.9	2.3
At2g05320	beta-1,2-N-acetylglucosaminyltransferase II	na	2.3	na
At4g26270	phosphofructokinase family protein	na	2.2	na
At3g07690	glycerol-3-phosphate dehydrogenase (NAD+)	na	2.2	na
At2g22480	phosphofructokinase family protein	na	2.2	na
At5g40760	G6PD6 (GLUCOSE-6-PHOSPHATE DEHYDROGENASE 6)	na	2.1	na
At1g35580	CINV1 (CYTOSOLIC INVERTASE 1); beta-fructofuranosidase	na	2.1	na
At1g12000	pyrophosphate--fructose-6-phosphate 1-phosphotransferase beta subunit, putative / pyrophosphate-dependent 6-phosphofructose-1-kinase, putative	na	2.0	na
At3g19930	STP4 (SUGAR TRANSPORTER 4); carbohydrate transmembrane transporter/ sugar:hydrogen ion symporter	na	na	2.2

At1g71880	SUC1 (SUCROSE-PROTON SYMPORTER 1); carbohydrate transmembrane transporter	na	na	2.2
At2g18700	ATTPS11 (Arabidopsis thaliana trehalose phosphatase/synthase 11); transferase	na	na	2.0
At2g39930	ATISA1/ISA1 (ISOAMYLASE 1); alpha-amylase/ isoamylase	na	0.5	na
At1g03310	ATISA2/BE2/DBE1/ISA2 (DEBRANCHING ENZYME 1); alpha-amylase/ isoamylase	na	0.5	na
At2g36390	STARCH BRANCHING ENZYME 2.1	na	0.5	na
At4g38300	glycosyl hydrolase family 10 protein	na	0.4	na
At5g51460	ATTPPA (Arabidopsis thaliana trehalose-6-phosphate phosphatase); trehalose-phosphatase	na	0.4	na
At4g17090	CT-BMY (BETA-AMYLASE 3, BETA-AMYLASE 8); beta-amylase	na	0.4	na
At1g45191	glycosyl hydrolase family 1 protein	na	0.3	na
At5g40610	glycerol-3-phosphate dehydrogenase (NAD+) / GPDH	na	0.3	0.5
At1g79410	ATOCT5 (ARABIDOPSIS THALIANA ORGANIC CATION/CARNITINE TRANSPORTER5); carbohydrate transmembrane transporter/ sugar:hydrogen ion symporter	na	0.3	0.5
At4g15210	BETA-AMYLASE 5	na	0.2	na

4.2.3.4 Transcripts involved in amino acid transport in infested leaves

While no changes were observed in the amino acid pools of infested leaves in this study, transcripts encoding several amino acid and ammonium transporters were differentially regulated (Table 4.2). For example, *LYS/HIS TRANSPORTER 7* encoding an amino acid transmembrane transporter (Lee and Tegeder, 2004) was induced at 6 hpi and remained up-regulated until the end of the experiment. Similarly, transcripts encoding LYSINE HISTIDINE TRANSPORTER 1 (LHT1) were increased at 24 and 48 hpi. The gene product of LHT1 is found in root epidermis as well as the leaf mesophyll. It is a high-affinity transporter, whose main substrate appears to glutamine. Mutants affected in *LHT1* show broad SA-dependent disease resistance, early senescence and an altered redox-status (Liu et al., 2010). Also, overexpression of *LHT1* increased nitrogen use efficiency (Hirner et al., 2006). These findings would suggest aphid-induced responses in leaf amino acid transport (but not metabolism) that occur early in the infestation process. Other evidence in support of this view is provided by the observation that transcripts encoding a plasma membrane localized ammonium transporter, AMMONIUM TRANSPORT 1 (AMT1;1), were increased at the last two time points of the experiment (Table 4.2). AMT1;1 plays an important role in the ammonium uptake in Arabidopsis roots under nitrogen-deficiency conditions and is a common marker gene for sensing nitrogen deficiency (Loqué et al., 2006). However, transcripts encoding a high-affinity ammonium transporter AMMONIUM TRANSPORTER 1;4 (AMT1;4), which has been shown to contribute to the nitrogen uptake of the pollen (Yuan et al., 2009), were decreased at 24 and 48 hpi. AMT1;4. In addition, a gene encoding a high-affinity nitrate transporter induced by nitrate, WOUND-RESPONSIVE 3, was also up-regulated at 24 hpi. WOUND-RESPONSIVE 3 has been implicated in a JA-independent signalling cascade (Okamoto et al., 2006). Transcripts encoding *CATIONIC AMINO ACID TRANSPORTER 1* (*CAT1*), a member

of the cationic acid subfamily of amino acid polyamine choline transporters, were increased at 24 hpi in the infested leaves. CAT1 mediates the uptake of lysine, arginine and glutamine in yeast (Su et al., 2004).

While there was no evidence of changes in expression of genes encoding enzymes of primary nitrogen assimilation in the infested leaves, transcripts encoding some enzymes or components of amino acid metabolism were altered in abundance in the infested leaves. For example, *ISOPROPYLMALATE DEHYDROGENASE 1*, which is involved in chain-elongation in leucine biosynthesis, was decreased in the attacked leaves at 24 hpi (see Chapter 3 Section 3.2.4.9), as was a monofunctional aspartate kinase (*ASPARTATE KINASE 3*), which is a key enzyme in the biosynthesis of aspartate family amino acids such as lysine, methionine and threonine (Curien et al., 2007). In addition, the expression of a member of the GATA transcription factor family (GATA, nitrate-inducible, carbon metabolism-involved (GNC)) was repressed in the infested tissues at 24 hpi. Interestingly, *GNC* was simultaneously induced in the systemic leaves at 24 hpi. GNC has been implicated in the coordination of carbon and nitrogen metabolism (Bi et al., 2005).

Accession	Description	Fold change		
		6h	24h	48h
At4g35180	LYS/HIS TRANSPORTER 7	8.0	21.7	11.5
At4g13510	AMT1;1 (AMMONIUM TRANSPORT 1)	na	3.1	2.5
At4g21120	AAT1 (CATIONIC AMINO ACID TRANSPORTER 1)	na	5.9	na
At5g50200	WOUND-RESPONSIVE 3 ; nitrate transmembrane transporter	na	2.7	na
At4g28700	AMT1;4 (AMMONIUM TRANSPORTER 1;4)	na	0.3	0.4
At1g64780	AMT1;2 (AMMONIUM TRANSPORTER 1;2)	na	0.2	na
At3g02020	ASPARTATE KINASE 3	na	0.4	na
At5g56860	GNC (GATA, NITRATE-INDUCIBLE, CARBON METABOLISM-INVOLVED)	na	0.4	na

Table 4.2 Genes related to nitrogen transport and metabolism which expression was altered in the local leaves of *A. thaliana* Col0 at 6, 24 and 48 h upon *M. persicae* attack. Sixty aphids were confined to a clip cage attached to a mature rosette leaf and gene expression analysed with Agilent V4 arrays relative to non-infested caged controls. Statistically significant differentially expressed genes were identified on volcano plots with a cut-off of fold change ≥2 and Student's t-test p-value ≤0.05. Genes were attributed to this category based on either literature evidence or Gene Ontology (GO). GO classification was made according to Biological Process (ammonium transport and ammonium transmembrane transport) as found at The Arabidopsis Information Resource (TAIR; www.arabidopsis.org).

4.2.3.5 Transcripts involved in myristoylation in infested leaves

In this study, the content of myristic acid was increased 10-fold at 48 hpi in the local leaves (Fig. 4.3). The observed increase in myristic acid in the infested leaves was accompanied by increases in transcripts encoding a number of proteins that are known to undergo N-myristoylation (Table 4.3). For example, the *thioredoxin H2* was increased at 24 hpi. Like glutaredoxins, thioredoxins can undergo N-myristoylation. Similarly, glutathione-dependent dehydroascorbate reductases and glutathione peroxidases have been predicted to be myrisoylated (Traverso et al., 2008). The observed increase in myristic acid in leaves infested with aphids is intriguing. This finding might suggest that N-myristoylation is an important component of the aphid response that could interface with changes in the cellular redox signalling. For example, it may influence the membrane binding properties of thioredoxins and glutaredoxins and so alter signalling functions (Pierre et al., 2007).

Accession	Description	Fold change		
		6h	24h	48h
At3g01290	band 7 family protein	3.7	5.5	4.8
At5g25440	protein kinase family protein	na	5.6	4.2
At4g04695	CPK31 (calcium-dependent protein kinase 31)	na	4.8	3.6
At5g01540	lectin protein kinase, putative	na	4.5	3.2
At1g02440	ATARFD1A (ADP-ribosylation factor D1A); GTP binding	na	3.2	2.6
At2g48150	ATGPX4 (GLUTATHIONE PEROXIDASE 4)	na	3.2	3.2
At4g20110	vacuolar sorting receptor, putative	na	3.2	na
At2g26190	calmodulin-binding family protein	na	2.2	2.4
At5g39950	ATTRX2 (ARABIDOPSIS THIOREDOXIN H2)	na	2.6	2.1
At4g35600	CONNEXIN 32; kinase	na	2.1	na
At2g47730	ATGSTF8 (GLUTATHIONE S-TRANSFERASE 8)	na	2.0	na
At5g24270	SOS3 (SALT OVERLY SENSITIVE 3)	na	0.5	na
At1g01740	protein kinase family protein	na	0.4	na
At4g27560	glycosyltransferase family protein	na	0.4	0.4
At1g02940	ATGSTF5 (Arabidopsis thaliana Glutathione S-transferase (class phi) 5)	na	0.3	0.4
At1g33770	protein kinase family protein	na	0.2	na
At4g11310	cysteine proteinase, putative	na	0.2	0.1
At1g52320	unknown protein	na	na	0.3

Table 4.3 Transcripts encoding proteins undergoing N-myristoylation which expression is affected upon *M. persicae* attack in the infested leaves. Sixty aphids were confined to a clip cage attached to a mature rosette leaf and gene expression analysed with Agilent V4 arrays relative to non-infested caged controls. Statistically significant differentially expressed genes were identified on volcano plots with a cut-off of fold change ≥2 and Student's t-test p-value ≤0.05. Genes were attributed to this category based on either literature evidence or Gene Ontology (GO). GO classification was made according to Biological Process (N-terminal protein myristoylation) as found at The Arabidopsis Information Resource (TAIR; www.arabidopsis.org).

4.2.3.6 Transcripts involved in carbohydrate metabolism in systemic leaves

Aphid attack did not provoke any significant changes in the carbohydrate content or the pools of TCA intermediates in the systemic leaves (Fig. 4.2). However, transcripts encoding enzymes involved in carbon metabolism were differentially expressed as early as 6 hpi in the systemic leaves (Table 4.4). These include a carbohydrate transmembrane transporter (*ATOCT6*) and *CYTOSOLIC INVERTASE 2*. The latter enzyme plays an important role in coordinating carbon fluxes in non-photosynthetic tissues (Barratt et al., 2009). Similarly, a gene (*At2g21590*) encoding one of the large subunits of the heterotetramer ADP-glucose pyrophosphorylase, the enzyme which catalyzes the first and limiting step in starch biosynthesis (Ventriglia et al., 2008), was induced in the systemic tissues at 24 hpi. Similarly, the expression of a cytoplasmic fructose-bisphosphate aldolase (*At4g26520*), a putative sucrose-phosphate synthase (*At4g10120*) and *PHOSPHORIBULOKINASE* (Marri et al., 2009), were enhanced at 48 hpi in the non-infested leaves. These findings may suggest a stimulation of carbon metabolism in the systemic leaves at 24 hpi but these changes did not result in a detectable change in photosynthetic carbon assimilation rates at this time point. The finding that transcripts encoding *GLYCINE DECARBOXYLASE P-PROTEIN 1* (*AtGLDP1*) forming the glycine decarboxylase complex (GDC), a key enzyme of the photorespiratory cycle (Palmieri et al., 2010) were induced in systemic leaves at the same time point, also supports the hypothesis that aphid feeding may favour enhancement of photosynthetic processes in leaves.

Accession	Description	Fold change		
		6h	24h	48h
At1g16370	ATOCT6; carbohydrate transmembrane transporter/ sugar:hydrogen ion symporter	2.8	na	na
At5g39790	5'-AMP-activated protein kinase beta-1 subunit-related	2.3	na	na
At4g09510	CYTOSOLIC INVERTASE 2	2.2	na	na
At2g21590	APL4 (large subunit of AGP 4); glucose-1-phosphate	na	3.6	na
At4g26520	fructose-bisphosphate aldolase, cytoplasmic	na	na	2.9
At4g10120	ATSPS4F; sucrose-phosphate synthase/ transferase, transferring glycosyl groups	na	na	2.7
At1g10150	ATPP2-A10 (Phloem protein 2-A10); carbohydrate binding	na	na	2.6
At1g32060	PRK (PHOSPHORIBULOKINASE); ATP binding / phosphoribulokinase/ protein binding	na	na	2.1

Table 4.4 Genes related to carbohydrate metabolism which expression was altered in the systemic leaves of *A. thaliana* Col0 at 6, 24 and 48 h upon *M. persicae* attack. Sixty aphids were confined to a clip cage attached to a mature rosette leaf and gene expression analysed with Agilent V4 arrays relative to non-infested caged controls. Statistically significant differentially expressed genes were identified on volcano plots with a cut-off of fold change ≥2 and Student's t-test p-value ≤0.05. Genes were attributed to this category based on either literature evidence or Gene Ontology (GO). GO classification was made according to Biological Process (carbohydrate metabolic process, transmembrane carbohydrate transport or carbohydrate biosynthetic process) as found at The Arabidopsis Information Resource (TAIR; www.arabidopsis.org).

4.2.3.7 Transcripts involved in nitrogen metabolism in systemic leaves

In these studies, infestation by *M. persicae* significantly decreased the levels of the branched chain amino acids leucine, isoleucine and valine in the systemic leaves (Fig. 4.2). The observed changes in the transcript profile alone do not provide any clues regarding the mechanisms that drive in the observed changes in leucine, isoleucine and valine. This suggests that the aphid-induced regulation of branched chain amino acid synthesis does not occur at the level of transcription, suggesting that these changes may occur because of post-transcriptional mechanisms for example enzyme activation/deactivation. However, transcripts encoding some enzymes involved in amino acid biosynthesis were modified in the systemic leaves as a result of aphid feeding (Table 4.5). For example, glutamine-dependent asparagine synthetase (*ASN1*) mRNAs were increased at 6 hpi. The expression of *ASN1* is considered to be controlled by the tissue carbon and nitrogen status (Lam et al., 1994). For example *ASN1* expression is induced by sucrose starvation and is repressed in the presence of exogenous sucrose. This may suggest that the carbon/nitrogen balance is changed in systemic leaves as a result of aphid feeding in a manner that cannot be detected by the metabolite profiling analysis conducted in the present study. Genes encoding the nitrate transporter (NTP3) and a member of the glutamine dumper family proteins, ARABIDOPSIS THALIANA GLUTAMINE DUMPER 4, which is involved in amino acid export (Pratelli et al., 2010), were up-regulated at 24 hpi. Transcripts encoding delta1-pyrroline-5-carboxylate synthase (P5SC1), the enzyme that catalyzes the rate–limiting step in proline biosynthesis (Székely et al., 2008), were increased at 6 hpi in the systemic leave , as were *PROLINE TRANSPORTER 3* mRNAs. This transporter can use proline, glycine betain and γ-aminobutyric acid as substrates (Grallath et al., 2005). These findings may suggest that proline synthesis and transport is enhanced in the systemic leaves, consistent with the perception of aphid-induced stress.

Accession	Description	Fold change		
		6h	24h	48h
At3g47340	ASN1 (DARK INDUCIBLE 6)	3.3	na	na
At2g39800	P5CS1 (DELTA1-PYRROLINE-5-CARBOXYLATE SYNTHASE 1)	2.9	na	na
At2g36590	ProT3 (PROLINE TRANSPORTER 3); amino acid transmembrane transporter	2.8	na	na
At3g21670	nitrate transporter (NTP3)	na	2.4	na
At2g17660	nitrate-responsive NOI protein, putative	na	2.4	na
At2g24762	ATGDU4 (ARABIDOPSIS THALIANA GLUTAMINE DUMPER 4)	na	2.2	na
At5g56860	GNC (GATA, NITRATE-INDUCIBLE, CARBON METABOLISM-INVOLVED)	na	2.2	na
At4g33010	ATGLDP1 (ARABIDOPSIS THALIANA GLYCINE DECARBOXYLASE P-PROTEIN 1)	na	na	2.4

Table 4.5 Genes related to nitrogen metabolism and transport which expression was altered in the systemic leaves of *A. thaliana* Col0 at 6, 24 and 48 h upon *M. persicae* attack. Sixty aphids were confined to a clip cage attached to a mature rosette leaf and gene expression analysed with Agilent V4 arrays relative to non-infested caged controls. Statistically significant differentially expressed genes were identified on volcano plots with a cut-off of fold change ≥2 and Student's t-test p-value ≤0.05. Genes were attributed to this category based on either literature evidence or Gene Ontology (GO). GO classification was made according to Biological Process (amino acid catabolic process, oligopeptide transport and regulation of amino acid export) as found at The Arabidopsis Information Resource (TAIR; www.arabidopsis.org).

4.3 Discussion

4.3.1 Effect of aphid feeding on primary metabolism

A key goal of the studies described in this chapter was to provide new information on the response of the leaf metabolome to aphid infestation. Furthermore, transcriptome data were added in order to provide a greater insight into the mechanisms that may be involved in aphid-induced re-direction of metabolism. The metabolite profiling studies reported here revealed that only a small number of metabolites were affected in the infested leaves within the first 48h of aphid feeding. Thus, the transcriptional re-programming occurred largely in the absence of global changes in metabolism, suggesting that the aphid induced changes in gene expression led to alterations in metabolism and not vice versa. The observed changes in gene expression patterns may also result in more pronounced alterations of the metabolism at stages of the infestation process that occur later than those measured in these experiments. It is entirely possible that aphids provoke localized changes in metabolite contents in the vicinity of stylet penetration that were below the level of detection in the assays used here which involved whole leaves. Similarly, the content of the phloem sap could be altered as a result of aphid feeding in ways that have not been detected in these experiments. The enhanced expression of *AMT1;1*, which is commonly used as a marker for nitrogen deficiency (Yuan et al., 2007), in infested leaves might suggest a localised depletion of nitrogen metabolites. However, no significant aphid-induced changes in leaf amino acids were observed in the infested leaves. Nevertheless, several other amino acid transporters, ammonium transporters and nitrate transporters were differentially regulated suggesting that the transport of nitrogen metabolites is perturbed as a result of aphid feeding. In this context it is worth noting that that the expression of these genes was increased in the infested leaves at the last two time points and may reflect alterations of the nitrogen status related to senescence. In line with this hypothesis the

expression of *CAT1* and *LHT1*, characterized as senescence-induced nitrogen transporters, was up regulated locally (Su et al, 2004; Hirner et al., 2006).

4.3.2 Senescence-related changes upon aphid attack

Aphids act as "sinks" for assimilates because they withdraw large amounts of phloem sap, which is rich in amino acids as well as sucrose. This may result in nitrogen deficiency and low nutrient supply which has been linked to premature leaf senescence (Thomas and de Villiers, 1996). An early onset of senescence might be beneficial for the phloem feeders because enhanced nitrogen mobilization might lead to enrichment of the phloem sap (Himelblau and Amasino, 2001). However, aphid reproduction does not seem to be influenced by the availability of nutrients in the phloem stream (Hunt et al., 2010), possibly because of the presence of endosymbiotic bacteria (*Buchnera*). Attempts to correlate the dietary content of the nitrogen (and other metabolites) to aphid behaviour and reproduction may therefore be an oversimplification, as it must be viewed within the overall context of plant responses and defences. Even though it is tempting to speculate that aphids affect plant metabolism for their own benefit the observed changes can be a side effect and reflect specific alterations of plant physiology such as senescence.

A recent study has showed that sucrose accumulates gradually during aphid infestation and this is linked with increased starch content (Singh et al., 2011). It is worth mentioning that in certain cases leaf senescence also affects sugar levels and their pools represent a major hub for integrating environmental signals (Buchannan-Wollaston et al., 2003; Wingler et al., 2006). Nitrogen deficiency often is followed by accumulation of sugars (Paul and Driscoll, 1997). Hexoses accumulate during developmental senescence, as the sucrose pool remains relatively stable (Quirino et al., 2001). Combination of 2% glucose and low nitrogen supply were able to induce senescence with a transcription profile close to a developmental senescence (Pourtau et al., 2004).

Aphid feeding causes callose deposition on the sieve plates resulting in impaired phloem flow. This may lead to local accumulation of metabolites in the infested leaves due to their reduced phloem loading. Indeed callose deposition results in an age-dependent sugar accumulation (Jongebloed et al., 2004). Despite the fact that *M. persicae* attack occurred in the absence of changes in sugar levels, the expression of transcripts encoding sugar transporters such as SUC1 and SPT4 was induced in the infested leaves implying that carbon partitioning is affected.

4.3.3 Role of photosynthesis during plant-aphid interactions

Photosynthesis is considered to play a pivotal role in the plant–aphid interactions (Kerchev et al., 2011a). Aphids feeding on phloem sap act as external sinks competing for resources required for growth and development with the rest of the plant. Thus a shutdown of the primary metabolism, limiting the growth and the availability of resources seems like a way to minimize the effects of aphid feeding. Several studies have shown that a reduction in sink demand results in gradual inhibition of photosynthesis and down-regulation of the expression of genes involved in photosynthesis. Such regulation of photosynthesis has been frequently associated to accumulation of carbohydrate in photosynthetic leaves, which in turn would exert a negative feedback on photosynthesis (Paul and Foyer, 2001). While aphid infestation is frequently associated with plant directed down-regulation of photosynthesis, insect resistant genotypes tend to maintain a high photosynthetic capacity and the potential links between day length, light signalling and the plant response to insect infestation remain to be explored. Insect herbivory also has a profound impact on photosynthesis both through physiological mechanisms (Nabity et al., 2009) and shifts in the transcriptional profile of genes associated with photosynthetic reactions (Bilgin et al., 2010). While photosynthesis was not measured in the infested leaves, the rates of photosynthetic carbon assimilation in systemic leaves were similar in both infested and

uninfested plants. However, a number of genes involved in starch metabolism were repressed upon *M. persicae* attack in the infested leaves. Starch metabolism is an intrinsic part of the primary photosynthetic metabolism, affecting plant growth and carbon metabolism (Stitt et al., 2010). Moreover, starch can have a role in the overall resistance to *M. persicae* and Arabidopsis mutant plants deficient in starch (*pgm1*) have been shown to support higher aphid population (Singh et al., 2011). Starch acts as a deterrent compound against *M. persicae* (Campbell et al., 1986). Accumulation of starch was shown after yellow sugarcane aphid infestation on *Sorghum bicolor* (Gonzales et al., 2002).

4.3.4 Aphid feeding results in decreased levels of branched-chain amino acids in the systemic leaves

The branched-chain amino acids (valine, leucine and isoleucine) are an essential part of the insect diet and their availability can have a direct effect on herbivore success. The initial step of isoleuicine synthesis requires threonine deaminase activity (TD). TD is also involved in the degradation of threonine. Insect larvae feeding on tomato accumulated TD in the midgut like its activity was enhanced by the removal of the C-terminus responsible for the feedback inhibition of isoleucine (Chen et al., 2005).

The synthesis of branched-chain amino acids occurs in the chloroplasts and it is possible that the decrease is a result of aphid-induced alterations in the redox status of the chloroplasts. For example, isopropylmalate dehydrogenase 1 (IMD1) involved in leucine biosynthesis and methionine chain elongation of aliphatic glucosinolates is subject to thiol-based redox regulation (He et al., 2009). Interestingly, the expression of *IMD1* was decreased in the infested leaves. A number of transcripts involved in glucosinolate biosynthesis were differentially regulated in both local and systemic tissues implying a complex interplay between the metabolism of branched-chain amino acids and glucosinolates which can result in a complex spatial regulation of their levels.

Similarly, it is possible that the degradation of branched-chain amino acids in the mitochondria and peroxisomes (Binder et al., 2007) is enhanced as a result of infestation. In Arabidopsis branched-chain aminotransferases (BCATs) encode enzymes involved in the final transamination step leading to both biosynthesis and degradation of branched-chain amino acids. For example, the mitochondrially-localized member BCAT1 plays an important role in leucine degradation (Schuster and Binder, 2005). Proteins from this family are also crucial for the synthesis of aliphatic glucosinolates (Knill et al., 2008; Schuster at al., 2006). Thus the plastid-located BCAT3 and the cytosolic BCAT4 have a dual role in primary and secondary metabolism.

4.3.5 Rapid accumulation of myristic acid in leaves directly subjected to *M. persicae* attack

The observed increase in myristic acid levels in *M. persicae*-challenged leaves suggests that N-myristoylation might be an important component of the aphid response. Moreover, the expression of a number of genes encoding proteins that undergo N-myristoylation was differentially regulated in the local leaves simultaneously. Among the redox proteins, which expression is increased by aphid infestation, several have been shown to be modified by attachment of myristic acid, including thioredoxins, glutathione peroxidases and GSTs (Pierre et al., 2007). The expression of *THIOREDOXIN H2*, *GLUTATHIONE PEROXIDASE 4*, and *GLUTATHIONE S-TRANSFERASE 8* was induced upon aphid attack. N-myristoylation of redox proteins might therefore also have an impact on redox signalling, particularly in the infested leaves, where myristic acid was found to increase as a result of aphid feeding.

4.3.6 The stearic acid pool in the systemic leaves diminishes upon *M. persicae* attack

Stearic acid is important for the synthesis of polyunsaturated fatty acids such as chloroplastic generated 18:3 fatty acids, serving as jasmonate precursors. The induction

of JA-markers genes in the systemic leaves suggests that aphid attack might lead to enhanced JA levels and partly explain the depletion of stearic acid content. However, the link is likely to be more complex and involves other defense components. The resistance against *M. persicae* in Arabidopsis is modulated by a plastid-localized fatty acid desaturase *SUPPRESSOR OF SALICYLIC ACID INSENSITIVITY2* (*SSI2*). SSI2 desaturates stearic acid to oleic acid (18:1). The *ssi2* mutants accumulate high levels of stearic acid and are hyper-resistant to *M. persicae* attack (Louis et al., 2010). This is accompanied with increase levels of SA and enhanced resistance to pathogens (Kachroo et al., 2001). However, high levels of stearic acid in artificial diets or after leaf infiltration did not alter aphid fecundity (Louis et al., 2010).

Chapter 4: Summary

- Microarray analysis, presented in Chapter 3, identified that *M. persicae* attack on Arabidopsis leads to substantial transcriptome changes implying the role of primary metabolism in plant defense. Targeted metabolite profiling was used to explore changes in major leaf metabolites in infested and non-infested leaves, at 6, 24 and 48 hours after the onset of the infestation.
- More than sixty compounds were identified and quantified by GC-MS, including major amino acids, organic acids involved in the tricarboxylic acid cycle, sugars, fatty acids, long-chain alcohols and various non-polar compounds.
- Aphid-challenged leaves accumulated high levels of myristic acid involved in N-myristoylation of various proteins implicated in redox signaling.
- Transcriptome data from the microarray experiment described in Chapter 3 related to carbon and nitrogen metabolism and transport is presented in order to provide a greater insight into the mechanisms that may be involved in aphid-induced re-direction of metabolism.

- Photosynthetic CO_2 assimilation upon *M. persicae* attack was measured at 24 hpi in non-infested Arabidopsis leaves.
- Leaf transcriptome changes occur in the absence of marked changes in photosynthesis and leaf metabolites. The findings presented here suggest that if large changes in leaf metabolism are induced in response to the presence of the phloem feeders they occur only after 48 hpi.

Chapter 5. Characterization of mutants that are deficient in ascorbate (altered redox status) or insensitive to ABA (altered ABA signalling)

5.1 Introduction

Cellular redox homeostasis is considered to be a convergence regulator for signals from metabolism and the environment as well as biotic and abiotic stress responses (Fujita et al., 2006). While the signaling roles of ROS in regulating plant responses to environmental and metabolic triggers have been extensively studied (Mittler, 2002; Mittler et al., 2004; Mhamdi et al., 2010) much less information is available concerning the roles of antioxidants in the control of plant growth and defense processes.

5.1.1 Roles of ascorbic acid beyond its antioxidant function

Ascorbic acid is an essential component of the cellular antioxidant network that buffers the production of ROS while allowing essential signaling that regulates plant growth, development and defense (Gong et al., 2007; Chaouch et al., 2010; Foyer and Noctor, 2011). Ascorbate and ascorbate oxidase exert a strong influence on plant growth and development (Chinoy, 1984; Conklin and Barth, 2004; Pignocchi et al., 2003; Barth et al., 2006). The vitamin C-defective (*vtc*) mutants are impaired in ascorbate synthesis. The *vtc1* and *vtc2* mutants have a lowered abundance of ascorbate (less than 30% of the wild type levels) and they show a slow growth phenotype (Conklin et al., 1999; Veljovic-Jovanovic et al., 2001; Pastori et al., 2003). The *vtc1* mutant harbors a point mutation in the ascorbate biosynthetic enzyme GDP-Man pyrophosphorylase (Conklin et al., 1999). The *VTC2* gene encodes the enzyme GDP-L-galactose phosphorylase, which catalyzes the conversion of GDP-L-galactose to L-galactose 1-phosphate in the

first committed step of the ascorbate synthesis pathway in Arabidopsis leaves (Smirnoff, 2000). The leaves of the *vtc* mutants have smaller cells (Pavet et al., 2005) and they show enhanced basal resistance to biotrophic pathogens (Pavet et al., 2005). Ascorbate also participates in the synthesis (Sommer-Knudsen et al., 1998) and cross-linking of cell wall components (Smirnoff, 2000; Pellny et al., 2009) and thus participates in the redox control of cell expansion (Esaka, 1998; Kato and Esaka, 2000). Ascorbate fulfils crucial roles in photosynthesis and chloroplast function and it has been implicated in the control of the expression of genes encoding chloroplast proteins (Kiddle et al., 2003). In the chloroplast, ascorbate protects the photosynthetic machinery by removing ROS (Foyer and Noctor, 2011). It also reduces tocopheroxyl radicals produced in the thylakoid membranes as a result of reactions associated with photosystem II function (Havaux, 2003). Ascorbate in the thylakoid lumen can act as an emergency electron donor to photosystem II when the oxygen evolving complex is inactivated in stressful conditions (Toth et al., 2009). The ascorbate pool in the lumen also protects photosystem II because ascorbate is a cofactor for violaxanthin de-epoxidase, which is component of the xanthophyll cycle. The *vtc* mutants are impaired in non photochemical quenching under high light (Muller-Moulé et al., 2003; 2004; Smirnoff, 2000).

Ascorbate is accumulated to high levels in growing tissues where it plays a key role in cell growth via regulation of the cell cycle (Potters et al., 2002; 2004) whereas ascorbate depletion is associated with quiescence (Kerk and Feldman, 1995; Potters et al., 2002; 2004). The complex antioxidant network of the plant cell has ascorbic acid and glutatione at its centre that act together in high capacity ROS processing pathways. However, ascorbate and glutathione have distinct roles in the control of cell proliferation (Noctor et al., 2000; Potters et al., 2002; Pellny et al., 2009; Diaz Vivancos et al., 2010). The *rm1* (*rootmeristemless1*) Arabidopsis mutant, which has less than 5%

of wild-type glutathione contents, fails to develop a root apical meristem because the cells arrests at the G1 phase of the cell cycle (Vernoux et al., 2000). Combining the *rlm1* mutation with mutations in the two genes encoding cytosolic/mitochondrial NADPH-thioredoxin reductase (NTRA, NTRB) results in even more severe shoot phenotype because of redundancy between glutathione and thioredoxins in the control of shoot apical meristem function (Reichheld et al., 2007). Reduced glutathione cycle between the cytoplasm and nucleus during the plant cell cycle and co-localizes with nuclear DNA in the early stages of plant cell proliferation (Diaz Vivancos et al., 2010).

The availability of ascorbate can influence growth through effects on GA synthesis because ascorbate is the co-factor involved in the catalysis of 2-oxoacid-dependant dioxygenase (2ODD) reactions (Arrigoni and de Tullio, 2000). Dioxygenases are important in the final stages of GA synthesis, where GA12-aldehyde is converted to bioactive GA (Hedden and Kamiya, 1997). The activities of these enzymes are enhanced by the addition of ascorbate in vitro (Graebe, 1987; Hedden, 1992; Lange, 1994; Prescott and John, 1996; Lukacin and Britsch, 1997). Moreover, the *vtc* leaves have enhanced ABA levels compared to the wild type with alterations in gene expression patterns that are characteristic of altered ABA signaling (Pastori et al., 2003; Kiddle et al., 2003). ABA and GA are considered to act antagonistically in the control of plant growth and defense, the ratio of ABA to GA being a fundamental determinant of cell growth or quiescence (Finkelstein and Rock, 2002).

5.1.2 Signalling pathways requiring ABI4

ROS are produced as secondary messengers in many hormone signalling pathways. For example, the ABA signaling pathway involves ROS production via the activation of NADPH oxidases (RbohD and RbohF; Kwak et al., 2003; Torres and Dangl, 2005; Torres et al., 2002). ABA signaling pathways have been implicated in plastid-derived retrograde signaling (Koussevitzy et al., 2007) pathways and in plant-pathogen

interactions (Ton and Mauch-Mani, 2004). Of the different components of the ABA-signalling pathway that have been characterised the nuclear localized ABI4 has been shown to have roles in the control of a diverse range of processes such as plant development, pathogen resistance, sugar and nitrogen signalling, and chloroplast and mitochondrial retrograde signalling (Kaliff et al., 2007; Koussevitzky et al., 2007; Giraud et al., 2009). ABI4 has therefore been suggested to be the 'master-switch' required for the regulation of nuclear genes in response to environmental and developmental cues (Koussevitzky et al., 2007).

ABI4 has been commonly identified in sugar screens supporting the key role of this transcription factor in sugar signalling. The expression of *ABI4* is tightly developmentally regulated and plays a crucial role in seed maturation and in seedlings shortly after germination when its transcript abundance is highest (Bossi et al., 2009). In seedlings *ABI4* expression declines with the establishment of autotrophic growth and reaches low but detectable levels at later development stages (Arroyo et al., 2003; Brocard et al., 2002). The expression of ABI4 is induced by glucose, threhalose and ABA (Arroyo et al., 2003; Ramon et al., 2007; Arenas-Huerto et al., 2000). Many genes responsive upon exogenous application of sugars and ABA require a functional ABI4 for their regulation. For example, the sugar activation of the genes involoved in strach metabolism ADP-glucose pyrophosphorylase (*APL3*) and STARCH BRANCHING ENZYME 2.2 (*SBE2.2*) is abolished in the *abi4* background (Rook et al., 2001). ABI4 directly binds the promoter sequence of *SBE2.2* and activates its glucose-mediated expression (Bossi et al., 2009). Moreover, ABI4 is implicated in the repression of many photosynthetic genes and their sugar-mediated repression is not observed in the *abi4* mutant (Acevedo-Hernandez et al., 2005; Arenas-Huertero et al., 2000; Dijkwel et al., 1997). The reduced expression of *Lhcb* (encoding a light-harvesting chlorophyll a/b-

binding protein) in glucose-treated wild type Arabidopsis plants was significantly attenuated in the *abi4* mutants (Koussevitzky et al., 2007).

Through photosynthesis and respiration, sugars play a central role in cellular energy metabolism and in ROS generation through the associated electron transport and metabolic pathways (Foyer and Noctor, 2009). The availability of sugars not only regulates the expression of genes encoding proteins involved in the photosynthetic and respiratory pathways but sugars also exert a major influence over plant stress responses (Sulmon et al., 2004, Couée et al., 2006). Like sugars, ROS are considered to be part of the repertoire of chloroplast signals that participate in the metabolite crosstalk regulating nuclear gene expression and are responsible for the fine-tuning of the expression of photosynthesis-related nuclear genes (Fey et al., 2005). Sucrose has a negative impact on both photosynthetic gene expression (Van Oosten et al., 1997) and on ascorbate levels (Yabuta et al., 2007). Moreover, the negative effect of sucrose on asocrbate accumulation is abolished in the *abi4* mutant (Yabuta et al., 2007).

The analysis of the global transcriptome changes triggered in Arabidopsis Col0 leaves upon *M. persicae* attack (Chapter 3) revealed that the abundance of numerous redox and ROS-related transcripts was changed rapidly in response to aphid feeding. For example, transcripts encoding *REDOX RESPONSIVE TRANSCRIPTION FACTOR 1* showed the greatest increase in abundance at 6 hpi in the infested leaves. The size and rapidity of the response implies that the cellular redox hub plays an important role in plant responses to aphids and that redox signalling may be crucial to the adaptive response to aphid attack. In addition, there was a rapid response in transcripts encoding components of hormone metabolism and signalling. Of these the abundance of a suite of genes associated with ABA metabolism and signalling was rapidly changed in infested and systemic leaves in response to aphid attack. This strongly suggested that ABA

signalling pathways are important in aphid triggered responses and like the cellular redox hub, ABA signalling might also be an important player in plant – aphid interactions. The following experiments were performed to test this hypothesis using mutants lacking ascorbate (*vtc2*), or a crucial component of the ABA-signalling pathway (ABI4). However, firstly it was important to characterise how gene expression pathways and associated signalling were changed by the inability to accumulate wild type levels of ascorbate in the *vitamin C defective 2* (*vtc2*) mutant and signal trough ABI4 in the *abi4* mutant, in the absence of stress (aphids). Since the *vtc* mutants show altered ABA signal transduction pathways it was pertinent to ask the question whether the low ascorbate levels are sensed via ABA-dependent signalling relays. Introducing the *abi4* mutation in a low ascorbate background (*vtc2*) could answer this question and provide further insight in the complex interplay between redox and ABA signalling pathways, particularly revealing the role of ABI4 in redox signalling which was lacking. The information obtained from the double *abi4vtc2* mutants was further needed to properly evaluate the individual roles of redox and ABA mediated signals since they are highly intertwined and are triggered simultaneously upon aphid attack. Thus these studies were designed to provide new information on the master hub where hormone (ABA) and redox signalling converge. The following experiments were therefore performed to characterize each of the mutants in terms of signalling pathways and the regulation of growth and to gain deeper insights into the relationships between redox and ABA signalling in double mutants (*abi4vtc2*) that are both defective in ascorbate synthesis (*vtc2*) and lack a functional ABI4 (*abi4*). The findings described in this chapter are also fundamental to understanding how the plant perception of aphid attack is modified in the mutants, as described in the next chapter.

5.2 Results

5.2.1 Characterisation of *abi4, vtc2,* and *abi4vtc2* double mutants

The ascorbate-defective *vtc2* mutant showed a marked slow growth phenotype (Fig. 5.1 A and B) associated with lower levels of leaf ascorbate relative to Col0 (Fig. 5.1 C), as previously reported (Pavet *et al.,* 2005). In contrast, the *abi4* mutants had similar ascorbate levels (Fig. 5.1 C) and growth phenotypes (Fig. 5.1 A) to Col0. When the *abi4* mutation was introduced into the *vtc2* background, the slow growth phenotype of the ascorbate-defective mutants was reverted (Fig 5.1 A). The *abi4vtc2* double mutants had a similar growth phenotype (Fig. 5.1 A) to Col0, despite having similar ascorbate levels to the *vtc2* mutants (Fig. 5.1 C). Rosette biomass accumulation in the *abi4vtc2* mutants resembled that of Col0 (Fig. 5.1 B).

Like ascorbate, the tri-peptide thiol antioxidant, glutathione is a modulator of abiotic and biotic stress signalling pathways in plants (Noctor et al., 2011). The leaf glutathione pool was significantly increased in the *vtc2* mutants (Fig. 5.1 D), the *abi4* mutant (Fig. 5.1 D) and the *abi4vtc2* double mutants relative to Col0 (Fig. 5.1 D).

No significant differences in the total protein and chlorophyll content and photosynthetic rates between the studied genotypes were detected by applying two-way ANOVA analysis. The leaves of the wild type, *abi4*, *vtc2* and *abi4vtc2* double mutants had similar levels of total protein ($p = 0.541$) and chlorophyll ($p = 0.208$) at 4 and 6 weeks. The photosynthetic CO_2 assimilation rates measured in rosette leaves at 6 weeks were also similar in all genotypes ($p = 0.374$).

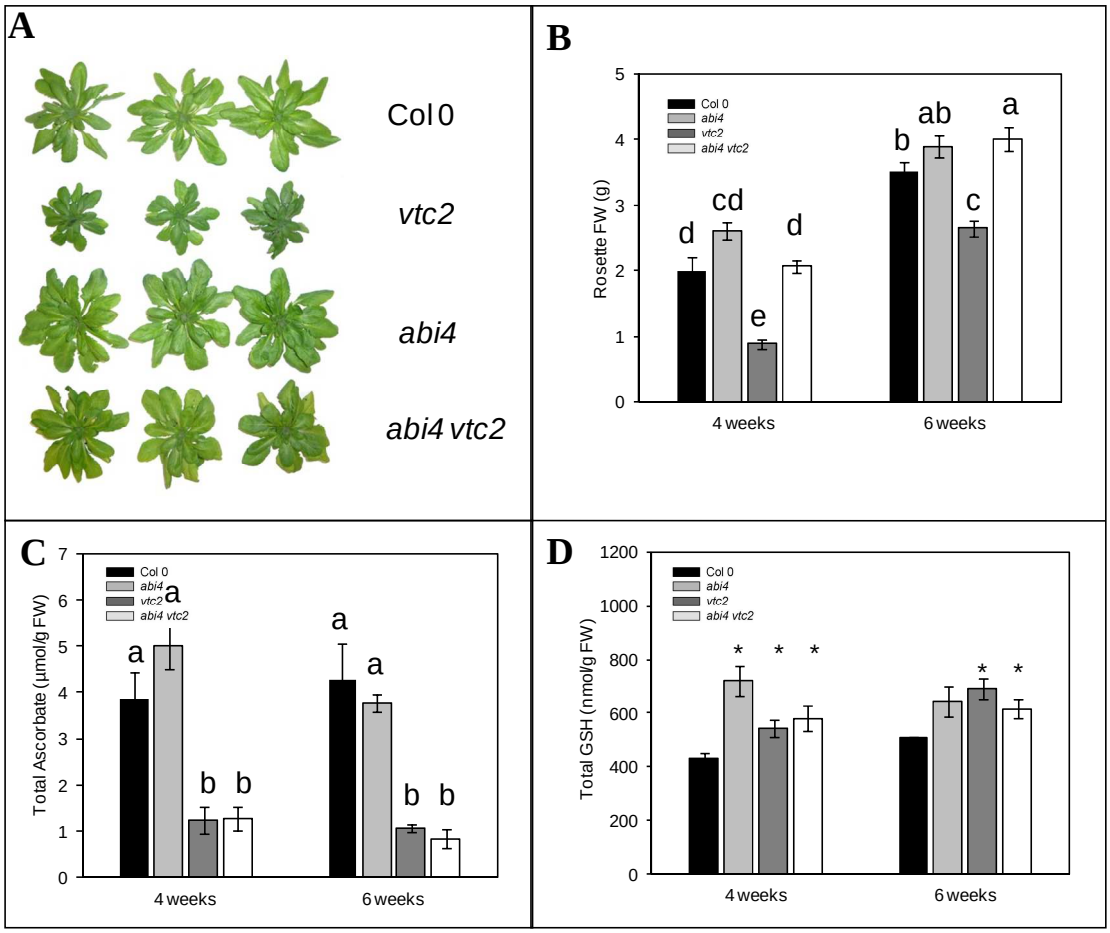

Figure 5.1 The growth phenotype and the leaf ascorbate and glutathione levels of the *abi4*, *vtc2* and *abi4vtc2* double mutants relative to Col0. A - Representative images showing the growth phenotype of 4 week-old plants; B - Biomass of fresh Col0, *abi4*, *vtc2*, and *abi4vtc2* rosettes at 4 and 6 weeks. Data represent average rosette fresh weights ± SE (n=15); C – Rosette ascorbic acid content in 4 and 6-week-old plants ± SE (n=4); C – Rosette glutathione content in 4 and 6-week-old plants ± SE (n=4). Significant differences in biomass fresh weight and ascorbate content were analysed with two-way ANOVA with post hoc analysis by Student-Newman-Keuls' pairwise comparison. Means with different letters are significantly different at the 5% level. Asterisks indicate significant differences in glutathione content between the mutants and wild type according to the Student's t-test ($p<0.05$).

5.2.2 Sugar-signalling

The following experiments were performed to determine whether the ABA and redox signalling pathways were linked. One way that a link might be demonstrated, was if low ascorbate had an effect on glucose signalling pathways that use ABI4. Sugar (glucose) signalling regulates a wide range of metabolic and developmental processes in plants, including germination. *ABI4* plays an important role in sugar signalling, as well as ABA responses with *ABI4* required for the regulation of many ABA and sugar responsive genes (Bossi et al., 2009). The *abi4* mutant seeds were able to germinate on media containing high levels of glucose whereas Col0 seeds showed a poor germination under these conditions (Fig 5.2 A, B). The germination of the *vtc2* mutant seeds showed a similar sensitivity to high glucose as observed in Col0 (Fig 5.2 A, B). However, like *vtc2*, the *abi4vtc2* double mutants exhibited a glucose-sensitive phenotype (Fig 5.2 A).

The quantitative comparison of seed germination rates (Fig 5.2 B) revealed that all genotypes showed high germination (>80%) on MS media. The germination of Col0 seeds was decreased to less than 5% when the MS media was supplemented with 6% glucose (Fig 5.2 B). However, the germination rate of the *abi4* seeds remained high (>80%) on glucose-containing media (Fig 5.2 B). The germination rates of the *vtc2*, and the *abi4vtc2* double mutants were similar to Col0 under the high glucose growth conditions (Fig 5.2 B). Thus, the glucose-insensitive phenotype of the *abi4* seeds was repressed in the low ascorbate (*vtc2*) background.

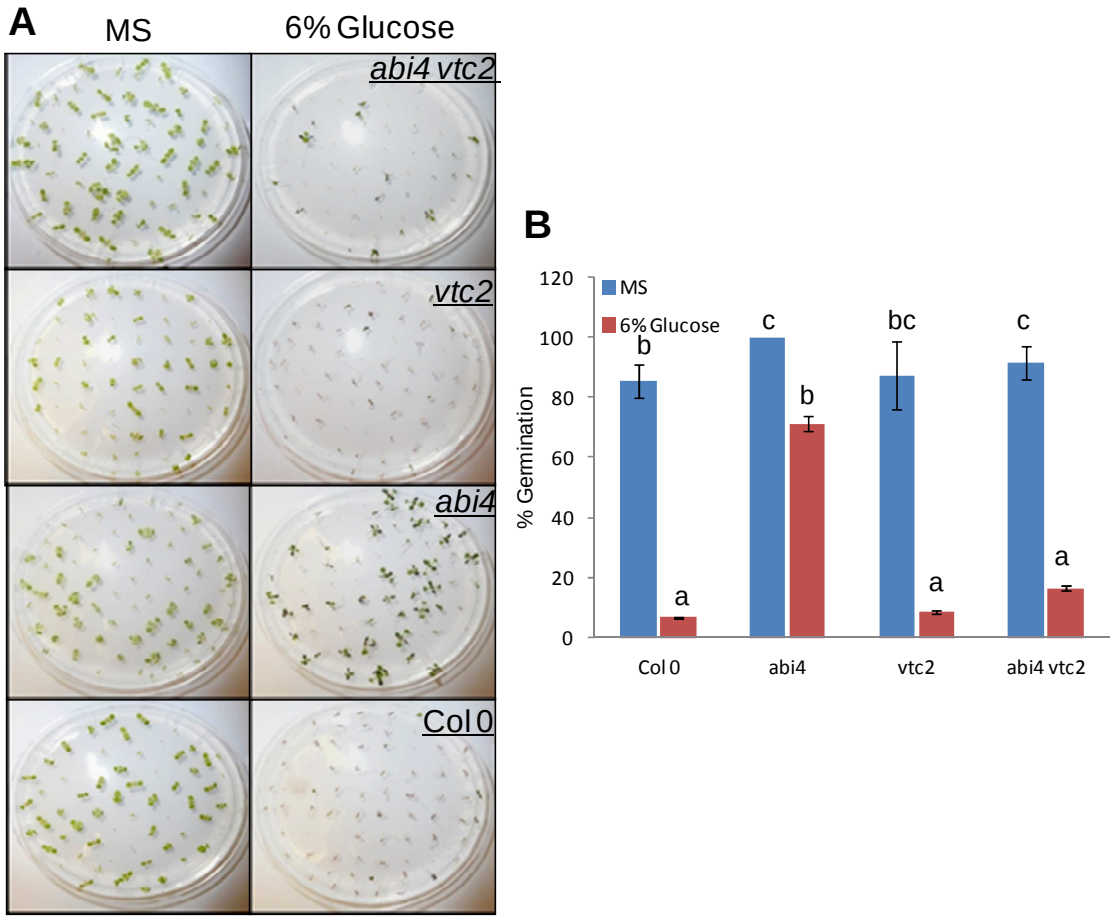

Figure 5.2 Phenotypes of *abi4*, *vtc2*, *abi4vtc2* and Col0 (A) ten days after sowing on MS media alone or MS media supplemented with 6% glucose (w/v). A quantitative comparison (B) of the germination rates of three individual batches of *abi4*, *vtc2*, *abi4vtc2* and Col0 mature seeds ten days after sowing on MS media alone (blue bars) or MS media containing 6% glucose (red bars). Plates were incubated in controlled environment chambers under a light intensity of 30 µmol m^{-2} s^{-1}, with a 12h photoperiod. Data are presented as mean ± SE (n = 3), and different letters indicates values that were significantly different within a treatment group according to Fisher's protected LSD test.

5.2.3 Comparisons of the *abi4*, *vtc2* and *abi4vtc2* leaf transcriptomes

The transcriptomes of the *abi4*, *vtc2*, and *abi4vtc2* leaves were compared to Col0 at the 6 week stage. A total of 547 transcripts were differentially expressed in *abi4* relative to Col0 with 283 of these increased relative to Col0 and 264 decreased, while 968 (617 up- and 351 down-regulated) transcripts were differentially expressed in *vtc2 (*Fig. 5.3). The nature of the genes that were either common or differentially expressed in the mutants relative to Col0 was broadly comparable in terms of functional categories (Fig. 5.4). The transcriptome analysis of the *abi4vtc2* double mutants revealed 768 transcripts constitutively induced and 515 repressed relative to the wild type (Fig 5.3). There was a significant overlap (up to 73%) between the transcripts that were differentially expressed in *abi4*, *vtc2*, and *abi4 vtc2* (Fig. 5.3).

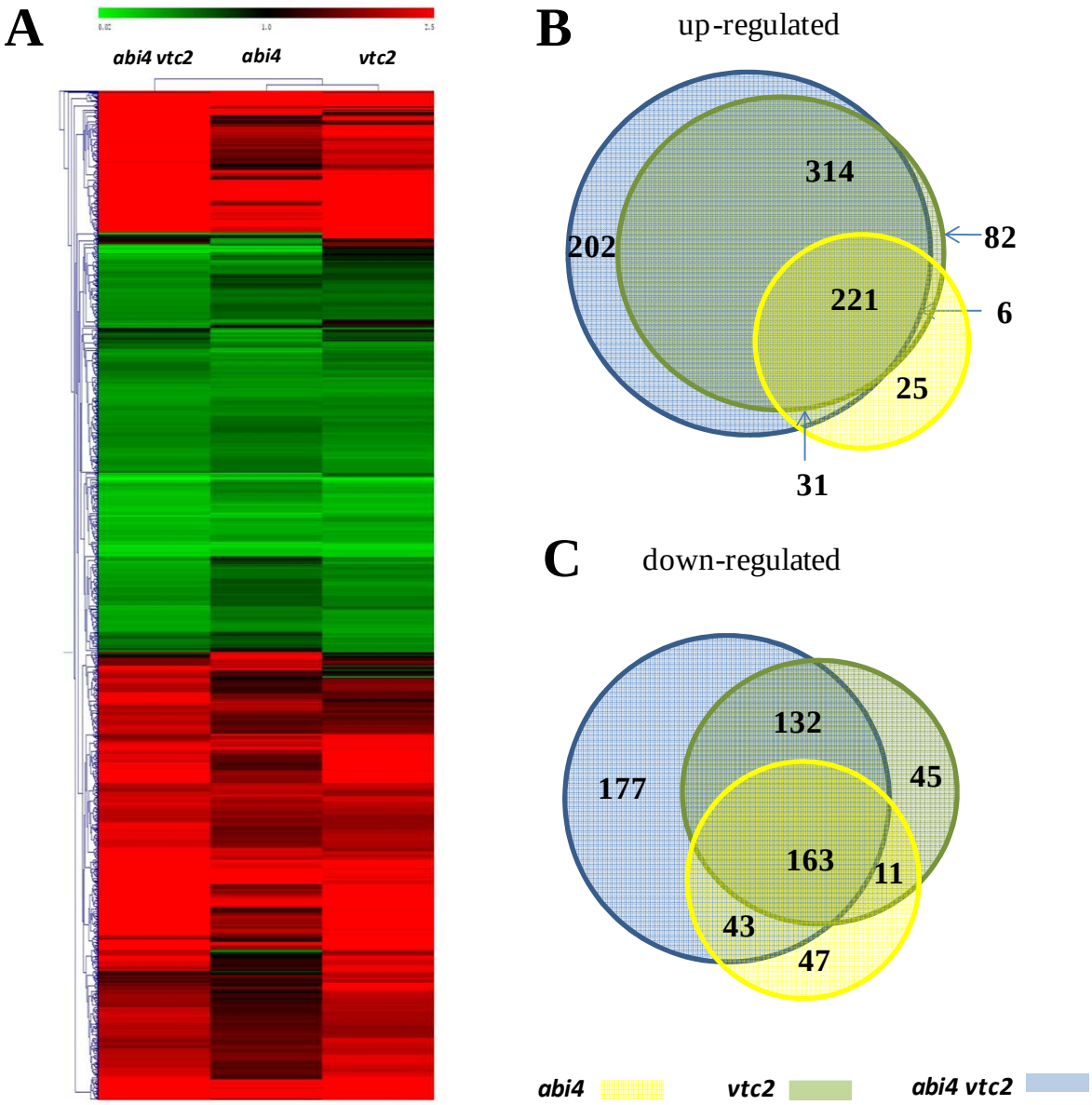

Figure 5.3 Hierarchical clustering of differentially expressed transcripts in *abi4*, *vtc2* and *abi4vtc2* relative to Col0 (A), together with venn diagrams showing common and unique genes differentially regulated between *abi4*, *vtc2* and *abi4 vtc2* relative to the wild type; B) Common and unique up-regulated genes. C) Common and unique down-regulated genes. Whole Arabidopsis rosettes were harvested following six weeks growth in controlled environment chambers, RNA extracted and microarray analysis performed as described in Material and Methods Section 2.8.2.

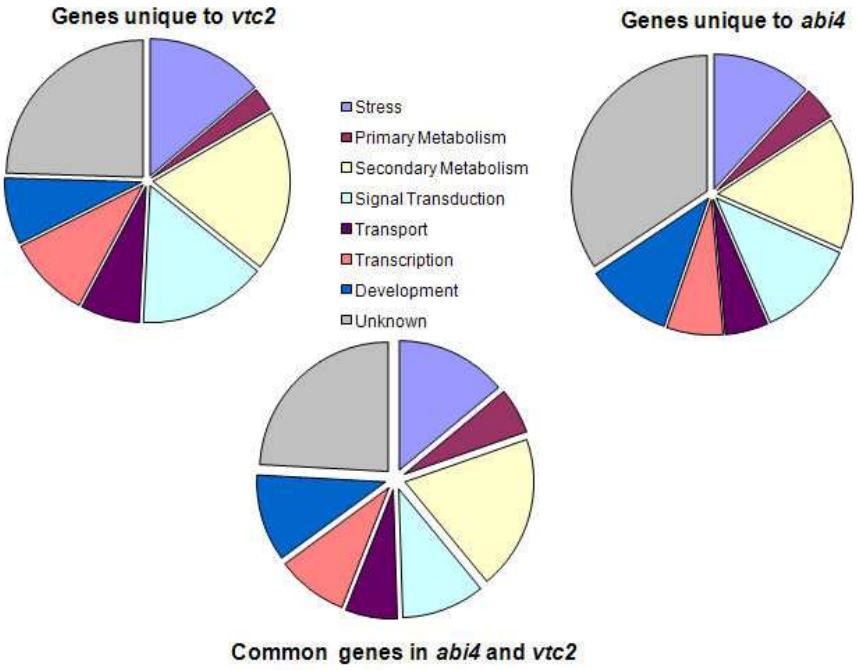

Figure 5.4 Functional groups of transcripts unique to *vtc2* and *abi4* relative to Col0, together with functional groups of identical transcripts in *abi4* and *vtc2* relative to Col0. Whole Arabidopsis rosettes were harvested following six weeks growth in controlled environment chambers, RNA extracted and microarray analysis performed with three to six biological replicates for each of the genotypes using Affymetrix ATH1 whole genome arrays. The analysis was performed at the Nottingham Arabidopsis Stock Centre (NASC) and the raw data analyzed using GeneSpring GX 11.00. To identify statistically significant gene expression in the mutant genotypes relative to the wild type a cutoff with p value <0.05 and log2 expression ratio ±1 was adopted. P values were calculated by asymptotic unpaired t test and subjected to multiple testing correction (Benjamini Hochberg false discover rate). Gene Ontology was used to assign the genes to functional categories.

5.2.4 Genes that are expressed in a similar manner in *abi4, vtc2* and *abi4vtc2* relative to Col0

5.2.4.1 Transcription factors

Of the transcripts that were repressed or induced in a similar manner in all three mutants (*abi4*, *vtc2* and *abi4vtc2*), a large number are either transcription factors or involved in signalling (Table 5.1; Appendix 8-10). This includes several WRKY transcription factors (40, 47, 53), NAC transcription factors (*anac036, anac090*) and *ZAT 10*, which are significantly over-expressed in all three mutants compared to Col0 (Table 5.1). Moreover several ethylene-responsive transcription factors such as *ERF104* were also highly expressed in the *abi4* and *vtc2* mutants relative to Col0 (Table 5.1; Appendix 8 and 9).

Accession	Expression ratio			Description
	abi4 vtc2	***vtc2***	***abi4***	
At4g23810	+6.09	+ 5.94	+ 4.91	WRKY53; transcription factor
At2g17040	+3.28	+ 3.92	+ 2.73	anac036 (Arabidopsis NAC domain containing protein 36); transcription factor
At1g80840	+3.17	+ 2.81	+ 3.04	WRKY40; transcription factor
At1g27730	+3.18	+ 3.84	+ 3.28	ZAT10__STZ (salt tolerance zinc finger); transcription factor
At5g22380	na	+ 2.85	+ 3.19	anac090 (Arabidopsis NAC domain
At4g01720	na	+ 2.20	+ 2.37	WRKY47; transcription factor
At1g74930	na	+ 3.01	+ 2.77	ORA47; transcription factor
At5g61600	na	+ 2.14	+ 2.27	ERF104; ethylene-responsive element-binding family protein
At5g62165	-3.21	- 2.83	- 2.69	AGL42 (AGAMOUS LIKE 42); transcription

Table 5.1 Transcripts encoding transcription factors commonly expressed in *abi4vtc2, vtc2* and *abi4* relative to Col0. Whole Arabidopsis rosettes were harvested following six weeks growth in controlled environment chambers, RNA extracted and microarray analysis performed with three to six biological replicates for each of the genotypes using Affymetrix ATH1 whole genome arrays. To identify statistically significant gene expression in the mutant genotypes relative to the wild type a cut-off with p value <0.05 and log2 expression ratio ±1 was adopted. P values were calculated by asymptotic unpaired t test and subjected to multiple testing correction (Benjamini Hochberg false discover rate). na (not applicable) indicates that the corresponding transcript did not fulfil the adopted statistical criteria for differential expression. All ratios are expressed on a linear scale relative to Col0. Genes were attributed to this category based on Gene Ontology (GO). GO classification was made according to Molecular Function (sequence-specific DNA binding transcription factor activity) as found at The Arabidopsis Information Resource (TAIR; www.arabidopsis.org).

5.2.4.2 Defence-related genes

Transcripts encoding a large number of defence-related proteins were increased in abundance in the mutants compared to Col0 (Table 5.2). The list shown in Table 5.2 includes transcripts involved in both SA-dependent and SA-independent defence responses. Among the most up-regulated genes in all genotypes were the SA-responsive *PR1*, *PR2*, *PR4*, and *PR5* transcripts (Fig. 5.5). Important components of the SA-defence pathways such as *PAD4, EDS1*, and *EDS5* were also induced in *vtc2* and *abi4vtc2*. The expression of *ISOCHORISMATE SYNTHASEI (ICS1*), which is required for the pathogen-induced biosynthesis of SA, was also up-regulated in the *abi4vtc2* double mutant. Interestingly, the marker genes for the JA/ET defence signalling pathways, *PDF1.2* and *PDF1.2b*, were induced in all genotypes. The abundance of *PDF1.2* and *PDF1.2b* transcripts in the mutants was even higher than some of the *PR* genes in *abi4* and *abi4vtc2* (Fig. 5.5). However, *PDF1.2* and *PDF1.2b* transcripts were lower in *vtc2* than in *abi4* or *abi4vtc2* (Fig. 5.5). Similarly, ET-responsive transcripts, such as the member of the ET response factor subfamily B-3 of the ERF/AP2 transcription factor family (*ATERF-2*) were induced in all mutants (Appendix 8-10). ATERF-2 is a positive regulator of JA-responsive genes, such as *PDF1.2* (Brown et al., 2003).

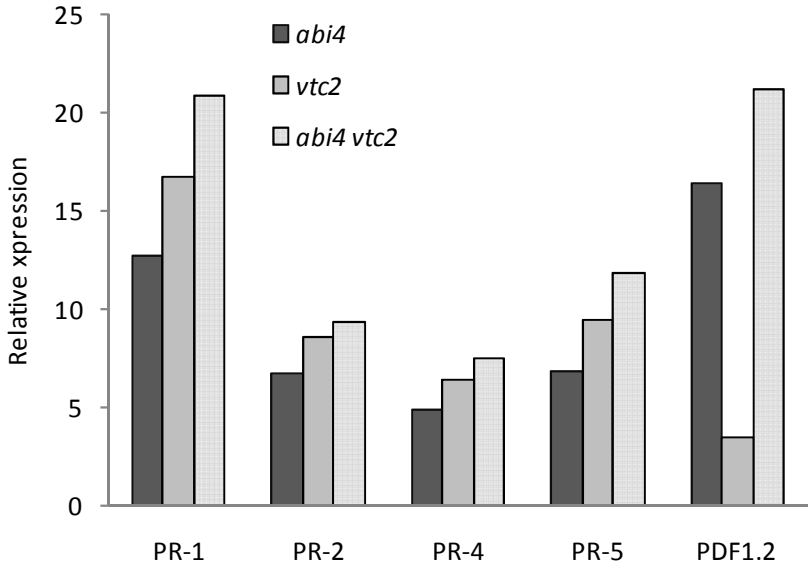

Figure 5.5 Expression of transcripts encoding defense related proteins responsive to SA (*PR1*, *PR2*, *PR4*, *PR5*) or JA/ET (*PDF1.2*) in *abi4*, *vtc2* and *abi4vtc2* Arabidopsis mutants relative to Col0. Whole Arabidopsis rosettes were harvested following six weeks growth in controlled environment chambers, RNA extracted and microarray analysis performed with three to six biological replicates for each of the genotypes using Affymetrix ATH1 whole genome arrays. The analysis was performed at the Nottingham Arabidopsis Stock Centre (NASC) and the raw data analyzed using GeneSpring GX 11.00. To identify statistically significant gene expression in the mutant genotypes relative to the wild type a cutoff with p value <0.05 and log2 expression ratio ±1 was adopted. P values were calculated by asymptotic unpaired t test and subjected to multiple testing correction (Benjamini Hochberg false discover rate).

Table 5.2 Differentially expressed transcripts in *abi4, vtc2* and *abi4vtc2* mutants encoding defence-related proteins. Whole Arabidopsis rosettes were harvested following six weeks growth in controlled environment chambers, RNA extracted and microarray analysis performed with three to six biological replicates for each of the genotypes using Affymetrix ATH1 whole genome arrays. The analysis was performed at the Nottingham Arabidopsis Stock Centre (NASC) and the raw data analyzed using GeneSpring GX 11.00. To identify statistically significant gene expression in the mutant genotypes relative to the wild type a cutoff with p value <0.05 and log2 expression ratio ±1 was adopted. P values were calculated by asymptotic unpaired t test and subjected to multiple testing correction (Benjamini Hochberg false discover rate). na (not applicable) indicates that the corresponding transcript did not fulfil the adopted statistical criteria for differential expression. All ratios are expressed on a linear scale relative to Col0. Genes were attributed to this category based on their involvement in pathogen infection and hormones associated defence signalling pathways.

Accession	**Expression ratio**			**Description**
	abi4	***vtc2***	***abi4vtc2***	
At2g14610	+12.8	+16.8	+20.9	PR1; PATHOGENESIS-RELATED GENE 1
At3g57260	+6.8	+8.6	+9.4	PR2; BETA-1,3-GLUCANASE 2
At3g04720	+4.9	+6.5	+7.5	PR4; PATHOGENESIS-RELATED 4
At1g75040	+6.8	+9.5	+11.8	PR5; PATHOGENESIS-RELATED GENE 5
At3g52430	na	+2.7	+2.7	PAD4; PHYTOALEXIN DEFICIENT 4
At3g48090	na	na	+2.4	EDS1; enhanced disease susceptibility 1
At4g39030	na	na	+2.3	EDS5; ENHANCED DISEASE SUSCEPTIBILITY 5
At2g13810	+2.11	+3.0	+5.1	ALD1; AGD2-LIKE DEFENSE RESPONSE PROTEIN1
At5g67160	-2.2	-2.2	-2.4	EPS1; ENHANCED PSEUDOMONAS SUSCEPTIBILTY 1
At1g74710	na	na	+2.4	ICS1; isochorismate synthase 1

At5g40780	+3.5	+6.3	+6.1	LHT1; amino acid transmembrane transporter
At5g24530	+2.0	+4.7	+3.7	DMR6; DOWNY MILDEW RESISTANT 6
At2g04450	+3.6	+3.8	+7.4	ATNUDT6; Arabidopsis thaliana Nudix hydrolase homolog 6
At5g55170	+2.7	+4.4	+5.0	SUMO3; SMALL UBIQUITIN-LIKE MODIFIER 3
At5g44420	+16.4	+3.5	+21.2	PDF1.2A__PDF1.2
At2g26020	+14.1	+4.7	+13.0	PDF1.2b; plant defensin 1.2b
At1g19640	-2.2	-2.6	-3.3	JMT (JASMONIC ACID CARBOXYL METHYLTRANSFERASE
At4g39090	+2.8	+2.9	+3.0	RD19; RESPONSIVE TO DEHYDRATION 19
At1g05570	+2.3	+3.1	+3.5	CALS1; CALLOSE SYNTHASE 1
At3g11840	+2.0	+3.5	+3.7	PUB24; PLANT U-BOX 24
At2g19190	+2.1	+2.8	+3.3	FRK1; FLG22-INDUCED RECEPTOR-LIKE KINASE 1
At5g10380	+2.5	+3.3	+3.0	RING1; protein binding / ubiquitin-protein ligase/ zinc ion binding
At4g39800	-2.6	-3.1	-2.8	MIPS1;MYO-INOSITOL-1-PHOSTPATE SYNTHASE 1
At5g52640	-2.4	na	na	ATHSP90.1; HEAT SHOCK PROTEIN 90.1
At2g26150	-2.4	na	na	HSFA2__ATHSFA2; transcription factor
At3g16470	-3.1	na	na	JASMONATE RESPONSIVE 1
At3g19930	na	+2.1	na	STP4; SUGAR TRANSPORTER 4
At5g59320	na	-3.8	na	LTP3; LIPID TRANSFER PROTEIN 3)
At2g37710	na	+2.1	na	RLK (receptor lectin kinase); kinase
At4g01720	na	+2.2	na	WRKY47; transcription factor
At2g23560	na	-2.2	na	MES7; METHYL ESTERASE 7
At2g40000	na	+2.2	na	HSPRO2; ARABIDOPSIS ORTHOLOG OF SUGAR BEET HS1 PRO-1 2
At5g47120	na	+2.1	na	ATBI1; BAX INHIBITOR 1

At1g33950	na	na	+2.4	avirulence induced gene (AIG1) family protein
At1g15520	na	na	+4.6	PDR12; PLEIOTROPIC DRUG RESISTANCE 12
At4g31800	na	na	+3.6	WRKY18; transcription factor
At1g70690	na	na	+2.2	HWI1; HOPW1-1-INDUCED GENE1
At5g13320	na	na	+2.6	PBS3; AVRPPHB SUSCEPTIBLE 3
At5g58940	na	na	+2.1	CRCK1;CALMODULIN-BINDING RECEPTOR-LIKE CYTOPLASMIC KINASE 1
At3g25780	na	na	+2.7	AOC3; ALLENE OXIDE CYCLASE 3
At2g34500	na	na	+2.4	CYP710A1; cytochrome P450, family 710, subfamily A, polypeptide 1
At5g20480	na	na	+2.2	EFR; EF-TU RECEPTOR

5.2.4.3 Redox-related genes

Transcripts encoding components involved in cellular redox homeostasis were altered in abundance in *abi4*, *abi4vtc2*, and *vtc2* mutants. Many of the genes that showed differential expression in the mutants relative to Col0 encode proteins that can be classed as "glutathione-associated gene expression" (Mhamdi et al., 2010; Table 5.3).

Two transcripts encoding tau-type glutathione peroxidases (GSTtau4 and GSTtau10) and two encoding glutaredoxins (GRX13 and GRX480) were increased in the *vtc2, abi4* and *abi4vtc2* double mutants relative to Col0 (Table 5.3). GSTtau8 transcripts were also increased only in *abi4* but not in the *vtc2* mutants or the *abi4vtc2* double mutants (Table 5.3). The expression of more genes encoding glutathione S-transferases (GSTs), glutathione peroxidases and glutaredoxins was changed relative to Col0 in the *vtc2* mutant than in *abi4* (Table 5.3). Moreover, the altered expression of these genes was retained in the *abi4vtc2* double mutants (Table 5.3).

The transcript levels of *VTC2* were lower not only in the plants carrying the *vtc2-1* mutation but also in the *abi4* mutants. Another gene, *VTC5*, encoding an enzyme with substrate specificity and enzymatic properties similar to VTC2 was similarly repressed in *abi4* and *abi4vtc2*. The abundance of transcripts encoding L-ascorbate oxidase (*At5g21100*) was decreased in all genotypes.

Accession	Expression ratio			Description
	abi4	*vtc2*	*abi4vtc2*	
At2g02390	na	+ 2.0	+ 2.2	Glutathione S-transferase zeta 1
At2g29470	na	+ 3.6	na	Glutathione S-transferase tau 3
At2g29460	+ 2.8	+ 2.4	+ 3.1	Glutathione S-transferase tau 4
At2g29450	na	- 2.0	- 2.2	Glutathione S-transferase tau 5
At2g29440	na	- 2.7	- 2.5	Glutathione S-transferase tau 6
At3g09270	+ 3.8	na	na	Glutathione S-transferase tau 8
At1g74590	+ 2.9	+ 4.0	+ 5.9	Glutathione S-transferase tau 10
At1g69930	na	na	+ 2.1	Glutathione S-transferase tau 11
At1g10370	na	- 4.0	- 4.0	Gutathione S-transferase tau 17
At4g02520	na	+ 5.4	+ 4.9	Glutathione S-transferase phi 2
At2g47730	na	- 13.9	- 6.7	Glutathione S-transferase phi 8
At1g03850	+ 2.3	+ 5.4	+ 8.2	Glutaredoxin, GRX13
At1g28480	+ 2.0	+ 2.6	+ 3.0	GRX480
At4g11600	na	na	+ 2.3	Glutathione peroxidase 6
At4g31870	na	na	-2.7	Glutathione peroxidase 7
At1g63460	na	+ 2.0	na	Glutathione peroxidase 8

Table 5.3 Differentially expressed transcripts in *abi4*, *vtc2* and *abi4vtc2* mutants encoding glutathione s-transferases, glutaredoxins (GRX) and glutathione peroxidases. Whole Arabidopsis rosettes were harvested following six weeks growth in controlled environment chambers, RNA extracted and microarray analysis performed with three to six biological replicates for each of the genotypes using Affymetrix ATH1 whole genome arrays. The analysis was performed at the Nottingham Arabidopsis Stock Centre (NASC) and the raw data analyzed using GeneSpring GX 11.00. To identify statistically significant gene expression in the mutant genotypes relative to the wild type a cutoff with p value <0.05 and log2 expression ratio ±1 was adopted. P values were calculated by asymptotic unpaired t test and subjected to multiple testing correction (Benjamini Hochberg false discover rate). na (not applicable) indicates that the corresponding transcript did not fulfil the adopted statistical criteria for differential expression. All ratios are expressed on a linear scale relative to Col0.

5.2.4.4 Cell suicide genes

A number of senescence-related transcripts were enhanced in the three genotypes (Table 5.4). For example, a gene encoding a type I metacaspase (*AtMC2*), which negatively regulates cell death (Coll et al., 2010), was constitutively induced in the *abi4*, *vtc2* and *abi4vtc2* mutants relative to Col0. The expression of *SYNTAXIN OF PLANTS 122* (*SYP122*), which is a member of the SNARE super-family of proteins, was induced in all mutants. SYP122, together with SYP121, acts as a negative regulator of programmed cell death in SA-, JA-, and ET-signalling pathways (Zhang et al., 2007). The expression of *STP13* (*SUGAR TRANSPORT PROTEIN 13*), which encodes a high affinity hexose-specific H^+-symporter, was strongly induced in all mutants. *STP13* is up-regulated during programmed cell death and its over-expression improves nitrogen use efficiency (Norholm et al., 2006; Schofield et al., 2009). Similarly, a gene encoding glutamine synthetase (*GSR1*), which may also be involved in nitrogen remobilization (Li et al., 2006) was up-regulated in the three mutants relative to the Col0.

Table 5.4 Differentially expressed senescence-related transcripts in *abi4*, *vtc2* and *abi4vtc2* mutants. Whole Arabidopsis rosettes were harvested following six weeks growth in controlled environment chambers, RNA extracted and microarray analysis performed with three to six biological replicates for each of the genotypes using Affymetrix ATH1 whole genome arrays. The analysis was performed at the Nottingham Arabidopsis Stock Centre (NASC) and the raw data analyzed using GeneSpring GX 11.00. To identify statistically significant gene expression in the mutant genotypes relative to the wild type a cutoff with p value <0.05 and log2 expression ratio ±1 was adopted. P values were calculated by asymptotic unpaired t test and subjected to multiple testing correction (Benjamini Hochberg false discover rate). na (not applicable) indicates that the corresponding transcript did not fulfil the adopted statistical criteria for differential expression. All ratios are expressed on a linear scale relative to Col0. Genes were attributed to this category based on either literature evidence or Gene Ontology (GO). GO classification was made according to Biological Process (aging or apoptotic process) as found at The Arabidopsis Information Resource (TAIR; www.arabidopsis.org).

Accession	**Expression ratio**			**Description**
	abi4	***vtc2***	***abi4vtc2***	
At4g25110	+2.9	+4.2	+3.2	AtMC2 (metacaspase 2); cysteine-type endopeptidase
At3g52400	+2.3	+3.8	+3.7	SYP122 (SYNTAXIN OF PLANTS 122)
At5g37600	+2.7	+4.1	+4.4	ATGSR1; copper ion binding / glutamate-ammonia ligase
At1g64780	-3.8	+2.2	+2.3	ATAMT1;2 (AMMONIUM TRANSPORTER 1;2)
At5g26340	+5.3	+8.0	+10.8	STP13; carbohydrate transmembrane transporter
At5g47060	+2.2	na	+2.3	senescence-associated protein-related

At1g22160	-2.5	-2.1	-2.3	senescence-associated protein-related
At1g08230	na	-2.1	na	GAT1; amino acid transporter family protein
At4g02380	na	+3.1	+4.3	SAG21;SENESCENCE-ASSOCIATED GENE
At1g78020	na	-2.3	-2.1	senescence-associated protein-related
At1g53885	na	+2.4	na	senescence-associated protein-related
At3g02040	na	na	+2.12	SRG3; senescence-related gene 3

5.2.4.5 Starch breakdown genes

The abundance of transcripts encoding genes involved in starch breakdown and maltose metabolism was uniformly altered in the leaves all mutants relative to the wild type (Table 5.5). *Starch Excess 4* (*SEX4*), which encodes a phosphoglucan phosphatase required for starch breakdown (Niittylä et al., 2006), was up-regulated in the three mutant genotypes. The abundance of transcripts encoding *ISA3* (*ISOAMYLASE 3*), which is also involved in starch breakdown (Delatte et al., 2006), was also increased in the mutants relative to the wild type, as were mRNAs encoding a UDP-glycosyltransferase superfamily protein. However, the *BETA-AMYLASE 3* (*BAM3*) mRNAS were decreased relative to the wild type. BAM3 is a chloroplast-localized beta-amylase that is involved in starch breakdown (Fulton et al., 2008).

Maltose, which produced from starch, is the predominant form of carbon exported from the chloroplasts during the night (Weise et al., 2011). The expression levels of two genes encoding proteins involved in maltose metabolism *DISPROPORTIONATING ENZYMES 1* and *2 (DPE1* and *DPE2*) were increased in the mutants relative to the wild type. DPE2 is a cytosolic protein with transglucosidase and amylomaltase activities (Chia et al., 2004). It plays an essential role in converting starch to sucrose in leaves at night by metabolizing maltose and water-soluble heteroglycans. Similarly, DPE1 is a chloroplastic enzyme with maltotriose-metabolizing and α-1,4-glucanotransferase activities (Critchley et al., 2001).

Accession	Expression ratio			Description
	abi4	*vtc2*	*abi4vtc2*	
At3g52180	+2.1	+2.1	+2.5	SEX4; STARCH-EXCESS 4
At4g09020	+4.8	+3.9	+5.8	ISA3; ISOAMYLASE 3
At1g32900	-4.2	-4.5	-4.4	starch synthase, putative
At2g40840	+2.1	+2.3	+2.4	DPE2; DISPROPORTIONATING ENZYME 2
At5g64860	+4.9	+3.7	+4.8	DPE1; DISPROPORTIONATING ENZYME 1
At4g17090	-2.5	-2.2	-2.7	BAM3; CHLOROPLAST BETA-AMYLASE

Table 5.5 Differentially expressed transcripts in *abi4*, *vtc2* and *abi4vtc2* mutants encoding proteins involved in starch breakdown and maltose metabolism. Whole Arabidopsis rosettes were harvested following six weeks growth in controlled environment chambers, RNA extracted and microarray analysis performed with three to six biological replicates for each of the genotypes using Affymetrix ATH1 whole genome arrays. The analysis was performed at the Nottingham Arabidopsis Stock Centre (NASC) and the raw data analyzed using GeneSpring GX 11.00. To identify statistically significant gene expression in the mutant genotypes relative to the wild type a cutoff with p value <0.05 and log2 expression ratio ±1 was adopted. P values were calculated by asymptotic unpaired t test and subjected to multiple testing correction (Benjamini Hochberg false discover rate). na (not applicable) indicates that the corresponding transcript did not fulfil the adopted statistical criteria for differential expression. All ratios are expressed on a linear scale relative to Col0. Genes were attributed to this category based on either literature evidence or Gene Ontology (GO). GO classification was made according to Biological Process (starch catabolic process or maltose catabolic process) as found at The Arabidopsis Information Resource (TAIR; www.arabidopsis.org).

5.2.4.5 Flowering time, circadian rhythms and light signalling genes

Genes that are involved in the control of flowering time, circadian rhythms and light signalling were differentially expressed in the *abi4*, *vtc2*, and *abi4vtc2* mutants relative to the wild type (Table 5.6).

For example, mRNAs encoding *CONSTANS-like 2* (homologous to the flowering-time gene CO) were decreased in all three mutants. Moreover, *NUCLEAR FACTOR Y, SUBUNIT B2* (*NF-YB2*) transcripts that encode a transcription factor regulating flowering time (Kumimoto et al., 2008) was also repressed as were mRNAs encoding *CIRCADIAN CLOCK ASSOCIATED 1*, which is a major regulator of the circadian rhythm and is involved in phytochrome control (Lu et al., 2009). Similarly, *WNK6* transcripts encoding a protein kinase were also repressed (Table 5.6). The transcription of *WNK6* is under circadian control (Nakamichi et al., 2002) as is *Glycine-rich RNA-binding protein 8 (GRP8). GRP8* transcripts were increased in the three mutants. Interestingly, the protein product of *GRP8* is a substrate of a type III *P. syringae* effector and affects flowering time (Fu et al., 2007).

The expression of *HY5-HOMOLOG (HYH)*, which is involved in phytochrome (phy) A signalling (Desai and Hu, 2008), was decreased in all three mutants (Table 5.6), as was *cryptochrome 3 (CRY3)*, which encodes a photoreceptor that recognizes and repairs UV lesions of DNA (Pokorny et al., 2008). However, *HEME OXYGENASE 3* (*HO3*) transcripts were increased relative to wild type. *HO3* encodes a member of the heme oxygenase family that is involved in the biosynthesis of the phy chromophore (Emborg at al., 2006).

Table 5.6 Differentially-expressed transcripts in *abi4*, *vtc2* and *abi4vtc2* mutants encoding proteinss involved in controlling flowering time, circadian rhythms and light signalling. Whole Arabidopsis rosettes were harvested following six weeks growth in controlled environment chambers, RNA extracted and microarray analysis performed with three to six biological replicates for each of the genotypes using Affymetrix ATH1 whole genome arrays. The analysis was performed at the Nottingham Arabidopsis Stock Centre (NASC) and the raw data analyzed using GeneSpring GX 11.00. To identify statistically significant gene expression in the mutant genotypes relative to the wild type a cutoff with p value <0.05 and log2 expression ratio ±1 was adopted. P values were calculated by asymptotic unpaired t test and subjected to multiple testing correction (Benjamini Hochberg false discover rate). na (not applicable) indicates that the corresponding transcript did not fulfil the adopted statistical criteria for differential expression. All ratios are expressed on a linear scale relative to Col0. Genes were attributed to this category based on either literature evidence or Gene Ontology (GO). GO classification was made according to Biological Process (regulation of flower development, circadian rhythm, flowering, response to UV-B, response to red light, response to blue light) as found at The Arabidopsis Information Resource (TAIR; www.arabidopsis.org).

Accession	**Expression ratio**			**Description**
	abi4	***vtc2***	***abi4vtc2***	
At3g02380	-3.9	-5.8	-6.1	COL2; constans-like 2
At5g47640	-2.1	-2.1	-2.3	NF-YB2; NUCLEAR FACTOR Y, SUBUNIT B2
At2g46830	-4.5	-4.8	-6.4	CCA1;CIRCADIAN CLOCK ASSOCIATED1
At3g18750	-2.3	-2.7	-2.8	WNK6 (WITH NO K (=LYSINE) 6)
At4g39260	+2.1	+2.3	+2.9	GR-RBP8; RNA binding / nucleic acid binding
At3g17609	-3.1	-3.5	-3.3	HYH; HY5-HOMOLOG
At5g24850	-2.6	-2.1	-3.3	CRY3; cryptochrome 3

At1g69720	+2.7	+2.7	+2.8	ho3; HEME OXYGENASE 3
At5g24120	-3.0	-2.9	-4.4	SIG5; SIGMA FACTOR E
At1g78600	+4.0	+4.3	+4.2	LZF1; LIGHT-REGULATED ZINC FINGER PROTEIN 1
At1g10370	-2.5	-5.1	-4.9	ERD9; EARLY-RESPONSIVE TO
At4g27440	+4.6	+3.7	+4.6	PORB; PROTOCHLOROPHYLLIDE OXIDOREDUCTASE B
At4g13250	+2.2	+2.2	+2.1	NYC1; NON-YELLOW COLORING 1
At4g14690	-4.2	-3.9	-5.0	ELIP2; EARLY LIGHT-INDUCIBLE PROTEIN 2
At5g52570	-4.9	-6.7	-6.0	BETA-OHASE 2; BETA-CAROTENE HYDROXYLASE 2
At3g15270	-2.2	na	na	SPL5; SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 5
At1g25560	na	+2.2	na	TEM1; TEMPRANILLO 1
At5g15850	na	-2.1	na	COL1; Constans-like 1
At1g76570	na	-2.0	na	chlorophyll A-B binding family protein
At3g04910	na	na	+2.0	WNK1; WITH NO LYSINE (K) 1
At5g62430	na	na	-2.3	CDF1; CYCLING DOF FACTOR 1
At2g30520	na	na	-2.2	RPT2; ROOT PHOTOTROPISM 2
At4g37590	na	na	-2.5	NPY5; NAKED PINS IN YUC MUTANTS 5
At4g17230	na	na	+2.0	SCL13; Scarecrow-like 13
At4g25420	na	na	-2.2	GA20ox1; gibberellin 20-oxidase
At3g05120	na	na	+2.4	GID1A; GA INSENSITIVE DWARF1A
At2g40100	na	na	-2.4	LHCB4.3; light harvesting complex PSII
At1g16720	na	na	-2.5	HCF173; high chlorophyll fluorescence phenotype 173

Transcripts that are involved in light signalling pathways such as *SIGMA FACTOR E* (*SIGE*) and *LIGHT-REGULATED ZINC FINGER PROTEIN 1 (LZF1*) were altered in expression in the mutants relative to Col0 (Table 5.6). However, while *SIGE* transcripts, which encode a sigma factor that is regulated by blue light and governs the expression of plastid genes (Onda et al., 2008) were decreased, the *LZF1* mRNAs, which encode a protein acting as a positive regulator of photomorphogenesis (Datta et al., 2008), were increased in the mutants relative to Col0. Similarly, *EARLY-RESPONSIVE TO DEHYDRATION 9* (*ERD9*) transcripts that encode a glutathione S-transferase, were decreased in the three mutants. ERD9 modulates Arabidopsis development via light signalling and affects ABA-mediated signal transduction pathways (Jiang et al., 2010; Table 5.6).

5.2.4.6 Plastid genes

The abundance of *PROTOCHLOROPHYLLIDE OXIDOREDUCTASE B* (*PORB*) transcripts was increased in all three mutants relative to Col0 (Table 5.6). *PORB* encodes a NADPH:protochlorophyllide oxidoreductase that catalyzes the light-dependent reduction of protochlorophyllide during the chlorophyll biosynthesis (Paddock et al., 2010). Similarly, *NON-YELLOW COLORING 1* (*NYC1*) mRNAs were increased in all three mutants relative to Col0 (Table 5.6). *NYC1* encodes a chlorophyll b reductase that is involved in the degradation of chlorophyll b and light harvesting complex II (Horie et al., 2009) However, *EARLY LIGHT-INDUCIBLE PROTEIN 2* (*ELIP2*) transcripts were decreased in all three mutants relative to Col0 (Table 5.6) as were *BETA-CAROTENE HYDROXYLASE 2* (*BETA-OHASE 2*) *mRNAs. ELIP2* modulates chlorophyll biosynthesis (Tzvetkova-Chevolleau et al., 2007) while *BETA-OHASE 2* encodes an enzyme converting β-carotene to zeaxanthin (Fiore et al., 2006). Taken together, these findings may suggest that the pathways of chloroplast to nucleus retrograde signalling are altered in the mutants.

5.2.5 Genes that are differentially expressed in the *abi4, vtc2* and *abi4vtc2* mutants

The above discussion has focussed on the considerable amount of overlap in the transcriptional re-programming that was observed in all three mutants relative to the wild type. However, each of the mutants showed suites of differentially expressed genes that were genotype-specific. The majority of genotype-specific transcripts observed in *abi4*, *vtc2*, and *abi4vtc2* were attributed to the categories shown in Fig. 5.5.

5.2.5.1 Genes which expression in vtc2 was reversed in the abi4vtc2 double mutants

During the characterisation of the *abi4, vtc2,* and *abi4vtc2* double mutants, it was found that the slow growth phenotype of *vtc2* was not observed in the *abi4* background (section 5.2.1). This suggested that the low ascorbate-dependent control of growth required a functional ABI4. One way to determine the mechanisms and pathways that were involved in the control of growth through ABI4, is to examine the transcripts patterns in *vtc2,* and search specifically for genes whose expression pattern in *vtc2* relative to Col0 is reversed in the *abi4vtc2* double mutants. This analysis revealed that only a small number of transcripts showed this trend (Table 5.7). For example, *ABI5 binding protein 3* (*AFP3*) was strongly repressed in the *abi4vtc2* double mutant but enhanced in *vtc2* (Table 5.7). Similarly, the expression of an AP2 type transcription factor of the DREB family, called *ORA47* (*At1g74930*) was enhanced in the *abi4* and *vtc2* single mutants relative to Col0 (Table 5.1) but it was repressed in the *abi4vtc2* double mutants (Table 5.7). Similarly, the expression of *histone H3* was strongly repressed in *abi4vtc2* double mutant but enhanced in *vtc2* (Table 5.7). Conversely, the expression of *SULPHUR DEFICIENCY-INDUCED 1* (*ATSDI1*) was repressed in *vtc2* but enhanced in the *abi4vtc2* double mutant. ATSDI1 is induced by sulphur starvation and is involved in regulation of sulphur homeostasis (Howarth et al., 2009).

Table 5.7 Transcripts with reversed expression in *abi4vtc2* relative to *vtc2*.

Whole Arabidopsis rosettes were harvested following six weeks growth in controlled environment chambers, RNA extracted and microarray analysis performed with three to six biological replicates for each of the genotypes using Affymetrix ATH1 whole genome arrays. The analysis was performed at the Nottingham Arabidopsis Stock Centre (NASC) and the raw data analyzed using GeneSpring GX 11.00. To identify statistically significant gene expression in the mutant genotypes relative to the wild type a cutoff with p value <0.05 and log2 expression ratio ±1 was adopted. P values were calculated by asymptotic unpaired t test and subjected to multiple testing correction (Benjamini Hochberg false discover rate). Genes which transcript abundance was enhanced in *vtc2* leaves compared to Col0 while transcript abundance was repressed in *abi4vtc2* leaves relative to wild type controls are shown in the table. All ratios are expressed on a linear scale relative to the corresponding control.

Accession	**Expression ratio**		**Description**
	vtc2	***abi4vtc2***	
At3g48080	+ 4.39	- 1.94	Lipase class 3 family protein
At1g76960	+ 4.23	- 20.26	Unknown protein
At3g50480	+ 4.05	- 4.05	Homolog of RPW8 4 (HR4)
At3g48650	+ 3.55	- 1.57	Pseudogene, At14a-related protein, similar to At14a
At1g74930	+ 3.01	- 1.23	ORA47, DREB subfamily A-5 of ERF, AP2 transcription factor family
At5g36930	+ 1.86	- 3.25	Disease resistance protein (TIR-NBS-LRR class)
At1g66970	+ 1.75	- 2.19	SHV3-like 2 (SVL2)
At3g26230	+ 1.70	- 3.71	Putative cytochrome P450
At3g16420/ At3g16430	+ 1.43	- 1.19	Jacalin-related lectin 30 (JAL30), PYK10-binding protein 1 (PBP1)/Jacalin-related lectin 31 (JAL31)
At1g80960	+ 1.36	- 2.64	F-box protein-related
At3g43740	+ 1.24	- 8.57	Leucine-rich repeat family protein
At5g44580	+ 1.24	- 2.95	Unknown protein

At3g47250	+ 1.23	- 19.02	Unknown protein
At3g29575	+ 1.94	- 1.61	ABI5 binding protein 3 (AFP3)
At5g39030	+ 1.18	- 3.16	Protein kinase family protein
At5g23010	+ 1.17	- 2.29	2-Isopropylmalate synthase 3 (IMS3), Methylthioalkylmalate synthase 1 (MAM1)
258246_s_at	+ 1.16	- 2.10	Hypothetical protein similar to putative transposase of transposable element Ac GB:CAA25635 [Zea mays]
At1g28290	+ 1.15	- 3.56	AGP31, Arabinogalactan-protein 31 (AGP31)
At3g27360	+ 1.12	- 3.21	Histone H3
At3g46980	+ 1.10	- 2.48	Phosphate transporter 4.3
At3g46530	+ 1.07	- 2.61	Recognition of Peronospora parasitica 13 (RPP13)
At3g26290	+ 1.04	- 4.09	CYP71B26, Cytochrome P450, family 71, subfamily
At1g68050/ At5g53410	+ 1.04	- 3.37	Flavin-binding kelch repeat F box 1 (FKF1)/unknown protein
At5g28500	+ 1.03	- 2.90	Unknown protein
At5g24240	- 1.03	+ 4.54	Phosphatidylinositol 3- and 4-kinase family protein,
At3g52390	- 1.05	+ 1.75	TatD-related deoxyribonuclease family protein
At5g38260	- 1.08	+ 2.05	Serine/threonine protein kinase, putative
At3g49580	- 1.16	+ 2.86	Response to low sulfur 1 (LSU1)
At3g14990	- 1.29	+ 1.18	4-Methyl-5(β-hydroxyethyl)-thiazole monophosphate
At5g48850	- 2.10	+ 2.02	Homologous to the wheat sulphate deficiency-
At5g65080	- 3.23	+ 1.04	Agamous-like 68 (AGL68), MADS affecting flowering 5 (MAF5)

5.2.6 Metabolomic profiles of *abi4*, *vtc2* and *abi4vtc2* mutants

To further analyse the effects of the mutations, the targeted metabolomics approach was used to determine the levels of major amino acids, sugars, TCA intermediates and various fatty acids and long-chain alcohols (Fig. 5.7; Appendix 11). The *vtc2* and *abi4vtc2* leaves had significantly lower amounts of threonic acid (a breakdown product of ascorbate) that the wild type or the *abi4* leaves, consistent with the low levels of ascorbate in these mutants. The *vtc2* leaves also accumulated more sucrose than the other genotypes. Proline accumulated in the *abi4* leaves (Fig. 5.8; Appendix 11). Conversely, the cinnamic acid and lignoceric acid derivatives were lower in the *vtc2* and *abi4vtc2* leaves than the wild type. Two metabolites associated with phytol chain turnover (phytil methyl ether and phytil methyl ether 2) were significantly increased in all mutant lines relative to the wild type. Similarly, leaf glutamate contents were significantly lower in the leaves of mutant genotypes relative to those of the wild type. The levels of various fatty-acids and long-chain alcohols were altered in the leaves of the three genotypes (Fig. 5.8 and Appendix 11)

Succinate and fumarate, which are TCA cycle intermediates, were decreased only in *vtc2* leaves compared to the wild type (Fig. 5.8; Appendix 11). Interestingly, inositol levels were decreased in the *vtc2* and *abi4vtc2* leaves relative to the wild type.

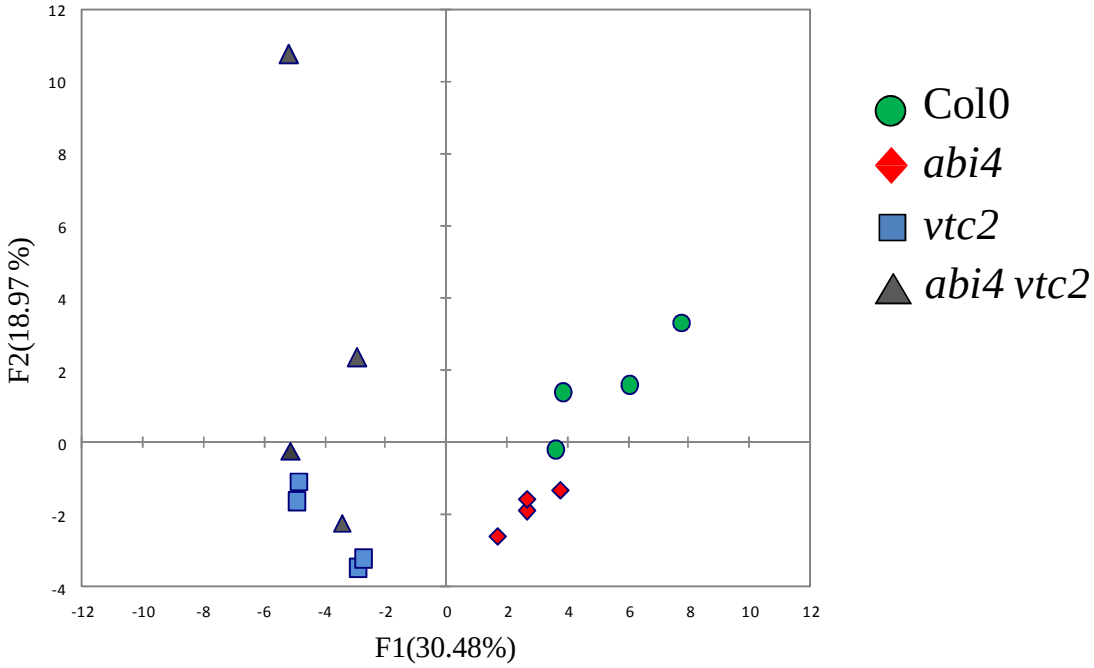

Figure 5.6 Principal component analysis of GC-MS profiles of Col0, *abi4*, *vtc2* and *abi4vtc2* Arabidopsis mutant plants in four biological replicates. The plot represents the first (PC1) and second components (PC2) of the metabolite profiles of rosette leaves from 4-week-old plants grown under controlled environmental conditions. The first and second components together explain 49.45% of the total variation between the genotypes.

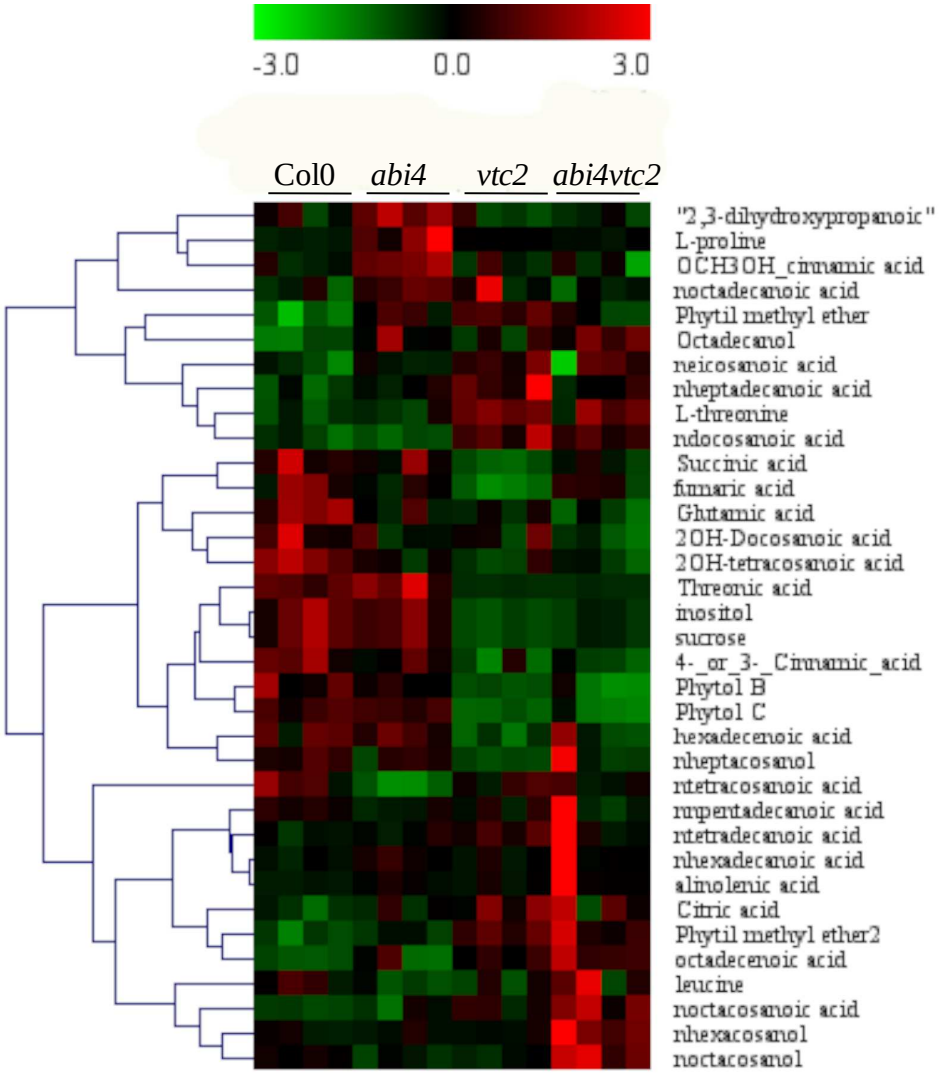

Figure 5.7 Hierarchical clustering analysis of metabolite profiles of Arabidopsis Col0, *abi4*, *vtc2*, and *abi4vtc2*. Plants were grown for four weeks under controlled environments and rosette leaves harvested from four replicate plants and immediately frozen on liquid nitrogen. Following lyophilisation, leaves were extracted and metabolites were derivatised for GC-MS. Only metabolites with significantly different contents between the studied genotypes according to one-way ANOVA ($p<0.05$) are displayed. Metabolite abundance is shown in red (high abundance) or green (low abundance). The figure shows reduced metabolite content in each of the four replicates relative to the centered mean across all samples. The metabolites were clustered according to Euclidean distances.

5.2.7 Constitutive expression of salicylic acid responsive *PR1* transcripts

Glutathione and thioredoxins participate in the regulation of the SA-dependent NONEXPRESSOR OF PATHOGENESISRELATED GENES 1 (NPR1) pathway (as discussed in Noctor et al., 2011). To determine the extent to which SA-dependent signalling pathways are modified in the *vtc2*, *abi4* and the *abi4vtc2* double mutants, the abundance of transcripts encoding PR1 was examined. The leaves of *vtc2*, *abi4* and *abi4*vtc2 genotypes had higher basal levels of *PR1* mRNAs than Col0 (Fig. 5.8).

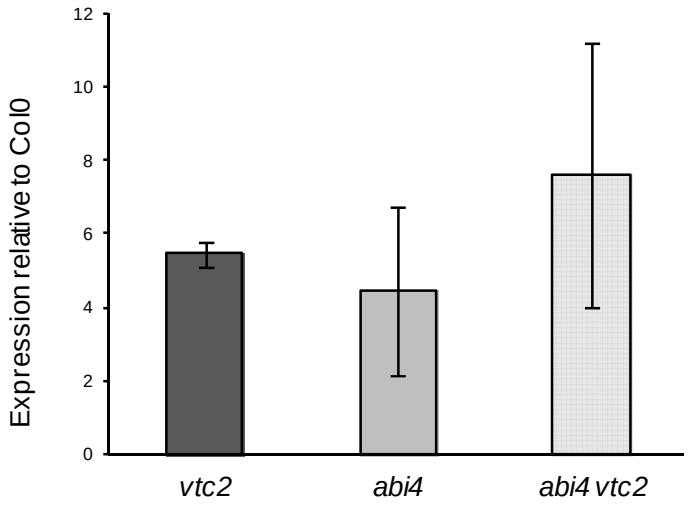

Figure 5.8 Expression of *PR1* transcripts in *abi4*, *vtc2*, and *abi4 vtc2* relative to Col0. All plants were grown under controlled environments for 4 weeks prior to analysis of gene expression. RNA was isolated from homogenized Arabidopsis rosettes and following reverse transcription used to quantify expression levels relative to wild type by qRT-PCR (3 biological replicates ± SE) according to the ΔΔCt method. *Actin-2* (*At3g18780*) was used as a reference gene in order to normalize the qRT-PCR data.

5.2.8. Effects of the mutations on flowering time

The flowering time of the mutants was altered relative to Col0 when grown under short day conditions, with *abi4* and *abi4vtc2* mutants flowering before the wild type and *vtc2* mutants (10h daylight; Fig. 5.11).

The flowering time of the *vtc2* mutant was delayed in comparison to Col0, as reported previously by Pavet et al. (2005). This observation may be related to the altered expression of genes that encode proteins that are involved in the control of flowering time and circadian rhythms (Table 5.6) in the mutant relative to the wild type, as discussed above.

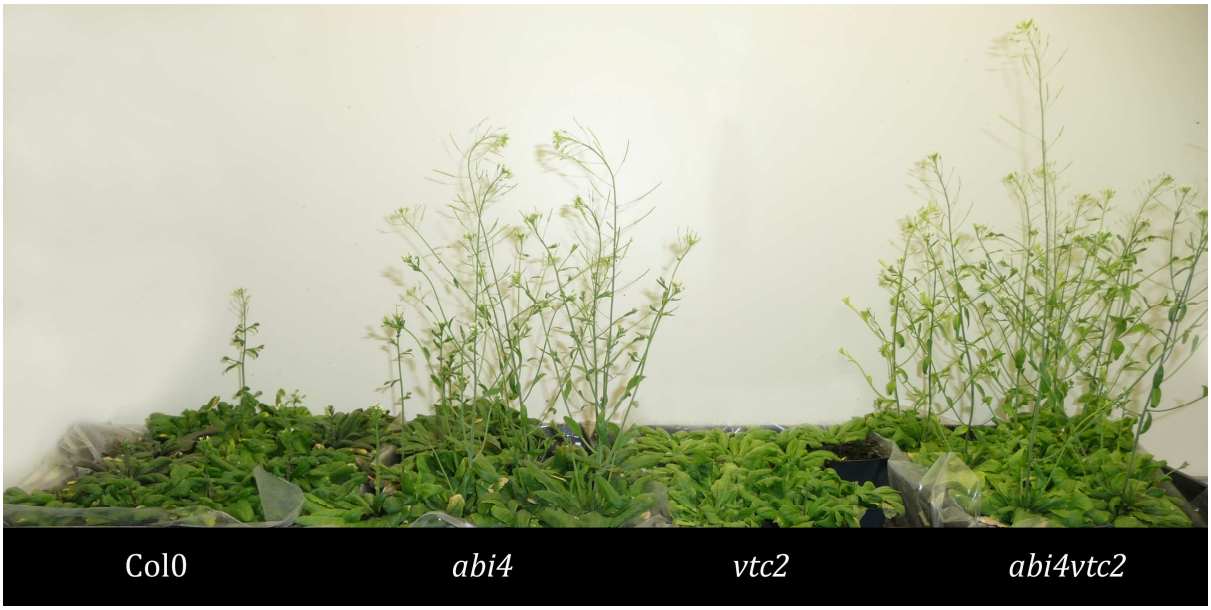

Figure 5.9 Flowering phenotype of *abi4*, *vtc2*, *abi4vtc2* and Col0 grown under short day conditions (10/14 light/dark) for 7.5 weeks.

5.2.9 Gibberrelic acid metabolism in the *abi4, vtc2* and *abi4vtc2* mutants

Gibberellic acid (GA) is involved in the control of flowering and in organ development (Liu et al., 2010). GA can act antagonistically to ABA in the control of organ development (Liu et al., 2010). The *vtc2*, *abi4* and *abi4vtc2* mutants accumulate more ABA than Col0 (Kerchev et al., 2011). The following experiments were therefore undertaken to determine whether GA metabolism was altered in the mutant genotypes relative to Col0.

The transcript abundance of several genes involved in GA biosynthesis, such as *GA20ox1*, is feedback regulated by bio-active GAs and thus the abundance of transcripts like GA20ox1 can be used as an indicator of GA levels in different tissues (Phillips et al., 1995; Xu et al., 1999). The abundance of *GA20ox1*, *GA20ox2*, *GA20ox3* and *GA3ox1* transcripts, which encode proteins involved in GA synthesis, and of *GA2ox2* and *GA2ox6* mRNAs that are encode proteins of GA degradation was comparable in all genotypes (Fig. 5.10). However, *GA2ox2* transcripts were higher in the *vtc2* mutants than Col0 (Fig. 5.10).

The abundance of the various GA forms was compared in the *vtc2*, *abi4* and *abi4vtc2* mutants (Table 5.8). The abundance of bioactive GAs (GA_1 and GA_4) and that of other GA forms was similar in all genotypes (Table 5.8).

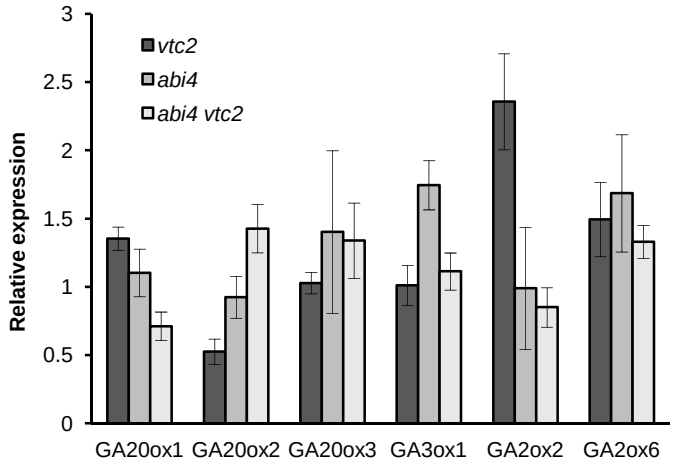

Figure 5.10 Expression profiles of GA oxidases involved in the synthesis (*GA20ox1*, *GA20ox2*, *GA20ox3* and *GA3ox1*) or degradation (*GA2ox2* and *GA2ox6*) of GAs in the leaves of *abi4*, *vtc2*, *abi4vtc2* mutants. All plants were grown under controlled environments for 4 weeks prior to analysis of gene expression. RNA was isolated from homogenized Arabidopsis rosettes and following reverse transcription used to quantify expression levels relative to wild type by qRT-PCR (3 biological replicates ± SE) according to the ΔΔCt method. *Actin-2* (*At3g18780*) was used as a reference gene in order to normalize the qRT-PCR data.

	GA_1	GA_{19}	GA_{44}	GA_{53}	GA_4	GA_{34}	GA_9	GA_{51}	GA_{24}
Col 0	0.3 (0.1)	2.9 (0.0)	0.7 (0.4)	9.1 (0.5)	2.5 (1.8)	3.1 (0.7)	1.2 (0.8)[a]	1.5 (1.6)	20.6 (6.0)
abi4	0.4 (0.1)	2.6 (0.6)	0.5 (0.3)	9.4 (1.9)	2.2 (1.8)	2.9 (0.8)	2.7 (0.4)[a]	1.5 (1.1)	24.3 (4.7)
vtc2	0.5 (0.2)	2.1 (0.2)	0.5 (0.2)	5.4 (1.0)	2.9 (1.9)	2.4 (0.6)	0.5 (0.1)	2.1 (1.2)	20.4 (2.7)
abi4 vtc2	0.4 (0.2)	3.1 (0.5)	1.0 (0.2)	10.1 (0.6)	4.4 (2.5)	3.0 (0.7)	1.3 (1.4)	1.2 (1.1)	24.0 (3.5)

Table 5.8 Concentrations of GAs in ng g^{-1} dry weight. All plants were grown under controlled environments for 4 weeks prior to analysis of gibberellin contents. Freeze-dried Arabidopsis rosettes (Col0, *abi4*, *vtc2* and *abi4vtc2*) were ground to powder and aliquots derivatized and analysed by GS-MS. Values are means of 3-5 biological replicates (±SD), except where indicated. a) Mean of two biological replicates. Gibberellins A_8, A_{20} and A_{29} were also analysed, but were below the level of detection in all samples.

5.3 Discussion

5.3.1 The slow growth phenotype of *vtc2* is modulated by ABI4

The *vtc2* mutants have constituvely lower levels of ascorbate relative to the wild type but they do not show symptoms of oxidative stress under optimal growth conditions (Veljovic-Jovanovic et al., 2001; Colville and Smirnoff, 2008). The reduced overall abundance of asorbate can be a limiting factor for the growth of *vtc2* as it is required as a cofactor in various metabolic pathways such as GA biosynthesis (Arrigoni and de Tullio, 2000). The in-depth analysis of the *vtc2*, *abi4* and *abi4vtc2* mutants demonstrates unequivocally that the low levels of ascorbic acid are not a limitation for normal growth and the ascorbate-dependent control of plant growth requires the ABI4 transcription factor. In the absence of a functional ABI4 transcription factor the ascorbate-dependent slow growth phenotype is not expressed. Thus, like other ABA signalling components such as ABI1 and ABI2, which have long been known to function in stress signalling cascades involving ROS as second messengers (Allen et al., 1999), the data presented here also implicate ABI4 in redox signalling.

Altered transcripts levels of genes related to ABA biosynthesis and signaling have been shown in the *vtc* mutants (Pastori et al., 2003). Moreover, ABA accumulates in these mutants suggesting that ABA-dependent signaling pathways play an important role in the execution of the growth phenotype of *vtc2*. The restoration of the wild type growth rates in the *abi4 vtc2* mutant suggests that an intact ABA signaling pathway is required for the expression of the slow growth phenotype. The absence of a functional ABI4 in the double mutant may result in ABA insensitivity and hence limit the impact on growth. However *abi4* was initially isolated in screens for sugar insensitive mutants and since then many roles in carbon and nitrogen signaling, ABA-dependent pathways, retrograde signaling, and defense has been shown (Kallif et al., 2007; Koussevitzky et al., 2007; Giraud et al., 2009). The diverse roles of ABI4 suggest that other functions

apart from its role in ABA mediated signaling should be considered when discussing its growth reverting effect in the *abi4 vtc2* mutants.

5.3.2 Ascorbic acid and sugar signalling pathways are tightly interconnected

High levels of sucrose and glucose have been shown to suppress the germination of wild type but not *abi4* seeds (Arenas-Huertero et al., 2000). The low levels of ascorbic acid in the *abi4* background abolished the sugar insensitive germination implying that the signalling pathways utilizing ABI4 and ascorbic acid are highly intertwined and normal levels of ascorbic acid are required for the sugar insensitive phenotype of *abi4*.

Whereas, high sucrose negatively affects photosynthesis and ascorbate levels in wild type Arabidopsis, theses effects are not observed in *abi4* plants (Yabuta et al., 2007). Ascorbate levels regulate the expression of nuclear encoded photosynthetic genes such as those encoding light harvesting proteins of PSI and PSII, and stromal enzymes including glucose 6-phosphate dehydrogenase (Kiddle et al., 2003). Interestingly, sucrose levels were elevated in the *vtc2* mutant, further suggesting that ascorbate play a role in sugar signalling pathways.

5.3.3 Role of ascorbate and ABI4 in plastid-derived retrograde signaling

ABI4 is part of an organelle to nucleus retrograde signaling cascade (Koussevitzy et al., 2007; Giraud et al., 2009; Kakizaki et al., 2009). The significant overlap between the transcriptome profiles of *abi4* and *vtc2* implies a common signaling pattern that is triggered under low ascorbate and in the absence of a functional ABI4. In line with this hypothesis, ascorbate has also been implicated in plastid-derived retrograde signaling (Kerchev et al., 2011). Although some components of the retrograde signaling cascades have been identified, there is no conclusive evidence about the actual chemicals involved in the communication between the chloroplasts and the nucleus (Galvez-Valdivieso and Mullineaux, 2010). Compounds that are partially synthesized in the

chloroplast have been proposed to act as retrograde signals. In Arabidopsis GSH synthesis is split between the chloroplasts and the cytosol (Pasternak et al., 2008). The activity of the chloroplastic enzyme γ-glutamylcysteine synthase (γ-ECS) involved in GSH biosynthesis is activated by oxidizing conditions *in vitro* (Jez et al., 2004). Environmental conditions leading to perturbation of the cellular redox status can stimulate GSH synthesis and its export to the cytosol thus acting as a retrograde redox signal (Mullineaux and Rausch, 2005). Elevated GSH levels were observed in the rosette leaves of *abi4*, *vtc2*, and *abi4 vtc2* together with altered expression of numerous transcripts that can be classified as 'glutathione-associated' gene expression (Mhamdi et al., 2010).

Interestingly, the three genotypes displayed altered sugar levels and differentially regulated genes involved in starch metabolism. The synchronized induction of transcripts encoding enzymes involved in starch breakdown and maltose metabolism implies enhanced rates of sugar export from the chloroplast since maltose is the predominant form of carbon exported from the chloroplast at night. This finding further implicates ascorbate and ABI4 in control of the plastid processes.

5.3.4 Ascorbic acid is involved in a complex interplay governing defense-related gene expression through hormonal pathways

A large number of transcripts associated with SA-dependent defence responses were more highly expressed in *abi4*, *vtc2* and the *abi4vtc2* double mutants than the wild type. For example, the mutant genotypes displayed higher basal levels of *PR1* transcripts than Col0, suggesting that SA signalling is primed in all the mutant genotypes.

In addition to increasing SA and SA signalling pathways, low ascorbate results in an increase in ABA levels in the Arabidopsis *vtc2* mutants relative to the wild type, together with alterations in gene expression patterns that are characteristic of altered

ABA signalling (Pastori et al., 2003; Kiddle et al., 2003). It is therefore likely that the low ascorbate-induced increases in leaf ABA signalling pathways also influence redox signalling pathways that might contribute to the ability of the plants to mount an effective defence against aphids.

The 'glutathione-associated' associated gene expression observed in the three mutant genotypes is characteristic of characteristic of H_2O_2-mediated signalling pathways that interact with JA, SA and inositol signalling pathways that control cell death (Mhamdi et al., 2010). Glutathione is considered to be a modulator of redox-triggered SA and JA signalling pathways (Noctor et al., 2011). For example, a suite of JA-responsive genes are repressed in the *gr1* mutants that lack the cytosolic/peroxisomal form of glutathione reductase (GR). Moreover, *gr1cat2* double mutants that lack both GR and the major leaf form of catalase show H_2O_2-induced expression of these and other JA-associated genes (Mhamdi et al., 2010).

The increased expression of *GSTtau4*, *GSTtau10* and *GRX13* was observed in all mutant genotypes relative to the wild type. The expression of *GSTU4* and *GRX13* has been linked to expression of JA/COI1-signaling pathways (Armengaud et al., 2010; Tamaoki et al., 2008). Taken together, these data suggest that low ascorbate and defective ABI4 signaling drive gene expression through common glutathione/SA/JA-mediated signaling pathways to regulate defense gene expression. The higher levels of glutathione observed in all the mutant genotypes would serve to repress JA signalling through SA-dependent induction of NPR1 and GRX480, both of which interact with TGA transcription factors. However the expression of marker genes characteristic of the JA/ET was also elevated implying that the JA signalling is uncoupled.

5.3.5 Modulation of flowering time

The flowering time of *vtc2*, *abi4*, and *abi4 vtc2* was altered in comparison to the wild type in the absence of significant differences in the levels of major bioactive GAs and GA related gene expression. Despite the fact that floral induction has been related to ascorbate and GA levels (Barth et al., 2006) and ascorbate biosynthesis is regulated by GA, the low levels of ascorbate and the lack of functional ABI4 most probably modulate flowering time via GA-independent pathways since GA signalling is only one of the signals controlling flowering time in Arabidopsis (Corbesier and Coupland, 2005).

For example, the expression of *CONSTANS-like 2*, homologous to the flowering-time gene *CO*, was repressed in the three genotypes (Ledger et al., 2001). Interestingly, the transcript levels of *EARLY-RESPONSIVE TO DEHYDRATION 9* (*ERD9*) that modulates Arabidopsis development via light signalling and affects ABA-mediated signal transduction pathways were induced in *abi4, vtc2,* and *abi4 vtc2* (Jiang et al., 2010). *ERD9* encodes glutathione S-transferase implying a complex interaction between light signalling, redox homeostasis, and hormonal pathways (Jiang et al., 2010).

Summary Chapter 5

- The *vitamin C defective 2* (*vtc2*) and *abscisic acid insensitive 4* (*abi4*) Arabidopsis mutants were phenotypically and biochemically characterised together with the corresponding double mutant *abi4vtc2*. The slow-growth phenotype of the *vtc2* mutant was abolished in the *abi4* background despite the low ascorbate contents. Moreover, wild type ascorbate levels were required for the sugar-insensitive germination of the *abi4* mutants.

- The transcriptome profiles of the *abi4*, *vtc2*, and *abi4vtc2* leaves were compared to Col0 at the 6-week stage using Affymetrix ATH1 whole genome arrays. There was a significant overlap between the transcriptomes of the mutant genotypes implying that low ascorbate and the absence of a functional ABI4 trigger common signalling patterns.
- The metabolite profiles of the *abi4*, *vtc2*, and *abi4vtc2* rosettes were analysed by GS-MS using a targeted metabolomics approach tailored to detect major amino acids, sugars, organic acids, fatty acids, long chain alcohols, and various non-polar metabolites.
- The constitutive expression of the SA-marker gene *PR1* in the *abi4*, *vtc2*, and *abi4vtc2* double mutants was evaluated relative to wild type using qRT-PCR.
- The *vtc2*, *abi4*, and *abi4vtc2* mutants displayed altered flowering phenotype at short day conditions (10/14 light/dark) with the *vtc2* mutant flowering later and *abi4* and *abi4vtc2* before the wild type plants.
- The expression profiles of several transcripts encoding GA oxidases involved in the synthesis and degradation of GAs were measured in the *abi4*, *vtc2*, and *abi4vtc2* mutants relative to Col0 together with the contents of various GAs. No differences were observed between the studied genotypes implying that GA homeostasis is not perturbed by the lack of ascorbate and functional ABI4.

Chapter 6. Effects of altered redox and ABA-signalling pathways on the transcriptional changes in the local and systemic *A. thaliana* leaves in response to aphid attack

6.1 Introduction

Understanding the molecular basis of the plant–aphid interaction requires the identification of genes and pathways that play an important role in aphid perception and defence. Numerous microarray experiments exploring various plant–aphid combinations identified large numbers of differentially regulated genes with potential role in modulating aphid success. Nevertheless, only a small number of these genes have been individually studied for their role in plant resistance against aphids (De Vos et al., 2007; Singh et al., 2011; Kusnierczyk et al., 2011), possibly partly due to the lack of high-throughput methods for screening for aphid resistance. The majority of the studies were conducted with Arabidopsis and involved the *Brassica*-feeding specialist *B. brassicae* and the broad generalist *M. persicae*. Only a few of the tested genes were shown to affect aphid reproduction or behaviour when the corresponding loss or gain of function lines was tested.

The main selection criteria for genes seem to follow the historical trend of identifying important components of the plant response triggered upon aphid attack. Not surprisingly genes implicated in SA-mediated signalling pathways were among the first and most extensively studied for their role in plant-aphid interactions since aphid attack has long been know to elicit hallmarks of SA defence response and induce the expression of *PR* genes (van der Westhuizen et al., 1998; Fidantsef et al., 1999). Moran and Thompson (2001) exploited Arabidopsis mutants affected in components of the SA

signalling pathway (*eds5* and *eds9*) but failed to observe significant changes in aphid reproduction on these mutants. Similarly, the lack of functional NPR1 in the *npr1* mutant background did not compromise aphid resistance in this study (Moron and Thompson, 2001). Given the importance of the NPR1 transcription factor in induction of SAR and *PR* gene expression, *npr1* mutants were further tested for aphid resistance in other studies but the reported result are contradictory with both negative or no effect on aphid reproduction and do not show unequivocally its role in plant-aphid interactions (Mewis et al., 2006; Pegadaraju et al., 2005). Consistent with the role of the SA signal transduction pathway in plant-aphid interactions, the effect of impaired SA accumulation following aphid attack was explored in the Arabidopsis *sid2* mutant affected in the SA biosynthetic enzyme isochorismate synthase 1 but no effects were observed (Pegadaraju et al., 2005). Moreover, the presence of the bacterial SA-degrading salicylate hydroxylase (*NahG*) in Arabidopsis negatively effected the reproduction of both *M. persicae* and *B. brassicae* (Mewis et al., 2005) or had no effect on *M. persicae* fecundity (Pegadaraju et al. 2005). PAD4 is an important component of the SA signalling cascade during pathogen infection and acclimation to high light intensities (References). A number of microarray experiments identified *PAD4* as a significantly induced gene upon aphid attack (De Vos et al., 2005; Couldridge et al., 2006; Pegadaraju et al., 2005; Kusnierczyk et al., 2008) suggesting an important role for *PAD4* in aphid resistance. Indeed, Arabidopsis plants carrying the *pad4* mutation support bigger aphid colonies and display premature leaf senescence upon *M. persicae* attack (Pegadaraju et al., 2005). In contrast, overexpression of PAD4 enhances aphid resistance by modulating a phloem-based defence mechanism (Pegadaraju et al., 2005). However, the function of PAD in aphid resistance is most probably not related to its role in SA mediated defence. EDS1, the interacting partner of PAD4 in plant innate immunity is not required for aphid resistance (Pegadaraju et al., 2007). Similarly,

mutants affected in *SAG101*, a stabilizing and signalling partner of the EDS1 protein, do not show altered aphid fecundity (Pegadaraju, 2005).

The leaf surface is an important first line of defence against aphids with the epicuticular wax layer playing a significant role in restricting the probing activities of the stylet. Different Arabidopsis mutants affected in ECERIFERUM (CER) genes involved in the wax biosynthesis have been used to evaluate the effects of altered wax composition on aphid success. Whereas aphid fecundity was not affected on *cer1* and *cer2* mutant plants, the *cer3* mutants showed elevated resistance (Jenks et al., 2002). However, the *cer3cer4* double mutant had no effect on aphid offspring as well as the *cer4* mutation on its own.

Despite the fact that aphid attack differentially regulates numerous genes involved in cell wall remodelling, the number of cell wall associated genes that affect aphid reproduction and settlement in the literature is limited. The Arabidopsis xyloglucan endotransglycosylase/hydrolase *XTH33* alters the settling behaviour of *M. persicae* (Divol et al., 2007). Aphids preferentially choose *xth33* mutant plants when given a choice between them and the wild type. Nevertheless, ectopic expression of *XTH33* does not result in elevated resistance.

Analysis of mutants with altered JA synthesis and signalling demonstrated that Arabidopsis resistance to *M. persicae* is largely jasmonate-dependent. The Arabidopsis *cev1* mutant display constitutive activation of JA responses and is a poor host for *M. persicea* (Ellis et al., 2002) Similarly, the *fou2* mutant that accumulates higher jasmonate levels relative to wild type supports smaller aphid colonies. (Kusnierczyk et al., 2011). Aphid reproduction on mutants insensitive to jasmonate as a result of the *coi1* mutation on the other hand was significantly increased (Ellis et al., 2002).

The significant role of glucosinolates in plant-aphid interactions was also demonstrated by manipulating single glucosinolate-related genes. The overexpression of *IQD1*, implicated in regulation of glucosinolate accumulation, negatively affected *M. persicae* host preference in choice experiments (Levy et al., 2005). Arabidopsis mutants carrying mutant alleles of *CYP812F2*, a gene encoding cytochrome P450 monooxygenase that catalyzes the conversion of indole-3-yl-methyl to 4-hydroxy-indole-3-yl-methyl glucosinolate, were more susceptible to *M. persicae* attack (Pfalz et al., 2009). Myrosinases are crucial for glucosinolates degradation but double *tgg1 tgg2* Arabidopsis mutants with no myrosinase activity *in vitro* did not alter *M. persicae* and *B. brassicae* reproduction suggesting that enzymatic glucosinolate degradation does not play a significant role in plant-aphid interactions (Barth and Jander, 2006).

Given the impact of aphid feeding on plant growth and the intrinsic link between defence processes and primary metabolism it is not surprising that manipulation of genes fulfilling important metabolic functions modulates aphid success. For example, Arabidopsis mutants affected in *TREHALOSE PHOSPHATE SYNTHASE11*, involved in trehalose biosynthesis, displayed enhanced susceptibility to *M. persicae*, whereas the resistance was higher in the trehalose accumulating *tre1* mutant (Singh et al., 2011). Like trehalose levels, starch content was also shown to affect aphid resistance in the starch-deficient *pgm1* mutant that supported bigger *M. persicae* colonies in comparison to the wild type (Singh et al., 2011). However, manipulation of the phloem amino acid content did not affect aphid reproduction both in the Arabidopsis *aap6* mutants affected in an amino acid permease and knock out mutants of the *ATN1* amino acid transporter (Hunt et al., 2006; Hunt et al., 2009).

Despite the fact that the cellular redox homeostasis and ABA signalling have been shown to modulate plant defence responses, the information regarding their roles in

plant – aphid interactions is limited. The findings described in Chapters 3 and 4 suggested that cellular redox homeostasis and ABA signalling plays an important role in both local and systemic responses in the *A. thaliana–M. persicae* interaction. To characterise the role of redox homeostasis and ABA signalling in the plant response to aphids in more detail, two mutants, *vtc2* and *abi4*, where selected for further study based on the low redox buffering capacity and deficiency in the ABI4 signalling pathways respectively. The effects of the *vtc2* and *abi4* mutations on the leaf transcriptome profiles, particularly with respect to the control of growth were described in Chapter 5. The findings reported in Chapter 5, demonstrate strong links between ascorbic acid- and ABA-dependent signal transduction pathways. Moreover, the *abi4vtc2* mutants are a useful new tool with which to explore the effects of the combined defects on plant responses to aphids. Based on this background information, the following study was performed to determine how alterations in redox buffering capacity and altered ABA signalling pathways alter the perception and responses of local and systemic leaves to aphids.

The effects of low ascorbic acid and impaired ABI4-signalling on aphid fecundity and reproductive success were also analysed. In the following studies, the transcriptome and metabolome analyses were compared in the local and systemic leaves of the *vtc2*, *abi4* and the *abi4vtc2* mutants to the responses in the wild type in order to determine how aphid-dependent transcriptional and metabolome re-programming was modified in the mutant genotypes. These studies have not only allowed the identification of key components that modulate aphid resistance in Arabidopsis and also provide novel insights into the interplay between the leaf redox hub (here explored through low redox buffering capacity) and ABA-mediated signalling networks in the responses of *A. thaliana* to *M. persicae*.

6.2 Results

6.2.1 Effect of low levels of ascorbate on aphid fecundity

M. persicae reproduction rates were measured on three different ascorbate-deficient mutants (*vtc1*, *vtc2* and *vtc4*) that have different low leaf ascorbate levels relative to the wild type (Col0). The *vtc4* mutants have about half the leaf ascorbic acid of Col0 (Conklin et al., 2006), whereas the *vtc1* and *vtc2* mutants have about 30% and 25% of the Col0 levels of leaf ascorbate respectively (Conklin et al., 1999).

In these experiments single 1-day old nymphs were placed on each rosette of the mutant genotypes and the wild type and the aphids were allowed to feed and reproduce for 15 days. At this point the rosettes were harvested and aphid numbers were counted. Aphid numbers measured after 15 days were significantly lower on the *vtc2* mutant relative to the wild type, whereas aphid numbers found on the *vtc1*, *vtc4* and wild type plants were similar (Fig. 6.1).

6.2.2 Comparisons of aphid fecundity on the *vtc2*, *abi4* and the *abi4vtc2* mutants

The data shown in Chapter 5 provide evidence that the signalling pathways activated by low levels of ascorbate and lack of a functional ABI4 are closely intertwined in the control of growth and sugar signalling. The following experiments were performed in order to determine whether low ascorbate and lack of a functional ABI4 are also linked in the control of stress responses. Aphid success rates were determined on all genotypes (Col0, *vtc2*, *abi4* and *abi4vtc2* mutants) as described in Section 6.2.1. Aphid numbers measured after 15 days were significantly lower on the *vtc2* mutants in these experiments (Fig. 6.2), as they were in the experiments shown in Fig. 6.1, even though the absolute numbers of aphids obtained after 15 days showed some variation between experiments. The numbers of aphids were significantly higher on the *abi4* mutants than Col0 (Fig. 6.2). However, when the *abi4* mutation was introduced into the *vtc2* background aphid fecundity was similar to that observed in the wild type (Fig. 6.2).

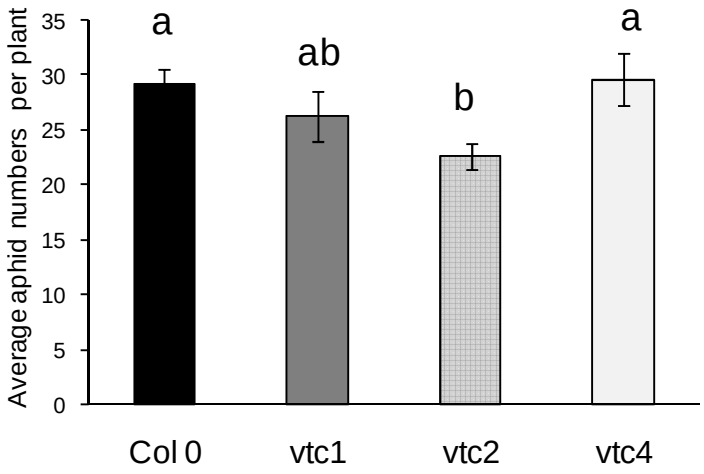

Figure 6.1 Aphid reproduction on ascorbate defective Arabidopsis mutant plants. One-day-old nymphs were placed in the middle of 3-week old rosettes of *vtc1*, *vtc2* and *vtc4* mutant plants and the newly formed aphids were counted after 15 days. Col0 plants were used as references. To retain aphids, each plant was covered with a meshed capped clear cage. The numbers represent means of at least 15 replicates ± SE. Significant differences were analysed with one-way ANOVA with post hoc analysis by Student-Newman-Keuls' pairwise comparison. Means with different letters are significantly different at the 5% level.

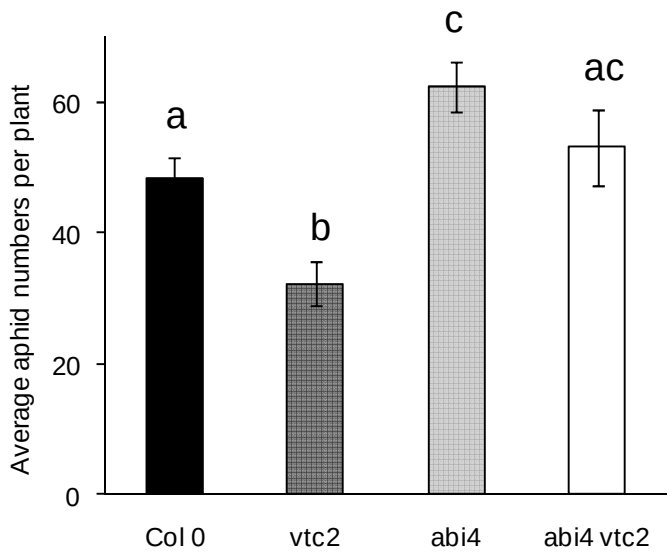

Figure 6.2 Aphid reproduction on Arabidopsis mutants carrying the *vtc2* and *abi4* mutations. One-day-old nymphs were placed in the middle of 3-week old rosettes of *abi4*, *vtc2* and *abi4vtc2* double mutant plants and the newly formed aphids were counted after 15 days. Col0 plants were used as references. To retain aphids, each plant was covered with a meshed capped clear cage. The numbers represent means of 16 (Col0 and *abi4*) or 15 (*vtc2* and *abi4 vtc2*) replicates ± SE. Significant differences were analysed with one-way ANOVA with post hoc analysis by Student-Newman-Keuls' pairwise comparison. Means with different letters are significantly different at the 5% level.

6.2.3 Transcriptome changes triggered in the Col0, *abi4*, *vtc2*, and *abi4vtc2* genotypes following *M. persicae* attack

6.2.3.1 Aphid-dependent changes in leaf transcriptome profiles

In these experiments, global transcript profiles were determined at 6 hpi in leaves directly infested with sixty aphids (local leaves) and non-infested leaves from the same rosette (systemic leaves) of the wild type and *abi4*, *vtc2*, and *abi4vtc2* double mutants (Fig. 6.3). Similar to the transcriptome data reported in previous chapters, a cut-off of a minimum of a 2-fold change was applied with p-value ≤ 0.05. Transcripts that were significantly changed in abundance relative to the non-infested controls of each genotype are described in the following sections.

The total numbers of transcripts that were modified in the infested leaves and systemic leaves of the Col0 plants at 6 hpi were similar in these experiments (Fig. 6.3) to those reported in Chapter 3. The numbers of transcripts that were increased in abundance in the infested leaves was greatly decreased in the mutant genotypes (Fig. 6.3). However, the numbers of transcripts that were decreased in abundance in the infested leaves varied with the mutant genotype relative to Col0 (Fig. 6. 3). The *abi4vtc2* double mutant showed the lowest number of differentially regulated genes (48) as a result of aphid infestation. In comparison, the local leaves of the wild type had much large numbers (162) of differentially regulated transcripts (Fig. 6.3).

There was little overlap between the transcripts that were modified in the infested leaves and those in the systemic leaves of the Col0 plants at 6 hpi (Fig. 6.3) as observed in the earlier experiments reported in Chapter 3. Moreover, the degree of overlap in the differentially-regulated transcripts between the local and systemic leaves was largely unchanged in the mutant genotypes (Fig. 6.3). Only two genes (*ARIADNE 15* and P-loop containing nucleoside triphosphate hydrolases superfamily protein; *At5g63630*)

were similarly increased in expression in the infested leaves and the leaves distant from the site of attack in *abi4* (Fig. 6.3; Appendices 14 and 15). Similarly, aphid infestation led to a decreased abundance of different transcripts in local and systemic leaves (Fig. 6.3). However, there was a small degree of overlap in the mutant genotypes, with a maximum of five transcripts (*SYP72*, *ARF-GAP DOMAIN 1*, unknown protein; *At1g52618*, unknown protein; *At1g11120* and *EF183305*) in the *abi4* mutant that were decreased in expression in a similar manner in the infested and systemic leaves (Fig. 6.3; Appendices 14 and 15).

6.2.3.2 Genotype-dependent comparisons of transcriptome responses to aphids in the infested leaves

As shown in Fig 6.3 aphid infestation resulted in an increase in the abundance of a large number (138) of transcripts in the infested Col0 leaves at 6 hpi.

In comparison, relatively few transcripts were increased in abundance of the infested leaves of the mutants at 6 hpi (Fig. 6.3). For simplicity, the changes in the transcript profiles at 6 hpi observed in the mutants relative to their respective controls were compared to those observed in Col0 at the same time point (Fig. 6.4).

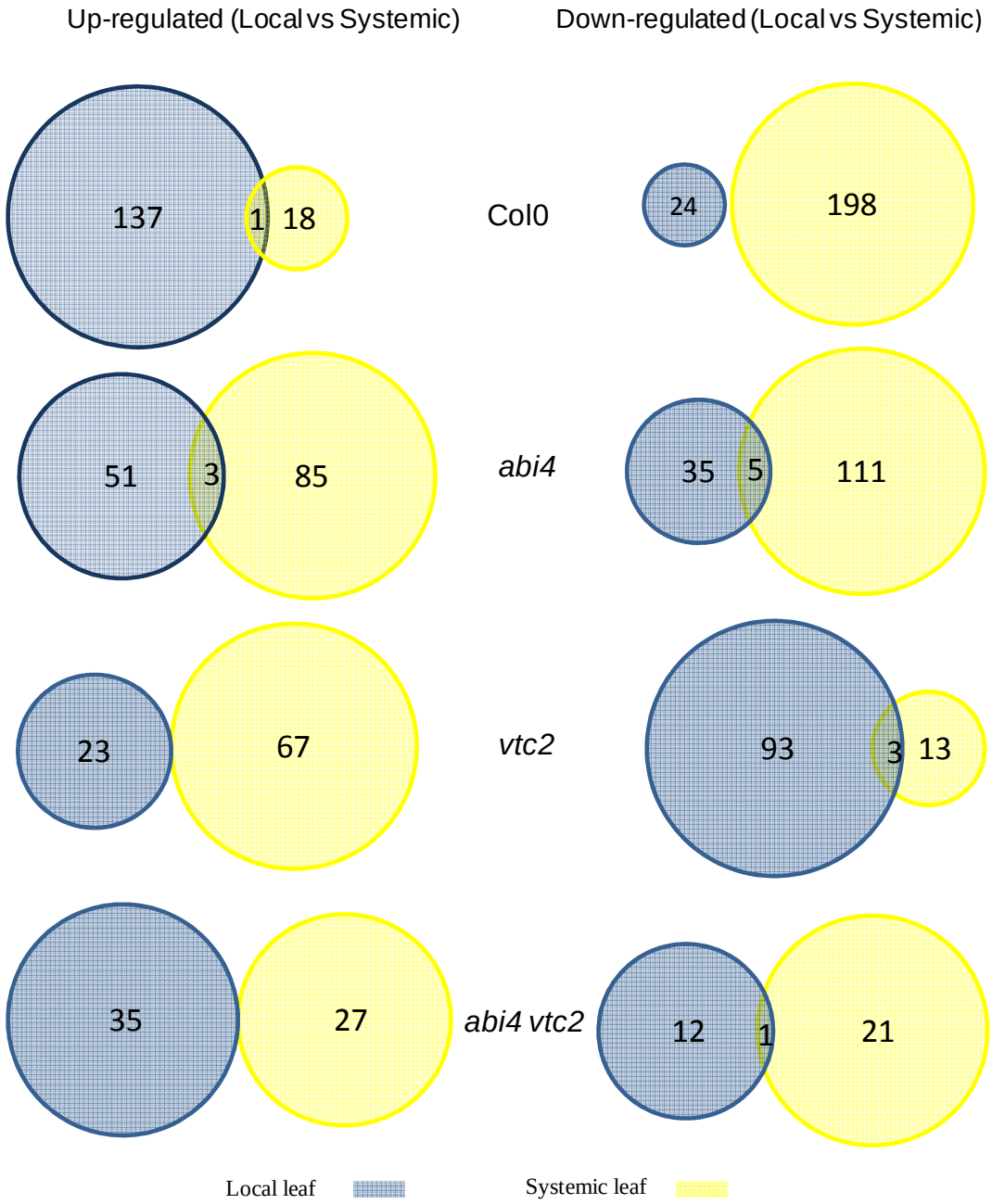

Figure 6.3 Venn diagrams showing common and unique genes that were differentially regulated in the local and systemic leaves of Arabidopsis Col0, *abi4*, *vtc2* and *abi4vtc2* plants at 6 hpi upon *M. persicae* attack.

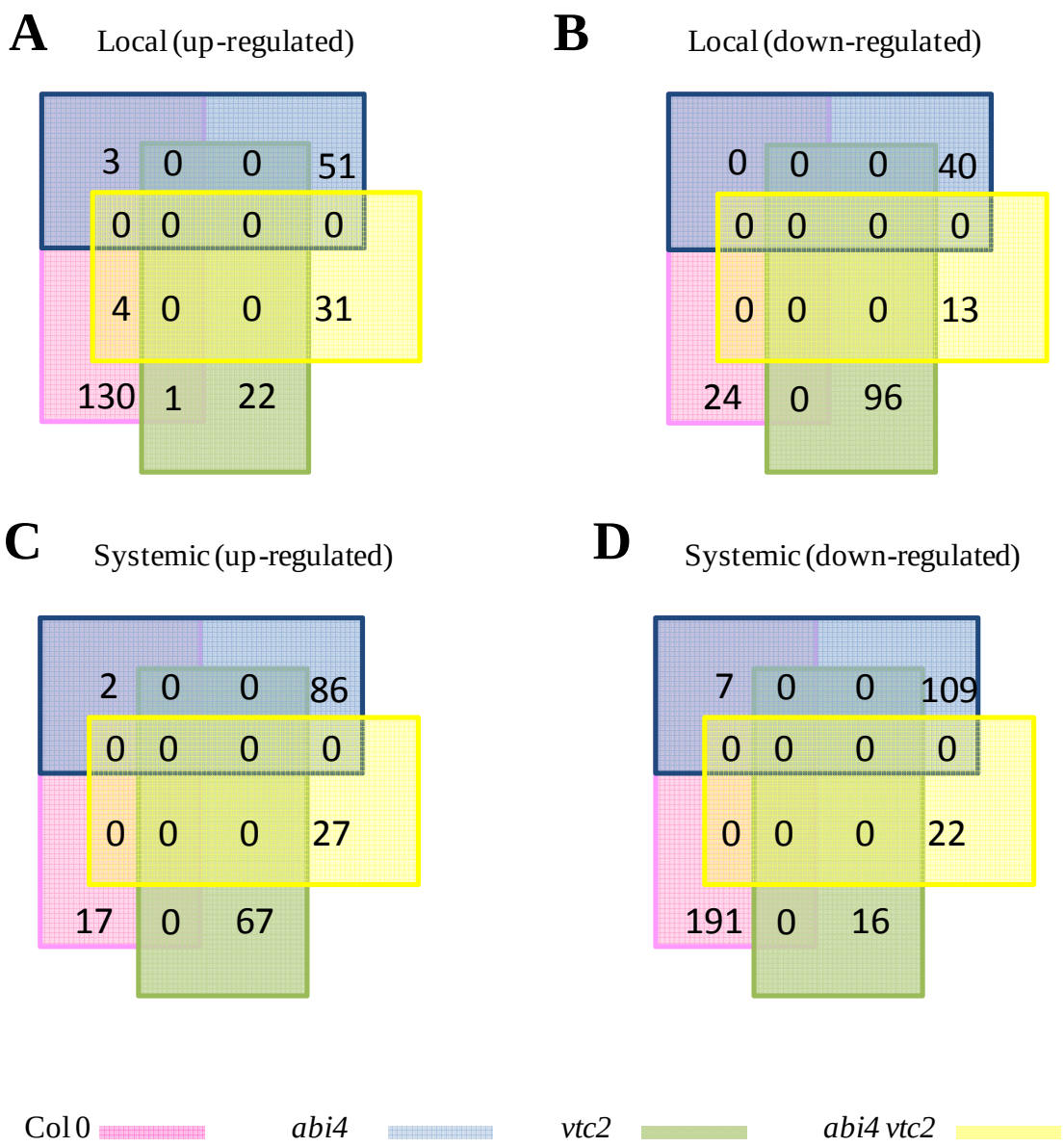

Figure 6.4 Venn diagrams showing common and unique genes that were differentially regulated at 6 hpi in Arabidopsis Col0, *abi4*, *vtc2* and *abi4vtc2* plants upon *M. persicae* attack. A) Common and unique up-regulated genes in the local leaves of the studied genotypes. B) Common and unique genes down-regulated in the local leaves of the studied genotypes. C) Common and unique genes up-regulated in the systemic leaves of the studied genotypes. D) Common and unique genes down-regulated in the systemic leaves of the studied genotypes.

Figure 6.5 shows the 10 most highly increased and the 10 most decreased transcripts in each genotype at 6 hpi. The transcript profile of the infested Col0 leaves at 6 hpi shows a pronounced redox signature that is absent from all other genotypes. In the different mutant backgrounds between 86 and 114 transcripts are no longer induced by aphid feeding. At least 18 of the transcripts that are absent in the mutant genotypes are linked to redox processes (Appendix 12). The number of transcripts that were induced in the *abi4* mutant was significantly decreased (about 60% less) compared to those induced in Col0 at 6 hpi (6.4A). Moreover, of the 54 transcripts that were increased in abundance in *abi4*, only 3 were similarly expressed (*XTH11*, UDP-Glycosyltransferase superfamily protein; *At2g36780*, and PQ-loop repeat family protein; *At4g36850*) in Col0 (Fig. 4A; Appendices 12 and 14). However, the overall change in the abundance of these transcripts was less than that of the transcripts shown in Fig. 6.5. The number of transcripts that were induced in the infested leaves of the *vtc2* mutant was significantly decreased (over 80% less) compared to those induced in Col0 at 6 hpi (Fig. 6.4A). Of the 23 transcripts that were induced in *vtc2* at 6 hpi, only one (unknown protein; *At5g57760*) was expressed in a similar manner in Col0 (Fig. 6.4A; Appendices 12 and 16), but the overall change in the abundance was less than that of the transcripts shown in Fig. 6.5.

The number of transcripts that were induced in the infested leaves of the *abi4vtc2* mutant was significantly decreased (about 75 % less) compared to those induced in Col0 at 6 hpi (Fig. 6.4 A). In this case, 4 transcripts were similarly expressed in the *abi4vtc2* double mutant (*MAPKKK19*, *XTH23*, Homeodomain-like superfamily protein; *At5g01380* and FAD-binding Berberine family protein; *At1g30700*) (Appendices 12 and 18).

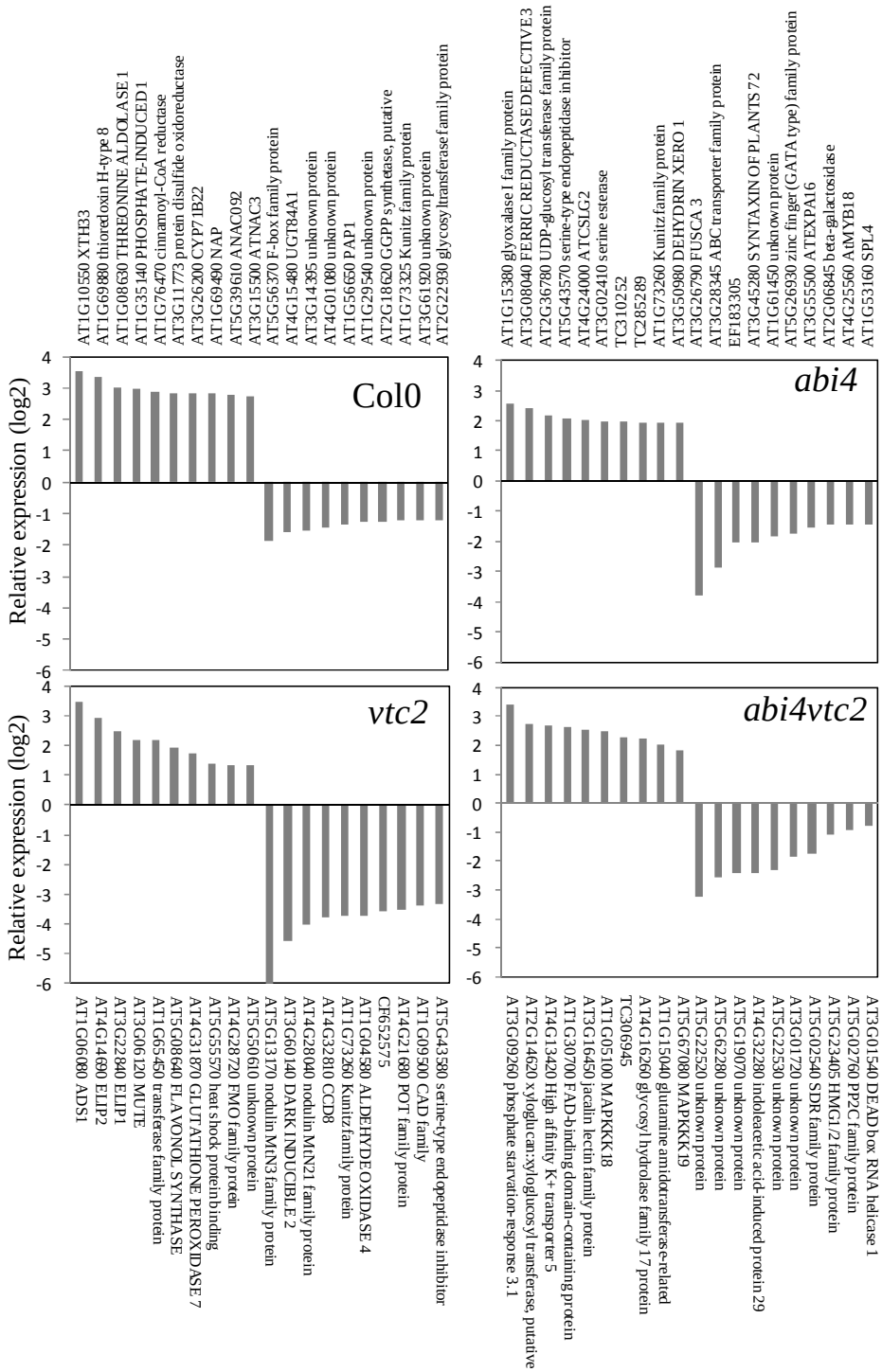

Figure 6.5 Top 10 most induced or repressed transcripts in the infested leaves of Col0, *abi4*, *vtc2* and *abi4vtc2* double mutant plants relative to the aphid-free controls of the corresponding genotype.

However, the overall change in the abundance of these transcripts was less than that of the transcripts shown in Fig. 6.5. Of the 24 transcripts that were decreased in abundance in the infested leaves of Col0 at 6 hpi, none was expressed in a similar manner in the *vtc2*, *abi4*, or *abi4vtc2* genotypes at 6 hpi (Fig. 6.4B).

6.2.3.3 Genotype-dependent comparisons of transcriptome responses to aphids in the systemic leaves

In marked contrast to the situation in infested leaves, aphid infestation resulted in an increase in the number of transcripts induced in the systemic leaves of the mutants at 6 hpi relative to their respective controls, compared to that observed in the Col0 leaves (Figs 6.3 and 6.4). For example, in comparison, more (450% more) transcripts were increased in abundance in the systemic leaves of the *abi4* mutants at 6phi than in Col0 at the same time point (Figs 6.3 and 6.4).

For simplicity, the changes in the transcript profiles at 6 hpi observed in the mutants relative to their respective controls were compared to those observed in Col0 at the same time point (Fig 6.6). It should be noted that there was no overlap in the transcripts that were increased in expression in the systemic leaves in these experiments on 4-week old plants and in those described in chapter 3, with 9-week old plants. This difference may be due to variations in the experimental conditions. However, in both series of experiments the numbers of transcripts that were enhanced in abundance were very low, 91 in the experiments reported in Chapter 3 and 19 in the present experiments. Many of the transcripts that were enhanced in expression in the systemic leaves of Col0 at 6phi were of unknown function. However, of the functional categories that were represented at 6 hpi, defence, cell wall and redox signalling features were evident (Appendix 13).

Of the 19 transcripts that were higher in abundance in the systemic leaves of Col0 at 6 hpi (Fig. 6.4C), 2 were similarly expressed in the *abi4* mutant (*ATSOFL2* and Protein

kinase superfamily protein; *At4g13190*) (Appendices 13 and 15). However, the overall change in the abundance of these transcripts was less than that of the transcripts shown in Fig. 6.6. Of the 67 transcripts that were induced in the *vtc2* mutant at 6 hpi and the 35 transcripts that were induced in *abi4vtc2*, none was expressed in a similar manner in Col0 (Fig. 6.4C).

Unlike the transcripts that were increased in abundance in the systemic leaves, which showed consistently higher numbers in the *abi4* and *vtc2* mutants than the Col0 leaves at 6hpi, the numbers of transcripts that were decreased in abundance were lower in all mutants genotypes than on Col0 (Figs 6.3 and 6.4D). Of the 198 transcripts that were lower in abundance in the systemic leaves of Col0 at 6 hpi, 7 were similarly expressed in the *abi4* mutant (*PCR2*, *Peroxidase 71*, alpha/beta-Hydrolases superfamily protein; *At5g67050*, *HEME OXYGENASE 4*, *WRKY62*, glutamine-fructose-6-phosphate transaminase; *At3g24090* and *PDI-LIKE 1-3*) (Fig. 6.4 D; Appendices 13 and 15). However, the overall change in the abundance of these transcripts was less than that of the transcripts shown in Fig. 6.6. Of the 16 transcripts that were decreased in *vtc2* at 6 hpi, and the 22 transcripts that were decreased in *abi4vtc2*, none was expressed in a similar manner in Col0 (Fig. 6.4 D).

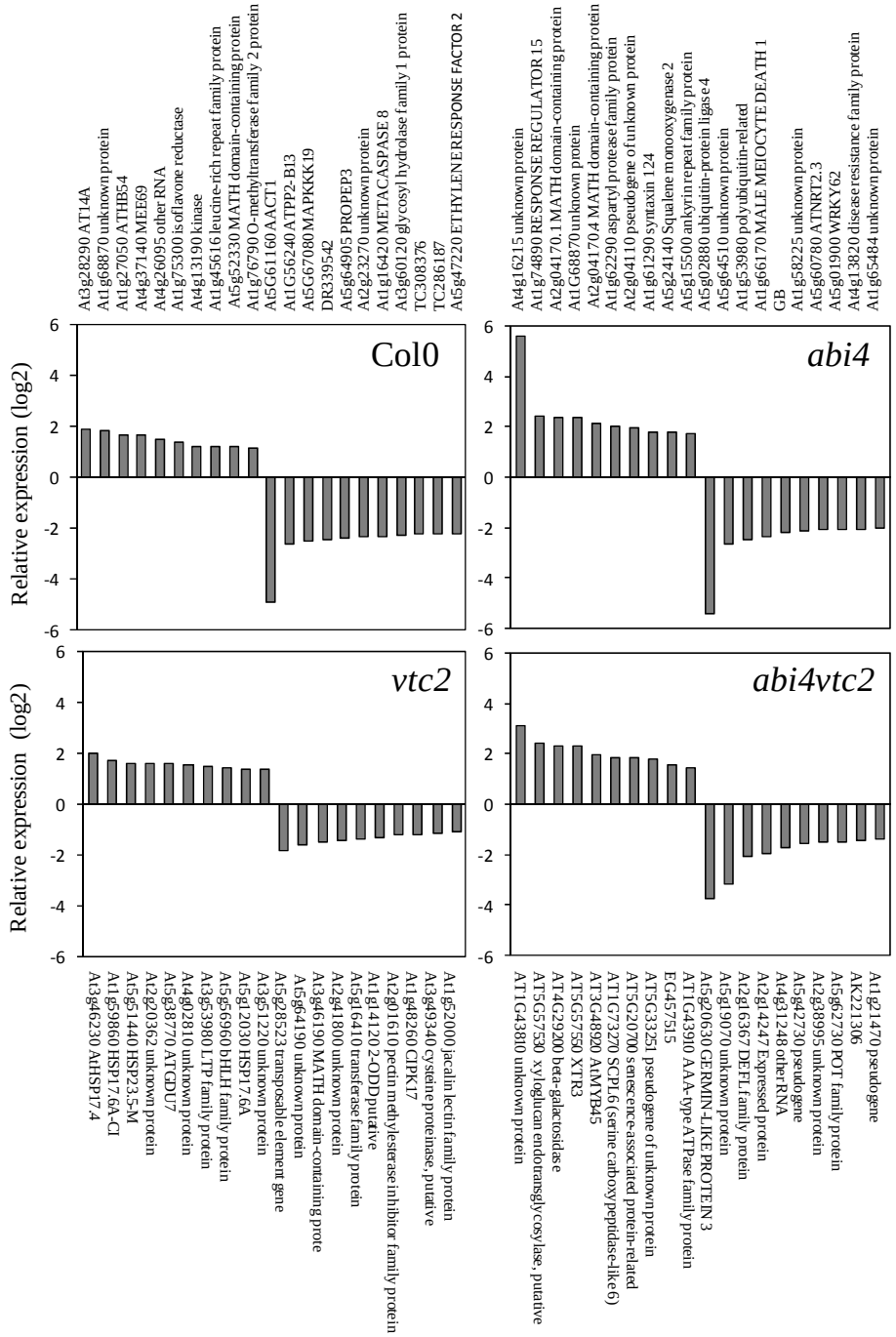

Figure 6.6 Top 10 most induced or repressed transcripts in the systemic leaves of Col0, *abi4*, *vtc2* and *abi4vtc2* double mutant plants relative to the aphid-free controls of the corresponding genotype.

6.2.4 Effect of aphid feeding of the abundance of defence-related transcripts in *abi4, vtc2* and *abi4vtc2*

6.2.4.1 Expression profiles of defence-related genes in the infested leaves

The genes described in the following section can be broadly assigned to the category defence-related transcripts based on their involvement in pathogen infection and hormones associated defence signalling pathways. The abundance of *HEAT SHOCK PROTEIN 18.2* (*HSP18.2*) transcripts was decreased in the *abi4* mutants at 6 hpi, whereas *SENESCENCE-RELATED GENE 3* (*SRG3*), and *CALCINEURIN B-LIKE PROTEIN 5* (*CBL5*) transcripts were increased (Appendix 14). *CBL5* encodes a calcium sensor protein (Cheong et al., 2010). Whereas *CBL5* transcripts were increased in a*bi4*, they were decreased in *vtc2* (Appendix 16). Similarly, a gene encoding a Kunitz family protein (*At1g73260*) that plays a role in programmed cell death during plant-pathogen interactions (Li et al., 2008) was induced in *abi4* but repressed in the *vtc2* background (Appendices 14 and 16). Several glutathione S-transferases (*ATGSTU11*, *ATGSTU24*, *ATGSTU25*, *ATGSTU12*, *ATGSTU2*, *ATGSTU3*), senescence-related (*SRG1*), and thioredoxin (*ATH8*) encoding genes were also repressed in the *vtc2* background (Appendix 16). However, transcripts encoding another glutathione S-transferase (*ATGSTU2*) and a cytochrome P450 (*CYP76C2*), which is associated with the hypersensitive response and cell death (Godiard et al., 1998), were increased in *abi4vtc2*. Perhaps the most interesting aspect of the response of the transcriptomes of the infested leaves of the mutants is the marked decrease in the number of induced transcripts. Large numbers of induced genes are missing from the profiles of the mutants. For example, *REDOX RESPONSIVE TRANSCRIPTION FACTOR 1, ACIREDUCTONE DIOXYGENASE, PHYTOALEXIN DEFICIENT 4, ORA47, ENHANCED DISEASE SUSCEPTIBILITY 1*, etc. were no longer induced in the mutant genotypes upon *M. persicae* attack.

6.2.4.2 Expression profiles of defence-related genes in the systemic leaves

The response of the transcriptomes of the systemic leaves of the mutants to infestation (in terms of absolute transcript numbers) was greater than that of Col0 (Fig. 6.3 and 6.4). For example, transcripts encoding heat shock proteins (*ATHSP17.4*, *HSP17.6A-CI*, *HSP23.5-M*, *AT-HSP17.6A*, *HSP70*, *HSP17.6B-CI* and *ATHSFA2*), *WRKY30*, and a glutathione S-transferase (*ATGSTF4*) accumulated in the systemic leaves of *vtc2* but not of Col0 (Appendix 17). Interestingly, *EDS5* and *ATMYC2* transcripts were increased following aphid feeding in the systemic leaves of *abi4vtc2* but not in any other genotype (Appendix 19). However, WRKY transcription factors (*WRKY 38* and *62*), PR proteins (*PR1* and *PR2*) and disease resistance proteins (*At4g04220*, *At1g71390*, *At2g32680* and *At4g13820*) in the systemic leaves were decreased in abundance in *abi4* but not in Col0 (Appendix 15).

6.2.5 Effect of aphid feeding on the abundance of transcripts related to cell wall remodelling in *abi4, vtc2* and *abi4vtc2*

6.2.5.1 Expression profiles of cell wall associated transcripts in the local leaves

Cell wall-associated transcripts were among the most overrepresented transcripts differentially regulated upon aphid infestation in all genotypes (Appendices 14, 16 and 18). Xyloglucan endotransglucosylase/hydrolases (XHTs) that catalyzes the reformation of xyloglucan chains are important for cell wall remodelling (Campbell and Braam, 1999). *XTH11* was induced upon aphid attack in the infested tissues of *abi4*, whereas transcripts encoding other XHT forms (*XTH10* and *XTH23*) were up-regulated in infested leaves only in the *abi4vtc2* background. Similarly, transcripts encoding two expansin-like proteins (EXPA16 and EXPA14) were decreased in abundance in infested *abi4* leaves of during *M. persicae* feeding (Appendix 14). In the infested *vtc2* leaves *XTH11* and *XTH24* transcripts were increased whereas two expansin-like family

proteins (*EXPL1* and *EXPL3*), and *Cellulose synthase-like G2* (*ATCSLG2*) transcripts were decreased (Appendix 16).

6.2.5.2 Expression profiles of cell wall associated transcripts in the systemic leaves

Transcripts encoding a *SECONDARY WALL-ASSOCIATED NAC DOMAIN PROTEIN 3* (*SND3*) were increased in the systemic tissues of *vtc2* at 6hpi (Appendix 17). The expression of several cell wall-associated genes was increased in the systemic leaves of the *abi4* mutants at 6 hpi. For example, transcripts encoding ARABIDOPSIS THALIANA EXPANSIN A3 (ATEXPA3) and ARABIDOPSIS THALIANA EXPANSIN-LIKE A3 (ATEXLA3) were increased, but ARABIDOPSIS THALIANA EXPANSIN A14 (ATEXPA14) was decreased (Appendix 15). Transcripts encoding XTH25 and XTH12 were increased in the systemic leaves of the *abi4vtc2* double mutant (Appendix 19).

It is of interest to note that a number of transcripts that were increased in abundance in the infested leaves of the *abi4* mutants at 6 hpi were decreased in abundance in the infested leaves of the *vtc2* mutant (Table 6.1).

Similarly, there were a small number of transcripts whose expression was reversed in the infested *abi4vtc2* leaves compared to local *vtc2* leaves following *M. persicae* attack (Table 6.2).

Accession	Expression ratio		Description
	abi4	*vtc2*	
At5g43570	4.2	0.1	PR peptide that belongs to the PR-6 proteinase inhibitor family
At1g73260	3.8	0.1	Trypsin inhibitor involved in modulating programmed cell death in plant-pathogen interactions.
At5g22860	2.5	0.4	Serine carboxypeptidase S28 family protein
At3g48580	3.7	0.1	Xyloglucan endotransglucosylase/hydrolase 11
At4g24000	4.1	0.3	ATCSLG2; Cellulose synthase-like G2
At3g21500	3.1	0.4	DXPS1; 1-deoxy-D-xylulose-5-phosphate synthase
At4g01420	3.1	0.4	CBL5; Calcineurin B-like protein 5

Table 6.1 Transcripts with reversed expression in infested *abi4* leaves in comparison to infested *vtc2* leaves. Sixty aphids were confined to a clip cage attached to a mature rosette leaf of either *abi4* or *vtc2* 4-week-old Arabidopsis plants and allowed to feed for 6 h. Non-infested caged leaves from the corresponding genotype were used as controls. Four biological replicates were collected and gene expression analysed with Agilent V4 arrays. Statistically significant differentially expressed genes in either *abi4* or *vtc2* infested leaves were identified on volcano plots with a cut-off of fold change ≥2 and Student's t-test p-value ≤0.05 relative to caged controls from the corresponding genotype. Genes which transcript abundance was enhanced in infested *abi4* leaves compared to non-infested *abi4* controls while transcript abundance was repressed in *M. persicae*-challenged *vtc2* leaves relative to non-infested *vtc2* controls are shown in the table. All ratios are expressed on a linear scale relative to the corresponding control.

Accession	Expression ratio		Description
	vtc2	*abi4vtc2*	
At2g29480	0.3	2.6	ATGSTU2; GLUTATHIONE S-TRANSFERASE 20
At4g13420	0.4	6.4	HAK5 (High affinity K+ transporter 5); potassium ion transmembrane transporter
At4g13790	0.5	2.4	auxin-responsive protein, putative
At2g35730	0.2	2.1	heavy-metal-associated domain-containing protein

Table 6.2 Transcripts with reversed expression in infested *abi4vtc2* leaves in comparison to infested *vtc2* leaves. Sixty aphids were confined to a clip cage attached to a mature rosette leaf of either *abi4vtc2* or *vtc2* plants and allowed to feed for 6 h. Non-infested caged leaves from the corresponding genotype were used as controls. Four biological replicates were collected and gene expression analysed with Agilent V4 arrays. Statistically significant differentially expressed genes in either *abi4vtc2* or *vtc2* infested leaves were identified on volcano plots with a cut-off of fold change ≥2 and Student's t-test p-value ≤0.05 relative to caged controls from the corresponding genotype. Genes which transcript abundance was enhanced in infested *abi4vtc2* leaves compared to non-infested *abi4vtc2* controls while transcript abundance was repressed in *M. persicae*-challenged *vtc2* leaves relative to non-infested *vtc2* controls are shown in the table. All ratios are expressed on a linear scale relative to the corresponding control.

6.2.6 Metabolomic changes in the local and systemic leaves of *abi4*, *vtc2*, and *abi4vtc2* following aphid attack

Aphid-induced changes in the metabolite profiles in local and systemic leaves of the *abi4*, *vtc2*, and *abi4vtc2* mutants were quantified using a targeted metabolomic approach (Fig. 6.7). In these experiments, 60 aphids were confined on single rosette leaves for 6, 24 and 48 h. Infested (local) leaves and non-infested (systemic) leaves from the same rosettes were harvested as in previous experiments. It is interesting to note that in comparison to the data shown in Chapter 3 (Fig. 4.1), where relatively few aphid-induced changes in metabolites were observed over the first 48 hpi, the global patterns of metabolite change were much greater in the mutant lines.

For simplicity, the aphid-induced changes in leaf metabolite profiles are discussed in the following sections according to the metabolic pathways that were affected.

6.2.6.1 Organic acids and amino acids in infested leaves

Relatively few aphid-induced changes in amino acids were observed in the mutant lines. However, a marked increase of the leaf serine pool was observed in local *vtc2* leaves at 48 hpi (Fig. 6.8). The content of valine was also significantly higher in the infested *vtc2* leaves than controls at 48 hpi. Citrate accumulated in the infested *abi4* leaves at 24 and 48 hpi in comparison to the control leaves (Fig. 6.8).

6.2.6.2 Myristic acid in infested leaves

The levels of myristic acid were significantly increased at 6, 24 and 48 hpi in the *abi4vtc2* double mutants and at 48 hpi in the *abi4* mutant (Fig.6.8).

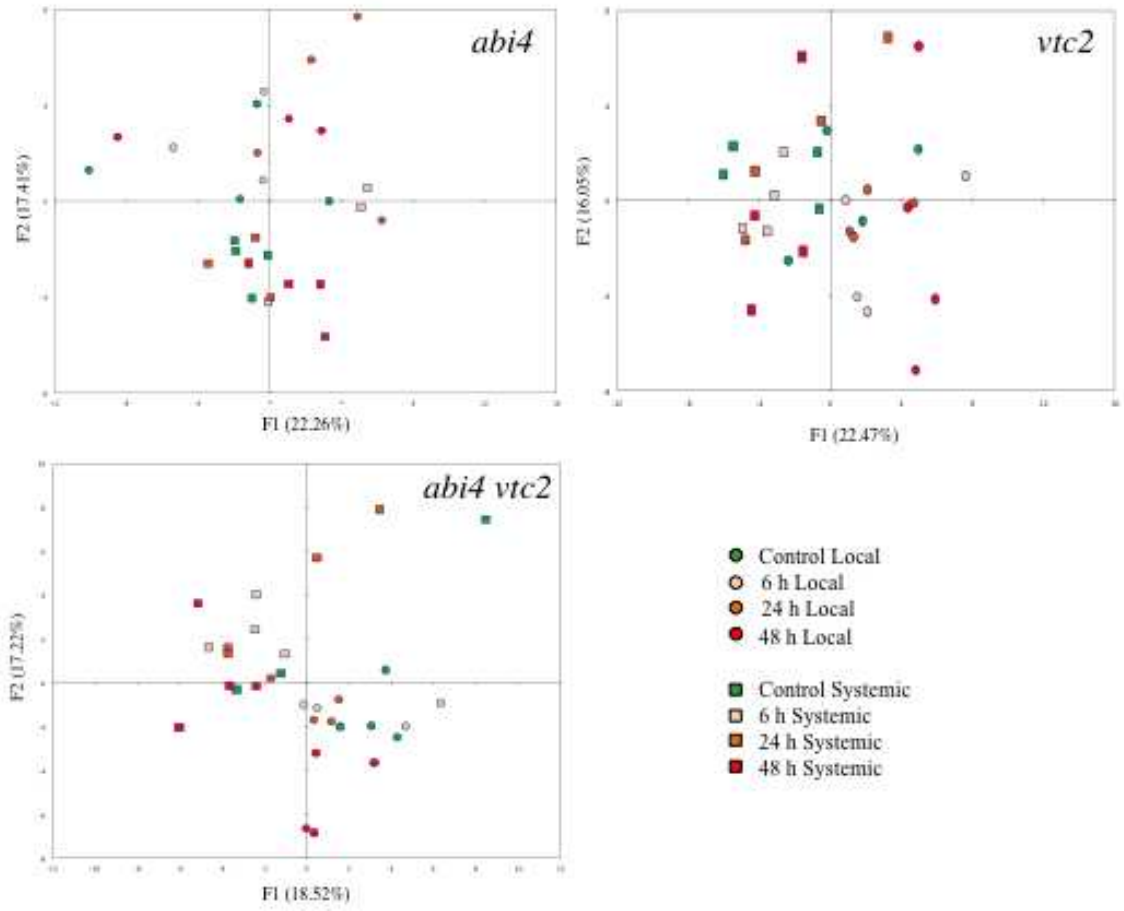

Figure 6.7 Principal component analysis of GC-MS profiles of *abi4*, *vtc2* and *abi4vtc2* Arabidopsis mutant plants infested with *M. persicae* for 6, 24 and 48 h in four biological replicates. The plots represent the first (PC1) and second components (PC2) of the metabolite profiles of infested (local) and uninfested (systemic) leaves from *M. persicae*-challenged *abi4*, *vtc2* and *abi4vtc2* Arabidopsis mutant plants and corresponding controls. The first and second components together explain 39.67% of the total variation in *abi4*, and 38,52% and 35,74% in *vtc2* and *abi4vtc2* respectively.

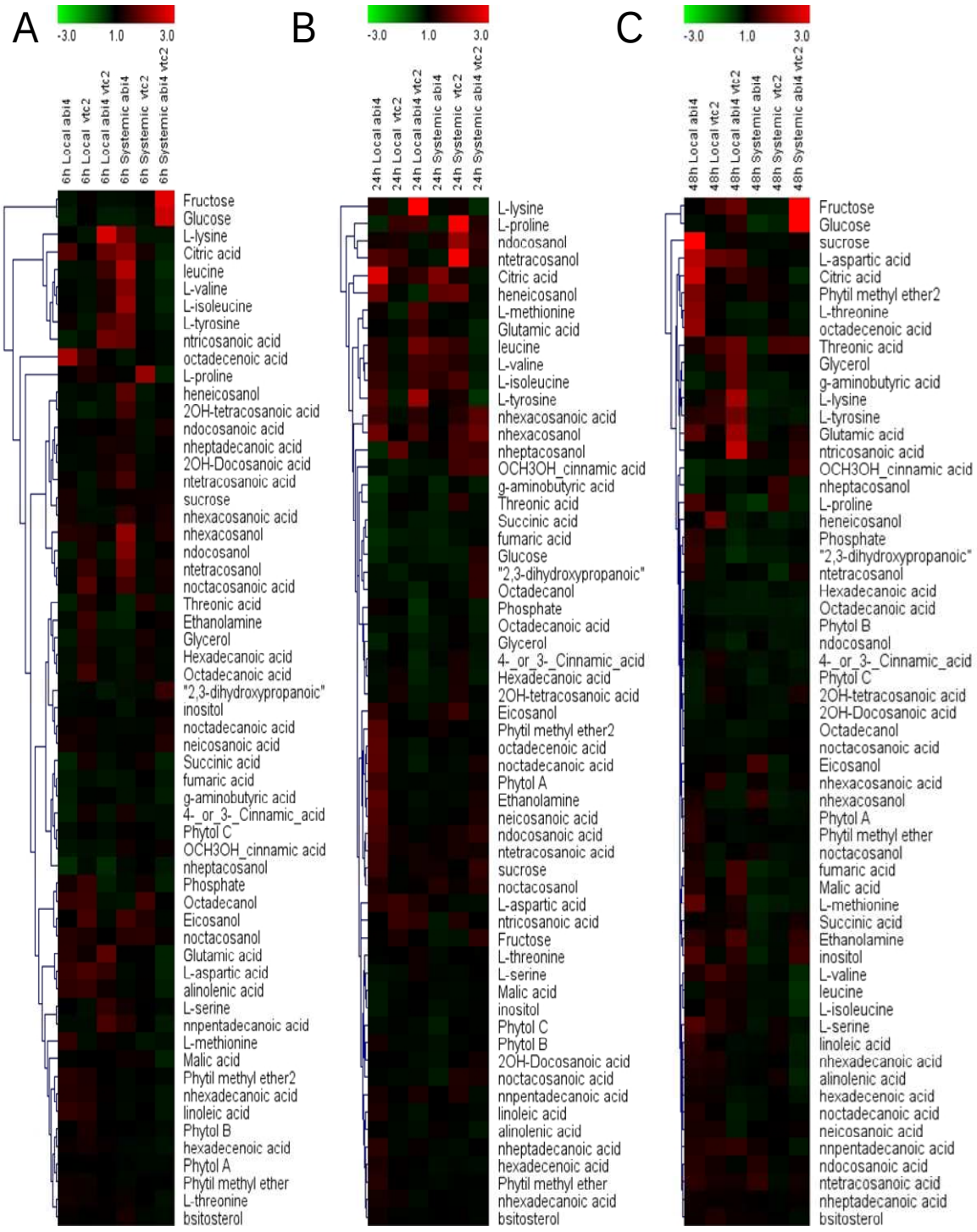

Figure 6.8 Hierarchical clustering analysis of metabolite profiles of local and systemic leaves of *abi4*, *vtc2*, and *abi4vtc2* plants infested with *M. persicae* at 6, 24, and 48 hpi. The values represent ratios relative to control samples. The metabolites were clustered according to Euclidean distances. A) Metabolite profiles at 6 h following aphid attack. B) Metabolite profiles at 24 h following aphid attack. C) Metabolite profiles at 48 h following aphid attack.

6.2.6.3 Fatty acids and related metabolites in infested leaves

The levels of palmitic, oleic, linoleic, and α-linoleic acid were significantly increased in the infested *abi4* and *vtc2* leaves (Fig. 6.8; Appendices 20 and 21). In contrast, aphid feeding depleted the pools of palmitic, linoleic, oleic, and stearic acid in the *abi4vtc2* double mutants (Fig. 6.8 and Appendix 22).

6.2.6.4 Cinnamic acid in infested leaves

A compound identified as either 4- or 3-cinnamic acid was significantly decreased in the infested *abi4* leaves of relative to the non-infested controls (Fig. 6.8 and Appendix 20).

6.2.6.5 Organic acids, amino acids and sugars in systemic leaves

The aspartate and threonine levels of *abi4* leaves were significantly lower at 48 hpi (Fig. 6.8). The proline levels of the systemic leaves of the *abi4vtc2* double mutant were decrased at 48 hpi.

Similar to the local leaves (Fig 6.8), citrate was increased in the systemic leaves (Fig 6.8) of the *abi4* mutant. Moreover, glucose levels were significantly decreased in the systemic tissues of the *abi4* mutants at 6, 24 and 48 hpi (Fig. 6.8).

6.2.6.6 Inositol in systemic leaves

The levels of inositol in the systemic tissues of the *abi4* were differentially regulated following aphid attack with a decrease observed at 48 hpi.

6.2.6.7 Fatty acids in systemic leaves

The levels of tricosanoic acid significantly increased in the systemic leaves of the *abi4* mutants at 6 hpi

6.2.7 Nymph survival on primed local and systemic leaves of the *abi4*, *vtc2* and *abi4vtc2* mutants

The local and systemic effect of priming of plants carrying the *abi4* and *vtc2* mutations on nymph survival was determined as described previously (Chapter 3, Section 3.2.6). Sixty aphids were caged on a mature fully expanded rosette leaf from 4-week old Arabidopsis plants and allowed to feed for 24 h. After their removal, 10 one-day old nymphs were placed on previously infested (local) leaves or non-infested (systemic) leaves from the same rosette. Non-infested caged plants from the corresponding genotype were used as controls. Nymph survival rate was counted after three days. No statistically significant differences were observed on the numbers of nymphs present on the local or systemic leaves of pre-infested *abi4*, *vtc2* and *abi4 vtc2* and control plants according to the Kruskal-Wallis test (*abi4* p=0.307; *vtc2* p=0.211; *abi4 vtc2* p=0.650).

6.3 Discussion

6.3.1 Effect of low ascorbic acid levels on aphid fecundity

In the present study, it was shown that aphid colony numbers were lower on the Arabidopsis *vtc2-1* mutants than the wild type plants. The *vtc2* mutants constitutively express *PR* genes and accumulate high levels of SA resulting in enhanced resistance against the bacterial pathogen *P. syringae* (Pavet et al., 2005). However, defence mediated through the SA signalling pathway is ineffective against aphid infestation (Pegadaraju et al., 2005) and most probably phloem-feeding insects can manipulate the hormonal cross talk by suppressing the effective JA defence pathway through the activation of SA triggered signalling cascades (Zarate et al., 2007). The reproduction rates of *M. persicae* were similar on the *vtc1-1* and *vtc4-1* mutants to those on the wild type plants. However, these mutations have less severe effects on the leaf ascorbate contents than the *vtc2* mutation. Furthermore, the *vtc4-1* mutant does not exhibit

elevated SA levels (Mukherjee et al., 2010), implying that the effects of low ascorbate are only executed under a certain threshold.

6.3.2 Lack of functional ABI4 results in enhanced susceptibility to *M. persicae*

ABA has been implicated in responses to adverse environmental conditions such as drought or high salinity and was shown to modulate pathogenesis responses (Shinozaki et al., 2003; de Torres-Zabala et al., 2007). The little information available on the role of ABA in plant response to insect herbivory however suggests that this hormone plays a crucial role in plant defence (Thaler and Bostock, 2004). The loss of ABI4 function in the *abi4* mutant resulted in increased susceptibility to *M. persicae*. This finding suggests that ABA transduction pathways are important in plant responses to aphids since a functional ABI4 is required for the full expression of aphid resistance in Arabidopsis. However, ABI4 fulfils a range of diverse yet important roles in plant development and defence suggesting that a mechanism different from ABA mediated signalling may be responsible for modulation of aphid success. The data shown in Chapter 5 demonstrate that ABI4 is required for the ascorbate-dependent control of growth. Similarly, the enhanced aphid susceptibility observed in the *abi4* mutant was not observed in a low asorbate (*vtc2*) background. The low ascorbate background abolished the susceptible phenotype of the *abi4* mutant and restored the wild type levels of resistance.

The transcriptome profiles of *abi4*, *vtc2* and the corresponding double mutant *abi4 vtc2* are highly overlapping with numerous defence related genes being induced. The results presented in Chapter 5 show that the signalling events mediated by low levels of ascorbic acid and triggered by the absence of ABI4 are highly intertwined and their interplay is most probably involved in modulating plant responses against aphids in the *abi4 vtc2* background. However, it is possible that in terms of aphid resistance both mutations act independently and their antagonistic effects determine the overall aphid success. If the factor limiting aphid reproduction that is present in the *vtc2* mutant acts

independently of the *abi4* mutation in the double mutant, it can also compromise aphid success to a certain extent in the double mutant *abi4 vtc2*. It follows that the presence of the *vct2* mutation in the *abi4* background can compensate partially for the enhanced susceptibility of the *abi4* mutants to aphid attack.

6.3.3 Cell wall and aphid resistance

The cell wall structure and stability have been suggested to play an important role in plant-aphid interactions (Divol et al., 2007). Aphid feeding differentially regulated genes involved in cell wall reorganization and remodelling in all studied genotypes. Interestingly, a suite of cell wall associated transcripts was repressed in the *vct2* mutant upon aphid attack. Ascorbic acid is important for conditioning the right redox balance in the apoplast required for cell expansion and thus affects cell wall reorganization (Smirnoff, 2000; Kato and Esaka, 2000). The ascorbic acid deficient *vtc1* and *vtc2* mutants display smaller cells in the leaf mesophyll (Pavet et al., 2005). Moreover, certain steps of the ascorbic acid biosynthetic pathways deliver intermediates for cell wall biosynthesis (Smirnoff, 2000). Thus constitutive changes in the cell wall structure resulting from impaired ascorbate biosynthesis can be related to the enhanced aphid resistance of the *vtc2* mutant.

6.3.4 Interplay between ABI4 and sugar signalling in plant-aphid interactions

The ABI4 transcription factor may be linked to aphid resistance, because it is a key component of sugar signalling pathways in plants. Aphid feeding has been shown to increase trehalose levels, which has a role in aphid resistance (Singh et al., 2011). The expression of *TREHALOSE PHOSPHATE SYNTHASE11* was induced in Arabidopsis by *M. persicae* feeding and this was accompanied by trehalose accumulation (Singh et al., 2011). Like ascorbate, trehalose is an important signalling molecule regulating plant growth and development (Fernandez et al., 2010). In the present study, aphid feeding

resulted in increased expression of both *TREHALOSE-6-PHOSPHATE PHOSPHATASE* and *TREHALOSE PHOSPHATE SYNTHASE11* in wild type *A. thaliana* plants. Exogenous application of trehalose affects starch biosynthesis and breakdown and Arabidopsis wild type plants plants grown on trehalose display a strong down-regulation of genes important for starch breakdown (Ramon et al., 2007). Such repression was not observed in *abi4* mutants, suggesting that the ABI4 transcription factor orchestrates transduction pathways affecting starch metabolism down-stream of trehalose accumulation (Ramon et al., 2007). Interestingly, aphid feeding on wild type plants resulted in starch accumulation (Singh et al., 2011). It is tempting to speculate that ABI4 mediates signalling events triggered by trehalose during aphid attack. Moreover, it is possible that starch accumulation will be compromised in the *abi4* mutants upon aphid attack. Interestingly, starch present in artificial diets was shown to deter *M. persicae* (Campbell et al., 1986).

6.3.5 Low ascorbate may modulate aphid success via JA-dependent signalling pathways

The transcripts differentially regulated in *vtc2* which expression was reversed in the *abi4vtc2* double mutant (Table 5.7) provides new insights into the signalling pathways by which low ascorbate enhances the resistance to *M. persicae* in the *vtc2* mutant. Of these, the ORA47 transcription factor and the ABI5-binding protein (AFP3) are candidates for the low ascorbate-dependent control of aphid resistance in the *vtc2* mutants. These transcripts were enhanced in *vtc2* relative to Col0 and they were strongly repressed in the *abi4vtc2* double mutant. It is possible that the ability of *vtc2* mutant plants to execute aphid resistance depends on affects on *ORA47* and/or *AFP3*. The stability of ABI5 is regulated by ABA through ubiquitin-related events (Lopez-Molina et al., 2003). It has recently been shown that a member of the ABI5 binding protein family called Novel Interactor of JAZ (NINJA) functions as an adaptor protein

that interacts with the ZIM domain of most JA ZIM-domain (JAZ) proteins that act as substrates of the E3 ubiquitin ligase SCF complex in the repression of JA-response genes (Pauwels et al., 2008). Moreover, NINJA contains an ERF-associated amphiphilic repression (EAR) motif that recruits the co-repressor TOPLESS, which interacts with an EAR motif on Auxin/IAA substrates of TIR1 to repress auxin responses (Pauwels et al., 2010). The ORA47 transcription factor functions downstream of the MYC2 transcription factor in the JA signalling pathway (Pauwels and Goossens, 2008). Of all transcription factors, which are expressed in a similar manner in the *vtc2* and *abi4* single mutants, ORA47 is the only one which expression is reversed in the *abi4vtc2* leaves compared to *vtc2* rosettes. It is possible that the over-expression of ORA47 in *vtc2* confers a JA-sensitive phenotype and contributes to the enhanced resistance to *M. persicae*. This situation will also favor the induction of a subset of MYC2-controlled genes in the JA pathway (Pauwels and Goossens, 2008). The repression of *ORA47* together with *AFP3* in the *abi4vtc2* leaves is consistent with the restoration of the wild type levels of resistance in *abi4vtc2*. In contrast, transcripts encoding arabinogalactan protein 31, which expression is repressed by methyl jasmonate, were increased in *vtc2* but repressed in the *abi4vtc2* leaves. Similarly, transcripts encoding JA methyltransferase were decreased in all mutant genotypes relative to Col0. Taken together, these seemingly conflicting findings reveal a complex interplay between ABA, JA and redox signaling pathways.

Chapter 6: Summary

- Aphid fecundity was studied on ascorbate deficient Arabidopsis mutants (*vtc2*, *vtc1* and *vtc4*). Enhanced aphid resistance was observed on the *vtc2* mutant, whereas *M. persicae* reproduction on the *vtc1* and *vtc4* mutants was similar to that on the wild type plants.

- The lack of functional ABI4 in the *abi4* mutant resulted in increased susceptibility to *M. persicae*. The enhanced aphid fecundity observed on the *abi4* mutant was abolished in a *vtc2* background. The low ascorbate background (*vtc2*) abolished the susceptible phenotype of the *abi4* mutant and restored the wild type levels of resistance in the *abi4 vtc2* mutants.
- Microarray analysis was employed to identify genes responsive to *M. persicae* attack in *abi4*, *vtc2*, *abi4 vtc2* and wild type Arabidopsis plants at 6 hpi both in infested leaves and leaves distant from the site of attack.
- Aphid-induced changes in the metabolite profiles of local and systemic leaves of *abi4*, *vtc2*, and *abi4vtc2* mutants were quantified using a targeted metabolomic approach at 6, 24 and 48 hpi.
- Priming of *abi4*, *vtc2* and *abi4vtc2* Arabidopsis plants did not significantly alter nymph mortality on previously infested leaves. Similarly, nymph mortality was not affected when nymphs were placed on the systemic leaves.

Chapter 7. Effects of altered redox homeostasis on the interaction between *M. persicae* and *Solanum tuberosum* L.

7.1 Introduction

Potato (*Solanum tuberosum* L.) is a globally important source of carbohydrates. After wheat, rice and maize, it is the forth most important crop species worldwide. The potato originates in South America and currently there are 235 recognised wild *Solanum* species (Hawkes, 1990). This wide genetic diversity represents an excellent pool to identify mechanisms underlying natural abilities to withstand extreme environmental conditions. So far more than 15 genes have been incorporated in modern commercial varieties (Alvarez et al., 2006). Nevertheless, potato varieties with specific resistance to insects have not received much attention (Flanders et al., 1999).

7.1.1 *M. persicae* as an important virus vector

M. persicae is a major insect pest of crop plants worldwide, particularly seed potatoes (Blackman and Eastop, 2000; Kuroli and Lantos, 2006). Much of the damage associated with aphid feeding occurs because of the transmission of viruses such as potato leaf roll virus (PLRV), potato virus A and potato virus Y (Van Toor et al., 2009). For example, the intensity of PLRV distribution in potato fields is positively correlated with the number of aphids that previously fed on infected plants (Beekman, 1987). Thus in the absence of *M. persicae* low degree of PLRV infestation does not pose a risk for the yield. It follows that a strict control of *M. persicae* population can insure low levels of PLRV. The predominant way to control aphid density in the field is the use of insecticides. However, the emergence of insecticide-resistant clones and ever increasing

legislative pressure to limit pesticide use necessitates alternative control strategies (Fenton et al., 2010).

7.1.2 Resistance against *M. persicae* in wild *Solanum* species

Resistance against *M. persicae* has been observed in more than 60 wild *Solanum* species (Tingey and Sinden, 1982; Gibson and Pickett, 1983). Key genes from these species can be potentially used in breeding strategies aiming at enhancing resistance of commercial varieties to *M. persicae*. Nevertheless, the information concerning the biochemical and molecular responses of potato leaves to aphid attack is limited and is a perquisite to developing resistant varieties. Moreover, despite the substantial number of aphid-resistant potato species, the only defence strategy described so far is related to glandular trichomes. The presence of glandular trichomes producing various secondary metabolites is an effective strategy against aphid infestation observed in wild potatoes such as *Solanum tarijense* and *Solanum bethaultii* (Tingey and Laubengayer, 1981; Lapointe and Tingey, 1984). These metabolites might be deterrent to the aphids, cause entrapment and digestive disorders resulting in reduced overall performance (Gibson and Turner, 1977; Gibson and Pickett, 1983). Despite the fact that glandular trichome-based resistance has been extensively studied, its use in breeding strategies is limited because of the associated yield penalty and complex genetics (Bonierbale et al., 1994).

7.1.3 Polyphenols and peroxidase activities in plant-aphid interactions

ROS are considered to have direct roles in defence against insect attackers that involves changes in the cell wall properties and composition (Chaman et al., 2001). This role is achieved by the concerted action of various cell wall peroxidases that catalyse cross-linking of cell wall compounds such as lignin, phenolics, callose and hydroxyproline-rich glycoproteins (Whetten and Sederof, 1995; McDougall, 1991). For example, deposition of newly formed lignin is one of the first reactions upon fungal penetration

and wounding typically leads to oxidation of phenolic compounds (León et al., 1993). The synthesis of lignin from monolignols is mediated by peroxidases and laccases in H_2O_2-dependent reactions leading to reinforcement of the cell wall (O'Malley et al., 1993). Peroxidase activity and ROS production have previously been linked to the activities of NADPH oxidases or xanthine oxidases in the Russian wheat aphid–wheat interaction (Moloi and van der Westhuizen, 2006; Berner and van der Westhuizen 2010). Similarly, peroxidase activity and total polyphenol levels were increased in alfalfa (*Medicago sativa*) following infestation by the spotted alfalfa aphid (*Therioaphis trifolii maculata*). Feeding of the same aphid species on alfalfa resulted in browning of the cells surrounding the feeding sites and was related to accumulation of phenolic conjugates (Jiang and Miles, 1993). Furthermore, it has been proposed that oxidized polyphenols have a direct feeding deterrent effect on aphids (Miles and Oertli, 1993). The effective defence is hypothesized to be achieved by maintaining a deterrent level of monomeric quinones, while sufficient phenolics are channelled in final oxidation products deposited in the apoplast.

7.1.4 Transcriptome changes in potato upon aphid attack

Despite the urgent need to understand in details the molecular basis of potato response to aphid attack currently such information is limited. An attempt to identify genes responsive to *M. persicae* and *M. euphorbiae* feeding in potato was made by Alvarez (2007). The wild potato *Solanum stoloniferum* Schlechtd used in this study allowed the identification of genes involved in both compatible and incompatible interaction because this variety possesses an effective resistance mechanism against *M. persicae*. Using custom made cDNA microarray (3564 clones) enriched with pathogen-responsive clones the author identified numerous stress related genes differentially regulated following aphid attack. Significantly higher numbers of genes were differently regulated following *M. euphorbiae* attack than upon *M. persicae* infestation reflecting a stronger

transcriptional response in the compatible interaction. A role for transcripts involved in hormonal signalling (SA, JA and ET), calcium homeostasis, regulatory processes and general metabolism was found. Both aphid species induced the expression of *PR* genes and negatively affected photosynthesis-related transcripts. *M. euphorbiae* attack increased the transcriptional abundance of the ET biosynthetic enzyme 1-aminocyclopropane-1-carboxylate oxidase implying a significant role for ET production in compatible plant-aphid interactions.

The transcriptional and metabolic responses observed in wild type and mutant *A. thaliana* genotypes were described in the previous chapters of this thesis. ROS-related transcriptome signatures observed upon *M. persicae* attack imply that the redox homeostasis plays an important role in transmitting information about aphid perception and governs down-stream responses that may be crucial for developing effective resistance. Moreover, lack of ascorbic acid in the Arabidopsis *vtc2* mutant negatively affected *M. persicae* fecundity reflecting the important role of this antioxidant in plant-aphid interactions. The studies described in this chapter address the question of how potato (*S. tuberosum*) leaves respond to *M. persicae* attack, with a particular focus on redox-mediated processes and the role of asorbate on aphid fecundity. The findings presented in this chapter provide insights into the generic importance of the data obtained in *A. thaliana* and crucially allow the evaluation of the data with regard to translational aspects of the work from model to crop species. The transfer of information from model species is crucial for development of new aphid resilient potato cultivars and the identified generic role of the redox hub is a step in this direction.

7.2 Results

7.2.1 Impact of *M. persicae* feeding on redox metabolism in potato leaves

7.2.1.1 Metabolite levels

While the H_2O_2 content of infested leaves was similar to those of uninfested controls at 8 hpi and 24 hpi, the levels of this metabolite were increased significantly in infested leaves at 48 hpi (Fig. 7.1 A). Similarly, the GSH pool was higher in the infested leaves at 48 hpi (Fig. 7.1 C). However, leaf ascorbate levels were not changed as a result of aphid feeding (Fig. 7.1 B). Moreover, the leaf ascorbate pool of the infested plants was more oxidised at 48 hpi than that of the leaves of the uninfested control plants (Fig. 7.1 B).

7.2.1.2 Antioxidant enzyme activities

The Halliwel-Asada cycle is crucial for maintaining the reduced forms of ascorbate and GSH during H_2O_2 scavenging (Asada, 1994). First, H_2O_2 is removed by the action of ascorbate peroxidase, which requires ascorbate. The oxidized monodehydroascorbate generated in this reaction is converted back to ascorbate by monodehydroascorbate reducatase. The rest is rapidly oxidised to dehydroascorbate which is a substrate for the enzyme dehydroascorbate reductase (DHAR). DHAR requires GSH as a reducing power and the later is regenerated from GSSG by the action of glutathione reductase (GR).

The activities of two key enzymes from the Halliwell-Asada cycle DHAR and GR were comparable in uninfested and infested leaves at 8, 24 and 48 h following *M. persicae* attack. There were no significant differences analysed by two-way ANOVA between the experimental conditions in the DHAR assay ($p = 0.275$) and the GR assay ($p = 0.223$).

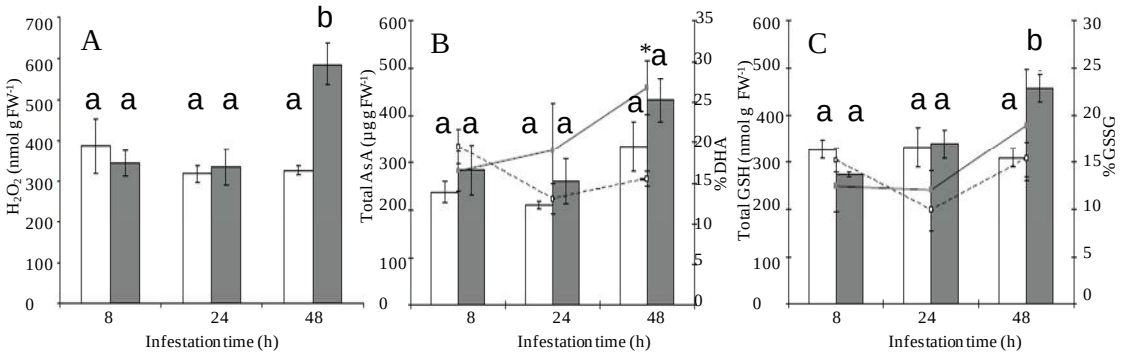

Figure 7.1 Impact of aphid herbivory on leaf oxidants and antioxidants. Mature potato leaves were infested with 60 wingless aphids confined to the abaxial leaf surface in clip cages. Uninfested controls had empty cages on. Metabolites were extracted and quantified as described in Materials and Methods (Section 2.5). A – H_2O_2 content, B – Total ascorbate content (left hand y-axis) and % total ascorbate pool as DHA (right hand y-axis), C – Total glutathione content (left hand y-axis) and % total glutathione pool as GSSG (right hand y-axis). Grey and empty bars represent data for infested and control leaves respectively. Data are the means ± SE (n=4). Significant differences in the levels of hydrogen peroxide, total AsA and total GSH were analysed with two-way ANOVA with post hoc analysis by Student-Newman-Keuls' pairwise comparison. Means with different letters are significantly different at the 5% level. Black and dotted lines represent % DHA and % GSSG in infested and uninfested leaves respectively. A significant difference between the experimental conditions according to two-way ANOVA ($p<0.05$) was found only at 48h in the levels of DHA and is indicated by an asterisk.

7.2.1.3 Levels of transcripts encoding antioxidant enzymes

The abundance of *FeSOD* transcripts were progressively decreased during the course of aphid infestation (Fig. 7.2 A). In contrast, transcripts encoding a putative cytosolic copper-zinc superoxide dismutase were relatively unchanged compared to those of uninfested control leaves (Fig. 7.2 B). However, transcripts encoding a catalase 2 isoform (*CAT2*) were repressed at the early stages of the infestation process (8 and 24 hpi) but then increased once more at the later stages of the infestation process (Fig. 7.2 C).

7.2.1.4 Visual appearance of infested tissue under the light microscope

Visual inspection of infested leaves revealed the presence of dark brown deposits along the major veins in the immediate area of feeding aphids (Fig. 7.3 A) that were most pronounced at 48 h following aphid infestation (Fig. 7.3 A). The apoplast/cell wall compartments of cells in the infested area were also filled with brown compounds that were insoluble in hot ethanol (Fig. 7.3 C).

7.2.1.5 Leaf polyphenol content and non-specific peroxidise activities

Following on from the above observations, the area of infestation sites (within the cage) was compared to the caged areas from uninfested leaves. The total polyphenol content of the infestation sites was increased by 50% relative to uninfested leaves at 48 hpi (Fig. 7.4 A). Similarly, non-specific peroxidase activities were higher in uninfested leaves than the controls at this time point (Fig. 7.4 B).

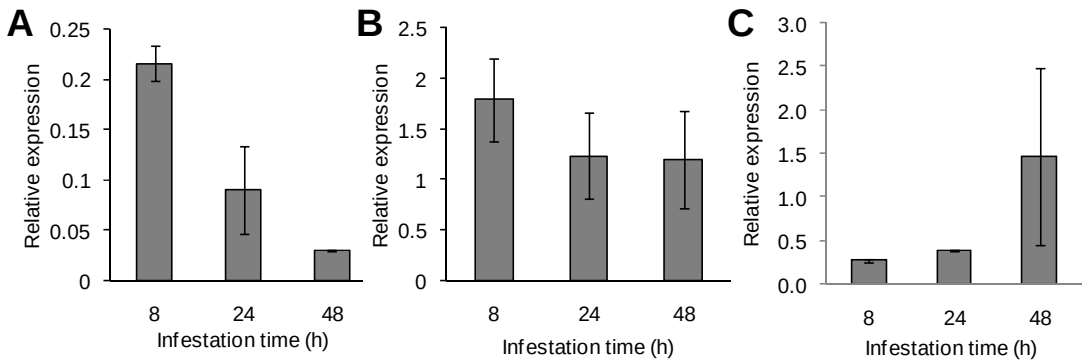

Figure 7.2 Impact of aphid herbivory on the abundance of *FeSOD* (*EU545469*, A), *Cu/ZnSOD* (*AF354748*, B), and *CAT2* (*AY500290*, C) transcripts in potato leaves. Sixty wingless aphids were confined to the abaxial leaf surface of a mature potato leaf with a clip cage and allowed to feed for 48 h. Leaf material was collected from the immediate area of infestation at 8, 24 and 48 hpi and from uninfested caged controls. RNA was extracted and transcript expression estimated as described in Material and Methods (Section 2.4). Data are the means of 3 replicates ± SE calculated relative to uninfested caged controls according to the Livak method using *elongation factor 1-α* (*AB061263*) as a reference gene.

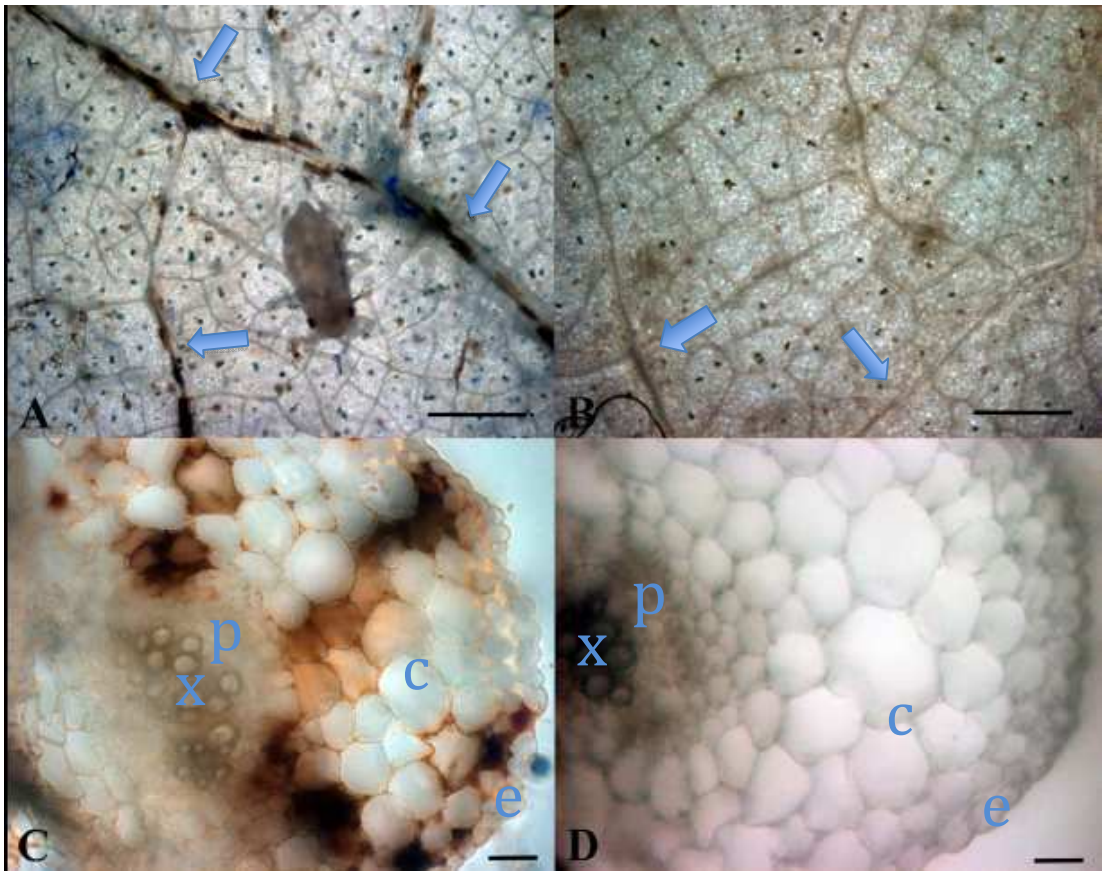

Figure 7.3 Brightfield images of potato leaves infested with *M. persicae*. Leaves were infested for 48 h with 60 wingless aphids confined to the abaxial surface of fully developed mature leaves in a clip cage. A and B are representative images of equivalent areas from the abaxial leaf surface of leaves decolorized in 70% ethanol on which aphids were allowed to feed (A) or with an empty cage on (B). Arrows indicate major veins. C and D are representative images of transverse sections through a major vein of infested (C) or non-infested (D) leaf decolorized in 70% ethanol. x – xylem; p – phloem; e – epidermis; c – collenchyma. Scale bars in A and B represent 500 μm and in C and D represent 100 μm.

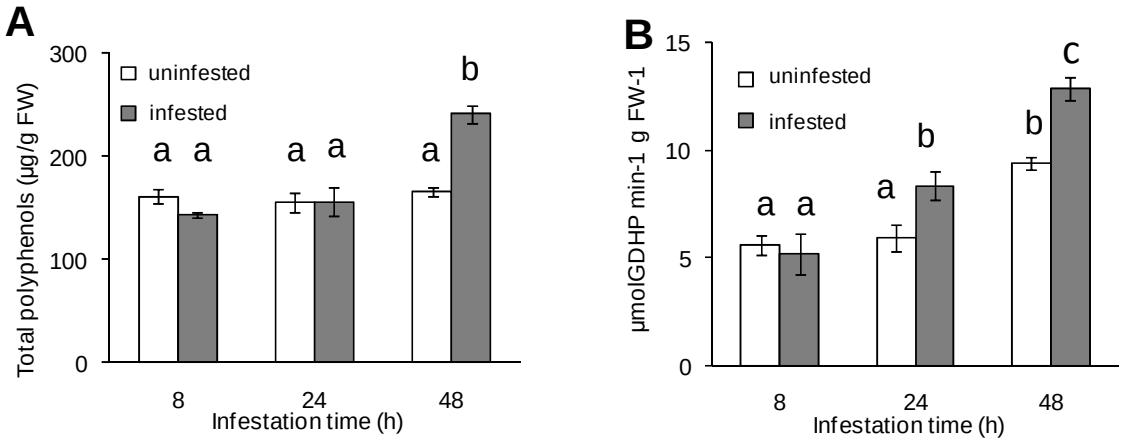

Figure 7.4 Impact of aphid herbivory on potato leaf polyphenol contents (A) and on non-specific peroxidase activities (B). Mature potato leaves were infested with 60 wingless aphids confined to the abaxial leaf surface in clip cages. Uninfested controls had empty cages on. Polyphenols and peroxidase activity were extracted and quantified as described in Materials and Methods (Section 2.9). Grey and empty bars represent data for infested and control leaves respectively. Data are the means ± SE (n=4). Significant differences were analysed with two-way ANOVA with post hoc analysis by Student-Newman-Keuls' pairwise comparison. Means with different letters are significantly different at the 5% level. GDHP - guaiacol dehydrogenation product.

7.2.2 Effect of aphid feeding on SA and JA associated transcripts

To study the effect of aphid feeding on SA- and JA-dependent signalling pathways, *PR1* expression was used a marker for SA signalling (Yoshimoto et al., 2009) and that of *JAZ1* was used a marker for the JA signalling pathway (Sheard et al., 2010). *PR1* transcripts were increased infested leaves at all time points relative to uninfested controls, but the aphid-induced increase in the abundance of *PR1* transcripts was most marked at 48 hpi (Fig. 7.5 A). *JAZ1* transcripts were increased in infested leaves at 8 hpi and values remained at a higher level than those detected in uninfested controls at all time points (Fig. 7.5 B).

To examine the effects of aphid feeding on *PR1* expression in more detail, the abundance of *PR1* transcripts was compared in the caged area with that in parts of the leaf that were distant to the caged area. In the experiment shown in Fig. 7.6, aphid feeding resulted in the accumulation of *PR1* transcripts (330-fold induction relative to the non-infested controls) at 48 hpi, but the values in the non-infested leaf areas were similar to those of the control leaves (Fig. 7.6).

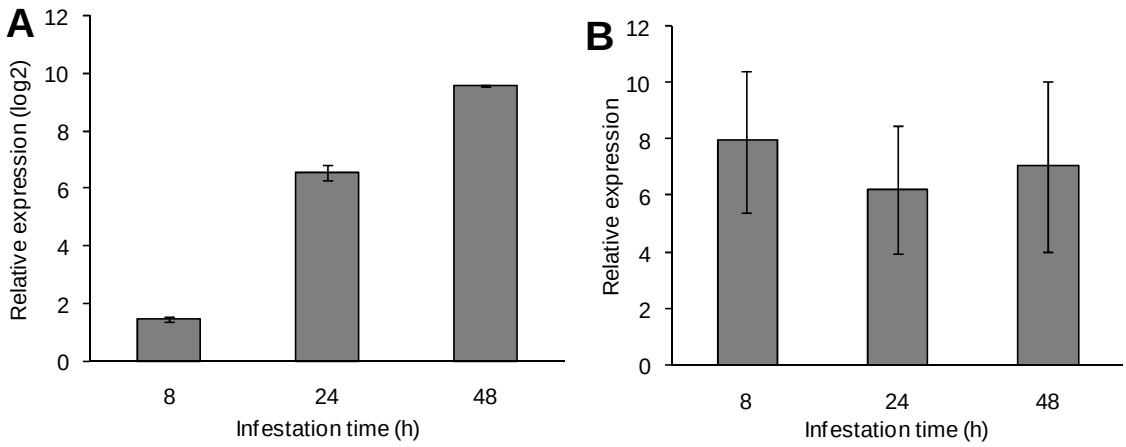

Figure 7.5 Impact of aphid herbivory on expression of phytohormone responsive transcripts. A – *PR1* (*AJ250136*), B – *JAZ1* (*EF591123*). Sixty wingless aphids were confined to the abaxial leaf surface of a mature potato leaf with a clip cage and allowed to feed for 48 h. Leaf material was collected from the immediate area of infestation at 8, 24 and 48 hpi and from uninfested caged controls. RNA was extracted and transcript expression estimated as described in Material and Methods (Section 2.4). Data are the means of 3 replicates ± SE calculated relative to uninfested caged controls according to the Livak method using *elongation factor 1-α* (*AB061263*) as a reference gene.

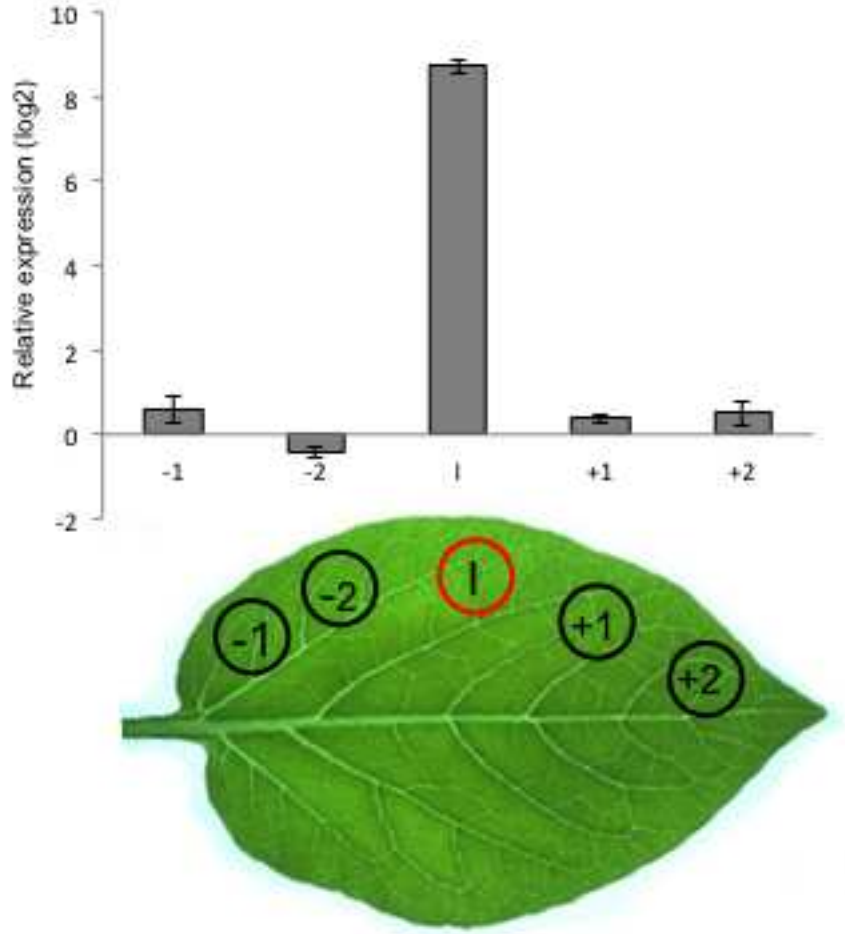

Figure 7.6 *PR1* expression in different areas of infested potato leaves. Sixty wingless aphids were confined to the abaxial leaf surface of a mature potato leaf with a clip cage and allowed to feed for 48 h. Leaf material was collected from the immediate area of infestation (I) and non-infested leaf areas (-1,-2, +1,+2). RNA was extracted and transcript expression estimated as described in Material and Methods (Section 2.4). Data are the means of 3 replicates ± SE calculated relative to uninfested caged control according to the Livak method using *elongation factor 1-α* (*AB061263*) as a reference gene.

7.2.3 Effects of leaf ascorbate on aphid fecundity

To determine how the leaf ascorbate pool might affect aphid fecundity, leaves were supplied with L-Galactono-1,4-lactone (L-GalL), which is the immediate precursor of ascorbate in the biosynthetic pathway, prior to infestation. In these experiments, L-GalL (50 mM) or water (controls) were supplied to excised potato leaves through the transpiration stream for 15 days. The ascorbate content of the L-GalL-fed leaves was greatly increased compared to that of leaves supplied with water alone (Table 7.1). Aphids cultured on high ascorbate leaves produced more offspring than those cultured on the control leaves with the lower ascorbate pool size (Table 7.1).

To determine whether the aphids feeding on the high ascorbate leaves retained more ascorbate than those feeding on the control leaves with the lower ascorbate pool size, the amount of ascorbate present in the aphids was determined after 15 days of infestation. At this time point, the ascorbate pool of the aphids was comparable regardless of the ascorbate content of all leaves on which they had been feeding (Table 7.1). The ascorbate content exuded with the honeydew was then determined in other experiments that were performed after 3 days of the onset of infestation. At this time-point the ascorbate content of the honeydew produced by the aphids feeding on the high ascorbate leaves was significantly higher than that of the aphids fed on the control leaves with the lower ascorbate pool size. However, the feeding rates were similar on the high ascorbate leaves and on the control leave (Table 7.1).

	Treatment	
	water	**50 mM L-GalL**
Leaf ascorbate (µg/gFW)[a]	661 ± 56	10888 ± 230*
Leaf DHA (%)[a,c]	21.7 ± 1.3	10.9 ± 1.4*
Aphid ascorbate (µg/gFW)[a]	566 ± 69	636 ± 71
Honeydew ascorbate [b,d]	5.2 ± 2.4	242.3 ± 58.8*
Honeydew production (drops/aphid h)[b]	0.54 ± 0.06	0.44 ± 0.05
Colony size (numbers)[a]	13.7±1.7	26.3 ± 3.6*

Table 7.1 Impact of leaf ascorbic acid contents on aphid fecundity. Experiments were conducted as described in Materials and Methods (Section 2.3.3) with either 10 ([a]) or 6 ([b]) biological replicates. Leaf ascorbate denotes the ascorbate pool of excised potato leaves supplied with water or 50 mM L-GalL, whereas leaf DHA (%) shows the oxidation status of ascorbate in both conditions. The ascorbate content of aphids fed on leaves supplied with water or 50 mM L-GalL is displayed as Aphid ascorbate. The ascorbate content exuded with the honeydew is shown as Hodeydew ascorbate. Honeydew production was calculated as honeydew drops per aphid per hour on control and high ascorbate leaves respectively. Colony size and represents the number of newly formed nymph originated from a single one-day-old nymph placed on leaves supplied with water and 50 mM L-GalL. Data are the means ±SE. Significant differences ($P < 0.05$) as estimated by the Student's t-test, are indicated by asterisks.

[c] DHA was calculated as a percentage of the total pool (ascorbate plus DHA).

[d] Relative values of ascorbate peak areas determined by HPLC.

7.3 Discussion

In potato leaves, the infestation by *M. persicae* resulted in enhanced levels of H_2O_2 and glutathione. ROS generation and ROS/hormone crosstalk have long been considered to be important in restricting insect herbivory. Microarray studies including those presented in this thesis, demonstrate that one of the earliest events occurring following aphid attack is the expression of genes that maintain cellular redox homeostasis. Transcripts encoding a chloroplast localized FeSOD and a major leaf catalase (CAT2) isoform were repressed rapidly upon aphid attack further supporting the idea that aphid perception affects key ROS metabolizing enzymes. Moreover these findings suggest that the capacity of the chloroplasts and peroxisomes to remove superoxide and hydrogen peroxide is rapidly impaired in response to the perception of the presence of aphids. Initial aphid-induced perturbations of the redox status may be localized to organelles such as the chloroplasts since the spatial and temporal regulation of ROS production is crucial for their signalling functions. Transcript abundance of a gene encoding a putative cytosolic Cu/ZnSOD (Accession number: AF354748) remained relatively stable and close to control values at any time point. The predicted cytosolic localization of Cu/ZnSOD encoded by AF354748 further supports the hypothesis that aphid feeding results in selective transcriptional reprogramming of antioxidant enzymes targeted to organelles. Changes in the expression of organelle-targeted antioxidant enzymes leading to accumulation of H_2O_2 have been reported in tobacco leaves in response to the bacterial elicitor, harpin (Garmier et al., 2007). Moreover, a previous bioinformatic analysis demonstrated that genes encoding chloroplast-targeted ROS scavenging enzymes are over-represented in the down-regulated group following a broader range of biotic interactions (Bilgin et al., 2010).

In line with this hypothesis is the observation that increases in the H_2O_2 and glutathione pools, together with a slight increase in the oxidation state of the ascorbate pools were

only observed at the later stage (48 hpi) of infestation. Moreover, the increased GSH levels may be linked to earlier redox perturbations occurring in the chloroplasts. The first enzyme of the GSH biosynthetic pathway, γ-glutamyl cysteine synthetase, resides in the chloroplasts and is activated by oxidation of crucial cysteine groups. The absence of a general compensatory increase in the activities of antioxidant enzymes such as DHAR or GR suggests that aphid-induced perturbations to cellular redox homeostasis favour enhanced oxidation. Previous work has similarly suggested perturbation of the cellular redox status in the absence of observed changes in global ROS. For example, Kuśnierczyk et al. (2008) observed up-regulation of a range of known ROS-responsive transcripts but were unable to detect ROS accumulation using the 3,3′-diaminobenzidine staining method. Similarly, Kempema et al. (2007) observed significant accumulation of a large number of oxidative stress-related transcripts but failed to observe ROS accumulation.

The appearance of brown staining in the immediate area of infestation was associated with enhanced polyphenol content, increased peroxidase activity and hydrogen peroxide. Similar pattern of brown staining was observed in alfalfa leaves (*Medicago sativa*) infested by the spotted alfalfa aphid (*Therioaphis trifolii maculata*). Moreover, during the interaction peroxidase activity and polyphenol levels were increased (Jiang and Miles, 1993). The synthesis of polyphenolic domains in potato requires free radical coupling process mediated via H_2O_2-dependent peroxidase activity (Bernards et al., 2004) implying that brown deposits may have a phenolic nature. Given the limited information it is difficult to speculate about the exact role of the deposits in the plant-aphid interaction. It is possible that they might be deterrent to the aphids or involved in cell wall strengthening. Increase of the cell wall peroxidases activities in barley following infestation with the aphid *Schizaphis graminum* (Rondani) was suggested to be involved in cell wall remodeling (Chaman et al., 2001).

PR1 gene expression was rapidly induced in the immediate area of infestation suggesting that SA signaling cascades are involved the response of potato to *M. persicae* attack. *PR1* gene expression requires the reductive activation of NPR1 that has been linked to changes in cellular GSH and S-nitroso-l-glutathione (Mou et al., 2003; Tada et al., 2008). Numerous studies have demonstrated intimate crosstalk between ROS and SA signalling in plant responses to pathogens (Mori et al., 2009), and SA is known to induce ROS accumulation (Chen et al., 1993).

Plants are able to mount an effective defense against aphids utilizing the JA transduction pathway. It has been suggested that aphids are able to manipulate the negative crosstalk between the SA and JA signalling pathways and suppress the effective defense mediated by JA (Verhage et al., 2010). In the present study, *JAZ1* was induced already at the first time point of the experiment implying an early activation of JA signaling events upon aphid perception. *JAZ1* is a negative regulator of the JA defense pathway but JA also induces its expression through a positive feedback loop (Chini et al., 2007). However, in contrast to the *PR1* transcripts which accumulated progressively, *JAZ1* expression remained stable throughout the infestation. The balance between the SA and the JA signalling pathways thus may alter during the infestation period, thereby modulating the nature of the defence responses of the plant as observed in other types of herbivory (Diezel et al., 2009).

The findings that aphid fecundity was increased on potato leaves with enhanced ascorbate contents provide further evidence in support of the view that cellular redox state plays a fundamental role in plan-aphid interactions. The amount of ascorbate retained in the aphids did not increase as a result of higher dietry ascorbate uptake. Rather, more ascorbate was excreted with the honeydew when aphids were feeding on potato leaves that were enriched in ascorbate. These results would suggest that ascorbate is not a limiting factor in the aphid diet, at least under the conditions used in

these experiments. In addition, the observation that more ascorbate was excreted with the honeydew when aphids were feeding on high-ascorbate leaves would suggest that the ascorbate present in the phloem is increased as a result of providing the leaves with the ascorbate precursor. It is possible that the high phloem ascorbate levels facilitate sap ingestion and increase the overall nutrient availability but the overall feeding rate did not appear to vary with the availability of ascorbate. The accumulation of ascorbate observed in these experiments after feeding with L-GalL is significantly higher than the levels previously reported for glasshouse grown plants (Keller et al., 1999; Tedone et al., 2004). However, high levels of ascorbate are common in plants grown in the field under high light (Rajashekar et al., 2009) and the level of ascorbate in potato leaves is significantly shifted by illumination (Tedone et al., 2004). It is therefore likely that in the field, leaf ascorbate levels will markedly change in response to prevailing environmental conditions and hence, the effects of leaf ascorbate content on aphid colony expansion reported here have physiological and agricultural relevance. While further work is required to characterize the precise mechanisms that link aphid fecundity to leaf ascorbate, the data presented here are consistent with the view that high leaf ascorbate modulates the plant immune responses to aphids.

Summary: Chapter 7

- The effect of *M. persicae* feeding on the redox homeostasis of potato leaves was studied up to 48 hpi by following the changes of major antioxidants (ascorbate and GSH), hydrogen peroxide content, enzymatic antioxidant activities (GR and DHAR), and the transcriptional abundance of *SOD* and *CAT2*.
- Aphid attack rapidly induced SA- and JA-responsive marker genes expression in the immediate area of infestation as determined by qRT-PCR analysis.

- Dark brown deposits along the major veins were observed in the infested leaves. This was accompanied by increased total polyphenol content and non-specific peroxidase activity.
- To determine how the leaf ascorbate pool might affect aphid fecundity, the ascorbate content of potato leaves was greatly increased by supplying them with L-GalL. High ascorbate leaves supported bigger aphid colonies. The ascorbate pool of the aphids was comparable regardless of the ascorbate content of all leaves on which they had been feeding and the excess amounts were excreted with the honeydew.

Chapter 8. General discussion

8.1 SA is a well recognized player in plant-aphid interactions but its role remains elusive

Plants utilize a limited number of transduction pathways and signalling molecules and the crosstalk and convergence of various internal and external stimuli at specific hubs have been shown to be crucial for the correct implementation of the environmental and developmental input signals (Mittler, 2006; Koussevitzky et al., 2008). The role of the SA mediated defence during pathogen attack have been extensively studied and numerous components involved in fine-tuning of SA synthesis and transmission of signalling events down stream of its accumulation have been shown (Loake and Grant, 2007, Tada et al., 2008). Moreover, the position of the SA transduction pathway in relation to MAPK, ROS and ET signalling have started to emerge as a result of extensive efforts to elucidate the early events following pathogen attack (Qiu et. al., 2008; Gao et al., 2008). However, SA action is not limited to pathogen attack and the involvement of SA mediated signalling in processes such as programmed cell death, photosynthesis, and high light acclimation has been shown implying a versatile and fundamental role for this plant hormone in plant growth, development and defence (Mateo et al., 2004; Rivas-San Vicente and Plasencia, 2011; Gallego-Giraldo et al., 2011). Despite the fact that the molecular mechanisms of the SA transduction cascade in various processes have received substantial attention, the pathways utilizing SA and their involvement in the plant response to aphid attack are far from elucidated. Moreover, even though aphids might have indeed evolved to orchestrate the naturally occurring negative cross talk between the SA and JA hormonal pathways in their benefit (Kempema et al., 2007), the massive induction of the SA defence transduction pathway implies a more profound impact on plant transcriptional and metabolomic reprogramming.

Numerous studies published in the last decade have reported that aphid attack activates the SA defence signalling pathway shedding some light on the molecular mechanisms of the interaction between plants and phloem feeders (De Vos et al., 2005; Moran and Thompson, 2001). Despite the fact that this response is now well recognized, its role in modulating plant physiology following aphid attack and the efficiency of the triggered defence response remains elusive. Experimental evidence failed to show unequivocally the effective role of the SA mediated defence in aphid resistance and the results reported so far are largely inconsistent (De Vos et al., 2007). The findings presented in Chapter 3 further support the involvement of the SA signal transduction pathways in response to aphid attack with numerous crucial signalling components such as *PAD4*, *EDS1* and *EDS5* being induced. PAD4 is the only SA signalling component that have been shown to affect aphid resistance but it is very likely that this mechanism is independent of its role in SA-mediated defence. For example, PAD4 mediates a phloem-based resistance against *M. persicae* that is not modulated by the absence of EDS1, an interacting partner of PAD4 in plant immunity (Pegadaraju et al., 2005; Pegadaraju et al., 2007). Similarly, aphid success is not affected on *eds1* Arabidopsis mutants suggesting that the mechanisms required for an effective pathogen resistance are not sufficient to limit aphid attack (Pegadaraju et al., 2007). Although the interaction between PAD4 and EDS1 is not required for the phloem-based resistance dependent on PAD4, most probably both protein still interact and execute SA related signalling events triggered upon aphid attack. It is inevitable that these signalling cascades will result in significant alterations of whole plant physiology during the course of infestation. Moreover, it is possible that under environmental conditions such as those observed in the field, the SA defence pathways would have a significant effect on aphid fitness and plant yield.

The majority of studies looking into aphid resistance so far was performed under control laboratory conditions where apart from the imposed aphid pressure all other parameter were kept optimal and did not restrict growth and development. This situation does not reflect the actual conditions that occur in the field where crops are often subjected to a combination of different abiotic and biotic stresses with highly variable temporal and spatial patterns of occurrence. In order to develop plants with increased aphid fitness under field conditions we need to take into account the highly unpredictable and constant interaction between biotic and abiotic stresses that occur in nature. Unravelling the wiring and interaction of biotic and abiotic stress responses occurring in plants' natural habitats is crucial to achieving a good predictive value whether the observed phenotype can be reproduced in the field. A significant number of transgenic plants displaying enhanced resistance to adverse conditions in the laboratory failed to show increased fitness in the field. Moreover, mutant plants with prominent phenotypes in controlled conditions behaved like wild type when grown in the open environment. A focus on molecular and metabolic aspects of stress combination is needed to bridge this gap leading to development of crops with enhanced tolerance to aphids in field stress conditions.

Below is a discussion of how SA mediated signalling events triggered upon aphid attack might modulate plant physiology and defence while interact with other components of the metabolic and defence machineries required for integrating the plethora of stimuli perceived simultaneously by the plant. Such systems approaches are urgently needed in order to interpret the experimental results at different levels and obtain a global view of the system. Several lines of evidence position the chloroplast at the forefront of the plant response to aphid attack with SA playing an important role in the interplay between ROS homeostasis and photosynthetic capacity.

8.2 Role of chloroplasts during aphid attack

The photosynthetic electron transport chain generates ROS even under optimal conditions (Foyer and Shigeoka, 2011). In the chloroplasts EDS1 regulates steps down-stream of superoxide accumulation that coordinate cell death by balancing the activities of SA and the ROS producing enzyme NAPDH oxidase (Straus et al., 2010). The production of ROS by the photosynthetic electron transport chain is greatly enhanced under high light, a condition that has been termed excess excitation energy (EEE). Plants are considered to experience EEE when the absorption of light energy by the photosystems exceeds the capacity of the regulated photosynthetic electron transport system, a condition that can arise as a result of changing light intensity or via limitations in carbon fixation (Karpinski et al., 2003). Acclimation to EEE is associated with cell death and is orchestrated by redox changes of the plastoquinone pool (Roden and Ingle, 2009). EDS1 and PAD4 are required for the propagation of the EEE-induced programmed cell death in the Arabidopsis LESION SIMULATING DICEASE (*lsd1*) mutant that is deregulated for EEE acclimation (Mateo et al., 2004). The cell death phenotype is induced by ET, like PAD4 and EDS1 act upstream of its accumulation, and executed through SA accumulation via redox signal relay. Similarly, transfer of wild-type Arabidopsis plants grown under low light (100 mmol m^{-2} s^{-1}) to excess light (2200 mmol m^{-2} s^{-1}) for 2 h caused significant induction of cell death (Mateo et al. 2004; Mühlenbock et al. 2008). The cell death response was exacerbated by fumigation of the plants with low levels (7.5 ppb) of ethylene and was not observed in ethylene-insensitive *ein2* mutants. Therefore, ethylene perception was considered to be a requirement for the excess-light-induced cell death response. Regulation of the redox state of the plastoquinone pool was also implicated in this response. Treatments designed to alter the redox state of the plastoquinone pool were implicated in a number of downstream responses including accumulation of hydrogen peroxide and the immediate ethylene precursor 1-

aminocyclopropane-1-carboxylate, as well as the accumulation of free and bound SA, the expression of biotic stress responsive genes and altered susceptibility to the biotrophic pathogen *P. syringae* (Mühlenbock et al., 2008).

The findings reported in this thesis show that aphid attack triggers ET signalling events. Moreover, one of the most highly up-regulated transcripts in the infested leaves throughout the experiment was *ACIREDUCTONE DIOXYGENASE* implying enhanced rates of ET biosynthesis. Thus ET synthesized during aphid infestation might be involved in processing of ROS signalling events via EDS1/PAD4-dependent relay leading to accumulation of SA and eventual cell death. Previously PAD4 has been shown to be required for *M. persicae*-induced leaf senescence (Pegadaraju et al., 2005) and this role is in line with the above shown signalling cascades executing cell death. It is possible to speculate that aphid attack impacts pathways similar to those observed under high light with the chloroplast playing a major role in the initiation of the signalling process. While aphid infestation is frequently associated with plant directed down-regulation of photosynthesis, insect resistant genotypes tend to maintain a high photosynthetic capacity and the potential links between light signalling and the plant response to aphid infestation remain to be explored. The repression of photosynthetic capacity might be related to genetically predetermined events triggered by aphid perception that result in cell death events orchestrated by the chloroplasts with ET and SA-mediated pathways playing a major role in their execution. Thus photosynthesis appears to play a pivotal role in the plant–aphid interaction presumably because it is the major driver of metabolism and it is intrinsic to light, carbon and nitrogen signalling pathways.

Links between photosynthetic efficiency and SA accumulation were postulated following the analysis of a range of Arabidopsis mutants exhibiting either elevated or reduced SA levels (Mateo et al., 2006). When Arabidopsis plants carrying mutations leading to enhanced SA levels were grown under low light (100 mmol m^{-2} s^{-1}) conditions biomass accumulation was

decreased relative to the wild type. However, the limitation on biomass production was largely overcome when the mutants were grown under high light (450 mmol m-2 s-1) conditions. The SA-accumulating mutants appeared to exhibit lower photosynthetic efficiencies than the wild-type plants. SA accumulation under low light growth conditions was associated with higher hydrogen peroxide accumulation in the leaves, together with a greater abundance of glutathione. Conversely, low SA levels were associated with low leaf hydrogen peroxide accumulation and a failure to accumulate glutathione on transfer to high light. Glutathione and nitric oxide participate in the reductive activation of the major regulatory hub of the SA mediated signalling cascade NPR1, which is required for downstream expression of a range of PR genes (Mou et al. 2003). The results of the experiments described in Chapter 7 clearly demonstrate that aphid attack increases the glutathione and hydrogen peroxide contents and this is accompanied with increased abundance of *PR1* transcripts implying enhanced rated of SA biosynthesis. Taken together these results can be related to aphid-induced repression of photosynthesis executed via SA-mediated signalling events.

The link between high light signalling pathways and those triggered upon aphid attack merits a further investigation. Whether or not aphid-induced symptoms can be attenuated when the infestation is performed under high light is of paramount interest. Moreover, studying the mechanism by which aphid resistant genotypes maintain enhanced photosynthetic rates is a promising avenue for manipulating resistance against aphids without negatively impacting plant yield.

The crucial role of the chloroplast in plant-aphid interactions is most probably not exclusively limited to SA-mediated responses. Mutant studies have also revealed significant crosstalk between photosynthetically generated ROS and jasmonate signalling. The Arabidopsis *flu* mutant accumulates protochlorophyllide in the dark, which results in the generation of plastid

localized singlet oxygen after transfer to light (op den Camp et al., 2003). The *flu* mutants display reduced growth and enhanced cell death phenotypes following a shift from dark to light that is dependent on the plastid localized proteins EXCUTER1 and EXECUTER2 (Wagner et al., 2004; Lee et al., 2007) suggesting that singlet-oxygen dependent cell death is genetically programmed and not merely the result of oxidative damage to the plastid and plasma membranes. Furthermore, *flu* mutants exhibited a specific transcriptional response that was distinct from that following treatment with the superoxide-generating herbicide paraquat. This transcriptional signature appears to be at least in part mediated by the enzymatic synthesis of a series of bioactive oxylipins including jasmonic acid and its precursor, 12-oxo-phytodienoic acid (Przybyla et al., 2008). Further evidence for crosstalk related to plastid generated singlet oxygen was provided following analysis of the transcriptional signature of the *npq4* Arabidopsis mutant, which lacks PsbS, an essential component of the system required for the thermal dissipation of EEE (Frenkel et al., 2009). PsbS is a chlorophyll-binding protein of PSII essential for the non-photochemical quenching of chlorophyll a fluorescence (Li et al., 2000), which de-excites the triplet state of chlorophyll, thereby limiting production of singlet oxygen. Field grown *npq4* plants exhibited a transcriptional profile resembling that of plants treated with MeJA and furthermore, transcripts encoding four MeJA biosynthetic enzymes were up-regulated as were lipoxygenase and allene oxide synthase (Frenkel et al., 2009). Although *npq4* mutants did not exhibit any difference in JA content when compared with wild-type plants in the absence of insect pressure, only the *npq4* mutant was shown to increase JA levels following herbivory.

8.3 Interplay between redox and hormonal homeostasis in plant-aphid interactions

Extensive experimental evidence implicate the perturbation of redox-homeostasis as one of the early events following aphid attack. The findings presented in Chapter 3, 6 and 7 of this thesis further corroborate the idea that aphid feeding results in ROS accumulation and leads

to transcriptional responses indicative of redox signalling. Given the dynamics of the infestation process and the inducible nature of the defence response it is tempting to speculate that plants utilize to a full extent their limited resource in the defence against aphids. ROS are intrinsically harmful and can cause potential damage to the aphid if ingested with the phloem sap. A highly coordinate spatial and temporal regulation of ROS production is required in this case in order to provoke localized accumulation of ROS in the vicinity of the stylet tip while allowing specific signalling events. A growing body of evidence supports a direct role for ROS in plant defences against insect herbivores. Several studies have revealed significant differences in the capacity of insects to detoxify ROS within the gut lumen. The gut lumen of herbivorous insects has high activities of a range of antioxidant enzymes, including superoxide dismutase, catalase, and all of the enzymes of the ascorbate-glutathione cycle (Ahmad, 1992; Felton and Duffey, 1992; Barbehenn et al., 2001). Variations in gut antioxidant concentrations can account for tannin-tolerance and tannin-sensitivity in grasshoppers (Barbehenn et al., 2003). Such observations suggest that significant oxidation occurs in the insect gut as a result of the dietary intake of plant metabolites and that some insect species have evolved efficient antioxidative defence mechanisms to limit oxidative stress. It is possible that some insects depend on plant antioxidants to prevent uncontrolled oxidation within the gut. Furthermore, it could be that the plant is able to influence insect performance by shifting cellular redox status i.e. to a more oxidized state.

A few studies have examined the impact of manipulating plant antioxidant status on insect performance. Larval weight gain of *S. littoralis* was significantly enhanced when feeding on the *pad2-1* (glutathione deficient) or *vtc1-1* (ascorbate deficient) Arabidopsis mutants in comparison to wild type plants (Schlaeppi et al., 2008). Enhanced insect growth was associated with a reduced capacity for glucosinolate accumulation in the *pad2-1* mutant. The mechanism that led to enhanced resistance in *vtc1-1* mutants could not be attributed to

changes in these defence compounds and therefore remains to be elucidated. In the present study, it was shown that aphid colony numbers were lower on the Arabidopsis *vtc2-1* mutants than the wild type plants (Chapter 6). Moreover, the findings reported in Chapter 7 show that aphid fecundity was increased on potato leaves with enhanced ascorbate contents. Taken together, these data show that low ascorbate has a negative impact on aphid fecundity. However, earlier experiments on *Medicago sativa* had suggested that enhancing antioxidant levels could increase resistance to aphids (Miles and Oertli, 1993). In this case, supplying low levels (1 mM) of ascorbate or glutathione reduced the expansion of *Therioaphis trifolii* maculata colonies by 30% and expansion of *Acyrthosiphon kondo* colonies by 50% (Miles and Oertli, 1993).

Currently, the mechanism by which leaf ascorbate influences aphid fecundity is hard to elucidate but given the findings describe in this thesis a likely hypothesis is that the abundance of ascorbate in the leaves alters the poise of redox signalling. For example, it is possible that the greater aphid fecundity observed in the high ascorbate potato leaves is due to a different poise or suppression of defence signalling pathways. However, apart from being a major redox buffer within the plant cells, ascorbate is also required for the activities of many enzymes including components in the biosynthetic pathways of GAs (Arrigoni and de Tullio, 2000). Moreover, the ability to accumulate ascorbate within the leaves has a major impact on growth and defence (Pastori et al., 2003; Pavet et al., 2005). Thus altered levels of ascorbate might have profound pleiotropic effects which are difficult to account for. Within this context it is not surprising that low ascorbate levels result in enhanced leaf ABA and SA contents and influence numerous signalling pathways. A detailed characterization of one of those pathways is presented in Chapter 5 of this thesis that demonstrates unequivocally that the ascorbate-dependent control of growth requires the ABI4 transcription factor. Moreover, the enhanced resistance against *M. persicae* observed in the *vtc2* mutants also requires the

presence of a functional ABI4. The *abi4vtc2* mutant showed wild type levels of resistance suggesting that the enhanced susceptibility of the plants lacking a functional ABI4 can be at least partly counteracted by the effects of the low ascorbate. Whether or not this can be attributed to ABA-mediated signalling independent of ABI4 remains an open question that requires further experiments. The role of other ABA signalling components in modulating aphid resistance such as ABI1 and ABI2, which have been long known to function in stress signalling cascades involving ROS as second messengers (Allen et al., 1999), is especially promising to be explored on its own and in low ascorbate background. There is some evidence to suggest that this hormone plays a crucial role in plant defence against insects. While the application of ABA to barley plants infested with Russian wheat aphid did not alter aphid-induced damage (Miller et al., 1994), tomato mutants with low ABA levels were less resistant to *S. exigua* feeding (Thaler and Bostock, 2004). Similarly, *S. littoralis* larvae gained more weight on Arabidopsis mutants carrying the *aba2-1* mutation and thus having impaired ABA synthesis, accumulating only 9 % of the wild type ABA levels (Bodenhausen and Reymond, 2007). The gene product of *ABA2* converts xanthoxin to ABA-aldehyde in ABA biosynthesis (Gonzalez-Guzman et al., 2002).

The role of ABI4 in chloroplast to nucleus retrograde signalling have been described and emerging evidence is supporting the view that ascorbate transmits signals originating in the chloroplasts (Kiddle et al., 2003; Koussevitzky et al., 2007). The findings displayed in Chapter 5 show that the lack of ascorbic acid and ABI4 trigger largely comparable transcriptome profiles suggesting overlapping participation in chloroplast to nucleus signalling. In the absence of a functional ABI4 chloroplast regulated nuclear genes under the control of ABI4 will be insensitive to perturbation of chloroplast function. Thus if indeed chloroplasts play a crucial role in plant-aphid interaction the enhanced susceptibility of the

abi4 mutants can be explained by the inability of the chloroplast to signal the effects of aphid attack and translate them to nuclear expression and effective resistance.

8.4 Future perspectives

Huge resources are currently directed at the control of insect pests through the application of synthetic insecticides, which have been proven highly effective in the reduction of specific pests and resulted in accompanying yield increases (Cooper and Dobson, 2007). However, both consumers and politicians in Europe have increased their demand for food to be produced in an environmentally sound manner. Farmers are asked to not only produce high quality food at low prices, but are also required to minimize their impact on the environment. Among the requirements is the reduction of pesticides. This makes aphid pest management harder than ever. Additional factors contributing to that include a switch to crop varieties that have inherently lower aphid resistance, the destruction of natural aphid enemies through a lack of insecticide specificity and the development of insecticide resistance (Fenton et al., 2010). Improved strategies to control pest numbers are urgently needed. These include appropriate selection of aphid-resistant cultivars, the use of crop rotations, the encouragement of aphid predators and the use of selective agrochemicals when all else has failed. Key to such strategies is the development of crop varieties that exhibit specific resistance and/or tolerance traits allowing maintenance of crop yield. Despite that research has made considerable progress towards our understanding of the complexity of plant responses to aphid infestation and the molecular bases of resistance and tolerance traits, more efforts are needed to reconstruct the signalling cascades activated during aphid attack. Currently, our knowledge about plant-aphid interactions is fragmentary and limited to particular signalling pathways such as those mediated by SA and JA. Systems biology is the key to understand the plant response from the point of view of the whole organism and pinpoint important regulatory hubs orchestrating growth, development and defence. Manipulation of such hubs

is likely to modulate aphid resistance while allowing enhanced annual yields. The crucial roles of the redox homeostasis and ABA-mediated signalling cascades in aphid resistance identified in the current study are ideal candidates for future exploration. Moreover, as described in Chapter 5 of this thesis they are highly intertwined in the control of plant growth. Given the complex nature of these hubs it is important to identify the role of other components functioning alongside ascorbic acid and ABI4 in manipulating aphid resistance. Ascorbic acid is only one of the many antioxidants contributing to the cellular redox homeostasis and experiments tailored to examine the role of other non-enzymatic ROS scavenging molecules such as GSH during aphid attack will be especially useful to reveal whether the observed effects are unique to ascorbic acid. Similarly, more information is needed about the components of the signalling pathways utilizing ABI4 in plant-aphid interactions in order to understand in more details the role of ABA-mediated signalling pathways in orchestrating defence and yield related phenotypes.

Of crucial importance to identify the underlying mechanism at the molecular level is to develop high-throughput screening methods for aphid success. They can be utilized to identify sources of resistance in germplasm collections of agricultural and model species. Moreover, screening programs taking advantage of gain- or loss-of-function Arabidopsis mutants will inevitably expand our understanding of the molecular basis of aphid resistance. Although not of agricultural importance, studying the basis of resistance in model species such as Arabidopsis is more timely than ever. The complete genome sequence of over 80 Arabidopsis accession have been released so far as a part of the ‘1001 Genomes Project’ aiming at identifying the sequence variation in 1001 Arabidopsis inbred lines (http://1001genomes.org/). This sequence information can be used in combination with studies looking into the natural variations of aphid resistance and pave the way for novel breeding programmes.

Breeding programmes to develop crop varieties resistant to aphids will greatly benefit from the availability of information underlying the molecular basis of resistance. This will not only speed up the development of novel varieties through marker-assisted selection but also provide information on how aphids overcome plant resistance that is of particular importance for achieving stable traits. Genes from wild crop species that display elevated natural levels of resistance are especially promising for breeding strategies aiming at enhancing aphid resistance of commercial high yield varieties.

Much of the threat associated with aphid attack is related to the transmission of viruses. Many of the agricultural important viruses are acquired during brief probing periods, remain attached to the stylet and can be transmitted to another plant. Knowing the exact timing of the interaction of the stylet with the plant tissue is crucial to estimate the extent of aphid-borne virus infection in the field. Moreover, resistance mechanisms that minimize stylet probing activities will be especially useful in an agricultural context. In contrast, phloem based resistance will be of little value for crops for which aphid infestation is predominantly related to viral dissemination. Electrical penetration graph (EPG) techniques are a powerful tool to localize the basis of resistance and should be more widely used in a high-throughput mode.

The existence of naturally occurring chemical compounds that are deterrent to aphids implies that the metabolic diversity found in plant species can be utilized to identify new resistance mechanisms. Metabolite profiling of aphid resistant cultivars has the potential to implicate previously unknown compounds as natural insecticides. One of the major drawbacks of the modern metabolomics techniques is the structural elucidation of the huge number of unknown peaks. Chemical informatics tools are emerging that can narrow down the number of structures corresponding to a chromatographic peak. Such approaches will greatly facilitate metabolite discovery and in combination with whole genome studies will inevitably result in elucidating new biochemical pathways important for aphid resistance.

To fully understand plant-aphid interactions we should not exclusively focus on the plant response but also monitor aphid behavior and physiology during the attack. Overcoming plant resistance requires an active approach tailored to the specific plant chemical composition and physiological status and is most probably highly coordinated process. Until recently the molecular mechanisms underlying aphid abilities to utilize plants as food source have been largely unknown. The sequencing of the pea aphid genome (The International Aphid Genomics Consortium, 2010) and the growing amount of genetic information for other aphid species such *M. persicae* opens a new avenue of research. Silencing of aphid genes using RNA interference (RNAi) technology has the potential to change the way we study plant-aphid interactions. The first example of RNAi-mediated gene knockdown in aphids revealed that C002, a gene with high transcript abundance in the salivary glands of *A. pisum*, is vital for establishing a successful interaction with the plant host (Mutti et al., 2006). Moreover, aphids with silenced C002 were able to survive on artificial diets, but not on plants (Mutti et al., 2008). Microinjection and artificial diets can be an efficient way to perform RNAi in aphids but both of them do not represent a natural treatment. A breakthrough in this direction was the down-regulation of *M. persicae* gene expression using a plant-mediated RNAi method that delivers double-stranded RNA synthesized by the plant to the aphid during phloem sap ingestion (Pitino et al., 2011). How aphids modulate plant defenses and the role of potential effector proteins injected in the plant tissue is crucial for our understanding of the molecular interaction between plants and aphids. RNAi techniques will play a major role in the elucidation of the function of genes essential for aphid success. Moreover, revealing the role of specific genes important for acquiring resistance against major insecticides will be of particular importance to develop novel strategies for aphid control.

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