Induced defence in wild cabbage: integrating genes, volatiles & insect behaviour

(Stirrup, 2003)

Timothy J Stirrup

Thesis submitted for the degree of Doctor of Philosophy

University of Southampton, UK

Faculty of Medicine, Health & Life Sciences

School of Biological Sciences

March 2007

University of Southampton

NATURAL ENVIRONMENT RESEARCH COUNCIL

I would feel more optimistic about a bright future for man if he spent less time proving that he can outwit Nature and more time tasting her sweetness and respecting her seniority.

E. B. White (1899 - 1985)

ABSTRACT
UNIVERSITY OF SOUTHAMPTON
FACULTY OF MEDICINE HEALTH AND LIFE SCIENCES
SCHOOL OF BIOLOGICAL SCIENCES

Doctor of Philosophy

INDUCED DEFENCE IN WILD CABBAGE: INTEGRATING GENES, VOLATILES AND INSECT BEHAVIOUR

By Timothy J Stirrup

This study integrates three methods for detection of an induced response to aphid feeding. Firstly, it is shown that volatile organic compounds emitted from wild cabbage infested with the cabbage aphid *Brevicoryne brassicae* attract the aphid parasitoid *Diaeretiella rapae* and could constitute a reliable and detectable cue during parasitoid host foraging. It is found that there is no difference in attraction to aphid-infested plants when comparing field-collected and lab-reared parasitoids. Secondly, collection and analysis of compounds emitted from the aphid and *Plutella xylostella* damaged plants reveals several compounds exhibiting significant induction when compared to uninfested plants. Univariate techniques and multivariate canonical discriminant analysis reveal distinct volatile profiles contain several terpenes and 3-butenyl isothiocyanate as induced compounds that could be involved in the attraction of the parasitoid. It is proposed that this multivariate technique may closer resemble the method of signal recognition used by foraging insects.

Finally, global gene expression analysis using *Arabidopsis thaliana* microarrays coupled to a *Brassica oleracea* specific software filter is used to assay the expression of genes induced by aphid and *Plutella* feeding. These experiments reveal the aphid- and *Plutella*-related induction of genes involved in isoprenoid biosynthesis, involvement of the octadecanoid pathway following *Plutella* feeding and that although the genes induced are different, number of genes induced by the different herbivores is roughly equal. AFLP analysis illustrates the high degree of genotypic variation in wild *Brassica* populations when compared to crop cultivars and *Arabidopsis thaliana*. This suggests that the wild populations are segregating with a high level of gene flow between populations.The importance of an integrated approach to studying these research themes is discussed and a novel mechanism for the release of glucosinolate derivatives due to aphid-myrosinases is proposed.

Table of Contents

ABSTRACT ii

Table of Contents iii

Abbreviations ix

List of figures 11

List of tables 13

Declaration of authorship 14

Acknowledgements 15

Chapter 1 – Introduction 16

1.1 – Plant defence 16

1.2 The tritrophic system 18

1.2.1 The first trophic level - Brassica oleracea (L) subsp. oleracea 18

1.2.2 The second trophic level - Brevicoryne brassicae (L) (Hemiptera: Aphididae) 24

1.2.3 Diaeretiella rapae (MacIntosh) (Hymenoptera: Aphidiinae) 29

1.3 Mechanisms of induced plant defence 32

1.3.1 The Aliphatic Glucosinolate pathway 33

1.3.2 Octadecanoid / Jasmonic acid pathway 38

1.3.3 Salicylic acid pathway 41

1.4 Volatile organic compound emission following herbivory 42

1.4.1 Genes encoding terpene synthases and induced defence components 44

1.5 Thesis aims and hypotheses 46

1.5.1 Chapter 2 - Aims and Hypotheses 46

1.5.2 Chapter 3 – Aims and Hypotheses 46

1.5.3 Chapter 4 – Aims and Hypotheses 47

Chapter 2 – Response of the parasitoid *Diaeretiella rapae* to aphid-induced volatiles from *Brassica oleracea*. 49

2.1 Introduction 49

2.1.1 Hypotheses: 53

2.2 Materials and Methods 54

2.2.1 Plants and insects 54

2.2.2 Parasitoids 54

2.2.3 Y-tube olfactometer bioassays 55

2.2.4 Y-tube olfactometer bioassays - Experiment 1 – May 2005 56

2.2.5 Y-tube olfactometer - Experiment 2 – July 2005 57

2.2.6 Statistical Analysis 57

2.3 Results 58

2.3.1 Y-tube olfactometer bioassays - Experiment 1 – May 2005 58

2.3.2 Y-tube olfactometer bioassays - Experiment 2 – July '05 60

2.4 Discussion 62

2.4.1 Comparison of the foraging behaviour of wild and lab-reared parasitoids 62

2.4.2 Parasitoid response to plant odours supplemented with 3-butenyl isothiocyanate 63

2.5 Summary 66

Chapter 3 Volatile organic compound emissions from the wild cabbage *Brassica oleracea* 67

3.1 Introduction 67

3.1.1 Hypotheses 70

3.2 Materials and Methods 71

3.2.1 Plants and insects 71

3.2.2 Air entrainments from treated plants 71

3.2.3 Volatile analysis using Gas Chromatography 73

3.2.4 Compound identification using GC-MS 73

3.2.5 Experiment 1 – Identification and quantification of volatiles released from aphid-infested plants (January 2006) 73

3.2.6 Experiment 2 – Identification and quantification of volatiles released from aphid-infested and Plutella-infested plants (July 2006) 74

3.2.7 Statistical analysis 74

3.3 Results **75**

3.3.1 Experiment 1 - Identification and quantification of volatiles from aphid-infested plants (January 2006) 75

3.3.2 Experiment 2 – Identification and quantification of volatiles released from aphid-infested and Plutella-infested plants (July 2006) 82

3.3.3 Discriminant analysis 86

3.3 Discussion **89**

3.4.1 Common plant volatiles emitted following herbivory 89

3.4.2 Species-specific, aphid-mediated volatile emission 91

3.4.3 Treatments can be grouped by using canonical analysis of discriminance 93

3.4.4 Cross-spectrum volatile analysis can identify potential infochemicals 94

3.5 Summary **95**

Chapter 4 - Analysis of gene expression in aphid challenged *Brassica oleracea* ... **96**

4.1 Introduction **96**

4.1.1 Global gene expression analysis using microarrays 99

4.1.2 Hypotheses: 100

4.2 Materials and Methods **101**

4.2.1 Global gene expression analysis using Affymetrix microarrays 101

4.2.2 Experimental design 103

4.2.3 Plant growth and insect culture conditions *105*

4.2.4 B. brassicae infestation *105*

4.2.5 P. xylostella larvae infestation *105*

4.2.6 Plant tissue collection *106*

4.2.7 RNA extraction *106*

4.2.8 Hybridisation of Arabidopsis ATH1 GeneChip® arrays *107*

4.2.9 Analysis of microarray data *107*

4.2.10 Gene Ontology classification using Genemerge *108*

4.2.11 Primer design for qPCR *108*

4.2.12 cDNA synthesis *109*

4.2.13 Real-time quantitative polymerase chain reaction (qPCR) *109*

4.2.14 Analysis of qPCR data *109*

4.2.15 Correlation of gene expression levels measured using qPCR and microarrays *110*

4.3 Results **112**

4.3.1 Probe selection for cross-species hybridisation *112*

4.3.2 ANOVA analysis using the R statistical environment *113*

4.3.3 Ontological classifications *115*

4.3.4 Real-time quantitative PCR analysis *119*

4.4 Discussion **122**

4.4.1 Genes induced by aphid or Plutella feeding *123*

4.4.2 Genes involved in isoprenoid biosynthesis are induced by both aphid and Plutella feeding *126*

4.4.3 Correlation of gene expression ratios from real-time PCR and microarray analysis *129*

4.4.4 Herbivore-induced genes in a wild multitrophic system *134*

4.5 Summary **135**

Chapter 5 - Population genetics of the *Brassicaceae* **136**

5.1 Introduction **136**

5.1.1 Genetic diversity of the Brassicaceae 136

5.1.2 Amplified fragment length polymorphism analysis 139

5.1.3 Hypotheses: 141

5.2 Materials and Methods 142

5.2.1 Plant preparation 142

5.2.2 DNA Extraction 143

5.2.3 Gradient PCR 143

5.2.4 AFLP Protocol 143

5.2.5 Analysis 1 – Proportion of polymorphic loci 146

5.2.6 Analysis 2 – Construction of genetic distance matrix and phylogram 147

Results 149

5.3.1 AFLP Fragment analysis 149

5.3.2 Analysis 1 – Proportion of polymorphic loci 150

5.3.3 Analysis 2 – Construction of genetic distance matrix and phylogram 151

5.4 Discussion 154

5.4.1 Brassica oleracea var. oleracea has a highly diverse genotype 154

5.4.2 Arabidopsis thaliana exhibits limited sequence similarity to Brassica species studied 157

5.4.3 The importance of genetic diversity in a natural context 158

5.5 Summary 159

Chapter 6 - General Discussion 160

The response to aphid-induced plant volatiles by parasitoid wasps 160

The origin of aphid-induced volatile compounds 162

Epicuticular wax formation following aphid-feeding on wild B. oleracea 165

The need for an interdisciplinary approach to studying plant-aphid interactions 166

Appendix A 170

Appendix B 170

Appendix C **172**

Bibliography **174**

Abbreviations

AITC	Allylisothiocyanate
CaMV	Cauliflower Mosaic Virus
CATMA	Complete Arabidopsis Transcriptome MicroArray
CDA	Canonical Discriminant Analysis
cM	Centimorgan
DMNT	(E)-4,8-dimethyl-1,3,7-nonatriene
DTT	Dithiothreitol
EAG	Electroantennography
EDTA	Ethylene diamine tetraacetic acid
EST	Expressed sequence tag
FID	Flame ionisation detector
GLV	Green leaf volatiles
GPP	Geranyl pyrophosphate
GSL	Glucosinolate
GST	Gene-specific Sequence Tags
HPLC	High pressure liquid chromatography
IDV	Integrated density value
IPM	Integrated pest management
IPMS	isopropyl malate synthetase
IPP	Isopentenyl pyrophosphate
ITCs	Isothiocyanates
JA	Jasmonic acid
MeJA	Methyl jasmonate
MeSA	Methyl salicylate
MGD	Mean genetic distance
MGED	Microarray Gene Expression Data (MGED) Society
MIAME	Minimum Information About a Microarray Experiment
NASC	Nottingham Arabidopsis Stock Centre
PHC	Plant Host Complex

qPCR	Real-time quantitative polymerase chain reaction
SA	Salicylic acid
SAR	Systemic acquired resistance
SNP	Single Nucleotide Polymorphism
TAIR	The *Arabidopsis* information resource
TD	Thermal desorption
TIGR	The Institute for Genomic Research
TuMV	Turnip Mosaic virus
UPGMA	Unweighted Pair Group Method Analysis
VOC	Volatile organic compound

List of figures

Chapter 1

1.1 A graphical representation of the tritrophic interaction
1.2 Distribution map of European *Brassicaceae*
1.3 Photographs of *B. oleracea* in ther natural habitat
1.4 Ancestral tree detailing evolution of the Brasssicaceae
1.5 *Brevicoryne brassicae* feeding on a *B. oleracea* leaf vein
1.6 Cross-sectional diagram showing aphid feeding
1.7 Drawing of *D. rapae* ovipositing in an aphid host
1.8 Life cycle of the parasitoid *D. rapae*
1.9 Photo series showing emergence of parasitoid larva from aphid mummy
1.10 Chemical structure of volatile organic compounds that cause a behavioural or physiological response in *D. rapae*
1.11 Biochemical pathway illustrating glucosinolate hydrolysis by myrosinase
1.12 Major gene loci involved in synthesis or modification of GSLs
1.13 The octadecanoid pathway detailing enzymes involved in reaction steps
1.14 The volatile organic compound cis-jasmone

Chapter 2

2.1 Y-tube olfactometer apparatus setup
2.2 Initial and final selections by *Diaeretiella rapae* in a y-tube olfactometer
2.3 Boxplot showing the times taken for an initial and final parasitoid choice
2.4 Parasitoid y-tube choice analysis
2.5 Boxplot showing the times taken for an initial and final parasitoid choice

Chapter 3

3.1 Air entrainment apparatus
3.2 Example chromatogram

3.3 Experiment 1 - Mean total quantity of volatiles released from control and aphid-infested plants
3.4 Experiment 1 - VOC emission quantity for significantly different compounds
3.5 Experiment 1 - Linear representation of the discriminant scores (a)
3.6 Experiment 1 - Linear representation of the discriminant scores (b)
3.7 Experiment 2 - Total volatiles emitted from herbivore infested plants when compared to an undamaged control plant
3.8 Experiment 1 - VOC emission quantity for significantly different compounds
3.9 Scatter plot showing the position of control and treated samples according to the two discriminant functions

Chapter 4

4.1 Diagram of experimental design for global gene expression analysis
4.2 Graph showing the percentage of genes included in the microarray analysis at different intensity thresholds
4.3 Frequency of ANOVA p-values in microarray analysis
4.4 Venn diagram summarising the number of genes upregulated in response to treatment
4.5 Single gene expression ratio comparison
4.6 Correlation of qPCR expression ratios with microarray expression ratios
4.7 Pathway illustrating the production of terpene precursors
4.8 Glucosinolate hydrolysis pathway

Chapter 5

5.1 Photographs showing the differing morphologies of the Brassicaceae
5.2 An example image taken from the AFLP fragment profile
5.3 Frequency distribution of sample fragments from AFLP analysis
5.4 Distance matrix showing pairwise distances between AFLP samples
5.5 Phylogram constructed from AFLP data showing genetic distance between samples

List of tables

Chapter 1

1.15 Aphid species parasitised by the parasitoid *D. rapae*

Chapter 3

3.1 Experiment 1 - Quantity of volatiles detected from treated and control plants

3.2 Experiment 1 - Canonical analysis of discriminance statistics (a)

3.3 Experiment 1 - Canonical analysis of discriminance statistics (b)

3.4 Experiment 2 - Quantity of volatiles detected from treated and control plants

3.5 Experiment 2 - Eigenvalue statistics for the two discriminant functions

3.6 Experiment 2 – Discriminant function statistics

Chapter 4

4.1 Summary of sample treatments and identifiers

4.2 Number of genes represented by the Affymetrix microarray at different intensity thresholds

4.3 Gene ontology analysis of genes induced by aphid feeding

4.4 Gene ontology analysis of genes induced by *Plutella* larval feeding

4.5 Gene ontology analysis of genes induced by both treatments

4.6 Genes induced by plant treatments that are involved in isoprenoid biosynthesis

Chapter 5

5.1 Summary of plants used for AFLP analysis

5.2 Sequences of AFLP primers and adaptors

Acknowledgements

This PhD project was funded by the Natural Environment Research Council, UK. Additional funding for microarray experiments was generously donated by the Rank Prize Fund Nutrition Committee.

My sincere thanks and a great deal of respect go to my two supervisors Professor Guy Poppy and Dr Mark Dixon. Without their guidance, ideas and patience, none of this would have been achieved. I would like to thank Dr Alex Stewart-Jones for his friendship, advice and invaluable assistance with all things chemical, Dr Ed Hunt and Dr Katy Johnson for their friendship and for sharing all the ups and the downs. I thank Dr Martin Broadley and colleagues at the Nottingham Arabidopsis Stock Centre for their guidance and assistance with the microarray analysis. Also, Dr Bregje Wertheim and Dr Lex Kraaijeveld for their support and advice on bioinformatics questions. Thanks to my parents for inspiring me to go my own way and finally, I would like to thank my partner Nici for her love and support throughout. I look forward to sharing a lifetime of fun and adventures with her.

Chapter 1 – Introduction

1.1 – Plant defence

In their defence against phytophagous insects, plants have evolved a number of strategies. Constitutively produced and accumulated secondary metabolites are extremely widespread methods of defence throughout the Kingdom Plantae. These direct defences can be toxic to herbivores, result in lignification or resin production or deter feeding or egg deposition. Other plants minimise damage through rapid growth or evolutionary adaptation to a niche habitat that is inhospitable to herbivores (Strong et al., 1984). As opposed to constitutive defences, induced defence strategies involve a perception and response to the threat. Induced responses can be effective in reducing herbivory against a variety of insect herbivores depending on the stimulus received (Thaler et al., 2001). Many plants use indirect defence mechanisms involving the release of volatile organic compounds that promote the effectiveness of natural enemies of the attacking herbivore (Karban and Myers, 1989; Dicke and van Loon, 2000). One method of doing this is by releasing a blend of volatile organic compounds (VOCs) that are attractive to predatory or parasitoid insects. These insects may reduce the impact of the herbivore on the plant and result in an overall increase in fitness in which case the attraction may be considered a defensive mechanism by the plants. This kind of tritrophic interaction has been shown for many plant species, belonging to at least 12 plant families (Dicke et al., 2003a). This study will address the tritrophic system summarised in Fig. 1.1 involving the interaction between *Brassica oleracea* subsp. oleracea, the cabbage aphid - *Brevicoryne brassicae* and the aphid parasitoid - *Diaeretiella rapae*. The components and possible interactions will be introduced in this chapter.

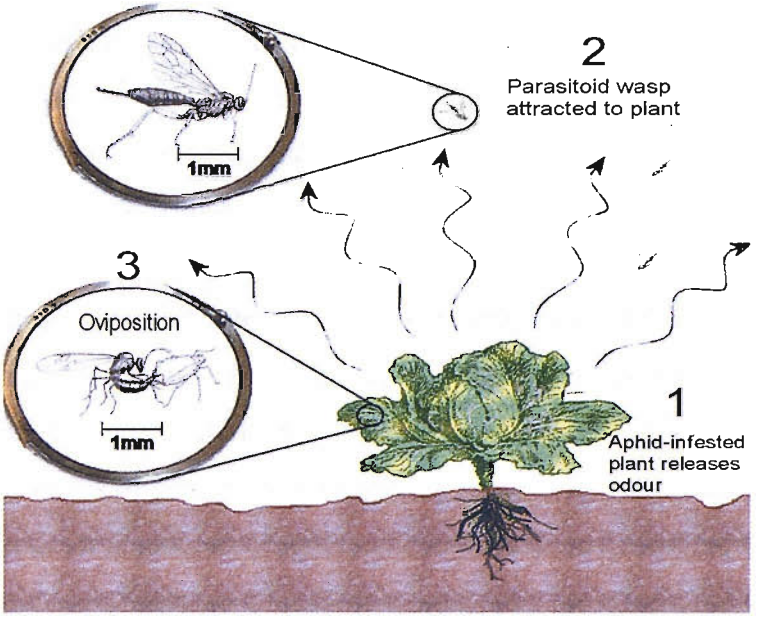


Fig. 1.1 – A graphical representation of the tritrophic interaction between *Brassica oleracea* subsp. oleracea, the cabbage aphid - *Brevicoryne brassicae* and the aphid parasitoid - *Diaeretiella rapae*.

An understanding of the defences that plants employ in a natural context could lead to more effective biological control and reduced or novel pesticide use through the elicitation of plant defence responses prior to large scale pest infestation. Functional characterisation of genes involved in plant defence may enable the improvement of crops by marker assisted breeding or gene introgression techniques.

Throughout this study, an holistic understanding of the system has been pursued through the integration of reductionist science with ecological observations. This overarching principle has been pursued in the acknowledgment that the natural system is an open one that is located in an environment affected by numerous factors about which we have a limited understanding.

1.2 The tritrophic system

There is a substantial body of evidence that suggests that *Brassica* species are able to indirectly defend themselves from attack by phytophagous arthropods by recruiting natural enemies of the attacking herbivore (Vaughn et al., 1996; Dicke and van Loon, 2000; Blande, 2004). This mutualistic relationship could have evolved through the natural selection of parasitoids that respond to the volatile compounds released following damage of the plant by the parasitoid larva hosts. The benefit of reducing aphid populations on the plant (and therefore the reduction in likelihood of contracting aphid-borne viruses) could in turn confer a selective advantage on those plants that produce high levels of synomone precursors. There is still some debate about whether a selective advantage is indeed conferred on the plant since parasitism does not immediately terminate consumption of plant resources by the attacking herbivore (Dicke and van Loon, 2000; van der Meijden and Klinkhamer, 2000; Kessler and Baldwin, 2001). A benefit in the form of increased seed production has been observed following induction of a direct defence in tobacco (Baldwin, 1998) and an indirect defence in maize (Hoballah and Turlings, 2001). More recent research conducted by Hoballah *et al.* (2004) assesses the costs of herbivore-induced volatile production and concludes that an overall fitness benefit would be observed in an environment with natural enemies present. This concept is an extremely difficult one to measure due to the number of variables affecting a plant in its natural context. Despite being beyond the scope of this study, the cost-benefit analysis of induced responses is an extremely important question and one that is fundamental to the field of herbivore-induced plant volatiles. This study instead focuses on the signals that mediate the interaction between the three trophic levels.

1.2.1 The first trophic level - *Brassica oleracea* (L) subsp. oleracea

Unless indicated otherwise, *Brassica oleracea* subsp. oleracea will also be referred to as wild *B. oleracea* or wild cabbage. A suffrutescent (having a woody base) perennial, wild *B. oleracea* is normally found in small demes and can grow 50–150cm tall when mature with a stem diameter of 30-50mm. It is a dicotyledenous diploid with diploid chromosome number of 2n=18. It has evolved to be able to survive in shallow soils and

exposed conditions, being able to withstand frosts down to -10^{O}C and highly halophytic conditions where few competitors can survive (Snogerup et al., 1990). Brassica *oleracea* subsp. oleracea is the original wild relative from which are descended many commercial crops used in the cuisine of a number of cultures. These are loosely categorised into oilseed, vegetable, and condiment crops. *B. napus, B. rapa* (formerly *campestris*), *B. juncea,* and *B. carinata* provide about 12% of the worldwide edible vegetable oil supplies (Paterson et al., 2001). Many crops of the species *B. oleracea* and *B. rapa* have been selectively bred to express extreme morphological characteristics such as the enlarged inflorescences of cauliflower (*B. oleracea* subspecies *botrytis*) and broccoli (*B. oleracea* subspecies *italica*). Other examples of such morphologies include; the enlarged stem of kohlrabi (*B. oleracea* subsp. gongylodes); the enlarged root of turnip (*B. rapa* subsp. rapifera); the enlarged single apical bud of cabbage (*B. oleracea* subsp. capitata) or the many axillary buds of Brussels sprout (*B. oleracea* subsp. gemmifera). Finally, the seed of *B. nigra* is utilised to make mustard (Paterson et al., 2001). *Brassica* species are a valuable source of dietary fibre, vitamin C, and are a source of glucosinolates which have been shown to have anti-carcinogenic properties (Talalay and Fahey, 2001). Estimates of the economic importance of *Brassica* species are conservative, because several cole crops such as collards are cultivated primarily for local or home use, but are nonetheless a dietary mainstay in low-income communities where other fresh vegetables can be prohibitively expensive (Paterson et al., 2001).

The family Brassicaceae is widely distributed and comprises about 340 genera and more than 3350 species (Alshehbaz, 1984). The family's major centres of diversity are south-western and central Asia and the Mediterranean region. Secondary centres of diversity are western North America and the mountains of South America (Price and Palmer, 1994). More specifically, wild *B. oleracea* is distributed among a variety of coastal habitats in northern Spain, western and northern France, the UK and the German island of Helgoland.

Fig. 1.2 – Distribution mapof European *Brassica* spp. (adapted from Snogerup 1990)

Mitchell (1976) and Mitchell and Richards (1979) propose that many of the present day *B. oleracea* crop cultivars grown in Western Europe were derived from wild plants introduced by humans from the centre of diversity in the Mediterranean region (Fig. 1.2). However, Snogerup et al.(1990) suggest that it is unlikely that all the populations and varieties have originated from these Mediterranean species. It is argued that it is highly improbable that *B. oleracea* - a plant of Mediterranean origin - had naturalised repeatedly and successfully in NW Europe while not currently being native to area of origin. They also report that the cultivated *B. oleracea* forms have an unusual glaucous leaf surface, common to the wild variety *oleracea* but not found in any of the Mediterranean species. Therefore it seems likely that the distribution is effected by a combination of natural seed dispersal by wind, water and animals and small scale introductions by humans. The isolated population on the island on Helgoland in the North Sea (Fig. 1.2) is likely to be introduced since there are not any reports of corresponding populations in coastal regions of mainland Germany or Denmark.

These habitats are usually limestone or chalk cliffs, in situations protected from grazing, often with some individuals below the cliffs. Also found in scree, among shrubs and some populations on steep, grassy slopes. In Helgoland, populations can be found on open, rocky ground (Snogerup et al., 1990).

Fig. 1.3 - *B. oleracea,* natural habitat. Kimmeridge, Dorset, UK (Stirrup, 2003)

Wild *B. oleracea* grows mostly in hard, limestone or chalk substrates, often in fissures of limestone cliffs and rocks with little soil. For commercial growth of other *Brassica* species, deep tillage is not required since most Brassica species are shallow rooted plants. Despite this, for optimum yield the soil needs to be worked well, with no clods or rocks and well-drained soils with high organic matter content are preferred. Crop rotation out of a cruciferous crop is needed to avoid persistent disease problems (Saskatchewan Agriculture Food and Rural Revitalisation, 2004).

Wild *B. oleracea* is a perennial plant that can live as long as ten years. Populations develop vegetatively for several years. They require vernalisation (a period of low winter temperature) to initiate or accelerate the flowering process. Therefore the length of the vegetative phase is often dependent on the weather. The first influorescence occurs when the plant is 50-100cm with 100-200 flowers, usually with only 2-5 branches. Unfortunately, the vegetative stage and the period of vernalisation required means that it is impractical to carry out transformation, inheritance or fitness studies that require collection of seeds. Rapid-cycling lines of *B. oleracea* have been developed that have a

shorter life cycle that is more akin to that of *Arabidopsis thaliana,* making them easier to study (Williams and Hill, 1986).

B. oleracea subsp. oleracea populations growing on the Dorset coast of England were employed as a model for this study of genomic and chemical plant responses for a number of reasons. From an ecological point of view, this model system has been well characterised and the cabbage aphid and its parasitoid are known to interact with this species. Wild cabbage populations at Durdle Door (grid ref. 380620,080235) and Kimmeridge (grid ref. 390920,078695) have undergone extensive investigation during studies conducted by Brockhouse (2005), Moyes (2000) and Mithen (1995a). The glucosinolate content for a number of individuals was studied and it was found that the mean level of aliphatic glucosinolates was significantly greater in plants from the Durdle Door population. Glucosinolate compounds have been shown to be involved in a number of insect-plant interactions and previous studies have shown that glucosinolate derivatives are used as cues by both the aphid and the parasitoid (Nottingham et al., 1991; Bradburne and Mithen, 2000; Blande, 2004). The Durdle Door population was deemed to be part of a multitrophic system that could prove to be a powerful model to gain ecological and genomic insights into induced plant defence systems. Wild *B. oleracea* produce higher levels of glucosinolates when compared to common crop cultivars due to artificial selection and breeding for plants with low glucosinolate levels (Mithen et al., 1987) (see section 1.4.1). Sustained and intensive crop breeding programmes often neglect pest and disease resistance and tend towards selection of high yield, high homology crops with low levels of glucosinolates (Bradburne and Mithen, 2000; Burton et al., 2004) resulting in a genome-wide loss of genetic diversity (Tanksley and McCouch, 1997). For this reason, it is believed that the surviving wild populations would retain a diverse genotype and conserved natural defences.

It is agreed that naturally occurring *Brassica* genetic variants may prove useful for exploring gene function in complex ecological systems such as responses to biotic and abiotic stimuli, particularly through the study of the effects of alleles that lack variation in *Arabidopsis thaliana* (Mitchell-Olds, 2001; Paterson et al., 2001). The knowledge gained

from studies on wild plants will be applicable to crop cultivars and could be useful for the breeding of crops with reduced vulnerability to pest and disease attack, or crops that are able to adapt to different environments. From a genomic point of view, there is great potential for using the molecular tools and genetic resources developed for *Arabidopsis thaliana* (hereafter referred to as *Arabidopsis*) to study wild *B. oleracea* due to their shared lineage and high genome homology (synteny) (Fig. 1.4). Extrapolation of the results of environmental or functional genomics studies is likely to enable accurate predictions to be made concerning other members of the *Brassicaceae* (Price and Palmer, 1994).

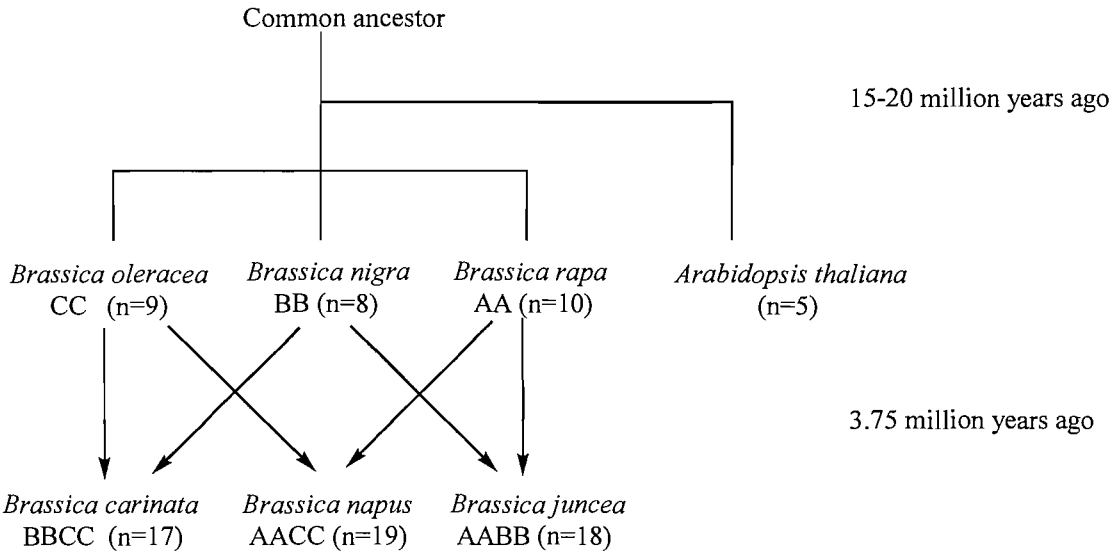


Fig. 1.4 - *Brassicaceae* evolution showing species genotype. Haploid chromosome number is shown in parentheses (based on data from Yang *et al.* 1999)

Brassica oleracea and *Arabidopsis thaliana* belong to the family Brassicaceae and are believed to have diverged from a common ancestor c.15-20 million years ago. Despite the nearly 5-fold difference in genome size – *A. thaliana* ~125 megabases, *B. oleracea* ~600 megabases – there is a high degree of homology in exon sequences (Yang et al., 1999). This genome size difference is believed to result from the *Arabidopsis* genome consisting of an exceptionally low amount of repetitive DNA and a high gene density (Bevan et al., 1998). The vast majority of the shared sequences are expected to be proteins encoded by a heterogeneous class of genetic elements able to insert at new

locations on chromosomes also known as transposable elements (Zhang and Wessler, 2004). Since divergence from a common ancestor (Fig. 1.4), it has been suggested that the three modern diploid *Brassica* species (*B. oleracea, B. nigra* and *B. rapa*) arose following a triplication of the *Arabidopsis* genome followed by significant chromosomal rearrangements and fusion events to reduce the chromosome number from 15 to 8 (Lagercrantz, 1998). More recently however, Lukens et al (2004) assert that there is no strong evidence supporting these suggested autopolyploid events. Similarly Li et al (2003) argue that the lack of even representation of all five *Arabidopsis* chromosomes in duplicated segments of the *Brassica* genome and the variable number of duplications and rearrangements is more consistent with events of higher complexity than autopolyploidy such as aneuploidy or chromosomal rearrangement.

Although *Arabidopsis* itself is not directly of agricultural significance, it has been used as a model system for a multitude of genomic and physiological studies due to its relatively small, fully sequenced genome (25,498 genes), small size and prolific seed production (AGI, 2000). Additionally, many aspects of plant defence that are employed by *A. thaliana* have been observed in other plant families and there exists huge numbers of accessions, mutants and transformed lines with which to study plant function (Mitchell-Olds, 1999, 2001). *Arabidopsis* is most often found in disturbed habitats and although it has naturalised in many parts of the world such as North America, its native range covers Eurasia and northern Africa (O'Kane Jr. and Al-Shehbaz, 1997; Sharbel et al., 2000; Mitchell-Olds, 2001). It is an annual plant which has a short lifecycle of approximately 2 months.

1.2.2 The second trophic level - Brevicoryne brassicae (L) (Hemiptera: Aphididae)

B. brassicae, commonly known as the cabbage aphid is a specialist herbivore that constitutes a world-wide problem causing significant yield losses and reducing the market value of *Brassica* crops (Australian Institute For Horticultural Development, 1995). Aphid infestations often occur in dense colonies and are usually seen on the leaf as small, bleached, necrotic spots at the feeding sites. The leaves turn yellowish and curl in, thus

protecting the aphid colonies (McKinlay, 1992). Infected plants may stop growing and if aphid populations are high enough, the plants wilt and die. A lower level of infestation can result in unmarketable heads, through the presence of both live and dead aphids. Contamination of plant heads with cast skins, wax and honeydew provides an ideal medium for fungal growth (Hely et al., 1982). In addition to direct crop damage, aphids transmit plant viruses. *B. brassicae* and other aphids such as *Myzus persicae* are vectors of persistent and non-persistent cruciferous viruses including Cauliflower Mosaic Virus (CaMV) and Turnip Mosaic Virus (TuMV) (McKinlay 1992). The apterous (wingless) form of the cabbage aphid grows to between 1.6 and 2.6 mm long at maturity and is grey-green in colour, with characteristic black, transverse, dorsal bars on its abdomen and thorax (Fig. 1.5). The alate (winged) form grows to between 1.6 and 2.8 mm long, with a dark coloured head and thorax and black, transverse, dorsal bars only on its abdomen (McKinlay, 1992).

Fig. 1.5 – *Brevicoryne brassicae* feeding on a *B. oleracea* leaf vein (Stirrup, 2006)

B. brassicae go through four instar stages taking 11-14 days to reach maturity. Their parthenogenetic reproduction strategy (reproduction from unfertilised ova) allows rapid expansion of the colony from a single female. Typically of aphids in temperate regions, overwintering *B. brassicae* can reproduce sexually whereby eggs laid in the autumn hatch in the spring (Hely et al., 1982; Walkey, 1991; McKinlay, 1992). As many as twenty-one generations per year can occur in warmer climates. Nymphs rapidly develop and reproduce to form a colony of apterous aphids. Colony overcrowding, reduction in host plant quality or adverse weather conditions can cause the aphids to migrate to adjacent plants or to produce alate forms, which can rapidly disperse and form new colonies on suitable host plants (Hely et al., 1982; Walkey, 1991).

B. brassicae feeds by inserting a stylet intercellularly to establish feeding sites in the phloem sieve elements that can be maintained for several days (Fig. 1.6)(Tjallingii,

B. brassicae feeds by inserting a stylet intercellularly to establish feeding sites in the phloem sieve elements that can be maintained for several days (Fig. 1.6)(Tjallingii, 2006).

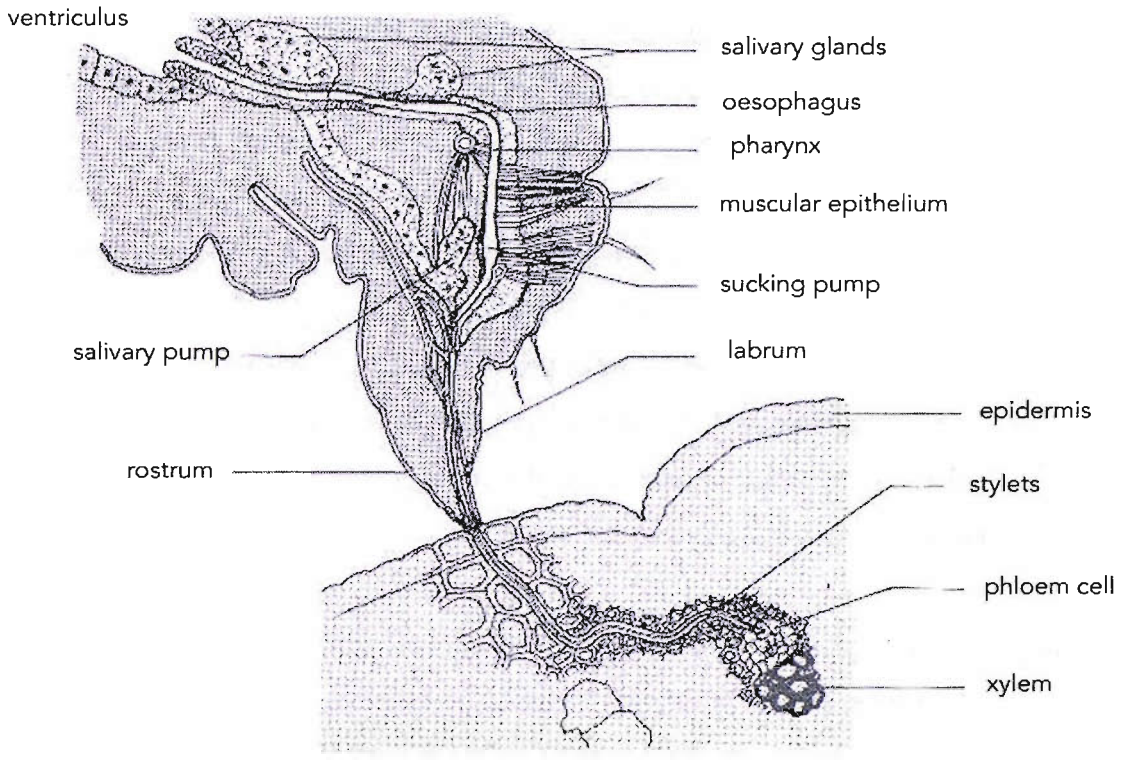


Fig. 1.6 – Cross-sectional diagram showing aphid feeding apparatus and stylet path in leaf epidermis (from Sorin 1966)

Although this intercellular probing minimises wounding and limits the local induction of defence responses, it does, however, result in cell wall disturbance, disruption of plasma membranes, and penetration of epidermal, mesophyll, and parenchyma cells (Thompson and Goggin, 2006). The phloem sap is either sucked or passively received and is rich in carbohydrates, excess of which is excreted as 'honeydew'. Aphids host intracellular bacterial symbionts of the genus *Buchnera* which are able to synthesise amino acids. Analysis of phloem sap has shown methionine and leucine to be present in low amounts and it has been suggested that there is a dependence on the symbiont for synthesis of these amino acids (Sandstrom and Moran, 1999).

Plants with thicker cuticles are less susceptible to heavy infestations; therefore infestations tend to concentrate on the younger, meristematic tissues. When the stylet probes a substrate, such as the leaf epidermis, it leaves behind a solid structure of salivary origin termed the stylet sheath. It is believed that this sheath may reduce hypersensitive reactions of wounded plant cells thereby preventing the release and/or production of autotoxic defensive compounds from surrounding cells. This sheath emerges from the tip of the stylet and is composed of a viscous mixture of sheath precursors that gel rapidly in air or liquid media (Miles, 1999). Enzymes detected in the stylet sheaths of aphids include polyphenol oxidase (Miles, 1964); peroxidase and 1,4-glucosidases (Miles, 1999). Concurrent with the sheath production is the intermittent discharge of watery saliva from the stylet tip. This saliva contains amino acids (Anders, 1958; Miles, 1999), shows significant reducing and surfactant properties (Miles and Harrewijn, 1991; Madhusudhan et al., 1994), has a pH of 8-9 and contains various enzymes including pectinases, cellulases and enzymes that hydrolyse phenolic glycosides and sucrose.

Whereas generalist herbivores feed on a wide range of plant taxa, specialist herbivores such as *B. brassicae* survive solely on a limited number of similar plant species. Defence responses by specialists and their hosts are believed to have co-evolved consistent with Ehrlich and Raven's model of co-evolution (1964). This states that the evolution of one species is partially dependent on the evolution of another. The Brassicaceae contain high levels of glucosinolates and a great diversity of other secondary plant compounds which can function as anti-feedants or repellents to certain herbivores (Chew, 1988; Rosa et al., 1997; Lambdon et al., 2003). Although phytochemicals are involved in many other systems apart from defence, co-evolution is likely to have contributed to this chemical diversity by the reciprocal evolutionary response of the plant/insect defence mechanisms (Harbourne, 1993). The exploitation of these plants by a specialist herbivore necessitates a metabolic system able to tolerate these levels. Further to simply tolerating these levels, it has been shown that specialists *B. brassicae* and *Lipaphis erysimi* produce a non-plant myrosinase which is stored in crystalline microbodies found in the head and thorax regions (MacGibbon and Beuzenberg, 1978; Bridges et al., 2002). This enzyme breaks down the glucosinolates sequestered from their host plants into toxic metabolites and it is

thought that this system may be a direct defence to generate metabolites which deter predators, parasitoids and protect against parasitic fungi (Bridges et al., 2002).

Current agronomical control of aphids is based mainly on the use of insecticides (Zhang and Hassan, 2003). This reliance on pesticides is driven by the consumer expectation that produce must be completely pest free. There is increasing concern amongst growers, markets and consumers about the high levels of pesticides required to achieve this goal. This concern coupled with the growing market for organic produce in the UK and internationally, means that development of effective biological control measures and integrated pest management (IPM) strategies has become a priority in agricultural research.

Biological control of the specialist *B.brassicae* in the field is mostly ineffective due to the aphid's high reproductive rate. Apart from the parasitic wasp *D. rapae*, natural predators include the common spotted ladybird, *Harmonia conformis*, the seven-spot ladybird, *Coccinella septempunctata*, and other predatory insects such as larvae of syrphid flies (hover fly and drone fly) (Metcalf and Metcalf, 1993). *B. brassicae* is susceptible to various fungal diseases and to Destruxin E, a toxin produced by the fungus *Metarhizium anisopliae* (Agrobiologicals, 2004). However, none of these organisms cause a significant reduction in aphid populations until a large amount of crop damage has already occurred (Hely et al., 1982).

Aphids parasitised by *Diaeretiella rapae* and other parasitoids undergo a decrease in fecundity (number of offspring and reproductive period) and activity which often cease completely at day 8-9 after parasitism as the larva grows and feeds inside the aphid. On day 9-10, the cuticle of the aphid turns a tan gold colour and becomes hard. On day 12-14, the adult parasitoid emerges. Aphids parasitised at age 1-4 days (first to third instars) produce no offspring. Aphids parasitised from days 5-6 (third-fourth instars) and 7 day old newly moulted adults produce only between 8% and 40% of the total reproduction in unparasitised aphids of the same age at 25-27°C (Zhang and Hassan, 2003). The average

reproductive period in unparasitised aphids was 14.9 days. This compares to 1.9 and 4.4 days in aphids parasitised on days 5-6 and 7+ respectively (Zhang and Hassan, 2003).

1.2.3 Diaeretiella rapae (MacIntosh) (Hymenoptera: Aphidiinae)

Diaeretiella rapae (MacIntosh) is a braconid wasp that parasitises a number of aphid species including the cabbage aphid *Brevicoryne brassicae* (L.) (Homoptera: Aphididae). Following oviposition into the abdomen of the host by the endoparasitoid, the larva develops, greatly reduces the host's reproductive capacity and eventually kills it (Godfray, 1994).

Table 1.1 - Aphid species parasitised by the parasitoid *D. rapae* (Zhang and Hassan, 2003).

Latin name	Common name
Brevicoryne brassicae	Cabbage aphid
Myzus persicae	Green peach aphid
Diuraphis noxia (Mordvilko)	Russian wheat aphid
Aphis gossypii	Cotton aphid
Rhopalosiphum padi	Bird cherry-oat aphid
Rhopalosphum maidis	Corn-leaf aphid

Female *D. rapae* parasitise their aphid hosts by laying a single egg inside the aphid abdomen (Fig. 1.7).

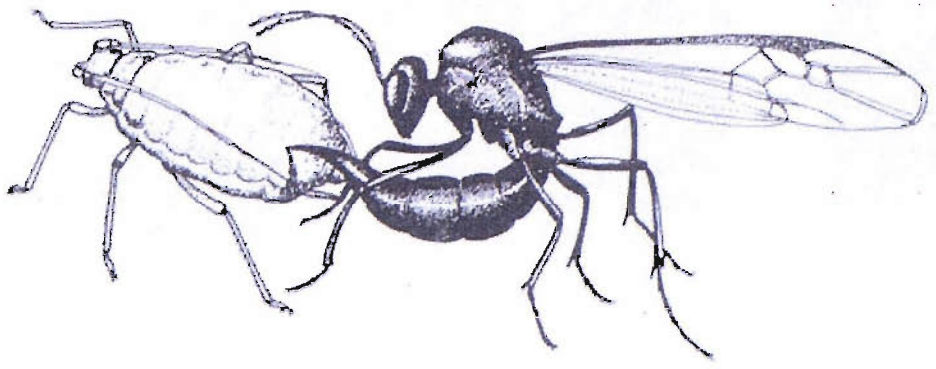


Fig. 1.7 – *D. rapae* ovipositing in an aphid host (Hoffmann and Frodsham, 1993)

The larva goes through four instars and consumes the aphid before finally pupating, the aphid dies, sclerotises (hardens) and turns a tan colour, and becomes what is referred to as a "mummy", which is easily distinguished from unparasitised aphids (Fig. 1.8 and 1.9).

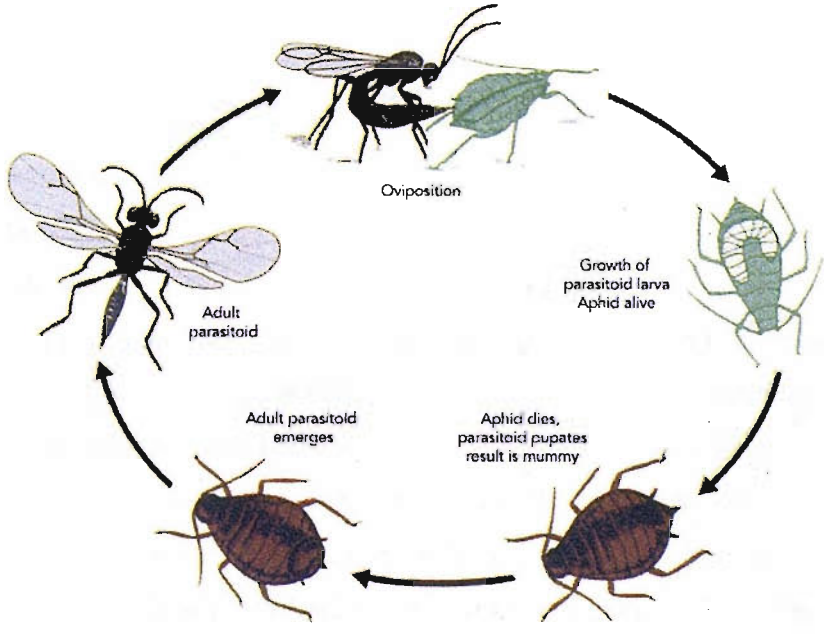


Fig. 1.8 – Life cycle of the parasitoid *D. rapae* from oviposition to emergence taking 12-14 days (Hoffmann and Frodsham, 1993).

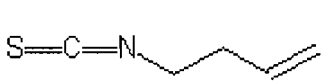
Fig. 1.9 – Photo series showing emergence of parasitoid larva from aphid mummy (IPM Images, 2004).

D. rapae attacks and develops in all stages of the cabbage aphid and shows no preference for aphid age. (Zhang and Hassan, 2003). Parasitoids locate and choose their hosts depending on a number of different cues depending on the distance from the host. Vinson (1976) proposed six steps based on either chemical, visual or physical cues that parasitoids follow during foraging behaviour in order to achieve successful host parasitism.

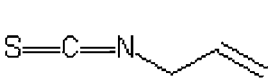
1. Host habitat location
2. Host location
3. Host recognition
4. Host acceptance
5. Host suitability
6. Host regulation

Each step serves to reduce the physical volume searched by the female. Host habitat location has been shown to be facilitated mainly by long range volatile compounds originating from either the herbivore, its food, organisms associated with the herbivores presence or from interactions between these sources (Vet and Dicke, 1992). Host location is carried out on a smaller scale and is also thought to be facilitated by volatile cues as well as visual stimuli (Mackauer, 1986; Mackauer et al., 1996) and textural examination of damaged plant areas with antennae. The antennae are used for the detection of non-volatile compounds during the process of host recognition. This study will utilise the propensity of parasitoids to respond to plant odours to attempt to characterise the compounds involved.

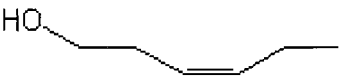
Vaughn et al. (1996) showed that there are clear differences between male and female sensory perception of semiochemicals and the potential for odour discrimination. Using electroantennography (EAG), it was demonstrated that female antennae responded to allylisothiocyanate (AITC) and (Z)-3-hexen-1-ol whereas males only responded to (Z)-3-hexen-1-ol. This fact, coupled with the knowledge that plants often release a volatile blend that is unique to the attacking herbivore (Turlings et al., 1998; Ozawa et al., 2000) suggests that females perceive specific compounds corresponding to specific herbivores (Vet and Dicke, 1992). Neither male nor female *D. rapae* showed any electroantennogram response to 20-25 crushed aphids (and aphid honeydew) suggesting that an aphid-mediated plant response is the component to which the parasitoid is attracted.



3-butenyl isothiocyanate



allyl isothiocyanate
(propenyl isothiocyanate)



(z)-3-hexen-1-ol

Fig. 1.10 – Volatile organic compounds that cause a behavioural or physiological response in *D. rapae*.

1.3 Mechanisms of induced plant defence

The induced plant response necessitates a network of signal transduction pathways to function within the plant resulting in an extracellular manifestation that affects either members of the same trophic level or those of others. Two such pathways identified as being important in induced plant defence mechanisms are those involving the plant hormone jasmonic acid (JA) (Karban and Baldwin, 1997; Dicke and van Poecke, 2002) referred to as the jasmonate or octadecanoid pathway and the salicylic acid pathway involving the plant hormone salicylic acid (SA) (Dempsey et al., 1999; Dicke and van Poecke, 2002). Ethylene has also been implicated in certain defence mechanisms (Kato et al., 2002). Both the salicylic acid and ethylene pathways have been shown to interact with the octadecanoid pathway (Turner et al., 2002) with SA having an inhibitory effect

on the octadecanoid pathway (Penacortes et al., 1993) and JA having an inhibitory effect on the SA pathway (Niki et al., 1998). JA and SA have also been reported to act synergistically (Xu et al., 1994). Much research done on the octadecanoid pathway involves studies on *Arabidopsis* and tomato - *Lycopersicon esculentum.* However, there are several discrepancies between the proposed JA signalling pathways of these species, and it is not clear whether these reflect gaps in knowledge or reveal fundamental differences in mechanism. For example the systemic induction of JA responses in tomato is through the well-characterised systemin signal pathway (Ryan et al., 2002) but in *Arabidopsis* there is no evidence for an equivalent pathway, even though systemic signalling can be demonstrated (Turner et al., 2002).

The aliphatic glucosinolate pathway is important in plant defence against insect herbivores, pathogens, nematodes, and other competing plants. It has been well characterised and is known to be involved in both constitutive and induced defence responses in members of the Brassicaceae (Agrawal et al., 2002b; Siemens et al., 2002; Traw and Dawson, 2002; Mewis et al., 2005). Glucosinolate derivatives are released from herbivore damaged plants and there is some evidence that these isothiocyanates cause behavioural responses in aphid parasitoids (Bradburne and Mithen, 2000; Blande, 2004). An increasing amount of research is now being dedicated to understanding the transcriptional control of enzymes involved in these pathways.

Comparative mapping studies, the extensive annotation and expression databases and sequence alignment search tools that have been developed for *Arabidopsis* are invaluable resources available to the plant science community. By exploiting the homology between the genomes and the knowledge on induced defence pathways in *Arabidopsis,* gene expression and functional genomics studies may be able to identify genes in wild species that have distinct ecological functions.

1.3.1 The Aliphatic Glucosinolate pathway

Glucosinolates (GSLs) are secondary metabolites synthesised almost exclusively in plants of the order Capparales, which contains 15 families, including the Brassicaceae,

Capparaceae, and Caricaceae (Rodman et al., 1996). Their extensive study has been brought about following discovery of their potential as cancer-prevention agents, crop-protection compounds, and biofumigants in agriculture. The Brassicaceae and in particular *Arabidopsis* has been extensively studied to elucidate the mechanisms of GSL synthesis and hydrolysis. GSLs are classified as either aliphatic (derived from methionine, alanine, leucine, isoleucine or valine), aromatic (derived from tyrosine or phenylalanine) and indolyl (derived from tryptophan) (Fahey et al., 2001). Their synthesis is catalysed by the action of a number of cytochrome P450 enzymes (Halkier and Gershenzon, 2006). *Brassica oleracea* contains mostly aliphatic and indolyl glucosinolates. There is substantial qualitative and quantitative variation among subspecies (Mithen et al., 1995b; Fahey et al., 2001) and particularly within and between wild populations (Moyes et al., 2000; Brockhouse, 2005).

GSLs are produced in the cytoplasm and stored primarily in the vacuole until the cells are disrupted by mechanical wounding or chewing insects (Fahey et al., 2001). Cell disruption causes mixing of the compartmentalised GSLs and myrosinase enzymes resulting in GSL hydrolysis (Bones and Rossiter, 1996). This reaction produces D-glucose, a sulphate ion and a number of possible products depending on hydrolysis conditions and substrate (Fig. 1.11). These products have diverse and important biological activities including carcinogen detoxification, inhibition of pathogenic fungal growth (Li and Quiros, 2002), activity against nematodes (Lazzeri et al., 1993) and tumour cell growth (Leoni et al., 1997). Some are also anti-nutritional and deter generalist herbivores from feeding (Mithen, 1992b; Giamoustaris and Mithen, 1995; Rosa et al., 1997). In studies where hydrolysis products have been specifically tested for biological activity, it is often found that the isothiocyanates (ITCs) are the mediators responsible. For example, Li *et al.*(2000) found that the hydrolysis product allyl isothiocyanate (Fig. 1.9) and its allyl glucosinolate precursor both were lethally toxic to the generalist herbivore *Spodoptera eridania*, but only the allyl isothiocyanate was lethally toxic to the crucifer specialist herbivore *Plutella xylostella*. Separate studies by Bradburne and Mithen (2000) and Blande (2004) have suggested that ITCs are involved

in the attraction of aphid parasitoids following aphid feeding. Also, Vaughn (1996)showed that 3-butenyl ITC elicits a physiological response in parasitoid wasps.

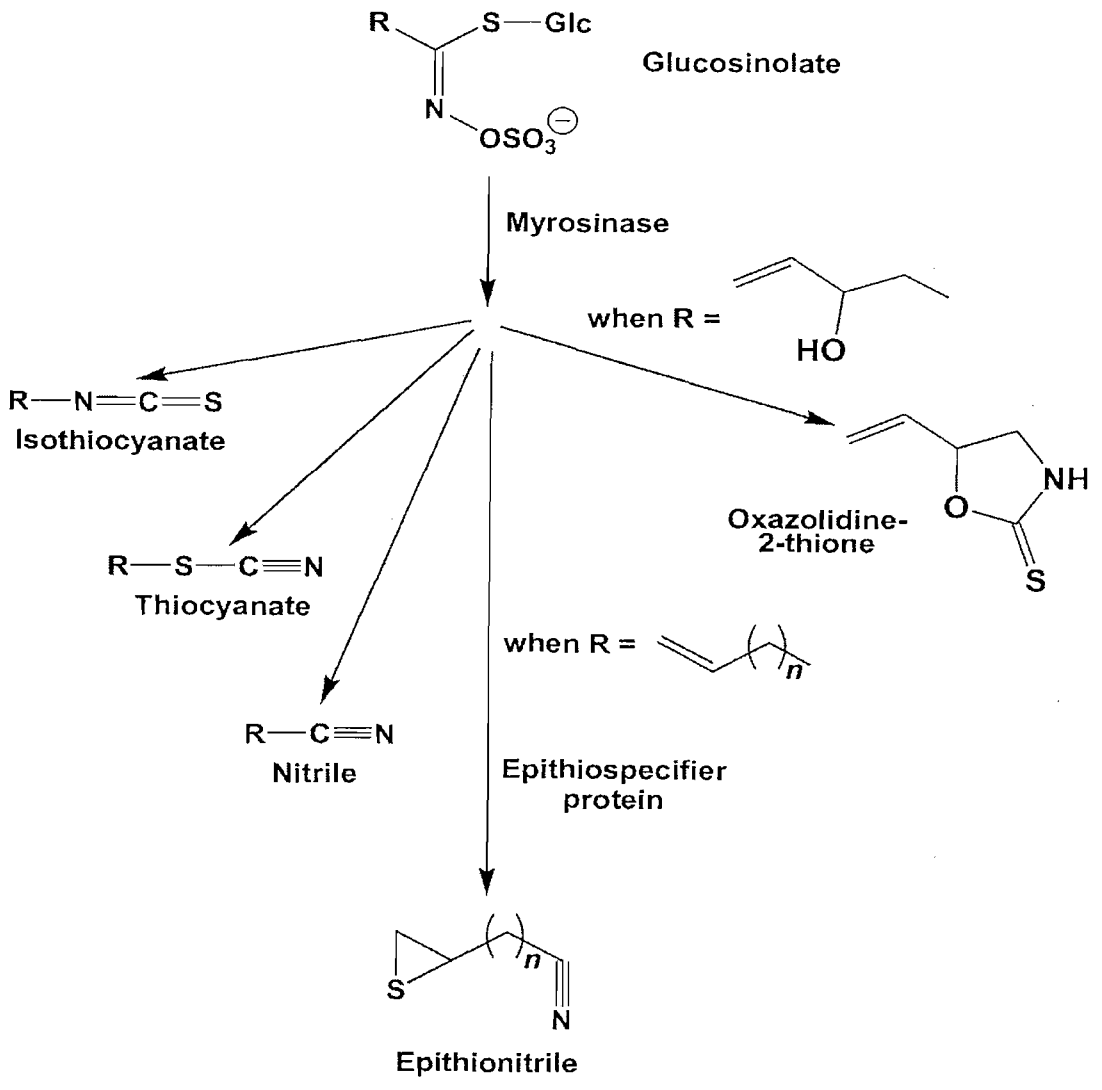


Fig. 1.11 - Biochemical pathway illustrating glucosinolate hydrolysis by myrosinase (thioglucosidase) enzymes to form a range of secondary metabolites (Lambrix et al., 2001).

The herbivore-induced systemic accumulation of glucosinolates has been reported in *Arabidopsis* (Mewis et al., 2005), black mustard (*Brassica nigra*) (Traw, 2002; van Dam et al., 2005) and wild radish (Agrawal et al., 2002a). Some specialist herbivores have evolved to tolerate or detoxify the glucosinolate products synthesised to defend against them, often by preventing the isothiocyanate being formed. For example, *Plutella*

xylostella – the diamondback moth – modifies the core glucosinolate by cleaving the sulphate residue with an endogenously produced broad-spectrum sulphatase which inhibits the myrosinase-catalysed hydrolysis (Ratzka et al., 2002). *Pieris rapae* – the cabbage white butterfly – redirects the hydrolysis towards the production of nitriles rather than isothiocyanates by secreting an epithiospecifier protein-like factor into its gut (Wittstock et al., 2004). Other specialists are able to sequester the defensive compounds and exploit them in self-protection. This has been observed in the harlequin bug - *Murgantia histrionica* (Hemiptera) (Aliabadi et al., 2002), the sawfly, *Athalia rosae* (Hymenoptera) (Müller et al., 2001), and the aphids, *Brevicoryne brassicae* and *Lipaphis erysimi* (Homoptera) (Bridges et al., 2002).

Although a number of gene loci have been identified that are involved in glucosinolate chain elongation and addition of functional groups, relatively few genes have been functionally characterised and sequenced. *MAM1 (Methylthioalkylmalate synthase)* has been identified as being responsible for the condensing reaction of the first two methionine elongation cycles in *Arabidopsis* (Kroymann et al., 2001). This gene has a high similarity to genes encoding isopropylmalate synthase, which catalyses chain elongation in leucine biosynthesis. Subsequently, *MAM-L* was identified which catalyses the remaining chain-elongation activities and the *MAM2* gene is associated with accumulation of glucosinolates having undergone only one round of elongation. Two further gene-products *AOP2* and *AOP3* (which encode 2-oxoacid-dependent dioxygenases) have been identified in *Arabidopsis* (Kliebenstein et al., 2001b). The gene products catalyse the stepwise oxidation of the sulphur atom in the methylthioalkyl side chain and homologues have been identified in *B. oleracea* (Gao et al., 2004).

Quantitative trait locus analysis of 39 *Arabidopsis* ecotypes has identified loci that control aliphatic glucosinolate side-chain length and functional groups. A high degree of variation was found in the glucosinolate profiles between ecotypes of which 61% was explained by a single locus (*GSL-OX)* that controlled the conversion of methylsulfinylalkyl glucosinolates into alkenyl or hydroxyalkyl glucosinolates (Fig. 1.12). Considering GSL accumulation, Kliebenstein *et al.* (2001a) identify six QTLs that determine total aliphatic glucosinolate accumulation, of which two are the biosynthetic loci *GSL-Elong* and *GSL-AOP*, six loci control total indole glucosinolates, and three loci regulate the less dominant aromatic glucosinolates. Five additional loci were specific to subsets of the indole glucosinolates.

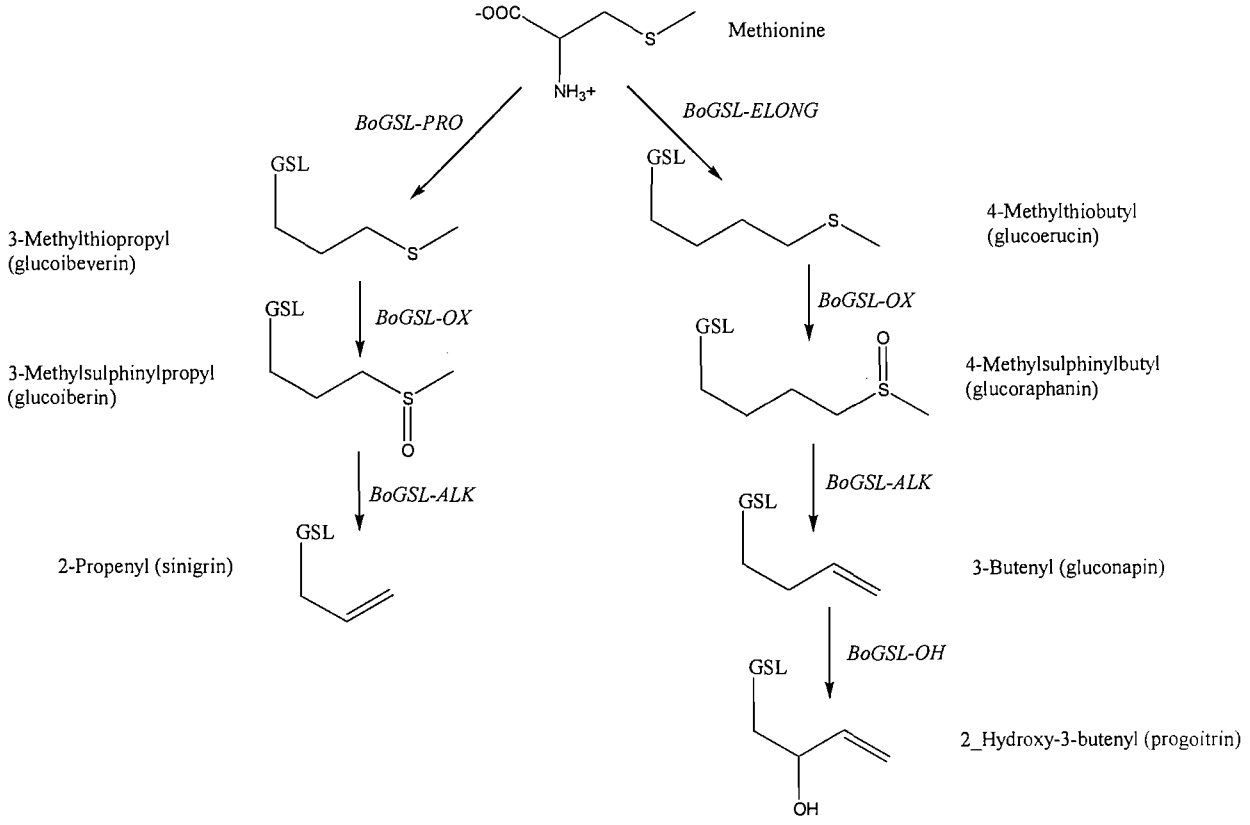


Fig. 1.12 – Major gene loci involved in synthesis or modification of GSLs (Adapted from Li and Quiros (2002).

Since glucosinolate structure and concentration is controlled by a small number of genes with a large effect. This enables rapid evolutionary responses to changing selection pressures (Kliebenstein et al. 2001). All of these gene loci may be important for

improvement of *Brassica* breeding lines with high content of desirable GSL. For example down-regulation or silencing of genes from the *BoGSL-ALK* loci could produce varieties of oilseed rape lacking the antinutrient progoitrin and would simultaneously produce plants accumulating glucoraphanin - a demonstrated precursor of anti-carcinogenic compounds. Conversely, *BoGSL-ALK* over-expression would produce crops high in sinigrin. Residues from these *Brassicas* with high biomass capacity could be used as biocontrol agents against soil-borne pathogens, weeds or nematodes (Li et al., 2001).

Indole glucosinolates have been shown to accumulate following JA and SA pathway induction in mutant studies suggesting that herbivore-induced JA and SA signals would elicit a similar response (Mikkelsen et al., 2003). Aliphatic glucosinolates however appear to be primarily developmentally regulated (Kliebenstein et al., 2002). Induction in this case could occur by the release or synthesis of myrosinase enzymes that catalyse the formation of the biologically active volatile compounds such as 3-butenyl isothiocyanate. Studies by Pontoppidan *et al.* (2003; 2005) report the induction of a myrosinase binding protein (*MBP*) and a myrosinase-associated protein (*MyAP*) following wounding and *Plutella* feeding on *B. napus*. However, it is unclear how these proteins are related to glucosinolate hydrolysis. After hydrolysis, the *epithiospecifier protein* (*ESP*), in the presence of myrosinase, has been shown to promote the formation of epithionitriles (Fig. 1.10) (Lambrix et al., 2001). Although nitriles are known to be toxic to insects (Peterson et al., 2000), and epthionitriles have been shown to be cytotoxic to mammals (Vansteenhouse et al., 1991), the importance of this protein in plant defence is unclear.

1.3.2 Octadecanoid / Jasmonic acid pathway

The octadecanoid pathway (Fig. 1.13) plays a key role in gene and metabolic regulation, responses to wounding and abiotic stress, reproduction, defence against insects and pathogens and possibly in communication (Liechti and Farmer, 2002).

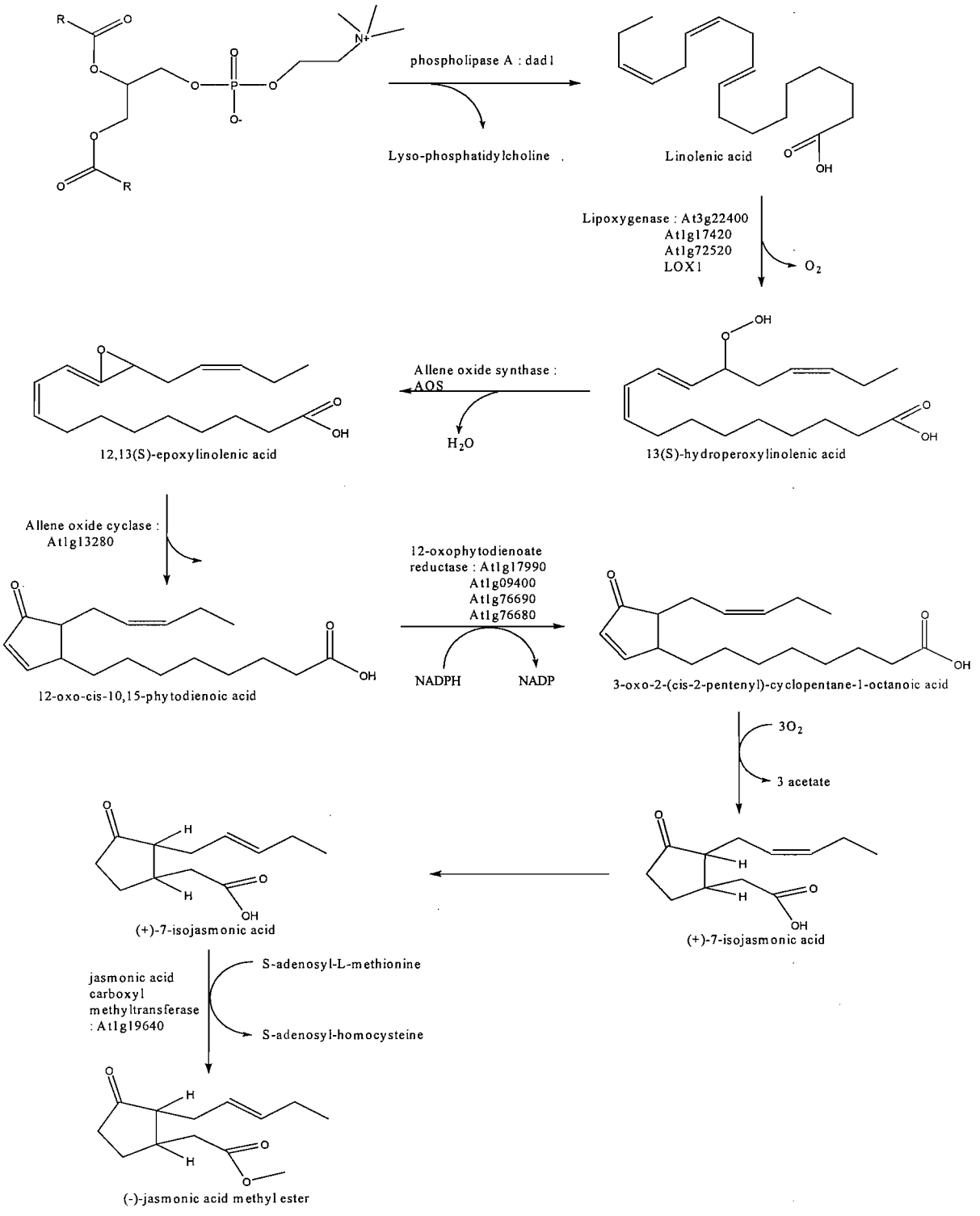


Fig. 1.13 – The octadecanoid pathway detailing enzymes involved in reaction steps. Specific genes involved in regulation of certain reaction steps are shown. Figure adapted from The Arabidopsis Information Resource (2004).

The term jasmonates (JAs) includes the biologically active intermediates in the pathway for jasmonic acid biosynthesis as well as the biologically active derivatives of jasmonic acid. A 12-carbon fatty acid derivative, jasmonic acid and its methyl ester (methyl jasmonate, MeJA) are synthesised via the octadecanoid pathway from the 18-carbon substrate linolenic acid (Fig. 1.13). These constituents are involved in protecting the plant against herbivores (Karban and Baldwin 1997) and microbial pathogens (Thomma et al., 1998).

The function of JAs in plant defence was proposed by Farmer and Ryan (1992), who provided evidence for a causal link between wounding (as caused by insect herbivores), the formation of JAs, and the induction of genes for proteinase inhibitors that deter insect feeding. They proposed that wounding caused the release of linolenic acid (Fig. 1.13) – the presumed precursor of JAs – from membrane lipids (Turner et al., 2002).

Wounding increases the endogenous levels of JA in many plant species, and exogenous application of JA stimulates production of proteinase inhibitors that deter insect feeding (Reinbothe et al., 1994; Staswick and Lehman, 1999). JA-treated wild-type *Arabidopsis* attract more of the parasitoid wasp *Cotesia rubecula* than untreated plants, as do *Pieris rapae* infested plants (van Poecke and Dicke, 2002). Although these interactions with the third trophic level have been demonstrated following damage by chewing insects, phloem-feeding insects including aphids do not have such a pronounced ecological effect. Studies on a number of plant species following exogenous application of jasmonic acid have, however, observed a decrease in fecundity, survival and host preference (Omer, 2001; Bruce et al., 2003; Cooper et al., 2004; Zhu-Salzman et al., 2004). Population growth of the green peach aphid (*Myzus persicae*) was significantly reduced on the *cev1* mutant in *Arabidopsis*, in which JA and ET signalling is constitutively activated (Ellis et al., 2002). Also, studies using the Jasmonate-insensitive *Arabidopsis* mutant *coi1* showed a modest increase in aphid population growth, suggesting that JA-dependent responses can limit aphid performance on wild-type plants (Mewis et al., 2005). These results suggest that defences regulated by JA and/or ET can effectively reduce aphid infestations, although the extent to which aphids induce these defences is unclear. The

potential of measuring gene transcript levels to assess the effect of aphid feeding has been exploited in recent years by the use of micro- and macro-arrays that assay the changes in expression of a number of genes simultaneously. A modest induction of genes encoding JA and ET pathway genes are reported in the wild *Nicotiana attenuata* (Heidel and Baldwin, 2004) and Moran and Thompson (2001) report the low-level induction of the JA pathway related genes *LOX2* (Voros et al., 1998) and *PDF1.2.*

Volatile jasmonates such a *cis*-jasmone (Fig. 1.14), have been shown to be active in both direct and indirect defences. Plants emitting *cis*-jasmone were repellent to the summer morph of *Nasonovia ribis nigri* and *Phorodon humuli. Cis*-jasmone has also been shown to induce plants to become significantly attractive to the parasitoid *Aphidius ervi* and aphid predator the seven-spot ladybird - *Coccinella septempunctata* (Birkett et al., 2000).

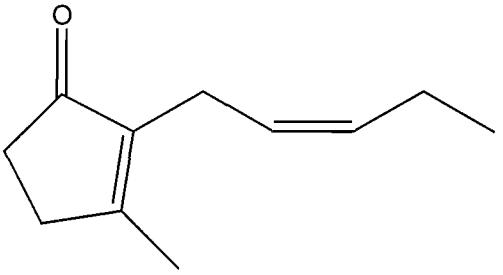


Fig. 1.14 – The volatile organic compound cis-jasmone

1.3.3 Salicylic acid pathway

With reference to direct defences, the salicylic acid pathway plays an important role in the protection of plants against many pathogen species (Dempsey et al., 1999). Herbivory can lead to increases in the endogenous levels of SA and activate SA-responsive genes in *Arabidopsis*, but there is no evidence that this has a direct negative effect on herbivore performance (Bi et al., 1997; Moran and Thompson, 2001; Thompson and Goggin, 2006). However, there have been a number of studies that report the aphid-induced expression of genes involved in the SA pathway in several plant species, including *Arabidopsis*, tomato, sorghum, and *Nicotiana attenuata* (Moran and Thompson, 2001; Moran, 2002; Heidel and Baldwin, 2004; Zhu-Salzman et al., 2004; Park et al., 2006).

There is evidence that SA is involved in the indirect defence of plants by semiochemicals. The volatile methyl salicylate (MeSA) is emitted by herbivore infested plants (van Poecke and Dicke, 2002), including caterpillar infested *Arabidopsis* (Van Poecke et al., 2001). Predatory mites are attracted by MeSA (De Boer and Dicke, 2004) and the chemoreceptors of the parasitoids *C. rubecula* and *C. glomerata* are sensitive to MeSA (van Poecke et al., 2003). Using caterpillar infested transgenic *Arabidopsis* (*NahG*) that do not accumulate SA, Van Poecke and Dicke (2002) found that the NahG plants were less attractive to *C. rubecula* than caterpillar infested wild-type plants. This suggests some level of interaction between SA and the emission of semiochemicals.

1.4 Volatile organic compound emission following herbivory

Plants produce a large variety of volatile organic compounds (VOCs) and the function for which they have been most studied is at the spatial scale of the ecosystem. VOCs at this level are active in defending the plants against phytophagous herbivores and pathogens or to providing a reproductive advantage by attracting pollinators and seed dispersers (Pichersky and Gershenzon, 2002; Reinhard et al., 2004). Following herbivory, a greater amount of volatiles are released and their identity varies with the plant and herbivore species (Pare and Tumlinson, 1999). The volatiles released have been shown to defend plants by repelling herbivores (Kessler and Baldwin, 2001), attracting predators or parasitoids of the herbivores (Arimura et al., 2004) and to occasionally affect the defences of neighbouring plants (Dicke et al., 2003b). The largest group of plant volatiles are low-molecular-weight terpenes, these include monoterpenes (10C), sesquiterpenes (15C) and homoterpenes (20C) (Fig. 1.16). These can attract pollinators, fruit-dispersing animals, and predators, parasitoids or herbivorous arthropods (Faldt et al., 2003). Loviamaki et al (2004) have shown that *Brassica napus* treated with methyl jasmonate produce higher levels of indolyl glucosinolates and also release the common plant volatile (*E*)-4,8-dimethyl-1,3,7-nonatriene suggesting that these MeJA treated plants could be less palatable to herbivores.

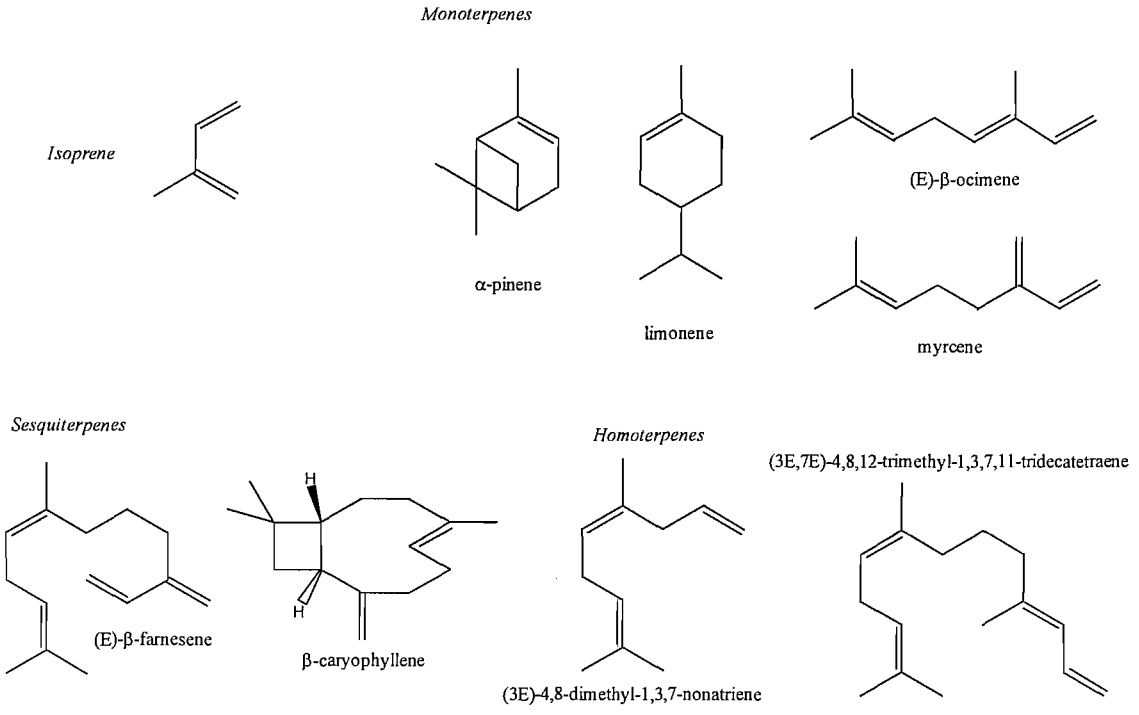


Fig. 1.16 – Monoterpenes (C10), homoterpenes (C11 or C16) and sesquiterpenes (C15) are composed of isoprene units and are synthesised via the isopentenyl pyrophosphate (IPP) intermediate.

Differences in the amount of volatiles released by individual plants can vary with environmental conditions that influence the plant's physiology. Several species, including corn, cotton, and lima bean, respond to reduced light (due to either lower light intensity or shorter daylength) with a decline in the release of herbivore-induced volatiles. Based on studies with lima bean, water stress also seems to directly affect volatile release (Takabayashi et al., 1994). With less water available for the plant, elevated levels of volatiles are released from infested individuals relative to non-water-stressed controls. Correlating this with insect preference showed that predatory mites selected plants that were infested and water-stressed over those that were infested but not water-stressed (Takabayashi et al., 1994).

Following herbivory, plants release a volatile blend that is sometimes qualitatively and quantitatively different to that released during wounding. This effect has been demonstrated in *Arabidopsis,* where herbivory or treatment with *P. rapae* caterpillar

regurgitant results in the emission of a different blend of volatiles that is emitted for a longer period after termination of the treatment when compared to mechanical wounding. Herbivore-infested or regurgitant-treated plants are more attractive to parasitoids than undamaged or mechanically damaged plants (van Poecke et al., 2003). Herbivore-derived elecitors have been identified in the regurgitant of caterpillars, β-glucosidase from *P. brassicae* and fatty acid-amino acid conjugates from *Spodoptera exigua* (volicitin), *Manduca sexta* and *Manduca quinquemaculata* (Mattiacci et al., 1994; Alborn et al., 2000; Halitschke et al., 2001).

Green leaf volatiles (GLVs) are released after herbivory or wounding of many plants. Many are synthesised from linolenic acid which is also the precursor for JA. Synthesis is catalyzed by the enzymes hydroperoxide lyase, isomerization factor, and alcohol dehydrogenase (Blee, 1998). *AtLOX2* and *AtHPL* – encoding lipoxygenase and hydroperoxide lyase respectively, are induced by *Pieris rapae* caterpillar feeding and are both involved in green leaf volatile production (Van Poecke et al., 2001). This indicates some overlap between responses to herbivory and generalised responses to wounding. Therefore it is important to understand the generalised responses to wounding in order to appreciate the specialised responses to herbivory.

1.4.1 Genes encoding terpene synthases and induced defence components

Determining the gene transcripts encoding enzymes involved in the biosynthetic pathways of volatiles released after herbivory will enable a better understanding of the process by which plant defences are induced. To date more than 100 terpene synthases have been isolated and characterized from different plant species (Dudareva et al., 2006). These *TPS* genes have been categorised into seven subfamilies (designated TPS-a through TPS-g) based on sequence relatedness, functional assessment, and gene architecture. *P. rapae* feeding can cause induction of the terpene synthase genes *AtTPS03* – encoding (E)-β-ocimene synthase gene and *AtTPS10* (Van Poecke et al., 2001; Faldt et al., 2003; van Poecke et al., 2003). *AtTPS03* catalyses the formation of *(E)-β*-ocimene from geranyl diphospate (GPP). *AtTPS10* has been isolated from a jasmonate-

induced cDNA library and encodes a monoterpene synthase enzyme, which converted GPP (C-10) into the acyclic monoterpenes - β-myrcene and (*E*)-β-ocimene and small amounts of cyclic monoterpenes (Bohlmann et al., 2000).

1.5 Thesis aims and hypotheses

This thesis aims to use several methods at to detect the response of wild cabbages to aphid feeding. By integrating the information obtained using different methods of detection the aim is to build a more holistic understanding of induced plant defences and their ecological interactions. On an ecological level, the behaviour of parasitoids will be used to convey an understanding of their response to plant volatiles. Parasitoids and other biological organisms have been shown to be extremely effective at sensing volatiles. However, the necessity of interpretation of their responses and the subjective filter with which we view their behaviour can lead to an inaccurate reading of the interaction. To augment the information obtained using this biological detector, chemical detection methods will be employed that enable the identification and quantification of VOCs. The VOCs emitted by the plant in response to the aphid will have specific synthesis pathways that may be transcriptionally regulated. To detect the plant transcriptional response, gene expression analysis techniques will be used and linked to previous knowledge on plant defence pathways and gene functional annotation.

1.5.1 Chapter 2 - Aims and Hypotheses

This chapter uses traditional odour choice bioassays to assess the odours that cause female parasitoids to exhibit host-searching behaviour. Behavioural bioassays are conducted to assess the effect of using field-collected or lab-reared parasitoids.

Hypotheses:

- *D. rapae* will exhibit a significant preference for the plant-host complex rather than the uninfested control plant odour.
- *D. rapae* will exhibit a significant preference to the plant-ITC complex rather than the control plant odour.
- Lab-reared *D. rapae* will respond more slowly to the plant-host complex.

1.5.2 Chapter 3 – Aims and Hypotheses

and multivariate statistical analysis techniques will be used in order to draw conclusions about the nature of the VOCs released.

Hypotheses:

- The blend of volatiles detected from herbivore-damaged plants will be qualitatively and quantitatively distinct to the blend emitted from undamaged plants.
- Plants that have undergone feeding by phloem-feeding insects will emit a quantitatively different VOC blend to that induced by a chewing insect.
- Canonical discriminant analysis is an effective technique for the analysis of VOCs and can aid in the biological interpretation of chemical data.

1.5.3 Chapter 4 – Aims and Hypotheses

Chapter 4 aims to identify the genes that are involved in the response to herbivore feeding. An understanding of the transcriptional regulation of such genes may enable links to be made between the herbivore-specific response and the volatiles released by the plant. This chapter will use full genome *Arabidopsis* microarrays analysed using a cross-species filter to assay many genes simultaneously.

Hypotheses:

- Phloem feeding by *Brevicoryne brassicae* will induce a unique suite of genes when compared to *Plutella* larvae infestation.
- Genes involved in pre-characterised plant defence pathways will be induced in aphid-infested plants.

1.5.4 Chapter 5 – Aims and Hypotheses

This chapter aims to investigate the genetic variation present in the populations of wild cabbage at Durdle Door and Kimmeridge. These wild species will be compared to cultivated varieties to provide an estimate of the biological diversity present in these populations.

Hypotheses:

- Wild populations of *B. oleracea* will show greater genetic diversity (as measured by proportion of polymorphic loci and genetic distance) than the cultivated varieties in line with previous studies on gene expression and glucosinolate phenotype.
- *Arabidopsis thaliana* ecotypes will be more genetically distinct than the *Brassica* species due to its earlier divergence.

Chapter 2 – Response of the parasitoid *Diaeretiella rapae* to aphid-induced volatiles from *Brassica oleracea.*

2.1 Introduction

Brassica oleracea species. release a complex blend of volatiles prior to as well as after aphid feeding (see chapter 3; Bradburne & Mithen 2000). The qualitative and quantitative differences in these blends can be measured by using chemical analysis techniques such as gas chromatography and mass spectrometry (see chapter 3). However, these analyses are limited by many factors such as the sensitivity of equipment and the variability of emissions from plants. Also, these chemical analysis methods cannot give us any idea of the significance of herbivore-induced changes to the ecosystem being studied. Our understanding is more fundamentally limited by our subjective, human-centric view of the system. As humans, we cannot appreciate the importance of how the compounds are blended and there are possibly important subtleties in the quality or quantity of the compound blend. By using y-tube bioassays to analyse odour preference, the parasitoid is used as a biological detector to indicate host preference, albeit in a laboratory rather than a natural context. This chapter addresses the tritrophic system by analysing the behavioural responses of foraging female parasitoids to volatiles released from aphid challenged plants.

Insect parasitoids seek out and oviposit into phytophagous insect hosts by using semiochemicals for habitat and host location (see section 1.2.3 and Vinson (1976)). These semiochemicals can arise from the plant, the host, host frass (excreta), or a combination of these (Vaughn et al., 1996). Many studies have demonstrated that aphid parasitoids are attracted to herbivore-damaged plants when compared to undamaged plants (Guerrieri et al., 1993; Du et al., 1996; Du et al., 1997; Guerrieri et al., 1997; Guerrieri et al., 2002; Birkett et al., 2003; Girling et al., 2006). This propensity of the parasitoid to detect compounds from the plant rather than those released by the herbivore is believed to be a solution to the reliability-detectability problem. The reliability of a

stimulus indicates the host presence, its accessibility, and its suitability, while its detectability refers to the degree to which it can be perceived (Vet and Dicke, 1992). Semiochemicals released from the parasitoid host or directly from its activities would be highly reliable. However, natural selection has favoured those hosts that do not emit such tell-tale signs and therefore the detectability of these semiochemicals will be minimal (Du et al., 1996; Vinson, 1998). Semiochemicals released from plants that assist parasitoid foraging ability and produce a benefit for the plant (synomones) are generally less reliable than semiochemicals from the host level, but are more detectable because plants have a bigger biomass than hosts (Vet and Dicke, 1992). To solve the reliability-detectability problem, parasitoids may use a hierarchical search sequence, in which the more detectable semiochemicals from the host plants are used for host habitat location, and the more reliable semiochemicals from the host level serve for host location within that habitat (see section 1.2.3).

Previous studies have focussed on the importance of constituents of the volatile blend in attracting parasitoids (Vaughn et al., 1996; Du et al., 1997; Bradburne and Mithen, 2000) or the differential attraction of distinct populations of wild *Brassica oleracea* plants (Brockhouse, 2005). Features of the volatile profile that could influence parasitoid foraging behaviour include qualitative and quantitative attributes of compounds present and previous experience of host odour. This study uses the aphid endoparasitoid *Diaeretiella rapae* (MacIntosh) (Hymenoptera: Braconidae) that is able to parasitise more than 60 aphid species including the cabbage aphid *Brevicoryne brassicae* (L.) (Homoptera: Aphididae) (Pike et al., 1999). Recent evidence from Girling *et al.* (2006) shows that *D. rapae* is attracted to aphid-infested Arabidopsis – a member of the Brassicaceae and a distant relative of wild cabbage. Additionally, Blande (2004) showed that *D. rapae* is attracted to aphid-infested turnip (*Brassica rapa* var. *rapifera*) under certain conditions. Considering the evidence from other members of the Brassicaceae it is hypothesised that the wild cabbage *Brassica oleracea* subsp. oleracea is induced to release plant volatiles following aphid infestation that are attractive to the parasitoid *D. rapae*. Wild *B. oleracea* produce higher levels of glucosinolates when compared to common crop cultivars which have undergone artificial selection to reduce levels of these

bitter tasting compounds (Mithen et al., 1987; Bradburne and Mithen, 2000; Moyes et al., 2000; Burton et al., 2004; Brockhouse, 2005). Glucosinolates have been shown to be involved in a host of both direct and indirect defences (Giamoustaris and Mithen, 1995; Dicke and van Loon, 2000) (see section 1.4.1). By using plants from a natural ecosystem, it is hypothesised that the evolved defence responses will be fully active and a response will be induced in the foraging parasitoid.

It is possible that the parasitoids respond to a complex mixture of volatiles rather than a single component. Attempts have been made to identify the active compound(s) and 3-butenyl isothiocyanate (Fig. 1.9) which is released from damaged cruciferous plant tissue (Cole, 1980; Blande, 2004) has been identified as a possible semiochemical used by *D. rapae* to locate its hosts (Vaughn et al., 1996; Bradburne and Mithen, 2000; Blande, 2004). Bradburne and Mithen (2000) used *Brassica napus* lines that exhibit allelic variation in the *GSL-ELONG* locus to imply that *D. rapae* flight was oriented towards plants that exhibited higher 3-butenyl isothiocyanate production. The *GSL-ELONG* locus contains a gene that determines whether the resulting glucosinolate contains a 3-carbon or 4-carbon side chain. The hydroysis of these compounds can lead to formation of either 3-butenyl or 2-propenyl isothiocyanate. However, they did not demonstrate that 3-butenyl isothiocyanate as a single compound (and not other, unmeasured volatiles) was involved in the behavioural response of the parasitoids. Blande (2004) used y-tube bioassays to show that *D. rapae* are significantly attracted to 3-butenyl isothiocyanate compared to an ether control. It will be an aim of this chapter to explore the behaviour of parasitoids when presented with plant odour versus plant odour plus synthetic 3-butenyl isothiocyanate (ITC) in order to ascertain which compounds may be of ecological significance.

The behavioural responses of the parasitoids will be measured using y-tube olfactometer bioassays. This method presents the foraging insect with a choice of odours from two treatments – typically an aphid-infested plant versus an uninfested plant. The foraging insect will choose to move towards the source of one of the two odours or not make a choice (Fig. 2.1). Following several independent experimental replicates, statistical

analysis is conducted on the insect choice data to determine whether a preference exists for one odour over the other. One of the problems inherent in this odour bioassay is that of distinguishing between the odour released by the plant and the odour originating from the herbivore or from herbivore-derived substances. Both of these odours will be present in the volatile blend presented to the forager since the herbivores cannot be removed from the plant without further affecting the volatile blend. Previous studies have addressed this issue while investigating the reliability-detectability problem and have found that the volatiles emitted from the herbivore or herbivore-derived substances do not attract the parasitoid (Vet and Dicke, 1992; Du et al., 1996; Morgan and Hare, 1998; Vinson, 1998). Recent research conducted by Girling *et al.* (2006) investigated this problem by carrying out bioassays comparing the attractiveness of *Myzus persicae* aphids and honeydew with no-odour controls. They found that the parasitoid did not display any preference for the herbivore or honeydew alone. Read and colleagues (1970) found that *B. brassicae* was attractive to parasitoids but only after recent removal from the plant. This suggests that plant compounds that are metabolised by the aphid are attractive to the parasitoid but naturally occurring aphid volatiles are not. To address this issue, the odour emitted from the plant and aphid will be referred to as the plant-host complex (PHC).

If chemical cues are found to mediate this tritrophic interaction, it will be an important step in advancing our knowledge of native biological control systems. It will also present an opportunity to examine further aspects of this indirect defence in plants that have not undergone artificial selective breeding which may have reduced the efficacy of naturally evolved defences. Possibilities for the commercial application of this research include the development of semiochemical assisted biological control strategies, and improved integrated pest management strategies by way of a deeper understanding of ecological interactions.

The proposed differences in fitness between lab-reared and 'wild' parasitoids will be addressed and whether this difference affects the parasitoid's ability to discriminate between odours from infested and uninfested plants. Brockhouse (2005) presents evidence that wild parasitoids attack aphid hosts at a significantly higher rate and in a

significantly shorter time than lab-reared individuals. The lab-reared individuals used were taken from stocks at Rothamsted Research, which have been continuously cultured since 1995 (Blande, 2004), whereas the 'wild' parasitoids were collected as mummies from Durdle Door (grid ref. 380620,080235) and used within 24 hours of emergence. Several studies have shown that lab-reared parasitoids that have a previous learning experience of a plant-host complex display enhanced location and attack ability (Powell and Wright, 1991; Vaughn et al., 1996; Du et al., 1997; Du et al., 1998; Blande, 2004). However, Brockhouse (2005) found that this learning ability is only evident in lab-reared parasitoids, wild parasitoids do not appear to need this experience to perform as well as experienced lab-reared parasitoids. Additionally, Brockhouse presents evidence that lab-reared parasitoids have significantly smaller hind tibia and a significantly lower oviposition rate. It is suggested that the lab reared insects are less fit due to the continued inbreeding or lack of environmental stimulation. The response to PHC odour will be examined to determine whether these differences may lead to a lower level of parasitoid response to the chemical cues. If this is found to be the case, it may suggest that a true representation of the ecological interactions would not be observed when using lab-reared parasitoids.

2.1.1 Hypotheses:

- *D. rapae* will exhibit a significant preference for the plant-host complex rather then the uninfested control plant odour.
- *D. rapae* will exhibit a significant preference to the plant-host complex plus 3-butenyl ITC rather than the control plant-host complex odour.
- Lab-reared *D. rapae* will respond more slowly than field-collected parasitoids to the plant-host complex

2.2 Materials and Methods

2.2.1 Plants and insects

Brassica oleracea subsp. oleracea were grown in a glasshouse compartment at 24±5°C with 60±25% R.H. Plants were sown in 0.3L square plastic pots filled with standard JS2 potting compost (Levingtons, Ipswich, Suffolk, UK) with no extra fertiliser added.

B. brassicae were obtained from wild *B. oleracea* subsp. oleracea at Durdle Door, Dorset, UK (grid ref. 380620,080235). They were continuously reared for 14 months on plants grown from seed in the glasshouse from the same population in clear Perspex cages 70cm x 60cm x 45cm high. Aphids selected for infestation treatments were of mixed instars and removed from different parts of the host plant using a fine paintbrush. The aphids were placed inside an open cone of filter paper next to the main stem of the plant to be infested. This method prevented the aphids from falling off the leaves into the soil or pot tray during transfer to the plant. Empty filter paper was placed in an identical position on all control plants. Four replicates of each treatment were prepared. Plants were either left untreated or infested with 350 aphids for 96 hours. All plants were covered with perforated plastic plant sleeves (30x50x10cm, 40 micron punched, clear plastic. Avoncrop, Bracknell, UK) and sealed at the top and around the pot with masking tape.

2.2.2 Parasitoids

Wild *D. rapae* were first generation cultures which were collected as mummies from wild *B. oleracea* var. oleracea at Durdle Door, Dorset, UK during May 2005. Laboratory reared parasitoids were taken from stocks at Rothamsted Research, which has been continuously culturing the parasitoids since 1995 (Blande, 2004; Brockhouse, 2005). The parasitoids were reared using a culture of *B. brassicae* for two years (as described above) in a separate Perspex cage of similar dimensions. A 5% honey solution was provided in a Petri-dish. Parasitoid response to induced volatiles has been shown to be greatest from 1-4 days after emergence (Blande, 2004). In view of this, only parasitoids that were 2-4 days old were obtained for bioassays.

Parasitoids were collected by placing leaves with large numbers of mummies present into darkened plastic containers with a 2cm diameter hole leading into a clear closed Perspex cylinder. The parasitoids emerged and moved into the jar by means of phototaxis. This allowed collection of parasitoids of a known age. Following collection of parasitoids, females were selected and kept in sample tubes with cotton plugs for no longer than 3 hours prior to introduction into y-tube. The plugs were moistened with distilled water.

2.2.3 Y-tube olfactometer bioassays

Entire plants were placed inside 5L glass jars with lids fastened using bulldog clips. Air was filtered through activated charcoal then hydrated by bubbling through distilled water. Filtered hydrated air was pumped into the vessel through an inlet pipe at the bottom of the vessel at a flow rate of 0.3L/min. All tubing was Teflon 2mm diameter and connected using brass Swagelok connectors (Ohio, USA). Air passed over the plant, through an outlet at the top of the vessel and into one end of the y-tube (Fig. 2.1). The flow rates at the point of entry to the y-tube were checked simultaneously using flow meters. The y-tube was housed in a blackened box with a diffused fluorescent lamp at constant brightness directly above the centre point of the y-tube. Female *D. rapae* were introduced into the end of the tube via the glass sample tubes (Fig. 2.1) and parasitoid activity was monitored for up to five minutes. If the insect passed from section A (Fig. 2.1) to section B, the time and choice of arm was recorded as the 'first choice'. If the insect passed from section B to section C and stayed in this section for at least 15 seconds, the time and choice of arm was recorded and this was designated a 'final choice'. If the parasitoid did not leave section A for 5 mins, it was recorded as 'no choice'. All glassware was washed daily with Decon90 (Decon Laboratories, East Sussex, UK), followed by distilled water then acetone to remove any contaminating substances. Glassware was baked overnight at 220°C to remove volatile compounds. The charcoal filter was cleaned by baking overnight at 180°C with a flow of nitrogen passing through it. Temperature was maintained at 26±2°C with 60±15% R.H.

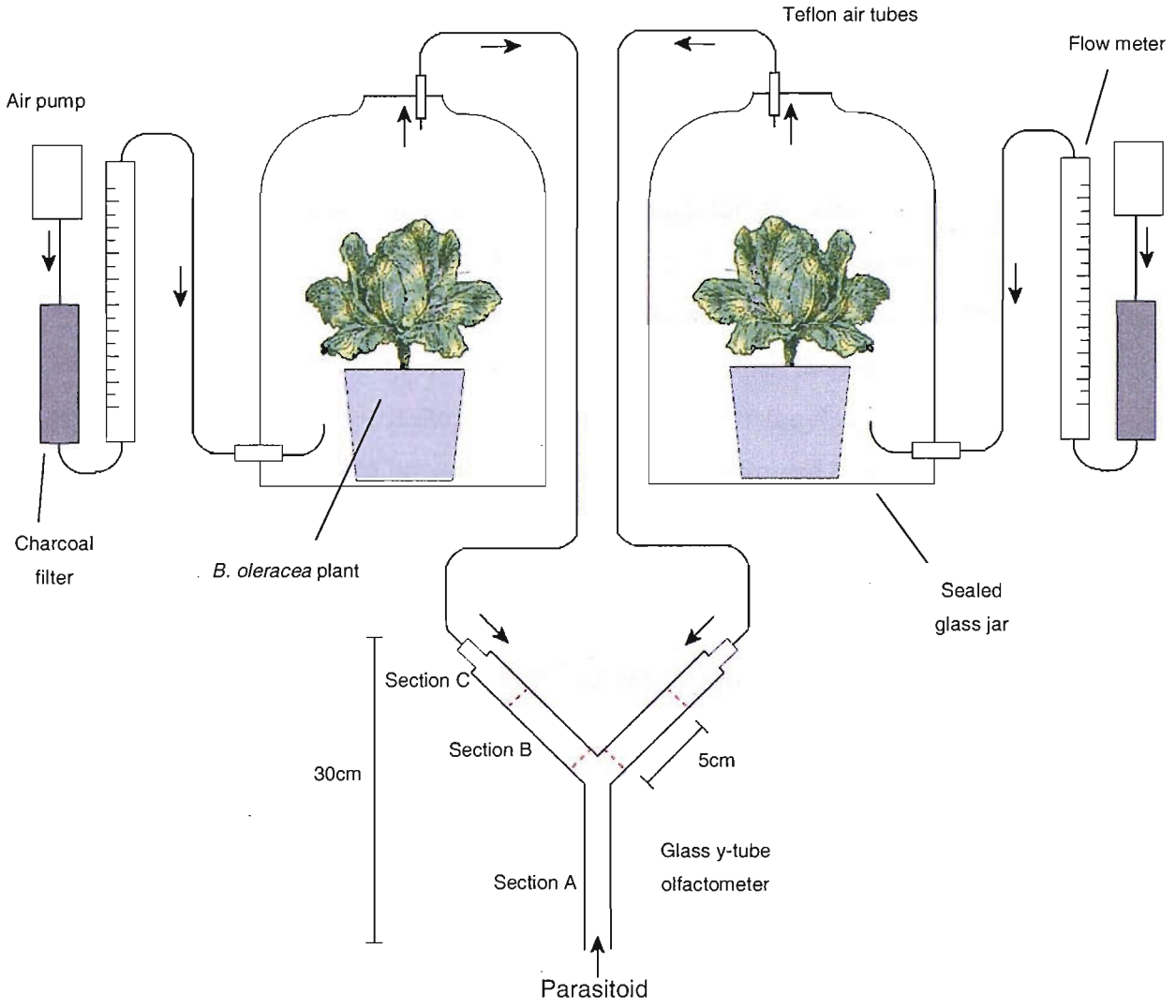


Fig. 2.1 – Y-tube olfactometer apparatus setup. Arrows indicate direction of air flow. Labels indicate entry route of parasitoid (section A), 'first choice' section (section B) and 'final choice' (section C) section.

The y-tube inlets were swapped every five replicates and the air pipes exiting the plant vessels were swapped every ten replicates to control for any bias in experimental conditions.

2.2.4 Y-tube olfactometer bioassays - Experiment 1 – May 2005

'Naïve' female *D. rapae* parasitoids were compared using y-tube olfactometer bioassays presenting either the plant-aphid complex from plants infested for four days with 350 *B.*

brassicae aphids or the odour from uninfested control plants. Individual parasitoids were used from both wild and lab-reared populations.

2.2.5 Y-tube olfactometer - Experiment 2 – July 2005

A small vessel was added to the apparatus between the top of the plant vessel and the y-tube. A $2cm^2$ piece of Whatman filter paper was impregnated with either 2µl 3-butenyl isothiocyanate (50ng/µl in hexane) or 2µl of hexane. The filter paper was left for 10 seconds before being put into the vessel to allow the solvent to evaporate. This procedure was carried out prior to each parasitoid replicate. Individual, lab-reared parasitoids were given the choice of plant + hexane (control) or plant + 3-butenyl isothiocyanate (ITC). The concentration of the compound to be tested was determined by estimating the amount of compound collected during GC analysed air entrainments (see chapter 3). These entrainments collect volatile compounds that are released from plants over a period of several hours so the concentrations applied to the filter paper were determined to consist of the amount released during one minute of plant compound emission.

2.2.6 Statistical Analysis

Initial and final choice data was analysed separately using a Chi-squared test. Yates' correction was applied to adjust for the data with only one degree of freedom. Response rate was calculated by the percentage of parasitoids making a first choice divided by the total number introduced to the y-tube.

A one-way ANOVA was used to compare the times taken for the parasitoids to respond. Homogeneity of variance was tested using Levene's test. All calculations were carried out using SPSS (v.13.0). Parasitoids that did not make a choice within the 5 minutes of experimentation were not included in the analysis.

2.3 Results

2.3.1 Y-tube olfactometer bioassays - Experiment 1 – May 2005

A total of 191 parasitoids (88 lab-reared, 103 wild) were assayed for odour preference over three consecutive days. Of the 158 parasitoids that responded and made a choice of odour (83% response), 78 were wild (75% response) and 80 lab-reared (90% response). Lab-reared exhibited a significant initial and final preference for the plant-aphid complex over the control plant odour (chi-squared with Yates' correction, $p=0.01$) (Fig. 2.2). Equally, wild parasitoids also showed a significant initial and final preference for the plant-aphid complex over the control ($p<0.01$) (Fig. 2.3).

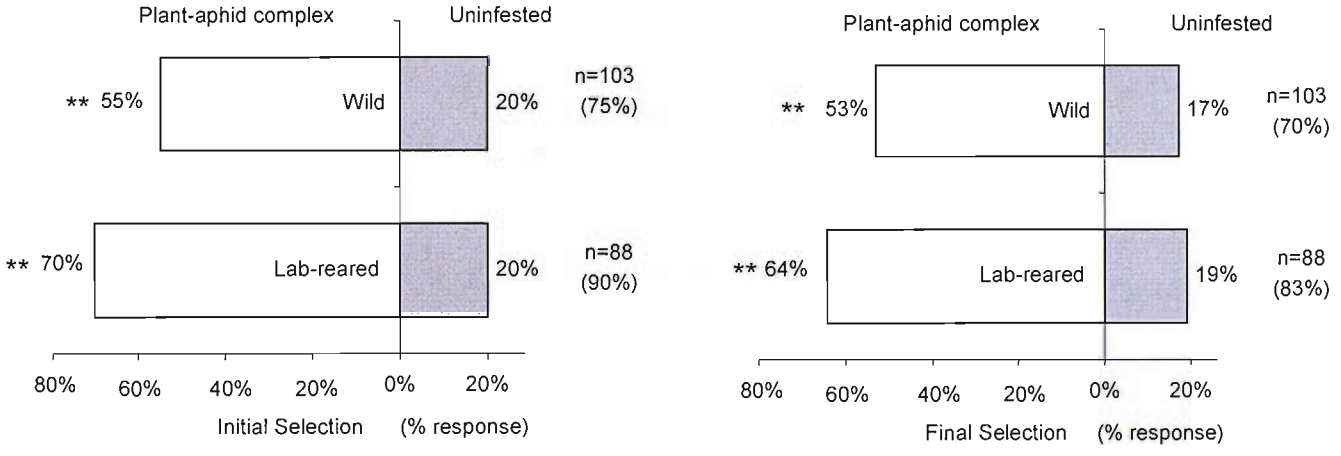


Fig. 2.2 – Initial (left) and final (right) selections by *Diaeretiella rapae* in a y-tube olfactometer of either plant-aphid complex infested for 96 hours or an uninfested plant. n for each treatment is displayed to the right with the % response in parentheses underneath. ** indicates a significant odour preference by the parasitoid ($p<0.01$).

The time taken for parasitoids to make an initial and final choice of y-tube arms was recorded and analysed. However, the frequency distribution of this data was not normal according to a Kolmogorov-Smirnoff test. Also, the variances were not equal according to Levene's test for homogeneity of variance. Although ANOVA is robust to unequal variances where the sample size is similar, the violation of both of these assumptions

requires a non-parametric analysis to be used. A non-parametric Kruskal-Wallis test showed that time taken for lab-reared parasitoids to make the initial choice of odour was not significantly different to the time taken by wild parasitoids (p=0.09) (Fig. 2.3a). Similarly, lab-reared parasitoids did not differ significantly when compared to wild parasitoids in the time taken for a final choice (p=0.95) (Fig. 2.3b).

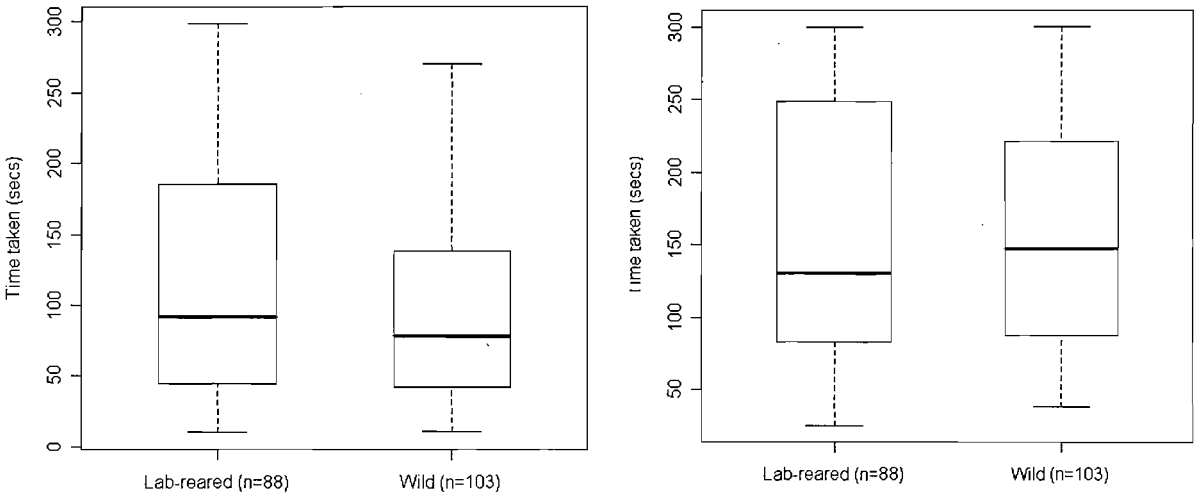


Fig. 2.3 – Boxplot showing the times taken for an initial (left) and final (right) parasitoid choice in the y-tube olfactometer. Plots show median (bold line), upper and lower quartiles and minimum and maximum data values.

It may be expected that those parasitoids that choose the plant-aphid complex may make the choice more quickly than those choosing the plant only odour. This could be expected as the parasitoid may be actively foraging for a host and respond more readily than a parasitoid that is not actively foraging. However, the parasitoids that chose the infested plant did not make a choice (initial or final) more quickly than those that chose the uninfested plant (ANOVA; initial choice, p=0.97; final choice, p=0.99).

2.3.2 Y-tube olfactometer bioassays - Experiment 2 – July '05

Lab-reared parasitoids were assayed for their preference of odour from an uninfested plant plus 3-butenyl isothiocyanate versus an uninfested plant with a hexane control. 73 parasitoids were assayed over 5 consecutive days of which only 61% made a choice of odour. There was no significant initial or final preference for either odour (Fig. 2.4).

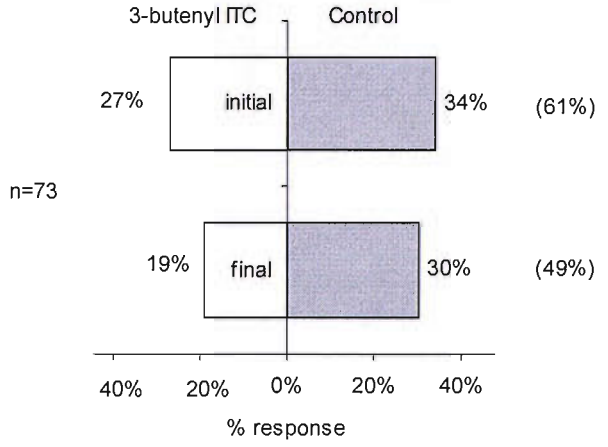


Fig. 2.4 – Analysis of *D. rapae* response when presented with one uninfested control plant (grey) versus one uninfested plant with 3-butenyl isothiocyanate added (unfilled bars). Number of replicates (n) is shown on the left. % of individuals that show a response is shown in parentheses.

The time taken for the parasitoids to make an initial or final choice was analysed for normality using a Kolmogorov-Smirnoff test. This showed that the data were normally distributed and a one-way ANOVA showed that there was no significant difference in the time taken to respond to the odour containing 3-butenyl isothiocyanate compared to that of the control (Fig. 2.5) (initial choice $p=0.40$, final choice $p=0.540$).

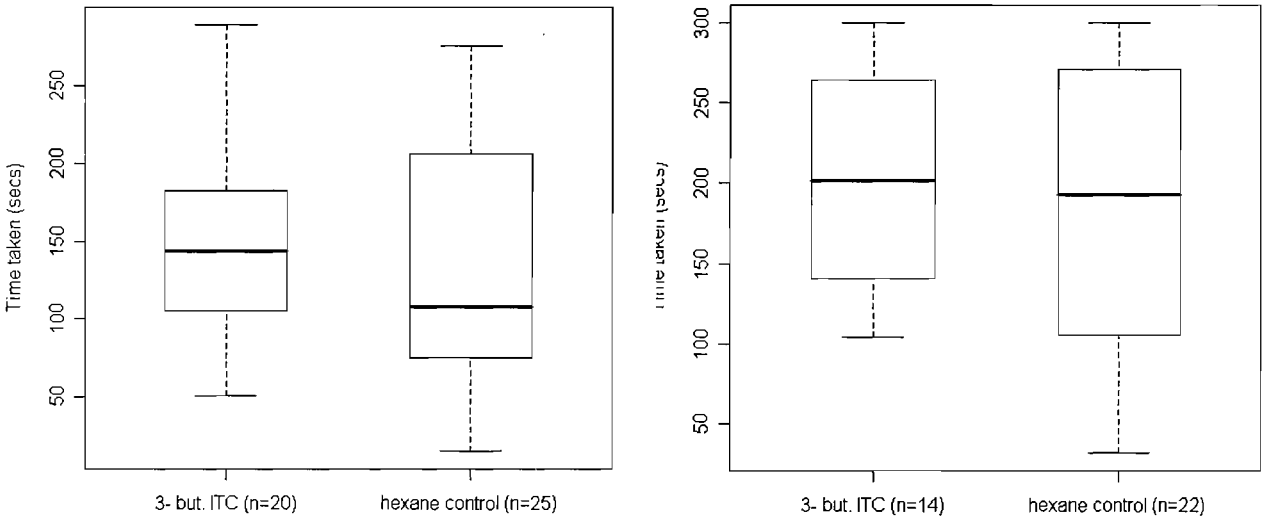


Fig. 2.5 – Boxplot showing the times taken for an initial (left) and final (right) parasitoid choice in the y-tube olfactometer. Plots show median, upper and lower quartiles and minimum and maximum data values.

2.4 Discussion

Volatile compounds are known to be important cues used by parasitoids in the location of the host habitat and the location of a host within that habitat (Vinson, 1976). This research has demonstrated for the first time in a wild plant that female parasitoids show a preference for a plant-aphid complex compared to an uninfested control. Although this study has been conducted in a laboratory context and further work will be needed to confirm the results in a field-situation, it is likely that this multitrophic interaction involving the plant, aphid and parasitoid would occur in their natural habitat. This interaction could increase the fitness of the parasitoid by improving habitat and host location capability and thus reducing flight times, energy expenditure and improving oviposition rate. The fitness of the plant may also be increased as a result of this interaction by limiting the damage done by the feeding aphids. The evolution of this interaction will be addressed in depth in the final discussion (chapter 6).

2.4.1 Comparison of the foraging behaviour of wild and lab-reared parasitoids

Y-tube olfactometer bioassays showed that both naïve wild and naïve lab-reared parasitoids preferred the plant-aphid complex and that there was no significant difference in the time taken to respond to the odours. This result is in contrast to research presented by Brockhouse (2005) which found that laboratory-reared *D. rapae* with an 'experience' of the host-plant complex displayed enhanced attack activity when compared to 'naïve' lab-reared insects. Evidence from Brockhouse (2005) and previous studies (Bryant and Cowles, 2000; Mohan and Gujar, 2003) has led to the suggestion that laboratory rearing may decrease the insect's fitness and foraging efficiency. The contrasting result observed in this study suggests that the attack behaviour and the habitat/host location ability of the parasitoid are separate physiological functions. A decrease in the parasitoid's attacking rate when presented with a number of aphids in an open arena (Petri-dish) does not necessarily impute a decrease in the foraging efficiency using host-plant odours. This study shows that the host-plant location ability using semiochemicals and the rate of taxis to the odour source appears to be unchanged. This result suggests that the foraging

capability using chemical cues is just as important in a restricted environment as in a natural context. It is possible that the decrease in hind tibia length observed by Brockhouse (2005) in laboratory reared parasitoids may be as a result of evolution selection for parasitoids with smaller body sizes that require less nutrient intake. In the natural environment, larger muscle mass and energy resources would be selected for by the increased ability of the parasitoid to forage and oviposit and the wider area with over which its offspring can be spread. The first three of the steps proposed by Vinson (1976) when considering foraging behaviour of parasitoid wasps - host habitat location, host location and host acceptance – are believed to require both visual and chemosensory input to the parasitoid. The decreased forage area under laboratory conditions would suggest that the parasitoids may be able to rely solely on visual cues to locate suitable hosts. However, evidence from this study suggests that visual cues may be less important since the host location behaviour using chemical cues is not adversely affected despite many generations of high host density and low forage area. The importance of chemical cues is not limited to the host location behaviour and cues may also be used to discriminate between previously parasitised hosts. This ability to discriminate would be equally important in a highly competitive restricted environment and could explain the results obtained.

2.4.2 Parasitoid response to plant odours supplemented with 3-butenyl isothiocyanate

Experiment one shows that there is evidence that parasitoids are attracted to the plant-aphid volatile complex over an uninfested control plant. However, this confirmation tells us nothing about the difference between the blend of volatiles released by from the plant-aphid complex and the uninfested plant. Whether the difference in behaviour is due to a specific compound, or due to a number of compounds has not been determined and further analysis of blend constituents will be conducted in chapter 3. Experiment two addresses this question by using a compound added to the blend from an undamaged plant in order to study whether a similar response is observed. However, the treatment applied did not elicit any significant preference for 3-butenyl isothiocyanate or the hexane control and supports results obtained by Brockhouse (2005) which found that the

addition of 3-butenyl isothiocyanate to the plant-aphid complex did not attract *D. rapae*. This is contrary to the indications from Blande (2004) which suggest that *D. rapae* are preferentially attracted to the 3-butenyl isothiocyanate when compared to the ether control odour. The amount of isothiocyanate solution used by Blande in each assay was 50μg compared to the 0.1μg used in this study. This reduction in the amount of compound present could explain the differences in the results although in an ecological situation, the concentration and amounts of isothiocyanate would almost certainly be a tiny fraction of both of these volumes. Research conducted by Bradburne and Mithen (2000) uses plants which show allelic variation at the *GSL-ELONG* gene locus which controls the structure of the isothiocyanates produced. The plants differ in the amount of 3-butenyl isothiocyanate being produced. Headspace volatile analysis of these plants, combined with electrophysiological analyses of the parasitoid to emitted volatiles would be useful to gain further insight into parasitoid response and odour preference. This experiment has shown under the conditions presented, the parasitoids did not prefer one of the odour sources over the other. Whether parasitoids in a natural context are attracted to 3-butenyl isothiocyanate in order to facilitate improved foraging efficiency remains to be investigated. To address this question, further experiments must be conducted that investigate not only whether the parasitoid is attracted to this compound but at which concentrations the odour may be attractive in a natural or laboratory environment.

The inactivity and low level of response of parasitoids can be considered a major factor in this experiment. It was observed that on some days, the parasitoids are less responsive to foraging cues and the parasitoids do not appear to exhibit foraging and host orientation behaviour. 61% of parasitoids making an initial choice and 49% for final choice combined with a relatively high mean response time (141 secs and 189 secs respectively) are indicative of a lack of active host locating behaviour. It can be observed by the experimenter that the behaviour during host location foraging behaviour and non-active 'wandering' is different and it is hypothesised that the response rates obtained would not be very different to those of parasitoids introduced to a y-tube without any odour or even air flow. The low response could be due to the fact that the plants are completely uninfested and so are not releasing compounds that are recognised by the parasitoid as

host location infochemicals. This would indicate that although 3-butenyl isothiocyanate may be an important component of the volatile blend, it is not the sole component that the parasitoids are responding to. Additionally, the low level of response could be due to a number of environmental factors that are hard to control for that affect either parasitoid response or the emissions of the plant-aphid complex. These factors include time of day, temperature, barometric pressure, barometric pressure change or natural circadian rhythms (Steinberg et al., 1992; Takabayashi et al., 1994; Fournier et al., 2005). Insect flight and dispersal is triggered or hindered either by intrinsic factors (genetic or physiological) and/or by extrinsic factors (population interactions or the abiotic factors noted above) (Fournier et al., 2005). The hymenopteran egg parasitoid *Trichogramma* spp. is used as a biological control agent and temperature has been shown to have a strong influence on flight initiation (Suverkropp, 1997). Temperature thresholds for flight initiation have been established for a number of *Trichogramma* species and their dispersal is improved in warmer, sunny weather (Keller et al., 1985; Fournier et al., 2005). However, by conducting the experiments indoors at 26±2°C, it is unlikely that this factor affects the propensity of the parasitoids to exhibit foraging behaviour. Rapid changes in barometric pressure have been shown to reduce flight initiation and the response of parasitoids to volatile infochemicals (Steinberg et al., 1992; Marchand and McNeil, 2000). These responses to abiotic conditions are likely to have evolved in order to restrict flight to periods with suitable conditions for host location and to avoid the risk of dispersal to unsuitable habitats (Fournier and Boivin, 2000). The environment inside a glass y-tube is not representative of a natural environment due to the texture of the surfaces, the enclosed space and the fluorescent lighting. In view of this, it is possible that there is a decrease in the likelihood of flight initiation or active host foraging behaviour. Carrying out the same experiment using wind-tunnel apparatus may result in a scenario that more closely matches that which the parasitoid encounters in a natural context. Another factor that could affect parasitoid response is that the odour concentration would be abnormally high since the odour from an entire plant is presented to the parasitoid and this may cause the parasitoid to exhibit arrested or avoidance behaviour.

Further work in this area may include carrying out bioassays using a different method of compound release and different concentrations of compound. Another method for the elucidation of biologically active plant volatiles may be to use a combinatorial chemistry approach. Several compound mixtures are assayed and those inducing odour preference behaviour from the parasitoids can then be further analysed to give an indication as to whether a specific compound is involved or a compound blend. This combinatorial approach hugely reduces the number of experiments necessary to identify active compounds.

2.5 Summary

- Female *Diaeretiella rapae* exhibit foraging behaviour that is oriented towards the odour of *B. brassicae* infested *B. oleracea* var. oleracea when compared to the odour from uninfested control plants.
- Wild parasitoids have the same preference for the plant-aphid as lab-reared parasitoids and there is no difference in the time taken to respond.
- Under laboratory conditions, lab-reared parasitoids do not exhibit a preference for wild cabbage supplemented with a specific quantity of 3-butenyl isothiocyanate when compared to wild cabbage alone.

Chapter 3 Volatile organic compound emissions from the wild cabbage *Brassica oleracea*

3.1 Introduction

Undamaged plants release a range of volatile organic compounds (VOCs or volatiles) including monoterpenes, sesquiterpenes and aromatic compounds. These compounds accumulate and are released from leaf stomata and specialised glands known as trichomes in the leaf epidermis (Pare and Tumlinson, 1997). They often function as direct plant defences and are repellant, distasteful or toxic to attacking herbivores (Mithen, 1992a; Giamoustaris and Mithen, 1995; Shields and Mitchell, 1995; Schoonhoven, 1998). This defence is not always effective however. The specialist herbivores *Lipaphis erysimi* and *Brevicoryne brassicae* have evolved to tolerate and exploit their host plant direct defence compounds and are even attracted to the glucosinolate derivative 3-butenyl isothiocyanate (Fig. 1.10) (Nottingham et al., 1991). In some plant systems, indirect defences have evolved where a natural enemy of the attacking herbivore is 'recruited' to nullify the threat. This indirect defence can take the form of volatile semiochemicals that communicate the threat via airborne chemical signals. Following either mechanical wounding or herbivory, the plant's volatile profile is changed by the autolytic oxidation of membrane lipids to yield a range of green-leaf volatiles (Pare and Tumlinson, 1999). During herbivory, the profile of the volatiles emitted is markedly different from those of undamaged or mechanically damaged plants and in some cases is unique for specific herbivores (Turlings et al., 1998; Ozawa et al., 2000). It is this change in volatile profile that is used by predators or parasitoids of phytophagous insects to enhance their foraging success. If this indirect response is shown to decrease herbivore preference or performance, it is termed induced resistance and if this increases plant fitness, it is termed an induced defence (Karban and Myers, 1989).

Previous studies (Du et al., 1998; Weissbecker et al., 2000; Smid et al., 2002) have sought to identify the compounds released from plants in response to herbivory in the

hope of identifying which of these compounds influence the third trophic level. In studies on differing plants and systems, a number of compounds have repeatedly been identified as being induced by herbivory (Du et al., 1998; Vuorinen et al., 2004b) with some being linked to specific genes involved in their synthesis (Frey et al., 2000; Shen et al., 2000). These studies use cultivated crops which have been selected for their marketable characteristics such as taste and yield. In a wild system, natural selective pressures from herbivory, environmental conditions and resource competition are present and co-evolutionary relationships between original adversaries are ongoing. The wild plant may exhibit a higher level of resistance to herbivory than crops which have been artificially bred and their survival assisted by the addition of pesticides. This loss of defensive capabilities following artificial plant breeding is likely to have occurred in plants of the family *Brassicaceae.* Members of this family contain high levels of glucosinolate compounds and cultivated varities have been shown to contain lower levels of these defensive compounds (Mithen et al., 1987; Burton et al., 2004) (see section 1.2.1). Glucosinolates have been shown to be precursors to herbivore-induced plant volatiles and plant secondary metabolites that increase direct resistance to herbivory. It will be the aim of this chapter to investigate how herbivory affects the emission of herbivore-induced plant volatiles from wild plants.

There is evidence that plants release a qualitatively and quantitatively different blend of volatiles depending on the attacking herbivore and the method of feeding (Takabayashi et al., 1995; De Moraes et al., 1998; Dicke, 1999). These differences are believed to be one of the methods by which herbivore parasitoids locate their hosts (see chapter 2). This study aims to use chemical and statistical analysis techniques to distinguish between the volatiles emitted from plants that have been infested with either a phloem-feeding insect (*Brevicoryne brassicae*) or a chewing insect (*Plutella xylostella* larvae). Volatiles will be collected using air entrainment apparatus and the headspace collected will be analysed using gas chromatography (GC) or GC linked mass spectrometry (GC-MS). Comparing the volatile profile from infested and control plants may highlight compounds that induce a behavioural or physiological response in aphids or their parasitoids. The entrainment method employed during these experiments detects volatiles released from specific parts

of the growing plants and does not damage the plant or require potentially stress inducing manipulations of the plant such as removal from the soil or the use of clip-cages. These manipulations have been shown to affect leaf development and may affect the expression of genes and the volatile emissions from the plant (Moore et al., 2003).

The accuracy and sensitivity of the methods employed to detect plant volatiles has improved considerably in recent years. However, this improvement has also required an increase in the scope and power of the data analysis techniques. This was realised many years ago in groups studying global gene expression profiles and a number of bioinformatics techniques are used to extract usable information from datasets with more than one response variable for observation. Cluster analyses are used to cluster variables with a similar response and can be used to infer functional relationships between distinct and previously unrelated entities. Principal component analysis (PCA) describes a linear function that is composed of variables with maximal variance. Discriminant analysis is used to create a linear function that is composed of the variables that allow maximal discrimination between pre-defined treatment groups. Degen *et al.* (2004) used both cluster analysis and PCA during analysis of volatiles released from maize treated with caterpillar saliva. These techniques extracted data showing that there is considerable influence of genetic factors on volatile emissions and biosynthetic relationships were highlighted between individual compounds. Although the techniques and equipment used to detect plant volatiles has improved, the insects themselves have been shown to be highly sensitive biological detectors. Chapter 2 highlights this fact whereby a parasitoid is able to distinguish between an infested and uninfested plant in a very short time frame by processing the odour signals effectively. When considering the method of signal recognition that is employed by foraging parasitoids, electrophysiological studies have shown that the insects respond to specific volatiles (Bruce et al., 2005). By using this method of detection, it is possible that the insect has evolved to detect only those compounds that have a bearing on its life history and is able to 'filter out' the other compounds that are unimportant during host location (van Dam and Poppy, 2007).

In this study, compounds that are emitted from plants in significantly different quantities between treatments will be identified using a one-way analysis of variance. However, although ANOVA is useful to indicate differences in individual compounds, the ecological interactions with other trophic levels may be elicited by the detection of particular volatiles as described above. In this study, canonical discriminant analysis (CDA) (also called multiple discriminant analysis or discriminant factor analysis) is used to extract information from the data on the volatile blend as a whole. This multivariate data analysis technique discriminates between the treatment groups by creation of a canonical discriminant function. The compounds that are most effective in distinguishing between treatment groups are added to the linear function with different loadings to indicate the importance of that compound. This method may enable a more insect-centric perspective of the compounds being emitted and suggest those that are most important in mediating ecological interactions.

3.1.1 Hypotheses

- The blend of volatiles detected from herbivore-damaged plants will be qualitatively and quantitatively distinct to the blend emitted from undamaged plants.
- Plants that have undergone feeding by phloem-feeding insects will emit a different VOC blend to that induced by a chewing insect.
- Canonical discriminant analysis is an effective technique for the analysis of VOCs and can aid in the biological interpretation of chemical data.

3.2 Materials and Methods

3.2.1 Plants and insects

Brassica oleracea subsp. oleracea were grown in a glasshouse compartment for 5 weeks at 24±5°C with 60±25% R.H. Plants were sown in 0.3L square plastic pots filled with standard JS2 potting compost (Levingtons, Ipswich, Suffolk, UK) with no extra fertiliser added.

B. brassicae were obtained from wild *B. oleracea* var. oleracea at Durdle Door, Dorset, UK (grid ref. 380620,080235). They were reared on plants grown from seed in the glasshouse from the same population in clear Perspex cages 70cm x 60cm x 45cm high. Aphids selected for infestation treatments were of mixed instars and removed from different parts of the host plant using a fine paintbrush. The aphids were placed inside an open cone of filter paper next to the main stem of the plant to be infested. This method prevented the aphids from falling off the leaves into the soil or pot tray during transferal to the plant. Empty filter paper was placed in an identical position on all control plants.

Plutella xylostella larvae were reared on Chinese cabbage (*Brassica rapa* var. pekinensis) in clear Perspex cages 70cm x 60cm x 45cm high. Larvae selected were of 2nd to 4th instars.

3.2.2 Air entrainments from treated plants

All air lines were baked at 180°C and the charcoal filters were baked at the same temperature with a stream of filtered nitrogen being passed through them to reduce background volatile contamination. The apparatus was assembled as shown in Fig. 3.1. Entrainment bags (Multi-purpose cooking bags, Sainsburys, UK) were oven baked at 110°C for at least 2 hours. The use of pre-baked oven bags has been shown to be an efficient and versatile method for collection of plant volatiles with minimal background contamination (Stewart-Jones and Poppy, 2006). Plants and air inlets were carefully inserted into the open end of bags then left for 15 mins at a flowrate of 0.9L/min. The bags were closed around the stem of the plant using gardening wire (Homebase, UK).

Thermal desorption (TD) tubes (Anatune Optic PTV liner) were packed with a small plug of silanised glass wool, 50mg tenax TA 60/80 (Supelco, UK) then a further plug of silanised glass wool. Prior to use, the tubes were cleaned at 250°C in the HP 6890 GC. Air filtered through activated charcoal was pumped into the bag through an inlet tube passed through the neck of the bag at a flow rate of 0.9L/min. Air was drawn out through the tenax TD tube inserted through a snipped off corner of the bag at a rate of 0.8L/min. The bag was secured around the shaft of the TD tube using gardening wire.

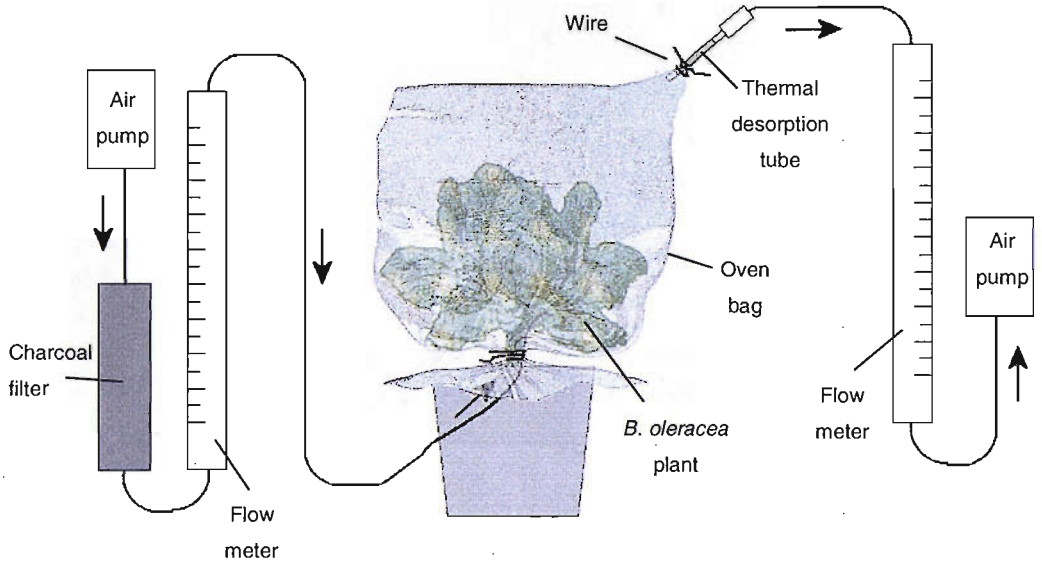


Fig. 3.1 – *B. oleracea* air entrainment apparatus. Arrows indicate air flow direction.

VOCs adsorbed onto the tenax were analysed using gas chromatography following the method outlined below. TD tubes were stored at all times in baked glass tubes which had been sealed prior to storage at -20°C. All tubing was 2mm diameter Teflon and connected using brass Swagelok (Crewe, UK) connectors. Sampled leaf area was measured using an ADC BioScientific AM200 (Hoddesdon, UK) area meter scanner.

3.2.3 Volatile analysis using Gas Chromatography

Volatiles adsorbed onto tenax during entrainments were desorbed using an Optic 2 programmable injector (Anatune, Cambridge, UK) fitted to a Hewlett-Packard 6890N GC with flame-ionisation detector (FID). Injector temperature (30°C) and pressure (18psi) were equilibrated for 1 min then ramped to 220°C at 16°C/sec whilst operating in splitless mode (6 min). A non-polar fused silica capillary column (50m x 0.32mm i.d.) was used. The carrier gas was helium (constant 18 psi) and oven temperature was held at 30°C for 2 min then increased by 5°C/min to 150°C then at 10°C/min to 250°C and held for 6 min. Detector temperature was 260°C. The ionisation current was measured in picoAmps (pA) and analysed using Agilent ChemStation Plus (Rev. B.01.01).

3.2.4 Compound identification using GC-MS

Air entrainments samples were collected from aphid-infested, *Plutella*-infested and control plants for 32 hours. This length of collection period ensured sufficient quantity of material was collected for a positive identification. The samples were analysed using a Hewlett Packard 6890 GC linked to a Hewlett Packard 6890 mass selective detector. The area of GC peaks gives an indication of the amount of compound collected during the entrainment and is measured in picoAmps^2 (pA^2). Compounds were identified by spectra comparison to libraries. Unique compounds were identified by assessing which peaks with a mean area over 10 pA^2 could be consistently distinguished from background peaks.

3.2.5 Experiment 1 – Identification and quantification of volatiles released from aphid-infested plants (January 2006)

Eight replicate samples were analysed. Plants were either left untreated or infested with 200 aphids. Both control and infested plants were left for 96 hours in a glasshouse compartment at least two metres apart. All aphids moved from the filter paper to the plant within four hours. Air entrainments were conducted for four hours each.

3.2.6 Experiment 2 – Identification and quantification of volatiles released from aphid-infested and *Plutella*-infested plants (July 2006)

Eight replicate samples were analysed. Control plants were left untreated. Aphid infested plants had 150 aphids present on them for 12 days. *Plutella* infested plants had five larvae present for three days. All aphids moved from the filter paper to the plant within four hours. Air entrainments were conducted for five hours each.

3.2.7 Statistical analysis

Unique compound peak areas were extracted from the chromatogram and normalised to the leaf area sampled. The values were $\log_2$ transformed in order to reduce the effect of outliers on the subsequent analysis and to homogenise the variance. Peaks with an area less than $1pA^2$ (picoAmps) were assigned an area of 0.5.

For each compound, transformed peak area was subjected to a one-way analysis of variance (ANOVA). Each compound was treated as an independent variable grouped by the different treatments. Each variable was tested for normality using a Kolmogorov-Smirnov test.

Canonical analysis of discriminance (CAD) was used to discriminate between treatment groups and highlight the compounds that contribute most to this discrimination. CAD involves deriving linear combinations (canonical functions) of the variables that will best discriminate among the a priori identified groups. The canonical functions are defined as weighted linear combinations of the original variables, where each variable is weighted according to its ability to discriminate among groups (McGarigal et al., 2000). All statistical procedures were carried out using SPSS (v. 13.0).

3.3 Results

3.3.1 Experiment 1 - Identification and quantification of volatiles from aphid-infested plants (January 2006)

Analysis was conducted on clean control plants and on wild cabbage infested with 200 aphids for 96 hours. Volatile organic compounds released from both treated and control plants were collected for 4 hours and analysed using gas chromatography.

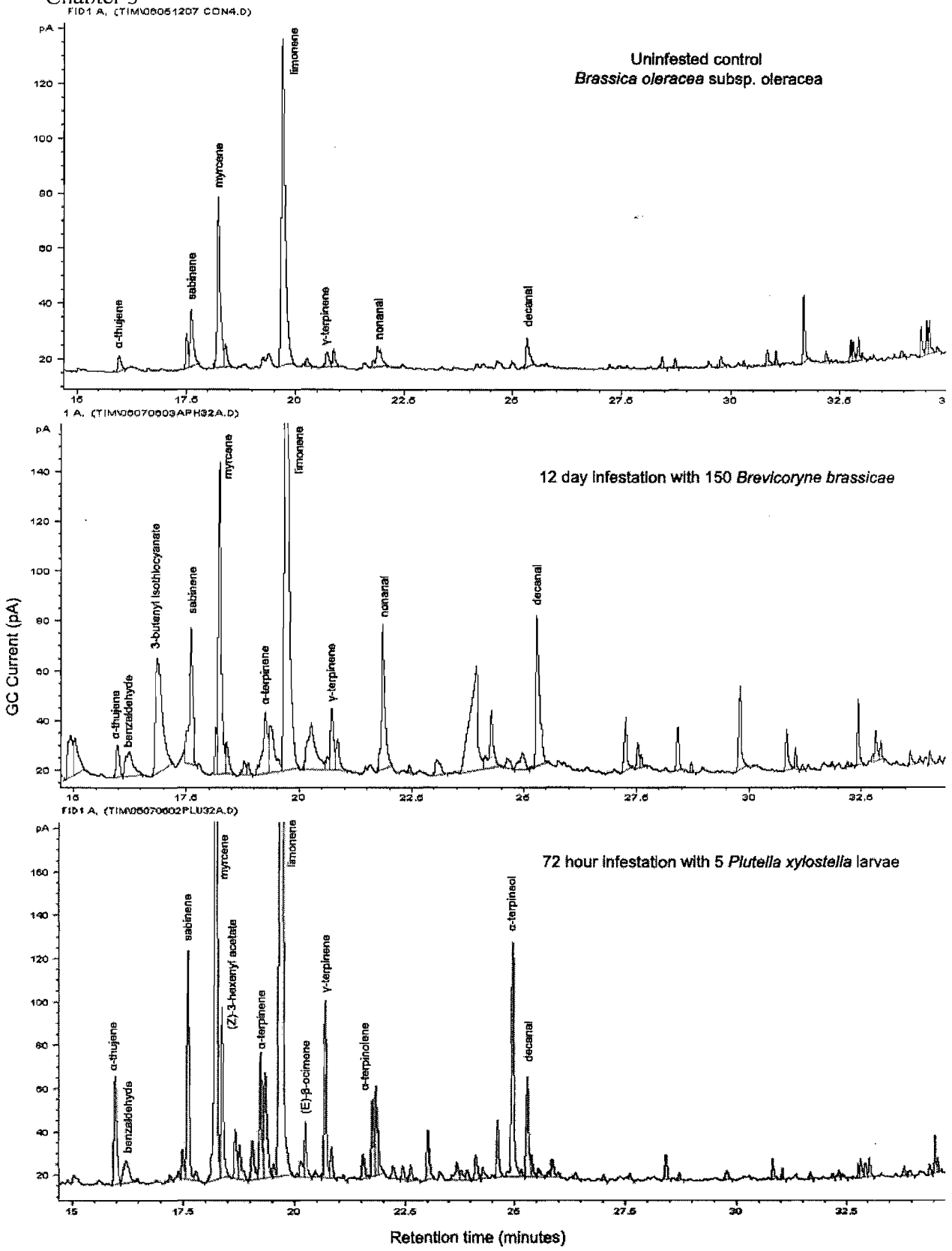


Fig. 3.2 – Example chromatogram illustrating the compounds emitted following different plant treatments.

Analysis of variance

The amount of compound (as measured by the flame ionisation current peak area) collected from the headspace of both treated and control plants was compared using a one-way analysis of variance (Table 3.1). This method will detect the compounds that are emitted from the plant-host complex only following aphid infestation.

Table 3.1 – Emission of VOCs from aphid-infested and control plants. * indicates a significant difference where $p<0.05$, ** indicates $p<0.01$.

Significance	Compound ID	Retention time (mins)	Mean transformed peak area ± s.error		
			Control (n=8)	Aphid Infested (n=8)	ANOVA p-value
	α-thujene	15.964	0.7 ± 0.63	1.96 ± 0.89	0.295
	benzaldehyde	16.203	1.34 ± 0.78	1.87 ± 0.92	0.679
**	3-butenyl ITC	16.833	-1 ± 0	4.07 ± 1.21	0.002
*	9a	17.493	3.35 ± 0.33	4.61 ± 0.32	0.020
	Sabinene	17.608	4.89 ± 0.42	5.86 ± 0.33	0.104
**	Myrcene	18.227	5.64 ± 0.27	6.86 ± 0.23	0.005
	(z)-3-hexenyl acetate	18.384	4.54 ± 0.97	5.47 ± 1.05	0.544
	hexyl acetate	18.685	-0.57 ± 0.4	0.66 ± 0.59	0.125
	10a	19.238	0.73 ± 0.46	0.91 ± 0.75	0.844
*	10b	19.355	4.95 ± 0.19	5.52 ± 0.15	0.039
	1,8-cineole	19.547	4.44 ± 0.39	4.63 ± 0.17	0.664
	Limonene	19.690	6.88 ± 0.38	7.48 ± 0.47	0.353
	(E)-β-ocimene	20.254	2.74 ± 0.59	3.64 ± 0.35	0.220
	10c γ-terpinene	20.701	0.59 ± 0.54	0.83 ± 0.69	0.800
	trans sabinene hydrate	20.849	2.37 ± 0.46	3.6 ± 0.42	0.077
	10d linalool oxide	21.557	4.88 ± 1.22	3.25 ± 0.95	0.320
	α-terpinolene	21.771	0.99 ± 1.2	1.12 ± 0.64	0.927
	11a branched monoterpene	23.046	-1 ± 0	-0.61 ± 0.39	0.369
	11b	23.694	0.98 ± 0.71	1.65 ± 0.7	0.523
	11c	23.961	0.21 ± 0.55	1.27 ± 0.68	0.269
	11d monoterpene alcohol	24.122	2.79 ± 0.34	2.81 ± 0.63	0.975
	11e	24.289	4.3 ± 0.98	3.23 ± 0.9	0.451
	11f	24.460	2.61 ± 0.73	3.87 ± 0.72	0.252
**	terpinen-4-ol	24.620	2.23 ± 0.31	3.81 ± 0.33	0.005
	11g α-terpineol	24.964	0.61 ± 0.71	1.36 ± 0.74	0.492
	decanal	25.399	2.55 ± 0.9	3.37 ± 0.69	0.493
**	13a	28.724	2.82 ± 0.2	3.91 ± 0.24	0.005
**	tetradecane	31.040	4.23 ± 0.13	5.21 ± 0.16	0.001
	benzyl isothiocyanate	31.183	4.01 ± 0.53	5.08 ± 0.19	0.080
	β-ionone	32.433	4.42 ± 0.42	4.3 ± 0.82	0.908
	14c	32.765	3.86 ± 0.41	4.32 ± 0.33	0.412
	α-farnesene	32.917	5.23 ± 0.39	6.2 ± 0.37	0.105
	15a	33.034	3.85 ± 0.78	5.19 ± 0.24	0.128
*	15c	33.827	0.62 ± 0.58	2.44 ± 0.51	0.041
	Total VOCs released		9.69 ± 0.21	10.24 ± 0.19	0.112

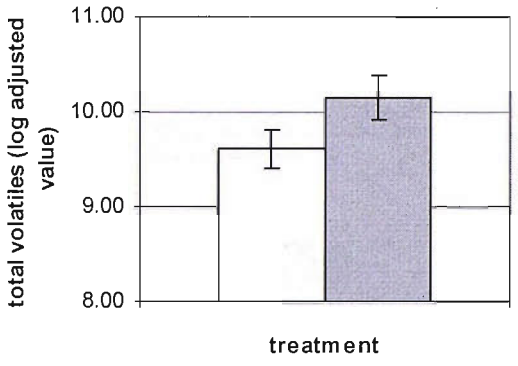


Fig. 3.3 – Mean total quantity of volatiles released from control (unshaded) and aphid-infested (shaded) plants. ANOVA indicates that the difference observed is not significant ($p<0.05$).

A number of common plant volatiles were detected in the headspace of the plant and some of these exhibited a significant increase following infestation. In particular, the *Brassica* specific glucosinolate derivative 3-butenyl isothiocyanate was emitted in much greater quantities following aphid infestation. The mean quantity of volatiles produced from plants infested with 200 aphids for 96 hours did show an increase, however, this difference was not significant (ANOVA, $p=0.112$). Although no significant overall quantitative difference was observed, significant increases were observed in a number of compounds when considered individually. Fig. 3.4 is a graphical representation of dat extracted from table 3.1 and shows the compounds that exhibit a significant difference.

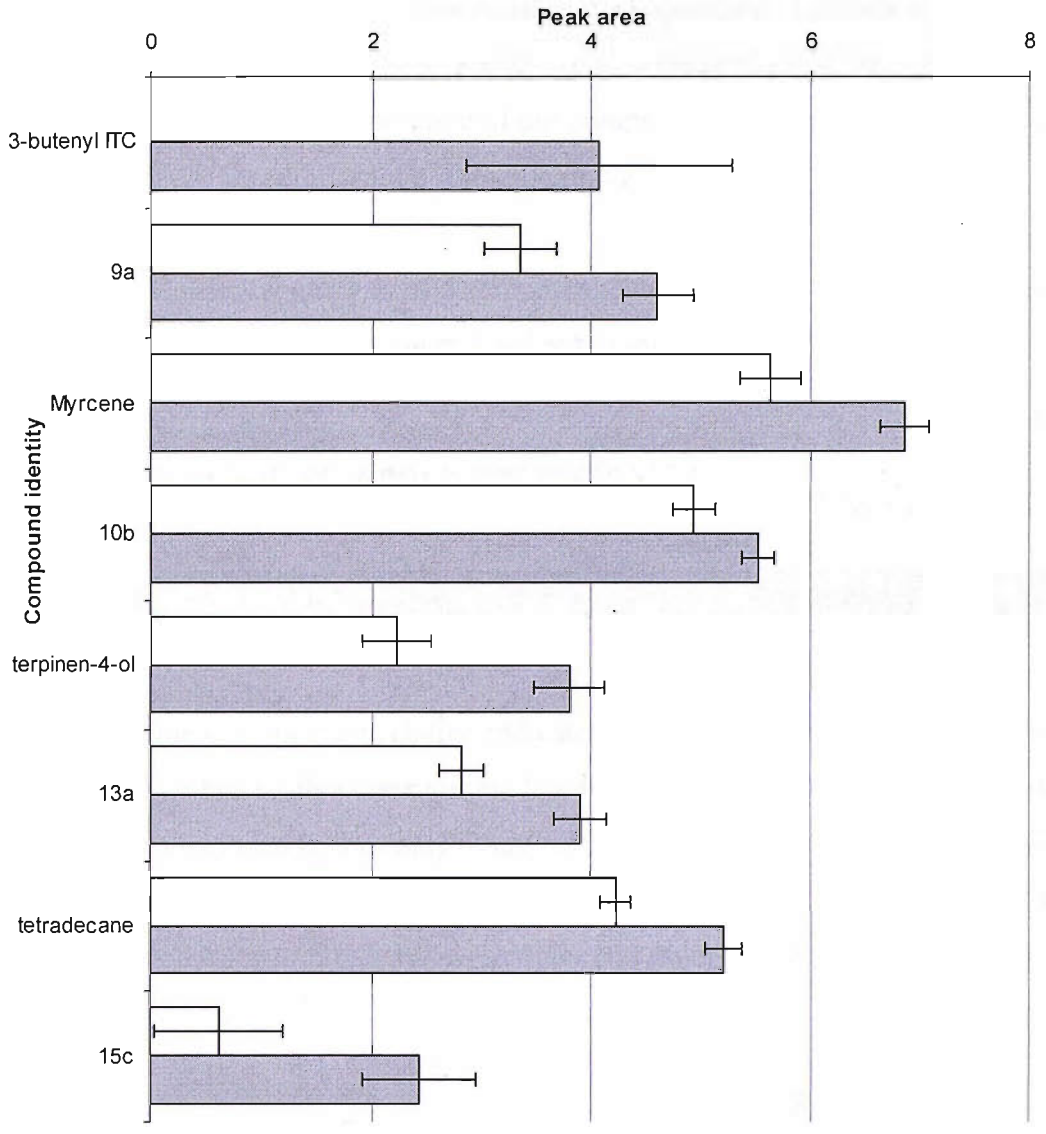


Fig. 3.4 – VOC emission quantity for compounds identified as exhibiting a significant difference in emission (ANOVA $p<0.05$) from aphid infested (shaded bars) and control (unshaded) plants.

Discriminant Analysis

Stepwise CAD was performed to discriminate between the treatment groups and to identify which compounds (variables) contribute most to this discrimination. Step-wise discriminant analysis tests all of the variables at each step to determine which variable minimises the Wilks' Lambda statistic. Wilks' lambda tests the null hypothesis that the

means of the pre-defined groups are equal in the population. Lambda approaches zero if the two groups are well separated. Further steps were performed until the Wilks' Lambda statistic (and therefore the separation of the groups) did not change by a significant amount ($p<0.05$) by addition of a further variable.

Table 3.2 – Canonical analysis of discriminance statistics for compounds that contribute to the discrimination between control and aphid-infested plants

Eigenvalue Canonical Correlation = 0.869

93.3% of original grouped cases correctly classified using this function

Step	Retention time	Compound	Structure co-efficient	Significance of Wilks' Lambda
1	31.040	tetradecane	0.696	0.0007
2	16.833	3-butenyl ITC	0.638	0.0002

The eigenvalue canonical correlation indicates that 86.9% of the variation between groups is described by this discriminant function. The analysis above shows that the first two variables included in the analysis are sufficient to classify 93% (14 out of 15) of the given cases correctly. Fig. 3.5 shows a linear representation of the discriminant function and indicates the separation between samples and groups when considering these two variables.

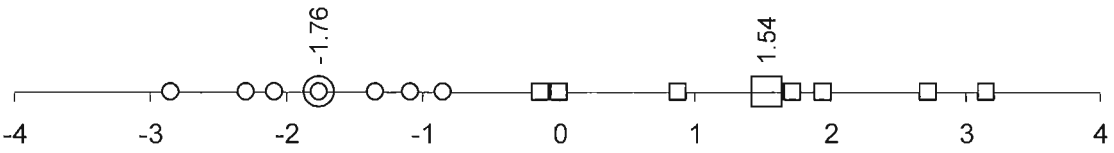


Fig. 3.5 – A linear representation of the discriminant scores for each sample as separated by the discriminant function. Circles represent control samples and squares aphid infested samples. The group centroid/mean is labelled and shown as a large icon.

Since only two variables were identified in the above function, a less stringent level of significance ($P<0.1$) was used to test whether the Wilks's Lambda varies following input of further variables. This analysis identifies further variables that contribute to the discrimination between groups:

Table 3.3 – Canonical analysis of discriminance statistics for compounds that contribute to the discrimination between control and aphid-infested plants

Eigenvalue Canonical Correlation = 0.9998

100% of original grouped cases correctly classified using this function

Step	Retention time	Compound	Structure co-efficient	Significance of Wilks' Lambda
1	31.040	tetradecane	0.02529294	0.00067783
2	16.833	3-butenyl ITC	0.02223916	0.00020743
3	24.620	terpene-4-ol	0.01930909	0.00016189
4	28.724	13a	0.01918867	0.00010772
5	33.827	15c	0.01295509	2.4419E-05
6	17.608	Sabinene	0.00997437	5.187E-06
7	23.961	11c	0.00659008	1.375E-06
8	19.690	Limonene	0.00549979	2.1049E-06
9	19.547	1,8-cineole?	0.00253879	2.1024E-06
10	32.433	B-ionone	-0.000671	2.6859E-06

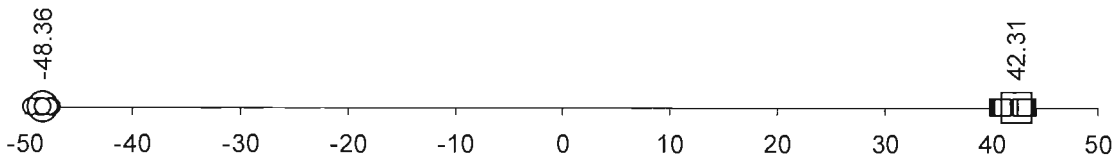


Fig. 3.6 - A linear representation of the discriminant scores for each sample as separated by the discriminant function. Circles represent control samples and squares aphid infested samples. The group centroid/mean value is labelled and shown as a large icon.

Fig. 3.6 shows the clear discrimination between treatment groups by using this discriminant function containing contributions from other variables. The structure co-efficients are all fairly small and exhibit little variation. This implies that each variable is similar in importance in contributing to the discriminant function. The high eigenvalue canonical correlation indicates that greater than 99% of the variation between the treatments can be defined by considering these ten compounds.

3.3.2 Experiment 2 – Identification and quantification of volatiles released from aphid-infested and *Plutella*-infested plants (July 2006)

VOCs were collected by air entrainment from eight plants from each of three treatment groups. The treatment groups were 'A' - 150 aphids for 12 days, 'P' – five *Plutella* larvae for three days and 'C' – control plants. The data from 34 unique compounds were measured from eight replicates of each treatment. Data was adjusted according to leaf surface area and log transformed to normalise the distribution.

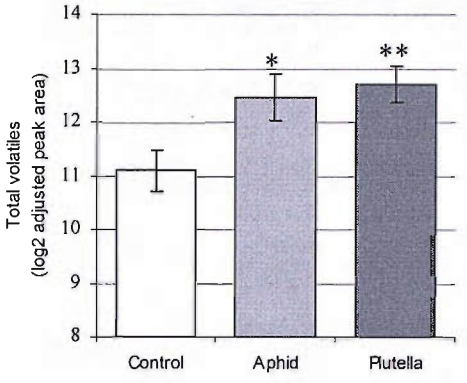


Fig. 3.7 – Total volatiles emitted from herbivore infested plants when compared to an undamaged control plant. * indicates a significant difference where $p<0.05$. ** indicates a significant difference where $p<0.01$.

The total amount of volatiles emitted from both aphid-infested and *Plutella*-infested plants were significantly greater when compared to the control plants (ANOVA, $p=0.035$ and $p=0.008$ repectively).

Table 3.4 - Emission of VOCs from aphid-infested, *Plutella*-infested and control plants. * indicates a significant difference where $p<0.05$, ** indicates $p<0.01$.

Significance			Retention time	Mean adjusted peak area ± s.error				
Aphid	Plut	Compound ID		Control (n=8)	Aphid Infested (n=8)	p-value	*Plutella* Infested (n=8)	p-value
	*	α-thujene[p]	15.964	3.78 ± 0.71	6.21 ± 0.63	0.077	6.87 ± 0.41	0.011
		Benzaldehyde	16.203	3.96 ± 0.69	5.45 ± 0.38	0.135	5.97 ± 0.29	0.031
**		3-butenyl isothiocyanate[a]	16.833	-0.48 ± 0.48	6.98 ± 0.56	0.000	2.18 ± 1.19	0.134
*		9a[a]	17.493	4.33 ± 0.26	5.6 ± 0.34	0.014	4.45 ± 0.37	0.893
*	**	Sabinene[a,p]	17.608	5.78 ± 0.34	8.34 ± 0.57	0.011	8.86 ± 0.39	0.000
*	**	Myrcene[a,p]	18.227	7.83 ± 0.48	9.81 ± 0.49	0.048	10.42 ± 0.36	0.004
		(Z)-3-hexenyl acetate	18.384	6.43 ± 0.81	7.59 ± 0.6	0.312	8.07 ± 0.41	0.097
		9b	18.685	3.49 ± 0.53	3.81 ± 0.57	0.862	3.59 ± 0.55	0.918
*	*	10a[a,b]	19.238	2.99 ± 0.85	6.04 ± 0.48	0.024	6.02 ± 0.33	0.017
*		10b[a]	19.355	5.13 ± 0.29	6.65 ± 0.33	0.032	6.09 ± 0.38	0.239
		1,8-cineole	19.547	1.23 ± 0.81	0.02 ± 0.55	0.361	2.82 ± 0.65	0.091
*	**	Limonene[a,p]	19.690	8.28 ± 0.7	10.92 ± 0.62	0.040	11.46 ± 0.37	0.005
		(e)-β-ocimene	20.254	5.07 ± 0.44	6.26 ± 0.43	0.138	6.36 ± 0.5	0.120
	*	10c γ-terpinene	20.701	4.21 ± 0.53	6.34 ± 0.6	0.084	6.68 ± 0.34	0.017
*	**	trans sabinene hydrate[a,p]	20.849	2.96 ± 0.71	6.08 ± 0.49	0.011	6.45 ± 0.39	0.003
		trans-Linalool oxide	21.557	4.33 ± 0.34	4.35 ± 0.36	0.866	5.00 ± 0.48	0.351
	*	α-terpinolene[p]	21.771	2.56 ± 0.79	5.08 ± 0.76	0.105	5.55 ± 0.24	0.014
	*	11a unknown branched monoterpene[p]	23.046	1.39 ± 0.67	4 ± 0.75	0.098	4.65 ± 0.37	0.013
		11b	23.694	2.31 ± 1.01	3.29 ± 0.78	0.559	3.94 ± 0.57	0.229
		11c	23.961	0.59 ± 0.61	1.19 ± 0.72	0.910	2.66 ± 0.63	0.129
		11d monoterpene alcohol	24.122	3.05 ± 0.73	4.42 ± 0.17	0.147	4.39 ± 0.27	0.173
		11e	24.289	3.96 ± 0.34	3.98 ± 0.64	0.407	4.31 ± 0.49	0.274
		11f	24.460	2.46 ± 0.96	2.55 ± 0.86	0.933	1.33 ± 1.13	0.452
	*	terpinen-4-ol[p]	24.620	3.05 ± 0.7	4.96 ± 0.72	0.137	5.11 ± 0.25	0.030
		α terpineol	24.964	3.8 ± 0.87	6.04 ± 0.42	0.064	6.07 ± 0.57	0.074
		decanal	25.399	4.59 ± 0.22	5.14 ± 0.25	0.156	5 ± 0.45	0.476
		13a	28.724	3.66 ± 0.28	3.45 ± 0.23	0.505	3.44 ± 0.32	0.563
		tetradecane	31.040	3.97 ± 0.32	4.08 ± 0.26	0.903	4.05 ± 0.33	0.969
		benzyl ITC	31.183	1.6 ± 0.37	2.05 ± 0.39	0.354	1.61 ± 0.33	0.895
		β-ionone	32.433	2.38 ± 0.44	3.03 ± 0.57	0.398	3.29 ± 0.35	0.120
		14c	32.765	4.46 ± 0.28	3.87 ± 0.36	0.316	3.99 ± 0.4	0.442
		α-farnesene	32.917	5.02 ± 0.44	5.54 ± 0.56	0.598	5.1 ± 0.36	0.930
		15a	33.034	3.79 ± 0.29	4.17 ± 0.42	0.451	4.19 ± 0.19	0.222
		15c	33.827	2.65 ± 0.45	4.26 ± 0.93	0.088	3.76 ± 0.5	0.110
*	**	Total volatiles		11.1 ± 0.38	12.46 ± 0.43	0.035	12.7 ± 0.34	0.008

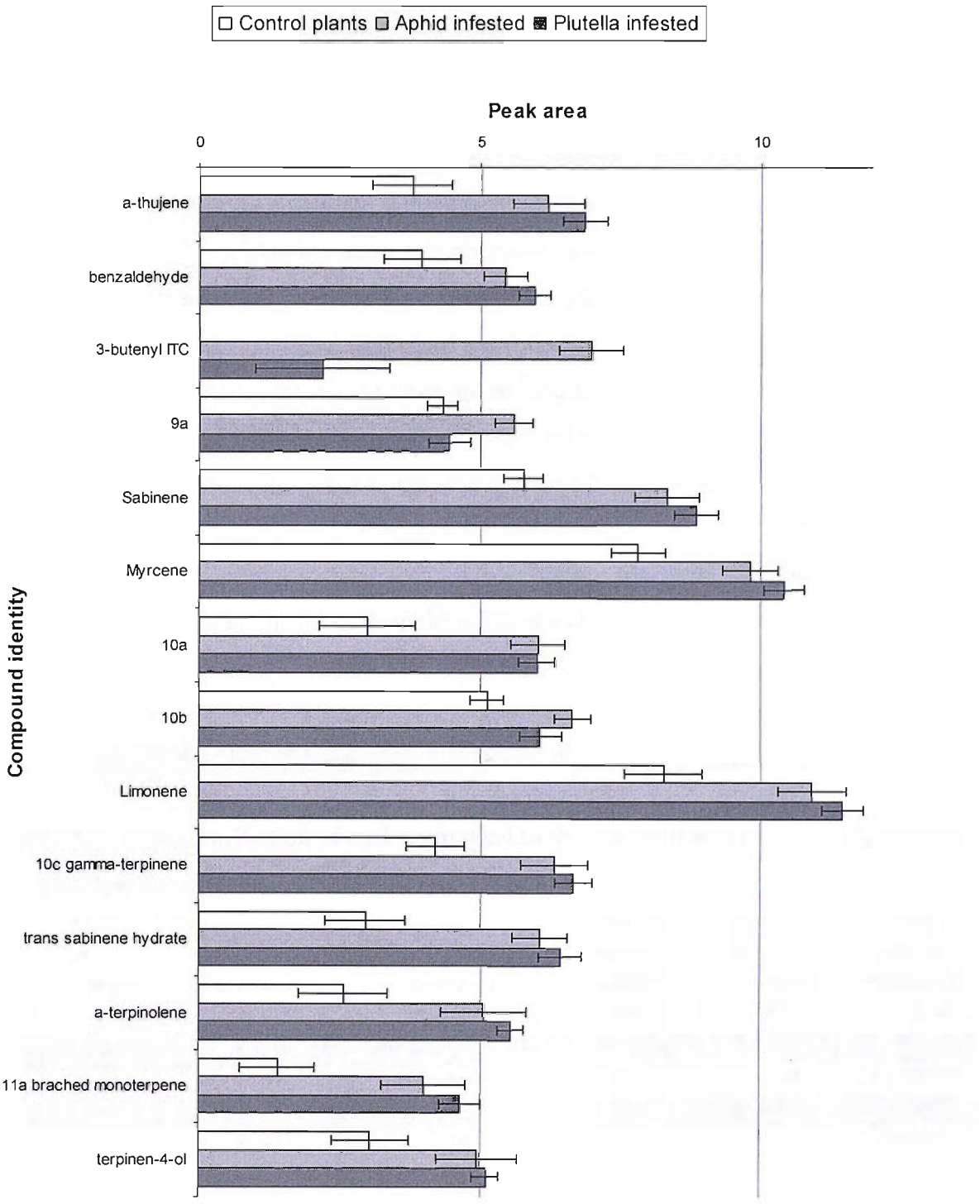


Fig. 3.8 – Quantity of compounds identified as exhibiting a significant difference in emission (ANOVA $p<0.05$) from control plants (unshaded), aphid infested plants (light shading) and *Plutella* infested plants (dark shading).

3.3.3 Discriminant analysis

Stepwise CAD by minimising Wilks' Lambda (see section 3.3.1) was performed to discriminate between the treatment groups and to identify which compounds (variables) contribute most to this discrimination. Further steps were performed until the Wilks' Lambda statistic (and therefore the separation of the groups) did not change by a significant amount ($p<0.05$) by addition of a further variable. Two discriminant functions (table 3.5) discriminated among the three groups and correctly classified the pre-defined group samples with 95.8% accuracy. The contribution of each variable (compound) to the discriminant function is given as the structure co-efficient in table 3.6.

Table 3.5 - Eigenvalue statistics for the two discriminant functions that allow discrimination between the control, aphid-infested and *Plutella*-infested plants.

Discriminant Function	Eigenvalue	% of Variance accounted for	Cumulative %	Canonical Correlation
1	4.2310091	70.29	70.29	0.899
2	1.7887672	29.715	100	0.801

Table 3.6 – The contribution of each compound to the discriminant functions as given by the structure co-efficient.

Step	Retention time	Compound	Function 1 Structure co-efficient	Function 2 Structure co-efficient	Wilks' Lambda significance
1	16.833	3-butenyl ITC	0.649	-0.087	2.059E-05
2	20.849	trans sabinene hydrate	0.320	0.469	1.95E-06
3	19.547	1,8-cineole?	-0.140	0.434	6.159E-07
4	17.493	9a	0.294	-0.165	4.179E-07
5	24.289	11e	0.078	0.184	2.458E-07

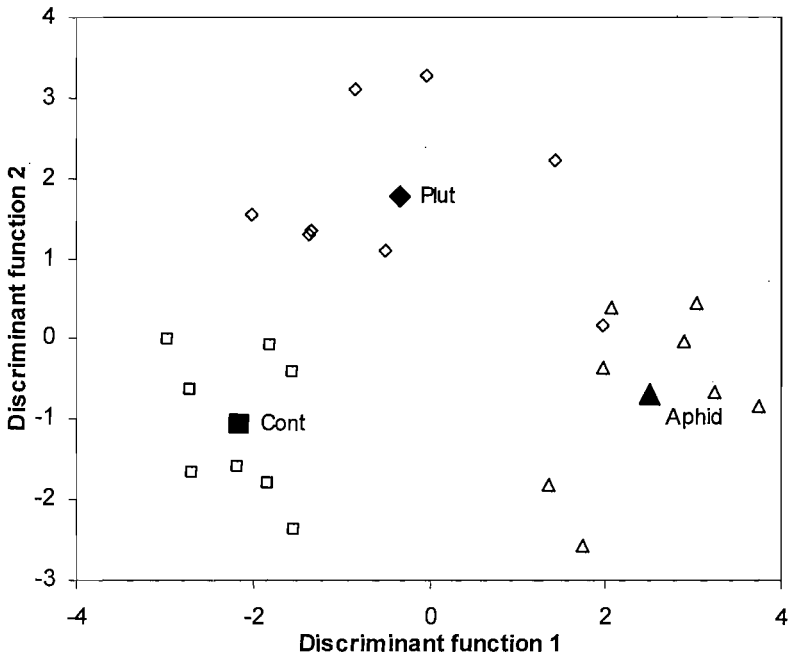


Fig. 3.9 – Scatter plot showing the position of control and treated samples according to the two discriminant functions. (□) denote control samples, (Δ) denotes aphid-infested samples and (◊) denotes *Plutella*-infested samples. The group centroid/mean is shown with a large, filled symbol.

A distinct separation of the three treatment groups is observed when samples were classified according to the two discriminant functions. The structure co-efficient for 3-butenyl ITC in discriminant function 1 is 0.649 which shows that this compound is by far the most important constituent that defines the function. Considering discriminant function 1 on Fig. 3.9, it is clear that this function allows complete discrimination between the control and aphid-infested treatments. This supports evidence from the above univariate analysis that the quantity of compound exhibits a highly significant difference following aphid infestation. The structure co-efficients for discriminant function 2 show that both trans-sabinene hydrate and 1,8-cineole are of almost equal importance in defining the function. These compounds therefore allow discrimination between the *Plutella* and control treatments, and the *Plutella* and aphid treatements on the y-axis of Fig, 3.9. They do not provide effective discrimination between the aphid and control treatments. Although trans-sabinene hydrate is identified during univariate

analysis as being highly significant, 1,8-cineole is not emitted in significantly different quantities. This suggests that the multivariate technique used here provides a different perspective to discriminate between the treatment applied to the plant.

3.3 Discussion

A number of plant species have been shown to release volatile compounds when under herbivore attack. In the multitrophic system being studied, parasitoids of the attacking herbivore have been shown to be preferentially attracted to aphid-damaged plants over undamaged control plants (Chapter 2). This response to the odour from infested plants must be facilitated by changes in the profile (qualitative or quantitative) of the volatile blend. These changes could be brought about by mechanical damage causing release of green leaf volatiles, by the action of components of the herbivore oral secretion inducing specific plant responses or by the method of feeding itself. In cotton (*Malvaceae gossypium*) breakage of leaf glands causes stored terpenes to be released in much higher levels. Emissions of lipoxygenase pathway green-leaf volatiles are increased due to the oxidative degradation of surface plant lipids by enzymes liberated after plant tissue damage (Loughrin et al., 1994). Further to this response, elicitors that may be present in the oral secretions of herbivores can result in the enhanced and prolonged emission of volatiles from treated plants. Volicitin (*N*-(17-hydroxylinolenoyl)-l-glutamine) was identified by Alborn *et al.* (1997) as an active substance in the regurgitant of *Spodoptera exigua* larvae and was shown to enhance the attractiveness of the maize plants to parasitoids. Concentrations of this elicitor alone cause the same reaction in maize plants as pure regurgitant and render the plants equally attractive to the parasitoids (Turlings et al., 2000). Similarly, a β-glucosidase in the regurgitant of *Pieris rapae* larvae causes a release of volatiles in brassica plants that is similar to the release observed after feeding by those larvae (Mattiacci et al., 1995).

3.4.1 Common plant volatiles emitted following herbivory

The results in this study show that there are significant changes in the volatile profiles observed and that the type of herbivory affects both the qualities and quantities of compounds released. Many plant volatiles that are commonly produced and released from a variety of plant species were detected in the odour profile. Sabinene, myrcene and limonene are the major constituents of the volatile blend and all three of these compounds are released in significantly higher amounts following herbivory. These compounds have

been reported as being active as pheromones, allomones or attractants across a range of insect species. They are synthesised from the monoterpene precursor geranyl diphosphate (GPP) by the action of various monoterpene synthases (Wise and Croteau, 1999). Sabinene is a compound that is a constituent of many plant oils and is induced by *Plutella* feeding in seedlings of white cabbage (*Brassica oleracea* subsp. *capitata* cv. Lennox) (Vuorinen et al., 2004a). Myrcene has been identified as being emitted constitutively from brassica species such as *Brassica napus* (McEwan and Smith, 1998) and *Brassica oleracea* ssp. *capitata* (Vuorinen et al., 2004b). Vuorinen et al. (2004a) report that levels of myrcene are significantly higher in cabbage plants infested with *Plutella* larvae or *Spodoptera litoralis* and Blakmeer et al (1994) report that it is induced in cabbage plants infested with *Pieris brassicae*. That these compounds have been shown to be involved in a number of insect-plant interactions in *Brassica* species suggests that they may also be an active component in the attraction of aphid parasitoids. Parasitoid hosts (including *B. brassicae*) live on a number of different *Brassica* species so a response to these induced compounds would prove to be a reliable and highly detectable signal for the forager. The genes encoding myrcene synthases have been characterised in *Snapdragon* (Dudareva et al., 2003) therefore if a homologous gene can be identified in *Brassica,* further experiments that assay expression of these genes could suggest whether the increase in emission correlates with an increase in production as would be expected in an induced response. Limonene has also been implicated as a semiochemical in many ecological interactions. *Diaphania nitidalis* (Stoll.) is attracted by limonene released from leaves of the field pumpkin (*Cucurbita pepo*) (Peterson et al., 1994). Most significantly, the larval parasitoid *Cotesia plutellae* (Kurdjumov) has been shown to be attracted to both the pure compound and to cabbage plants supplemented with limonene (Ibrahim et al., 2005). This evidence suggests that limonene may be a compound that facilitates the attraction of parasitoids to a plant damaged with either aphids or *Plutella* larvae. Further studies including Y-tube bioassays and electroantennography could support this hypothesis.

Benzaldehyde has been identified as a semiochemical in a plethora of insect and plant species. It attracts the soybean aphid (*Aphis glycines*) when used as bait in traps (Zhu and

Park, 2005) and has been shown to attract the cabbage white butterfly (*Pieris rapae*) (Honda et al., 1998). The presence of higher levels of α-thujene corroborates findings by Vuorinen et al. (2004a). Other terpenes emitted include γ terpinene, α terpinolene, tepene-4-ol, trans-sabinene hydrate and another unidentified terpene -11a.

It is likely that the significantly increased levels of terpenes and other volatiles observed in both treatments is due to a combination of a localised, wound response and also a systemic response initialised in response to the plant damage or to components of the oral secretions. It is possible that they are used as beacons to both predators and parasitoids to highlight which plants are undergoing attack from herbivores (Ibrahim et al., 2005).

3.4.2 Species-specific, aphid-mediated volatile emission

The level of 3-butenyl isothiocyanate emitted from aphid infested plants is significantly higher when compared to either the intact or *Plutella* damaged plants. This effect is observed after 3 days and 12 days of aphid feeding. Additionally, the discriminant analyses of data from experiment 1 and 2 identify 3-butenyl ITC as accounting for most of the variation between the groups. The compound is formed when myrosinase enzymes hydrolyse glucosinolates. In plants, myrosinases are localised in the myrosin cells in the parenchymatous tissue, especially in epidermal cells of leaves (Bones et al., 1991). On disruption of the tonoplast membrane, the myrosinase is activated and the glucosinolates are broken down to form various derivatives (Fig. 1.10) (Halkier and Gershenzon, 2006). A rise in the amount of 3-butenyl ITC is observed following *Plutella* damage but a much greater induction is observed during aphid feeding. The *Plutella* feeding method results in the localised disruption of cell walls and this could explain the higher levels of 3-butenyl ITC observed in the volatile blend. Conversely, aphids are known to feed primarily intercellularly and cause limited disruption to the intracellular membranes (Miles, 1999). This evidence strongly suggests that oral secretions from the aphid effect a myrosinase-catalysed hydrolysis of 3-butenyl glucosinolate in the plant to its volatile derivative. Jones et al. (2001) report the presence of myrosinase enzymes in the head and thorax region of the specialist herbivores *Lipaphis erysimi* and *B. brassicae* and Miles (1999) identifies β-glucosidase as a component of the saliva of the grain aphid

(*Sitobion avenae*) and the wheat aphid (*Rhopalosiphum padi*). Although β-glucosidase and β-thioglucosidase do not normally act on the same substrate, Husebye et al. (2005) state that the myrosinase isolated from *B. brassicae* does indeed hydrolyse glucosinolates despite having a global structure very similar to plant *O*-β-glucosidases. Tjallingii (1990) demonstrated that the insertion of aphid stylets can result in the injection of oral secretions into the plant. These secretions could be sufficient to hydrolyse locally stored glucosinolates but a further response would be required to realise the large-scale ITC release observed in this study. The phloem-mediated transport of the aphid myrosinase could precipitate this kind of systemic response.

The role of the aphid myrosinase is unknown although there has been speculation as to its function. A nutritional function for the myrosinase is possible but considered unlikely since the phloem itself is already rich in sugars (MacGibbon and Beuzenberg, 1978). *B. brassicae and L. erysimi* have been shown to sequester glucosinolates from their host plants and yet avoid the toxic degradation products by compartmentalisation of myrosinase in cyrstalline micro-bodies. Bridges et al. (2002) propose that disruption of these micro-bodies by attcking predators or disease could lead to the release of isothiocyanate that acts as a synergist to the aphid alarm pheromone *E*-β-farnesene.

Dicke (1999) states that *Brassica* species mainly employ a direct strategy of plant defence against herbivorous arthropods. Although this may be true when considering chewing insects that ingest the leaf cuticle and the associated stores of glucosinolates, aphids feed by piercing the phloem tubes and passively receiving the phloem sap. These direct defences may therefore be ineffective against an herbivore that is still capable of causing significant damage to the plant, albeit in a different manner. The evolution of an indirect defence strategy, facilitated by the release of HIPVs, specifically targeted against phloem feeding insects would be beneficial to the plant.

3.4.3 Treatments can be grouped by using canonical analysis of discriminance

Mixtures of volatile compounds may often have sensory properties that are different from the perceptual properties of their individual compounds (Chandra and Smith, 1998). The total amount of volatiles emitted varied from plant to plant across all treatments, and it has been shown that various environmental factors and the plant genotype affect the plant volatile phenotype (Takabayashi et al., 1994; Bradburne and Mithen, 2000). Considering the substantial variation between individuals and habitats, the cues that parasitoids use to locate their hosts may not necessarily be related to the absolute quantities of the compounds but more to the ratios between them. By employing this multivariate technique the compounds that allow the optimal discrimination between groups are extracted. This method may more closely resemble the strategy employed by foraging parasitoids during host habitat location and host location.

Clear differences are observed between treatments when the discriminant functions are used to define the treatment groups. In experiment 1, a much clearer distinction between groups is made when a further eight compounds are included in the discriminant function. This suggests that the sensory apparatus of parasitoids and other foraging insects would be more effective if 'tuned' to detect the properties of a range of compounds rather than the qualitative properties of a single compound. The separation of the *Plutella*-infested, aphid-infested and control groups defined by the two discriminant functions in experiment 2 (Table 3.6) reinforces the hypothesis that species specific volatile induction occurs in plant responses (Turlings et al., 1998; Ozawa et al., 2000).

Using standard stepwise parameters in experiment 1, only 2 compounds are included in the canonical function that describes 87% of the variation between treatment groups. The emission of tetradecane did not change significantly (ANOVA, $p<0.05$) but its presence confirms reports of emission from *Brassica oleracea* by Bergström et al. (1994) and it has previously been linked to pheromonal action in many species of the order hymenoptera and coleoptera (El-Sayed, 2006). Long-chain alkanes are known to be involved in the production of wax (Muller and Riederer, 2005) and aphid feeding has

been shown to induce wax production in *Beta vulgaris* (Bystrom et al., 1968). It can be observed in the wild brassicas used in this study that waxiness of the plant cuticle increases over time although this has not been measured and it is unclear whether this is linked to herbivory or other environmental factors. Increased wax production could be a means of physical defence employed by the plant and the tetradecane could be a by-product of its production. 3-butenyl ITC is the compound identified as accounting for a large amount of the variation between treatment groups in every analysis. This analysis, together with the evidence presented by Bradburne and Mithen (2000) and Blande (Blande, 2004), reinforces the hypothesis that it may be an important compound used by parasitoids during host habitat location and host location. If treatments such as mechanical wounding and infestations from different herbivores were incorporated into this analysis, a clearer picture would emerge of which compounds are used to discriminate and which treatments induce a similar plant response.

3.4.4 Cross-spectrum volatile analysis can identify potential infochemicals

In summary, this study uses a combination of linear and multivariate analysis techniques to identify plant volatiles that are induced in herbivore damaged plants and are likely to mediate plant-aphid-parasitoid tritrophic interactions. 3-butenyl isothiocyanate has been identified as the compound that is the most reliable and detectable indicator of herbivore infestation. Whether this reliability and detectability is also apparent to parasitoid chemosensory apparatus remains to be determined although previous studies suggest that this is the case. Discriminant analysis has been demonstrated to be a valid method for cross-spectrum analysis of plant volatiles and may be useful to define which herbivore-induced plant volatiles are important in mediating an indirect plant defence. Further studies should address the response of the parasitoid to individual and blends of plant volatiles, both from an electrophysiological and behavioural perspective. Also, analyses should be conducted into which components of the oral secretions affect the time/intensity of the volatile burst and whether mechanical damage produces a burst of a similar magnitude.

3.5 Summary

- Several commonly detected plant volatiles are emitted in significantly higher quantities following aphid and *Plutella* larvae infestation. This suggests that the compounds may be involved in induced plant defence systems.
- The glucosinolate derivative 3-butenyl isothiocyanate is emitted in much greater quantities following aphid and *Plutella* infestation. Canonical analysis of discriminance identifies this compound and also tetradecane as being of most use when discriminating between treatment groups.
- Canonical discriminant analysis is a powerful multivariate technique that is able to select compounds that accurately discriminate between herbivore treatments.

Chapter 4 - Analysis of gene expression in aphid challenged *Brassica oleracea*

4.1 Introduction

To understand how the plant responds on a molecular level to the external stimulus of aphid attack requires knowledge of the receptor apparatus, signal transduction mechanism, transcriptional regulation, gene function, protein function and protein metabolism. This chapter seeks to elucidate how a wild *Brassica* species responds to aphid phloem feeding on a genomic level by measuring transcriptional regulation using high density oligonucleotide microarrays. Knowledge on gene function and signal transduction pathways will be used to try to deduce the systems and mediators that are involved in the synthesis and release of volatile organic compounds. An understanding of the processes by which wild plants regulate resistance or susceptibility to phytophagous pests could enable steps to be taken to limit the impact of these pests in agricultural systems.

The signal pathways that are activated in response to chewing insects have been well documented and include the octadecanoid, salicylic and ethylene pathways as well as agonistic and antagonistic interactions or 'crosstalk' between them (see section 1.3) (Arimura et al., 2000; Reymond et al., 2000; Stotz et al., 2000; Kessler and Baldwin, 2002). Chewing insects cause extensive cellular disruption and the transcript profile induced has significant overlap with that induced by wounding (Reymond et al., 2000; Walling, 2000). In contrast, phloem feeding insects (PFIs) use their stylets to probe the plant tissue intercellularly in order to establish feeding sites in the phloem sieve elements which can be maintained for several days (Tjallingii, 2006). This method of feeding minimises damage but does result in cell wall disturbance, disruption of plasma membranes and penetration of epidermal, mesophyll and parenchyma cells (see section 1.2.2). As a result of this specialised mode of feeding, a distinct transcript profile is induced although few studies have yet attempted to define it (Thompson and Goggin,

2006). In common with the plant response to chewing insects, the studies that have addressed this question have implicated a variety of genes involved in the salicylic acid, octadecanoid and ethylene pathways in species including *Arabidopsis*, tomato, sorghum, *Nicotiana attenuata*, cotton and wheat. Moran and Thompson (2001; 2002) studied the effect of *Myzus persicae* and *Brevicoryne brassicae* feeding on *Arabidopsis* using micro- and macro-arrays and found that the induction patterns are not indicative of generalised stress responses. They are more likely a combination of localised responses to the insertion of the aphid stylet and a wider, systemic response involving signalling pathways. Specifically, they identify three pathogenesis-related (*PR*) and salicylic acid inducible genes – *BGL2, PR-1* and *PDF1.2* which are upregulated following aphid infestation and used as positive controls in their latter (2002) paper. Of unknown function, *PR-1* has been shown to be induced in resistant aphid-damaged tomato but to a lesser extent in susceptible plants (de Ilarduya et al., 2003). This suggests a role for *PR-1* in the SA-mediated resistance to aphids. The plant defensin gene *PDF1.2* has been implicated in a number of studies on transcriptional response to pathogens (Penninckx et al., 1998; Thomma et al., 1998; Pieterse and van Loon, 1999). Moran and Thompson (2001) report maximum *PDF1.2* induction after 72h aphid feeding although the effects were modest and delayed when compared to methyl JA and fungal infection treatments (Penninckx et al., 1998). Aphid feeding failed to induce *PDF1.2* in the jasmonate-insensitive *coi1-1* mutant, suggesting that elicitation of the JA/ethylene response pathway is activated in infested wild-type plants. Ellis *et al.* (2002) showed that aphid performance was reduced on the *cev1* mutant in *Arabidopsis* in which JA and ET signalling is constitutively activated. The involvement of the JA pathway is corroborated by a number of studies in which JA is exogenously applied to plants and a reduction in aphid fecundity and survival is observed (Omer, 2001; Bruce et al., 2003; Zhu-Salzman et al., 2004; Cooper and Goggin, 2005).

In addition to plant oriented responses, the components of the oral secretions of PFIs are thought to be important in determining the transcriptional changes. Although PFI oral secretions have not been definitively shown to induce transcriptional changes, Madhusudhan and Miles (1998) report that the transport of salivary components

contributes to the development of symptoms associated with aphid feeding. Thompson and Goggin (2006) outline research on a protein fraction derived from aphid saliva that induces symptoms such as leaf-rolling in susceptible plants whereas symptoms were absent in resistant plants. It is hoped that by controlling for the effects of leaf wounding, the experiments conducted in this study will move towards a more focussed analysis of the plant transcriptional regulation mediated by aphid oral secretions.

Several genes have been identified as key steps in the signalling pathways mentioned and it will be one of the aims of this study to investigate whether plant responses to phloem feeding by *Brevicoryne brassicae* involve the complex integration of multiple signalling pathways as seen in other species (Arimura et al., 2000; Reymond et al., 2000; Schenk et al., 2000; Stotz et al., 2000; Kessler and Baldwin, 2002; Moran, 2002). The activation of direct and indirect defence responses such as the production of volatile compounds are controlled by signal transduction pathways and attempts will be made to identify genes and pathways that are part of these systems. Also, comparisons will be made between aphid induced transcriptional responses and the responses induced by *Plutella xylostella* larval feeding. Identification of genes which are involved in the production of semiochemicals would increase our understanding of the signalling cascades and metabolic pathways. Following identification, key regulatory genes could be targeted for silencing or overexpression to confirm their role.

Although analysis of gene expression is an important step in understanding semiochemicals production, there are many cellular controls other than gene expression to regulate downstream protein levels or their effects. These include degradation of specific mRNA (making the mRNA unavailable for translation), protein degradation, and the level of enzyme activity. Additionally, environmental conditions can have an effect on the gene expression or transcription over very short periods. Levels of volatile organic compounds released by the plant are known to exhibit diurnal variation (Loughrin et al., 1994), therefore transcript abundance may also vary and time of sampling is crucial to obtaining accurate and relevant data.

4.1.1 Global gene expression analysis using microarrays

Global expression analysis using microarrays allows the parallel screening of gene expression levels for a large number of genes from an organism at a particular developmental stage or in response to a treatment. The lack of annotation and the incomplete sequencing of the *Brassica oleracea* genome mean that an *Arabidopsis*-specific array has to be used. Despite the relatedness and common ancestry of the two species (see section 1.2.1), many of the *Arabidopsis* probes will be non-homologous to the sequence encoded by the *Brassica* genome. Hammond *et al.* (2005) have developed a set of software-based filters which can be used to remove non-homologous probe data from the overall analysis. These filters were developed by hybridising *Brassica oleracea* genomic DNA to the ATH-1 array and measuring the hybridisation intensity for each probe. A range of filters have been developed ranging arbitrarily from 0000 to 1000 at 0100 intervals. 0000 includes all probes, regardless of their homology to the *Brassica* genome and 1000 includes only the probes which hybridised with the highest intensity.

To confirm the results obtained from microarray analysis, quantitative (or 'real-time') RT-PCR will be used. Real-time PCR measures gene transcript levels by measuring the intensity of a fluorescent dye that intercalates with DNA amplicons. As more product is formed, the intensity becomes greater and the quantity of transcript can be measured relative to a reference gene. In this study, five genes were selected based on previous studies on insect-plant interactions and three genes were selected based on the results of the microarray analysis (see results section for more details). Of the five genes selected based on the literature, hydroperoxide lyase - *HPL* - is involved in the production of green-leaf volatiles by mediating the breakdown of 13(S)-hydroperoxylinolenic acid to (z)-3-hexenal and other C_6 volatile derivatives (Donath and Boland, 1994; Pare and Tumlinson, 1999). *AtHPL* is also induced by *Pieris rapae* caterpillar feeding in *Arabidopsis* (Van Poecke et al., 2001). The gene *BoGSL-ELONG,* which was identified in *Brassica oleracea* (Li and Quiros (2002), controls the synthesis of glucosinolates with 4C sidechains such as 3-butenyl isothiocyanate (see section 1.3.1). Lipoxygenase *LOX2* is involved in the synthesis of jasmonic acid and has been identified in a number of

studies as being upregulated in response to wounding and herbivory (Reymond et al., 2000; Van Poecke et al., 2001). Myrosinase binding protein (*MBP*) and myrosinase activation protein (*MyAP*) are upregulated in *Brassica napus* following infestation by *B. brassicae* and code for proteins involved in the myrosinase-glucosinolate system (Pontoppidan et al., 2003). By measuring the expression of these genes from diverse pathways, a greater understanding of which pathways are activated will be obtained.

4.1.2 Hypotheses:

- Phloem feeding by *Brevicoryne brassicae* will induce a unique suite of genes when compared to *Plutella* larvae infestation.
- Genes involved in pre-characterised plant defence pathways will be induced in aphid-infested plants.

4.2 Materials and Methods

4.2.1 Global gene expression analysis using Affymetrix microarrays

The development of microarrays was pioneered by Schena *et al.* (1995) at Stanford University and, coupled with the advances in sequencing techniques and computing power, now allows quantitative expression profiling of an entire genome in one experiment. There is some ambiguity in the way in which people refer to the nucleic acid which is attached to the slide and the nucleic acid that is applied as a solution. For the sake of clarity, the former (the attached sequence) will be referred to as the probe and the latter (which is applied) as the target. Microarrays can be produced and treated in different ways but all are currently glass slides with an array of gene specific sequences for the particular organism/tissue attached to it. Following extraction, the mRNA is transcribed to produce the target - a complementary nucleic acid sequence - which is hybridised to the slide. A fluorescent dye is incorporated at some stage (either pre-hybridisation or post-hybridisation) and after washing off the unhybridised target, the intensity of fluorescence is measured following excitation by a laser at a specific wavelength. The intensity is proportional to the abundance of complementary target mRNA present in the sample.

Affymetrix arrays are produced using a patented method known as photolithography to build up nucleotide sequences one by one on a quartz wafer. This method uses a lithographic mask to block or transmit light onto specific locations on the wafer. The surface is then flooded with a solution containing either adenine, thymine, cytosine, or guanine, and coupling occurs only in those regions on the glass that have been deprotected through illumination. On the *Arabidopsis* ATH1 array, this process is repeated to build up about half a million sequences, each 25 nucleotides long. Each gene is represented by 11 probe pairs or 'spots' which correspond to unique (but occasionally overlapping) sequences within the open reading frame for that gene. The spots are located in different places on the chip so that any minor differences in hybridisation efficiency across the chip are averaged out. One probe of the pair matches the sequence

13th nucleotide changed to the complementary base and is known as the 'mismatch' probe. The fluorescence of the 'perfect match' probes are adjusted by the 'mismatch' fluorescence to get a more accurate picture of the mRNA concentration.

During preliminary experiments using wild cabbage, it was found (using real-time RT-PCR) that gene expression in two genes (*BoGSL-ELONG* and *HPL1)* varied among progeny from a single parent under identical treatment conditions. Therefore, in order to obtain an accurate analysis of gene expression in the absence of a large number of experimental replicates, samples must be taken from a genetically identical plant in order to compare between aphid challenged and unchallenged plants effectively. Since the plants are taken from a wild population and seed production is very slow, selfing to produce inbred lines would not be viable and tissue culture or cuttings may be unsuccessful. For this reason, samples will be taken from the same plant before and after infestation. This 'wounding' event of excising a leaf will no doubt cause many genes to be regulated even before the aphid challenge. For this reason, controls will be carried out in which a plant will undergo identical leaf excision treatment to aphid challenged plants but be unchallenged. The genes regulated in this control plant can also be considered in the final analysis to gain an insight into how wounding affects gene expression. The variation in gene expression among progeny also precludes the use of pooled samples as an effective technique for increasing the number of biological replicates. Pooling samples would be likely to mask any subtle expression changes that are designated as being statistically significant.

In order to confirm results obtained during the microarray studies, real-time quantitative PCR (polymerase chain reaction) analysis will be conducted on a number of characterised genes with diverse functions related to plant defence. PCR amplifies a primer defined nucleotide sequence present in a nucleic acid sample using DNA polymerase. Real-time quantitative PCR is used to give an indication of the amount of a gene transcript in a treated sample when compared to an endogenous reference gene (or 'housekeeping' gene) in the same cDNA sample. A fluorescent dye is added to the mastermix which is incorporated into the DNA amplicon. The intensity of fluorescence can be measured

following each amplification cycle within the PCR machine. Expression of the target gene is normalised relative to that of one or more reference genes, which are known to express stable levels of transcripts in most tissues. The selection of an appropriate reference gene is important for qPCR analysis in order to obtain consistent and reliable results. Current opinion and publication guidelines require that microarrays are validated using an independent expression profiling method (Kammenga et al., 2007). Real-time qPCR has been shown to provide a repeatable and reliable measurement of sample mRNA quantity.

4.2.2 Experimental design

The two-factor experimental design used is illustrated in Fig. 4.1. A pair of plants was used for each of the two biological replicates. The basal leaf was removed from each plant to measure the pre-treatment baseline expression. One of the plants was then immediately infested with the herbivore and left for a period of time (detailed below) after which another leaf sample was removed. Using a two-way analysis of variance, the interaction between the 'time' and 'treatment' factors can be considered in order to identify those genes that are upregulated following herbivore infestation but do not change in the control plant. This factorial design enables the gene induction effect of the leaf removal to be discounted from the analysis. The drawback of using this experimental design is that if a gene is significantly induced following leaf removal, the same gene would not be identified if similarly induced by herbivore damage.

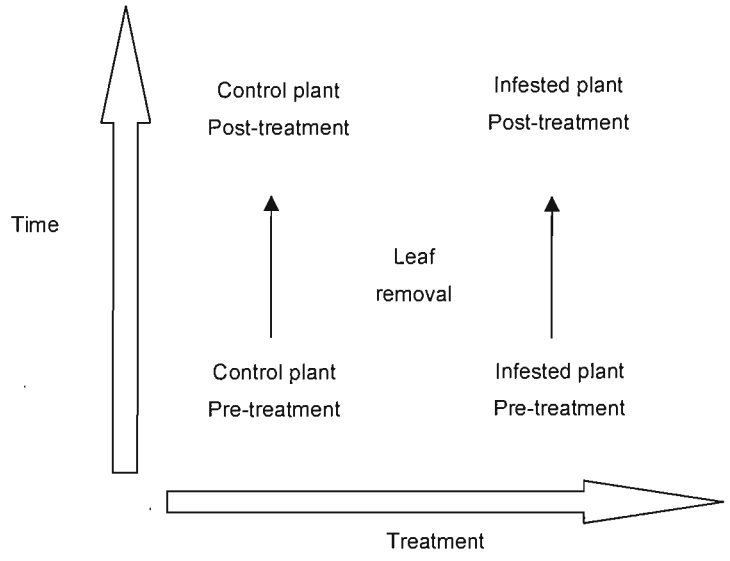


Fig. 4.1 – Two-way factorial experimental design for the global expression analysis of wild *Brassica oleracea* following herbivore feeding.

Table 4.1 – Summary of sample treatments and identifiers. Two plants were infested with aphids, two infested with *Plutella* larvae and two were controls.

Sample name	Plant number	Time of sampling	Treatment
1a	1	Pre-treatment	None
1b	1	Post-treatment	200 aphids, 96 hours (and leaf removed)
2a	2	Pre-treatment	None
2b	2	Post-treatment	None (but leaf removed)
3a	3	Pre-treatment	None
3b	3	Post-treatment	200 aphids, 96 hours (and leaf removed)
4a	4	Pre-treatment	None
4b	4	Post-treatment	None (but leaf removed)
P1a	P1	Pre-treatment	None
P1b	P1	Post-treatment	5 *Plutella* larvae, 72 hours (and leaf removed)
P2a	P2	Pre-treatment	None
P2b	P2	Post-treatment	5 *Plutella* larvae, 72 hours (and leaf removed)

Gene-expression analysis using microarrays and real-time quantitative PCR was carried out on all samples individually.

4.2.3 Plant growth and insect culture conditions

Seeds collected from wild plants at Durdle Door, Dorset, UK were sown in 0.3L square pots filled with Levingtons FS2 potting compost (Fisons Ltd. Loughborough, UK) with no extra fertiliser added. Plants were grown in a glasshouse for 6 weeks under 16h light, 24°C ±2°C and 60% R.H. *B. brassicae* aphids were obtained from wild *B. oleracea* var. oleracea at Durdle Door, Dorset, UK (grid ref. 807802) in May 2004. They were reared continuously on glasshouse grown plants from the same population in clear Perspex cages 70cm x 60cm x 45cm high, 22-26°C . *P. xylostella* larvae were reared on commercially grown cabbage in clear Perspex cages 70cm x 60cm x 45cm high.

4.2.4 *B. brassicae* infestation

Aphids selected for infestation treatments were of mixed instars and removed from different parts of the host plant using a fine paintbrush. Plants were infested with 200 aphids for 96 hours. This time period was chosen considering previous studies on transcriptional and response to aphid feeding (Moran and Thompson, 2001; Moran, 2002). The aphids were placed inside an open cone of filter paper (Whatman no.9) next to the main stem of the plant at least 5cm above the soil and above the basal leaf. This method prevented the aphids from falling off the leaves into the soil or pot tray. Empty filter paper was placed in an identical position on all control plants. Plants were checked every 24 hours and any aphids that were present on the basal leaf were removed and placed on other leaves. This ensured that any gene induction was due predominantly due to systemic signals rather than a localized response. Any aphids that were on the leaf to be excised were removed prior to excision using a fine paintbrush.

4.2.5 *P. xylostella* larvae infestation

Five *P. xylostella* larvae approx 8mm in length were placed on separate leaves and allowed to feed for 72 hours. Plants were checked twice a day and if a larva was found to have fallen off or pupated, it was replaced. All larvae were placed on, and remained on, leaves distal to that which was excised for analysis.

4.2.6 Plant tissue collection

The basal leaf (not including cotyledons) was excised using a razor blade before treatment, wrapped in aluminium foil and immediately immersed in liquid nitrogen. Following plant treatment and (if necessary) removal of insects, the leaf above the original basal leaf was excised, wrapped in foil and immersed in liquid nitrogen. All plant material was stored at -80°C. Control plants were grown under identical conditions to the treated plants and samples taken at the same times. Control plant tissue samples were conducted to control for changes in gene expression following the removal of the leaf (i.e. wounding) as well as any developmental changes over the treatment time period.

4.2.7 RNA extraction

Total RNA was extracted using a phenol-chloroform extraction and LiCl precipitation based on the method by Verwoerd et al (1989).

0.5g frozen tissue was ground to a powder and added to 5 ml of a 1:1 vol. of extraction buffer and phenol mixture (extraction buffer: 0.1M LiCl, 100mM Tris-HCl (pH 8.0), 10mM EDTA (ethylene-diamine-tetraacetic acid), 1% SDS (w/v)). The mixture was vortexed for 2 mins after which 2.5ml chloroform was added. The mixture was further vortexed for 30 secs then centrifuged at 3000 x g for 10 minutes. The aqueous phase was added to an equal volume of 4M LiCl and incubated for 2 hours at -20°C then decanted into baked corex tubes and centrifuged at 9500 x g for 20 minutes at 4°C. The supernatant was removed and the pellet washed with 70% and then 100% ethanol with 5 mins centrifugation after each wash. After drying, the pellet was dissolved in 1.5ml sterile dist. water. 0.15ml 3M NaAc (pH 5.2) and 3.75ml 100% ethanol were added and the solution was incubated at -20°C for four hours. The samples were centrifuged at 9500 x g for 20mins at 4°C then consecutively washed with 70% and 100% ethanol. The pellet was air dried for 30 mins then dissolved in Ambion RNA storage solution. RNA was stored at -80°C. Residual genomic DNA was digested and removed using the Ambion DNA-Free kit according to manufacturer's instructions. The yield and RNA purity was determined spectrophotometrically and using an Agilent 2100 Bioanalyser (Agilent Technologies Inc.) according to manufacturers instructions.

4.2.8 Hybridisation of *Arabidopsis* ATH1 GeneChip® arrays

Leaf tissue samples taken before and after each treatment from three replicate samples per treatment group were hybridised. Hybridisations were carried out at Nottingham Arabidopsis Stock Centre (NASC) using the Affymetrix Service (Craigon et al., 2004). Labelling, cleanup and hybridisation protocols are detailed in the Affymetrix GeneChip expression analysis technical manual (Affymetrix, 2005). Approximately 5 µg of total RNA was used to produce 15 µg of fragmented labelled cRNA. This was hybridised to *Arabidopsis thaliana* ATH1 GeneChip® arrays (Affymetrix) for 16h at 45°C. Arrays were scanned with a G2500A GeneArray Scanner (Affymetrix). Following scanning, nonscaled RNA signal intensity (CEL) files were generated using Microarray Analysis Suite (MAS version 5.0; Affymetrix). Nonscaled RNA CEL files contain the raw signal intensity values for more than 500 000 individual probes on the ATH1 GeneChip® array. Sample quality and hybridisation controls were validated prior to data anaylsis.

4.2.9 Analysis of microarray data

RNA CEL files were analysed using the Robust Multichip Average (RMA) preprocessor in GeneSpring GX (Agilent Technologies Inc.). The processing ensures that probe values from a number of chips can be compared accurately. The X-Species probe masking files (available at http://affymetrix.arabidopsis.info/xspecies/) (Hammond et al., 2005) were used for probe selection. A variety of hybridisation intensities were used in order to optimise the *Brassica* genome coverage and the probe homology. Individual probe data was normalised to the median gene expression value for that chip and $\log_2$ transformed. This normalisation procedure ensures that genes on the same chip can be compared and that the frequency distribution is approximately normal. A two-way ANOVA analysis was conducted using the R statistics environment to calculate the interaction term between the independent plant samples. To account for the multiple testing of several thousand hypotheses, the False Discovery Rate (FDR) multiple-testing correction was applied to all results (Benjamini and Hochberg, 1995). This method yields a q-value for each test carried out i.e. each gene. The q-value gives the probability that the genes with either the same or lower q-value are falsely identified as being significantly different. For

example a genelist of 10 genes yields a range of q-values from 0.05 to 0.2. The maximum q-value is used to predict the number of false positives in that list i.e. 0.2*10 = 2 genes. For cases with sufficient statistical power and replication, the q-value is analogous to the p-value for a single test (Wertheim, B. Pers. Comm.. 2006).

4.2.10 Gene Ontology classification using Genemerge

Genelists detailing the significantly up- or down-regulated genes were subjected to functional classification and analysis using the free software Genemerge (Castillo-Davis and Hartl, 2003). By using the gene ontology functional classifications found at <http://www.geneontology.org>, the list of significant genes (where ANOVA $p<0.05$) was analysed and a statistical rank score given for over-representation of particular functions or categories in the dataset. Rank scores for functional or categorical overrepresentation within the study set of genes are obtained using the hypergeometric distribution. This distribution gives a quantification of the level of one's 'surprise' at finding over-representation for a particular item in a given sample of size *k* drawn from a larger population, size *n* (Sokal and Rohlf, 1995). This analysis gives clues as to which pathways and processes are involved in the response to plant treatment.

4.2.11 Primer design for qPCR

Appendix A details the primer sequences and the gene accessions from which these sequences were derived. Since many of the genes being investigated have not yet been characterised in *Brassica oleracea* species, primer sequences were derived from a number of sources. Most were derived from *Arabidopsis* ESTs using the Washington University Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990) at http://www.arabidopsis.org. The full length cDNA sequence for each gene was aligned to known *Brassica* sequences then the sequence with highest homology was used to design primers to amplify the sequence. The 'Primer3' web interface (Rozen and Skaletsky, 2000) was used to design primers as close as possible to a length of 21 nucleotides with a calculated T_m of 61°C. Due to differences between the *Arabidopsis* and *Brassica* genomes, many of the primers selected to amplify *Arabidopsis* homologues in the *Brassica* genome were unsuccessful.

4.2.12 cDNA synthesis

cDNA synthesis reactions were carried out in 200µl PCR tubes. 0.5 µg total RNA was transcribed to cDNA using the Promega ImProm-II™ Reverse Transcription System according to the manufacturer's instructions. The cDNA product was diluted to 1 in 20 for all downstream applications and frozen at -20°C.

4.2.13 Real-time quantitative polymerase chain reaction (qPCR)

All samples that underwent microarray analysis were also used for qPCR analysis. The reference gene for all experiments was deemed to be polyubiquitin (*UBQ10*) based on previously accepted methods and an analysis of the most stably expressed 'housekeeping' genes carried out by Czechowski *et al.* (2005). qPCR was carried out using an MJ Research Chromo4 Real Time 4-color 96-well PCR system with Opticon 3 analysis software. 7.5µl 2x qPCR MasterMix (PrimerDesign, Southampton, UK), 0.75µl SYBR® green intercalating dye, 0.75µl of forward and reverse primers (4µM), 0.8µl cDNA template and 4.5µl H_2O were combined in single wells of a white 96-well plate (MJ Research). Optical grade caps were used. The plate with caps firmly pressed down was centrifuged at 3000 x g for 1 min. to ensure that all reaction mixture was at the bottom of the well and no bubbles were present. The following programme was used for qPCR:

Incubate at 95.0°C for 10 mins
Incubate at 94.0°C for 30 secs
Incubate at 58.0°C for 1 min
Incubate at 72.0°C for 1 min
Plate Read
Goto line 2 for 39 more times
Incubate at 72.0°C for 10 mins
Melting Curve from 50.0 °C to 92.0 °C, read every 0.2 °C, hold 1 sec
End

4.2.14 Analysis of qPCR data

Real-time analysis was conducted according to the method outlined by Pfaffl (2001). A threshold fluorescence level is applied to a graph of time versus well intensity. This level is set at the point at which the increase in intensity becomes exponential. The number of cycles required to reach the threshold level (C(t)) gives a quantitative prediction of the initial levels of mRNA in the sample. The threshold value for C(t) measurement was set to 0.025 relative fluorescence units (rfu) and the baseline average over the first 10 cycles was subtracted in order to account for any well intensity anomalies. C(t) values were the mean of three technical replicates amplified in separate wells with solutions drawn from a mastermix including all reaction components.

The amplification efficiency (E) of the target and reference genes were generated using the Opticon Monitor software and are based on the gradient of the linear phase of amplification. The difference between the C(t) values of the treatment versus reference gene is then used to give a ratio or 'fold-change' value which can be used as a standalone comparison over several samples. The equation for calculation of expression ratios between a control and treated cDNA sample (Pfaffl, 2001) is as follows:

$$\text{Ratio} = \frac{(E_{target})^{\Delta C(t)\ \text{target (control-treated)}}}{(E_{ref})^{\Delta C(t)\ \text{ref (control-treated)}}}$$

Where: E = efficiency

$\Delta C(t)$ = difference in threshold cycle value

The mean expression ratio will be analysed using a one-way ANOVA to determine which genes exhibit significantly different expression levels.

4.2.15 Correlation of gene expression levels measured using qPCR and microarrays

Microarray expression ratios were calculated using Genespring for each plant (i.e. a and b samples) by normalising the b value to the a value to give b in terms of a. Expression ratios calculated using the above qPCR method were compared to the microarray values to give a measure of the correlation between the two methods.

4.3 Results

Two biological replicates were carried out for each of three treatments – control, aphid-infested and *Plutella* larvae infested. This involved hybridising 12 microarrays. All control transcripts on the arrays were validated before analysis to ensure efficient hybridisation and scanning. It should be noted that only two replicates were carried out due to financial considerations of carrying out microarray experiments.

4.3.1 Probe selection for cross-species hybridisation

Raw data from the microarray scan is processed using the Robust Multi-array Average (RMA) algorithm. This method of probe response analysis detects probes that do not change across the dataset and uses these to normalise all probes. Millenaar *et al.* (2006) report that out of six algorithms tested, RMA gave most reproducible results and showed the highest correlation coefficients with Real Time RT-PCR data. The X (cross) -species software filter (Hammond et al., 2005) is applied during the RMA analysis and discounts probes present on the array that are non-homologous to the *Brassica oleracea* genome. Table 4.2 details the options for stringency of the X-species filter.

Table 4.2 – Number of genes represented by the Affymetrix microarray at different intensity thresholds.

Intensity Threshold	Number of genes included in analysis
0	22810 (100%)
50	22810 (100%)
100	22806 (99.98%)
150	22799 (99.95%)
200	22770 (99.82%)
300	22527 (98.76%)
400	21682 (95.05%)
500	20121 (88.21%)
600	18118 (79.43%)
700	15993 (70.11%)
800	14014 (61.44%)
900	12040 (52.78%)
1000	10371 (45.47%)

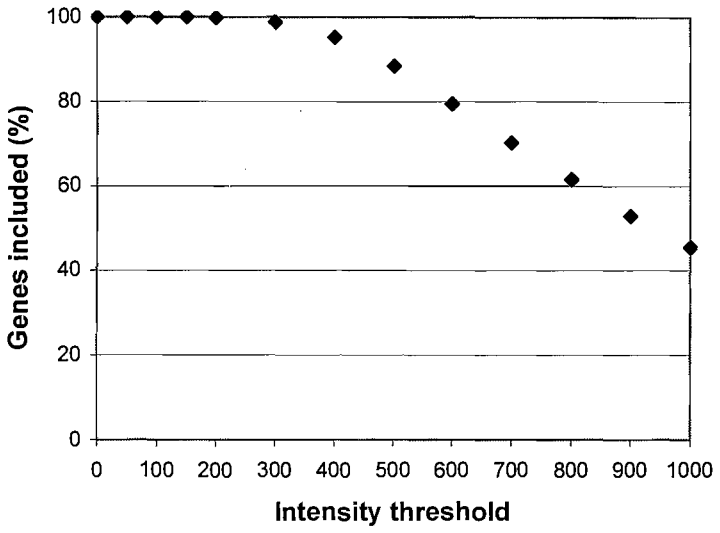


Fig. 4.2 – Graph showing the percentage of genes (out of the entire set of 22810 genes) included in the analysis at different thresholds. Analysis was carried out using the 500 threshold.

In order to optimise the number of genes considered and the homology of the probes to the Brassica target transcripts, an intensity threshold of 500 was used during all subsequent analyses. .

4.3.2 ANOVA analysis using the R statistical environment

Data for 20121 genes across three treatments and two biological replicates were analysed using a two-factor ANOVA analysis to identify the between-groups interaction p-value for significantly upregulated genes ($p<0.05$). The two factors considered were time (pre- and post-treatment) and treatment (infested and control). Only genes that were upregulated when comparing the treated with the control sample were considered.

The frequency distribution of p-values for aphid-infested and *Plutella* infested plants is displayed in Fig. 4.3. The higher frequency of p-values where $p<0.05$ is expected since a number of genes are significantly changed due to the treatment.

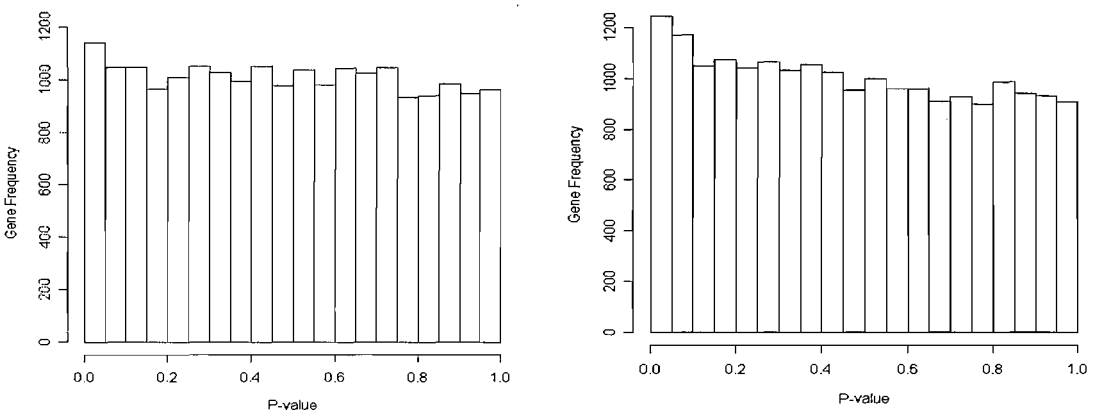


Fig. 4.3 – Frequency of ANOVA p-values comparing control with *B. brassicae*-infested plants (left) and comparing control with *P. xylostella* larvae-infested plants (right).

Following aphid infestation, 1139 genes exhibited significantly different expression (ANOVA $p<0.05$). 611 were upregulated (Fig. 4.4), 528 downregulated. The false discovery rate multiple testing correction gives a maximum q-value of 0.64 for the upregulated genelist. This indicates that 391 genes are statistically false positives. Following infestation by *Plutella xylostella* larvae, 1245 genes exhibited significantly different expression (ANOVA $p<0.05$). 616 genes were upregulated (Fig. 4.4) and 629 downregulated. The maximum q-value for this genelist is 0.62 indicating that 382 genes are false positives. 94 genes that were upregulated were common to both treatments (Fig. 4.4).

Following *Plutella* larval feeding, it may be expected that a much larger suite of genes would be upregulated due to the physical damage done to the plant. The lower number of genes upregulated supports previous microarray analyses carried out by De Vos *et al.* (2005) which found that when *Arabidopsis* was challenged with aphids, pathogens, thrips and caterpillars, the highest number of genes was induced by the aphid *Myzus persicae*. This relatively low number could be explained by the fact that the genes identified in this study are those that are only due to *Plutella* feeding and not due to leaf removal. Due to the experimental design, any genes that are regulated to the same extent due to the removal of a leaf (which is effectively a wounding event) as due to the *Plutella* feeding

would not be detected in the analysis. This kind of analysis is used in order to increase the specificity of the detection to evaluate solely the plant response to the insect feeding. A general trend can be observed in the *Plutella* data showing a greater number of genes with lower p-values when compared to the frequency distribution of the aphid gene p-values which has a flatter distribution. This trend could be due to the greater damage inflicted by the *Plutella* feeding and therefore a larger suite of genes will be affected by the signalling cascades. The number of replicate microarrays carried out means that their expression is not regulated sufficiently to be significantly different.

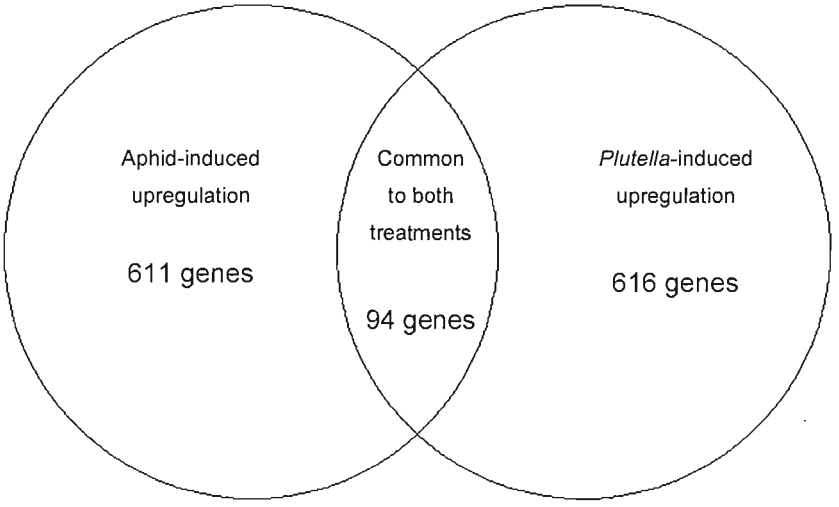


Fig. 4.4 – Venn diagram summarising the number of genes upregulated in response to aphid infestation, *Plutella* infestation and genes common to both treatments.

4.3.3 Ontological classifications

The genes identified as being significantly upregulated following aphid infestation, were classified according to their gene ontology functional classifications (http://www.geneontology.org). Using GeneMerge (Castillo-Davis and Hartl, 2003), the functional and categorical data for the upregulated given set of genes (the study group) is analysed and a statistical rank score is given for over-representation of particular functions or categories when considering the entire dataset (the population group). This genelist will include both true and false positives since a number of the genes identified as being significant will be identified due to chance alone. However, the chance that the

GeneMerge over-representation analysis of the genes yields false positives is extremely low.

Genemerge analysis of aphid induced genes

The biological processes that are likely to be activated following aphid feeding are detailed in table 4.3. 611 genes that were significantly upregulated following *Plutella* infestation (ANOVA $p<0.05$) were used as the study group out of a population group of 20121 genes.

Table 4.3 – Biological processes which are statistically over-represented by genes identified as significantly upregulated following 96 hours aphid feeding. The study fraction is the number of genes out of the entire study set. The p-value gives the probability that the result was obtained by chance alone. Contributing genes details the AGI identifiers for the *Arabidopsis* genes that are associated with this term in the study set.

Study fraction	p-value	Biological process	Contributing_genes
2/611	0.0053	histone methylation	AT1G79730;AT2G06210
2/611	0.01747	arginine biosynthesis	AT2G19940;AT1G29900
3/611	0.01879	negative regulation of transcription	AT5G22250;AT2G32070;AT2G39810
5/611	0.02041	DNA repair	AT2G02550;AT3G10140;AT3G54320 AT1G02670;AT3G18630
2/611	0.02283	RNA modification	AT5G22250;AT2G32070
4/611	0.02991	mitochondrial transport	AT5G64970;AT2G47490;AT5G26200;AT5G56450
4/611	0.03514	translational initiation	AT4G18040;AT5G57870;AT1G34360;AT2G04520
2/611	0.03524	phenylpropanoid metabolism	AT4G24520;AT1G51680
3/611	0.03903	negative regulation of flower development	AT1G79730;AT5G65070;AT2G06210
3/611	0.03903	isoprenoid biosynthesis	AT2G18640;AT1G31910;AT5G47770
2/611	0.04222	cytokinesis	AT4G02980;AT4G39920
2/611	0.04222	gravitropism	AT2G18790;AT1G61720
2/611	0.04967	chromatin assembly or disassembly	AT3G51880;AT5G44800

Genemerge analysis of Plutella induced genes

The biological processes that are likely to be activated following *Plutella* larvae feeding are detailed in table X. 616 genes that were significantly upregulated following aphid infestation (ANOVA $p<0.05$) were used as the study group out of a population group of 20121 genes.

Table 4.4 - Biological processes which are statistically over-represented by genes identified as significantly upregulated following 72 hours of *Plutella* larvae feeding. The study fraction is the number of genes out of the entire study set. The p-value gives the probability that the result was obtained by chance alone. Contributing genes details the AGI identifiers for the *Arabidopsis* genes that are associated with this term in the study set.

Study fraction	p-value	Biological process	Contributing_genes
4/616	0.0001	protein secretion	AT3G54860;AT2G17980;AT4G12120;AT1G12360
4/616	0.0015	phospholipid biosynthesis	AT3G45040;AT3G55030;AT4G04870;AT4G16700
5/616	0.0045	photosynthesis	AT4G12800;AT3G54890;AT4G10340;AT5G01530;AT3G47470
3/616	0.0065	photosynthesis light harvesting	AT4G10340;AT5G01530;AT3G47470
3/616	0.0165	nucleobase, nucleoside, nucleotide and nucleic acid metabolism	AT4G10760;AT5G35170;AT5G47840
2/616	0.0177	response to freezing	AT5G66000;AT2G26140
9/616	0.0231	lipid metabolism	AT5G18630;AT3G48080;AT1G02660;AT3G14225;AT5G37710; AT3G48090;AT2G30550;AT4G16230;AT2G42450
2/616	0.0292	jasmonic acid mediated signalling pathway	AT3G23240;AT2G39940
2/616	0.0358	response to water	AT3G50970;AT1G76180
2/616	0.0358	protein amino acid N-linked glycosylation	AT1G27520;AT1G12990
3/616	0.0398	isoprenoid biosynthesis	AT2G18640;AT1G31910;AT5G47770
2/616	0.0428	tip growth	AT5G51060;AT5G57590
2/616	0.0429	regulation of meristem organization	AT1G62360;AT2G42620
3/616	0.0440	cellulose biosynthesis	AT5G05170;AT2G39770;AT4G24000
2/616	0.0504	cold acclimation	AT2G15970;AT1G35515

From this over-representation analysis, hypotheses can be formed to suggest which processes may be activated in the plant following herbivory. Genes involved in

isoprenoid biosynthesis are over-represented in both *Plutella* and aphid-infested plants. This category includes genes classified as being involved in the chemical reactions and pathways resulting in the formation of any isoprenoid compound, isoprene (2-methylbuta-1,3-diene) or compounds containing or derived from linked isoprene (3-methyl-2-butenylene) residues. This includes all terpene compounds (see chapter 3), many of which are common plant volatiles and have been identified as semiochemicals in insect-plant interactions.

Genes defined as exhibiting significant difference in expression following both treatments underwent functional classification using Genemerge. Table 4.5 shows that the only category to be significantly over-represented was that containing genes involved in isoprenoid biosynthesis.

Table 4.5 – Biological processes identified during Genemerge analysis of significant genes commonly upregulated following both aphid infestation and *Plutella* larvae feeding.

Study fraction	p-value	Biological process	Contributing_genes
3/95	0.00022	isoprenoid biosynthesis	AT2G18640;AT1G31910;AT5G47770
3/95	0.13024	transport	AT5G20380;AT1G20840;AT2G47490

This result is exciting since it is driven by an objective, statistical analysis of the data observed rather than being driven by a subjective review of the significant genes based on prior knowledge. The three genes identified as being induced by both aphid and *Plutella* feeding are summarized in Table 4.6.

Table 4.6 – Genes involved in isoprenoid biosynthesis induced by both aphid and *Plutella* feeding.

AGI identifier	Gene name	Description
AT2G18640	GGPS4	Encodes an endoplasmic reticulum-targeted geranylgeranyl pyrophosphate synthase
AT5G47770	FPS1	farnesyl diphosphate synthase precursor (FPS1) mRNA,
AT1G31910	F5M6.9	GHMP kinase family protein, contains TIGRFAM profile TIGR01219: phosphomevalonate kinase; contains Pfam PF00288: GHMP kinases putative ATP-binding protein domain; similar to Phosphomevalonate kinase (EC 2.7.4.2) (Swiss-Prot:P24521) (Saccharomyces cerevisiae)

A list of genes that exhibit a significant upregulation of expression following insect treatment was formed. From this list, genes that have been previously identified as being involved in one of the following processes were extracted for further study:

- Glucosinolate production or metabolism (including myrosinase synthases/binding proteins etc)
- Response to or synthesis of jasmonic acid or genes involved in the octadecanoid pathway
- Genes involved in the ethylene or salicylic acid synthesis or response pathways
- Genes involved in terpene synthesis or other common volatile organic compound synthesis
- Genes identified as being involved in defence or response to herbivory

Appendix B and appendix C detail the significant genes that fall into these categories following aphid feeding and *Plutella* feeding respectively.

4.3.4 Real-time quantitative PCR analysis

The expression levels of eight genes were analysed relative to the reference gene *UBQ10*. cDNA was transcribed from the same RNA samples used for the microarray analysis. Expression ratios were calculated from the post-treatment expression value divided by the pre-treatment value. Values are the mean of three technical replicates and two biological replicates per treatment. *HPL1, MBP, GSL, LOX2* and *MyAP* are genes involved in pre-characterised plant defence pathways. *ESP, CER1* and *XTH6* were selected due to their large change in expression according to the microarray analysis and their possible involvement in an aphid-mediated defence response.

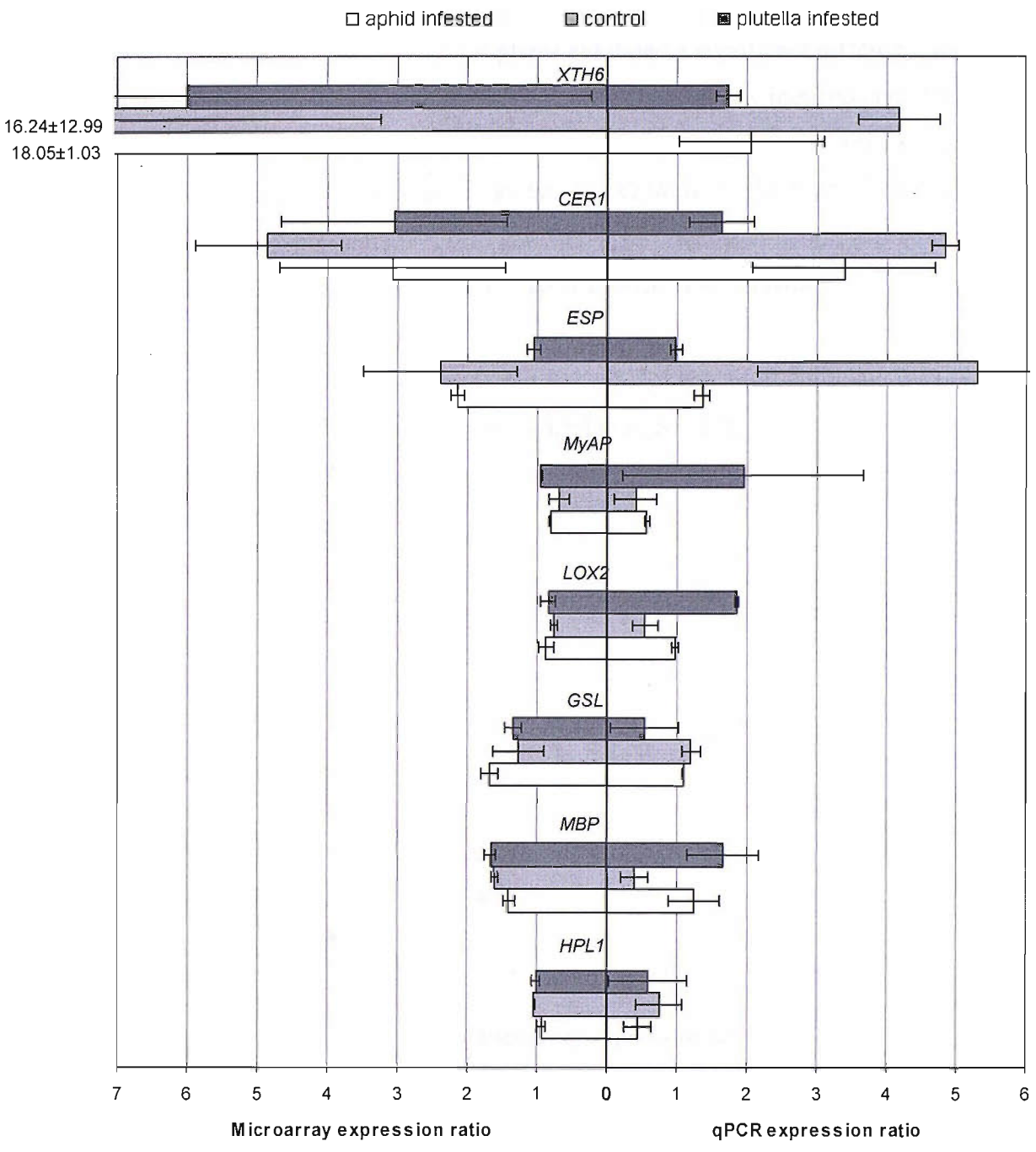


Fig. 4.5 – Mean expression ratios of *B. oleracea* genes following 96 hours aphid infestation (clear bars), no treatment (light shaded bars) or 72 hours *Plutella* larvae feeding (dark shaded bars). Expression ratio is given as post-treatment expression divided by pre-treatment value. Standard error is based on the two biological replicates carried out.

None of the genes in aphid infested plants exhibited a significant difference in expression when compared to the control plants. The only significantly induced gene following *Plutella* larval feeding when compared to uninfested controls was *LOX2* (ANOVA, $p<0.05$). Microarray expression ratio data for *XTH6* is in text due to the ratio being off the scale. The expression of XTH6 is massively upregulated in all the post-treatment samples suggesting a transcriptional response to the leaf removal.

The correlation between the microarray and qPCR expression ratio data is visually displayed in Fig. 4.6. Spearman's rho correlation co-efficient for all 48 bivariate correlations was 0.52 which was a statistically significant correlation ($p=0.0001$).

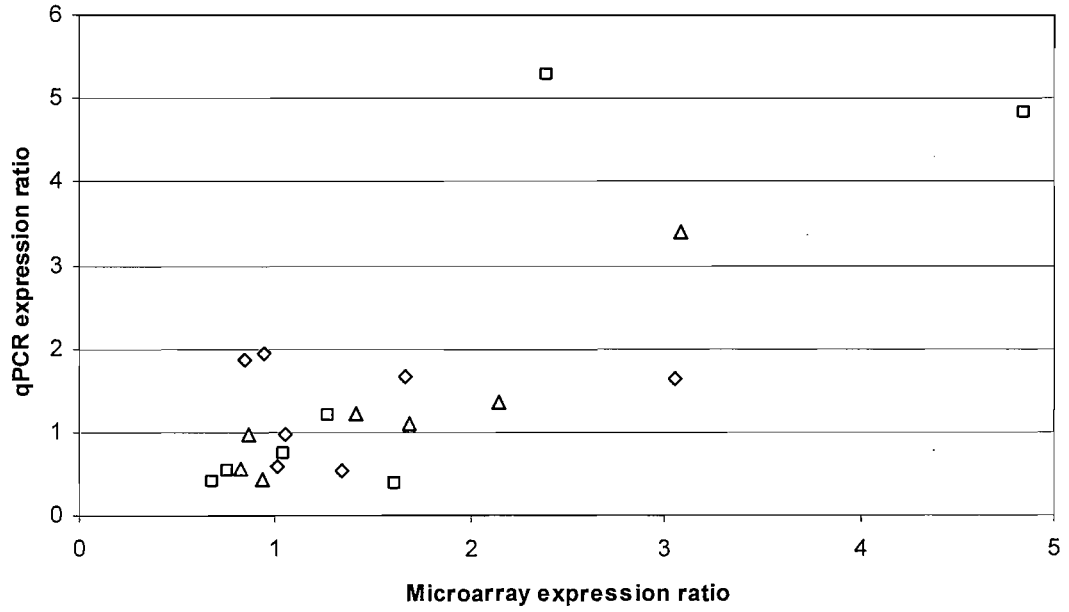


Fig. 4.6 – Correlation of qPCR expression ratios with microarray expression ratios. Ratio is given as post-treatment expression value normalised to pre-treatment expression value. (□) denote control samples, (Δ) denote aphid-infested samples and (◊) denote *Plutella*-infested samples. The values for XTH6 are not illustrated on this figure due to the extremely high microarray expression ratio.

4.4 Discussion

Full genome Affymetrix *Arabidopsis* microarrays were used to measure the changes in gene expression following *B. brassicae* and *Plutella* larvae infestation in wild cabbage – *B. oleracea*. Analysis was carried using probe selection according to the method developed by Hammond *et al.* (Hammond et al., 2005). A software filter was used to remove array probes that were non-homologous to the *Brassica* genome sequence and therefore increase the specificity and accuracy of the expression measured. By using this novel analysis method, it is possible to open up a range of wild plants to analysis using the extensive resources developed for model systems. Although a complete picture is not yet possible due to the differences in sequence and possibly uncharacterised genes in *Brassica*, this method is useful to deduce the pathways and gene families that may be involved in response to a variety of biotic stimuli. By using a wild plant that is involved in multitrophic interactions, and has retained the defence characteristics necessary to survive under constant pressure from herbivorous challenges, it is hoped that a clearer picture of the genetic determinants behind the ecological interactions will be elucidated.

By conducting the analysis at a threshold level of 0500, 88.2% of the *Arabidopsis* genes represented on the array were considered - 20121 genes. A two-factor ANOVA measured expression changes before and after removal of the leaf sample in either treated or control plants. This experimental design ensured that the removal of the leaf for RNA analysis (i.e. a wounding event) was accounted for during the analysis. It also ensured that there was a direct comparison of gene expression within the same plant. This was identified as an important factor during early gene expression studies of wild *B. oleracea* where different plants exhibited very different levels of expression. A comparison among plants was therefore deemed to introduce too much error into the measurements. This method ensures that only genes involved in a specific response to aphid or *Plutella* larvae feeding are identified, not those induced during the generalised wound response. Differentiating between these responses, especially following *Plutella* feeding is an

important step in understanding how the plant perceives the challenge and may lead to the identification of genes that are induced by oral secretions of the *Plutella* larvae.

The false discovery rate q-value gives us an indication of the number of genes that we would expect to be false positives in the list. For this study however, the lack of resources to ensure large-scale replication has reduced the statistical power to such an extent that more than half of the genes identified as significantly upregulated are identified by chance. For this reason, it is statistically improbable that a straightforward analysis of all the genes where $p<0.05$ would yield any useful information. It would be possible to use a q-value cutoff that is lower in order to reduce the number of false positives although this would also increase the number of false negatives and result in a loss of information from the analysis. Further replication is essential in this area of research in order to be able to have statistical confidence in the results obtained. The gene ontology analysis attempts to extract more information from the genelist by classifying the genes and calculating the probability that the category is over-represented by chance alone.

4.4.1 Genes induced by aphid or *Plutella* feeding

96 hours of aphid infestation caused a total of 1139 genes to exhibit a significant ($p<0.05$) difference in expression. Of these, 611 genes were upregulated. Following functional classification using GeneMerge (Castillo-Davis and Hartl, 2003), a number of ontologies were identified as being over-represented. Many of the over-represented ontologies appear to be related to DNA/RNA modification and transcriptional regulation. It is unclear how these ontologies could be related to the activation of a plant defence response although they could be involved in the transcriptional regulation of pathways that are addressed below.

The phenylpropanoid pathway is involved in the induced defence response to pathogens and is one of the pathways by which salicylic acid is produced (Dixon et al., 2002). Aphid feeding has been shown to induce the expression of *PR* genes and other transcripts

associated with SA-mediated signalling in several plant species, including *Arabidopsis*, tomato, sorghum, and *Nicotiana attenuata* (Moran and Thompson, 2001; Moran, 2002; de Ilarduya et al., 2003; Zhu-Salzman et al., 2004). SA promotes the development of systemic acquired resistance which confers resistance to some pathogen and aphid species. It is also crucial in the activation of the hypersensitve response in plants (Smith and Boyko, 2007). Although detailed studies have not been carried out on this wild *Brassica,* it is possible that the upregulation of genes controlling the phenylpropanoid pathway may be driving a similar SA induction.

Plutella larvae feeding for 72 hours induced significant upregulation of 616 genes. The jasmonic acid-mediated signalling pathway is significantly over-represented by three of these significant genes. This ontology consists of genes that are involved in mediating responses to jasmonic acid. JAs induce the production of secondary metabolites in several plant species (Gundlach et al., 1992) and the jasmonate pathway, which modulates the lipoxygenase pathway producing green leaf volatiles, is known to be activated following challenge by a number of insects (Liechti and Farmer, 2002). Also, jasmonate-deficient plants have reduced direct and indirect defences against herbivores (Thaler et al., 2002). The genes induced following *Plutella* feeding may play an important role in the mediation of these defences and therefore the volatiles released.

ERF1 (ethylene response factor 1 - At3g23240) is significantly upregulated in *Plutella* damaged plants and a number of *ERF* genes are upregulated in aphid-infested plants (Appendix B). ERFs are a large gene family of transcription factors which have a variety of functions involved in the developmental and physiological processes in plants. Nakano et al. (2006) classify the *ERF* gene family in *Arabidopsis* into 12 groups containing a total of 122 genes. *ERF1* (also called *ERF#092*) is classified in group 9 which contains genes linked to defensive gene expression and *ERF1* has been identified as a convergence point between the ethylene and jasmonate pathways (Devoto and Turner, 2005). The overexpression of *Arabidopsis ERF1* and a tomato ERF - *Pti4* enhanced resistance to necrotic and biotrophic pathogens (Berrocal-Lobo et al., 2002; Gu et al., 2002). Furthermore, defence-related phytohormones such as ethylene, jasmonate,

and salicylic acid have been shown to differentially induce the expression of genes in group 9 (Gu et al., 2000; Onate-Sanchez and Singh, 2002). Although there is no direct link, overexpression of several other members of the *ERF* family results in the enhanced accumulation of epidermal wax (Aharoni et al., 2004; Broun et al., 2004; Zhang et al., 2005). Wax accumulation would be an effective method of defence against both pests and pathogens and evidence in chapter 3 suggests that this process could be occurring.

Also upregulated is the gene *COI1* (coronatine insensitive 1 - At2g39940), required for jasmonic acid (JA)-induced growth inhibition, resistance to insect herbivory, and resistance to pathogens. *COI1* is also required for transcription of several genes induced by wounding or by JA (Devoto et al., 2005). Upregulation of this gene suggests an activation of the jasmonic acid signalling pathway in response to the feeding by *Plutella* larvae. *coi1* knockout mutants are deficient in JA signalling and, as a result have diminished resistance to the *P. xylostella* and are also more susceptible to aphid infestation (Ellis et al., 2002). The jasmonate and ethylene pathways have been shown to act synergistically via the two genes that are identified in this study - *COI1* and *ERF1* – to regulate the expression of the plant defensin fusion gene *PDF1.2* (Devoto and Turner, 2005). *PDF1.2* is upregulated in response to a variety of pathogens (Penninckx et al., 1998; Thomma et al., 1998; Pieterse and van Loon, 1999) and Moran and Thompson (2001) report *PDF1.2* induction after 72h aphid feeding. Since these two genes are such major regulators in the ethylene and jasmonate signal transduction pathways in *Arabidopsis*, this evidence suggests that a similar wound-inducible response occurs in wild *B. oleracea.* The nature of the defences that are induced by these pathways is yet to be determined but is likely to include both a direct and indirect component in much the same way as in *Arabidopsis* (Thaler et al., 2002).

COI1 has also been shown to affect *MBP* (myrosinase binding protein) expression in *Arabidopsis* (Capella et al., 2001). This interaction suggests a further link with the production of secondary compounds and the release of volatile organic compounds such as isothiocyanates (see chapter 2).

4.4.2 Genes involved in isoprenoid biosynthesis are induced by both aphid and *Plutella* feeding

Of particular interest is the over-representation of genes involved in isoprenoid biosynthesis. Genes in this category are classified as being involved in the chemical reactions and pathways resulting in the formation of any isoprene compound or derivative (isoprenoid). This includes all terpene compounds (see chapter 3), many of which are common plant volatiles and function as semiochemicals, insect attractants, phytoalexins and antifeedants as defences against various predators (Liu et al., 2005). Also, isoprenoids play essential roles in plants as hormones (abscisic acid, cytokinins, gibberellins, and brassinosteroids), photosynthetic pigments (carotenoids), electron carriers (cytochrome *a*, quinones, chlorophylls), and membrane components (steroids). Isoprenoid synthesis is initiated via one of two pathways (Fig. 4.7) – the cytosol-localised mevalonate pathway (Chappell, 1995) or the plastid-localised 1-deoxyxylulose-5-phosphate (DXP) pathway (Rodriguez-Concepcion and Boronat, 2002). These pathways yield isopentenyl diphosphate (IPP) and its close relative dimethylallyl pyrophosphate (DMAPP) which are the activated intermediates during the synthesis of downstream terpene and terpenoid products. Subsequent conjugation and cyclisation steps controlled by separate enzymes proceed in a variety of tissue types and cellular compartments.

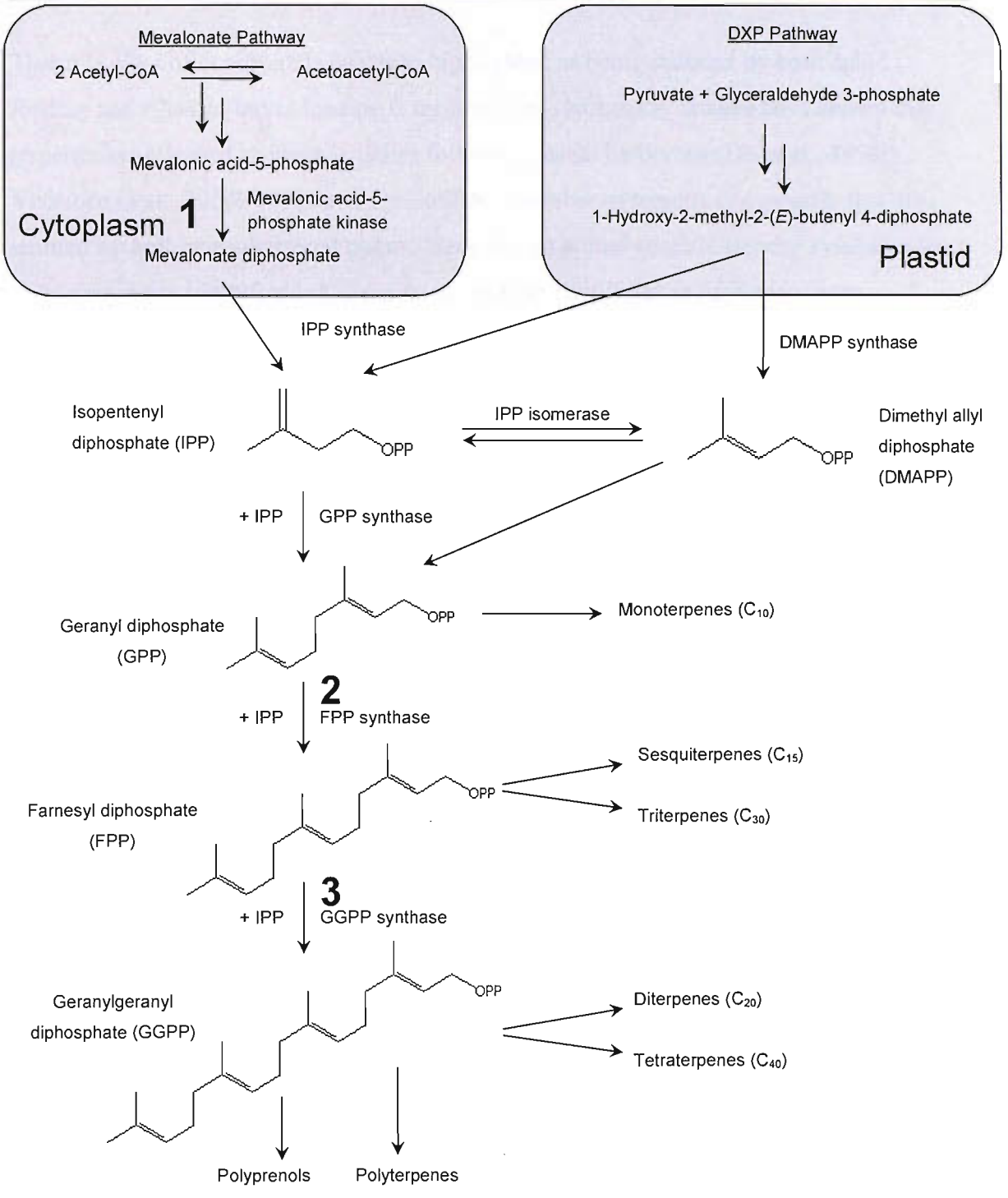


Fig. 4.7 – Synthesis pathway for terpene precursors isopentenyl pyrophosphate (IPP) and dimethyl pyrophosphate via the cytoplasm-localised mevalonate pathway and the plastid-localised DXP pathway. Subsequent synthesis steps occur in either the cytoplasm, the plastid or Labeling of enzymes refers to genes identified as significantly upregulated following aphid and *Plutella* infestation: 1=*F5M6.9*; 2 = *FPS1*; 3 = *GGPS4*.

That this class of compounds has been highlighted as being induced by both aphid feeding and *Plutella* larval feeding is unsurprising since many studies have shown that terpenes are released as plant volatiles following insect herbivory (Du et al., 1998; Vuorinen et al., 2004b). Chapter 3 identifies a number of terpene compounds that are emitted by herbivore-damaged plants. Several compound specific terpene synthases have previously been identified but these do not exhibit significant induction in these experiments. The reason for this could be that the rate-determining step in the reaction is controlled by one of these inducible components and synthesis of downstream compounds may be catalysed by constitutively produced and stored terpene synthases. It is also possible that many of the genes involved in the production of plant volatiles in this wild plant are as yet uncharacterised or do not exhibit sufficient homology with *Arabidopsis* genes to be identified.

The genes identified as being induced include a geranylgeranyl pyrophosphate synthase (*GGPS4*), farnesyl diphosphate synthase precursor (*FPS1*) and a putative phosphomevalonate kinase (*F5M6.9*). *GGPS4* has been identified as being expressed in the flowers of *Arabidopsis* using RNA-blot and promoter-β-glucuronidase (GUS) analysis. Synthetic green fluorescent proteins (sGFP) were expressed by the cauliflower mosaic virus 35S promoter in *Arabidopsis* and GGPS4-sGFP was shown to be localised in the endoplasmic reticulum (Okada et al., 2000). It is suggested that GGPS4 catalyses the production of plastoquinones which are used in the electron transport chain in the light-dependent reactions of photosynthesis. Since this class of compound lacks comprehensive study in either *Arabidopsis* or other members of the Brassicaceae, it is possible that GGPS4 catalyses the synthesis of a variety of diterpenes or carotenoids.

The enzyme farnesyl-diphosphate synthase (FPS1) catalyses the synthesis of farnesyl diphosphate (FPP) from isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). The contribution of FPS1 to the overall control of the isoprenoid biosynthetic pathway in plants has not yet been fully evaluated but it is believed to be a rate-limiting step in this metabolic pathway (Cunillera et al., 1996). This assumption is based on the fact that FPP is the starting point of a number of different branched pathways leading to

the synthesis of a variety of isoprenoid end-products (phytosterols, dolichols, ubiquinone, heme a, sesquiterpenoid phytoalexins and prenylated proteins) (Bach, 1995; McGarvey and Croteau, 1995). It is further supported by the observation that FPS plays a regulatory role in plant sesquiterpenoid phytoalexin biosynthesis (Liu et al., 1999). The FPS1:GUS reporter gene is widely expressed in all *Arabidopsis* plant tissues throughout development. The gene *F5M6.9* has been characterised as being involved in the transfer of phosphorus containing groups in the mevalonate pathway but further information is lacking. By subjecting the transgenic *Arabidopsis* plants in the above studies to aphid and *Plutella* feeding challenges, a confirmed pattern of aphid-induced gene expression could be built up. Further knowledge could be gained on whether this plant response is systemic or localized and linked to data on volatile emissions to deduce the identities of particular terpene synthases.

4.4.3 Correlation of gene expression ratios from real-time PCR and microarray analysis

Results from the quantitative PCR analysis of individual genes and their correlation with the microarray expression ratios illustrates that there is a significant correlation between the two datasets. However, the Spearman's correlation co-efficient of 0.52 does not indicate a high correlation and this may be explained by the way that primers for the qPCR were designed. Many of the genes that have been characterised in *Arabidopsis* do not have an homologue in *Brassica* that has been sequenced; therefore the primers for qPCR were designed to amplify the gene sequence with highest homology. This method of primer design was necessary due to the substantial nucleotide insertion/deletions and substitutions in the *Brassica* genome compared to the *Arabidopsis* genome. Although the method has clearly worked to a certain extent (illustrated by the significant correlation result), some of the genes that are amplified using qPCR may be different to those represented by the probes on the microarray. Fig. 4.6 illustrates that there may be a difference in magnitude measured by the differing methods of expression analysis. In control plants, the expression ratio for the gene *XTH6* using microarray analysis was around 16. This ratio is substantially larger than the qPCR ratio of around 4. However, the results are both considerably greater than the other genes assayed which suggests that

the same gene is being assayed. This difference in magnitude may be due to the data transformations applied to the data. The microarray analysis method involves a number of normalisation steps to ensure commutability between different arrays and probe sets but does not use a single reference gene; instead using a normalisation to the average intensity per chip. The qPCR analysis compares gene expression data to a single reference gene while taking into account the efficiency of the PCR. If there are changes in the expression of the reference gene due to the treatments, this could result in inaccurate measurements of target gene expression. This criticism was considered during experimental planning by considering both the 'traditional' reference genes used in previous studies and the data presented by Czechowski *et al.*(2005) suggesting 'novel' reference genes. Czechowski *et al.* present data that *UBQ10* was stably expressed in *Arabidopsis* following a number of treatments including biotic stress although whether it is also stably expressed in wild *Brassica oleracea* remains unknown. The difference in magnitude between gene expression ratio measurements could also be due to the manner in which the gene is assayed. With microarrays, a number of 25bp oligos hybridise to homologous mRNA transcripts and are assayed to represent the gene while using qPCR, two individual primers are used that amplify a section of that gene. If we consider gene families such as the xyloglucan endotransglycosylase-hydrolase (*XTH*) family that has 33 members (Rose et al., 2002), often with high homology, it is likely that the qPCR amplifies a number of these genes. Although melting-curve analysis was carried out to assert the specificity of the PCR product, it is possible that the gene products exhibited a similar melting point. This would 'dilute' the intensity signal detected from the *XTH6* gene with the lower intensities of other gene family members.

The high standard errors observed for many of the gene measurements are illustrative of the high degree of variation between samples. This variation could be due to the genetic nature of the wild plants. The plants are segregating lines and, although the seeds were taken from the same parent plant, the progeny may not be isogenic and different alleles will inevitably be present at some gene loci resulting in differing measurements of gene expression. This topic will be covered in more depth in Chapter 5. Overall, the analysis suffered from a lack of replication thus resulting in large standard errors and a low

probability of obtaining statistically significant results. However, to have used further replicates for correlation would have invalidated the comparison with the microarray analysis. The key to reducing the errors inherent in these kinds of analyses is to increase the replication of both the qPCR and the microarray analyses. Unfortunately, this was beyond the financial resources available for this project.

On an individual basis, most of the genes assayed did not exhibit a significant change in expression. *LOX2* exhibited a significant up-regulation following *Plutella* larvae feeding when analysed using qPCR and this result adds evidence to the conclusion from microarray ontological analysis that a similar induction of the jasmonic acid pathway is occurring as observed in previous studies (Reymond et al., 2000; Van Poecke et al., 2001). It is unusual however that the results from microarray analysis do not support this observation. The qPCR analysis of the three genes identified from microarray studies as exhibiting high expression ratios corroborates the evidence that wounding alone is causing the change in expression. This is shown by the similarly high levels of induction following each treatment. The gene *XTH6* encoding a xyloglucan endotransglycosylase-hydrolase is involved in the cleavage and subsequent transfer of xyloglucan molecules into plant cell walls by transglycosylation (Emons and Mulder, 2000; Hui et al., 2003). Xyloglucans tether cellulose microfibrils into a strong but extensible cell wall and are essential for growth and the tensile strength of cells (Pena et al., 2004). Voelckel *et al.*(2004) report the induction of a *Nicotiana attenuata XTH* that is upregulated in a similar fashion following feeding by a trio of phytophagous herbivores including a leaf chewer (*Manduca sexta*), a cell-content feeder (*Tupiocoris notatus*) and a phloem-feeder (*Myzus nicotianae*). From the evidence presented by Voelckel *et al.* together with evidence presented above it is hypothesised that this generalised response to leaf damage is triggered in order to strengthen the cell walls and reduce the damage caused by herbivorous arthropods or mechanical damage. To test this hypothesis, leaf tensile strength and activity of *XTH* genes could be measured following differing levels and types of wounding at both distal and proximate locations.

Concurrent with, but on a smaller scale than the *XTH6* gene, a 3-4 fold induction of the *CER1* is seen across all treatments. *CER1* encodes an eceriferum gene which plays a role in the secretion and transport of epicuticular wax components in a range of plants (Jenks et al., 1995). Evidence was presented in Chapter 3 of the release of long-chain hydrocarbons that may be a by-product of increased wax production. The production of epicuticular wax in *Brassica* spp. and *Pisum sativum* L. has been shown to reduce herbivory and prevent attachment by leaf-feeding beetles while *cer* mutants with reduced waxiness were more susceptible to damage (Stoner, 1990; Bodnaryk, 1992). Research carried out using *Arabidopsis cer* mutants has shown that *Plutella xylostella* moths oviposit significantly more eggs on *cer4* plants than on *cer2* plants and this difference was due to the chemical composition of the waxes produced (Jenks et al., 2002). Jenks *et al.* also conducted experiments using *B. brassicae* and found evidence of an antixenotic reaction to waxes from the *cer3 Arabidopsis* mutant. The aphids probed less and walked more on the stems of *cer3* plants (Rashotte, 1999). This response was associated with reduced fecundity of the aphids on *cer3,* suggesting that reduced acceptance of the host leads to decreased performance. Although these experiments found that different *cer* mutants were involved in the insect-plant interaction, the evidence suggests that an induced increase in wax production or modification of constitution could be an effective generalised response against a multitude of herbivores. This topic is addressed in further detail in the final discussion (chapter 6).

ESP encodes an epithiospecifier protein which is a major determinant in the structural specificity of glucosinolate derivatives. Further detail on the role of the *ESP* can be found in section 1.3.1.

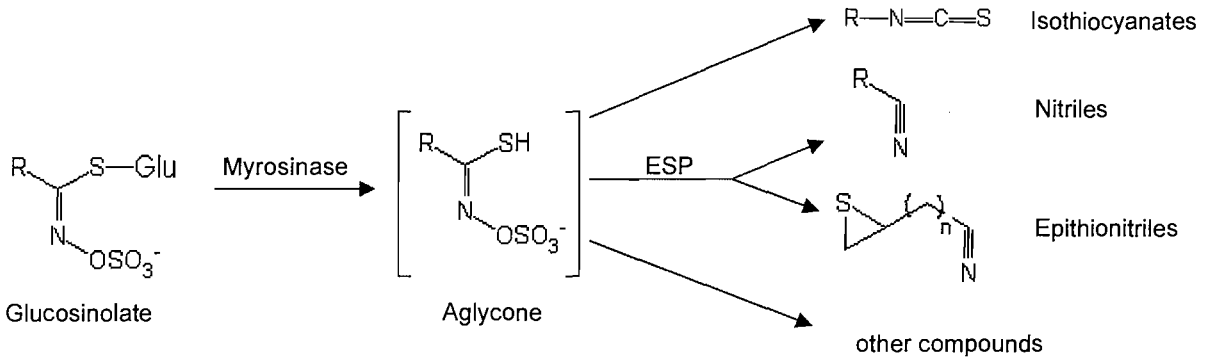


Fig. 4.8 – Glucosinolates are broken down to yield isothiocyanates, epithionitriles and a variety of other compounds.

During hydrolysis of alkenyl glucosinolates, ESP promotes epithionitrile/nitrile formation while the lack of ESP favours the spontaneous production of isothiocyanates (Fig. 4.8). Lambrix *et al.* (2001) demonstrated that *Arabidopsis* ecotypes with high *ESP* activity and consequently high nitrile production were preferred by *Trichoplusia ni* (cabbage looper moth) larvae over ecotypes that produced predominantly isothiocyanates (ITCs). Although ITCs are known to be antifeedants for a number of insect herbivores (Li et al., 2000), Lambrix *et al.* suggest that the role of *ESP* may be to block the production of volatile ITCs that function as an host-finding attractant for specialist Brassicaceae herbivores. Although the reduction of allyl ITC production could make the plant more susceptible to generalist herbivores to whom it is an antifeedant, a selective advantage may be realised if there is a significant reduction in pressure from specialist herbivores. In order for this strategy to be most effective, it would have to be an inducible defence response that depended on the challenge to the plant. Although the results detailed in this study are not conclusive, they suggest that both aphid infestation and control treatments induce the *EPS* gene but not *Plutella* larval feeding. Since isothiocyanates are an effective antifeedant for generalist herbivores while specialist herbivores are attracted to and able to tolerate the ITCs (section 1.2), this species-specific induction strategy could function as an effective inducible direct defence response and provide an evolutionary

advantage for the plant. This subject is discussed in detail with reference to other results from this thesis in chapter 6.

4.4.4 Herbivore-induced genes in a wild multitrophic system

Herbivore-induced plant volatiles are derived mainly from the jasmonic acid signalling pathway, and the two isoprenoid pathways detailed above (Dicke et al., 2003a). Since both of these gene ontologies have been shown to be significantly over-represented in a completely objective statistical analysis, we conclude that this method has considerable potential for the functional characterisation of *Arabidopsis* genes. Using this cross-species analysis on a wild plant with a poorly characterised genome has previously been outside the scope of most global gene expression analyses. However, cross-species analysis is not without limitations. The non-homologous elements of the *Arabidopsis* and *Brassica* genomes restrict the number of genes considered and any specialised components of the *Brassica* defensive function evolved since divergence may remain unidentified from a genomic perspective. Further experimental replicates will help to identify which genes are involved with a greater degree of statistical power and accuracy. By targeting genes for silencing or overexpression then measuring both gene expression and volatile output, it should be possible to deduce the mechanisms by which the release of volatiles is triggered and implemented. Research using transgenic plants or elicitors that enhance the attractiveness of plants to natural enemies would result in a decrease in pesticide use and possible increases in local biodiversity. Unfortunately, the vegetative stage and the period of vernalisation required for the wild *Brassica* means that collecting seeds from transformed plants for expression, inheritance or fitness studies is extremely time-consuming and impractical for laboratory studies. Rapid-cycling strains of *B. oleracea* have been developed that have a shorter life cycle which makes them a viable alternative (Williams and Hill, 1986).

4.5 Summary

- Global gene expression analysis on a wild plant using this unique experimental design and X-species filter identifies previously characterised herbivore-induced pathways confirming the utility of this technique.
- The major jasmonic acid / ethylene signalling pathway genes *ERF1* and *COI1* were significantly upregulated in *Plutella* damaged plants suggesting that these pathways are activated in a similar manner to those in *Arabidopsis.*
- Genes involved in isoprenoid biosynthesis were significantly upregulated following both aphid and *Plutella* feeding. These genes may be directly involved in the synthesis of volatile organic compounds.
- The number of genes exhibiting a significant increase in expression following aphid feeding was approximately equal to the number induced by *Plutella* feeding which supports conclusions by De Vos *et al.* (2005)
- Genes induced following wounding may be involved in cross-spectrum induced defences against a multitude of herbivores.

Chapter 5 - Population genetics of the *Brassicaceae*

5.1 Introduction

The family Brassicaceae has been shown to be composed of a diverse group of plant species brought about by both natural and artificial selection (see section 1.2.1). The majority of studies investigating the phenotypic and genotypic variation in this family has focused on the fully sequenced model plant *Arabidopsis thaliana* (AGI 2000). This research has allowed the characterisation of many different ecotypes and represents a unique genomic resource for understanding evolutionary adaptations and, alongside genetic modification, for the functional analysis of genes (Breyne et al., 1999). Although this research effort has been highly successful it is becoming apparent that wild relatives have a major role to play in identifying and exploring complex systems such as defensive function and multitrophic interactions (Mitchell-Olds, 2001). By understanding the genetic structure of natural populations of crop relatives, an understanding can be gained of their phylogenetic relationships, the possibilities and limitations of genomic analyses and the factors that may account for differences in phenotype. Additionally, this knowledge could be useful for the breeding of crops with reduced vulnerability to pest and disease attack, or crops that are able to adapt to different environments.

5.1.1 Genetic diversity of the Brassicaceae

The family Brassicaceae is composed of about 340 genera and more than 3350 species (Alshehbaz, 1984). Many members of the family have been cultivated over many generations to use as food crops and the diversity of the family as a whole has provided a variety of desirable characteristics that may be introduced by selective breeding. Examples of such morphologies include the enlarged inflorescence of cauliflower (*B. oleracea* subspecies *botrytis*) and broccoli (*B. oleracea* subspecies *italica*); the enlarged root of turnip (*B. rapa* subspecies *rapifera*); the enlarged single apical bud of cabbage (*B. oleracea* subspecies *capitata*) and the many axillary buds of Brussels sprout (*B. oleracea*

subspecies *gemmifera*) (Kalloo and Bergh, 1993). The seed of *B. nigra* is utilised to make mustard. Fig. 5.1 illustrates four members of the *Brassicaceae* species used in this study.

Fig. 5.1 – Morphology of the Brassicaceae studied (clockwise from top left) - Derby Day roundhead cabbage, Montgomery brussel sprout, *Arabidopsis thaliana* and wild *B. oleracea*.

The domestication of crop plants and selective breeding for desirable traits is typically accompanied by a genome-wide loss of genetic diversity (Tanksley and McCouch, 1997). This lack of diversity in cultivated plant varieties is believed to occur due to the small initial population and the intense self-fertilisation used to fix the desirable traits. This study (chapter 2) has demonstrated the ecological importance of a wild *Brassica* that deploys an indirect defence system in response to aphid feeding. Many other such

ecological interactions may be occurring in crop wild relatives that may contain beneficial traits that are yet to be discovered. Although wild germplasm is recognised as an important resource for plant breeding, one of the main factors limiting its use in crop improvement efforts is the fact that crosses with wild taxa typically result in the introduction of agronomically undesirable traits (Liu and Burke, 2006). However, recent advances in gene introgression techniques and gene association maps mean that a more directed approach to crop improvement can now be pursued. These genomic techniques require a great deal of knowledge of the gene function and genome organisation and this is one of the major impediments to their use with wild plants. An understanding of the genetic structure and diversity in these wild plants is essential if we are to utilise the wild germplasm for crop improvement and achieve a reduction in the amount of externally applied pesticides.

The high level of variation in gene expression among wild *Brassica oleracea* progeny grown under identical conditions from a single plant from Durdle Door has been demonstrated through the expression analyses in Chapter 4. Also, Brockhouse (2005) noted that individual plants appear to differ in their susceptibility to aphid infestation and this can be observed by examining the plants in their natural context. Some plants appear heavily infested with aphids while others that are growing next to the infested plant have escaped infestation altogether. This suggests that the plants manifest their defences in different ways, with some more effective than others. There is evidence for genotypic variation between populations and individuals in the differing quantities and nature of glucosinolates identified in the plants (Mithen et al., 1995a; Moyes, 1997; Brockhouse, 2005). All *Brassica* species have undergone several genome duplication and rearrangement events (chapter 1), and the outcrossing in wild *Brassica* could produce a large amount of phenotypic variation. In order to be able to study and understand the differences between individuals, species and families, analytical methods have been developed which estimate or directly measure the polymorphisms in genome sequences. Molecular marker technologies such as restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPD), amplified fragment length polymorphisms (AFLPs) and simple sequence repeat (microsatellite) polymorphisms are

increasingly being used to characterise levels of genetic diversity in cultivated and wild populations (Warwick et al., 2004). AFLP analysis (Vos et al., 1995) offers a highly reproducible method for measuring random polymorphims over the entire genome and can give an estimate of the genetic relatedness of individuals within and between populations.

5.1.2 Amplified fragment length polymorphism analysis

The AFLP technique is based on the selective ampification of restriction fragments from a total restriction digest of genomic DNA (gDNA). The technique involves three steps. Firstly, the genomic DNA is cut using restriction endonucleases and oligonucleotide adaptors are ligated to the resulting 'sticky' ends. A subset of restriction fragments is amplified using selective PCR primers in order to reduce the complexity of the fragment mixture. This subset is reproducible and represents fragments randomly distributed across the entire genome. A further selective amplification step is performed to label the selected fragments and further reduce complexity. Finally, the fragment lengths are determined by capillary gel analysis.

The amplified fragments constitute random markers which correspond to a unique genome locus. The differences in fragment lengths can be attributed to base changes in the restriction site or the primer extension site, or to insertions or deletions in the body of the DNA fragment. The number of bands that can be amplified in one AFLP reaction can be very high so optimisation of the PCR reaction and using a reasonable threshold value during fragment analysis is necessary to select a fragment number that can be resolved accurately and repeatedly by the capillary gel electrophoresis system. Since fragments cannot be associated with specific gene loci (when using gDNA), AFLP analyses are performed in order to determine relationships and discriminate between individuals or populations (Halliburton, 2004). The fragments produced have been shown to be spread over the entire nuclear genome as expected from the distribution of sites for the restriction endonucleases used (Nilsson et al., 1999). Once binary data (i.e. presence/absence) is collected on the polymorphic loci, this data can be used to calculate the genetic distance between variants and the proportion of polymorphic loci. The proportion of polymorphic

loci (*P*) is used to give an estimate of sequence diversity. However, because the loci being investigated are random and 'anonymous' (i.e. not sequenced), it cannot be determined whether at each locus there are two or multiple alleles and *P* makes no distinction between them. A locus with many alleles would be classed as more highly variable when using the more sensitive indicator of observed heterozygosity as a measure of variability.

The aim of this chapter will be to explore the genetic variation in a subset of the family Brassicaceae considering wild and cultivated varieties. By comparison to inbred cultivated lines, a relative quantification of the level of diversity will be achieved. This quantification may assist in the explaining the observed differences between populations, individuals and progeny from the same individual. Several recent studies have employed the AFLP method to study the genetic diversity in *Brassica* species including breeding lines of *Brassica juncea* (Srivastava et al., 2001; Burton et al., 2004), *B. napus* (Lombard et al., 2000) and *B. rapa* (Huh and Huh, 2001). These studies were used to determine the agronomical origin and seed quality of plants grown for commercial purposes. In wild plants, the technique can be used to assess the sequence diversity relative to commercial cultivars and the phylogenetic relationships between populations. AFLP studies are able to generate many hundreds of markers to distinguish between samples depending on the reaction conditions and numbers of primers used. For this reason, the technique is versatile enough to be used as a tool to answer a variety of questions relating to sequence similarity such as forensic identification (DNA fingerprinting) and studies on the migration and geographical origin of species.

Since a large proportion of research into plant genomics has been dedicated to *Arabidopsis thaliana* and the genome is the most well understood plant genome, it is relevant to quantitatively express the relatedness of certain *Brassica* species to each other and to *Arabidopsis*. To provide a reference for these previously unperformed comparisons, two fully sequenced *Arabidopsis thaliana* ecotypes – Columbia (Col-0 NASC stock number N1092) and Landsberg *erecta* (L*er*-0 NASC stock number NW20) will be included in which the single nucleotide polymorphisms (SNPs) are known and

quantified. This approach may enable the use of AFLP analysis to estimate the number of SNPs in unsequenced organisms by taking into account the size of genome. By using this method of SNP estimation and comparing the results to actual sequence SNP data, it may be possible to identify hypervariable regions or regions that exhibit fewer changes that normal. This data would be of use in genome mapping and the understanding of genome rearrangement events.

5.1.3 Hypotheses:

- Wild populations of *B. oleracea* will show greater genetic diversity (as measured by proportion of polymorphic loci and genetic distance) than the cultivated varieties in line with previous studies on gene expression and glucosinolate phenotype.
- *Arabidopsis thaliana* ecotypes will be more genetically distinct than the *Brassica* species due to its earlier divergence.

5.2 Materials and Methods

5.2.1 Plant preparation

Plants (detailed in table 5.1) were grown in a glasshouse compartment for at L16-D8 photoperiod, 22-26 °C and 40-80% R.H. Seeds were sown in 0.3L square pots filled with standard potting compost (Levingtons JS2) with no extra fertiliser added. Table. 5.1 details the plants that were used in the AFLP analysis. To investigate the genetic diversity from a single parent, eight seeds were grown from each of two Durdle Door and two Kimmeridge plants. Six more seeds were grown from each population to investigate diversity among individuals from different plants from the same population (see experimental design). Plants were labeled with the sample prefix corresponding to the species or population followed by the plant identifier number. The plants grown from the same parent were included a further suffix of a letter from A-H.

Table 5.1 – Summary of plants used for AFLP analysis. The sample prefix letter refers to the species (or population in the case of wild cabbage). The sample number refers to the plant number. Samples with a letter suffix are taken from the same parent plant.

Sample	No. of plants	Species	Ecotype / Line	Origin
A1-A2	2	*Arabidopsis thaliana*	Columbia	Missouri (USA), seeds obtained from Nottingham Arabidopsis Stock Centre (NASC)
L1-L2	2	*Arabidopsis thaliana*	Landsberg *erecta*	NASC
B1-B8	8	*Brassica oleracea* var. gemmifera (Brussel sprout)	F1 Montgomery	Moles Seeds, Colchester UK
C1-C8	8	*Brassica oleracea* var. capitata (Cabbage)	Derby day	Moles Seeds, Colchester UK
D3,D11,D12,D 21,D23,D24,	6	*Brassica oleracea* var. oleracea (wild cabbage)	N/A	Durdle Door, Dorset, UK
D13A-D13H	8	*Brassica oleracea* var. oleracea (wild cabbage)	N/A	Durdle Door, Dorset, UK
D6A-D6H	8	*Brassica oleracea* var. oleracea (wild cabbage)	N/A	Durdle Door, Dorset, UK
K0,K2,K3, K11,K12,K15	6	*Brassica oleracea* var. oleracea (wild cabbage)	N/A	Kimmeridge Bay, Dorset, UK
K1A-K1H	1	*Brassica oleracea* var. oleracea (wild cabbage)	N/A	Kimmeridge Bay, Dorset, UK
K4A-K4H	8	*Brassica oleracea* var. oleracea (wild cabbage)	N/A	Kimmeridge Bay, Dorset, UK

5.2.2 DNA Extraction

1 to 2 cm^3 young leaf tissue was added to a small amount of ground glass in a 1.5ml tube. 500µl nuclear extraction buffer (0.38% (w/v) sodium bisulphate, 120mM Tris HCl pH 7.5, 30mM EDTA, 1.2M NaCl, 1.2% (w/v) CTAB (cetyl trimethylammonium bromide)) and 1µl Rnase was added and ground thoroughly. 100µl 5% (w/v) sarkosyl was added and the reaction was incubated at 65°C for 30 mins. 500µl phenol:chloroform mix (1:1) was added and the phases separated by centrifugation at 13000 x *g* for 2 mins. 500µl of the upper phase was removed and added 300µl isopropanol before a further centifugation at 13000 x *g* for 1 min to recover the DNA. The supernatant was discarded and the pellet washed with 70% ethanol. A final centrifugation for 1 min was followed by removal of the supernatant and drying of the pellet in air. Each pellet was resuspended in 100µl TE.

5.2.3 Gradient PCR

A gradient PCR reaction was carried out to identify the optimum annealing temperature for the pre-selective primers. A pre-selective amplification reaction was carried out (see below) in a PCR block that ranged in temperature from 40°C to 60°C across 12 lanes. Each lane represented a rise in temperature of 1.67°C.

5.2.4 AFLP Protocol

Table 5.2 - Sequences of AFLP primers and adaptors.

Primer/Adaptor	Sequence (5'→ 3')
*Mse*I adaptor	5'-GAC GAT GAG TCC TGA G 3'- TA CTC AGG ACT CAT
*Mse*I universal primer (M0)	GAT GAG TCC TGA GTA A
*Mse*I pre-selective primer	M0 + A
*Mse*I selective primer (F06)	M0 + A + CA
*Mse*I selective primer (F40)	M0 + A + AC
*Mse*I selective primer (G01)	M0 + A + AG
*Mse*I selective primer (G04)	M0 + A + GA
*Pst*I adaptor	5'-CTC GTA GAC TGC GTA CAT GCA 3'-CAT CTG ACG CAT GT

*Pst*I universal primer (P0)	GAC TGC GTA CAT GCA G
*Pst*I 91T23 pre-selective primer (G)	P0 + G
*Pst*I* WellRED labelled 91T23 primer	(dyeD3) + P0 + GT

Restriction-ligation reaction

gDNA was digested using two restriction enzymes: a frequent cutter (*Mse*I) and a rare cutter (*Pst*I). The restriction sites for these endonucleases are shown below:

*Pst*I restriction site

5'-C TGCA|G
3'-G |ACGT C

*Mse*I restriction site

5'-T|TA A
3'-A AT|T

Double stranded adaptors are then ligated to the enzyme-specific restriction sites:

5x Restriction-Ligation (R-L) (50mM Tris acetate pH 7.5, 250mM KAc, 50mM MgAc, 25mM DTT and 250 ng/µl BSA). Each 25µl reaction contained 1.25µg gDNA, 5µl 5x R-L buffer, 12.5U *Pst*I, 6.25U *Mse*I plus water to 25µl. The reactions were incubated at 37°C for 2 hours. The adaptors were heated to 95°C for 5mins then allowed to cool to room temp. Immediately afterwards, 2.5pmol *Pst*I adaptor, 25pmol *Mse*I adaptor, 0.6µl ATP (10mM), 1µl 5x RL Buffer and 2.5U T4 DNA ligase were added and incubated overnight at 37°C. The product was diluted 1/8 (1 product:7 H_2O) and stored at -20°C. The product was visualised by EtBr agarose gel electrophoresis at this stage.

Pre-selective amplification

Primers used in this step consist of a core sequence, an adaptor specific sequence and a selective single-base extension at the 3' end. This base ensures a subset of restriction fragments are amplified thus achieving a 16-fold reduction in complexity of the PCR fragment mixture.

A mastermix was prepared consisting of 5µl Bioline clear PCR mix, 0.5µl Mse (A) pre-selective primer (20µM), 0.5µl Pst (G) pre-selective primer (20µM), 1µl 1/8 restricted-ligated DNA and 3.5µl H_2O.

The following PCR programme was used for amplification:

Incubate at 72.0°C for 20secs
Incubate at 50.0°C for 1 min
Incubate at 72.0°C for 2 min
Repeat for 25 cycles
Incubate at 72.0°C for 2 mins
Incubate at 60.0°C for 30 mins

The product was diluted 1/25 (1µl product:24µl TE).

Selective amplification

WellRED Dye labeled primers (Sigma-Proligo, UK) and another selective unlabelled primer are used to amplify the diluted pre-selective products and enable visualisation of the fragments by laser fluorescence.

The following components were added to a 20µl PCR tube; 0.5µl *Mse*I selective primer (either F06, F40, G01 or G04), 0.5µl *Pst*I* labeled primer, 5µl Bioline clear PCR mix, 1µl 1/25 diluted gDNA template (from pre-selective amplification) and 3µl H_2O.

The PCR amplification programme used was as follows:

Incubate at 94.0°C for 30 secs
Incubate at 66.0°C for 2 mins
Incubate at 72.0°C for 2 mins
Repeat for 25 cycles lowering annealing temp. by 0.6°C each cycle until at 51°C
Incubate at 94.0°C for 30 secs
Incubate at 56.0°C for 2 mins

Incubate at 72.0°C for 2 mins

Repeat 20 times

Incubate at 60.0°C for 30 mins

The amplification product was diluted to 1/10 concentration.

A mastermix of 320µl sample loading solution (SLS) (Beckman Coulter, UK) and 3.5µl of a 400nt size standard was prepared. Each 200µl well contained 32µl of mastermix and 1µl amplification product from two different Mse primers (F06 & F40 or G01 & G04). This was mixed by pipetting then overlaid with a drop of mineral oil (Beckman Coulter, UK). Fragment analysis was carried out using the FRAG-4 programme on a Beckman Coulter CEQ 2000XL capillary gel electrophoresis system according to manufacturer's instructions. Fragment data was analysed using the Beckman Coulter CEQ fragment analysis software. Fragments underwent a binning analysis to determine presence/absence of fragments across the different samples and was checked by eye (Vos et al., 1995).

5.2.5 Analysis 1 – Proportion of polymorphic loci

Plant AFLP profiles were compared to identify polymorphisms within several groups of samples to provide a measure of genetic diversity. The samples were grouped and polymorphisms measured between samples within the following groups.

- A1, A2 (*Arabidopsis* - Col-0)
- L1, L2 (*Arabidopsis* - L*er*)
- A1, A2, L1, L2 (*Arabidopsis* - Col-0 and Ler)
- B1-B8 (Brussel sprout)
- C1-C8 (Derby day cabbage)
- D13A-D13H (Durdle Door wild cabbage) 8 seeds from plant 13
- D6A-D6H (Durdle Door wild cabbage) 8 seeds from plant 6
- K1A-K1H (Kimmeridge wild cabbage) 8 seeds from plant 1
- K4A-K4H (Kimmeridge wild cabbage) 8 seeds from plant 4

- K0, K1D, K2, K3, K11, K12, K15, K4B (Kimmeridge wild cabbage) 8 seeds from different plants
- D3, D11, D6C, D12, D13F, D21, D23, D24 (Durdle door wild cabbage) 8 seeds from different plants

The proportion of polymorphic loci was scored by eye across each set of eight samples (except for *Arabidopsis* where only four were used). Fragments over 500 relative fluorescence units (rfu) were included in the analysis and the presence or absence of the fragments from other samples (whether above or below 500rfu) was recorded. Data from the four Mse primers was pooled.

5.2.6 Analysis 2 – Construction of genetic distance matrix and phylogram

AFLP profiles from the following samples were analysed and fragments scored as being present (1) or absent (0):

- A1 (*Arabidopsis* - Col-0)
- L1 (*Arabidopsis* - L*er*)
- B2 (Brussel sprout)
- C5 (Derby day cabbage)
- D3 (Durdle Door wild cabbage) plant 3
- D12 (Durdle Door wild cabbage) plant 12
- D13D (Durdle Door wild cabbage) plant 13D
- D24 (Durdle Door wild cabbage) plant 24
- K0 (Kimmeridge wild cabbage) plant 0
- K1D (Kimmeridge wild cabbage) plant 1D
- K2 (Kimmeridge wild cabbage) plant 2
- K3 (Kimmeridge wild cabbage) plant 3

Data from the four Mse primers was pooled. PAUP* 4.0b10 (Swofford, 1998) was used for all phylogenetic analyses. A genetic distance matrix was constructed using the binary data matrix and a neighbor-joining (NJ) cluster analysis (da Silva et al., 2005) was used to construct a phylogram. 1000 bootstrap replicates were carried out to give an estimation of branch reliance (Efron and Gong, 1983). Bootstrapping resamples the

original dataset to create pseudoreplicates and outputs the percentage of times that the tree node is placed in an identical position.

Results

Different taxa and individuals from the family Brassicaceae were analysed using AFLP analysis then their marker profiles compared. The polymorphisms between samples were analysed to map the phylogenetic relationships and the amount of genetic diversity between them. Fig. 5.2 illustrates the analysis method to detect polymorphic fragments.

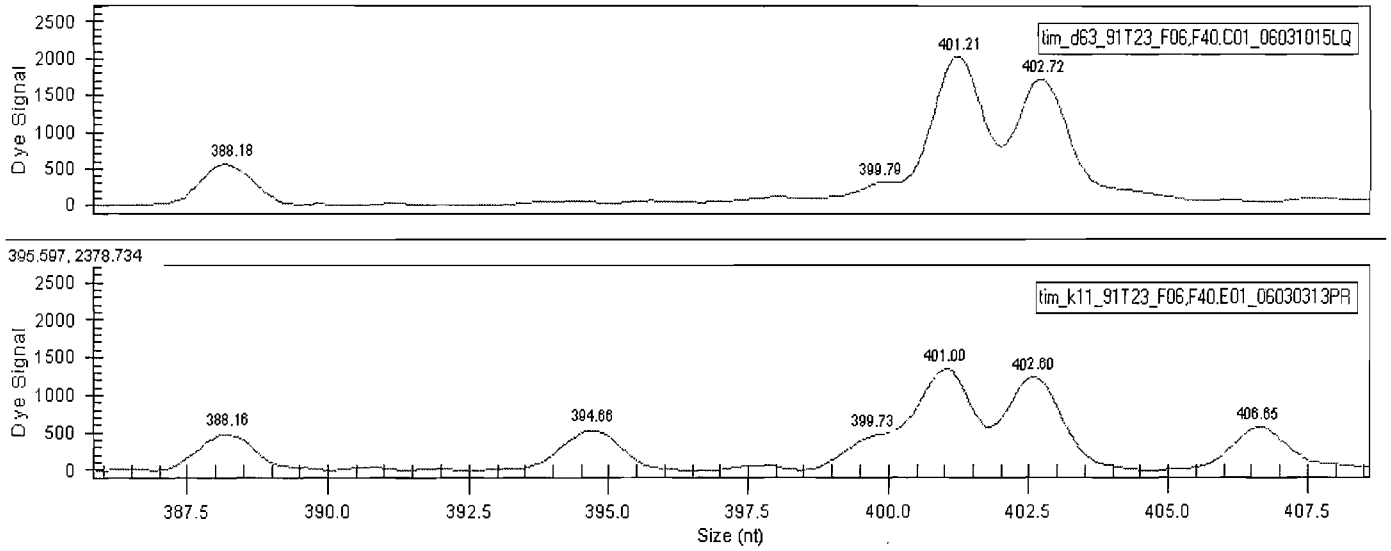


Fig. 5.2 – An example image taken from the AFLP fragment profile of plant Durdle Door D63 and Kimmeridge K11. Out of the six fragments, two polymorphic fragments can be observed at lengths of 394.66 and 406.65 nucleotides.

5.3.1 AFLP Fragment analysis

The four Mse primers selected produced a good range of fragments and were analysed using capillary gel electrophoresis. Extensive PCR optimisation was necessary in order to attain the optimum primer concentrations. It was found that high primer concentrations and multiple primers used in the same PCR reduced the final fluorescence signal of the fragments and the size standard. This effect is believed to be due to excess primer in solution causing competition between ions during uptake into the gel. Size standard fluorescence was also reduced in the presence of high primer concentrations. For these reasons, the primer concentration and the PCR dilution steps are vitally important during the AFLP reactions. Multiplexing of primer dyes was found to also

cause a reduction in fragment and size standard uptake (i.e. relative fluorescence) and was not used.

5.3.2 Analysis 1 – Proportion of polymorphic loci

AFLP analysis revealed polymorphic loci from a number of plant groups (table 5.3). The proportion of polymorphic loci gives an idea of the amount of genetic diversity within the group.

Table 5.3 – Summary of intra-specific polymorphic loci detected using AFLP

Samples compared	No. of samples	Plant species	Total fragments	Percentage of polymorphic loci
A1, A2	2	*Arabidopsis thaliana* (Col-0)	67	0
L1, L2	2	*Arabidopsis thaliana* (Ler)	81	1.7
A1, A2, L1, L2	4	*Arabidopsis thaliana* (Col-0 and Ler)	107	14.0
B1-B8	8	*Brassica oleracea* var. gemmifera (Brussel sprout)	105	0
C1-C8	8	*Brassica oleracea* var. capitata (Cabbage)	92	0
D13A-D13H	8	*Brassica oleracea* var. oleracea (Durdle Door)	170	13.5
D6A-D6H	8	*Brassica oleracea* var. oleracea (Durdle Door)	138	21.7
K1A-K1H	8	*Brassica oleracea* var. oleracea (Kimmeridge)	187	12.8
K4A-K4H	8	*Brassica oleracea* var. oleracea (Kimmeridge)	141	11.3

Comparison of the *Arabidopsis* ecotypes reveals a substantial level of genetic variation. A low level of genetic variation (1.7%) detected within the Landsberg *erecta* genome may be a real phenomenon or may be as a result of experimental error (incomplete disgestion or a problem with fragment separation/analysis). There is a high level of sequence variation in the wild species collected from Durdle Door and Kimmeridge. This level of variation is to be expected in a wild, segregating population with a number of possible alleles at each gene locus. The Brassicaceae crop cultivars are homogenous and do not exhibit any sequence variation in the loci analysed.

The number of fragments detected using the four primer pairs was variable between the different species. This variation may be due in part to the variation in genome size between *Arabidopsis* (157Mbp) and *B. oleracea* (696Mbp) (Johnston et al., 2005). The extensive duplication of genes and the polyploid nature of *B. oleracea* may explain the

lack of a large difference in the number of fragments detected. However, the electropherograms generated following fragment analysis exhibit some variation in fluorescence intensity within and between plant groups that is believed to be dependent on ion uptake by the capillaries. The relative fluorescence threshold above which fragments were counted was not controlled for between groups since a direct comparison was not conducted. Although identical volumes of PCR products and size standard were used, the size standard cannot be used as an rfu standard, only as a sequence length indicator. Without extensive analysis of PCR product and size standard ion uptake, inferences based on the number of fragments observed above a certain threshold are not considered possible. Only inferences based on presence or absence of a fragment (which can be deduced down to very low rfu values) are considered as these can be expressed as proportions and in relation to all other samples. The issue of ion uptake during capillary gel electrophoresis is a complex one and appears to be affected by primer/product concentration, PCR efficiency and other unknown factors.

5.3.3 Analysis 2 – Construction of genetic distance matrix and phylogram

A total of 376 fragments were detected using four primer combinations analysing 12 plant gDNA samples. 348 fragments (92.5%) were polymorphic while 28 (7.4%) were present in all samples. Fig. 5.3 illustrates the frequency distribution of fragment lengths from the eight plant samples where the mean fragment length was 211.2 nts. Fragments were analysed in the range of 58-500 nt.

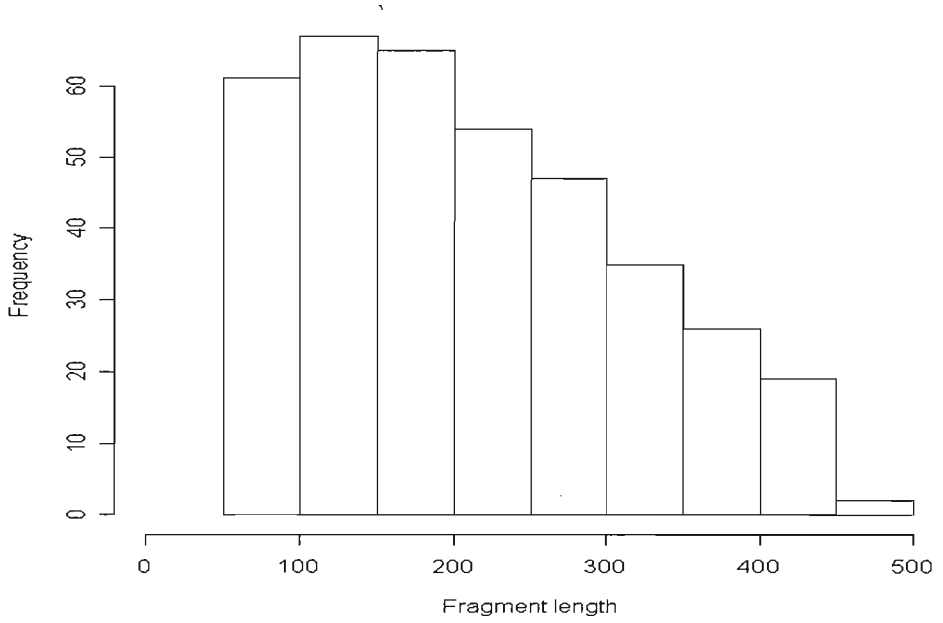


Fig. 5.3 – Frequency distribution of sample fragments from AFLP analysis of eight plant gDNA samples.

Genetic distance analysis was used to investigate the genealogical lineages and population structure, based on pairwise comparisons of the total number of DNA polymorphisms (AFLPs) between plants. Fig. 5.4 shows the pairwise distances between species and individuals.

A1										
199	B2									
213	88	C5								
206	89	81	D12							
208	97	89	78	D13D						
202	97	99	56	66	D24					
219	98	98	73	63	73	D3				
226	99	93	80	70	86	79	K0			
214	99	95	88	66	86	89	54	K1D		
213	92	100	91	75	97	96	59	53	K2	
230	115	117	102	94	110	105	72	70	57	K3
91	226	222	213	219	213	222	227	213	220	227	L1

Fig. 5.4 - Distance matrix showing pairwise distances between samples by total number of sequence polymorphisms.

The neighbor-joining genetic distance algorithm (Saitou and Nei, 1987) was implemented using PAUP* version 4.0b10 (Swofford, 1998) to construct a phylogram (Fig. 5.5) that graphically represents the genetic distance between taxa and individuals within taxa.

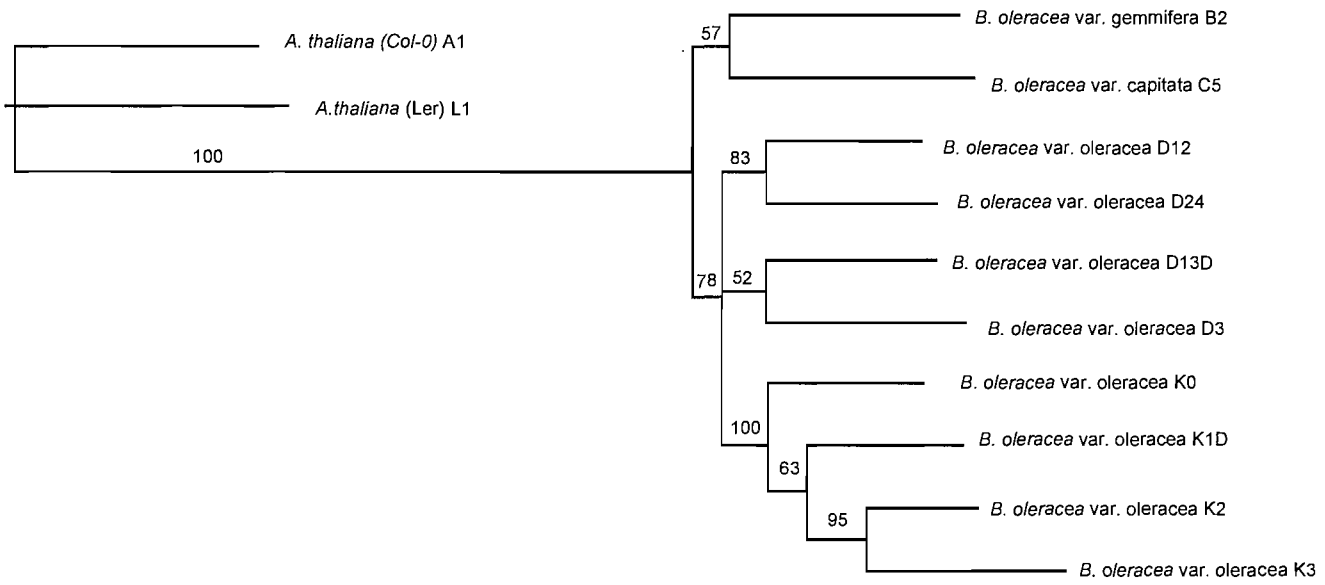


Fig. 5.5 – Phylogram constructed from AFLP data showing a scale representation of genetic distance between samples. Numbers above branches give the bootstrap values from 1000 resamplings.

This phylogram illustrates the intra- and inter-population genetic distance of plants from wild cabbage populations at Kimmeridge and Durdle Door. The populations are genetically distinct with a mean genetic distance (MGD) between populations of 88.4. The intrapopulation MGD for Durdle Door was 68.2 compared to 60.8 for Kimmeridge. This suggests that the Kimmeridge population is more inbred than the Durdle Door population. The MGD from Durdle Door cabbage to Brussel sprout was 95.3 compared to 101.3 for the Kimmeridge population. These values were almost identical to the MGDs from wild cabbage to Derby day cabbage which were 91.7 and 101.3 for the Durdle Door and Kimmeridge populations respectively. In contrast, the MGD from all *B. oleracea* species to the *Arabidopsis* ecotypes was 216.6.

The genetic distance between *Arabidopsis* ecotypes (91) is similar to the distance between the crop cultivars Brussel sprout and Derby Day cabbage (88). Also very similar is the genetic distance between populations of wild cabbage (88.4). This result is surprising since the visual phenotypic differences between crop cultivars would suggest a higher genetic distance than that between the visually similar wild cabbage populations.

5.4 Discussion

The results presented have demonstrated the small-scale use of AFLP analysis to assess genetic diversity and estimate phylogenetic relationships among wild and cultivated Brassicaceae. It has been shown that there exists a high number of polymophisms between individuals of the same wild species which suggests a segregating population and a high level of gene flow between populations. The wild plant is a useful model for ecological analyses and the investigation of insect-plant interactions. However, when analysing a phenotypic character of the plant, such as the volatile phenotype, both the genotype and the environmental context must be considered. Since the phenotype is a product of these variables, both of them should be known or standardised in order to allow a valid comparison of experimental treatments.

5.4.1 Brassica oleracea var. oleracea has a highly diverse genotype

AFLP analysis comparing the fragment profiles from plants from a single parent has revealed a high proportion of polymorphic loci (Fig. 5.3) demonstrating the sequence variability of the wild species. The proportion of polymorphic loci in the cultivated species studied is zero which is to be expected from an inbred line that is commercially available. This monomorphic character ensures that the resulting crop is homogenous both in growth characteristics and in response to pests and pesticides. The phylogram (Fig. 5.5) and genetic distance matrix (Fig. 5.4) contribute further evidence to the conclusion of high genetic diversity in the wild plants. This high level of variation is beneficial in wild populations since the plant is able to survive and 'adapt' on an evolutionary timescale to changes in environment and selection pressures.

It is interesting in this instance that the genetic distance between different commercial cultivars of *B. oleracea* – Derby Day cabbage and Brussel sprout – is less than the distance between some members of the same wild variety (e.g. D24:K3). The phenotypic differences between the two crops are visually very obvious (Fig. 5.1), while the wild plants exhibit extremely homogenous morphologies. It is proposed that this disparity could be explained by the neutral equilibrium model that is derived from Kimura's neutral genetic theory (Kimura, 1968, 1983). NE theory posits that in a population that is neutral with respect to selection, sequence polymorphisms are more likely to occur in non-coding DNA sequences (introns) than coding sequences (exons). In addition to NE theory, positive selection or balancing selection may occur. Positive selection for a desirable or beneficial allele results in a reduction in levels of nucleotide diversity (Smith and Haigh, 1974). Balancing selection results in no single allele being selected for and therefore multiple alleles are maintained in the population by antagonistic forces. This results in increased nucleotide diversity (Kaplan et al., 1988). Considering this study, artificial positive selection has occurred in order to fix the desirable mutations that differentiate the crop cultivars and a relatively lower level of sequence diversity is observed. In the wild populations, it is unclear whether there the neutral theory holds or whether balancing selection maintains the nucleotide diversity in the population. Since it is estimated that >90% of DNA sequence is non-coding intron sequence (Halliburton, 2004), it is likely that the variation observed is mostly due to naturally occurring mutations that are neutral with respect to the amino acids being synthesised. However, despite the lack of visual phenotypic variation, Brockhouse (2005) found considerable differences in glucosinolate content and Mithen (1995a) found allelic variation at loci believed to control glucosinolate production. The habitat at each site also varies and may result in selective forces acting on the plants. At Kimmeridge, the plants are found in a highly unstable, predominantly shale substrate, cliff-top environment and as such are exposed to sea spray and high winds. This hostile environment also decreases the diversity of herbivores present to feed on them. At Durdle Door, the plants are found at the bottom of the cliffs in a calcareous substrate and are exposed to more sea spray but lower wind levels. The plants at Durdle Door exist in a more halophytic environment so their biology may have adapted to this and their defences may be more geared towards

resistance to halophytic herbivores. These different selective forces may account for the variation in glucosinolate content between populations and the substantial sequence dissimilarity observed during isozyme studies (Mithen et al., 1995a) and AFLP analysis. Whether these forces result in balancing selection is unclear although the limited studies carried out so far suggest this could be the case. Mithen *et al.* (1995a) and Moyes (1997) suggest that founder effects and random genetic drift could account for the variation observed between sites. However, these factors depend on the populations being spatially distinct with relatively low gene flow. This study has shown that the inter-population genetic variability is only slightly greater than the intra-population genetic variability. This suggests a high degree of inter-population gene flow (i.e. high level of outbreeding) which has been shown to be facilitated by foraging insects (Warman, 1994). Consequently a degree of balancing selection may be maintained at the sites by the selective forces acting. This may explain the variation rather than founder effects or genetic drift which imply genetically isolated populations.

The possibility of selective forces acting on the populations means that some of the segregating polymorphisms observed are present in coding regions of DNA (exons). Statistical tests that measure the presence of sequence polymorphisms within and between populations (or species) are available (Wright and Gaut, 2005) but require extensive DNA sequence data which AFLP analysis does not provide. Analysis based on the sequence polymorphisms within quantitative trait loci known to be associated with a proposed selective factor may elucidate further information. The non-specific nature of the AFLP loci analysed has previously been identified as a problem when using binary data (i.e. presence/absence) to infer phylogenetic relationships. A particular problem is the violation of the assumption of independence between polymorphisms. If a new restriction site evolves between two existing sites, one (longer) fragment disappears and two (shorter) fragments appear. In this case, even though the two species may share two of three restriction sites and have a high sequence similarity, they have no fragments in common. Insertions and deletions are also problematic since they also alter the fragment lengths but may have no effect on the map-based gene position or gene function

(Swofford et al., 1996). One of the problems encountered during this study involved the possible presence of different fragments having the same nucleotide length and therefore not being flagged as polymorphic. Fig. 5.3 illustrates the frequency distribution of fragments over the nucleotide length range and it can be observed that 68 fragments are present in the range 100-150nts. 8.5 fragments per sample are to be distributed in a 50nt range which suggests that some overlap may occur. This error will result in the species being deemed to be more closely related than they actually are. The error rate could be reduced by analysing the fragment mixture from each primer pair separately instead of combining the amplified products in the final capillary gel electrophoresis step. In summary, these problems with AFLP analysis result in the data obtained being relative approximations that give an idea of distinctions between relatives and of sequence similarity/diversity. They are not substitutes for sequence based phylogenetic techniques such as SNP analysis and allozyme studies that yield information on gene mapping and allelic variation.

5.4.2 *Arabidopsis thaliana* exhibits limited sequence similarity to Brassica species studied

The genetic distance between *Arabidopsis* and the *Brassica oleracea* illustrates the sequence changes that have occurred since divergence an estimated 20 million years ago (section 1.2.1) (Yang et al., 1999). Microsynteny studies involving comparative genetic and physical mapping of specific chromosome segments have shown largely conserved gene order in *Arabidopsis* and *Brassica,* but some disruption in gene content by insertions or deletions (Conner et al., 1998; Grant et al., 1998). Studies have found extensive replication and rearrangement of segments of the genome when comparing the two species (Town et al., 2006). The phenotype of the two plants is also very different and this can be observed in Fig. 5.1. The AFLP fragments observed from the *Brassica oleracea* genome were often present in considerably different quantities (measured by relative fluorescence units - rfu) compared to the *Arabidopsis* fragments. This could be due to the fragments coming from different loci but measuring the same length. Alternatively, the fragments could be part of a replicated gene family so multiple copies were amplified. As stated above, it is not possible to provide a quantitative analysis of

the fragment abundance since the controls were not comparable. Despite the differences, it is agreed that naturally occurring Brassica *ge*netic variants may prove useful for new investigations into plant development, particularly through the study of the effects of alleles that lack variation in *Arabidopsis.* Chapter 4 has demonstrated the potential of using *Arabidopsis* resources for research into Brassica genomics, albeit in a manner limited by the homology between genes. In this chapter, it has been shown that there exists a substantial number of polymorphisms between the two genomes. However, it is unknown how many of these polymorphisms are in exon regions and so would affect the interpretation of gene expression data.

5.4.3 The importance of genetic diversity in a natural context

The benefit to a species of a having a diverse genotype can be summarised by considering the drawbacks of having a monomorphic genotype. Well known cases such as the Irish potato famine (1846), the loss of 18 million citrus fruit trees in Florida (1984) and more recently the Black Sigatoka virus of *Musa* spp. (banana) illustrate the problems when monomorphic cultivars are pursued to the detriment of their diverse relatives. These cases and a host of others demonstrate the need for diversity in cultivated varieties and the same need is manifested in natural populations. A diverse gene pool allows a degree of evolutionary adaptability to changes in environment and other selective pressures. Genetic diversity is also being increasingly recognized as an essential factor in future food security when producing more productive varieties (Tanksley and McCouch, 1997), those that have higher resistance to pests and diseases or those that are adapted to tolerate environmental extremes such as high salt or low water. The genome of wild *Brassica oleracea* exhibits a high degree of diversity when compared to cultivated Brassicaceae. This suggests that wild *B. oleracea* has potential as a model plant used not just for the investigation of multitrophic interactions but also for a molecular population genetics analysis of selective traits, testing of the neutral equilibrium model and as a resource for plant breeders. The difficulties in using the plant for genomic studies however have been highlighted by the results from this study. For this reason, homogenous cultivars such as Derby Day cabbage may be more suitable for genomic studies where stable comparisons between plants are necessary. This study could be expanded to include further

populations from different geographical areas to provide information on the origin and evolution of *Brassica oleracea* var. oleracea.

5.5 Summary

- Wild *Brassica oleracea* exhibit a high number of polymorphisms between individuals of the same wild species which suggests a segregating population and a high level of gene flow between populations.
- The cultivated *B. oleracea* subspecies did not exhibit sequence diversity thus confirming they are from pure-breeding lines.
- The genetic distance between the cultivated *B. oleracea* subspecies is (in some cases) equal to the genetic distance between individuals of the same wild *Brassica*. This provides further evidence of the substantial sequence diversity in wild species.
- The *Arabidopsis* ecotypes studied exhibit some sequence similarity to the *Brassica* species although the high genetic distance between species supports previous studies estimating their divergence some 15 million years ago.

Chapter 6 - General Discussion

This aim of the research described in this thesis is to expand on the knowledge and understanding of the mechanism of induced plant defences by using a novel wild tritrophic interaction. The details of this interaction were investigated from different perspectives by using behavioural, chemical and transcriptomic analysis techniques. By integrating the knowledge gained through the use of these techniques, a clearer picture of the mechanisms mediating this interaction can be constructed. This general discussion will address aspects of the research where an integrated perspective is advantageous and necessary to convey a more holistic understanding of the subject.

The response to aphid-induced plant volatiles by parasitoid wasps

By studying the behaviour of parasitoid wasps when presented with odours from infested and control plants, it has been demonstrated that volatile components emitted by the plant-host complex are attractive to the parasitoid (chapter 1). This attraction is likely to facilitate the first two of the steps proposed by Vinson (1976) when considering foraging behaviour of parasitoid wasps - host habitat location and host location. The question of whether this host searching behaviour constitutes an induced indirect defence system depends on whether an increase in fitness of the plant is observed.

The emission of plant volatiles following herbivore feeding has in many cases been shown to be an active plant process whereby the compounds are synthesised *de novo* following induction of biochemical pathways such as the octadecanoid pathway (Dicke et al., 1990; Pare and Tumlinson, 1997). Following release of these volatiles, it has been shown that predators or parasitoids of the attacking herbivores are attracted to the induced compounds and this has been classified as an indirect defence system 'activated' by the plant (Dicke and Vet, 1999). The definition of a plant defence requires that a net gain in fitness is achieved following induction in the plant (Karban and Myers, 1989). This may come about as a decrease in the negative consequences of herbivory while also taking into account the costs in producing further secondary metabolites. The driving force

behind the evolution of these indirect defences is the mutual benefit to both the plant and the predator/parasitoid. The plant supposedly benefits through a reduction in herbivory and the associated risks of virus transmission. The predator/parasitoid overcomes the reliability/detectability problem (see section 2.1) by responding to a reliable and detectable indicator of host presence (Vet and Dicke, 1992). In chapter 3, it is demonstrated that following aphid infestation, there is a significantly greater amount of 3-butenyl isothiocyanate detected in the headspace of the plant-host complex. Additionally, following discriminant analysis, this compound is identified as one that is the most reliable indicator of aphid infestation. It would seem logical therefore, that the parasitoid would be attracted to this compound. The traditional view is that the plant responds to the aphid infestation by way of an induced defence pathway to facilitate the systemic breakdown and release of the plant volatile. However, in this case the predominant signal (3-butenyl isothiocyanate) has also been shown to be attractive to *B. brassicae* (Nottingham et al., 1991). It would seem to be contrary to the welfare of the plant to release an attractant that may also be an aggregation signal for the aphid. It is hypothesised therefore, that the high level of 3-butenyl isothiocyanate does not come about as a result of an induced plant response but as a result of the aphid-synthesised myrosinase. By increasing the emission of the volatile 3-butenyl isothiocyanate during feeding, the aphids would be communicating to other members of the species the message that a suitable host plant has been located. Previous evidence has suggested that this compound is involved in the attraction of the parasitoid wasp *D. rapae* (Bradburne and Mithen, 2000; Blande, 2004) although the results from this study suggest otherwise (see section 2.4.2). If it were proved that 3-butenyl isothiocyanate is attractive to parasitoids, it would provide support for the aphid-driven release hypothesis by providing an efficient solution to the reliability/detectability problem. The signal produced would be highly reliable due to the aphid being directly involved in the production of the compound. Also, the signal would be highly detectable due to its volatility and the substantial quantities being produced.

The origin of aphid-induced volatile compounds

Aphid myrosinase has been detected in the sarcoplasm of the muscle tissue surrounding the head, thorax and abdominal regions and is stored in structures described by Bridges *et al.* (2002) as crystalline microbodies. They suggest that the compatmentalisation of an aphid myrosinase is a defence mechanism and is released following death or damage in order to facilitate glucosinolate breakdown. It is stated that the release of isothiocyanates may act as a synergist to the aphid alarm pheromone *E*-β-farnesene. However, there is no evidence that this compound does act as a synergist in *B. brassicae* and if this was the case, it would seem counter-intuitive that *B. brassicae* is also attracted to an alarm-pheromone synergist. The evidence would suggest that a more likely scenario would be the case that 3-butenyl isothiocyanate is released from the host-plant complex as an aggregation pheromone or as a by-product of the degradation of toxic glucosinolates. An aggregation pheromone could provide fitness benefits to the aphid through 'dilution effects' whereby the probability of being attacked is less when surrounded by others (Bertram, 1978; Turchin and Kareiva, 1989). Additionally, this gregarious behaviour would be selected for by the increased survivorship of clonal/related individuals (McCauley, 1994; Fellowes, 1998).

It is known that intact glucosinolates are loaded into and transported by the phloem in *Arabidopsis* (Chen et al., 2001) therefore the aphid myrosinase-catalysed emission of 3-butenyl isothiocyanate could arise by either or both of two routes. Assuming that a similar mechanism of glucosinolate translocation occurs in *B. oleracea,* the catalysis could occur endogenously within the aphid gut or exogenously following enzyme secretion. The sheath material and watery saliva of the grain aphid (*Sitobion avenae*) and the bird cherry-oat aphid (*Rhopalosiphum padi*) have been reported to contain a *β*-glucosidase (Miles, 1999) that has the potential to hydrolyse glucosinolate compounds (Iori et al., 1999). Prado & Tjallingii (1997) report that when the black bean aphid (*Aphis fabae*) feeds on *Vicia faba*, the stylet's entry into a phloem sieve tube is followed by discharge of saliva for 10 minutes or more. These salivary secretions are believed to limit the plant response to wounding and to prevent the plugging of sieve elements (Miles, 1999; Tjallingii, 2006; Will and van Bel, 2006). If a similar feeding method is

employed by *B. brassicae,* the aphid-myrosinase could be discharged directly into the phloem sieve tubes where it would hydrolyse the glucosinolates. A release of volatile glucosinolate derivatives would be expected and this can be observed in results observed in chapter 3. This transport of salivary components has previously been suggested to explain the systemic induction of defence-related genes (Divol et al., 2005) and could also result in the systemic emission of volatiles (Guerrieri et al., 1999). It should be stressed however that these previous studies have been conducted on different species of plant and aphid.

Alternatively, endogenous catalysis of glucosinolates could occur within the aphid gut as the phloem passes through. Either of these methods may provide a further fitness benefit to the aphid by emission of isothiocyanates that are toxic to a range of fungi and non-specialist insects (Chew, 1988; Bones and Rossiter, 1995; Li et al., 2000). The hypothesis that the emission of 3-butenyl isothiocyanate is an aphid-driven rather than plant-driven mechanism would imply that evolutionary pressure has not necessarily been exerted on the plant to release plant 'defence' compounds. The idea that *D. rapae* attraction to wild *Brassica* confers a fitness benefit has not been proved and experiments investigating the effectiveness of the parasitoid as a method of biological control reveal severe limitations. Zhang *et al.* (2003) demonstrated that in order to control an aphid infestation in confined (cage or greenhouse) conditions required three releases of artificially reared parasitoids at a total wasp to aphid ratio of 1.2:1. It is extremely unlikely that this ratio would be reached under natural conditions with a widely distributed host and host-plant population.

Additional evidence for this proposed model of aphid-plant interaction comes from the comparison of the aphid-induced 3-butenyl isothiocyanate and the *Plutella*-induced quantity of the same volatile. The quantity released following *Plutella* larvae feeding is about half that of the aphid-feeding (chapter 3). Although it could be argued that the time of feeding and numbers of insects were not comparable, the chewing action of *Plutella* is likely to cause more indiscriminate cell disruption due to the chewing method of feeding compared to the piercing-sucking method employed by the aphid. This increased

disruption would lead to the contact of glucosinolates from cell vacuoles and myrosinase from myrosin cells and a release of glucosinolate derivative products. Conversely, the aphid feeds primarily by intercellular probing (Tjallingii, 2006) that is believed to cause minimal direct damage to other cells (Thompson and Goggin, 2006). The results obtained in this study suggest that enzymes from other sources may also be acting on phloem-borne glucosinolates.

This hypothesis also fits with the observed induction of the *EPS* gene during gene expression studies (chapter 4). The EPS protein directs the myrosinase hydrolysed catalysis of glucosinolates towards the production of epithionitriles rather than isothiocyanates (Bernardi et al., 2000). It was found that both aphid infestation and control treatments induce the *EPS* gene but not *Plutella* larval feeding. This directed catalysis to form a different hydrolysis product would be contrary to what would be expected if the volatile induction was a plant-driven defence response. Li *et al.* (2000) suggest that induction of the *EPS* gene could be a plant response to reduce the emission of isothiocyanates or to block the production of volatile ITCs that function as an host-finding attractant for specialist Brassicaceae herbivores (Lambrix et al., 2001). It is possible that *EPS* could be induced via an endogenous feedback mechanism to control quantities of myrosinase present in the phloem.

To test the hypothesis that an aphid myrosinase is injected into the plant, confocal microscopy could be used to track the myrosinase translocation. A myrosinase-specific fluorescent dye could be added to the food-plant during aphid development then the aphid would be removed to a clean plant to track the location of the aphid-myrosinase.
A more straightforward method would be to allow a generalist aphid to feed on the plant and analyse the differences in the volatile profile emitted. If an aphid-driven system was present, it would be expected that the level of 3-butenyl isothiocyanate would be very low or absent. However, it must be considered that the 3-butenyl isothiocyanate emitted may be an aphid-specific response to another component in the saliva of *Brevicoryne brassicae.*

Epicuticular wax formation following aphid-feeding on wild *B. oleracea*

Evidence is presented in chapter 4 that two genes involved in epicuticular wax formation are induced following aphid feeding and leaf removal. The gene *CER-1* is involved in the conversion of long chain aldehydes to alkanes, a key step in wax biosynthesis (Aarts et al., 1995). Also, a gene from the same family as the induced *ERF1* gene has been shown to be involved in the enhanced accumulation of epidermal wax (Aharoni et al., 2004; Broun et al., 2004; Zhang et al., 2005). The production of epicuticular wax in *Brassica* spp. and *Pisum sativum* L. has been shown to reduce herbivory and prevent attachment by leaf-feeding beetles while *cer* mutants with reduced waxiness were more susceptible to damage (Stoner, 1990; Bodnaryk, 1992). The ability of *D. rapae* to forage, locate and attack its host is also decreased where heavy wax blooms are present on the leaf surface (Gentry and Barbosa, 2006). They groomed more often and for longer periods of time, fell from the leaves more often, took longer to find colonies of aphids, and attacked them at a lower rate than wasps foraging on the variety with a lighter wax bloom. When epicuticular wax was removed from the leaves, the wasp's foraging efficiency and efficacy improved significantly on the cauliflower variety with a heavy wax bloom. It is unclear whether the induction of these genes would cause a change in the amount of epicuticular wax present on the leaf although it is clear from previous studies that this may have a bearing on the performance of herbivores and their natural enemies. Rashotte *et al.* (1999) demonstrated that *B. brassicae* shows evidence of an antixenotic reaction to waxes from the *cer3 Arabidopsis* mutant that is characterised by reduced stem waxiness. The aphids probe less and walk more on the stems of *cer3* plants and this response is associated with reduced fecundity of the aphids on *cer3*. It was found that a chemical component of the wax (a C_{30} primary alcohol - triacontanol) specific to the *cer3* mutants was the probable reason for the reduced host acceptance and it is suggested that this compound is a chemical repellent. Additional evidence indicating the involvement of long-chain hydrocarbons as a response to aphid feeding comes from Chapter 3 where tetradecane was found to be released as a volatile compound. This could be a by-product of increased or modified wax production that could be a direct

defence response to deter feeding by the aphid. During discriminant analysis, tetradecane was found to be the compound that could be used to discriminate most clearly between aphid infested and uninfested plants. This could indicate that tetradecane may be a compound to which parasitoids would be able to respond to both reliably and detectably as an indicator of host presence.

The use of *Arabidopsis* knockouts has been an important step in the functional annotation of genes involved in epicuticular wax formation. However, as demonstrated with the *cer3* gene knockout mutant, wax precursors produced due to gene disruption may have unforeseen ecological effects that may be different to the natural waxes produced as a result of biotic or abiotic stresses (Eigenbrode, 1996; Jenks et al., 2001; Jenks et al., 2002). Using variants of species that differ in their natural production of waxes may be a more effective way to investigate the ecological effects of waxiness on herbivore feeding. Additionally, experiments could be undertaken to examine whether wax production is induced in herbivore damaged plants.

The need for an interdisciplinary approach to studying plant-aphid interactions

It has been demonstrated in this and many previous studies that the fundamental mechanisms of plant defence can be uncovered by answering questions in a reductionist fashion. By formulating and testing hypotheses that address the mechanistic aspects of defence from induction to final response is a tried and tested method of building knowledge and filling in gaps in our understanding. This study has used three major avenues of research to explore the interaction that has been observed between the aphid, plant and parasitoid.

Firstly, experiments were conducted to investigate the behavioural response of the parasitoid to aphid-induced plant volatiles. These behavioural bioassays revealed that female parasitoids ready to oviposit are preferentially attracted to aphid-infested plants if given a choice of plant odours. It could be argued that this proves that odour cues are an

important resource utilised by the parasitoid during foraging for hosts. However, these experiments are conducted under controlled conditions that immediately render the situation different to the natural context and may affect our interpretation of results. For example, the concentration of the plant odour delivered to the parasitoid is much higher than that would be encountered in a natural situation. One of the fundamental problems encountered when using controlled environment y-tube bioassays for studying biological responses in insects was the variability of the parasitoid's response levels. From the subjective (but experienced) point of view of the experimenter, at some times active foraging behaviour could be observed while at others the parasitoid would appear to be unresponsive. This unresponsiveness could be attributed to environmental factors and the results of the experiment ignored although this attribution diminishes the power of the analysis. There is a strong tendency in science to only report positive/significant results and other experiments are discounted or considered 'failed' due to 'extraneous' or 'complicating' factors. If this method of discovery is employed then the robustness of the statistical tests is severely compromised since there is a much higher probability that the result was obtained by chance. If the 'positive' result is to be accepted, it should be wholly and repeatably confirmed under identical conditions. Equally, if the 'positive' result cannot be refuted by a 'negative' result under seemingly identical conditions then the experiment violates the fundamental tenet of falsification as set out by Popper (1959). In the wider scientific arena, the trend has been to publish only studies with 'significant' results and to ignore studies that seem uneventful. This may lead to a biased, perhaps untrue, representation of what exists in nature.

Secondly, the compounds released by the plants were analysed to identify differences between emission from control plants, aphid-infested plants and *Plutella*-infested plants. By using statistical methods, a number of important compounds were identified and it was shown that by using only a few variable compounds, the source of the damage could be detected. This implies that foraging insects would be able to detect and identify similar species specific profiles. To test the efficacy of these methods, electrophysiological studies on the parasitoid would identify which compounds are

detected. Additional behavioural studies would be required to test the parasitoid response to any compounds identified.

Finally, *Arabidopsis* microarrays were used to identify genes and pathways that were significantly induced following aphid and *Plutella* infestation. These experiments showed that this method is a useful technique for the analysis of plants with unsequenced genomes and minimal functional gene annotation. By using this method on a wild perennial, a clearer picture of the similarities and differences to model organisms can be established. Also, this method is useful for suggesting the involvement of other plant responses (such as wax production) that may be induced to defend or resist the herbivore attack.

It is clear that the multitrophic interaction being studied is acting at many different levels and these reductionist techniques each consider only one aspect. The task of integrating these strands to construct an ultimate understanding of the relationships occurring is an immense one and one that is ongoing. It can only be achieved through collaboration in research, an amalgamation of ideas and sharing of knowledge. It is also generally agreed that there is a necessity for more studies that attempt to connect the ecological aspects of the interaction with the finely tuned and controlled mechanistic aspects. The experiments now possible with a large number of transformed plants and knockout mutants will go a long way towards addressing these ecological aspects. However, the utmost care must be taken when using these plants in a natural context to ensure that current species are not affected by the release of transformed species. Although advances have been rapid over the last decade, there are still areas of study about which we have little information. The herbivore-specific plant response detected during gene expression analyses and volatile analysis is an area of research that is yet to be investigated fully. The response may be to the aphid salivary components or the mechanism of feeding. Additionally, the plant perception of the aphid is an area about which very little is known. Maffei *et al* (2004; 2006) present research on the Egyptian cotton leaf worm (Spodoptera littoralis) showing that a wave of cell depolarisation spreads theough the leaf following wounding. It is proposed that this may be a signalling mechanism that causes the systemic release of

transcription factors leading to a change in gene expression. It is unclear whether a similar mechanism occurs following aphid infestation. Further to this scenario, there has been minimal research conducted on the transcription factors involved in the activation of even commonly characterised plant defence pathways. Finally, the relationship between gene expression and volatile release is a growing area of research with the recent characterisation of genes encoding terpene synthases (Dudareva et al., 2003; Faldt et al., 2003; Chen et al., 2004; Huber et al., 2005; Hyatt and Croteau, 2005), myrosinase-associated proteins (Pontoppidan et al., 2003, 2005) and epithiospecifier proteins (Lambrix et al., 2001; Zhang et al., 2006). Manipulating the volatile blend emitted from a plant by genetic means will afford a wealth of exciting experimental possibilities and a corresponding increase in understanding of the behavioural responses of higher trophic levels.

The wealth of research on plant mechanisms and defence pathways is often justified by the proposed benefits to crop protection and crop yield. Despite this, there appears to be a dearth of applied products being developed. This is probably due in part to the perceived lack of economic viability for novel products that employ methods other than pesticides and traditional plant breeding to control pests and diseases or increase yields. It is possible that future studies on the effects of these pesticide chemicals or large-scale studies on the safety of GM crops on humans and wildlife may indicate that alternatives are viable when taking into account the hidden costs of intensive farming practices. In many areas of cutting edge technology such as engineering and materials science, ideas are generated by taking cues from nature. By understanding the evolution of natural systems that have been trialled over millennia, our hand is guided to create sustainable, efficient and effective designs. During the coming biotech generation, it will become even more important to manage our natural resources in a sustainable manner so that we can continue to seek guidance in this way.

Appendix A

Primer sequences used to amplify gene products to be used for real-time RT-PCR. See section 4.2.10

Gene name	Left primer	Right primer	Product size	Total Gene length
HPL1	CAGTGTGCAGCCAAAGACAT	ATGATACGAACCCGTGGAAG	159	856
BoGSL-ELONG	ATGGTTGTCCGGTCATTCTTAC	GTTTCCCACGGTTTTGGCGAT	375	6120
MyAP	CGAAGCTGTGCGAGTATCAA	TTCACTGGCACTGACGACAT	236	1152
MBP	ATGGTCATGGCAAACAATCA	ACCCAATCGCTAGCTTTTCC	253	2967
LOX2	ATGCTTCTGGCTCTTCTCCA	TACTTTCCCAACCGACCAAC	211	857
UBQ10	ACCATCACACTTGAGGTGGAG	ACCATCCTCGAGCTGCTTT	126	855
EPS	GGAGCTGGATTCATTGTGGT	CCACTTCGGTCCCACTTTT	143	710
XTH-6	CGCAGCGTTGTGTTTATTGT	GTACACTCCCATCGCTTGGT	102	1018
CER-1	TGCCTCCAGTATCGCTTTCT	TTCTACGCCAGCCTCTTTGT	252	605

Appendix B

This appendix details the genes involved in defence pathways, identified during microarray analysis, that exhibit a significant difference following aphid infestation of wild *B. oleracea*.

AGI Identifier	P-value	Gene description
AT1G05460	0.0037087	RNA helicase SDE3 (SDE3), identical to RNA helicase SDE3 (Arabidopsis thaliana) GI:13811296
AT1G80840	0.0041283	WRKY family transcription factor, similar to WRKY transcription factor GB:BAA87058 GI:6472585 from (Nicotiana tabacum)
AT1G19230	0.0066822	respiratory burst oxidase protein E (RbohE) / NADPH oxidase, nearly identical to respiratory burst oxidase protein E GI:3242787 (gi:3242787) from (Arabidopsis thaliana)
AT1G28370	0.0068559	encodes a member of the ERF (ethylene response factor) subfamily B-1 of ERF/AP2 transcription factor family. The protein contains one AP2 domain. There are 15 members in this subfamily including ATERF-3, ATERF-4, ATERF-7, and leafy petiole.
AT2G24850	0.0124293	aminotransferase, putative, similar to nicotianamine aminotransferase from Hordeum vulgare (GI:6498122, GI:6469087); contains Pfam profile PF00155 aminotransferase, classes I and II
AT3G23010	0.0130792	disease resistance family protein / LRR family protein, contains leucine rich-repeat domains Pfam:PF00560, INTERPRO:IPR001611; contains similarity to Cf-2.2 (Lycopersicon pimpinellifolium) gi:1184077:gb:AAC15780
AT5G51700	0.0140324	RAR1 disease resistance protein (RAR1), Contains a 3 nt micro-exon at exon 2.
AT1G08810	0.0149415	myb family transcription factor (MYB60)
AT5G41750; AT5G41740	0.0235948	[AT5G41750, disease resistance protein (TIR-NBS-LRR class), putative, domain signature TIR-NBS-LRR exists, suggestive of a disease resistance protein.];[AT5G41740, disease resistance protein (TIR-NBS-LRR class), putative, domain signature TIR-NBS-LRR exists, suggestive of a disease resistance protein.]
AT1G25470	0.0264347	encodes a member of the ERF (ethylene response factor) subfamily B-6 of ERF/AP2 transcription factor family. The protein contains one AP2 domain. There are 12 members in this subfamily including RAP2.11.

		transcription factor family. The protein contains one AP2 domain. There are 7 members in this subfamily.
AT1G63030	0.0296042	encodes a member of the DREB subfamily A-1 of ERF/AP2 transcription factor family (DDF2). The protein contains one AP2 domain. There are six members in this subfamily, including CBF1, CBF2, and CBF3. Overexpression of this gene results in the reduction of gibberellic acid biosynthesis. This gene is expressed in all tissues examined, but most abundantly expressed in rosette leaves and stems. Overexpression of DDF1, a putative paralog of this gene, also reduces gibberellic acid biosynthesis and makes the plants more tolerant to high-salinity levels.
AT4G10270; AT4G10280	0.0297144	[AT4G10270, wound-responsive family protein, similar to wound induced protein (GI:19320) (Lycopersicon esculentum)];[AT4G10280, expressed protein]
AT4G10920	0.029937	transcriptional coactivator p15 (PC4) family protein (KELP), similar to SP:P53999 Activated RNA polymerase II transcriptional coactivator p15 (PC4) (p14) {Homo sapiens}; contains Pfam profile PF02229: Transcriptional Coactivator p15 (PC4); supporting cDNA gi:2997685:gb:AF053303.1:AF053303
AT1G52660	0.0312679	disease resistance protein, putative, similar to NBS/LRR disease resistance protein GI:9758302 from (Arabidopsis thaliana)
AT2G25000	0.0316964	WRKY family transcription factor, contains Pfam profile: PF03106 WRKY DNA -binding domain
AT5G51060	0.0336442	respiratory burst oxidase protein C (RbohC) / NADPH oxidase, nearly identical to respiratory burst oxidase protein C from Arabidopsis thaliana (gi:3242785)
AT1G49010	0.0364556	myb family transcription factor, contains Pfam profile: PF00249 myb-like DNA-binding domain
AT5G46470	0.0394755	disease resistance protein (TIR-NBS-LRR class), putative, domain signature TIR-NBS-LRR exists, suggestive of a disease resistance protein.
AT1G71400	0.0409966	disease resistance family protein / LRR family protein, contains leucine rich-repeat domains Pfam:PF00560, INTERPRO:IPR001611; similar to Hcr2-5D (Lycopersicon esculentum) gi:3894393:gb:AAC78596
AT1G47370	0.0414241	Toll-Interleukin-Resistance (TIR) domain-containing protein, domain signature TIR exists, suggestive of a disease resistance protein.
AT2G20100	0.0416963	ethylene-responsive family protein, similar to Ethylene-regulated ER33 protein (GI:5669656) (Lycopersicon esculentum); PMID: 12679534; putative bHLH133 transcription factor
AT1G76700; AT1G76690; AT1G76680	0.0440475	[AT1G76700, DNAJ heat shock N-terminal domain-containing protein, similar to SP:P39101 CAJ1 protein, Saccharomyces cerevisiae; contains Pfam profile PF00226 DnaJ domain];[AT1G76690, 12-oxophytodienoate reductase (OPR2), identical to 12-oxophytodienoate reductase OPR2 GB:AAC78441 (Arabidopsis thaliana)];[AT1G76680, 12-oxophytodienoate reductase (OPR1), identical to 12-oxophytodienoate reductase OPR1 GB:AAC78440 (Arabidopsis thaliana)]
AT1G28360	0.044693	encodes a member of the ERF (ethylene response factor) subfamily B-1 of ERF/AP2 transcription factor family (ERF12). The protein contains one AP2 domain. There are 15 members in this subfamily including ATERF-3, ATERF-4, ATERF-7, and leafy petiole.
AT1G51680	0.0461647	4-coumarate--CoA ligase 1 / 4-coumaroyl-CoA synthase 1 (4CL1), identical to SP:Q42524 4-coumarate--CoA ligase 1 (EC 6.2.1.12) (4CL 1) (4-coumaroyl-CoA synthase 1) {Arabidopsis thaliana}
AT5G40020	0.0467754	pathogenesis-related thaumatin family protein, similar to SP:P50699 Thaumatin-like protein precursor {Arabidopsis thaliana}, pathogenesis-related group 5 protein (Brassica rapa) GI:2749943; contains Pfam profile PF00314: Thaumatin family
AT3G14230	0.0500208	encodes a member of the ERF (ethylene response factor) subfamily B-2 of ERF/AP2 transcription factor family (RAP2.2). The protein contains one AP2 domain. There are 5 members in this subfamily including RAP2.2 AND RAP2.12.

Appendix C

This appendix details the genes involved in defence pathways, identified during microarray analysis, that exhibit a significant difference following *Plutella* infestation of wild *B. oleracea*.

AGI Identifier	P-value	Gene description
AT1G09090	0.0016486	respiratory burst oxidase protein B (RbohB) / NADPH oxidase, identical to respiratory burst oxidase protein B from Arabidopsis thaliana (gi:3242783)
AT1G05460	0.002752	RNA helicase SDE3 (SDE3), identical to RNA helicase SDE3 (Arabidopsis thaliana) GI:13811296
AT1G17615	0.0037766	disease resistance protein (TIR-NBS class), putative, domain signature TIR exists, suggestive of a disease resistance protein.
AT1G11310	0.00454	seven transmembrane MLO family protein / MLO-like protein 2 (MLO2), idenctical to membrane protein Mlo2 (Arabidopsis thaliana) gi:14091574:gb:AAK53795; similar to Mlo (Hordeum vulgare subsp. vulgare) gi:1877221:emb:CAB06083 SWISS-PROT:P93766
AT5G42500; AT5G42510	0.0053017	[AT5G42500, disease resistance-responsive family protein, similar to disease resistance response protein 206-d (Pisum sativum) gi:508844:gb:AAB18669];[AT5G42510, disease resistance-responsive family protein, similar to disease resistance response protein 206-d (Pisum sativum) gi:508844:gb:AAB18669]
AT2G43510	0.0056325	Encodes a defensin-like (DEFL) family protein.
AT2G23570	0.00739	hydrolase, alpha/beta fold family protein, similar to ethylene-induced esterase (Citrus sinensis) GI:14279437, polyneuridine aldehyde esterase (Rauvolfia serpentina) GI:6651393
AT3G23240	0.0082666	encodes a member of the ERF (ethylene response factor) subfamily B-3 of ERF/AP2 transcription factor family (ERF1). The protein contains one AP2 domain. There are 18 members in this subfamily including ATERF-1, ATERF-2, AND ATERF-5. EREBP like protein that binds GCC box of ethylene regulated promoters such as basic chitinases. Constitutive expression of ERF1 phenocopies ethylene over production. Involved in ethylene signaling cascade,downstream of EIN2 and EIN3.
AT2G38870	0.0099798	protease inhibitor, putative, similar to SP:P24076 Glu S.griseus protease inhibitor (BGIA) {Momordica charantia}; contains Pfam profile PF00280: Potato inhibitor I family
AT5G46000	0.0106237	jacalin lectin family protein, similar to myrosinase-binding protein homolog (Arabidopsis thaliana) GI:2997767; contains Pfam profile PF01419 jacalin-like lectin domain
AT1G31580	0.011076	expressed protein, identical to ORF1 (Arabidopsis thaliana) gi:457716:emb:CAA50905
AT3G44300; AT3G44310	0.0120358	[AT3G44300, nitrilase 2 (NIT2), identical to SP:P32962 Nitrilase 2 (EC 3.5.5.1) {Arabidopsis thaliana}];[AT3G44310, nitrilase 1 (NIT1), identical to SP:P32961 Nitrilase 1 (EC 3.5.5.1) {Arabidopsis thaliana}]
AT5G41750; AT5G41740	0.012997	[AT5G41750, disease resistance protein (TIR-NBS-LRR class), putative, domain signature TIR-NBS-LRR exists, suggestive of a disease resistance protein.];[AT5G41740, disease resistance protein (TIR-NBS-LRR class), putative, domain signature TIR-NBS-LRR exists, suggestive of a disease resistance protein.]
AT2G39940	0.0165326	coronatine-insensitive 1 / COI1 (FBL2), E3 ubiquitin ligase SCF complex F-box subunit; identical to LRR-containing F-box protein GI:3158394 from (Arabidopsis thaliana)
AT1G59870	0.0179715	ABC transporter family protein, similar to PDR5-like ABC transporter GI:1514643 from (Spirodela polyrhiza)
AT5G46070	0.018367	guanylate-binding family protein, contains Pfam domains PF02263: Guanylate-binding protein, N-terminal domain and PF02841: Guanylate-binding protein, C-terminal domain
AT5G51060	0.0210708	respiratory burst oxidase protein C (RbohC) / NADPH oxidase, nearly identical to respiratory burst oxidase protein C from Arabidopsis thaliana (gi:3242785)
AT5G05170	0.0221849	cellulose synthase, catalytic subunit (Ath-B), nearly identical to gi:2827143, cellulose synthase, catalytic subunit (Ath-B)
AT2G39770	0.02438	GDP-mannose pyrophosphorylase (GMP1), identical to GDP-mannose pyrophosphorylase from Arabidopsis thaliana (GI:3598958); updated per Conklin PL et al, PNAS 1999, 96(7):4198-203
AT5G25810	0.0245328	encodes a member of the DREB subfamily A-4 of ERF/AP2 transcription factor family (TINY). The protein contains one AP2 domain. There are 17 members in this subfamily

		including TINY. Ectopic or overexpression of this gene in a Ds tagged line has reduced cell expansion. The expression of this gene is induced by ethylene and light and appears to stimulate cytokinin biosynthesis.
AT5G55250	0.0253389	S-adenosyl-L-methionine:carboxyl methyltransferase family protein, similar to SAM:jasmonic acid carboxyl methyltransferase (JMT)(GI:13676829) and to SAM:benzoic acid carboxyl methyltransferase (BAMT)(GI:9789277)(Antirrhinum majus)
AT5G09810	0.0279167	actin 7 (ACT7) / actin 2, identical to SP:P53492 Actin 7 (Actin-2) {Arabidopsis thaliana}
AT3G61250	0.0343408	myb family transcription factor (MYB17), contains PFAM profile: Myb-like DNA-binding domain PF00249
AT5G45200	0.0343948	disease resistance protein (TIR-NBS-LRR class), putative, domain signature TIR-NBS-LRR exists, suggestive of a disease resistance protein.
AT5G67000	0.034743	encodes a member of the ERF (ethylene response factor) subfamily B-6 of ERF/AP2 transcription factor family. The protein contains one AP2 domain. There are 12 members in this subfamily including RAP2.11.
AT5G13370; AT5G13360	0.0370456	[AT5G13370, auxin-responsive GH3 family protein, similar to auxin-responsive GH3 product (Glycine max) GI:18591; contains Pfam profile PF03321: GH3 auxin-responsive promoter];[AT5G13360, auxin-responsive GH3 family protein, similar to auxin-responsive GH3 product (Glycine max) GI:18591; contains Pfam profile PF03321: GH3 auxin-responsive promoter]
AT3G24220	0.0394914	9-cis-epoxycarotenoid dioxygenase, putative / neoxanthin cleavage enzyme, putative / carotenoid cleavage dioxygenase, putative, similar to GB:CAB10168 from (Lycopersicon esculentum) (J. Exp. Bot. 47, 2111-2112 (1997)); similar to 9-cis-epoxycarotenoid dioxygenase (Phaseolus vulgaris)(GI:6715257)
AT2G02100	0.0411841	plant defensin-fusion protein, putative (PDF2.2), plant defensin protein family member, personal communication, Bart Thomma (Bart.Thomma@agr.kuleuven.ac.be); similar to SWISS-PROT:O65740
AT3G48090	0.0413668	disease resistance protein (EDS1), identical to disease resistance protein/lipase homolog EDS1 GI:4454567; contains Pfam profile PF01764: Lipase
AT3G23250	0.0420521	myb family transcription factor (MYB15), similar to myb-related transcription factor GB:CAA66952 from (Lycopersicon esculentum)
AT1G49010	0.0433632	myb family transcription factor, contains Pfam profile: PF00249 myb-like DNA-binding domain
AT5G63660	0.0491291	plant defensin-fusion protein, putative (PDF2.5), plant defensin protein family member, personal communication, Bart Thomma (Bart.Thomma@agr.kuleuven.ac.be)
AT5G40020	0.0497685	pathogenesis-related thaumatin family protein, similar to SP:P50699 Thaumatin-like protein precursor {Arabidopsis thaliana}, pathogenesis-related group 5 protein (Brassica rapa) GI:2749943; contains Pfam profile PF00314: Thaumatin family
AT3G04580	0.0501884	ethylene receptor, putative (EIN4), similar to ethylene receptor GB:AAC31123 (Malus domestica), identical to putative ethylene receptor GB:AAD02485 (Arabidopsis thaliana); Pfam HMM hit: response regulator receiver domain, signal C terminal domain

Bibliography

Aarts, M.G.M., Keijzer, C.J., Stiekema, W.J., and Pereira, A. (1995). Molecular Characterization of the CER1 Gene of Arabidopsis Involved in Epicuticular Wax Biosynthesis and Pollen Fertility. Plant Cell **7,** 2115-2127.

Affymetrix. (2005). Affymetrix Expression Analysis Technical Manual http://www.affymetrix.com/support/technical/manual/expression_manual.affx accessed 25/7/05.

AGI. (2000). Analysis of the genome sequence of the flowering plant Arabidopsis thaliana. Nature, 796-815.

Agrawal, A.A., Conner, J.K., Johnson, M.T.J., and Wallsgrove, R. (2002a). Ecological genetics of an induced plant defence against herbivores: Additive genetic variance and costs of phenotypic plasticity. Evolution **56,** 2206-2213.

Agrawal, A.A., Conner, J.K., Johnson, M.T.J., and Wallsgrove, R. (2002b). Ecological genetics of an induced plant defense against herbivores: Additive genetic variance and costs of phenotypic plasticity. Evolution **56,** 2206-2213.

Agrobiologicals. (2004). (www.agrobiologicals.com) accessed 22/3/04.

Aharoni, A., Dixit, S., Jetter, R., Thoenes, E., van Arkel, G., and Pereira, A. (2004). The SHINE Clade of AP2 Domain Transcription Factors Activates Wax Biosynthesis, Alters Cuticle Properties, and Confers Drought Tolerance when Overexpressed in Arabidopsis. Plant Cell **16,** 2463-2480.

Alborn, H.T., Jones, T.H., Stenhagen, G.S., and Tumlinson, J.H. (2000). Identification and synthesis of volicitin and related components from beet armyworm oral secretions. J. Chem. Ecol. **26,** 203-220.

Alborn, H.T., Turlings, T.C.J., Jones, T.H., Stenhagen, G., Loughrin, J.H., and Tumlinson, J.H. (1997). An Elicitor of Plant Volatiles from Beet Armyworm Oral Secretion. Science **276,** 945-949.

Aliabadi, A., Renwick, J.A.A., and Whitman, D.W. (2002). Sequestration of Glucosinolates by Harlequin Bug Murgantia histrionica. J. Chem. Ecol. **28,** 1749-1762.

Alshehbaz, I.A. (1984). The Tribes of Cruciferae (Brassicaceae) in the Southeastern United-States. Journal of the Arnold Arboretum **65,** 343-373.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. J. Mol. Biol **215,** 403–410.

Anders, F. (1958). Aminosa uren als gallenerregende Stoffe der
Reblaus (Viteus [Phylloxera] vitifolii Shimer). Experientia **14,** 62-63.

Arimura, G., Ozawa, R., Kugimiya, S., Takabayashi, J., and Bohlmann, J. (2004). Herbivore-Induced Defense Response in a Model Legume. Two-Spotted Spider Mites Induce Emission of (E)-ß-Ocimene and Transcript Accumulation of (E)-ß-Ocimene Synthase in Lotus japonicus1. Plant Physiol. **135,** 1976-1983.

Arimura, G., Ozawa, R., Shimoda, T., Nishioka, T., Boland, W., and Takabyashi, J. (2000). Herbivory-induced volatiles elicit defence genes in lima bean leaves. Nature **406,** 512-515.

Australian Institute For Horticultural Development. (1995). http://www.rirdc.gov.au/reports/AFO/chinesecabbage.doc accessed 7/4/04.

Bach, T.J. (1995). Some new aspects of isoprenoid biosynthesis in plant: a review. In Lipids, pp. 191-202.

Baldwin, I.T. (1998). Jasmonate-induced responses are costly but benefit plants under attack in native populations (National Acad Sciences), pp. 8113-8118.

Benjamini, Y., and Hochberg, Y. (1995). Controlling the False Discovery Rate - a Practical and Powerful Approach to Multiple Testing. Journal of the Royal Statistical Society Series B-Methodological **57,** 289-300.

Bergström, G., Rothschild, M., Groth, I., and Crighton, C. (1994). Oviposition by butterflies on young leaves: Investigation of leaf volatiles. Chemoecology **5,** 147-158.

Bernardi, R., Negri, A., Ronchi, S., and Palmieri, S. (2000). Isolation of the epithiospecifier protein from oil-rape (Brassica napus ssp. oleifera) seed and its characterization, pp. 296-298.

Berrocal-Lobo, M., Molina, A., and Solano, R. (2002). Constitutive expression of ETHYLENE-RESPONSE-FACTOR1 in Arabidopsis confers resistance to several necrotrophic fungi. Plant J. **29,** 23-32.

Bertram, B.C.R. (1978). Living in groups: predators and prey (Blackwell Scientific Publications, Oxford), pp. 64–96.

Bevan, M., Bancroft, I., Bent, E., Love, K., Goodman, H., Dean, C., Bergkamp, R., Dirkse, W., Van Staveren, M., Stiekema, W., Drost, L., Ridley, P., Hudson, S.A., Patel, K., Murphy, G., Piffanelli, P., Wedler, H., Wedler, E., Wambutt, R., Weitzenegger, T., Pohl, T.M., Terryn, N., Gielen, J., Villarroel, R., De Clerck, R., Van Montagu, M., Lecharny, A., Auborg, S., Gy, I., Kreis, M., Lao, N., Kavanagh, T., Hempel, S., Kotter, P., Entian, K.D., Rieger, M., Schaeffer, M., Funk, B., Mueller-Auer, S., Silvey, M., James, R., Montfort, A., Pons, A., Puigdomenech, P., Douka, A., Voukelatou, E., Milioni, D., Hatzopoulos, P., Piravandi, E., Obermaier, B., Hilbert, H., Dusterhoft, A., Moores, T., Jones, J.D.G., Eneva, T., Palme, K., Benes, V., Rechman, S., Ansorge, W., Cooke, R., Berger, C., Delseny, M., Voet, M., Volckaert, G., Mewes, H.W., Klosterman, S., Schueller, C., and Chalwatzis, N. (1998). Analysis of 1.9 Mb of contiguous sequence from chromosome 4 of Arabidopsis thaliana. Nature **391,** 485-488.

Bi, J.L., Murphy, J.B., and Felton, G.W. (1997). Does salicylic acid act as a signal in cotton for induced resistance to Helicoverpa zea? J. Chem. Ecol. **23,** 1805-1818.

Birkett, M.A., Chamberlain, K., Guerrieri, E., Pickett, J.A., Wadhams, L.J., and Yasuda, T. (2003). Volatiles from whitefly-infested plants elicit a host-locating response in the parasitoid, Encarsia formosa. J. Chem. Ecol. **29,** 1589-1600.

Birkett, M.A., Campbell, C.A.M., Chamberlain, K., Guerrieri, E., Hick, A.J., Martin, J.L., Matthes, M., Napier, J.A., Pettersson, J., Pickett, J.A., Poppy, G.M., Pow, E.M., Pye, B.J., Smart, L.E., Wadhams, G.H., Wadhams, L.J., and Woodcock, C.M. (2000). New roles for cis-jasmone as an insect semiochemical and in plant defense. Proc. Natl. Acad. Sci. U. S. A. **97,** 9329-9334.

Blaakmeer, A., Geervliet, J.B.F., Vanloon, J.J.A., Posthumus, M.A., Vanbeek, T.A., and Degroot, A. (1994). Comparative Headspace Analysis of Cabbage Plants

Damaged by 2 Species of Pieris Caterpillars - Consequences for in-Flight Host Location by Cotesia Parasitoids. Entomol. Exp. Appl. **73,** 175-182.

Blande, J.D. (2004). Differential signalling from specialist and generalist *Brassica* feeding aphids to differentially adapted aphid parasitoids. (University of Southampton).

Blee, E. (1998). Phytooxylipins and plant defense reactions. Prog. Lipid. Res. **37,** 33-72.

Bodnaryk, R.P. (1992). Distinctive Leaf Feeding Patterns on Oilseed Rapes and Related Brassicaceae by Flea Beetles, Phyllotreta-Cruciferae (Goeze) (Coleoptera, Chrysomelidae). Canadian Journal of Plant Science **72,** 575-581.

Bohlmann, J., Martin, D., Oldham, N.J., and Gershenzon, J. (2000). Terpenoid secondary metabolism in Arabidopsis thaliana: cDNA cloning, characterization, and functional expression of a myrcene/(E)-beta-ocimene synthase. Arch. Biochem. Biophys. **375,** 261-269.

Bones, A.M., and Rossiter, J.T. (1995). Glucosinolates in cruciferous crops, pp. 46–67.

Bones, A.M., and Rossiter, J.T. (1996). The myrosinase-glucosinolate system, its organisation and biochemistry. Physiol. Plant. **97,** 194-208.

Bones, A.M., Thangstad, O.P., Haugen, O.A., and Espevik, T. (1991). Fate of Myrosin Cells - Characterization of Monoclonal-Antibodies against Myrosinase. J. Exp. Bot. **42,** 1541-1549.

Bradburne, R.P., and Mithen, R. (2000). Glucosinolate genetics and the attraction of the aphid parasitoid Diaeretiella rapae to Brassica. Proc. R. Soc. Lond. Ser. B-Biol. Sci. **267,** 89-95.

Breyne, P., Rombaut, D., van Gysel, A., van Montagu, M., and Gerats, T. (1999). AFLP analysis of genetic diversity within and between Arabidopsis thaliana ecotypes. Molecular and General Genetics **261,** 627-634.

Bridges, M., Jones, A.M.E., Bones, A.M., Hodgson, C., Cole, R., Bartlet, E., Wallsgrove, R., Karapapa, V.K., Watts, N., and Rossiter, J.T. (2002). Spatial organization of the glucosinolate-myrosinase system in brassica specialist aphids is similar to that of the host plant. Proc. R. Soc. Lond. Ser. B-Biol. Sci. **269,** 187-191.

Brockhouse, S. (2005). The role of genetic variation on a tritrophic interaction. In PhD Thesis (University of Southampton).

Broun, P., Poindexter, P., Osborne, E., Jiang, C.-Z., and Riechmann, J.L. (2004). WIN1, a transcriptional activator of epidermal wax accumulation in Arabidopsis. PNAS **101,** 4706-4711.

Bruce, T., Martin, J., Pickett, J., Pye, B., Smart, L., and Wadhams, L. (2003). cis-Jasmone treatment induces resistance in wheat plants against the grain aphid, Sitobion avenae(Fabricius)(Homoptera: Aphididae). Pest Management Science **59,** 1031-1036.

Bruce, T.J.A., Wadhams, L.J., and Woodcock, C.M. (2005). Insect host location: a volatile situation. Trends Plant Sci. **10,** 269-274.

Bryant, E., and Cowles, J. (2000). Differential Responses of Wild and Laboratory Strains of the Housefly to PCBs. J. Chem. Ecol. **26,** 1001-1011.

Burton, W.A., Ripley, V.L., Potts, D.A., and Salisbury, P.A. (2004). Assessment of genetic diversity in selected breeding lines and cultivars of canola quality Brassica juncea and their implications for canola breeding. Euphytica **136,** 181-192.

Bystrom, B., Glater, R., Scott, F., and Bowler, E. (1968). Leaf Surface of Beta vulgaris-Electron Microscope Study. Botanical Gazette **129,** 133-138.

Capella, A., Menossi, M., Arruda, P., and Benedetti, C. (2001). COI1 affects myrosinase activity and controls the expression of two flower-specific myrosinase-binding protein homologues in Arabidopsis. Planta **213,** 691-699.

Castillo-Davis, C.I., and Hartl, D.L. (2003). GeneMerge - post-genomic analysis, data mining, and hypothesis testing. Bioinformatics **19,** 891-892.

Chandra, S., and Smith, B. (1998). An analysis of synthetic processing of odor mixtures in the honeybee (Apis mellifera). J. Exp. Biol **201,** 3113-3121.

Chappell, J. (1995). The Biochemistry and Molecular-Biology of Isoprenoid Metabolism. Plant Physiol. **107,** 1-6.

Chen, F., Ro, D.K., Petri, J., Gershenzon, J., Bohlmann, J., Pichersky, E., and Tholl, D. (2004). Characterization of a root-specific Arabidopsis terpene synthase responsible for the formation of the volatile monoterpene 1,8-cineole. Plant Physiol. **135,** 1956-1966.

Chen, S., Petersen, B., Olsen, C., Schulz, A., and Halkier, B. (2001). Long-Distance Phloem Transport of Glucosinolates in Arabidopsis. Plant Physiol. **127,** 194-201.

Chew, F.S. (1988). Biological Effects of Glucosinolates. Acs Symposium Series **380,** 155-181.

Cole, R.A. (1980). Volatile components produced during ontogeny of some cultivated crucifers. J. Sci. Food Agric. **31,** 549-557.

Conner, J.A., Conner, P., Nasrallah, M.E., and Nasrallah, J.B. (1998). Comparative mapping of the Brassica S locus region and its homeolog in Arabidopsis: Implications for the evolution of mating systems in the Brassicaceae. Plant Cell **10,** 801-812.

Cooper, W., and Goggin, F. (2005). Effects of jasmonate-induced defenses in tomato on the potato aphid, Macrosiphum euphorbiae. Entomol. Exp. Appl. **115,** 107-115.

Cooper, W.C., Jia, L., and Goggin, F.L. (2004). Acquired and R-gene-mediated resistance against the potato aphid in tomato. J. Chem. Ecol. **30,** 2527-2542.

Craigon, D.J., James, N., Okyere, J., Higgins, J., Jotham, J., and May, S. (2004). NASCArrays: a repository for microarray data generated by NASC's transcriptomics service. Nucleic Acids Res **32,** 575-577.

Cunillera, N., Arró, M., Delourme, D., Karst, F., Boronat, A., and Ferrer, A. (1996). Arabidopsis thaliana Contains Two Differentially Expressed Farnesyl-Diphosphate Synthase Genes. J. Biol. Chem. **271,** 7774-7780.

Czechowski, T., Stitt, M., Altmann, T., Udvardi, M.K., and Scheible, W.-R. (2005). Genome-Wide Identification and Testing of Superior Reference Genes for Transcript Normalization in Arabidopsis. Plant Physiol. **139,** 5-17.

da Silva, A.E.A., Villanueva, W.J.P., Knidel, H., Bonato, V., dos Reis, S.F., and Von Zuben, F.J. (2005). A multi-neighbor-joining approach for phylogenetic tree reconstruction and visualization. Genet. Mol. Res **4,** 525-534.

De Boer, J.G., and Dicke, M. (2004). The role of methyl salicylate in prey searching behavior of the predatory mite Phytoseiulus persimilis. J. Chem. Ecol. **30,** 255-271.

de Ilarduya, O., Xie, Q., and Kaloshian, I. (2003). Aphid-induced defense responses in Mi-1-mediated compatible and incompatible tomato interactions. Molecular Plant–Microbe Interactions **16,** 699–708.

De Moraes, C.M., Lewis, W.J., Pare, P.W., Alborn, H.T., and Tumlinson, J.H. (1998). Herbivore-infested plants selectively attract parasitoids, pp. 570-573.

De Vos, M., Van Oosten, V.R., Van Poecke, R.M.P., Van Pelt, J.A., Pozo, M.J., Mueller, M.J., Buchala, A.J., Metraux, J.P., Van Loon, L.C., and Dicke, M. (2005). Signal signature and transcriptome changes of Arabidopsis during pathogen and insect attack, pp. 923-937.

Degen, T., Dillmann, C., Marion-Poll, F., and Turlings, T.C.J. (2004). High Genetic Variability of Herbivore-Induced Volatile Emission within a Broad Range of Maize Inbred Lines1 (Am Soc Plant Biol), pp. 1928-1938.

Dempsey, D.A., Shah, J., and Klessig, D.F. (1999). Salicylic acid and disease resistance in plants. Crit. Rev. Plant Sci. **18,** 547-575.

Devoto, A., and Turner, J. (2005). Jasmonate-regulated Arabidopsis stress signalling network. Physiol. Plant. **123,** 161-172.

Devoto, A., Ellis, C., Magusin, A., Chang, H., Chilcott, C., Zhu, T., and Turner, J. (2005). Expression profiling reveals COI1 to be a key regulator of genes involved in wound-and methyl jasmonate-induced secondary metabolism, defence, and hormone interactions. Plant Mol.Biol. **58,** 497-513.

Dicke, M. (1999). Are herbivore-induced plant volatiles reliable indicators of herbivore identity to foraging carnivorous arthropods? Entomol. Exp. Appl. **91,** 131-142.

Dicke, M., and Vet, L.E.M. (1999). Plant-carnivore interactions: evolutionary and ecological consequences for plant, herbivore and carnivore (Blackwell Science), pp. 483-520.

Dicke, M., and van Loon, J.J.A. (2000). Multitrophic effects of herbivore-induced plant volatiles in an evolutionary context. Entomol. Exp. Appl. **97,** 237-249.

Dicke, M., and van Poecke, R.M. (2002). Signalling in plant-insect interactions: signal transduction in direct and indirect plant defence. In Plant signal transduction - Frontiers in molecular biology ; 38 (Oxford: Oxford University Press), pp. 289-316.

Dicke, M., van Poecke, R.M.P., and de Boer, J.G. (2003a). Inducible indirect defence of plants: from mechanisms to ecological functions. Basic Appl. Ecol. **4,** 27-42.

Dicke, M., Agrawal, A.A., and Bruin, J. (2003b). Plants talk, but are they deaf? Trends Plant Sci. **8,** 403-405.

Dicke, M., Sabelis, M.W., Takabayashi, J., Bruin, J., and Posthumus, M.A. (1990). Plant Strategies of Manipulating Predator-Prey Interactions through Allelochemicals - Prospects for Application in Pest- Control. J. Chem. Ecol. **16,** 3091-3118.

Divol, F., Vilaine, F., Thibivilliers, S., Amselem, J., Palauqui, J., Kusiak, C., and Dinant, S. (2005). Systemic response to aphid infestation by Myzus persicae in the phloem of Apium graveolens. Plant Mol.Biol. **57,** 517-540.

Dixon, R.A., Achnine L, Kota P, Liu CJ, Reddy MSS, and LJ, W. (2002). The phenylpropanoid pathway and plant defence - a genomics perspective. Molecular Plant Pathology **3,** 371-390.

Donath, J., and Boland, W. (1994). Biosynthesis of Acyclic Homoterpenes in Higher-Plants Parallels Steroid-Hormone Metabolism. J. Plant Physiol. **143,** 473-478.

Du, Y.J., Poppy, G.M., and Powell, W. (1996). Relative importance of semiochemicals from first and second trophic levels in host foraging behavior of Aphidius ervi. J. Chem. Ecol. **22,** 1591-1605.

Du, Y.J., Poppy, G.M., Powell, W., and Wadhams, L.J. (1997). Chemically mediated associative learning in the host foraging behavior of the aphid parasitoid Aphidius ervi (Hymenoptera: Braconidae). J. Insect Behav. **10,** 509-522.

Du, Y.J., Poppy, G.M., Powell, W., Pickett, J.A., Wadhams, L.J., and Woodcock, C.M. (1998). Identification of semiochemicals released during aphid feeding that attract parasitoid Aphidius ervi. J. Chem. Ecol. **24,** 1355-1368.

Dudareva, N., Negre, F., Nagegowda, D., and Orlova, I. (2006). Plant Volatiles: Recent Advances and Future Perspectives. Crit. Rev. Plant Sci. **25,** 417-440.

Dudareva, N., Martin, D., Kish, C.M., Kolosova, N., Gorenstein, N., Faldt, J., Miller, B., and Bohlmann, J. (2003). (E)-beta-ocimene and myrcene synthase genes of floral scent biosynthesis in snapdragon: Function and expression of three terpene synthase genes of a new terpene synthase subfamily. Plant Cell **15,** 1227-1241.

Efron, B., and Gong, G. (1983). A Leisurely Look at the Bootstrap, the Jackknife, and Cross-Validation. American Statistician **37,** 36-48.

Eigenbrode, S.D. (1996). Plant surface waxes and insect behaviour (Bios), pp. 201-222.

El-Sayed, A.M. (2006). The Pherobase: Database of Insect Pheromones and Semiochemicals <http://www.pherobase.com>.

Ellis, C., Karafyllidis, I., and Turner, J. (2002). Constitutive activation of jasmonate signaling in an Arabidopsis mutant correlates with enhanced resistance to Erysiphe cichoracearum, Pseudomonas syringae, and Myzus persicae. Mol Plant Microbe Interact **15,** 1025-1030.

Emons, A.M.C., and Mulder, B.M. (2000). How the deposition of cellulose microfibrils builds cell wall architecture. Trends Plant Sci. **5,** 35-40.

Fahey, J.W., Zalcmann, A.T., and Talalay, P. (2001). The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. Phytochemistry **56,** 5-51.

Faldt, J., Arimura, G., Gershenzon, J., Takabayashi, J., and Bohlmann, J. (2003). Functional identification of AtTPS03 as (E)-beta-ocimene synthase: a monoterpene synthase catalyzing jasmonate- and wound-induced volatile formation in Arabidopsis thaliana. Planta **216,** 745-751.

Farmer, E.E., and Ryan, C.A. (1992). Octadecanoid Precursors of Jasmonic Acid Activate the Synthesis of Wound-Inducible Proteinase-Inhibitors. Plant Cell **4,** 129-134.

Fellowes, M.D.E. (1998). Do non-social insects get the(kin) recognition they deserve? , pp. 223-227.

Fournier, F., and Boivin, G. (2000). Comparative Dispersal of Trichogramma evanescens and Trichogramma pretiosum (Hymenoptera: Trichogrammatidae) in Relation to Environmental Conditions (Entomological Society of America), pp. 55-63.

Fournier, F., Pelletier, D., Vigneault, C., Goyette, B., and Boivin, G. (2005). Effect of barometric pressure on flight initiation by Trichogramma pretiosum and

Trichogramma evanescens (Hymenoptera : Trichogrammatidae). Environ. Entomol. **34,** 1534-1540.

Frey, M., Stettner, C., Pare, P.W., Schmelz, E.A., Tumlinson, J.H., and Gierl, A. (2000). An herbivore elicitor activates the gene for indole emission in maize. Proc. Natl. Acad. Sci. U. S. A. **97,** 14801-14806.

Gao, M.Q., Li, G.Y., Yang, B., McCombie, W.R., and Quiros, C.F. (2004). Comparative analysis of a Brassica BAC clone containing several major aliphatic glucosinolate genes with its corresponding Arabidopsis sequence. Genome **47,** 666-679.

Gentry, G.L., and Barbosa, P. (2006). Effects of leaf epicuticular wax on the movement, foraging behavior, and attack efficacy of Diaeretiella rapae (Blackwell Synergy), pp. 115-122.

Giamoustaris, A., and Mithen, R. (1995). The Effect of Modifying the Glucosinolate Content of Leaves of Oilseed Rape (Brassica-Napus Ssp Oleifera) on Its Interaction with Specialist and Generalist Pests. Ann. Appl. Biol. **126,** 347-363.

Girling, R., Hassall, M., Turner, J., and Poppy, G. (2006). Behavioural responses of the aphid parasitoid Diaeretiella rapae to volatiles from Arabidopsis thaliana induced by Myzus persicae. Entomol. Exp. Appl. **120,** 1.

Godfray, H.C.J. (1994). Parasitoids: Behavioral and Evolutionary Ecology. (Princeton University Press).

Grant, M.R., McDowell, J.M., Sharpe, A.G., de Torres Zabala, M., Lydiate, D.J., and Dangl, J.L. (1998). Independent deletions of a pathogen-resistance gene in Brassica and Arabidopsis (The National Academy of Sciences).

Gu, Y.-Q., Yang, C., Thara, V.K., Zhou, J., and Martin, G.B. (2000). Pti4 Is Induced by Ethylene and Salicylic Acid, and Its Product Is Phosphorylated by the Pto Kinase, pp. 771-786.

Gu, Y.-Q., Wildermuth, M.C., Chakravarthy, S., Loh, Y.-T., Yang, C., He, X., Han, Y., and Martin, G.B. (2002). Tomato Transcription Factors Pti4, Pti5, and Pti6 Activate Defense Responses When Expressed in Arabidopsis. Plant Cell **14,** 817-831.

Guerrieri, E., Pennacchio, F., and Tremblay, E. (1993). Flight Behavior of the Aphid Parasitoid *Aphidius ervi* (Hymenoptera, Braconidae) in Response to Plant and Host Volatiles. European Journal of Entomology **90,** 415-421.

Guerrieri, E., Pennacchio, F., and Tremblay, E. (1997). Effect of adult experience on in-flight orientation to plant and plant-host complex volatiles in Aphidius ervi Haliday (Hymenoptera, Braconidae). Biol. Control **10,** 159-165.

Guerrieri, E., Poppy, G.M., Powell, W., Tremblay, E., and Pennacchio, F. (1999). Induction and Systemic Release of Herbivore-Induced Plant Volatiles Mediating In-Flight Orientation of Aphidius ervi (Springer), pp. 1247-1261.

Guerrieri, E., Poppy, G.M., Powell, W., Rao, R., and Pennacchio, F. (2002). Plant-to-plant communication mediating in-flight orientation of Aphidius ervi. J. Chem. Ecol. **28,** 1703-1715.

Gundlach, H., Muller, M., Kutchan, T., and Zenk, M. (1992). Jasmonic Acid is a Signal Transducer in Elicitor-Induced Plant Cell Cultures. Proceedings of the National Academy of Sciences **89,** 2389-2393.

Halitschke, R., Schittko, U., Pohnert, G., Boland, W., and Baldwin, I.T. (2001). Molecular interactions between the specialist herbivore Manduca sexta (Lepidoptera, Sphingidae) and its natural host Nicotiana attenuata. III. Fatty acid-amino acid conjugates in herbivore oral secretions are necessary and sufficient for herbivore- specific plant responses. Plant Physiol. **125,** 711-717.

Halkier, B.A., and Gershenzon, J. (2006). Biology and biochemistry of glucosinolates. Annu. Rev. Plant Biol. **57,** 303-333.

Halliburton, R. (2004). Introduction to Population Genetics. (Western Connecticut State University: Prentice Hall).

Hammond, J., Broadley, M., Craigon, D., Higgins, J., Emmerson, Z., Townsend, H., White, P., and May, S. (2005). Using genomic DNA-based probe-selection to improve the sensitivity of high-density oligonucleotide arrays when applied to heterologous species. Plant Methods **1,** 10.

Harbourne, J.B. (1993). Introduction to Ecological Biochemistry. (London: Academic Press).

Heidel, A.J., and Baldwin, I.T. (2004). Microarray analysis of salicylic acid- and jasmonic acid-signalling in responses of Nicotiana attenuata to attack by insects from multiple feeding guilds. Plant Cell Environ. **27,** 1362-1373.

Hely, P.C., Pasfield, G., and Gellatley, J.G. (1982). Insect pests of fruit and vegetables in N.S.W. (Inkata Press), pp. 312.

Hoballah, M.E., Koellner, T.G., Degenhardt, J., and Turlings, T.C.J. (2004). Costs of induced volatile production in maize (Blackwell Synergy), pp. 168-180.

Hoballah, M.E.F., and Turlings, T.C.J. (2001). Experimental evidence that plants under caterpillar attack may benefit from attracting parasitoids. Evol. Ecol. Res. **3,** 553-565.

Hoffmann, M.P., and Frodsham, A.C. (1993). Natural Enemies of Vegetable Insect Pests. (Ithaca, NY.: Cooperative Extension, Cornell University,), pp. 63.

Honda, K., Omura, H., and Hayashi, N. (1998). Identification of floral volatiles from Ligustrum japonicum that stimulate flower-visiting by CABBAGE butterfly, Pieris rapae. J. Chem. Ecol. **24,** 2167-2180.

Huber, D.P.W., Philippe, R.N., Godard, K.A., Sturrock, R.N., and Bohlmann, J. (2005). Characterization of four terpene synthase cDNAs from methyl jasmonate-induced Douglas-fir, Pseudotsuga menziesii. Phytochemistry **66,** 1427-1439.

Huh, M.K., and Huh, H.W. (2001). AFLP fingerprinting of Brassica campestris L. ssp. napus var. nippo-oleifera Makino from Korea. Korean J. Biol. Sci **5,** 101–106.

Hui, D., Iqbal, J., Lehmann, K., Gase, K., Saluz, H.P., and Baldwin, I.T. (2003). Molecular Interactions between the Specialist Herbivore Manduca sexta (Lepidoptera, Sphingidae) and Its Natural Host Nicotiana attenuata: V. Microarray Analysis and Further Characterization of Large-Scale Changes in Herbivore-Induced mRNAs. Plant Physiol. **131,** 1877-1893.

Husebye, H., Arzt, S., Burmeister, W.P., Hartel, F.V., Brandt, A., Rossiter, J.T., and Bones, A.M. (2005). Crystal structure at 1.1 angstrom resolution of an insect myrosinase from Brevicoryne brassicae shows its close relationship to beta-glucosidases. Insect Biochemistry and Molecular Biology **35,** 1311-1320.

Hyatt, D.C., and Croteau, R. (2005). Mutational analysis of a monoterpene synthase reaction: Altered catalysis through directed mutagenesis of (-)-pinene synthase from Abies grandis. Arch. Biochem. Biophys. **439,** 222-233.

Ibrahim, M.A., Nissinen, A., and Holopainen, J.K. (2005). Response of *Plutella xylostella* and its Parasitoid *Cotesia plutellae* to Volatile Compounds. J. Chem. Ecol. **31,** 1969-1984.

Iori, R., Jean-Paul Wathelet, J.-P., Leoni, O., Nicolas Mabon, N., Marlier, M., and Palmieri, S. (1999). Enzymatic transformation of desulf-glucosinolates in pure nitriles using a recombinant beta-O-glucosidase. In Proceedings of the 10th International Rapeseed Conference - 'New Horizons for an Old Crop' (Canberra, Australia).

IPM Images. (2004). Parasitised host mummy http://www.ipmimages.org/browse/subimages.cfm?sub=11333 accessed 16/4/04.

Jenks, M.A., Eigenbrode, S.D., and Lemieux, B. (2002). Cuticular Waxes of Arabidopsis. The Arabidopsis Book.

Jenks, M.A., Tuttle, H.A., Eigenbrode, S.D., and Feldmann, K.A. (1995). Leaf Epicuticular Waxes of the Eceriferum Mutants in Arabidopsis. Plant Physiol. **108,** 369-377.

Jenks, M.A., Andersen, L., Teusink, R.S., and Williams, M.H. (2001). Leaf cuticular waxes of potted rose cultivars as affected by plant development, drought and paclobutrazol treatments, pp. 62-70.

Johnston, J.S., Pepper, A.E., Hall, A.E., Chen, Z.J., Hodnett, G., Drabek, J., Lopez, R., and Price, H.J. (2005). Evolution of Genome Size in Brassicaceae. Ann Bot **95,** 229-235.

Jones, A.M.E., Bridges, M., Bones, A.M., Cole, R., and Rossiter, J.T. (2001). Purification and characterisation of a non-plant myrosinase from the cabbage aphid Brevicoryne brassicae (L.). Insect Biochemistry and Molecular Biology **31,** 1-5.

Kalloo, G., and Bergh, B.O. (1993). Genetic improvement of vegetable crops. (Pergamon Press Oxford, UK).

Kammenga, J., Herman, M., Ouborg, J., Johnson, L., and Breitling, R. (2007). Microarray challenges in ecology. Trends Ecol. Evol. **22,** 273-279.

Kaplan, N.L., Darden, T., and Hudson, R.R. (1988). The Coalescent Process in Models With Selection. Genetics **120,** 819-829.

Karban, R., and Myers, J.H. (1989). Induced Plant Responses to Herbivory. Annu. Rev. Ecol. Syst. **20,** 331-348.

Karban, R., and Baldwin, I.T. (1997). Induced responses to herbivory. (The Univ. of Chicago Press Ltd).

Kato, M., Kamo, T., Wang, R., Nishikawa, F., Hyodo, H., Ikoma, Y., Sugiura, M., and Yano, M. (2002). Wound-induced ethylene synthesis in stem tissue of harvested broccoli and its effect on senescence and ethylene synthesis in broccoli florets. Postharvest Biol. Technol. **24,** 69-78.

Keller, M.A., Lewis, W.J., and Stinner, R.E. (1985). Biological and practical significance of movement by Trichogramma species: A review, pp. 138-155.

Kessler, A., and Baldwin, I.T. (2001). Defensive Function of Herbivore-Induced Plant Volatile Emissions in Nature.

Kessler, A., and Baldwin, I.T. (2002). Plant responses to insect herbivory: The emerging molecular analysis. Annu. Rev. Plant Biol. **53,** 299-328.

Kimura, M. (1968). Evolutionary rate at the molecular level. Nature **217,** 624-626.

Kimura, M. (1983). The neutral theory of molecular evolution Cambridge University Press. Cambridge, UK.

Kliebenstein, D.J., Gershenzon, J., and Mitchell-Olds, T. (2001a). Comparative quantitative trait loci mapping of aliphatic, indolic and benzylic glucosinolate production in Arabidopsis thaliana leaves and seeds. Genetics **159,** 359-370.

Kliebenstein, D.J., Figuth, A., and Mitchell-Olds, T. (2002). Genetic architecture of plastic methyl jasmonate responses in Arabidopsis thaliana. Genetics **161,** 1685-1696.

Kliebenstein, D.J., Lambrix, V.M., Reichelt, M., Gershenzon, J., and Mitchell-Olds, T. (2001b). Gene duplication in the diversification of secondary metabolism: Tandem 2-oxoglutarate-dependent dioxygenases control glucosinolate biosynthesis in arabidopsis. Plant Cell **13,** 681-693.

Kroymann, J., Textor, S., Tokuhisa, J.G., Falk, K.L., Bartram, S., Gershenzon, J., and Mitchell-Olds, T. (2001). A gene controlling variation in arabidopsis glucosinolate composition is part of the methionine chain elongation pathway. Plant Physiol. **127,** 1077-1088.

Lagercrantz, U. (1998). Comparative mapping between Arabidopsis thaliana and Brassica nigra indicates that Brassica genomes have evolved through extensive genome replication accompanied by chromosome fusions and frequent rearrangements. Genetics **150,** 1217-1228.

Lambdon, P.W., Hassall, M., Boar, R.R., and Mithen, R. (2003). Asynchrony in the nitrogen and glucosinolate leaf-age profiles of Brassica: is this a defensive strategy against generalist herbivores? Agric. Ecosyst. Environ. **97,** 205-214.

Lambrix, V., Reichelt, M., Mitchell-Olds, T., Kliebenstein, D.J., and Gershenzon, J. (2001). The Arabidopsis Epithiospecifier Protein Promotes the Hydrolysis of Glucosinolates to Nitriles and Influences Trichoplusia ni Herbivory. Plant Cell **13,** 2793-2807.

Lazzeri, L., Tacconi, R., and Palmieri, S. (1993). Invitro Activity of Some Glucosinolates and Their Reaction- Products toward a Population of the Nematode Heterodera- Schachtii. J. Agric. Food Chem. **41,** 825-829.

Leoni, O., Iori, R., Palmieri, S., Esposito, E., Menegatti, E., Cortesi, R., and Nastruzzi, C. (1997). Myrosinase-generated isothiocyanate from glucosinolates: Isolation, characterization and in vitro antiproliferative studies. Bioorg. Med. Chem. **5,** 1799-1806.

Li, G., Gao, M., Yang, B., and Quiros, C.F. (2003). Gene for gene alignment between the Brassica and Arabidopsis genomes by direct transcriptome mapping. Theor. Appl. Genet. **107,** 168-180.

Li, G., Riaz, A., Goyal, S., Abel, S., and Quiros, C.F. (2001). Inheritance of three major genes involved in the synthesis of aliphatic glucosinolates in Brassica oleracea. J. Am. Soc. Hortic. Sci. **126,** 427-431.

Li, G.Y., and Quiros, C.F. (2002). Genetic analysis, expression and molecular characterization of BoGSL-ELONG, a major gene involved in the aliphatic glucosinolate pathway of Brassica species. Genetics **162,** 1937-1943.

Li, Q., Eigenbrode, S.D., Stringam, G.R., and Thiagarajah, M.R. (2000). Feeding and Growth of Plutella xylostella and Spodoptera eridania on Brassica juncea with Varying Glucosinolate Concentrations and Myrosinase Activities. J. Chem. Ecol. **V26,** 2401-2419.

Liechti, R., and Farmer, E.E. (2002). The jasmonate pathway. Science **296,** 1649-1650.

Liu, A., and Burke, J.M. (2006). Patterns of Nucleotide Diversity in Wild and Cultivated Sunflower. Genetics **173,** 321-330.

Liu, C., Heinstein, P., and Chen, X. (1999). Expression Pattern of Genes Encoding Farnesyl Diphosphate Synthase and Sesquiterpene Cyclase in Cotton Suspension-Cultured Cells Treated with Fungal Elicitors. Molecular Plant-Microbe Interactions **12,** 1095-1104.

Liu, Y., Wang, H., Ye, H.-C., and Li, G.-F. (2005). Advances in the Plant Isoprenoid Biosynthesis Pathway and Its Metabolic Engineering. Journal of Integrative Plant Biology **47,** 769-782.

Loivamaki, M., Holopainen, J.K., and Nerg, A.M. (2004). Chemical changes induced by methyl jasmonate in oilseed rape grown in the laboratory and in the field. J. Agric. Food Chem. **52,** 7607-7613.

Lombard, V., Baril, C.P., Dubreuil, P., Blouet, F., and Zhang, D. (2000). Genetic Relationships and Fingerprinting of Rapeseed Cultivars by AFLP - Consequences for Varietal Registration (Published in Crop Sci. 40: 1417-1425.).

Loughrin, J., Manukian, A., Heath, R., Turlings, T., and Tumlinson, J. (1994). Diurnal Cycle of Emission of Induced Volatile Terpenoids by Herbivore-Injured Cotton Plant. PNAS **91,** 11836-11840.

MacGibbon, D.B., and Beuzenberg, E.J. (1978). Location of glucosinolase in Brevicoryne brassicae and Lipaphis erysimi (Aphididae). New Zealand Journal of Science **21,** 389–392.

Mackauer, M. (1986). Growth and Developmental Interactions in Some Aphids and Their Hymenopterous Parasites. Journal of Insect Physiology **32,** 275-280.

Mackauer, M., Michaud, J.P., and Volkl, W. (1996). Host choice by aphidiid parasitoids (Hymenoptera: Aphidiidae): Host recognition, host quality, and host value. Can. Entomol. **128,** 959-980.

Madhusudhan, V., and Miles, P. (1998). Mobility of salivary components as a possible reason for differences in the responses of alfalfa to the spotted alfalfa aphid and pea aphid. Entomol. Exp. Appl. **86,** 25-39.

Madhusudhan, V.V., Taylor, G.S., and Miles, P.W. (1994). The Detection of Salivary Enzymes of Phytophagous Hemiptera - a Compilation of Methods. Ann. Appl. Biol. **124,** 405-412.

Maffei, M., Bossi, S., Spiteller, D., Mithofer, A., and Boland, W. (2004). Effects of Feeding Spodoptera littoralis on Lima Bean Leaves. I. Membrane Potentials, Intracellular Calcium Variations, Oral Secretions, and Regurgitate Components, pp. 1752-1762.

Maffei, M.E., Mithofer, A., Arimura, G., Uchtenhagen, H., Bossi, S., Bertea, C.M., Cucuzza, L.S., Novero, M., Volpe, V., Quadro, S., and Boland, W. (2006). Effects of Feeding Spodoptera littoralis on Lima Bean Leaves. III. Membrane Depolarization and Involvement of Hydrogen Peroxide, pp. 1022-1035.

Marchand, D., and McNeil, J.N. (2000). Effects of Wind Speed and Atmospheric Pressure on Mate Searching Behavior in the Aphid Parasitoid *Aphidius nigripes* (Hymenoptera: Aphidiidae) (Springer), pp. 187-199.

Mattiacci, L., Dicke, M., and Posthumus, M.A. (1994). Induction of Parasitoid Attracting Synomone in Brussels-Sprouts Plants by Feeding of *Pieris brassicae* Larvae - Role of Mechanical Damage and Herbivore Elicitor. J. Chem. Ecol. **20,** 2229-2247.

Mattiacci, L., Dicke, M., and Posthumus, M. (1995). {beta}-Glucosidase: An Elicitor of Herbivore-Induced Plant Odor that Attracts Host-Searching Parasitic Wasps 10.1073/pnas.92.6.2036. PNAS **92,** 2036-2040.

McCauley, D.E. (1994). Intrademic Group Selection Imposed by a Parasitoid-Host Interaction. The American Naturalist **144,** 1-13.

McEwan, M., and Smith, W.H.M. (1998). Identification of volatile organic compounds emitted in the field by oilseed rape (Brassica napus ssp. oleifera) over the growing season. Clinical and Experimental Allergy **28,** 332-338.

McGarigal, K., Cushman, S., and Stafford, S. (2000). Multivariate Statistics for Wildlife and Ecology Research. (Berlin: Springer).

McGarvey, D.J., and Croteau, R. (1995). Terpenoid Metabolism. Plant Cell **7,** 1015-1026.

McKinlay, R.G. (1992). Vegetable crop pests (CRC Press, Inc.), pp. 406pp.

Metcalf, L., and Metcalf, R.A. (1993). Destructive and Useful Insects: Their habits and control (McGraw, New York.

Mewis, I., Appel, H.M., Hom, A., Raina, R., and Schultz, J.C. (2005). Major signaling pathways modulate Arabidopsis glucosinolate accumulation and response to both phloem-feeding and chewing insects. Plant Physiol. **138,** 1149-1162.

Mikkelsen, M.D., Petersen, B.L., Glawischnig, E., Jensen, A.B., Andreasson, E., and Halkier, B.A. (2003). Modulation of CYP79 genes and glucosinolate profiles in Arabidopsis by defense signaling pathways. Plant Physiol. **131,** 298-308.

Miles, P.W. (1964). Studies on the salivary physiology of plant-bugs: oxidase activity in the salivary apparatus and saliva. Journal of Insect Physiology **10,** 121-129.

Miles, P.W. (1999). Aphid saliva. Biol. Rev. Cambridge Philosophic. Soc. **74,** 41-85.

Miles, P.W., and Harrewijn, P. (1991). Discharge by Aphids of Soluble Secretions into Dietary Sources. Entomol. Exp. Appl. **59,** 123-134.

Millenaar, F.F., Okyere, J., May, S.T., van Zanten, M., Voesenek, L.A.C.J., and Peeters, A.J.M. (2006). How to decide? Different methods of calculating gene expression from short oligonucleotide array data will give different results. BMC Bioinformatics **7.**

Mitchell-Olds, T. (1999). Genetics and Evolution of Insect Resistance in Arabidopsis. In Insect- Plant Interactions and Induced Plant Defence (Novartis Foundation Symposium 223) (Wiley, Chichester), pp. 239- 252.

Mitchell-Olds, T. (2001). *Arabidopsis thaliana* and its wild relatives: a model system for ecology and evolution. Trends Ecol. Evol. **16,** 693-700.

Mitchell, N.D. (1976). The status of *Brassica oleracea* L. subsp. oleracea (wild cabbage) in the British Isles. Watsonia . **11,** 97-103.

Mitchell, N.D., and Richards, A.J. (1979). Biological flora of the British Isles. *Brassica oleracea* subsp oleracea (B. sylvestris (L.) Miller). Journal of Ecology, 1087-1096.

Mithen, R. (1992a). Leaf Glucosinolate Profiles and Their Relationship to Pest and Disease Resistance in Oilseed Rape. Euphytica **63,** 71-83.

Mithen, R. (1992b). Leaf glucosinolate profiles and their relationship to pest and disease resistance in oilseed rape. Euphytica **63,** 71-83.

Mithen, R., Raybould, A.F., and Giamoustaris, A. (1995a). Divergent Selection for Secondary Metabolites between Wild Populations of Brassica-Oleracea and Its Implications for Plant-Herbivore Interactions. Heredity **75,** 472-484.

Mithen, R., Clarke, J., Lister, C., and Dean, C. (1995b). Genetics of Aliphatic Glucosinolates .3. Side-Chain Structure of Aliphatic Glucosinolates in Arabidopsis-Thaliana. Heredity **74,** 210-215.

Mithen, R.F., Lewis, B.G., Heaney, R.K., and Fenwick, G.R. (1987). Glucosinolates of Wild and Cultivated Brassica Species. Phytochemistry **26,** 1969-1973.

Mohan, M., and Gujar, G. (2003). Local variation in susceptibility of the diamondback moth, Plutella xylostella (Linnaeus) to insecticides and role of detoxification enzymes. Crop Protection **22,** 495-504.

Moore, J.P., Taylor, J.E., Paul, N.D., and Whittaker, J.B. (2003). The use of clip cages to restrain insects reduces leaf expansion systemically in Rumex obtusifolius. Ecol. Entomol. **28,** 239-242.

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

Moran, P.J., Cheng, Y.F., Cassell, J.L., Thompson, G.A. (2002). Gene Expression Profiling of *Arabidopsis thaliana* in Compatible Plant-Aphid Interactions. Archives of Insect Biochemstry and Physiology **51,** 182-203.

Morgan, D.J.W., and Hare, J.D. (1998). Volatile cues used by the parasitoid, Aphytis melinus, for host location: California red scale revisited. Entomol. Exp. Appl. **88,** 235-245.

Moyes, C.L. (1997). The role of glucosinolate production in populations of wild cabbage (PhD Thesis, University of Southampton).

Moyes, C.L., Collin, H.A., Britton, G., and Raybould, A.E. (2000). Glucosinolates and differential herbivory in wild populations of Brassica oleracea. J. Chem. Ecol. **26,** 2625-2641.

Muller, C., and Riederer, M. (2005). Plant surface properties in chemical ecology. J. Chem. Ecol. **31,** 2621-2651.

Müller, C., Agerbirk, N., Olsen, C.E., Boevé, J.L., Schaffner, U., and Brakefield, P.M. (2001). Sequestration of Host Plant Glucosinolates in the Defensive Hemolymph of the Sawfly Athalia rosae. J. Chem. Ecol. **27,** 2505-2516.

Nakano, T., Suzuki, K., Fujimura, T., and Shinshi, H. (2006). Genome-Wide Analysis of the ERF Gene Family in Arabidopsis and Rice. Plant Physiol. **140,** 411-432.

Niki, T., Mitsuhara, I., Seo, S., Ohtsubo, N., and Ohashi, Y. (1998). Antagonistic effect of salicylic acid and jasmonic acid on the expression of pathogenesis-related (PR) protein genes in wounded mature tobacco leaves. Plant Cell Physiol. **39,** 500-507.

Nilsson, N.O., Hansen, M., Panagopoulos, A.H., Tuvesson, S., Ehlde, M., Christiansson, M., Rading, I.M., Rissler, M., and Kraft, T. (1999). QTL analysis of Cercospora leaf spot resistance in sugar beet. Plant Breed. **118,** 327-334.

Nottingham, S.F., Hardie, J., Dawson, G.W., Hick, A.J., Pickett, J.A., Wadhams, L.J., and Woodcock, C.M. (1991). Behavioral and Electrophysiological Responses of Aphids to Host and Nonhost Plant Volatiles. J. Chem. Ecol. **17,** 1231-1242.

O'Kane Jr., S.L., and Al-Shehbaz, I.A. (1997). A synopsis of Arabidopsis (Brassicaceae). Novon, 323-332.

Okada, K., Saito, T., Nakagawa, T., Kawamukai, M., and Kamiya, Y. (2000). Five Geranylgeranyl Diphosphate Synthases Expressed in Different Organs Are Localized into Three Subcellular Compartments in Arabidopsis. Plant Physiol. **122,** 1045-1056.

Omer, A. (2001). Chemically-induced resistance against multiple pests in cotton. International Journal of Pest Management **47,** 49-54.

Onate-Sanchez, L., and Singh, K.B. (2002). Identification of Arabidopsis Ethylene-Responsive Element Binding Factors with Distinct Induction Kinetics after Pathogen Infection, pp. 1313-1322.

Ozawa, R., Arimura, G., Takabayashi, J., Shimoda, T., and Nishioka, T. (2000). Involvement of jasmonate- and salicylate-related signaling pathways for the production of specific herbivore-induced volatiles in plants. Plant Cell Physiol. **41,** 391-398.

Pare, P.W., and Tumlinson, J.H. (1997). De Novo Biosynthesis of Volatiles Induced by Insect Herbivory in Cotton Plants. Plant Physiol. **114,** 1161-1167.

Pare, P.W., and Tumlinson, J.H. (1999). Plant volatiles as a defense against insect herbivores. Plant Physiol. **121,** 325-331.

Park, S.-J., Huang, Y., and Ayoubi, P. (2006). Identification of expression profiles of sorghum genes in response to greenbug phloem-feeding using cDNA subtraction and microarray analysis. Planta **223,** 932-947.

Paterson, A.H., Lan, T.H., Amasino, R., Osborn, T.C., and Quiros, C. (2001). Brassica genomics: a complement to, and early beneficiary of, the Arabidopsis sequence. Genome Biol **2,** 1011-1014.

Pena, M.J., Ryden, P., Madson, M., Smith, A.C., and Carpita, N.C. (2004). The Galactose Residues of Xyloglucan Are Essential to Maintain Mechanical Strength of the Primary Cell Walls in Arabidopsis during Growth1. Plant Physiol. **134,** 443-451.

Penacortes, H., Albrecht, T., Prat, S., Weiler, E.W., and Willmitzer, L. (1993). Aspirin Prevents Wound-Induced Gene-Expression in Tomato Leaves by Blocking Jasmonic Acid Biosynthesis. Planta **191,** 123-128.

Penninckx, I.A., Thomma, B.P., Buchala, A., Metraux, J.P., and Broekaert, W.F. (1998). Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in Arabidopsis. Plant Cell **10,** 2103-2113.

Peterson, C.J., Cosse, A., and Coats, J.R. (2000). Insecticidal components in the meal of Crambe abyssinica. Journal of Agricultural and Urban Entomology **17,** 27-35.

Peterson, J.K., Horvat, R.J., and Elsey, K.D. (1994). Squash Leaf Glandular Trichome Volatiles - Identification and Influence on Behavior of Female Pickleworm Moth Diaphania-Nitidalis (Stoll) (Lepidoptera, Pyralidae). J. Chem. Ecol. **20,** 2099-2109.

Pfaffl, M.W. (2001). A new mathematical model for relative quantification in real- time RT-PCR. Nucleic Acids Res. **29,** art. no.-e45.

Pichersky, E., and Gershenzon, J. (2002). The formation and function of plant volatiles: perfumes for pollinator attraction and defense. Curr Opin Plant Biol **5,** 237-243.

Pieterse, C.M., and van Loon, L.C. (1999). Salicylic acid-independent plant defence pathways. Trends Plant Sci **4,** 52-58.

Pike, K.S., Stary, P., Miller, T., Allison, D., Graf, G., Boydston, L., Miller, R., and Gillespie, R. (1999). Host Range and Habitats of the Aphid Parasitoid Diaeretiella rapae (Hymenoptera: Aphidiidae) in Washington State. Environ. Entomol. **28,** 61-71.

Pontoppidan, B., Hopkins, R., Rask, L., and Meijer, J. (2003). Infestation by cabbage aphid (Brevicoryne brassicae) on oilseed rape (Brassica napus) causes a long lasting induction of the myrosinase system. Entomol. Exp. Appl. **109,** 55-62.

Pontoppidan, B., Hopkins, R., Rask, L., and Meijer, J. (2005). Differential wound induction of the myrosinase system in oilseed rape (Brassica napus): contrasting insect damage with mechanical damage. Plant Science **168,** 715-722.

Popper, K. (1959). The Logic of Scientific Discovery. (New York: Basic Books).

Powell, W., and Wright, A.F. (1991). The Influence of Host Food Plants on Host Recognition by 4 Aphidiine Parasitoids (Hymenoptera, Braconidae). Bull. Entomol. Res. **81,** 449-453.

Prado, E., and Tjallingii, W.F. (1997). Effects of previous plant infestation on sieve element acceptance by two aphids (Springer), pp. 189-200.

Price, R.A., and Palmer, J.D. (1994). Systemic Relationships of Arabidopsis: A Molecular and Morphological Perspective,. In Arabidopsis., E.M. Meyerowitz and C.R. Somerville, eds (New York: Cold Spring Harbor Laboatory Press), pp. 7-19.

Rashotte, A.M. (1999). Epicuticular wax in Arabidopsis thaliana: a study of the genetics, chemistry, structure, and interactions with insects. In Plant Science (Tucson: University of Arizona).

Ratzka, A., Vogel, H., Kliebenstein, D.J., Mitchell-Olds, T., and Kroymann, J. (2002). Disarming the mustard oil bomb. PNAS **99,** 11223-11228.

Read, D., Feeny, P., and Root, R. (1970). Habitat selection by the aphid parasite Diaeretiella rapae and its hyperparasitoid. Charips brassicae.. Canadian Entomologist **102,** 1567–1578.

Reinbothe, S., Mollenhauer, B., and Reinbothe, C. (1994). Jips and Rips - the Regulation of Plant Gene-Expression by Jasmonates in Response to Environmental Cues and Pathogens. Plant Cell **6,** 1197-1209.

Reinhard, J., Srinivasan, M.V., and ShaoWu, Z. (2004). Scent-triggered navigation in honey bees. Nature (London) **427,** 411.

Reymond, P., Weber, H., Damond, M., and Farmer, E.E. (2000). Differential gene expression in response to mechanical wounding and insect feeding in Arabidopsis. Plant Cell **12,** 707-719.

Rodman, J.E., Karol, K.G., Price, R.A., and Sytsma, K.J. (1996). Molecules, morphology, and Dahlgren's expanded order Capparales. Systematic Botany **21,** 289-307.

Rodriguez-Concepcion, M., and Boronat, A. (2002). Elucidation of the Methylerythritol Phosphate Pathway for Isoprenoid Biosynthesis in Bacteria and Plastids. A Metabolic Milestone Achieved through Genomics. Plant Physiol. **130,** 1079-1089.

Rosa, E.A., Heaney, R.K., Fenwich, G.R., and Portas, C.A.M. (1997). Glucosinolates in crop plants. Horticultural Review **19,** 99-215.

Rose, J.K.C., Braam, J., Fry, S.C., and Nishitani, K. (2002). The XTH family of enzymes involved in xyloglucan endotransglucosylation and endohydrolysis: Current perspectives and a new unifying nomenclature. Plant Cell Physiol. **43,** 1421-1435.

Rozen, S., and Skaletsky, H. (2000). Primer3 on the WWW for general users and for biologist programmers. Krawetz S, Misener S, eds. Bioinformatics Methods and Protocols: Methods in Molecular Biology, 365–386.

Ryan, C.A., Pearce, G., Scheer, J., and Moura, D.S. (2002). Polypeptide hormones. Plant Cell **14,** 251-S264.

Saitou, N., and Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol **4,** 406-425.

Sandstrom, J., and Moran, N. (1999). How nutritionally imbalanced is phloem sap for aphids? Entomol. Exp. Appl. **91,** 203-210.

Saskatchewan Agriculture Food and Rural Revitalisation. (2004). http://www.agr.gov.sk.ca/docs/crops/horticulture/cabbage01.asp?printerversion=1 accessed 1/4/04.

Schena, M., Shalon, D., Davis, R.W., and Brown, P.O. (1995). Quantitative Monitoring of Gene-Expression Patterns with a Complementary-DNA Microarray. Science **270,** 467-470.

Schenk, P.M., Kazan, K., Wilson, I., Anderson, J.P., Richmond, T., Somerville, S.C., and Manners, J.M. (2000). Coordinated plant defense responses in Arabidopsis revealed by microarray analysis. Proc. Natl. Acad. Sci. U. S. A. **97,** 11655-11660.

Schoonhoven, L.M., Jermy, T., Van Loon, J. A. A. (1998). Insect -Plant Biology, from Physiology to Evolution. (London: Chapman and Hall).

Sharbel, T.F., Haubold, B., and Mitchell-Olds, T. (2000). Genetic isolation by distance in Arabidopsis thaliana: biogeography and postglacial colonization of Europe. Mol. Ecol. **9,** 2109-2118.

Shen, B.Z., Zheng, Z.W., and Dooner, H.K. (2000). A maize sesquiterpene cyclase gene induced by insect herbivory and volicitin: Characterization of wild-type and mutant alleles. Proc. Natl. Acad. Sci. U. S. A. **97,** 14807-14812.

Shields, V.D.C., and Mitchell, B.K. (1995). Sinigrin as a Feeding Deterrent in 2 Crucifer-Feeding, Polyphagous Lepidopterous Species and the Effects of Feeding Stimulant Mixtures on Deterrency. Philos. Trans. R. Soc. Lond. Ser. B-Biol. Sci. **347,** 439-446.

Siemens, D.H., Garner, S.H., Mitchell-Olds, T., and Callaway, R.M. (2002). Cost of defense in the context of plant competition: Brassica rapa may grow and defend. Ecology **83,** 505-517.

Smid, H.M., van Loon, J.J.A., Posthumus, M.A., and Vet, L.E.M. (2002). GC-EAG-analysis of volatiles from Brussels sprouts plants damaged by two species of Pieris caterpillars: olfactory receptive range of a specialist and a generalist parasitoid wasp species. Chemoecology **12,** 169-176.

Smith, C.M., and Boyko, E.V. (2007). The molecular bases of plant resistance and defense responses to aphid feeding: current status. Entomol. Exp. Appl. **122,** 1-16.

Smith, J.M., and Haigh, J. (1974). Hitch-Hiking Effect of a Favorable Gene. Genetical Research **23,** 23-35.

Snogerup, S., Gustafsson, M., and von bothmer, R. (1990). *Brassica sect. Brassica* (Brassicaceae). Willdenowia **19,** 271-365.

Sokal, R., and Rohlf, R. (1995). Biometry: the principles and practice of statistics in biological research: WH Freeman & Sons. New York **887.**

Sorin, M. (1966). Physiological and morphological studies on the suction mechanism of plant juice by aphids. Bulletin of the University of Osaka (Ser. B.) **18,** 94-106.

Srivastava, A., Gupta, V., Pental, D., and Pradhan, A.K. (2001). AFLP-based genetic diversity assessment amongst agronomically important natural and some newly synthesized lines of Brassica juncea. TAG Theoretical and Applied Genetics **102,** 193-199.

Staswick, P.E., and Lehman, C.C. (1999). Induced Plant Defences against Pathogens and Herbivores (APS Press), pp. 117-137.

Steinberg, S., Dicke, M., Vet, L.E.M., and Wanningen, R. (1992). Response of the braconid parasitoid Cotesia (= Apanteles) glomerata to volatile infochemicals: effects of bioassay set-up, parasitoid age and experience and barometric flux. Entomol. Exp. Appl. **63,** 163-175.

Stewart-Jones, A., and Poppy, G.M. (2006). Comparison of Glass Vessels and Plastic Bags for Enclosing Living Plant Parts for Headspace Analysis (Springer), pp. 845-864.

Stirrup, T.J. (2003). Cover photo - *B. oleracea*, natural habitat, Dorset UK.

Stirrup, T.J. (2006). *Brevicoryne brassicae* (cabbage aphid).

Stoner, K.A. (1990). Glossy Leaf Wax and Plant-Resistance to Insects in Brassica-Oleracea under Natural Infestation. Environ. Entomol. **19,** 730-739.

Stotz, H.U., Pittendrigh, B.R., Kroymann, J., Weniger, K., Fritsche, J., Bauke, A., and Mitchell-Olds, T. (2000). Induced plant defense responses against chewing insects. Ethylene signaling reduces resistance of Arabidopsis against Egyptian cotton worm but not diamondback moth. Plant Physiol. **124,** 1007-1017.

Strong, D.R., Lawton, J.H., and Southwood, T.R. (1984). Insects on Plants: Community patterns and mechanisms. (Oxford: Blackwell Scientific Publications).

Suverkropp, B.P. (1997). Host-finding behaviour of Trichogramma brassicae in maize (Landbouwuniversiteit Wageningen).

Swofford, D.L. (1998). PAUP*. Phylogenetic Analysis Using Parsimony (* and Other Methods). Version 4. Sunderland, MA: Sinauer Associates.

Swofford, D.L., Olsen, G.J., Waddell, P.J., and Hillis, D.M. (1996). Phylogenetic Inference. In Molecular Systematics, D.M. Hillis, C. Moritz, and B.K. Mable, eds (Sunderland, Massachusetts: Sinauer Associates, Inc.), pp. 412-413.

Takabayashi, J., Dicke, M., and Posthumus, M.A. (1994). Volatile Herbivore-Induced Terpenoids in Plant Mite Interactions - Variation Caused by Biotic and Abiotic Factors. J. Chem. Ecol. **20,** 1329-1354.

Takabayashi, J., Takahashi, S., Dicke, M., and Posthumus, M.A. (1995). Developmental stage of herbivorePseudaletia separata affects production of herbivore-induced synomone by corn plants (Springer), pp. 273-287.

Talalay, P., and Fahey, J.W. (2001). Phytochemicals from cruciferous plants protect against cancer by modulating carcinogen metabolism. Journal of Nutrition **131,** 3027-3033.

Tanksley, S.D., and McCouch, S.R. (1997). Seed banks and molecular maps: Unlocking genetic potential from the wild. Science **277,** 1063-1066.

Thaler, J.S., Stout, M.J., Karban, R., and Duffey, S.S. (2001). Jasmonate-mediated induced plant resistance affects a community of herbivores. Ecol. Entomol. **26,** 312-324.

Thaler, J.S., Farag, M.A., Pare, P.W., and Dicke, M. (2002). Jasmonate-deficient plants have reduced direct and indirect defences against herbivores. Ecol. Lett. **5,** 764-774.

The Arabidopsis Information Resource (TAIR). (2004). http://www.arabidopsis.org:1555/ARA/NEW-IMAGE?type=PATHWAY&object=PWY-735 accessed 16/4/04.

Thomma, B., Eggermont, K., Penninckx, I., Mauch-Mani, B., Vogelsang, R., Cammue, B.P.A., and Broekaert, W.F. (1998). Separate jasmonate-dependent and salicylate-dependent defense- response pathways in Arabidopsis are essential for resistance to distinct microbial pathogens. Proc. Natl. Acad. Sci. U. S. A. **95,** 15107-15111.

Thompson, G.A., and Goggin, F.L. (2006). Transcriptomics and functional genomics of plant defence induction by phloem-feeding insects. J. Exp. Bot. **57,** 755-766.

Tjallingii, W.F. (1990). Continuous recording of stylet penetration activities by aphids. In Aphid-Plant Genotype Interactions, R.K. Campbell and R.D. Eikenbary, eds (New York: Elsevier), pp. pp 89–99.

Tjallingii, W.F. (2006). Salivary secretions by aphids interacting with proteins of phloem wound responses. J. Exp. Bot. **57,** 739-745.

Town, C.D., Cheung, F., Maiti, R., Crabtree, J., Haas, B.J., Wortman, J.R., Hine, E.E., Althoff, R., Arbogast, T.S., and Tallon, L.J. (2006). Comparative Genomics of Brassica oleracea and Arabidopsis thaliana Reveal Gene Loss, Fragmentation, and Dispersal after Polyploidy. The Plant Cell Online **18,** 1348.

Traw, M.B. (2002). Is induction response negatively correlated with constitutive resistance in black mustard? Evolution **56,** 2196-2205.

Traw, M.B., and Dawson, T.E. (2002). Reduced performance of two specialist herbivores (Lepidoptera : Pieridae, Coleoptera : Chrysomelidae) on new leaves of damaged black mustard plants. Environ. Entomol. **31,** 714-722.

Turchin, P., and Kareiva, P. (1989). Aggregation in Aphis Varians: An Effective Strategy for Reducing Predation Risk. Ecology **70,** 1008-1016.

Turlings, T.C.J., Alborn, H.T., Loughrin, J.H., and Tumlinson, J.H. (2000). Volicitin, an elicitor of maize volatiles in oral secretion of *Spodoptera exigua*: Isolation and bioactivity. J. Chem. Ecol. **26,** 189-202.

Turlings, T.C.J., Bernasconi, M., Bertossa, R., Bigler, F., Caloz, G., and Dorn, S. (1998). The induction of volatile emissions in maize by three herbivore species with different feeding habits: Possible consequences for their natural enemies. Biol. Control **11,** 122-129.

Turner, J.G., Ellis, C., and Devoto, A. (2002). The jasmonate signal pathway. Plant Cell **14,** S153-S164.

van Dam, N.M., and Poppy, G.M. (2007). Why plant volatile analysis needs bioinformatics – Detecting signal from noise in increasingly complex profiles. Plant Biology (in press).

van Dam, N.M., Raaijmakers, C.E., and van der Putten, W.H. (2005). Root herbivory reduces growth and survival of the shoot feeding specialist *Pieris rapae* on *Brassica nigra*. Entomol. Exp. Appl. **115,** 161-170.

van der Meijden, E., and Klinkhamer, P.G.L. (2000). Conflicting interests of plants and the natural enemies of herbivores. Oikos **89,** 202-208.

van Poecke, R.M.P., and Dicke, M. (2002). Induced parasitoid attraction by Arabidopsis thaliana: involvement of the octadecanoid and the salicylic acid pathway. J. Exp. Bot. **53,** 1793-1799.

Van Poecke, R.M.P., Posthumus, M.A., and Dicke, M. (2001). Herbivore-induced volatile production by Arabidopsis thaliana leads to attraction of the parasitoid Cotesia rubecula: Chemical, behavioral, and gene-expression analysis. J. Chem. Ecol. **27,** 1911-1928.

van Poecke, R.M.P., Roosjen, M., Pumarino, L., and Dicke, M. (2003). Attraction of the specialist parasitoid Cotesia rubecula to Arabidopsis thaliana infested by host or non-host herbivore species. Entomol. Exp. Appl. **107,** 229-236.

Vansteenhouse, J.L., Fettman, M.J., and Gould, D.H. (1991). The Effect of Glutathione Depletion by Buthionine Sulfoximine on 1-Cyano-3,4-Epithiobutane Toxicity. Food and Chemical Toxicology **29,** 153-157.

Vaughn, T.T., Antolin, M.F., and Bjostad, L.B. (1996). Behavioral and physiological responses of Diaeretiella rapae to semiochemicals. Entomol. Exp. Appl. **78,** 187-196.

Verwoerd, T.C., Dekker, B.M.M., and Hoekema, A. (1989). A Small-Scale Procedure for the Rapid Isolation of Plant Rnas. Nucleic Acids Res. **17,** 2362-2362.

Vet, L.E.M., and Dicke, M. (1992). Ecology of Infochemical Use by Natural Enemies in a Tritrophic Context. Annu. Rev. Entomol. **37,** 141-172.

Vinson, S.B. (1976). Host selection by insect parasitoids. Annu. Rev. Entomol. **21,** 109-135.

Vinson, S.B. (1998). The general host selection behavior of parasitoid hymenoptera and a comparison of initial strategies utilized by larvaphagous and oophagous species. Biol. Control **11,** 79-96.

Voelckel, C., Weisser, W.W., and Baldwin, I.T. (2004). An analysis of plant-aphid interactions by different microarray hybridization strategies. Mol. Ecol. **13,** 3187-3195.

Voros, K., Feussner, I., Kuhn, H., Lee, J., Graner, A., Lobler, M., Parthier, B., and Wasternack, C. (1998). Characterization of a methyljasmonate-inducible lipoxygenase from barley (*Hordeum vulgare* cv. Salome) leaves. European Journal of Biochemistry **251,** 36-44.

Vos, P., Hogers, R., Bleeker, M., Reijans, M., Vandelee, T., Hornes, M., Frijters, A., Pot, J., Peleman, J., Kuiper, M., and Zabeau, M. (1995). AFLP - a New Technique for DNA-Fingerprinting. Nucleic Acids Res. **23,** 4407-4414.

Vuorinen, T., Reddy, G.V.P., Nerg, A.M., and Holopainen, J.K. (2004a). Monoterpene and herbivore-induced emissions from cabbage plants grown at elevated atmospheric CO2 concentration. Atmos. Environ. **38,** 675-682.

Vuorinen, T., Nerg, A.M., Ibrahim, M.A., Reddy, G.V.P., and Holopainen, J.K. (2004b). Emission of Plutella xylostella-induced compounds from cabbages grown at elevated CO2 and orientation behavior of the natural enemies. Plant Physiol. **135,** 1984-1992.

Walkey, D. (1991). Applied plant virology (Chapman and Hall, Australia,), pp. 338.

Walling, L.L. (2000). The Myriad Plant Responses to Herbivores. J. Plant Growth Regul. **19,** 195-216.

Warman, E.A. (1994). Pollination biology, seed disperal and demography in Brassica populations. In Gene Flow in Natural Populations of Brassica and Beta, A.J. Gray, Raybould, A.F., Warman, E.A., Mogg, R.J., Gliddon, C & Goudet, J., ed (Department of Environment), pp. pp39-51.

Warwick, S.I., Gugel, R.K., McDonald, T., and Falk, K.C. (2004). Genetic variation of Ethiopian mustard (Brassica carinata) germplasm in western Canada. Genet. Resour. Crop Evol., 1-16.

Weissbecker, B., Van Loon, J.J.A., Posthumus, M.A., Bouwmeester, H.J., and Dicke, M. (2000). Identification of volatile potato sesquiterpenoids and their olfactory detection by the two-spotted stinkbug Perillus bioculatus. J. Chem. Ecol. **26,** 1433-1445.

Will, T., and van Bel, A.J.E. (2006). Physical and chemical interactions between aphids and plants, pp. 729-737.

Williams, P.H., and Hill, C.B. (1986). Rapid-Cycling Populations of Brassica. Science **232,** 1385-1389.

Wise, M.L., and Croteau, R.B. (1999). Monoterpene biosynthesis. In Isoprenoids Including Carotenoids and Steroids. Comprehensive Natural Products Chemistry, D.E. Cane, ed (Amsterdam: Elsevier Science), pp. 97–153.

Wittstock, U., Agerbirk, N., Stauber, E.J., Olsen, C.E., Hippler, M., Mitchell-Olds, T., Gershenzon, J., and Vogel, H. (2004). Successful herbivore attack due to metabolic diversion of a plant chemical defense. PNAS **101,** 4859-4864.

Wright, S.I., and Gaut, B.S. (2005). Molecular Population Genetics and the Search for Adaptive Evolution in Plants. Mol Biol Evol **22,** 506-519.

Xu, Y., Chang, P.F.L., Liu, D., Narasimhan, M.L., Raghothama, K.G., Hasegawa, P.M., and Bressan, R.A. (1994). Plant Defense Genes Are Synergistically Induced by Ethylene and Methyl Jasmonate. Plant Cell **6,** 1077-1085.

Yang, Y.W., Lai, K.N., Tai, P.Y., and Li, W.H. (1999). Rates of nucleotide substitution in angiosperm mitochondrial DNA sequences and dates of divergence between Brassica and other angiosperm lineages. J. Mol. Evol. **48,** 597-604.

Zhang, J.Y., Broeckling, C.D., Blancaflor, E.B., Sledge, M.K., Sumner, L.W., and Wang, Z.Y. (2005). Overexpression of WXP1, a putative Medicago truncatula AP2 domain-containing transcription factor gene, increases cuticular wax accumulation and enhances drought tolerance in transgenic alfalfa (Medicago sativa). Plant J. **42,** 689-707.

Zhang, W.Q., and Hassan, S.A. (2003). Use of the parasitoid Diaeretiella rapae (McIntoch) to control the cabbage aphid Brevicoryne brassicae (L.). J. Appl. Entomol. **127,** 522-526.

Zhang, X.Y., and Wessler, S.R. (2004). Genome-wide comparative analysis of the transposable elements in the related species Arabidopsis thaliana and Brassica oleracea. PNAS **101,** 5589-5594.

Zhang, Z., Ober, J.A., and Kliebenstein, D.J. (2006). The Gene Controlling the Quantitative Trait Locus EPITHIOSPECIFIER MODIFIER1 Alters Glucosinolate Hydrolysis and Insect Resistance in Arabidopsis. Plant Cell, tpc.105.039602.

Zhu-Salzman, K., Salzman, R., Ahn, J., and Koiwa, H. (2004). Transcriptional Regulation of Sorghum Defense Determinants against a Phloem-Feeding Aphid1. Plant Physiol. **134,** 420-431.

Zhu, J.W., and Park, K.C. (2005). Methyl salicylate, a soybean aphid-induced plant volatile attractive to the predator Coccinella septempunctata. J. Chem. Ecol. **31,** 1733-1746.