Discovering clubroot resistance genes in

*Brassica* vegetable crops

A thesis submitted in total fulfilment of the requirements for the degree of Doctor of Philosophy

By

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; and, any editorial work, paid or unpaid, carried out by a third party is acknowledged.

Stephane L. KONG KAW WA

23rd October 2009
Acknowledgements

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Thesis abstract

Clubroot, caused by the soil-borne obligate biotroph *Plasmodiophora brassicae* Woronin, is one of the most serious diseases of *Brassica* worldwide. In Australia, it is responsible for losses of at least 10% in crucifer yield causing more than AU$ 17 million in lost profits every year. Agricultural practices such as the application of lime to increase soil pH or control of the disease with agrochemicals may reduce the damage to crops but their effects are often insufficient to keep the plant healthy. Moreover, the cost and practicality of current control measures may be prohibitive. Hence, the breeding of resistant cultivars, especially for the susceptible Chinese cabbage, is an effective approach to eliminate the use of expensive and usually environmentally harmful fungicides and to minimise loss. Despite the identification of several sources of clubroot resistance, there have been few successful breeding programs for resistance since these defence genes were hardly expressed under high disease pressure. The lack of information on the complex genetic control of resistance in the hosts and the distribution and mixed infection of multiple pathogenic races in a single field, were other impediments. Therefore, the identification of the actual number of genes involved in clubroot resistance and their mechanisms of action could be important for effective breeding strategies.

This study documented the first report of the large-scale profiling of the transcriptional changes to the very early stages of *P. brassicae* infection in *Brassica* vegetable crops using a microarray approach. Firstly, a cost-effective ‘boutique’ *Brassica* oligonucleotide array of 150 *Arabidopsis*-/*Brassica*-derived features was constructed using nucleotide sequences from GenBank. This array, which was biased towards defence-associated and regulatory genes, was used to investigate the gene expressions of the partially-resistant Chinese cabbages ‘Tahono’ and ‘Leaguer’ and the susceptible ‘Granaat’ when challenged with aggressive clubroot isolate. The microarray data, validated by qRT-PCR, indicated a high number of constitutively and
differentially expressed genes in response to pathogen attack, prominently at 48 hai as opposed to 24 and 72 hai. The lack of transcriptional changes at 24 hai demonstrated that there is no strong hypersensitive response in those genotypes. However, the major responses at 48 hai may correlate with the timing of penetration by the primary zoospores of *P. brassicae* in the host root hair cells. This involved 10, 11 and 2 differentially expressed genes upon inoculation in ‘Granaat’, ‘Tahono’ and ‘Leaguer’ respectively and 8 and 21 constitutively expressed genes in untreated 30-day-old ‘Tahono’ and ‘Leaguer’ when compared to ‘Granaat’ respectively. The key observations were the constitutive over-expression and induction of a pathogenesis-related (PR) protein: chitinase as well as the up-regulation of a lignin biosynthesis enzyme: caffeoyl-CoA 3-O-methyltransferase in these partially-resistant varieties. However, the ‘closed architecture’ system of this oligoarray restricted the results to the number of transcripts and associated genes present on the array. Nonetheless, its construction and its use to investigate defence-related gene expressions was a viable option to avoid the costly and inefficient use of the Affymetrix genechip in subsequent experiments.

Due to the limitations of the ‘boutique’ *Brassica* oligoarray, a more sophisticated microarray platform, the Affymetrix *Arabidopsis* ATH1 genechip, was used as an exploratory tool for whole-genome transcriptional profiling of *B. rapa*. The gene expression profiles in the roots of the partially-resistant ‘Tahono’ and susceptible ‘Granaat’ as well as a clubroot resistant fodder turnip ‘ECD04’ were investigated at 48 hai. It revealed 17, 34 and 2 differentially expressed genes upon inoculation in ‘Granaat’, ‘Tahono’ and ‘ECD04’ respectively and 110 and 205 constitutively expressed genes in untreated 30-day-old ‘Tahono’ and ‘ECD04’ when compared to ‘Granaat’ respectively. A key observation was three major SA-dependent defence responses that were consistent with elevated level of salicylic acid (SA) in partially-resistant ‘Tahono’ and resistant ‘ECD04’. SA possibly induced by the accumulation of...
reactive oxygen species (ROS), appears to be an essential regulatory component in firstly, the constitutive over-expression of the PR protein endochitinase, possibly via the NPR1, WRKY and TGA factors. Secondly, the high basal level of lignification via a peroxidase-dependent pathway in the roots provided an enhanced physical barrier against clubroot. Lastly, the myrosinase / glucosinolate defence system may also be regulated via the SA signalling. The high basal level of myrosinase was most likely involved in the rapid hydrolysis of aliphatic glucosinolates into toxic antifungal by-products while the low basal glucosinolate root content (possibly indole glucosinolate) may have resulted in less severe gall formation in the partially-resistant / resistant Brassica varieties. The key deductions from both microarray experiments were: firstly, the limited number of differentially expressed defence-related genes may be due to the suppression of the host’s defence arsenals by P. Brassicae. Secondly, this virulence mechanism did not affect the transcriptional control mechanisms of constitutively expressed defences in the host and therefore, may represent an alternative strategy in Brassica breeding (rather than the use of dominant resistance genes).

These conclusions were however based on previous reports on the putative role of these genes and therefore, further validation via other techniques such as SuperSAGE, ‘knock-out’ mutants or over-expressing transgenics, is required. Ultimately, these candidate genes involved in defence may potentially be used in the development of functional molecular markers for the marker-assisted selection of clubroot resistant Brassica vegetable crops.
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Supplementary data

In the attached DVD, these files are provided:

- **Folder ‘Oligoarray Raw Data’**: Raw scan images and signal intensities of the ‘boutique’ Brassica oligoarray generated from Affymetrix Jaguar and analysed using the BioDiscovery ImaGene and GeneSight softwares.

- **Folder ‘Oligoarray Results’**: Selection process to identify genes significantly differentially and constitutively expressed in the ‘boutique’ Brassica oligoarray data.

- **Folder ‘RNA BioAnalyser Results’**: Spectrophotometric data indicating the quality and concentration of total RNA samples prior to Affymetrix experiments.

- **Folder ‘Affymetrix Raw Data’**: Raw scan images and computed scan images of Affymetrix genechips that can be analysed using the Affymetrix GCOS software.

- **Folder ‘Affymetrix Results’**: Selection process to identify genes significantly differentially and constitutively expressed in the Affymetrix genechip data.

- **Folder ‘qRT-PCR Results’**: Quantitative real-time PCR data and graphs to validate both microarray experiments.

- **Folder ‘PhD Documents’**: Miscellaneous files comprised of the PhD thesis, PhD seminar presentation and conference poster and refereed paper.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>µ</td>
<td>micro</td>
</tr>
<tr>
<td>A&lt;sub&gt;260&lt;/sub&gt;</td>
<td>absorbance at 260 nm</td>
</tr>
<tr>
<td>A&lt;sub&gt;280&lt;/sub&gt;</td>
<td>absorbance at 280 nm</td>
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<td>aaCy&lt;sub&gt;5&lt;/sub&gt;-10T</td>
<td>5'-amine-modified Cy5 dye coupled with 10 deoxythymidines</td>
</tr>
<tr>
<td>ABA</td>
<td>abscisic acid</td>
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<tr>
<td>ACC</td>
<td>1-aminocyclopropane-1-carboxylic acid synthase</td>
</tr>
<tr>
<td>AFLP</td>
<td>amplified fragment length polymorphism</td>
</tr>
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<td>AGRF</td>
<td>Australian Genome Research Facility</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<td>AU$</td>
<td>Australian Dollar</td>
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<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<td>cc</td>
<td>cubic centimetre</td>
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<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<tr>
<td>COS</td>
<td>conserved orthologous set</td>
</tr>
<tr>
<td>dai</td>
<td>days after inoculation</td>
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<tr>
<td>DAMP</td>
<td>damage-associated molecular pattern</td>
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<td>DEPC</td>
<td>diethylpyrocarbonate</td>
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<td>disease index</td>
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<td>DTT</td>
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<td>ECD</td>
<td>European clubroot differential series</td>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>ELISA</td>
<td>enzyme-linked immunosorbent assays</td>
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<td>EREBP</td>
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<td>ET</td>
<td>ethylene</td>
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<td>ETI</td>
<td>elicitor-triggered immunity</td>
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HEPES 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC high performance liquid chromatography
IAA indole-3-acetic acid
ITS internal transcribed spacer
JA jasmonic acid
L litre
LRR leucine rich repeat
m meter
MAPK mitogen-associated protein kinase
MAS marker-assisted selection
Mbp nucleotide mega base pairs
MES 2-[N-Morpholino]ethansulfonic acid
min minute
mm millimetre
mRNA messenger RNA
MW molecular weight
NADH reduced nicotinamide adenine dinucleotide N-hydroxysuccinimide ester
°C degrees Celcius
PAL phenylalanine ammonia-lyase
PAMP pathogen-associated molecular pattern
PCNB pentachloronitrobenzene
PCR polymerase chain reaction
PR pathogenesis-related protein
PRR pattern-recognition receptor
PTI PAMP-triggered immunity
QTL quantitative trait loci
RAPD random amplified polymorphic DNA
RFLP restriction fragment length polymorphism
RNA ribonucleic acid
ROS reactive oxygen species
rpm revolutions per minute
SA salicylic acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>SAPE</td>
<td>streptavidin phycoerytherin</td>
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<tr>
<td>SAR</td>
<td>systemic acquired resistance</td>
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<tr>
<td>SCAR</td>
<td>sequence characterised amplified region</td>
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<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<td>SEM</td>
<td>scanning electron micrograph</td>
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<tr>
<td>SFP</td>
<td>single feature polymorphism</td>
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<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
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<td>sodium chloride / sodium citrate</td>
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<td>single spore isolates</td>
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<td>tri(hydroxymethyl) aminomethane</td>
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<td>ultra violet</td>
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</tr>
<tr>
<td>VIC</td>
<td>Victoria, Australian State</td>
</tr>
<tr>
<td>QLD</td>
<td>Queensland, Australian State</td>
</tr>
<tr>
<td>NSW</td>
<td>New South Wales, Australian State</td>
</tr>
<tr>
<td>w/v</td>
<td>weight per volume</td>
</tr>
<tr>
<td>XTH</td>
<td>xyloglucan endotransglucosylate hydrolase</td>
</tr>
<tr>
<td>ZR</td>
<td>zeatin riboside</td>
</tr>
</tbody>
</table>
Chapter 1

Introduction: Literature review

Clubroot, caused by the soil-borne obligate biotroph *Plasmodiophora brassicae* Woronin (Figure 1.1), is one of the most serious diseases of *Brassica* worldwide. In this review, the current state of knowledge regarding clubroot and its impact on Australian and worldwide *Brassica* cultivation is described. Firstly, the clubroot disease and its detrimental effects on *Brassica* production is highlighted, followed by an assessment of the mechanisms of defence against pathogens in plants and efforts to improve disease resistance by classical breeding and molecular breeding. Lastly, an overview on microarrays, as tools of functional genomics, promise to modernise the understanding of clubroot defence mechanisms and change the way disease resistant genotypes are developed is presented. Additionally, the gaps in the current knowledge of clubroot resistance in *Brassica* are identified and reference to a few previous excellent reviews in the area are provided.

1.1 Clubroot disease

1.1.1 Origin of clubroot

As early as the 13\textsuperscript{th} century, the description of spongy and fungus-like roots of Brassicas by Albert The Great was most probably the first report of clubroot. Over the last 200 years, the spread of clubroot has been reported in 1750 in England, in 1820 in France and in 1853 in Germany (Karling, 1968). It is thought that the movement of the pathogen from Europe resulted from transport of diseased animal fodder taken by colonists travelling to America, Australasia and other similar centres of settlement. This disease was referred by many names, such as ‘Hernie du Chou’ in France, ‘Fingerkrankheit’ in Germany ‘Kapoustnaja kila’ in Russia and ‘Finger and Toe’ in North America (Dixon, 2009). By 1872, the incidence and the destruction caused by the disease was so disruptive that the Russian Gardening Society
Figure 1.1 Mature resting spores of *P. brassicae* in infected roots of Chinese cabbage visualised by (a) scanning electron microscopy (Ludwig-Muller *et al.*, 1999) and (b) differential interference contrast microscopy ([http://www.microscopyu.com/galleries/dicphasecontrast](http://www.microscopyu.com/galleries/dicphasecontrast), last modified 2009). Horizontal bar 10 µm.
offered a prize to identify the causative agent of clubroot. Three years later, a Russian scientist: Michael Woronin announced that clubroot is caused by a plasmodiophorous organism and named it as *Plasmodiophora brassicae*. It was originally believed that clubroot was caused by a worm, excrements of syphilitic patients or imbalances of the soil as well as being the source of cancer due to the resemblance of the disease’s symptoms to tumorous growth in mammals (Karling, 1968).

1.1.2 Taxonomy of clubroot

*P. brassicae* is classified in the order Plasmodiophorida (informally known as the Plasmodiophorids) (Table 1.1), a group of eukaryotic intracellular parasites composed of mostly pathogens of economically important crops or as vectors for disease-causing plant viruses (Braselton, 2002). A unique characteristic feature of the Plasmodiophorids is their ability to reproduce through a form of closed mitosis known as cruciform nuclear division. Other unusual features include the two anterior whiplash flagella and the ‘Rohr and Stachel’, a cellular protrusion used by Plasmodiophorid zoospores to penetrate host cells and the formation of multinucleate plasmodia inside their hosts (Archibald and Keeling, 2004; Braselton, 2005).

Traditionally, the Plasmodiophorids were classified in the kingdom fungi since they produce spores and have been intensively studied by mycologists (Waterhouse, 1972). Despite the available information on their life cycle, infection strategies and ultrastructures of these organisms, their resulting traits have made it difficult to position these organisms in terms of eukaryotic evolution. The phylogenetic analysis of small subunit ribosomal RNA genes have revealed that these organisms are not true fungi (Ward and Adams, 1998) and Cavalier-Smith and Chao (2003) suggested that the Plasmodiophorids belong to the protist phylum Cercozoa (formerly known as Rhizopoda). Archibald and Keeling (2004) indicated that the
plasmodiophorids are indeed related to the Cercozoa by analysing the sequences of the proteins: actin and ubiquitin but relationships of the organisms belonging to the Cercozoa remain unsolved. Recently, Siemens et al. (2009) reported that *P. brassicae* chromosomes, ranging from 0.7 to 2.2 Mb, have been characterised and the total genome size is estimated to be approximately 20 Mb. In addition, their sequence data have reinforced the inclusion of the Plasmodiophorids within the Cercozoa.

Table 1.1. Taxonomy of clubroot (Archibald and Keeling, 2004; Brands, 2005).

<table>
<thead>
<tr>
<th>Domain</th>
<th>Eukaryota</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kingdom</td>
<td>Protozoa</td>
</tr>
<tr>
<td>Sub-kingdom</td>
<td>Biciliata</td>
</tr>
<tr>
<td>Infra-kingdom</td>
<td>Rhizaria</td>
</tr>
<tr>
<td>Phylum</td>
<td>Cercozoa</td>
</tr>
<tr>
<td>Sub-phylum</td>
<td>Endomyxa</td>
</tr>
<tr>
<td>Class</td>
<td>Phytomyxea</td>
</tr>
<tr>
<td>Order</td>
<td>Plasmodiophorida</td>
</tr>
<tr>
<td>Family</td>
<td>Plasmodiophoridae</td>
</tr>
<tr>
<td>Genus</td>
<td>Plasmodiophora</td>
</tr>
<tr>
<td>Species</td>
<td><em>Plasmodiophora brassicae</em> Woronin</td>
</tr>
</tbody>
</table>

1.1.3 Life cycle of clubroot

There is still incomplete understanding of the life cycle of *P. brassicae* in terms of the frequency and nature of karyogamy and meiosis. It is generally known that it is composed of two generations (Figure 1.2 and 1.3) and has three stages in its lifecycle: survival in soil, root hair infection, and cortical infection (Kageyama and Asano, 2009). Clusters of the thick-walled resting spores are released into the soil when infected roots deteriorate and decay and are capable of surviving in the soil up for at least 7 years. The specific conditions that triggers their germination are still unknown but favourable conditions include temperature ranging from 6 to 27°C and acidic pH (<6.0) (Karling, 1968). In the presence of susceptible host and near-saturated soil moisture, these spores germinate to release the primary zoospores that
Figure 1.2. General life cycle of the Plasmodiophorids (Braselton, 2005).

Figure 1.3. Life cycle of clubroot caused by *Plasmodiophora brassicae* (Agrios, 2005).
possess two unequal, anterior, whiplash flagella (Brown, 1997). When the primary zoospore reaches a root hair or other epidermal cells, it loses its flagella and becomes ameboid (Roberts and Boothroyd, 1972). The contents of the zoospore are injected into the host cell and the development of the multinucleated plasmodium is initiated (Rush, 2003). Although the need for the root hair infection is not fully understood, it is generally accepted that root hair infection increases the *P. brassicae* populations in susceptible host and also serves to cause severe damage to Chinese cabbage (Mitani *et al.*, 2003). The resting spores and primary zoospore are the only stage that seems to be independent of host tissue.

During the primary infection, sporangial plasmodia develop in epidermal cells to give rise to a thin-walled zoosporangium containing between four to eight secondary zoospores (Agrios, 2005). These secondary zoospores will initiate a second round of infection. It is not clear whether these zoospores are released outside or within the host tissue (Ohi *et al.*, 2003), but the secondary phase takes place deeper in the cortex and stele of hypocotyls and roots of infected plants (Dixon and Page, 1998). This results in the abnormal proliferation of plant tissues and formation of galls during the development of the sporogenic plasmodia (Rush, 2003). These galls are produced by uncontrolled cell division (hyperplasia) and consequent elongation (hypertrophy) of the newly formed cells (Ingram and Tommerup, 1972). Upon decay of the clubs, large amount of resting spores are released into the soil where they can survive for many years.

In both sporangial and sporogenic development, cruciform nuclear division occurs in the early stages followed by non-cruciform division at later stages. There is no evidence that the non-cruciform divisions during sporangial development (primary infection) are meiotic division in contrast to the non-cruciform divisions in sporogenic plasmodia (secondary infection) (Braselton, 2005). It is unknown whether the primary zoospores are of different mating types.
and that their fusion gives rise to zygotes that are capable of entering the root (Roberts and Boothroyd, 1972). In brief, the primary root infection stage occurred at 6 days after infection (dai) (Ingram and Tommerup, 1972) while the secondary infection was initiated at 13 dai and appearance of the first galls at 21 dai (Devos et al., 2005) during the artificial inoculation of susceptible host plants.

1.1.4 Symptoms of clubroot

Although clubroot has been reported to be most severe in temperate regions, Brassica cultivations have also been decimated in regions with tropical conditions or at high altitudes. The first visible symptoms of clubroot can be observed on the aerial parts of the infected plants: wilting of the leaves on hot and sunny days and the partial recovery of turgidity during the night, appearing fresh the following day (Agrios, 2005). Young plants may die soon after an infection while older plants may survive. As the disease progresses, yellowing and shedding of the lower leaves occur and the development of the host is halted (Brown, 1997). These plants become stunted and may never produce marketable products.

The disease was named after its obvious underground symptoms that involve tumorous growth and subsequent gall formation on the root system (Figure 1.4). These galls can be spindle-shaped, spherical, knobby or club-shaped when swellings are few and isolated. When these occur in close proximity, they may coalesce and form irregular outgrowths or compound spindles, covering the entire root system (Brown, 1997). These galls not only consume the food required for the normal growth of the host, but they also interfere in the efficient absorption and translocation of mineral nutrients and water through the root system (Agrios, 2005). This results in the wilting of the above-ground parts of the plant due to the uncontrolled cell divisions and splitting of the woody cylinder by infection and enlargement of the medullary rays (Karling, 1968).
Figure 1.4. Visual differences between a healthy (left) and a naturally-infected (right) Chinese cabbage, collected from the same *Brassica* vegetable farm. The clubbed roots and stunted growth were obvious symptoms in the diseased plant.
As the infected roots become larger and older, they will disintegrate to release the resting spores due to the invasion of secondary parasitic or saprophytic microorganisms. There is however no evidence that these organisms have any symbiotic relationship or are involved in the infection strategy and development of *P. brassicae* (Karling, 1968).

1.1.5 Economic impact of clubroot

The stunted growth and possibly unmarketable heads of the *Brassica* crops have severe consequences to any *Brassica* industry. According to the most recent and comprehensive Australian horticultural statistics, this industry produces more than AU$ 167 million of crops annually and is predominantly made up of broccoli, Brussels sprouts, cabbage, cauliflower and Chinese cabbage (Table 1.2) (Horticulture Australia Limited, 2004). Due to the national production of crucifers mainly in South-eastern Australia and Perth (Figure 1.5), the importation of crucifer products is insignificant. Hence the export of fresh *Brassica* mainly towards Asian countries represents a substantial source of income (Table 1.3) (Horticulture Australia Limited, 2004).

It was estimated that at least 10% of *Brassica* crops are infected in Australia (Faggian et al., 1999) compared to an average mean infection of 11% worldwide (Dixon, 2009), resulting in more than AU$ 16 million in lost profits to the Australia *Brassica* industry. Other surveys have shown that over 70% of *Brassica* properties in Victoria are contaminated by clubroot and crop losses of up to 25 hectares / property have been reported (Lane, 2004). This has prompted four states of Australia and over 20 industries to commit substantial voluntary funds to support research designed to develop integrated strategies to control and prevent the spread of clubroot. Otherwise, the *Brassica* export industry may experience severe loss in revenue if clubroot disease is left uncontrolled.
Table 1.2. Australian vegetables production in 2002/03 (Horticulture Australia Limited, 2004).

<table>
<thead>
<tr>
<th>Vegetable</th>
<th>Volume (tonnes)</th>
<th>Gross value (AU$ m)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Broccoli</td>
<td>45,901</td>
<td>65.4</td>
</tr>
<tr>
<td>Brussels sprouts</td>
<td>5,305</td>
<td>9.7</td>
</tr>
<tr>
<td>Cabbages</td>
<td>76,093</td>
<td>27.4</td>
</tr>
<tr>
<td>Cauliflowers</td>
<td>87,586</td>
<td>56.3</td>
</tr>
<tr>
<td>Chinese Cabbage</td>
<td>11,513</td>
<td>7.8</td>
</tr>
<tr>
<td><strong>Total vegetables</strong></td>
<td><strong>3,438,958</strong></td>
<td><strong>2,268.5</strong></td>
</tr>
</tbody>
</table>

Table 1.3. Australian exports of Brassica vegetables in 2002/03 (Horticulture Australia Limited, 2004).

<table>
<thead>
<tr>
<th>Vegetables</th>
<th>Top 3 markets</th>
<th>Top 3 markets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Country</td>
<td>Tonnes</td>
</tr>
<tr>
<td>Broccoli</td>
<td>Singapore</td>
<td>2,764</td>
</tr>
<tr>
<td></td>
<td>Malaysia</td>
<td>1,399</td>
</tr>
<tr>
<td></td>
<td>Japan</td>
<td>770</td>
</tr>
<tr>
<td><strong>Total exports</strong></td>
<td><strong>6,428</strong></td>
<td><strong>Total exports</strong></td>
</tr>
<tr>
<td>Brussels sprouts</td>
<td>Netherlands</td>
<td>451</td>
</tr>
<tr>
<td></td>
<td>New Zealand</td>
<td>108</td>
</tr>
<tr>
<td></td>
<td>United Kingdom</td>
<td>40</td>
</tr>
<tr>
<td><strong>Total exports</strong></td>
<td><strong>653</strong></td>
<td><strong>Total exports</strong></td>
</tr>
<tr>
<td>Cabbage</td>
<td>Japan</td>
<td>508</td>
</tr>
<tr>
<td></td>
<td>Taiwan</td>
<td>419</td>
</tr>
<tr>
<td></td>
<td>Singapore</td>
<td>322</td>
</tr>
<tr>
<td><strong>Total exports</strong></td>
<td><strong>1,913</strong></td>
<td><strong>Total exports</strong></td>
</tr>
<tr>
<td>Cauliflower</td>
<td>Malaysia</td>
<td>10,200</td>
</tr>
<tr>
<td></td>
<td>Singapore</td>
<td>5,360</td>
</tr>
<tr>
<td></td>
<td>Hong Kong</td>
<td>369</td>
</tr>
<tr>
<td><strong>Total exports</strong></td>
<td><strong>16,567</strong></td>
<td><strong>Total exports</strong></td>
</tr>
<tr>
<td>Chinese Cabbage</td>
<td>Taiwan</td>
<td>1,460</td>
</tr>
<tr>
<td></td>
<td>Singapore</td>
<td>1,397</td>
</tr>
<tr>
<td></td>
<td>Hong Kong</td>
<td>576</td>
</tr>
<tr>
<td><strong>Total exports</strong></td>
<td><strong>3,827</strong></td>
<td><strong>Total exports</strong></td>
</tr>
<tr>
<td><strong>Total vegetables</strong></td>
<td><strong>195,237</strong></td>
<td><strong>194,752</strong></td>
</tr>
</tbody>
</table>
Figure 1.5. Major cultivations of (a) Broccoli, (b) Brussels sprouts, (c) Cabbage, (d) Cauliflower and (e) Chinese cabbage in Australia (Horticulture Australia Limited, 2004).

Note: The above tables and figures represent the most comprehensive Australian horticultural statistics available in October 2009 by Horticulture Australia Limited (2004).
1.2 Detection of clubroot

The significance of determining the presence and abundance of *P. brassicae* resting spores in the soil before the planting *Brassica* crops or the implementation of control measures is apparent. Several techniques have been developed to assess the degree of clubroot contamination in the fields and the genetic variation within and between field isolates. The knowledge on the race of clubroot in areas where *Brassica* crops are intensively cultivated will permit the effective deployment of durable plant resistance.

1.2.1 Bait method

The physiological specialisation of *P. brassicae* has been studied independently and researchers have described significant differences in the degree of infection and clubbing in several varieties of *Brassica* crops. This has led to the belief that *P. brassicae* is composed of several physiological races with varying degree of virulence and host specificity (Karling, 1968; Buczacki *et al.*, 1975). Several bait methods have been devised for the typing of clubroot races to identify the likelihood of new resistant crop varieties being suitable for use and to facilitate the identification of the source of infection. As reviewed by Chambers (1977), the results generated could not be correlated since each researcher used different differential hosts for classifying clubroot races. This is why Buczacki *et al.* (1975) previously attempted to rationalise experimental procedures and to develop an internationally acceptable system known as the European Clubroot Differential set (ECD) for describing the populations of *P. brassicae*. The proposed system consists of a collection of *Brassica* genotypes with different numbers and types of resistance genes to differentiate between isolates based on their virulence phenotype. Since it has been developed through the co-operation of previous independent research groups, the ECD set represents a considerable advancement over previous systems.
This ECD set and other differential hosts have been used as soil bioassays to detect the presence of *P. brassicae* in the fields, taking around 5-6 weeks to complete (Wallenhammar, 1996). Hence, the routine test offered by bioassays is laborious, requires considerable amount of glasshouse space and is not practical for large numbers of field samples (Manzanares-Dauleux *et al.*, 2000a). Kuginuki *et al.* (1999) have previously encountered some difficulties since the responses of the differential hosts were not clear when *P. brassicae* populations from Japan were used. They also suggested that both the heterogeneity of the isolates and the differential hosts might account for the variations in their results. Field screening is usually affected by environmental variations in the infection and symptoms of the hosts. Hence, the establishment of a more reliable screening method is required through the use of genetically uniform differential hosts.

### 1.2.2 Microscopy and serology

Detection of *P. brassicae* was performed through microscopy by Samuel and Garrett (1945) who estimated the activity of *P. brassicae* in the soil by counting the infected root hair in 1% aceto-carmine stain. Arie *et al.* (1988) developed a fluorescent antibody method for the detection of resting spores of *P. brassicae* from soil and root. Several other methods based on an immunological system have been investigated. For example, Lange *et al.* (1989) detected *P. brassicae* by dot immuno-binding and visualisation of the serological reaction in a scanning electron microscopy. Wakeham and White (1996) used indirect immuno-fluorescence and indirect enzyme-linked immunosorbent assays (ELISA) to detect spores in artificially and naturally-infected soils.

The use of microscopy, fluorescence and serological methods have not been completely developed for the routine testing of field soils since these methods are laborious, require experience or the availability of specialised equipment. Even if immunoassays are used
routinely for the detection of plant pathogens in many laboratories, the reliability of the assays depends greatly on the quality and specificity of the antibodies. Despite difficulties to produce antisera for the different races of *P. brassicae* (Ito *et al.*, 1999), there was recently a renewed effort to develop inexpensive and convenient serologic on-farm diagnostic kits based on monoclonal antibodies for growers (Faggian and Strelkov, 2009).

### 1.2.3 Polymerase chain reaction

Another approach for the rapid, sensitive and reliable detection of *P. brassicae* was by polymerase chain reaction (PCR). This methodology was however hampered due to the obligate parasitic nature of *P. brassicae*, i.e. this pathogen could not be cultured. This complicated its purification from infected roots and other contaminants like bacteria and host debris (Faggian *et al.*, 1999). *P. brassicae* has been reported to take up random segments of the host DNA and the design of specific PCR primers using clubroot sequences may not be reliable. Generally, detection of micro-organisms in the soil is problematic due to their thick cell walls, strong binding to soil particles or organic matter and the presence of inhibitors such as humic acid that hinders PCR (Kageyama *et al.*, 2003). Previously, strategies to overcome these problems involve the mechanical disruption of the native soil organisms in soil particles and organic matter (Cullen and Hirsch, 1998) or by enzymatically degrading the thick cell walls of the pathogen spores (Porteus *et al.*, 1994). Inhibitors can be removed by purifying the extracted DNA using gel filtration (Moran *et al.*, 1993) and by recovering the DNA after gel electrophoresis or by column chromatography (Zhou *et al.*, 1996). Rather than eliminating the inhibitors, Kreader (1996) devised a way to inactivate them through the addition of bovine serum albumin in DNA extracted from soil. PCR has been applied to detect plant pathogens in diseased and asymptomatic plants as well as in the soil and water using specific primers.
The nested PCR technique has been used by several researchers and is comprised of two rounds of amplification to overcome inhibition, low amplification efficiency and other factors affecting PCR. It requires two primer sets and the chances of cross-contamination occurring is increased due to the additional reagents and handling required (Kageyama et al., 2003). Ito et al. (1999) used a nested PCR system and were able to detect clubroot resting spores from naturally infected soil samples. Their primers could effectively amplify a specific fragment from a single-copy target DNA sequence in *P. brassicae*. In contrast, Faggian et al. (1999) detected *P. brassicae* in both artificially infected and field soil samples using nested primers designed from ribosomal repeat and internal transcribed spacer (ITS) regions of *P. brassicae*. The latter offers several advantages since the ribosomal DNA (rDNA) region can be present up to 220 copies per genome, hence providing a higher sensitivity. Although these regions can be conserved at the species level, the base sequence can be highly variable and thus allow the specific differentiation between and within fungi species. Recently, Kageyama et al. (2003) have designed a one cycle PCR system using another primer set based in the ITS region of rDNA of *P. brassicae*. Their results supported the use of ITS regions as a reliable sequence for the design of specific primer set.

The use of random 10-mer oligonucleotide primers in random amplified polymorphic DNA (RAPD) techniques has been used to study the variations within plant pathogens as another PCR-based approach. Although this technique was useful in a broad range of facultative pathogens, it could only be applied to a limited number of obligate pathogens such as in *Peronospora parasitica* (downy mildew) (Tham et al., 1994). The reason is due to a high possibility of contaminating the pathogen DNA with host DNA. The problems associated with RAPD are very significant when using *P. brassicae* spores since this obligate pathogen may take up host DNA during its infection cycle (Bryngelsson et al., 1988). To alleviate some of these problems, Manzanares-Dauleux et al. (2000a) analysed the RAPD profiles of 37
single-spore-derived isolates belonging to seven different pathotypes. These researchers designed a sequence characterised amplified region (SCAR) marker from a molecular pattern common to all the isolates from a particular virulent pathotype. They were able to detect isolates belonging to this one specific class of clubroot pathotype from infected clubs as opposed to previous PCR methods. For the better understanding of the genetic variability and structural organisation within populations of *P. brassicae*, other types of molecular markers are required.

In conclusion, farmers and researchers have access to a selection of clubroot detection systems that have permitted the extensive characterisations of *P. brassicae* around the world. These techniques may be cheap (bait method), convenient (serology) and sensitive (PCR-based method), but have shortcomings in being laborious, too specific and costly respectively. The knowledge gained from these methods was vital for the appropriate selection and deployment of resistant crops.

1.3 **Control measures for clubroot**

Once the degree of clubroot infestation has been established using the above detection methods, integrated strategies to control and prevent the spread of clubroot have been developed and are described below.

1.3.1 **Integrated pest management**

In recent years, the growing incidence and severity of clubroot has been associated with the increase use of transplants, narrow crop rotations, more intensive cropping of the soil and the suspected spread of the disease through livestock, machinery and bulk bins. Once growers are faced with clubroot problems in their plantations, they are left with few alternatives to deal with yield reductions upon clubroot infection (Tremblay *et al.*, 2005). It is almost impossible
to eradicate the disease and the soil may become unsuitable for *Brassica* cultivation unless costly methods and materials (described later) are used to treat the soil (Cheah and Falloon, 2000). Good farm hygiene procedures and an increased awareness of the need for better soil health management and sustainable production practices have been conducive to the development of an integrated approach to the management of clubroot in vegetable *Brassica* crops. Such approaches included the detection of *P. brassicae* and prediction of yield loss due to clubroot and identification of hygiene risks in nurseries. The development of methods to minimise these risks together with in-field cultural methods, manipulation of soil pH, calcium and boron amendment, strategic use of pesticides, and the integration of these methods, have been extremely effective in vegetable production systems (Donald and Porter, 2009). These authors also reported that further improvement in integrated control would rely on cultivar resistance coupled with better reduction of inoculum through manipulation of the nutrient / chemical changes in the soil, rhizospheres, root hairs and plant cells.

### 1.3.2 Decoy crops

Planting non-host crops as well as resistant varieties before susceptible crucifers is known to reduce the severity of clubroot disease. Murakami *et al.* (2001) has demonstrated that the disease severity of clubroot on Chinese cabbage can be reduced by growing plants such as oats, spinach and leafy daikon radish prior to Chinese cabbage in artificially infected soil. Depending on the previous crop, there was a reduction of 29-62% in the resting spore densities of *P. brassicae* in the soil even if root hair infection was detected in the decoy plants (Murakami *et al.*, 2001). These plants achieved this by promoted the germination of resting spores in the soil but the formation of galls and reproduction of new resting spores did not occur since *P. brassicae* is an obligate parasite of Brassicas. Hence, the reduction of resting spores by growing non-host plants in infected soil could suppress the severity of the disease in susceptible crops. Ludwig-Müller *et al.* (1999) previously suggested that clubroot can infect
non-crucifers and potentially completing its life cycle in producing resting spores. This complicates strategies for crop rotation.

### 1.3.3 Liming

Addition of lime has been used against clubroot for more than 200 years with varying success since soil pH is possibly the most significant factor influencing clubroot development (Karling, 1968). The application of lime does not eliminate the pathogen, but creates unfavourable conditions against the invasion, colonisation and symptoms formation in the host plants (Webster and Dixon, 1991) and the reduction of spore density in the soil (Murakami et al., 2002). Tremblay et al. (2005) reported that while clubroot may not practically be eradicated from a contaminated field, high concentration of calcium, boron and magnesium may however contribute to the control of clubroot by increasing the pH. For an efficient control of clubroot, both the resulting effects in neutralising the soil pH (7.2 or above) and the probability of the lime particles to be exposed to the pathogen spores should be considered. Hence, the particle size and proper mixing are both essential during the timely application of lime (Cheah and Falloon, 2000). Alternatively, the incorporation of a large amount of calcium-rich organic matter resulting in an increase in soil pH and calcium concentration was reported to be the primary cause of clubroot suppression (Niwa et al., 2007).

### 1.3.4 Calcium cyanamide

The application of Calcium cyanamide is one of the oldest methods of controlling clubroot disease that is still currently used in many countries. Upon contact with soil moisture, Calcium cyanamide decomposes into hydrogen cyanamide (which further degrades into urea and dicyandiamide) and hydrated lime. Hydrogen cyanamide, the intermediate compound, possesses fungicidal properties while the lime and calcium components have fungistatic
effects (described above) on clubroot (Klasse, 1996). The ability of Calcium cyanamide (`powder': 98% w/w particles < 300 µm and ‘standard’: 68% w/w > 850 µm and 31% w/w 300-850) to control clubroot has been evaluated in field trials by Donald et al. (2004). These researchers suggested that the effectiveness of Calcium cyanamide was dependent upon the size of the particles since the ‘powder’ class of the product could almost eliminate root galling as opposed to the ‘standard’ class. Smaller particles have greater surface areas to volume ratio, can react faster in the soil and hence are significantly more active against clubroot.

The current cost of calcium cyanamide is more than AU$ 1,200 per tonne (US$ 1,000 per ton, http://www.diytrade.com/china/4/products/3565748/Calcium_Cyanamide.html, last modified January 2008) and this makes its application unprofitable in most horticultural crops, particularly in soils with low disease levels, unless a better application method is devised (Donald et al., 2004). Recently, Neuweiler et al. (2009) reported that the effectiveness of Calcium cyanamide is limited at sites with an elevated infection pressure of clubroot, despite following a common strategy of application before (1000 kg/ha) and after (1500 kg/ha) planting. Both liming and application of Calcium cyanamide are potential alternatives to fungicides. However, these curative methods may also result in a slight decrease in yield compared to uninfected fields, but still remain economically viable (Tremblay et al., 2005).

1.3.5 Chemical control

Several studies have reported the activity of several fungicides against clubroot, but with limited effectiveness where there is a high density of resting spores and highly virulent populations of P. brassicae. Knowledge of the modes of action of these fungicides can still allow a more effective application strategy.
The activity of ‘flusulfamide’ (2’,4-dichloro-α,α,α-trifluoro-4’-nitro-m-toluenesulfonanilide) was investigated against *P. brassicae* on Chinese cabbage by Tanaka *et al.* (1999). These researchers suggested that ‘flusulfamide’ was ineffective against *P. brassicae* already established in the cortical cells of the host plant. Clubroot was affected at the early stages in its life cycle since prior-treatment of ‘flusulfamide’ suppressed both the root-hair infection and gall formation by inhibiting the germination of the resting spores. Another commonly used chemical ‘quintozene’ (pentachloronitrobenzene) showed a limited effect on resting spore germination, but could control the already established *P. brassicae* within the cortical tissue of the host roots (Naiki and Dixon, 1987). Suzuki *et al.* (1995) later reported that ‘fluazinam’ possessed fungicidal action while ‘quintozene’ showed fungistatic action on the resting spores of *P. brassicae*. Several trials in Australia and New Zealand showed that ‘fluazinam’ (1.5 kg ai/ha) and ‘flusulfamide’ (0.6 kg ai/ha) were good control of clubroot and ‘fluazinam’ was subsequently registered for use in Australia in 1996 (Cheah and Falloon, 2000). Recently, Mitani *et al.* (2003) suggested that ‘cyazofamid’ (4-chloro-2-cyano-N,N-dimethyl-5-p-tolylimidazole-1-sulfonamide) is a novel fungicide with high activity levels against clubroot by directly inhibits resting spore germination. This pesticide can inhibit root-hair infection and gall formation, hence suppressing an increase of clubroot populations.

1.3.6 Biological control

Arie *et al.* (1998) reported that clubroot can be suppressed by epoxydon (5-hydroxy-3-(hydroxymethyl)-7-oxabicyclo [4.1.1] hept-3-en-2-one), a substance extracted from the fungus *Phoma glomerata*. This filamentous fungus is known to possess anti-tumour activity, phytotoxicity and anti-auxin activity but no microbial activity. Since galls resulting from *P. brassicae* infection contains 50-100 times more IAA than uninfected roots, they hypothesised that an increase enzymatic hydrolysis of IAA by epoxydon and other anti-auxins could
suppress the disease. This could offer the possibility of the development of new agrochemicals effective against clubroot.

A new biological method for the control of clubroot has been developed by stimulating the germination of resting spores after the application of an agent such as Posidonia powder, caffeic acid, coumalic acid and corilagin (Ohi et al., 2003). These researchers suggested that the resulting primary zoospores would be short-lived due to the absence of a host and an increased fungicidal sensitivity. These chemicals will drastically reducing the spore density and hence preventing the formation of galls upon plating of crops. The practical application of some of these agents could be difficult.

Alternatively, Friberg et al. (2008) investigated the effect of earthworms and incorporation of perennial ryegrass in clubroot-infested soil. These authors (and other previous studies) postulated that upon passage of the *P. brassicae* spores through the alimentary canal of earthworms, these spores should lose some of their ability to infect plants. Their results did not support their hypothesis, possibly due to the differences in the effect on *P. brassicae* among earthworm species.

In conclusion, current control measures have had limited success in preventing and controlling clubroot disease in Australia. The breeding of clubroot resistant crops as well as advancements in sustainable agrochemicals are required for the long-term survivability of the Australian *Brassica* industry.

### 1.4 Responses of the *Brassica* host during clubroot infection

Due to the impractical, costly and environmentally harmful current control measures, the development of resistant *Brassica* varieties is a viable option to minimise loss (discussed later
in Section 1.5 and 1.6). A detailed understanding of what makes a plant resistant to clubroot could improve the effectiveness of breeding strategies. In this section, the novel and current insights on plant defence mechanisms in general as well as hormonal responses upon clubroot invasion, is reviewed.

1.4.1 Novel insights on the initiation of plant defence mechanisms

Plant defence responses against pathogens have been extensively studied for many years to reduce crop damage caused by pathogen attack. The introduction of the well-characterised *Arabidopsis*-pathogen model system has improved our understanding of the plant defensive arsenals. This model has helped in the discovery and identification of resistance genes whose responses have been categorised into three main pathways. These are (a) innate resistance, conferring a broad-spectrum non-specific defence, (b) gene-for-gene resistance, when the pathogen carries a particular avirulent gene that matches to a particular resistance gene in the host and (c) systemic acquired resistance (SAR) that initiates a strong ‘whole-plant’ defence against re-infection.

**Innate resistance in plants:**

In plant, inducible defence responses are triggered by two levels of microbial recognition. The first line of microbial recognition relies on pathogen-associated molecular patterns (PAMPs) in plant innate immunity. Their recognition leads to PAMP-triggered immunity (PTI), in which pattern-recognition receptors (PRRs) recognise molecules that are conserved among a large group or class of microbes (Bent and Mackey, 2007; Zipfel, 2009). The major PRRs and their association with other signalling components are illustrated in Figure 1.6. Of particular interest are the CERK1 receptors that possess the chitin high-affinity-binding site CEBIP, a transmembrane protein with extracellular LysM domains. Consequently, *Arabidopsis cerk1*
Figure 1.6. Plant pattern-recognition receptors (PRR) and their signalling adapters, involved in PAMP-triggered immunity (PTI aka innate resistance). FLS2 and EFR possess a kinase domain (BAK) and these receptors recognise flagellin peptides and bacterial elongation factors respectively, CeBiP is a chitin-binding protein that may be coupled to a kinase domain (CERK), LeEIX1/2 detect fungal ethylene-inducing xylanase (EIX), GBP are soluble glucan-binding protein and PERP1 recognises damage-associated molecular patterns (DAMP) (Zipfel, 2009).
mutants were more susceptible to adapted fungi such as *Alternaria brassicicola* (Wan *et al.*, 2008) and surprisingly, as well as to adapted bacterium (Gimenez-Ibanez *et al.*, 2009). This demonstrated that CERK1 is not only restricted to chitin perception but to other molecules carrying N-acetylglucosamine moieties (Zipfel, 2009). Many questions remain unsolved, in particular on the specificity of the interactions between PRRs and their adapters as well as the molecular events occurring down-stream of PRR activation. The targeting of PRRs and their signalling adapters by microbial effectors (discussed in following paragraphs) clearly demonstrates their major importance for plant innate immunity.

**Gene-for-gene resistance in plants:**
Successful pathogens are able to suppress PTI by secreting effectors in the apoplast or directly in the cytoplasm of host cells, leading to effector-triggered susceptibility. As a second line of defence, some plant cultivars have evolved resistance proteins (*R* proteins) to recognise particular effectors directly or indirectly (discussed later), leading to effector-triggered immunity (ETI or gene-for-gene resistance) (Bent and Mackey, 2007; Zipfel, 2009). In other words, *R* genes encode putative receptors that respond to the product of avirulence (‘avr’ or effector) genes expressed by the pathogen during infection (Flor, 1947; Hammond-Kosack and Parker, 2003). Many *R* genes recognise only a limited number of pathogen strains and do not provide protection against a wide range of pathogens (McDowell and Woffenden, 2003). Many *R* genes against many different pathogens have now been cloned from a variety of plants and can be grouped into several super-families, based on protein domains as illustrated in Figure 1.7. The vast majority of *R* genes contains a leucine rich repeat (LRR) motif domain and belongs to the NB-LRR or LRR-Kinase superfamilies that have initially been identified in tomato, tobacco and *Arabidopsis* by map-based cloning or transposon tagging (Dangl and Jones, 2001; Jones, 2001). One of the most studied pathogen receptor is the *N* gene, a member of the TIR / NBS / LRR class of disease resistance genes in tobacco. Of
Figure 1.7. Major families of intra- and extra-cellular R proteins, involved in elicitor-triggered immunity (ETI aka gene-for-gene resistance). The majority of R proteins contain tandem leucine-rich repeats (LRR, depicted in blue) that may be coupled to nucleotide-binding (NB) sites, toll and interleukin receptors (TIR), coiled-coil domain (CC), serine-threonine kinase domain (KIN), PEST (Pro-Glu-Ser-Thr) domain for receptor-mediated endocytosis (RME) (McDowell and Woffenden, 2003).
particular interest is their LRR domains that can be separated into a variable and highly conserved segment. The elongated and curved shape of the LRR structure creates an extended and concave surface that is topographically suited for molecular docking and provides an interface for protein-protein interactions (Stange et al., 2008). These authors also reported that an effective defence response involved the induction of alternative splicing of the N gene upon recognition of an effector molecule. The variations on the sequence length and composition resulted in new ranges of specificity and more efficient recognition LRR domain, thus increasing the plant resistance gene repertoire in an adaptive way. This complex situation resembles the clonal expansion of the major histocompatibility complex (MHC) genes of the mammalian immune system (Stange et al., 2008). A single R gene may provide complete resistance to one or more strain of particular pathogen, when transferred to a previously susceptible plant of the same species. For this reason, R genes have been used in breeding programs for decades (McDowell and Woffenden, 2003).

The ‘Guard’ hypothesis:

Although direct association between at least two R-Avr protein pairs has been demonstrated in vitro, current data indicate that such direct R-Avr protein interactions occur only rarely. Instead, recognition of pathogen-derived proteins within a complex is involved (Hammond-Kosack and Parker, 2003). The ‘Guard’ hypothesis, illustrated in Figure 1.8, describes R proteins as ‘antennae’ that perceive modified plant virulence target caused by the earlier binding of the Avr factor. This ‘Guard’ recognition system can not be circumvented by alterations of the Avr factor without affecting the virulence function of the pathogen as opposed to the classic receptor-ligand model.
Figure 1.8. The guarding of pathogen virulence targets by plant R proteins (Hammond-Kosack and Parker, 2003).

(a) Compatible interaction. A virulence target is present in a susceptible host plant. Upon pathogen infection, the Avr factor binds to its associated virulence target, resulting in modifications to the target. These changes lead to pathogen virulence and host susceptibility.

(b) Incompatible interactions in a resistant host takes place in two ways. (1) The R1 protein directly recognises the Avr factor or (2) The R2 is a guard protein, recognising the plant virulence target-Avr factor interaction and both resulting in the initiation of defence responses.
Host small RNAs in PTI and ETI in plants:

Emerging evidence indicates that host endogenous small RNAs represent an essential mechanism of control in plant immune responses (Figure 1.9). Based on their precursor structures, small RNAs can be divided into microRNAs (miRNAs) and small interfering RNAs (siRNAs). These small RNAs can either guide post-transcriptional gene silencing by mRNA cleavage / degradation or translational inhibition or guide transcriptional gene silencing by DNA methylation and / or chromatin modification (Baulcombe, 2004; Matzke et al., 2007). For example in PTI, miR393 was the first miRNA to be identified in Arabidopsis and is induced by bacterial flagellin peptide. This miRNA negatively regulates auxin signalling by targeting the mRNAs of auxin receptors for degradation. This is because auxin is a plant growth-promoting hormone that may repress the salicylic acid-mediated defence pathway and prioritising defence signalling over plant growth may activate a series of defence responses (Padmanabhan et al., 2009). Hence, this indicates mechanisms by which pathogens can overcome host PTI. Plant bacteria have developed certain effector proteins (known as bacterial silencing repressors, BSR) to suppress the miRNA processing or accumulation. To overcome these BSRs, plants have evolved more endogenous small RNAs to explicitly regulate R gene-mediated ETI (Padmanabhan et al., 2009).

1.4.2 Current knowledge on the inducible responses against plant pathogens

Recent advances in plant defence signalling research have revealed that plants are capable of differentially activating inducible, broad-spectrum defence mechanisms, depending on the type of pathogen encountered. The chronological events in elicitor-induced defence responses is illustrated in Figure 1.10 and an overview of the local signalling network controlling activation of local defence responses (Figure 1.11) is reviewed below. Additionally, plant hormones such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET), play major roles
Figure 1.9. Plant immune responses controlled by endogenous small RNAs (Padmanabhan et al., 2009).
(a) The recognition of PAMPs triggers small RNA pathways (innate resistance). Small RNAs are generated by RNase III ribonuclease Dicer-like (DCL) proteins and loaded into Argonaute (AGO) proteins to regulate gene expression transcriptionally and/or post-transcriptionally, which activates PTI and basal defences.
(b) Bacteria have evolved bacterial silencing repressors (BSRs) delivered by type III secretion system into host cells to suppress small-RNA pathway.
(c) Plants have evolved $R$ proteins (gene-for-gene resistance) and more small rRNAs to overcome the function of BSRs.
The sequential events in elicitor-induced defence responses can be organised as follows: perception of elicitor by receptor, reversible phosphorylation and dephosphorylation of plasma membrane and cytosolic proteins, spiking of cytosolic $\text{Ca}^{2+}$ ions, depolarisation of plasma membrane, $\text{Cl}^-$ and $\text{K}^+$ efflux / $\text{H}^+$ influx, extracellular alkalinisation and cytoplasmic acidification, mitogen-activated protein kinase (MAPK) activation, NADPH oxidase activation and reactive oxygen species (ROS) production, early defence gene expression, ethylene and jasmonate production, late defence gene expression and secondary metabolite production.
Figure 1.11. Overview of the local signalling networks controlling activation of local defence responses, reproduced from Hammond-Kosack and Parker (2003). Four main $R$-protein-dependent signalling cascades are shown as (a) Pto serine-threonine protein kinase, (b) CC-NB-LRR, (c) TIR-NB-LRR and (d) RPW8.

Note: The list of *Arabidopsis* mutant and transgenes known to compromise or enhance plant defence without causing spontaneous cell death are denoted in boxes and are comprehensively reviewed by the above authors.

(a) Pto-kinase-mediated resistance involves both RAR1 and direct interaction with the Pti4/5/6 transcription factors to directly activate PR protein gene expression.

(b, c) Most CC-NB-LRR-type $R$ proteins require NDR1, whereas TIR-NB-LRR proteins are dependent on EDS1.

(d) RPW8 operates through EDS1 and SGT1 proteins.

A possible convergence point of the four $R$-protein-triggered pathways is at RAR1 / SGT1, both operating upstream of the hypersensitive response (HR) and oxidative burst (OB). Another early defence signal generated is nitric oxide (NO), which can potentiate both the HR and OB.
Activation of defence responses by TIR-NB-LRR proteins involves the combined actions of EDS1 and PAD4, EDS5, SA and NPR1. EDR1, MAPK4 and SSI2 can each repress activation of the SA pathway, while various SA-binding proteins (SABP) located in distinct cellular compartments may modulate the local concentrations of available SA signal.

The OB can potentiate SA-mediated signalling directly and via the induction of various MAPK cascades. NPR1 is required downstream of SA, which also stimulates NPR1 translocation into the nucleus where it interacts with TGA transcription factors and induces the expression of PR genes.

The signalling cascades (a), (b), (c) and (d) are important for resistance biotrophic pathogens. A different signal transduction network (e) leads to the activation of parallel JA and ET signalling cascades. Steps upstream of JA and OPDA are negatively regulated by CET1 and CET3, while downstream, CO1 and JAR1 are required sequentially to activate resistance to necrotrophic pathogens. Transduction of the ET signal requires EIN2 and leads to expression of the PDF1.2 defence marker gene. (f) The signalling proteins EDR1, MPK4 and SSI2 have roles in communication between the SA and JA / ET signalling networks.

CET1/CET3, constitutive expression of thionin 1/3; COI1, coronatine insensitive 1; EDR1, enhanced disease resistance 1; EIN2, ethylene-insensitive 2; NDR1, non-race specific disease resistance 1; NDS1, non-race specific disease susceptibility 1; OPDA, 12-oxophytodienoic acid; PAD4, phytoalexin-deficient 4; PDF1.2, plant defensin 1.2; Pti4/5/6, Pto-interacting 4, 5 and 6; RAR1 (required for Mla-dependent resistance 1); SGT1, skp1-Cullin-F-box protein-mediated ubiquitination; SID2, SA induction deficient 2; SSI2, suppressor of salicylate insensitivity of NPR1-5.
in the network of defence signalling pathways and their synthesis and downstream reactions are included in this review.

1.4.2.1 Accumulation of secondary messengers

Following perception of PAMPs or Avr proteins of pathogens (elicitor signals), plant receptors (PPRs or R proteins) are activated and in turn, trigger their effectors, such as ion channels, GTP binding proteins (G-proteins) and protein kinases. These activated plant effectors generate secondary messengers, which further amplify the elicitor signal for downstream reactions.

GTP binding proteins:

G-proteins represent a class of eukaryotic proteins, including heterotrimeric complexes consisting of α-, β- and γ-subunits, and monomeric small G-proteins (Jones and Assmann, 2004). Increasing evidences show that G-proteins regulate various cellular processes related to growth, development, hormone signalling and defence responses. These G-proteins are activated by coupling with plant receptors to mediate the elicitor signal and to trigger further effectors, such as ion channels and phospholipases early in the signal transduction network (Zhao et al., 2005).

Ion fluxes and Ca^{2+} signalling:

Elicitor-induced ion fluxes, such as K^{+} / H^{+} exchange, Cl⁻ effluxes and Ca^{2+} influx are generally observed as the earliest responses of plant cells (Zhao et al., 2005). Among these ion fluxes, Ca^{2+} influx is regarded as one of the most significant event since this ion is a key second messenger for many diverse physiological changes and cellular processes (White and Broadley, 2003). Studies reported that activation of defence response by elicitors was more
effective in the presence of Ca\(^{2+}\) in plants by either the influx of Ca\(^{2+}\) across the plasma membrane or release of Ca\(^{2+}\) from internal stores (Vidhyasekaran, 2007).

Cellular Ca\(^{2+}\) levels are tightly regulated and small changes in Ca\(^{2+}\) concentration can provide information for the modification of enzyme activity and gene expression needed for subsequent responses. These changes are perceived by various intracellular Ca\(^{2+}\) binding proteins to regulate a series of signalling cascades (Vidhyasekaran, 2007). For example, calcium-dependent protein kinases (CDPKs) have protein kinase and calmodulin-like Ca\(^{2+}\)-binding domains that can be triggered by Ca\(^{2+}\). The calmodulin-like protein acts as a sensor relay and communicates its changed conformation upon binding with Ca\(^{2+}\) to interacting partners such as protein kinases, peroxidases, NADPH oxidases, and phospholipases (Cheng et al., 2002).

**Phospholipid-signalling system:**

Several phospholipids commonly found in plant cell membranes play important roles in signal transduction. Elicitor-induced phosphoinositide breakdown has been reported to occur in many different plants. One of the significant phosphatidylinositol turnover paths is phosphatidyl inositol 4,5-diphosphate (PIP\(_2\)) hydrolysis by phospholipase C (PLC). This enzyme activated by Ca\(^{2+}\) spikes, can cleave PIP\(_2\) to yield two secondary messengers, inositol-1, 4, 5-triphosphate (IP\(_3\)) and diacylglycerol (DAG) (Berridge and Irvine, 1989). IP\(_3\) can further mobilise Ca\(^{2+}\) from intracellular calcium stores, and hence in signal transduction to downstream reactions (Zhao et al., 2005; Vidhyasekaran, 2007).

**1.4.2.2 Oxidative burst and reactive oxygen species**

The oxidative burst is the fastest active defence response induced by pathogen attack or elicitor treatment. The reactive oxygen species (ROS), predominantly superoxide anion (O\(_2^{-}\))
and hydrogen peroxide (H$_2$O$_2$) are toxic intermediates resulting from the reduction of molecular O$_2$. Plants possess mechanisms for generating ROS including NADPH oxidase and apoplastic peroxidases. In many plant systems, biphasic ROS generation is reported; the first phase occurs at about 10-30 min and second at 1-3 h after fungal elicitation (Zhao et al., 2005). These superoxide anions are rapidly dismutated either non-enzymatically or via superoxide dismutase (SOD) (Hammond-Kosack and Jones, 1996). The ROS have various effects on plant defence responses, including cell wall re-enforcement, hypersensitive cell death, defensive gene activation, as well as defensive compound induction and secondary metabolite accumulation (Vidhyasekaran, 2007).

1.4.2.3 Salicylic acid-signalling system

It is well established that infection by pathogens leads to synthesis of salicylic acid (SA). H$_2$O$_2$ appears to be important for SA accumulation in plant-pathogen-interactions, which ultimately results in the induction of plant systemic acquired resistance (SAR). SA has been reported to act locally in intracellular signal transduction and systemic intercellular signal transduction (Zhao et al., 2005). SA quickly accumulates at the site of infection during plant attack and hypersensitive reactions before inducing a wide range of defence responses. The importance of SA-signalling system in the induction of host defences was studied by developing transgenic plants expressing the bacterial gene $NahG$ (Delaney et al., 1994). This genes encodes the enzyme salicylate hydroxylase that inactivates SA. Consequently, these transgenic plants were unable to accumulate SA and are unable to develop HR and demonstrated increased susceptibility to pathogens. In contrast, mutants with high constitutive levels of SA show enhanced resistance and spray treatments with SA has been used to induce disease resistance in plants (Delaney et al., 1994).
NPRI (nonexpressor of PRI) gene is an important regulator of responses downstream of SA in Arabidopsis (Eulgem, 2005). The NPR1 protein is stimulated by SA for translocation to the nucleus where it interacts with TGA transcription factors. The latter bind to promoter regions of defence genes, leading to their expression. NPRI ‘knock-out’ mutants are able to accumulate normal levels of SA in response to pathogen infection but fail to mount SAR. This indicates that NPR1 is a key regulatory factor that functions in the SAR signalling pathway (Delaney et al., 1995). SA may also enhance release of \( \text{H}_2\text{O}_2 \) and \( \text{H}_2\text{O}_2 \)-derived active oxygen species as well as suppress the \( \text{H}_2\text{O}_2 \) scavenging-enzyme such as catalase and peroxidase. The resulting elevated levels of \( \text{H}_2\text{O}_2 \) may trigger a series of other defence responses (Vidhyasekaran, 2007).

1.4.2.4 Jasmonate-signalling system

Jasmonates, including jasmonic acid (JA), are a family of cyclopentanone compounds synthesised from linolenic acid via the octadecanoic pathway and are members of a large class of oxygenated lipids called oxylipins. They inhibit plant growth generally but also promote diverse processes, including fruit ripening, senescence, tuber formation, tendril coiling, pollen formation, and defence responses against insect pests and pathogens (Ellis et al., 2002b). JA is produced ubiquitously in all plant tissues and JA synthesis is induced by a number of biotic and abiotic stresses, including wounding, water deficit, and pathogen attack via phosphorylation and calcium ion influx (Vidhyasekaran, 2007).

In Arabidopsis, the JA response pathway generally is required for defences against necrotrophic pathogens and chewing insects, while the salicylic acid (SA) response pathway is generally required for specific, \( R \) gene-mediated defences against biotrophic and possibly against necrotrophic pathogens (Ellis et al., 2002a). The importance of JA has been demonstrated by using Arabidopsis coil mutant that lacks the F-box protein (receptor) for JA.
synthesis or perception, by applying JA onto plants and by developing transgenic plants overproducing JAs. The constitutive production of JA in the Arabidopsis cev1 mutant was accompanied by constitutive expression of defensin PDF1.2, thionin Thi2.1 and chitinase and showed enhanced resistance against pathogens (Ellis and Turner, 2001).

1.4.2.5 Ethylene-signalling system

Ethylene (ET) production is one of the earliest chemically detectable events in pathogen-infected plants or in plants treated with elicitors. It is a phytohormone that regulates a wide range of plant processes, from growth and development to defence responses and its production can be induced by various stresses such as wounding, ozone, microbial pathogen and insect attack (Zhao et al., 2005). The role of ethylene is complex and stimulates defence mechanisms against several pathogens and also induces susceptibility to several pathogens. It is reported that ethylene production is often required for basal defence but its production may ambiguously aggravate symptom development (Pieterse et al., 2001).

Downstream of ET receptors, the Arabidopsis gene CTR1 is activated and encodes a protein that belongs to the Raf family of serine-threonine protein kinases that initiate MAPK-signalling cascades (Wang et al., 2002). Another gene in Arabidopsis is EIN3 (ET-insensitive 3) which acts downstream of CTR1. EIN3 proteins can bind to primary ET response elements in the promoters of ethylene-responsive factor (ERF1) gene, resulting in their strong activation. The ERF1 belongs to large family of plant-specific transcription factors referred to as ET-response-element binding proteins (EREBPs). EIN3 stimulates ERF1 expression and these EREBPs bind to the GCC box, a DNA motif associated with ET- and pathogen-induced genes expression in plants (Wang et al., 2002). GCC box is a general transcriptional regulatory element present in the promoter of pathogenesis-related (PR) genes such as
chitinase and β-1,3-glucanse and other genes like phenylalanine lyase (PAL) and osmotin-like proteins (Vidhyasekaran, 2007).

1.4.3 Current knowledge on the hormonal responses upon clubroot invasion

The most obvious symptom of clubroot is the formation of galls in the roots and in many plant-microbe interactions, this can be activated by alterations in the auxin and / or cytokinin metabolism. The potential role of these phytohormones during *P. brassicae* infection is well documented (Ludwig-Müller *et al.*, 2009). Recently, Devos *et al.* (2006) performed a differential protein analysis of infected versus non-infected *A. thaliana* roots and reported that 12% of the visualised proteins had altered abundance compared with non-infected plants. These changes involved proteins in metabolism, cell defence, cell differentiation and detoxification. The data on the plant hormone content of cabbage roots during clubroot infection remains conflicting and non-synchronised.

**Changes in auxin levels:**

Researchers have demonstrated that there was a higher level of indole-3-acetic acid (IAA) at 6 dai (Devos *et al.*, 2006), 10 and 12 dai (Ludwigmüller *et al.*, 1993), at 17 dai (Kavanagh and Williams, 1981), at 36 dai (Butcher *et al.*, 1974) and at 20, 25, 30, 40 and 60 dai (Raa, 1971) using bioassays and analytical techniques. In contrast, infected roots had lower levels of IAA than control roots at 14 dai (Ludwigmüller *et al.*, 1993), at 28 dai (Kavanagh and Williams, 1981) and at 24, 48 and 54 dai (Butcher *et al.*, 1974). The published data can be difficult to compare because different races of *P. brassicae* were used and infection is dependent on the clubroot pathotype (Siemens *et al.*, 2002).

IAA biosynthesis in the *Brassicaceae* occurs *via* the indole glucosinolates, which are produced during hypertrophy of the infected roots (Ludwig-Müller, 2009) and possibly
involves the up-regulation of auxin-biosynthesis enzymes: nitrilase (Ando et al., 2008) and myrosinase (Grsic et al., 1998; Halkier and Gershenzon, 2006). Devos et al. (2005) also reported that plant growth was temporarily stimulated at the early stages of infection when compared to the controls. This might be due to the initial accumulation of total IAA pool, as well as the induction of xyloglucan endotransglucosylate hydrolase (XTH) action, resulting in cell expansion and the early growth promotion in Chinese cabbages. Of particular interest was the clubroot resistance of an *A. thaliana alh1* mutant that is defective in the cross talk between ethylene and auxins. This mutant may be resistant because host IAA transport to the site of infection was hampered, resulting in a lack of gall development (Devos et al., 2006). It should be noted that an indole glucosinolate-free *Arabidopsis* mutant was not altered by clubroot development (Siemens et al., 2002). Mutants altering glucosinolate metabolism and plant hormone responses may provide alternative strategies for breeding purposes.

**Changes in cytokinin levels:**

As opposed to IAA, measurements for cytokinin level in root tissues are more consistent. Dekhuijzen and Overeem (1971) showed that cytokinin levels from extracts of clubbed turnip tissue were significantly higher than that of uninfected roots. This is supported by levels of zeatin and zeatin riboside (ZR) two to three times higher in 25 dai tissues and the cytokinin-independence of the roots when grown in tissue culture (Dekhuijzen, 1980). Dekhuijzen (1981) found that ZR and its glucose derivatives were present in plasmodia isolated from three weeks old clubroot-infected explants while the host cytoplasm contained ZR and only small amount of its derivatives. Müller and Hilgenberg (1986) demonstrated that secondary plasmodia from 23 dai roots could assimilate $^{14}$C-adenine *in vitro* and incorporate it into *trans*-zeatin. It was concluded that the plasmodia of *P. brassicae* could partially generate cytokinins.
During the early infection stage, there was initially a reduction in active cytokinins such as zeatin and its precursor probably due to the plasmodia acting as a cytokinin sink (Devos et al., 2005). Devos et al. (2006) indicated that *P. brassicae* also synthesises cytokinins since increased levels of these hormones in susceptible hosts may be beneficial for the growth of the pathogen. These plasmodial-produced cytokinins trigger a local re-initiation of cell division in the root cortex resulting in a *de novo* meristematic area that acts as a sink for host-derived IAA, carbohydrates, nitrogen and energy to maintain the pathogen and to trigger gall formation. Another study by Ando et al. (2005) supported the involvement of cytokinin due to the formation of galls in root tissues with high levels of isopentenyl transferase (IPT) activity. It remains unknown how plants control the reactivation of the cell cycle during clubroot development.

In conclusion, a key finding was the lack of information on how *Brassica* vegetable crops specifically recognise *P. brassicae* and initiate the signalling networks controlling activation of defence responses against infection. It is not known if the defence models (and the genes) derived from extensive functional genomic studies on *Arabidopsis*, may apply to this complex *Brassica*-clubroot interaction. Despite the well-documented proteomic studies on the development of galls in the roots, the gap in knowledge at the very early stage of infection has impeded the breeding of clubroot-resistant cultivars (described below).

### 1.5 Breeding for clubroot resistance in *Brassica*

In this section, an overview on the status of clubroot resistance breeding is provided. It might be helpful to consider the phylogenetic relationship among *Brassica* species (Figure 1.12): *B. napus* being a recent natural amphidiploid hybrid between *B. rapa* and *B. oleracea* (Nagaharu, 1935). Based on recent chromosome nomenclature, *B. rapa* has chromosomes A1
Figure 1.12. The ‘Triangle of U’ showing the genetic relationships between the six species of the genus *Brassica*. Chromosomes from each of the genomes A, B and C are represented by different colours (Nagaharu, 1935).
to A10 and *B. oleracea* chromosomes C1 to C9 which together form the *B. napus* genome (Diederichsen *et al.*, 2009).

### 1.5.1 Plant breeding: source of resistance

Plant breeding is the science of genetically modifying and selecting for plants with desirable trait/s for the benefit of man. While breeding objectives and approaches vary widely with species, most plant breeding programs rely on the screening of genetically variable populations by applying selection pressure. The latter allowed the selective inclusion of desirable or exclusion of undesirable genotypes from the population (Beversdorf and Kott, 1987). Breeding programs designed to produce disease resistant plant varieties should firstly begin with the search and identification of plants with resistance-conferring genes (Allard, 1960). However, breeding for disease resistance does not only involve finding major genes. Another approach is the accumulation or epistatic interactions of minor genes that give transgressive segregation for high disease resistance (*i.e.* formation of extreme phenotypes by segregating hybrid populations). Polygenic resistance is a valuable and durable form of resistance (Lindhout, 2002; Werner *et al.*, 2008).

### 1.5.2 Disease epiphytotics: the resistance test

The ability to correlate genotype and phenotype is an important criterion in breeding for any plant characteristics. This is a major concern in breeding for clubroot resistance since *Brassica* lines may not be distinguishable in the absence of the parasite. Clubroot resistance breeding programs may not proceed unless the pathogen is present to induce the symptoms that would allow the *Brassica* lines conferring adequate resistance to be distinguished from susceptible ones. Due to the irregular level of infection or other environmental conditions in nature, it may not be possible to accurately differentiate between the resistant, partially-resistant and susceptible types. Breeding for disease resistance are based on artificially
induced infections (Allard, 1960), thus permitting an accurate and reproducible test system. The specialised knowledge brought forward by plant pathologists was essential in the infection procedures of these resistance tests and as a result, this demonstrated the need for cooperative efforts between these two disciplines in programs for disease resistance (Allard, 1960; Niks et al., 1993). Hence, researchers / plant breeders around the world have used such methodology in the identification and breeding of Ascochyta blight-resistant chickpea (Cicer arietinum L.) (Singh et al., 1981; Reddy and Singh, 1990; Singh, 1993), yellow rust-resistant bread wheat (Triticum aestivum) (Robert et al., 2000) and in the genetic mapping of clubroot resistance genes in oilseed rape (Brassica napus L.) (Werner et al., 2008).

1.5.3 Difficulties in clubroot resistance breeding

Since no highly resistant varieties of Chinese cabbage was available, Yoshikawa (1981) bred clubroot resistant lines of Chinese cabbage by introducing a single dominant resistant gene from European turnip. Subsequently, more than 50 clubroot resistant F1 hybrid cultivars of Chinese cabbage have been released in Japan, but these Chinese cabbage cultivars becoming susceptible in many parts of Japan. In cabbage breeding programs for disease resistance, the identification of resistant sources are performed in parallel with the recovery of marketing type and the elimination of undesirable traits from the resistant source. This is particularly difficult when inter-specific crosses are made with resistant resources (Nomura et al., 2005) or during the incorporation of the resistance trait into desired morphotypes of B. oleracea (Baggett and Kean, 1985).

Another reason for the inefficient deployment of clubroot resistant varieties was due to the complex nature of interaction between the P. brassicae populations and Brassica resistance genes. The differences in the pathogenicity of P. brassicae isolates were determined by the extensive use of the differential series by William (1966) and Buczacki et al. (1975). Many
studies have recognised that considerable differences in pathogenicity exists between field populations and even within field isolates (Buczacki et al., 1975). During an infection, both non-specific and isolate-specific resistance responses have been reported (Manzanares et al., 1996). Most of these studies were performed using non-homogenous field isolates of clubroot since even single root gall might possess different pathotypes or mixture of clubroot genotypes. Hence, the race-specificity of the previously identified resistance gene is difficult to define or was not addressed (Rocherieux et al., 2004). In addition, the expression of some major and minor resistance genes or QTLs can be concealed in the event of a strong resistance in the host against a specific pathogenic factor in the pathogen (Rocherieux et al., 2004).

Lastly, the breeding for clubroot resistance was hampered since the evaluation of resistance to a pathogen is generally affected by the differences in resistant hosts, screening methods and pathogen isolates and environmental factors such as the humidity level and temperature of the soil. Hence it is difficult to compare the effects of clubroot resistant genes among the published studies since these factors can influence the outcome of inoculation (Hamilton and Crete, 1978).

1.5.4 Overcoming difficulties in clubroot resistance breeding

A homogenous *P. brassicae* isolate (spore isolates developed from a single resting spore) can be used to simplify and assist in the detection of resistance genes and in the study of their specificity. The advantage in using single spore isolates to study resistance is that interaction between different pathotypes is avoided and a clearer picture of the mechanism involved can be obtained (Rocherieux et al., 2004). Piao et al. (2004) reported different results when single spore isolates and contaminated soil were tested: there was a high resistance in the plant hosts when single spore isolates were used while those inoculated with field isolates were either high or intermediately resistant. Hence, the use of single spore isolates is a pre-requisite for
the better understanding of the complex interaction occurring during an infection and for the accurate scoring of clubroot resistance. The routine examination of the virulence of a large number of genetically uniform single spore isolates collections may not be feasible due to the time required for their isolation and characterisation and the variable success of the single spore isolates method since single spores may not produce genetically uniform progeny (Tinggal and Webster, 1981).

Plant breeders investigate resistance in related wild species or genera and also consider the advantages of inter-specific hybridisation (Allard, 1960). Alternatively, the induction of resistance through mutagenic agents may be attempted. The backcross or pedigree methods of breeding would usually be performed to overcome the usually unsuitable agricultural properties of these wild-type lines. With either method, one of the parents, chosen for its good agronomic characteristics, is crossed with the other parent that demonstrated high level of resistance, preferably due to multiple dominant genes against a wide range of clubroot pathotypes (Allard, 1960; Moreno-Gonzalez and Cubero, 1993).

Ultimately, the identification of a complete set of resistance genes and their linkage markers would provide valuable tools for the establishment of a successful clubroot resistant breeding system (Suwabe et al., 2003). DNA markers linked to desirable traits such as disease resistance, morphological and physiological features can be useful in the genetic analysis and selection of large number of individuals. The use of these markers offers many advantages in the marker-assisted selection (MAS) breeding of plants since they are unaffected by environmental factors and can assist in the analysis of polygenic traits (Asíns, 2002; Werner et al., 2008). Subsequently, the pyramiding of different disease resistance genes using DNA markers is one of the most promising fields in marker-assisted breeding (Piao et al., 2004). The breeding for durable resistance of phenotypically similar cultivars will not be easy;
however, this is an ideal strategy to overcome the susceptibility or breakdown of clubroot resistance for the long-term sustainability of the Australian *Brassica* industry.

1.6 Marker-assisted breeding for clubroot resistance in *Brassica*

In recent years, molecular-assisted breeding is one of the major advancements for crop improvement. Molecular-assisted breeding is comprised of two major areas, namely the transgenic crops and the molecular marker technology. While the production and commercialisation of transgenic crops have had significant success, molecular marker technology has yet to be fully utilised in plant breeding programs. The latter is user-friendly and does not raise biosafety or bioethics questions as transgenics (Gupta et al., 2001). There has been emphasis on the development of newer and more efficient molecular marker systems involving inexpensive non-gel-based assays with high throughput detection systems. A series of molecular marker systems can be classified into three classes: (a) the first generation including restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPDs) and their modifications; (b) the second generation involving simple sequence repeats (SSRs or microsatellites), amplified fragment length polymorphisms (AFLPs) and their modified forms and (c) the third generation comprised of expressed sequence tags (ESTs) and single nucleotide polymorphisms (SNPs). ESTs are generally used for functional genomics studies and SNPs remain the only new molecular marker system for individual genotyping needed for MAS (Gupta et al., 2001). The following is a review on the mapping and tagging of clubroot-resistant genes using a number of molecular markers, genetics of clubroot resistance in *Brassica* species and the current application of recently available genomics tools for identifying genes involved in clubroot resistance.
1.6.1 Overview of molecular markers for clubroot resistance

Several research groups have developed DNA markers linked to clubroot resistance loci in *Brassica* crops. For example, a number of RAPD and RFLP markers in *B. rapa* have been identified (Kuginuki *et al.*, 1997). Manzanares-Dauleux *et al.* (2000b) designed RAPD markers linked to a major gene and to QTL involved in *B. napus* clubroot resistance. Grandclement and Thomas (1996) designed RAPD markers for polygenic resistance against clubroot while Voorrips *et al.* (1997) mapped two resistance genes based on 92 RFLP and AFLP markers in *B. oleracea*. Since the AFLP technique allows the simultaneous study of a large number of locus-specific markers, it has been broadly used to target specific plant loci (Vos *et al.*, 1995).

Alternatively, a PCR-based marker is much simpler and affordable. Some researchers have converted their AFLP markers from *B. rapa* (Piao *et al.*, 2004) or both their RAPD and RFLP markers from *B. oleracea* (Nomura *et al.*, 2005) that were closely linked to major QTLs for clubroot resistance into SCAR markers. Recently, SSRs have been developed as DNA markers in various studies such as in MAS, linkage mapping and population analysis in various species (Gupta and Varshney, 2000). SSRs are repeated nucleotide motifs (1-6 bp) throughout the plant genome and are highly polymorphic due to the variations in the number of repeats (Suwabe *et al.*, 2003). Hence, SSRs are the preferred DNA marker compared with RFLPs, AFLPs and RAPDs since they are inherited co-dominantly and can be analysed in a PCR-based system (Morgante and Olivieri, 1993).

1.6.2 Genetics of clubroot resistance in *Brassica* species

To breed clubroot-resistant cultivars of *Brassica* species, a number of *Brassica* germplasms were evaluated using the above molecular markers. This allowed sources of resistance to be
identified using the above molecular markers and the varying genetics of resistance to clubroot is reviewed below.

**Nature of clubroot resistance:**

Crute *et al.* (1980) indicated the occurrence of clubroot resistance in different *Brassica* species, namely the commonly cultivated *B. napus*, *B. rapa* and *B. oleracea*. Subsequently, other studies suggested that clubroot resistance was under polygenic control and involves both recessive (Crute *et al.*, 1983; Voorrips and Visser, 1993) and dominant alleles (Grandclement *et al.*, 1996). Fuchs and Sacristan (1996) suggested that since a single dominant allele controlled clubroot resistance in *A. thaliana*, a single locus in *B. rapa* and *B. napus* would be adequate to convey resistance against clubroot. Yoshikawa (1981) indicated that clubroot resistance in *B. rapa* is due to a major gene and some other genes with minor effect. This statement was later supported by Kuginuki *et al.* (1997) when these researchers identified a major locus that confers resistance to *P. brassicae* and the need of additional genetic element(s) to exhibit complete resistance.

QTLs and resistance (*R*) genes have frequently been observed to co-localise, indicating that quantitative resistance could result from the action of “weak” *R* gene alleles and qualitative resistance from strong alleles. Genes involved in defence responses such as production of antimicrobial compounds, cell wall strengthening, callose formation, lignification, the oxidative burst and genes encoding for metabolic enzymes may also be conferring quantitative resistance (Jubault *et al.*, 2008). These results suggest that clubroot resistance involves a complex polygenic mechanism among *B. rapa* and other crucifers.
QTLs for clubroot resistance in *B. rapa*:

Clubroot resistance traits were found in *B. rapa* (Karling, 1968; Buczacki et al., 1975). Among the European turnips, Suwabe *et al.* (2003) have identified two clubroot resistance loci namely *Crr1* and *Crr2*, that may exist on different regions or on different chromosomes. When both loci were homozygous, clubroot resistance was stronger when compared to heterozygous loci. These researchers suggested that clubroot resistance in *B. rapa* is under oligogenic control and the cooperation of both loci (epistasis) is necessary to generate resistance in *B. rapa*. Only *Crr2* is a novel gene for clubroot resistance since the *Crr1* linkage marker used in Suwabe *et al.* (2003) is tightly linked to Kuginuki *et al.*’s (1999) clubroot resistance marker. A third novel dominant clubroot resistance locus named as *Crr3* in a European turnip was identified through the use of sequence tagged-site (STS) markers developed from RAPD markers (Hirai *et al*., 2004). Although the precise map position of *Crr3* is unknown, this locus has been shown to be independent of the previously found clubroot resistance loci *Crr1* and *Crr2*. Previously, Matsumoto *et al.* (1998) reported a clubroot resistance locus, *CRa* and its linkage markers in fodder turnip (ECD02 host). Since they are RFLP markers and not converted into commonly usable markers, it is not known whether *CRa* matches to any of *Crr1*, *Crr2* and *Crr3* or is another independent clubroot resistance locus. This suggested the need to map the RFLP loci for the precise relationship of the identified clubroot resistance loci. Piao *et al.* (2004) found another clubroot resistance locus, *CRb* derived from a hybrid Chinese cabbage cultivar that was independent of *Crr1*, *Crr2* and *Crr3*. Recently, Sakamoto *et al.* (2008) reported two clubroot resistance loci, *CRk* and *C Rc* identified from an F₂ population of Chinese cabbage. *CRk* was located close to *Crr3* while the other locus, *C Rc* was independent from any published clubroot resistance loci.

As reviewed by Hirai (2006) and Piao *et al.* (2009), the occurrence of multiple clubroot resistance loci, namely *Crr1* on R8, *Crr2* on R1, *Crr3*, *CRa*, *CRb* on R3 linkage groups and
CReq in B. rapa, was not surprising. These clubroot resistance loci may be derived from the same region of the ancestral genome. The Crr1, Crr2 and CRb clubroot resistance loci were found to be aligned to the same region in the central part of the long arm of chromosome 4 of A. thaliana that contains clusters of disease resistance genes, termed the major recognition complexes. Since Fuchs andSacristan (1996) have identified similar major recognition complexes on the top of chromosome 1 in A. thaliana, Hirai (2006) suggested that the Crr1, Crr2 and CRb clubroot resistance loci may be derived by triplication and rearrangement. This is because the genome size of diploid Brassica species are around 3 to 4 fold that of A. thaliana and also have an extensive triplicate nature. It remains unclear whether the clubroot resistance loci found in crucifers are homologous but the differing position of these clubroot resistance loci may widen the choice for Brassica breeders.

QTLs for clubroot resistance in B. oleracea:

Both classical genetic studies without molecular markers as well as QTL analysis revealed the polygenic nature of clubroot resistance in B. oleracea (Hirai, 2006). Moriguchi et al. (1999) identified three QTLs from naturally-infected crops in the field and the most effective QTL explained 30% of the total phenotypic variation. When another experiment was performed under controlled conditions using field isolates, Voorrips et al. (1997) found a QTL, pb-3 which explained 54% of the phenotypic variation at the end of the linkage group 3. Using a genetic map constructed by RFLP, random and specific PCR-based markers, Rocherieux et al., (2004) have identified a total of nine clubroot resistance–related genomic regions. Of the nine QTLs identified, one was effective against all the isolates while the others were specific to one, two or three isolates and the degree of the QTL effect ranged between 20 to 87% depending on the isolates. These authors suggested that once major clubroot resistance genes are defeated by a clubroot pathotype, these genes may still possess some residual effect and the accumulation of these residual effects can give rise to quantitative resistance in B.
oleracea. Since Rocherieux et al.’s (2004) QTLs were also located at the end of a linkage group, Hirai (2006) indicated that a clubroot resistance gene at the end of a linkage group has a major effect in B. oleracea. The understanding of the clubroot resistance loci and marker-assisted selection in B. oleracea were hampered since none of these studies published the specific primer sequences or sequence of RFLP markers linked to these genes.

**QTLs for clubroot resistance in B. napus:**

Several attempts were made to introduce clubroot resistance from the ancestral species into B. napus. Diederichsen and Sacristan (1996) compared the expression of clubroot resistance in resynthesised B. napus and reported a less efficient defence mechanism than the original B. rapa and B. oleracea parents. Clubroot resistance from B. oleracea appeared to be strongly diluted, suggesting the presence of epistatic factors in the B. rapa genome. When highly resistant parents were used in resynthesised B. napus, it was however resistant against all P. brassicae isolates in their study (Diederichsen and Sacristan, 1996). Werner et al. (2008) also attempted to identify race-independent clubroot resistant genes from B. oleracea in resynthesised B. napus. These authors detected 19 QTLs all showing race specificity but no indication of race-independent QTLs that were assumed to be present in the B. oleracea C genome parent. Werner et al. (2008) concluded that the QTLs with the broadest effects against clubroot in B. napus are located in the B. rapa A genome.

**QTLs for clubroot resistance in A. thaliana:**

Recently, four additive QTLs (one moderate-effect locus Pb-At5.2 and three minor-effect loci, Pb-At5.1, Pb-At1 and Pb-At4) controlling partial resistance to clubroot were identified from the partially resistant A. thaliana parent Bur-0 through a QTL approach using two segregating populations (F2 and recombinant inbred lines) (Jubault et al., 2008). Several R-genes (NBS-LRR) are located within the confidence interval defined for these Pb-Ats QTLs, making them
potential candidate genes. The confidence intervals found for these QTLs involve large genomic regions and until further fine mapping of these four regions, these positional candidate genes should be regarded as hypothetical.

For several qualitative traits in *Brassica* breeding programs in general, MAS strategies were developed by traditional mapping approaches. The mapping of QTL is however not sufficient for the development of efficient DNA markers to identify genes for quantitative traits, such as clubroot resistance. These markers derived from QTL are not necessarily transferable to other material and the genetic distance between the markers and the quantitative traits are usually physically very large (Snowdon and Friedt, 2004). The complexity and reliance on gel-based detection system also make these techniques unsuitable for high-throughput selection. Hence, MAS for clubroot resistance has not been successfully achieved to date and will require the development of the more reliable gene-based marker system for automated plant screening.

1.6.3 Functional molecular marker for clubroot resistance

Functionally characterised genes, EST and genome sequencing projects have facilitated the development of molecular markers from the transcribed regions of the genome. Among the more popular and important molecular markers that were developed from ESTs are SNPs, SSRs and conserved orthologous sets of markers (COS) (Rudd *et al.*, 2005). Putative functions can be deduced for these markers derived from ESTs or using gene homology searches (BLASTX) with protein databases. These molecular markers generated from gene sequence data are called ‘functional’ markers. Functional markers have some advantages over random markers that are generated from an anonymous region of the genome because the former are completely linked to the desired trait allele. These are also known as ‘perfect’ markers. A set of perfect markers allows breeders to track specific alleles within pedigrees and populations and to minimise linkage drag (reduction in fitness in a cultivar due to
deleterious genes introduced along with the beneficial gene) (Varshney et al., 2005; Varshney et al., 2009).

### 1.6.4 Development of SNP molecular marker

SNPs are derived from single-based substitutions in the DNA sequences and are the most common form of DNA polymorphism in most organisms (Ganal et al., 2009). They have great potential as a marker system due to their high abundance and the possibility for extremely fine genetic mapping (Snowdon and Friedt, 2004). SNPs can help in the discovery of allelic variation directly within expressed sequences of resistance genes and in the development of haplotypes based on gametic phase disequilibrium for analyses of quantitative traits. There is some evidence that the stability of SNPs and the relative fidelity of their inheritance is higher than that of other marker systems like SSRs and AFLPs (Gupta et al., 2001). SNPs have proved ideal for MAS since these molecular markers are not only efficient and cost-effective but also are amenable to automation and high throughput approaches to handle large segregating populations.

There are several SNP detection systems that are used for the identification of large numbers of SNPs in a given plant. One such approach is based on EST / gene sequence data compiled from functional genomics studies. The main challenge of functional genomics is the identification of genes underlying a trait of interest so that they can be exploited in crop improvement programs. Microarrays have been successfully used in many plant species to understand the basic physiology, developmental processes and environmental stress responses and to identify and genotype mutations (Aharoni and Vorst, 2002). Such arrays can not only be used to investigate gene expression but also for the identification of single feature polymorphisms (SFPs) containing SNPs when the hybridisation patterns generated with cDNA or DNA samples from different individuals are being compared (Borevitz et al., 2003;
Ganal et al., 2009). The microarray technology that will be used in this study to investigate transcriptional changes for clubroot resistance is overviewed below.

### 1.6.5 Microarray technology

Microarrays have revolutionised gene expression profiling and have become progressively more common in both biological and medical research. They enable the simultaneous investigation of thousands of genes and have the ability to provide gene expression information on a whole genome level (Yang et al., 2005). Detailed information on the biology, terminology and technology underlying microarrays may be obtained from Schena (2003) and Bowtell and Sambrook (2003). In brief, the objective of many microarray projects is to identify genes expressed at different abundances in complex samples of RNA extracted from different types of tissues or from the same tissues growing at different conditions (Schena and Davis, 1999). This technology is based on the immobilisation of the gene-specific sequences (probes) onto a solid matrix and the application of fluorescently-labelled nucleic acids (targets) from the biological samples (Holloway et al., 2002). Fluorescence is detected by laser scanning at the appropriate wavelength depending on the fluorophores used. The hybridisation intensities for each DNA sequence present on the array are calculated using an automated process and provide quantitative data of the individual gene expression level (Aharoni and Vorst, 2002). In general, the signal strength represents (a) target abundance (transcript level, if the samples were RNA) or (b) sequence similarity between probes and targets (Clarke and Zhu, 2006). Because microarrays can simultaneously measure thousands of targets in a high-throughput manner, this technique facilitates recognition of global gene expression patterns. Association between specific trait and changes in gene expression by comparing expression patterns across samples is possible and allows the identification of gene function that can be used for plant improvement (Freeman et al., 2000; Chen et al., 2002). Of significance to this review is the use of microarray technology to establish correlations...
between the patterns of gene expression to clubroot resistance and in symptoms development of diseased versus normal tissues upon clubroot infection (Siemens et al., 2006) and other pathogens like *Alternaria brassicicola* in *Arabidopsis* (Schenk et al., 2000) and *Ascochyta* blight in chickpeas (Coram and Pang, 2006; Coram et al., 2007).

### 1.6.6 Types of microarray platform

Two kinds of microarrays, based on the type of probe used, are commonly used in functional genomics studies, namely (a) the cDNA microarray and (b) the oligonucleotide array. There are advantages and disadvantages associated with each microarray platforms. With the widespread availability of microarray core technologies, planar glass have become the most widely used type of array, due to their general utility and moderate price (Bodrossy and Sessitsch, 2004).

**cDNA microarrays:**

In the cDNA array, PCR-amplified cDNA sequences are printed on a glass slide and are commonly prepared from two different approaches. Firstly, clones of the cDNA library or the subtracted cDNA library are randomly picked and sequenced. Clones of unique sequences are identified by bioinformatics software and PCR-amplified using vector-specific primers. Another approach is to identify ESTs from a database, design gene-specific primers and amplify gene specific sequences that are from either cDNA clones or a cDNA library (Bowtell and Sambrook, 2003; Schena, 2003). For this type of array, the control and experimental RNA samples (targets) are usually reverse transcribed into cDNA and labelled with fluorophores: Cyanine 3 (Cy3) and Cyanine 5 (Cy5) before hybridisation onto the array (on which the probes were printed).
Oligonucleotide microarrays:

Instead of PCR products, oligonucleotide arrays consist of synthetic single stranded base sequences that are either printed onto glass surface or synthesised \textit{in situ} on the slide. In this array, the probe sequences are designed based on sequences publicly available on databases such as GenBank of the National Centre of Biotechnology Information (NCBI). Hence, construction of oligoarrays is limited to organisms for which gene sequences are available. As the efficiency and economy of oligonucleotide arrays improves, they will probably become the platforms of choice for gene expression analysis.

Oligonucleotide arrays are sub-divided into two types based on the length of the oligonucleotide probe. Companies such as Agilent and other researchers (Draghici 	extit{et al.}, 2006; Thomassen 	extit{et al.}, 2006) have constructed long oligonucleotide arrays in which the probe sequences are 40-80 bases long. The benefits of such arrays are the ease of production and design compared to cDNA arrays and these arrays may also be readily modified as more genomic information become available about the organisms (Petersen 	extit{et al.}, 2005). In contrast, high-density oligonucleotide array (genechips developed and patented by Affymetrix) differ from other formats in that the 25-mer oligonucleotide probes are directly synthesised on the array surface using a photolithographic process. Due to the very short probe sequences, this may lead to non-specific hybridisation patterns and several internal controls are included (Affymetrix, 2004). On the Affymetrix \textit{Arabidopsis} ATH1 genechip, a gene is represented by 11 oligonucleotide probe pairs, \textit{i.e.} 11 perfect match (PM) and 11 mismatch (MM) sequences. The PM probes represent a perfectly complementary match to a specific gene whereas the MM probes have a single base difference to the perfect match in the middle of the oligonucleotide sequence (Affymetrix, 2004). The signal intensities of the PM and MM generated upon binding of the labelled targets, would permit a more robust analysis of the microarray data.
In conclusion, the genome-wide transcriptional changes in challenged *Brassica* vegetable crops may be investigated using microarrays. This technology will provide vital information on the gene expressions correlated with defence mechanisms and susceptibility against *P. brassicae* and allow the development of functional molecular markers like SNPs. These ‘perfect’ markers, when developed from the identified genes associated with defence, have several benefits over random markers and ultimately, may be used in the high-throughput and automated selection for clubroot resistance in MAS.

1.7 Rationale of the thesis project

Clubroot, caused by the soil-borne obligate biotroph *Plasmodiophora brassicae* Woronin, is responsible for losses of at least 10% in crucifer yield and causes more than AU$ 17 million in lost profits every year in Australia. As reviewed, current control measures are costly, impractical and environmentally harmful and are often insufficient to keep the plant healthy. Hence, the breeding of resistant cultivars, especially for the susceptible Chinese cabbage, is a valid alternative. There have however been few successful marker-assisted breeding programs for resistance despite the identification of several sources of clubroot resistance. This is because the complex genetic control of resistance in the hosts is still not fully understood. Since marker-assisted breeding is increasingly aiming towards the discovery of candidate genes, the identification of the actual number of genes involved in clubroot resistance and their mechanisms of action is an essential first step. This is possible due to the knowledge gained by investigating the transcriptional changes in ‘challenged’ plant hosts using the gene expression profiling technique such as microarrays. However, since microarray technology merely provide “guilt by association” inferences, functional characterisation of these genes via knockouts / TILLING-mutants / over-expressing transgenics is still necessary.
Analysis of the expression and function(s) of defence-related genes will facilitate the understanding of the molecular mechanisms underlying clubroot resistance. This approach has the potential to assist plant breeders in improving disease resistance by gene selection or genetic manipulation. It would be ideal to study the whole-genome expression profiles in Brassaica to gain a complete picture of a plant’s response to clubroot. Fortunately, the close phylogenetic relationship between Brassaica and Arabidopsis makes genome-wide analysis of Brassaica vegetables possible via the Affymetrix Arabidopsis genechip (until the Affymetrix Brassaica genechip is released). The timing of resistance in Brassaica vegetables against clubroot is not fully documented and hence, the timing for tissue collection representing defence responses is vague. The construction of a ‘boutique’ oligoarray for the preliminary investigation of the transcriptional changes in challenged Brassaica vegetables was considered a viable option to avoid the inefficient use of the Affymetrix genechips. This oligoarray is achievable in this current project due to the extensive gene sequence data on nucleotide databases from previous Brassaica and Arabidopsis functional genomics studies.

Considering the gaps in knowledge regarding the mechanisms of clubroot resistance in Brassaica vegetable crops and the opportunities for research identified in this review, the aims of this study were to:

1. To collect, identify and characterise some virulent P. brassicae populations. These isolates were used as sources of infection to permit reliable and reproducible identification of clubroot resistant Brassaica varieties and in the induction of defence-related gene expressions.

2. To identify and characterise the level of clubroot resistance of some commercially-available Brassaica vegetables and landraces. These cultivars were subsequently used as
sources of resistance / partial resistance to compare their transcriptional profiles upon infection with clubroot.

3. To develop a reliable and reproducible test system to study the defence mechanisms against clubroot in *Brassica* vegetables. Originally, a soil-based test system was used. Due to complications concerning the RNA samples, a novel hydroponic system was designed to generate representative tissue samples to study defence responses.

4. To profile the gene expression for clubroot resistance using a ‘boutique’ oligonucleotide microarray. Genes significantly differentiated and constitutively expressed were investigated at three time points: 24, 48 and 72 hours after inoculation (hai) in susceptible ‘Granaat’ and partially-resistant ‘Leaguer’ and ‘Tahono’ Chinese cabbage lines.

5. To profile the gene expression for clubroot resistance or susceptibility using the Affymetrix *Arabidopsis* ATH1 genome array. This more sophisticated microarray platform has allowed a more detailed and robust investigation of defence responses at 48 hai in susceptible ‘Granaat’, partially-resistant ‘Tahono’ Chinese cabbages and resistant ‘ECD04’ fodder turnip.

6. To postulate the nature of clubroot resistance and the genes needed for resistance, partial resistance and susceptibility to clubroot disease in *Brassica*. Identification of possible weaknesses (in susceptible plants) or strengths (in partially-resistant or resistant plants) in their defence mechanisms may provide vital information for breeding strategies.
Chapter 2

Phenotyping of Australian clubroot populations using the European Clubroot Differential series

2.1 Introduction

Several pathotypes (i.e. pathogen displaying host specificity) of *Plasmodiophora brassicae* have been reported Australia-wide and worldwide (Toxopeus *et al.*, 1986; Kuginuki *et al.*, 1999; Donald *et al.*, 2006; Dixon, 2009) and this was problematic in the search for resistance genes due to their differing reactions on the *Brassica* vegetable species. This specificity of *P. brassicae* was first demonstrated by Honig (1931), although earlier research indicated this possibility (Appel and Werth, 1910). Honig’s observations were later confirmed by other researchers (Lammerink, 1964; Karling, 1968). As a result, research groups around the world have developed several sets of differential hosts to classify *P. brassicae* races according to their reactions or gall size on a subjective scale (Williams, 1966; Karling, 1968). Other scientists even recorded the actual gall weight (Crute *et al.*, 1983) and shape (Lammerink, 1967). This created ambiguity and the inability to compare data due to a lack of cross-referencing and rationalisation between methods. It was not until the European Clubroot Differential (ECD) series was developed by Buczacki *et al.* (1975) that an internationally accepted standard method for the classification of *P. brassicae* populations became available. This system represented a considerable advancement over other systems since it was developed through the co-operation of several independent research groups as well as being relatively cheap and simple to set up.

The ECD set comprised of 15 differential *Brassica* hosts originated from three species: *Brassica rapa, B. napus* and *B. oleracea*. These hosts have different numbers and types of resistance genes (Buczacki *et al.*, 1975). This bioassay, based on the relative susceptibility of
the differential hosts and virulence of the clubroot isolates, has been widely used by Dobson
et al. (1983), Toxopeus et al. (1986) and Donald et al. (2006) to differentiate *P. brassicae*
populations across the west coast of the USA, Western Europe and Australia respectively. In
brief, ‘samples’ of *P. brassicae* either as resting spores in soil or in root tissues are collected
from naturally infected fields or crops. When the resting spores are extracted from each
sample, this constitutes the ‘isolate’ to be applied onto the differential hosts of the ECD set.
Once the ECD interaction is recorded, the *P. brassicae* sample is classified as a differentially
interacting population or simply, a ‘population’ or ‘pathotype’ (Buczacki et al., 1975). These
*P. brassicae* samples often involve a mixture of separate populations, each capable of
differential interaction. It is possible for populations derived from first inoculations to be sub-
divided into further populations when resting spores are extracted from developing galls and
re-differentiated onto the ECD series (Manzanares-Dauleux et al., 2001). Hence, the term
‘pathotype’ may only be used when further subsequent extractions and differentiations of a *P.
brassicae* population give similar results, indicating a homogenous population (Buczacki et
al., 1975).

A wide range of factors was relevant in differentiating *P. brassicae* populations and a
guideline was formulated for the collection, storage, extraction and application of the clubroot
isolates when conducting the ECD tests (Buczacki et al., 1975). According to Karling (1968),
several other critical factors such as moisture content, temperature, ion concentration,
nutritional and physical conditions of the soil as well as light and spore load may affect the
degree of clubroot infection and formation. To simulate conditions favourable for the invasion
and proliferation of *P. brassicae* resting spores, the potting mix was prepared according to
Yoshikawa (1981) and maintained at high moisture level throughout the experiment. Other
factors were controlled through the regular application of liquid fertiliser and by growing the
*Brassica* hosts in environmentally-controlled glasshouses/growth rooms while the preparation
and application of clubroot isolates was performed according to Manzanares-Dauleux et al. (2000) with some modifications. These measures would allow the reproducibility, reliability and cross-referencing of the data for the survey.

The aims of the experiments described in this chapter were:

1. **To collect, identify and characterise a number of virulent *P. brassicae* populations.** These isolates were needed as sources of infection in future experiments and thus would permit reliable and reproducible results in the search for clubroot-resistant *Brassica* varieties and the study of defence-related gene expressions in those varieties.

2. **To assess the emergence or occurrence of new pathotypes of *P. brassicae*, especially in Victoria, Australia.** The small-scale survey was intended to provide an insight to the genetic diversity in pathogenicity of clubroot populations and was thus valuable for the Australian *Brassica* breeders. Since there is no conventional breeding program to develop clubroot-resistant *Brassica* crops in Australia (Donald et al., 2006), new resistant varieties with the ability to yield in Australian conditions have to be imported. Hence, the identification of existing *P. brassicae* pathotypes in Australia is crucial before the selection of cultivars for cultivation in Australia.

### 2.2 Materials and Methods

#### 2.2.1 Clubroot sample collection and preparation of the isolates

Naturally-infected broccolis and Chinese cabbages were collected from *Brassica* vegetable farms in Victoria located at Anakie, Bacchus Marsh and Launching Place (Figure 2.1) and as frozen diseased root samples from Dr Caroline Donald (Department of Primary Industry (DPI), VIC). The infected roots were washed in tap water, dried on paper towels for 1 h and
Figure 2.1. The locations where naturally infected *Brassica* crops were collected (http://maps.google.com.au) A, Anakie (Latitude: -37.9, Longitude: 144.3); B, Bacchus Marsh (-37.7, 144.5) and C, Launching Place (-37.8, 145.6).
stored in labelled bags at -20°C for future use. To maintain the virulence of the clubroot spores, these root samples were used within 3 years of collection as recommended by Buczacki et al. (1975). Twenty *P. brassicae* samples were tested in this study: eight from DPI and 12 from fields. Each clubroot isolate, originating from a single frozen gall (weighing >8 g), was prepared by soaking the infected root in 70% ethanol for 2 min followed by 3 washes of 2 min each in sterile MilliQ water. The gall was ground using a hand-held blender and the slurry made up to 50 mL with sterile MilliQ water. The homogenate was filtered through three metallic sieves (500 µm, 250 µm and 100 µm pore diameter, Sigma®) and the concentration of the isolates was adjusted to $2 \times 10^7$ spores per mL of MilliQ water using a haemocytometer (*Figure 2.2a*). As suggested by Donald *et al.* (2006), these isolates may be stored at 4°C for up to 3 months before they started deteriorating.

### 2.2.2 Growth and inoculation of the European Clubroot Differential series

The European Clubroot Differential (ECD) set consisting of 15 *Brassica* lines (*Table 2.1*) was sown in 10-cm diameter pots filled with autoclaved (three steam cycles at 105°C for 60 min) potting mix. The latter was prepared by mixing General Purposes® potting mix (Yates™, NSW), Canadian Sphagnum peat moss (Sunshine™) and medium perlite (Chillagoe™, QLD) in a ratio of 2:1:1 (v/v) and had its pH adjusted to 5.5-6.5 with hydrated lime (Richgrow™). Each ECD line was sown at four seeds per pot and three pots were used per clubroot isolate, *i.e.* 12 plants per ECD line per clubroot isolate. In contrast, only one pot of four plants for each ECD line was used as a control per experiment since it was only intended to check for the absence of infection.

Ten days after germination, each ‘treatment’ seedling was inoculated by pipetting 1 mL of the isolate at the base of its stem (‘pipette’ method) (*Figure 2.2b*). The differential hosts infected with the same clubroot isolate were randomly placed in a black 65 L plastic crate to collect
Figure 2.2. The ECD test (a) Equipment used in the extraction and quantification of clubroot isolates, (b) ‘Pipette’ method to inoculate the *Brassica* hosts, (c) 15 *Brassica* lines of the ECD set and (d) ECD hosts grown in plastic crates to prevent cross-contamination and to collect flow-through.
Table 2.1. The European Clubroot Differential (ECD) series: host species with denary values, reproduced from Buczacki et al. (1975).

<table>
<thead>
<tr>
<th>Differential Number</th>
<th>Differential Host</th>
<th>Other Name</th>
<th>Denary value</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 chromosome group (Brassica rapa)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>var. rapifera line aaBBCC</td>
<td>Fodder turnip</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>var. rapifera line AAbbCC</td>
<td>Fodder turnip</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>var. rapifera line AABBcc</td>
<td>Fodder turnip</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>var. rapifera line AABBCC</td>
<td>Fodder turnip</td>
<td>8</td>
</tr>
<tr>
<td>5</td>
<td>var. chinensis cv. Granaat</td>
<td>Chinese cabbage Pe-Tsai</td>
<td>16</td>
</tr>
<tr>
<td>38 chromosome group (Brassica napus)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>var. napus line Dc101</td>
<td>Fodder rape Nevin</td>
<td>1</td>
</tr>
<tr>
<td>7</td>
<td>var. napus line Dc119</td>
<td>Giant rape commercial</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>var. napus line Dc128</td>
<td>Giant rape selection</td>
<td>4</td>
</tr>
<tr>
<td>9</td>
<td>var. napus line Dc129</td>
<td>New Zealand resistant rape</td>
<td>8</td>
</tr>
<tr>
<td>10</td>
<td>var. napus line Dc130</td>
<td>Swede Wilhelmsburger</td>
<td>16</td>
</tr>
<tr>
<td>18 chromosome group (Brassica oleracea)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>var. capitata cv. Badger Shipper</td>
<td>Cabbage</td>
<td>1</td>
</tr>
<tr>
<td>12</td>
<td>var. capitata cv. Bindsachsener</td>
<td>Cabbage</td>
<td>2</td>
</tr>
<tr>
<td>13</td>
<td>var. capitata cv. Jersey Queen</td>
<td>Cabbage</td>
<td>4</td>
</tr>
<tr>
<td>14</td>
<td>var. capitata cv. Septa</td>
<td>Cabbage</td>
<td>8</td>
</tr>
<tr>
<td>15</td>
<td>var. fimбриата cv. Verheul</td>
<td>Fimbriate kale</td>
<td>16</td>
</tr>
</tbody>
</table>

ECD seeds provided by Dr. Caroline Donald (Donald et al., 2006).
flow-through and to prevent cross contamination (Figure 2.2c and 2.2d). In contrast, 1 mL of sterile MilliQ water was applied to the ‘control’ seedlings and the control pots were placed in another crate. The seedlings were grown in a controlled environment at 20±3°C under 16 h light (250 W halogen bulbs about two meters above the plants at an intensity of ~270 µmoles m\(^{-2}\) h\(^{-1}\)) and 65-70% humidity. The potting mix was kept moist throughout the whole experiment with liquid fertiliser (20 mL Nitrosol™/10 L tap water). No other chemical or pesticide was applied.

2.2.3 Disease assessment of the ECD hosts

The plants were evaluated for clubroot resistance 9-10 weeks after inoculation. The extent and severity of the clubroot symptoms was assessed visually according to Figure 2.3 and was scored on the scale: 0, no visible clubbing; 1, small galls confined to lateral roots; 2, moderate swellings on both lateral roots and/or taproot and 3, severe clubbing. The disease index (DI) was calculated using the 4-grade scale according to the formula:

\[
DI = \frac{n_1 + 2n_2 + 3n_3}{3N_t} \times 100
\]

where \(n_1\) to \(n_3\) is the number of plants in the indicated class and \(N_t\) is the total number of plants tested. Differential hosts with DI = 0 were considered resistant and susceptible ones had a DI \(\geq 33\) while those between these values had an indeterminate resistance. The clubroot isolates were characterised using the denary values (a decimal system used internationally for the ECD hosts, Table 2.1). For example, if ECD hosts 05, 12, 13 and 14 were susceptible, the ECD code for this isolate would be 16/00/14, \(i.e.\) the addition of the denary values of the susceptible hosts for each Brassica group. Only those experiments that resulted in pathotypes of \(P.\ brassicae\) causing a DI \(\geq 80\) on at least one of the ECD hosts were analysed in this study. This arbitrary value, used by Crute et al. (1983), Toxopeus et al. (1986) and Donald et al. (2006), was adopted to ensure that the reported data were obtained from infective and highly viable clubroot isolates.
Figure 2.3. The 4-grade scale used to assess clubroot symptoms (a) 0 = no visible clubbing, (b) 1 = small galls confined to lateral roots, (c) 2 = moderate swellings on both lateral and/or tap root and (d) 3 = severe clubbing. Arrows indicate gall formation. Vertical white scale bars represent 10 mm.
2.3 Results

2.3.1 Disease assessment of the ECD hosts

Of the 20 *P. brassicae* isolates being tested using the ECD set, 13 isolates: C, D, E, F, K, L, M, N, O, P, Q and R (all eight frozen root samples collected from DPI were included) produced unreliable results. This was because these isolates failed to cause any infection or at least one susceptible reaction with DI ≥ 80 within the ECD hosts (Table 2.2) and were excluded from further analysis. Six triplet codes were identified for the remaining seven *P. brassicae* populations, with ECD codes 16/02/14 (Isolates A and B) assigned twice. These *P. brassicae* populations were all virulent on the highly susceptible differential host ECD05, with disease indices (DI) >78.8 (Refer to Appendix 1 for detailed symptoms).

For the *B. rapa* hosts, there was no variation in the results of the Australian *P. brassicae* populations and only the differential host ECD05 was susceptible to all tested isolates. In contrast, the virulence towards the *B. napus* hosts was restricted. Isolate G was the only one not to produce any ‘susceptible’ response on the *B. napus* group. In contrast, susceptible reactions with the remaining six isolates were established in only the differential hosts ECD06 and/or ECD07 at a frequency of two and six respectively, *i.e.* hosts ECD06 and ECD07 had a DI ≥ 33 for 29% and 86% of the time in the study. Of the three *Brassica* groups, the *B. oleracea* hosts had the most diverse reactions and isolate S was the only one that failed to produce any susceptible reaction. The remaining six isolates caused a susceptible response in the differential hosts ECD11, ECD12, ECD13 and/or ECD14 at frequencies of two, six, six and six respectively, *i.e.* 29%, 86%, 86% and 86% of the time in this study. In general, the hosts ECD11 and ECD15 had a high frequency (three and five respectively) of indeterminate reactions out of a total of 14 in the whole study. Lastly, it was not possible to compare the ECD codes generated from the current study to that reported by Donald *et al.* (2006)Dr Caroline Donald due to lack of/insufficient infection (Table 2.2).
Table 2.2. The ECD codes of the *Plasmodiophora brassicae* populations.

<table>
<thead>
<tr>
<th>Clubroot Isolate ID</th>
<th>Australian State</th>
<th>Location</th>
<th>Host</th>
<th>European Clubroot Differential hosts</th>
<th>Expected ECD code&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>VIC</td>
<td>Bacchus Marsh&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Broccoli</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>B</td>
<td>VIC</td>
<td>Bacchus Marsh&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Broccoli</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>C</td>
<td>VIC</td>
<td>Bacchus Marsh&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Broccoli</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>D</td>
<td>VIC</td>
<td>Bacchus Marsh&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Broccoli</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>E</td>
<td>VIC</td>
<td>Anakie&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Broccoli</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>F</td>
<td>VIC</td>
<td>Anakie&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Broccoli</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>G</td>
<td>VIC</td>
<td>Bacchus Marsh&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Broccoli</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>H</td>
<td>VIC</td>
<td>Bacchus Marsh&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Broccoli</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>I</td>
<td>VIC</td>
<td>Bacchus Marsh&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Broccoli</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>J</td>
<td>VIC</td>
<td>Anakie&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Broccoli</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>K&lt;sup&gt;a&lt;/sup&gt;</td>
<td>NSW</td>
<td>NA</td>
<td>NA</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>L&lt;sup&gt;a&lt;/sup&gt;</td>
<td>VIC</td>
<td>Trentham</td>
<td>Cabbage</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>M&lt;sup&gt;a&lt;/sup&gt;</td>
<td>VIC</td>
<td>Launching Place</td>
<td>Broccoli</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>N&lt;sup&gt;a&lt;/sup&gt;</td>
<td>VIC</td>
<td>Boisdale</td>
<td>Broccoli</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>O&lt;sup&gt;a&lt;/sup&gt;</td>
<td>VIC</td>
<td>Werribee</td>
<td>Broccoli</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>P&lt;sup&gt;a&lt;/sup&gt;</td>
<td>WA</td>
<td>Manjimump</td>
<td>Cauliflower</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>Q&lt;sup&gt;a&lt;/sup&gt;</td>
<td>VIC</td>
<td>Coralyn</td>
<td>Cauliflower</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>R&lt;sup&gt;a&lt;/sup&gt;</td>
<td>WA</td>
<td>Donnybrook</td>
<td>Cauliflower</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>S</td>
<td>VIC</td>
<td>Launching Place&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Chinese Cabbage</td>
<td>R</td>
<td>R</td>
</tr>
<tr>
<td>T</td>
<td>VIC</td>
<td>Launching Place&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Chinese Cabbage</td>
<td>R</td>
<td>R</td>
</tr>
</tbody>
</table>
Isolates prepared from infected roots provided by Dr Caroline Donald (DPI, VIC).
Samples collected from the same property.
ECD test previously performed by Dr Caroline Donald (DPI, VIC).
ECD code assigned on the basis of definite susceptible reactions only, i.e. with at least one susceptible reaction with DI ≥ 80.
The naturally infected Brassica hosts from which the isolates were extracted.
R, resistant (DI = 0); S, susceptible (DI ≥ 33); ?, indeterminate (0 < DI < 33); NA, data not available.
ECD tests producing unreliable results were shaded.

Note 1: Refer to Table A.1 in Appendix 1 for detailed information on the distribution of plants using the 4-grade scale of clubroot symptom severity and their respective disease indexes.

Note 2: Infected Chinese cabbages (B. rapa var. chinensis) were used as sources for clubroot isolates S and T while the remaining isolates were extracted from infected B. oleracea var. italica (broccoli), botrytis (cauliflower) and capitata (cabbage).
2.4 Discussion

From this small-scale survey of 20 samples, seven were virulent against the susceptible *B. rapa* Chinese cabbage, ECD05 and six triplet codes were generated. By contrast, the comprehensive survey by Donald et al.’s bioassay (2006) generated 23 triplet codes from 41 samples of *P. brassicae* originating from important vegetable *Brassica* production regions in Victoria, Western Australia, Tasmania, Queensland and New South Wales. Both results indicated a relatively diverse population of clubroot within the samples collected in Victoria and similar level of diversity was reported by Donald et al. (2006) for samples originating from Western Australia. This may be attributed to the fact that the national production of crucifers are located mainly in the South-eastern and Perth region of Australia (Horticulture Australia Limited, 2004). This small-scale survey indicated that there was no emergence of new clubroot pathotypes in Victoria; however, the identification of multiple *Brassica* varieties with resistances towards a wide range of Victorian clubroot populations was still required. Hence, the pyramiding of resistance genes may be the most effective method for durable resistance.

2.4.1 Reactions of the *B. rapa* differential hosts

Amongst the seven successfully tested *P. brassicae* isolates, there was no variation in the reaction to the *B. rapa* hosts (ECD01-05), i.e. the only susceptible variety is host ECD05 (Table 2.2). This concurred with other extensive surveys performed by Toxopeus et al. (1986) and Donald et al. (2006) on mainly Western European and Australian clubroot samples respectively. This lack of diversity to infect the *B. rapa* group may be because fodder turnips are not frequently cultivated in the main Australian *Brassica* vegetable production regions (Donald et al., 2006). Hence, emergence or occurrence of clubroot pathotypes with strong virulence towards this *Brassica* group is unlikely to exist in those regions. These observations may indicate favourable cultivation conditions for *B. rapa* crops in Victoria. The strong
virulence of the current clubroot populations, particularly towards host ECD05, remains a concern to the local Chinese cabbage farmers. This small-scale survey identified a few resistant lines within the *B. rapa* group against clubroot. Although it should be quite easy to cross Chinese cabbage and fodder turnip (both being *B. rapa*) to achieve viable resistant offspring, this may not be appropriate for Chinese cabbage breeding. It may take a long process of backcrosses to recover desirable characteristics of Chinese cabbage. This justifies for the search of new sources of resistances, preferably within other Chinese cabbage varieties.

As expected, ECD05 was the most commonly susceptible differential host within the *B. rapa* group. This line was included during the development of the ECD series to validate the reliability of clubroot spore inoculation (Buczacki *et al*., 1975) and operated as a susceptible control. The absence of infection by seven, and insufficient infection by six of the 20 clubroot isolates indicated that the results produced by the current assays were unreliable. The high rate of failure using the DPI’s frozen root samples suggested that degradation of the resting spores have occurred possibly due to their age and long-term storage. All future experiments were performed using freshly-prepared and highly viable clubroot isolates, which were extracted from naturally infected roots collected in this study.

### 2.4.2 Reactions of the *B. napus* differential hosts

All *P. brassicae* isolates in the current study either failed to produce a susceptible reaction or were limited to only hosts ECD06 and ECD07 (denary values of 0, 2 or 3) (*Table 2.2*) as opposed to 80.5% (denary values 0, 1, 2 or 3) of Donald *et al.* (2006). These differences were probably due to the current localised survey in contrast to the Australia-wide *P. brassicae* samples. The current results again concurred with Donald *et al.* (2006), who reported that only 1 out of 15 Victorian samples (out of 41 Australian samples) had susceptible reactions other than with hosts ECD06 and ECD07. There is a broader range of virulence towards the *B.*
napus group (ECD06-10) worldwide with denary values of 0, 2, 3, 6, 7, 10, 11, 13, 14, 15, 18, 19, 22, 23, 26, 27, 30 or 31 (Toxopeus et al., 1986). This indicates that most Australian clubroot samples have limited virulence towards the B. napus hosts and comparable susceptible reactions to those of American origin according to a survey from Dobson et al. (1983). Hence, Australian clubroot populations may be more closely related to populations in the west coast of the USA than to Western European populations. It is thought that the Australian and American P. brassicae populations were derived mainly from B. oleracea (Donald et al., 2006) and have not fully developed virulence towards the B. napus group due to limited exposure towards the B. napus crops. The documentation of clubroot resistance in B. napus should not be neglected due to the growing canola industry in Australia (www.australianoilseeds.com) and those lines may prove useful in the future for Brassica plant breeders.

The high number of susceptible reactions for host ECD07 was again expected. In addition to host ECD05, the B. napus group has the host ECD07 as another widely accepted universally susceptible host (Buczacki et al., 1975). These observations again coincided with that of Donald et al. (2006) and indicated that these tests were performed with virulent isolates.

2.4.3 Reactions of the B. oleracea differential hosts

The current P. brassicae samples were particularly aggressive and had the most diverse reactions to the hosts of the B. oleracea group (ECD11-15). Susceptible reactions involving the entire B. oleracea group has also been reported by Donald et al. (2006); in particular, hosts ECD13 and ECD14 were susceptible to 86% of the isolates in the current study as opposed to 93%. The lack of susceptible reactions on the host ECD15 in the current study was a concern and this may explain the inability to identify the most common Australian clubroot populations, with triplet codes of 16/3/12 and 16/2/31 as reported by Donald et al. (2006).
The poor discriminating abilities of the *B. oleracea* hosts in general (Crute et al., 1983; Toxopeus et al., 1986) or other deficiencies of the current ECD tests discussed in Section 2.4.4 may have contributed to these discrepancies. The current observations reiterate the threat of aggressive and diverse clubroot populations to major broccoli, cabbage and cauliflower production centres in Victoria.

**2.4.4 The ECD series to study variation in the *P. brassicae* population**

Despite the standardisation and wide acceptance of the ECD series, there were many inconveniences related to the differentiation of *P. brassicae* populations based on phenotypic reactions. Due to the time-consuming and space-demanding nature of the ECD tests, multiple experiments had to be carried out in a staggered manner to phenotype 20 clubroot samples. This allowed for a better handling of the ECD tests but at the expense of data reliability since the ECD tests were significantly affected by environmental factors in glasshouse tests. Other researchers have encountered and reported similar obstacles (Karling, 1968). Personal observations indicated that freshly-prepared *P. brassicae* inocula had no or a very low infection rate during autumn and winter on the ECD set and other *Brassica* hosts in this study. Shortening of day-length in glasshouse tests may also have affected the success and reproducibility of the ECD tests due to increasing number of uninfected host ECD05 (data not shown) and the unreliable results of five out of 13 clubroot samples that were collected from Victorian *Brassica* farms (Table 2.2). Siemens et al. (2002) reported that two of their *Arabidopsis* lines, Ta-0 and rhd-31 switched from resistant and partially-resistant in long-day conditions to susceptible in short-day conditions. Seasonal changes in terms of temperature are well documented (Jones et al., 1982; Kuginuki et al., 1999). As yet, it is still unclear how photoperiod or light may affect the virulence of *P. brassicae* resting spores or cause changes in the host’s physiology that are unfavourable to clubroot invasion.
Another disadvantage of using the ECD series is the genetic heterogeneity of the differential hosts, whose clubroot resistance genes were maintained by mass selection because of self-incompatibility (Kuginuki et al., 1999). This resulted in 14 out of 105 reactions (13%) having indeterminate resistances (0 < DI < 33) due to the occurrence of one or two susceptible reactions from a predominantly resistant differential host. Kuginuki et al. (1986) have encountered similar difficulties in interpreting their results since the responses of the differential hosts were not clear using Japanese isolates and this led them to develop an entirely different set of differential hosts. According to Kuginuki et al. (1999), this outcome may also be due to the presence of more than one pathotype in the clubroot samples, even when the isolates were extracted from a single gall. In the current study, a crude extract was used rather than single-spore isolates and this source of genetic heterogeneity may explain the observed indeterminate host reactions if a gene-for-gene interaction was involved in the host-pathogen interaction (Dobson et al., 1983). The use of genetically uniform single-spore isolates and breeding of internationally-compatible homozygous differential hosts would be ideal in the study of this particular host-parasite interaction. This would provide a more reliable screening system and support the interpretation of data worldwide. Otherwise, Dobson et al. (1983) and Toxopeus et al. (1986) instructed that differential hosts showing indeterminate reactions should be reinoculated with spores from the same infected ECD host to prevent potentially susceptible hosts from being overlooked. This would increase the length of the already long bioassay and was not undertaken in the current survey.

The current small-scale survey provided a valuable source of information for the Brassica plant breeding industry in Victoria, especially to the local farmers from where the samples were collected. It is well documented that P. brassicae is composed of several pathotypes and are virulent against specific range of Brassica hosts. From the ECD codes generated in Table 2.2, the isolates (A to J) originating from broccoli (B. oleracea) were particularly aggressive.
to the *B. oleracea* group of the ECD series as opposed to isolates (S and T) extracted from Chinese cabbages (*B. rapa*). This was an indication that *P. brassicae* was host specific and the resulting galls became enriched with the invading clubroot pathotype. The contention that *P. brassicae* is composed of several pathotypes may not be fully supported from the current study. The observed range of ECD codes even amongst clubroot samples originating from the same *Brassica* vegetable farm may indicate the distribution and mixed infection in a single field. An infected field as well as a single gall may be composed of different pathotypes of clubroot (Donald *et al.*, 2006). No new pathotype of *P. brassicae* was encountered in this small-scale survey when compared to the Australian-wide survey of Donald *et al.* (2006). This suggested that once *Brassica* varieties with dominant resistance genes have been identified, their implementation in other *Brassica* crops may result in a significant increase in yield. However, polygenic resistance (from several minor genes) may be the most effective type of resistance in the long term. Finally, performing a larger number of collections under a range of environmental conditions and *Brassica* cultivations may provide a better insight to the genetic diversity in pathogenicity of *P. brassicae* populations in Victoria.

### 2.5 Summary

The ECD series is an internationally accepted standard method used for the classification of *Plasmodiophora brassicae* populations. In the current study, seven isolates prepared from naturally infected *Brassica* roots were analysed on the ECD series. This small-scale survey identified six triplet codes but no new pathotype. It confirmed that the *P. brassicae* populations possess a certain degree of host specificity since the current isolates were more aggressive on the *Brassica rapa / oleracea* species from which they were extracted. The current observations was not able to fully support the contention that *P. brassicae* is composed of several pathotypes. These results supported the conclusions of several previous publications and may indicate the involvement of seasonal changes and day-length in the
reproducibility of the ECD tests. Despite the limitations of the ECD series, this relatively cheap and simple system was a valuable source of information to both the *Brassica* plant breeders and local farmers. It indicated that current Victorian clubroot isolates had limited diversity in the infection of the *B. rapa* and *B. napus* groups of the ECD set but were highly aggressive to ECD05 (Chinese cabbage ‘Granaat’). Hence, the search and introduction of dominant resistance genes in *B. rapa* vegetables may be an effective short-term breeding strategy. Due to the diverse reactions to the hosts of the *B. oleracea* group, this survey justified the identification of multiple *Brassica* varieties with resistances towards a wide range of Victorian clubroot populations and that pyramiding these major or minor resistance genes may be the most effective method for durable resistance. Finally, the identification and characterisation of virulent *P. brassicae* populations, as a source of infection in future experiments, would allow reliable and reproducible tests in the search for clubroot-resistant *Brassica* varieties and the study of defence-related gene expressions in those varieties.
Chapter 3

Phenotyping of *Brassica* vegetables for resistance against clubroot

3.1 Introduction

For the Australian *Brassica* industry, plant breeders will have to improve crop yield as well as its quality, develop varieties with both wide and specific environmental adaptation and produce varieties with better resistance to both biotic and abiotic and hence, reduce the reliance on agrochemicals. Breeding strategies for the short-term production of elite *Brassica* varieties, intermediate-term improvement of adapted *Brassica* populations to Australian conditions and long-term development of genetic resources from wild-type *Brassica* collections are the backbone for successful classical breeding programs (Bosemark, 1993).

Breeding programs designed to produce disease resistant varieties should firstly begin with the search and identification of plants with resistance-conferring genes. The ability to correlate genotype and phenotype is an important criterion in breeding for any plant characteristics. This is a major concern in breeding for clubroot resistance since *Brassica* lines may not be distinguishable in the absence of the parasite. Therefore, clubroot resistance breeding programs may not proceed unless the pathogen is present to induce the symptoms that would allow the *Brassica* lines conferring adequate resistance to be distinguished from susceptible ones. Due to the irregular level of infection or other environmental conditions in nature, it may not be possible to accurately differentiate between the resistant and susceptible types. Therefore, modern programs of breeding for disease resistance are based on artificially induced infections (Allard, 1960), thus permitting an accurate and reproducible test system.
The aims of the experiments described in this chapter were:

1. **To assess the level of* P. brassicae* resistance of some commercially-available* Brassica* vegetables originating from Asian and Australian suppliers.** After thorough characterisation, selected* Brassica* lines would be used as a source of resistance in future experiments and thus, in the study of defence-related gene expressions against clubroot disease.

2. **To identify and characterise some worldwide* Brassica* landraces for new sources of resistance against* P. brassicae*.** This small-scale survey was intended to provide an insight to the genetic diversity in clubroot resistance of the* Brassica* vegetables. This information was essential for Australian* Brassica* breeders due to the severe susceptibility and apparently non-existent clubroot resistance within the Chinese cabbage varieties.

3. **To assess the heritability of clubroot resistance in* F₁* hybrids between selected partially-resistant and susceptible* Brassica* lines provided by the Henderson Seed Group Pty Ltd (Templestowe, VIC).** These preliminary tests may determine the feasibility and effectiveness of pyramiding their clubroot resistance genes.

### 3.2 Materials and Methods

#### 3.2.1 Seed material

Twenty* F₁* *B. rapa* hybrids (*Table 3.1*) were provided by the Henderson Seed Group Pty Ltd (Templestowe, VIC) and were putatively resistant to clubroot disease according to their* Brassica* breeders/seed collectors. Seeds of four commercially-available *B. oleracea* varieties (*Table 3.2*) were acquired from Mr Fothergill’s Seeds Pty Ltd (NSW) and 18* Brassica* landraces (*Table 3.3*) were collected from the Australian Temperate Field Crops Collection (DPI, Horsham, VIC) in sufficient amount.
Table 3.1. The 20 F<sub>1</sub> *Brassica rapa* hybrids provided by Henderson Seed Group Pty Ltd<sup>a</sup> (Templestowe, VIC).

<table>
<thead>
<tr>
<th>Plant ID</th>
<th>Accession&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Source&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>H01</td>
<td>CC04-145-2</td>
<td>CR Ohken 65 (65-day)-2-2</td>
<td></td>
</tr>
<tr>
<td>H02</td>
<td>CC04-161*CC04-145-2</td>
<td>CR Ohken 65 (65-day)*-2-2)-1cms</td>
<td></td>
</tr>
<tr>
<td>H03</td>
<td>01CC69-1</td>
<td>Tahono CR-1-1</td>
<td></td>
</tr>
<tr>
<td>H04</td>
<td>H010928</td>
<td>Tokita</td>
<td>CR Kaioh</td>
</tr>
<tr>
<td>H05</td>
<td>HQ010968</td>
<td>Kobayashi</td>
<td>Stylish CR 973</td>
</tr>
<tr>
<td>H06</td>
<td>H990915 (CC0033)</td>
<td>Tohoku</td>
<td>Tahono CR</td>
</tr>
<tr>
<td>H07</td>
<td>H010965</td>
<td>Takayama Seeds</td>
<td>CR Seiga 65</td>
</tr>
<tr>
<td>H08</td>
<td>H010966</td>
<td>Takayama Seeds</td>
<td>CR Seiga 75</td>
</tr>
<tr>
<td>H09</td>
<td>H010969</td>
<td>Kobayashi</td>
<td>Leaguer (CR 1052)</td>
</tr>
<tr>
<td>H10</td>
<td>Hyb 01-7</td>
<td>Kobayashi</td>
<td>Moonflower CR</td>
</tr>
<tr>
<td>H11</td>
<td>H020911</td>
<td>Kyowa Seed</td>
<td>CR Ohken 65</td>
</tr>
<tr>
<td>H12</td>
<td>H020912</td>
<td>Kyowa Seed</td>
<td>CR Ohken 75</td>
</tr>
<tr>
<td>H13</td>
<td>H020943</td>
<td>Mikado</td>
<td>CR 13C-1171</td>
</tr>
<tr>
<td>H14</td>
<td>HQ040918</td>
<td>Tohoku</td>
<td>Tah One CR</td>
</tr>
<tr>
<td>H15</td>
<td>H990920</td>
<td>Somsak</td>
<td>Tropical Delight</td>
</tr>
<tr>
<td>H16</td>
<td>H990902</td>
<td>Siam Shemical</td>
<td>Early Op</td>
</tr>
<tr>
<td>H17</td>
<td>HQ040912</td>
<td>Chia Tai</td>
<td>Bo-Phloi</td>
</tr>
<tr>
<td>H18</td>
<td>HQ040938</td>
<td>Tokita</td>
<td>CR Quinedao 65</td>
</tr>
<tr>
<td>H19</td>
<td>HQ040909</td>
<td>Primasid</td>
<td>Masano</td>
</tr>
<tr>
<td>H20</td>
<td>HQ040910</td>
<td>Ung Nong</td>
<td>Spring King</td>
</tr>
</tbody>
</table>

<sup>a</sup> Contact Dr Kiang Lee (kiang.lee@hendersonseed.com.au).

<sup>b</sup> Henderson Seed Group Pty Ltd’s serial number/source to track seed breeding lines.

Table 3.2. The commercially-available *Brassica oleracea* varieties purchased from Mr Fothergill’s Seeds Pty Ltd (NSW).

<table>
<thead>
<tr>
<th>Plant ID</th>
<th>Name</th>
<th>Taxon</th>
<th>Common name</th>
</tr>
</thead>
<tbody>
<tr>
<td>C01</td>
<td>Troika</td>
<td><em>B. oleracea</em> var. <em>gemmifera</em></td>
<td>Brussels Sprouts</td>
</tr>
<tr>
<td>C02</td>
<td>Italian Sprouting</td>
<td><em>B. oleracea</em> var. <em>italica</em></td>
<td>Broccoli</td>
</tr>
<tr>
<td>C03</td>
<td>Quickheart</td>
<td><em>B. oleracea</em> var. <em>botrytis</em></td>
<td>Cauliflower</td>
</tr>
<tr>
<td>C04</td>
<td>Green Sprouting</td>
<td><em>B. oleracea</em> var. <em>italica</em></td>
<td>Broccoli</td>
</tr>
</tbody>
</table>
Table 3.3. The 18 *Brassica* landraces provided by Australian Temperate Field Crops Collection\(^a\) (DPI, Horsham, VIC).

<table>
<thead>
<tr>
<th>Plant ID</th>
<th>ATC(^b)</th>
<th>Name</th>
<th>Alternate Name(^c)</th>
<th>Taxon</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>D01</td>
<td>95364</td>
<td>Rapa sponsa</td>
<td>Bra1234/87</td>
<td><em>B. rapa</em> subsp. <em>oleifa</em></td>
<td>Italy</td>
</tr>
<tr>
<td>D02</td>
<td>95193</td>
<td>Mizuna</td>
<td>K-159</td>
<td><em>B. rapa</em> subsp. <em>nipposinica</em></td>
<td>Japan</td>
</tr>
<tr>
<td>D03</td>
<td>90218</td>
<td>PI 207465</td>
<td>B561</td>
<td><em>B. rapa</em></td>
<td>Afghanistan</td>
</tr>
<tr>
<td>D04</td>
<td>90220</td>
<td>PI 257241</td>
<td>B422</td>
<td><em>B. rapa</em></td>
<td>China</td>
</tr>
<tr>
<td>D05</td>
<td>90310</td>
<td>PI 179652</td>
<td>B548</td>
<td><em>B. rapa</em></td>
<td>India</td>
</tr>
<tr>
<td>D06</td>
<td>92145</td>
<td>Dang Valley Tori</td>
<td>21695</td>
<td><em>B. rapa</em></td>
<td>Nepal</td>
</tr>
<tr>
<td>D07</td>
<td>92820</td>
<td>Pusa Kalyani</td>
<td>22535</td>
<td><em>B. rapa</em></td>
<td>India</td>
</tr>
<tr>
<td>D08</td>
<td>90537</td>
<td>Brutor</td>
<td>B504</td>
<td>*B. napus var. <em>napus</em></td>
<td>Spain</td>
</tr>
<tr>
<td>D09</td>
<td>90638</td>
<td>Nosovskijy</td>
<td>B015</td>
<td>*B. napus var. <em>napus</em></td>
<td>Ukraine</td>
</tr>
<tr>
<td>D10</td>
<td>90708</td>
<td>Tokiwa</td>
<td>B293</td>
<td>*B. napus var. <em>napus</em></td>
<td>Japan</td>
</tr>
<tr>
<td>D11</td>
<td>90070</td>
<td>Narc 82</td>
<td></td>
<td><em>B. napus</em></td>
<td>Pakistan</td>
</tr>
<tr>
<td>D12</td>
<td>90600</td>
<td>Lenora</td>
<td>B333</td>
<td><em>B. napus</em></td>
<td>South</td>
</tr>
<tr>
<td>D13</td>
<td>92455</td>
<td>Norde</td>
<td>22024</td>
<td><em>B. napus</em></td>
<td>Korea</td>
</tr>
<tr>
<td>D14</td>
<td>92963</td>
<td>Wen You 1</td>
<td></td>
<td><em>B. napus</em></td>
<td>China</td>
</tr>
<tr>
<td>D15</td>
<td>94674</td>
<td><em>B. montana</em></td>
<td>3607</td>
<td><em>B. montana</em></td>
<td>Spain</td>
</tr>
<tr>
<td>D16</td>
<td>94701</td>
<td><em>B. insularis</em></td>
<td>7347</td>
<td><em>B. insularis</em></td>
<td>Italy</td>
</tr>
<tr>
<td>D17</td>
<td>94696</td>
<td><em>B. incana</em> Ten.</td>
<td>6560</td>
<td><em>B. incana</em></td>
<td>Italy</td>
</tr>
<tr>
<td>D18</td>
<td>94690</td>
<td><em>B. cretica</em> Lam.</td>
<td>5971</td>
<td><em>B. cretica</em></td>
<td>Greece</td>
</tr>
</tbody>
</table>

\(^a\) Contact Dr Bob Redden ([bob.redden@dpi.vic.gov.au](mailto:bob.redden@dpi.vic.gov.au)).

\(b, c\) Australian Temperate Field Crops Collection’s serial number to track seed breeding lines.

These *Brassica* landraces, originating from great centres of diversity such as China, Asia Minor and the Mediterranean regions as well as from some secondary centres of origin, were tested for new sources of clubroot resistance. These locations were identified by the prominent Russian botanist and geneticist Nikoli Ivanovich Vavilov and still remains promising areas for future explorations (Allard, 1960).
3.2.2 Propagation of the Brassica hybrids by self- and cross-pollination

To bulk enough seeds for future experiments, the 20 F₁ Brassica rapa hybrids provided by Henderson Seed Group Pty Ltd (Templestowe, VIC) were propagated. The potting mix was prepared by mixing 100 L of General Purpose potting mix (Yates™, NSW), 150 L of PotMate Premium® potting mix (Debco™, VIC), 240 g of Osmocote Plus® garden beds fertiliser (Scotts™, NSW) and 1 L of 5 g/L Mancozeb Plus® contact fungicide (Yates™, NSW). This mixture was covered with a black plastic sheet and solarised for 1 week outdoors (Stapleton, 2000) before being distributed in twenty 30-cm diameter pots. The Brassica rapa seeds were treated in 5 g/L of Mancozeb Plus® contact fungicide (Yates™, NSW) for 60 min and 3 seeds per line were sown equidistantly in pots at a depth of about 0.5 cm. To facilitate germination and growth of the seedlings, the pots were kept in glasshouse under controlled temperature of 20 ± 3 °C. After two weeks, the above procedures were repeated so that the timing for flowering, pollination and seed collection became more manageable.

Maintenance of the growing plants involved spraying their leaves alternately with 5 g/L Mancozeb Plus® contact fungicide (Yates™, NSW) or 1 g/L of Bayleton® systemic fungicide (Yates™, NSW) or 2 g/L Fungus Fighter® Copper contact fungicide (Yates™, NSW) every two weeks. In addition, Bayer Confidor™ (systemic) or Bayer Baythroid™ (contact) insecticide spray aerosol (Yates™, NSW) or Defender™ snail & slug pellets (Scotts™, NSW) were applied alternately to control/treat insect, caterpillar or snail slug attacks every 2-3 weeks. To encourage healthy growth, each pot was fertilised with 2 mL of Garden King Nitrosol™ liquid fertiliser (Amgrow®, NSW) per L of tap water every 2 weeks until the onset of flowering. Finally, the plants were watered moderately about twice (winter) or 4-5 times (summer) per week and subsequently, at half rate during flowering and seed formation.
Vernalisation was required for the *Brassica* vegetables to develop flowers (Downey et al., 1980). This was achieved either by leaving the pots outdoors during winter or in a 4°C growth room at night for several days. Self- and cross-fertilisation were performed using the bud pollination technique (Downey et al., 1980). Initially, all open flowers were removed and the whole plant or branch with potential flower buds was covered with a transparent porous plastic bag (Figure 3.1a) for up to 3 days. The bag allowed air circulation while preventing the entry of pollen and hence resulted in flower buds free of exogenous pollen (Figure 3.1b). Anthers carrying fresh pollen from flowers inside the bag were collected and kept cool while each inflorescence was trimmed until 3-5 flower buds with strong stalks and fat ovaries remained (Figure 3.1c). The stigmas were exposed by removing part of the sepals and the undeveloped anthers with a fine-pointed pair of forceps (Figure 3.1d). Fresh pollen collected earlier was applied on the immature stigma. To maximise the fertilisation rate, these procedures were carried out during cool mornings. The pollinated buds were covered with labelled paper bags to protect the developing seed pods (Figure 3.1e). Once the maturing seed pods turned yellow/brown, they were harvested and dried further on the bench at room temperature for a few weeks. The resulting *Brassica* seeds were stored in labelled paper envelopes and kept in a dry and dark environment until future use. These five-to-six-months-long procedures were initiated twice in late summers and about 6-10 inflorescences were used per plant for self- and/or cross-pollination (3 plants × 2 pots × 2 years = 12 plants per *Brassica* line).

### 3.2.3 Preparation and propagation of the *Plasmodiophora brassicae* isolates

*P. brassicae* isolates I (ECD code: 16/03/15), J (16/03/14), Q (16/00/04), S (16/02/00) and T (16/00/00) (Table 2.2) were used as sources of infection in the resistance tests. These isolates were selected based on their differing virulence onto the ECD series and to represent current clubroot pathotypes infecting Victorian *Brassica* vegetable farms. They were prepared and
Figure 3.1. Bud pollination technique: (a) Developing flowers and buds free from exogenous pollen inside the porous plastic bag, (b) Collection of fresh pollen within the plastic bag, (c) Inflorescence trimmed of small and under-developed buds, (d) Stigma exposed before the application of the fresh pollen and (e) Development of the fertilised buds into seed pods in labelled paper bags.
quantified according to Section 2.2.1 from galls of infected Brassica plants from previous tests and/or from the highly susceptible host ECD05. The latter was grown as bait according to Section 3.2.2 and, once infected with the desired clubroot isolate, all chemical application was stopped and hot, humid and water-logged conditions were simulated to promote clubroot infection and gall formation. The resulting galls were cleaned and stored in labelled bags at -20 ºC for future use.

3.2.4 The resistance test: growth and inoculation of the Brassica lines

Nineteen Brassica rapa hybrids (Plant ID: H01-H20, Table 3.1) propagated by self-pollination, together with the 4 commercially-available Brassica oleracea varieties (C01-C04, Table 3.2) and the 18 Brassica landraces (D01-D18, Table 3.3) were tested for clubroot resistance according to Section 2.2.2 with some modifications. In brief, the seeds were firstly treated in 5 g/L of Mancozeb Plus® contact fungicide (Yates™, NSW) for 60 min and surface-sterilised by a 5 min wash in bleach (4% active chlorine), followed by a 5 min wash in 70% ethanol and finally 5 washes of 2 min in sterile MilliQ water. Each Brassica line was grown at four plants per 10-cm diameter pots filled with autoclaved potting mix and three pots were used per Brassica line per clubroot isolate, i.e. 12 plants per Brassica line per clubroot isolate. In contrast, only one pot of four plants for each Brassica line was used as the negative controls. The 10-day-old seedlings were inoculated with 2×10^7 spores of isolates I, J, Q, S or T using the ‘pipette’ method and maintained in separate black 65 L plastic crates. In addition, the highly susceptible host ECD05 was included in those experiments as the positive controls. The setup details were summarised in Table 3.4.

3.2.5 The resistance test: disease assessment

The severity of the clubroot symptoms was assessed 9-10 weeks after inoculation according to Section 2.2.3 with some modifications. The average disease index (average DI) for each
Brassica line was calculated using the DI values whose isolates resulted in susceptible reactions on the host ECD05, i.e. using those DI from virulent isolates. These Brassica lines were further classified as immune (average DI = 0), partially-resistant (0 < average DI < 20) and susceptible (average DI ≥ 20). According to Toxopeus et al. (1986), this stringent average DI cut-off value of 20 for partially-resistant lines ensured that most combination of host reactions fell within symptom grade scales of 0 and 1 (Figure 2.3). Therefore, indeterminate reactions, caused by of one or two susceptible reactions from predominantly resistant hosts, were mostly represented in partially-resistant lines. The level of resistance for each Brassica line was reported against each clubroot isolate as well as against the whole set of isolates used.

3.2.6 Preliminary study on the heritability of clubroot resistance

From the 20 F₁ Brassica rapa hybrids provided by Henderson Seed Group Pty Ltd, six lines (H06, H07, H08, H09, H12 and H16, Table 3.1) were selected according to their varying level of clubroot resistance. These lines were used in partially-resistant × partially-resistant crosses: H06×H09, H07×H08 and H08×H12 and resistant × susceptible crosses: H06×H16 and H09×H16 and were assessed for clubroot resistance according to Section 3.2.4 and 3.2.5. The setup details were summarised in Table 3.4.

Table 3.4. Details on the resistance tests performed.

<table>
<thead>
<tr>
<th>Seed material</th>
<th>Location</th>
<th>Date of inoculation</th>
<th>Season in Victoria</th>
</tr>
</thead>
<tbody>
<tr>
<td>19 B. rapa hybrids</td>
<td>Glasshouse</td>
<td>6-Jan-06</td>
<td>Mid Summer</td>
</tr>
<tr>
<td>4 B. oleracea varieties</td>
<td>Glasshouse</td>
<td>6-Jan-06</td>
<td>Mid Summer</td>
</tr>
<tr>
<td>18 Brassica landraces</td>
<td>Glasshouse</td>
<td>17-Jul-06 and 1-Dec-06</td>
<td>Mid Winter and Early Summer</td>
</tr>
<tr>
<td>5 cross-pollinated lines</td>
<td>Glasshouse</td>
<td>15-May-08</td>
<td>Late Autumn</td>
</tr>
</tbody>
</table>


3.3 Results

3.3.1 Propagation of *Brassica* lines by self- and cross-pollination

From the 20 F$_1$ *Brassica rapa* hybrids provided by Henderson Seed Group Pty Ltd (Templestowe, VIC), 19 *Brassica* lines were successfully self-pollinated to provide enough seed material while 93 (out of 190 possible crosses) *Brassica* lines were generated by cross-pollination (Table 3.5). Their fertilisation rates were above 80% while their germination rates were above 90% (data not shown).

3.3.2 The resistance test: Disease assessment

The isolates used onto the 19 *B. rapa* hybrids and four *B. oleracea* lines were all virulent due to susceptible reactions on host ECD05 with an average DI of 71. In particular, isolate S demonstrated 17 susceptible (74%), 4 (17%) indeterminate and no resistant reaction. In contrast, there were 9, 10, 5 and 9 susceptible; 10, 10, 12 and 11 indeterminate and 4, 3, 6 and 2 resistant reactions for isolates I, J, Q and T respectively.

The average DI suggested that 14 of the Henderson Group Pty Ltd’s hybrids (Table 3.6) were susceptible (Plant ID: H07, H03, H12, H13, H19, H04, H11, H18, H20, H01, H14, H15, H16 and H17) while five were partially-resistant (H06, H05, H08, H09 and H10) (in ascending order of average DI). None of them were however highly resistant to the current clubroot isolates. The average DI of the *B. oleracea* varieties (Table 3.7) showed that the cauliflower and both broccoli varieties (C03, C02 and C04) were susceptible while the Brussels sprouts (C01) had some partial-resistance to clubroot disease.

The resistance tests for the *Brassica* landraces encountered some difficulties due to the lack of any infection when inoculation was performed in mid winter. Despite these tests were again prepared in early summer, the reactions from isolate J and S (Table 3.8) were unreliable due
Table 3.5. Successful self- and cross-pollination of the Henderson Seed Group Pty Ltd’s *Brassica rapa* hybrids.

<table>
<thead>
<tr>
<th></th>
<th>H01</th>
<th>H02</th>
<th>H03</th>
<th>H04</th>
<th>H05</th>
<th>H06</th>
<th>H07</th>
<th>H08</th>
<th>H09</th>
<th>H10</th>
<th>H11</th>
<th>H12</th>
<th>H13</th>
<th>H14</th>
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</thead>
<tbody>
<tr>
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<td>Y</td>
<td>–</td>
<td>–</td>
<td>X</td>
<td>X</td>
<td>X</td>
<td>–</td>
<td>X</td>
<td>X</td>
<td>X</td>
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X, Seeds generated by cross-pollination; Y, Seeds generated by self-pollination; –, pollination either failed or was not carried out.

Note: Refer to Table 3.1 for the identity of the *Brassica* hybrids used.
Table 3.6. The level of *P. brassicae* resistance demonstrated by the Henderson Seed Pty Ltd’s self-pollinated *Brassica rapa* hybrids.

<table>
<thead>
<tr>
<th>Clubroot isolate ID</th>
<th>ECD code</th>
<th>Henderson Seed Group Pty Ltd</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>H01</td>
</tr>
<tr>
<td>S 16/02/00</td>
<td>S NA</td>
<td>S</td>
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<tr>
<td><strong>Average DI</strong></td>
<td>42</td>
<td>21</td>
</tr>
</tbody>
</table>

Table 3.7. The level of *P. brassicae* resistance demonstrated by commercially-available *Brassica oleracea* varieties.

<table>
<thead>
<tr>
<th>Clubroot isolate ID</th>
<th>ECD code</th>
<th>Mr Fothergill’s Seeds Pty Ltd</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>C01</td>
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<tr>
<td>I 16/03/15</td>
<td>?</td>
<td>S</td>
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<td>J 16/03/14</td>
<td>S</td>
<td>S</td>
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<td>Q 16/00/04</td>
<td>R</td>
<td>S</td>
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<td>S 16/02/00</td>
<td>?</td>
<td>?</td>
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<tr>
<td>T 16/00/00</td>
<td>?</td>
<td>R</td>
</tr>
<tr>
<td><strong>Average DI</strong></td>
<td>18</td>
<td>36</td>
</tr>
</tbody>
</table>
Table 3.8. The level of *P. brassicae* resistance demonstrated by selected worldwide *Brassica* landraces.

<table>
<thead>
<tr>
<th>Clubroot isolate ID</th>
<th>ECD code</th>
<th>Australian Temperate Field Crop Collection</th>
<th>Wild <em>Brassica rapa</em></th>
<th><em>Brassica napus</em></th>
<th>Wild <em>Brassica oleracea</em></th>
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<td></td>
<td>D01</td>
<td>D02</td>
<td>D03</td>
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<tr>
<td>I</td>
<td>16/03/15</td>
<td>NA</td>
<td>S</td>
<td>S</td>
<td>S</td>
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<tr>
<td></td>
<td></td>
<td><strong>DI</strong></td>
<td>NA</td>
<td>36</td>
<td>44</td>
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</table>

R, resistant (DI = 0); S, susceptible (DI ≥ 33); ?, indeterminate (0 < DI < 33); NA, data not available due to seed unavailability or lack of germination. unreliable results were shaded due to lack of susceptible reaction in host ECD05. Resistant/partially-resistant lines (average DI < 20) had their average DI values written in **bold**.

Note:
Refer to **Table 3.1**, **Table 3.2** and **Table 3.3** for the identity of the *Brassica* lines used.
Refer to **Table A1.2**, **Table A1.3** and **Table A1.4** in Appendix 1 for detailed information on the distribution of plants using the 4-grade scale of clubroot symptom severity and disease index.
to failed susceptible reactions on the host ECD05. These results were excluded from further analysis and the average DI values were not calculated. Moreover, the current DI of 33 on host ECD05 was a concern since DI value >66, as reported from previous tests, was expected. The reactions of isolate I suggested that the B. rapa landraces as well as the wild species of B. oleracea were completely susceptible to clubroot disease. Oddly, the symptoms reported on these lines were more severe than those upon the highly susceptible host ECD05. In contrast, four out of seven B. napus landraces were either resistant or had indeterminate reactions to isolate I.

The resistance tests of the five B. rapa lines generated by cross-pollination also encountered some difficulties when inoculated in late autumn. No infection was observed, even on the hosts ECD05 (data not shown).

3.4 Discussion

From this small-scale survey of 20 Chinese cabbage hybrids, five were partially-resistant to the current Victorian isolates; in particular, hybrid H06 (Tahono CR, average DI of 5). These partially-resistant B. rapa hybrids demonstrated diverse responses against these isolates and may indicate the presence of both pathotype-specific and non-pathotype specific resistance. In contrast, the reactions of the broccoli and cauliflower varieties suggested that these commercially-available lines were susceptible to Victorian isolates. New sources of clubroot resistance were investigated using worldwide Brassica landraces. Similarly to the ECD tests, these resistance tests were significantly affected by environmental factors such as seasonal changes. None of the wild B. rapa and wild B. oleracea lines was resistant from virulent isolates. The identification of only a few partially-resistant B. napus lines demonstrated the severity of this disease in Victoria and supported the ongoing research for clubroot resistance.
3.4.1 Resistance test of the *B. rapa* hybrids and *B. oleracea* varieties

Despite the lack of resistant genotypes against the Victorian isolates used, the diverse resistant and indeterminate reactions observed in the five partially-resistant *B. rapa* hybrids (provided by the Henderson Seed Group Pty Ltd) may indicate the presence of both vertical and horizontal resistance. This concurred with other researchers that the *Brassica* A genome (*B. rapa*) carries at least three dominant, race-specific resistance genes (Matsumoto *et al.*, 1998; Hirai *et al.*, 2004; Piao *et al.*, 2004). This showed that the highly partially-resistant genotype H06 (‘Tahono CR’ with average DI of 5) is composed of at least one dominant clubroot-resistant gene effective against isolates I, Q and T. Since indeterminate reactions were caused by one or two susceptible reactions from predominantly resistant host, this may also indicate the presence of dominant or recessive alleles for the genes involved in Tahono’s partial resistance against isolates J and S. Such QTL have previously been reported by Suwabe *et al.* (2006) in *B. rapa*. The resistance profiles of ‘Tahono’ and the other partially-resistant hybrids (H05, H08, H09 and H10) provided valuable information for the *Brassica* plant breeders as sources of clubroot resistance. The most important progress in the last few decades in *Brassica* breeding has been the implementation of F1 hybrids as a means to improve vigour, productivity, earliness, uniformity and quality (King, 1990). Selection within these existing commercial F1 *B. rapa* hybrids should provide the easiest and most satisfactory method of developing resistant *Brassica* lines due to their marketable agronomic traits. Further experimentation to differentiate the defence mechanisms involved in these partially-resistant hybrids may demonstrate the feasibility and effectiveness in pyramiding their resistance genes and overcoming the high susceptibility of Chinese cabbages against current Victorian isolates.

Another small-scale survey indicated that the commercially-available broccoli and cauliflower varieties did not possess sufficient resistance against Victorian isolates. Major Australian *Brassica* farmers however do not usually buy their seed stock from small retailers, but rather
from reputable seed companies (J. Erceg, Victorian Brassica farmer, pers. comm.). Clubroot resistance in *B. oleracea* (C genome) is often quantitative and race-independent (Figdore *et al.*, 1993; Voorrips, 1996; Rocherieux *et al.*, 2004) and strong resistance responses against any particular isolate was not expected. This lack of dominant resistance genes complicates the breeding for clubroot resistance in *B. oleracea* vegetables and justifies the development of durable resistance using the pyramiding technique.

Similarly to the ECD tests, the susceptibility of the hosts ECD05 in the current resistance tests indicated that virulent isolates were used. Despite the lack of variation of these isolates in their reactions to the *B. rapa* hosts of the ECD series (*i.e.* all the isolates had an ECD code of 16/-/-), isolate S was the most aggressive. Its specificity towards *B. rapa* vegetables was of particular interest in future experiments as a source of infection to permit accurate and reproducible tests.

### 3.4.2 Resistance test of the Brassica landraces and *B. rapa* cross-pollinated lines

New sources of clubroot resistance were investigated in landraces but all the wild *B. rapa* and *B. oleracea* lines tested were susceptible to isolate I in spite of their diverse genetic backgrounds. These landraces originating from mainly Southern/Eastern Asia and the Mediterranean regions may be unsuitable for cultivation in Victoria due to compatible clubroot isolates. Since isolate I was extracted from infected broccoli roots, its aggressiveness towards the wild *B. oleracea* lines may be due to the host-specific nature of clubroot. The susceptibility of all the wild *B. rapa* lines was unusual according to surveys using the ECD set from Toxopeus *et al.* (1986) and Donald *et al.* (2006) and may suggest the lack of genetic variations between these wild lines and commercialised Chinese cabbage varieties. This therefore complicates Chinese cabbage breeding programs if no new sources of resistance can be identified.
Three of the wild *B. napus* genotypes (D08, D10 and D13) were of particular interest as sources of clubroot resistance. In general, clubroot resistance in *B. napus* (AC genome) involves a complex type of inheritance with dominant genes from *B. rapa* and recessive genes from *B. oleracea* (Diederichsen and Sacristan, 1996). The combined resistance from both ancestral species of *B. napus* may explain the varying degree of resistance with a race-specific manner. Despite the limited success (insufficient infection by other isolates) and scale (number of landraces too small to represent the gene pool of an area) of this survey, these observations demonstrated the difficulties in looking for new sources of clubroot resistance that is vital for plant breeding purposes. Hence, a more thorough investigation with a wider range of landraces from the Australian Temperate Field Crops Collection (DPI, Horsham, VIC) may provide a comprehensive genetic database for clubroot resistance as well as other agronomic traits suitable for Australian conditions.

The absence or insufficient infection on the hosts ECD05 observed in these surveys supported the perception that both the ECD and resistance tests were adversely affected when inoculation was performed in autumn or winter. Another possibility for this lack of disease was the loss of virulence of the clubroot isolates during storage, even if they were used prior to 3 month after extraction as recommended by Buczacki *et al.* (1975) and Donald *et al.* (2006). The heritability of the *B. rapa* cross-pollinated lines may still be investigated at the right time with fresh clubroot isolates and may provide valuable information on the nature of clubroot resistance in the current *Brassica* lines.

### 3.5 Summary

The breeding of clubroot-resistant cultivars is an effective approach to eliminate the use of expensive and usually environmentally harmful fungicides and to minimise loss. Breeding programs designed to produce these resistant cultivars should firstly begin with the search and
identification of plants with clubroot resistance-conferring genes. In the current study, five previously characterised *P. brassicae* isolates (I, J, Q, S and T) determined the level of resistance of 19 *B. rapa* hybrids, four commercially-available *B. oleracea* varieties, 18 *Brassica* landraces and five *B. rapa* cross-pollinated lines. This small-scale survey identified genotype H06 (Chinese cabbage, Tahono CR) as the most potential source of clubroot resistance, but no highly resistant *Brassica* line was observed. The mostly susceptible reactions of the landraces demonstrated the severity of this disease and the difficulties being encountered in breeding clubroot-resistant varieties. The current resistance tests were significantly affected by environmental factors and were the cause of limited success in some experiments. The diverse reactions of ‘Tahono’ and other partially-resistant *Brassica* lines (H05, H08, H09, H10, D08, D10 and D13) may indicate the presence of both vertical (specific *R*-mediated resistance) and horizontal (effective against a range of pathotypes) resistance against the current Victorian isolates. The identification of these resistance genes and their linkage markers would provide valuable tools for the establishment of a successful clubroot breeding system. The susceptibility of *Brassica* vegetables, especially Chinese cabbages, may be addressed against current Victorian clubroot isolates by pyramiding these resistance genes.
Chapter 4

Gene expression profiling for clubroot resistance using the ‘boutique’ RMIT Brassica oligoarray

4.1 Introduction

As described in earlier chapters, germplasm have been screened to improve clubroot resistance in breeding and marker-assisted selection of Brassica crops. These breeding techniques have mainly relied on the selection of dominant genes involved in clubroot resistance and the hormones cytokinin (Siemens et al., 2006) and auxin (Ludwig-Müller, 2004; Ludwig-Müller et al., 2009). Yet durable resistance to Plasmodiophora brassicae has not been achieved in susceptible crops through their genetic manipulation. This may be partly due to an insufficient understanding of the host/pathogen interaction at the molecular level. Small-scale studies have focused on changes in only one or a few molecules; this limitation is due to difficulties in distinguishing host from pathogen molecules in this complex biotrophic interaction (Cao et al., 2008). The identification of the number of genes involved in clubroot resistance and their mechanisms of action is essential for effective breeding strategies.

Plants have evolved mechanisms involving developmental, morphological, physiological and biochemical strategies to survive against different biotic and abiotic stresses (Vidhyasekaran, 2007). Studies on the mechanisms of plant defence have reported the transcriptional activation and repression of genes from pathogen detection to response/adaptation. Speed, coordination and magnitude of detection, signal transduction and activation of the relevant defence genes are critical for effective disease resistance. The complexity and diversity of these responses to different stages of clubroot infection has not been previously documented in Brassica. The understanding of this coordinated response network may be improved by investigating gene expression profiles in response to clubroot disease. One such approach involves the use of
high-throughput microarray techniques. This valuable tool has been exploited in a number of plants, such as in *Arabidopsis* (Siemens et al., 2006) and canola (Cao et al., 2008, proteomics-based approach) against clubroot and in *Arabidopsis* (Schenk et al., 2000) and chickpea (Coram and Pang, 2006) against other plant pathogens. The availability of the Affymetrix™ *Arabidopsis* genechip would permit detailed analysis of selected clubroot-resistant crops. The onset of resistance in *Brassica* vegetables against clubroot is however not fully understood and may be at least partly constitutive and partly inducible. Hence the timing for the extraction of total RNA in defence responses is vague. The construction of a ‘boutique’ oligonucleotide array (oligoarray) and its use to investigate defence-related gene expression was a viable option to avoid costly and inefficient use of Affymetrix™ chips in future experiments.

The aims of the experiments described in this chapter were:

1. **To develop a reliable and reproducible test system to study the defence mechanisms against clubroot in *Brassica* vegetables, especially Chinese cabbage.** Initially, a soil-based system was used to challenge the partially-resistant *Brassica rapa* varieties ‘Tahono’ and ‘Leaguer’ as well as the susceptible ‘Granaat’ and to compare their transcript levels from root tissues at different time points. Due to complications with the quality of total RNA samples, a novel hydroponic system in the study of clubroot disease was later designed to provide a ‘clean’ and consistent source of total RNA for future experiments.

2. **To investigate the gene expression profiling for clubroot resistance using microarray technology.** The construction of a ‘boutique’ oligonucleotide array (oligoarray) and its use to investigate defence-related gene expressions at different time points in ‘Tahono, ‘Leaguer’ and ‘Granaat’ after inoculation with clubroot isolate S was a viable option to
narrow down key enzymes and times to use the Affymetrix *Arabidopsis* genechip. This system minimised the issue of pathogen RNA interfering with the results by using non-inoculated controls and *Brassica*-specific probes on the oligoarray.

3. **To postulate possible nature of the defence responses and the genes needed for resistance, partial-resistance and susceptibility to clubroot disease.** In this study, both the inducible and constitutive nature of clubroot resistance was investigated.

### 4.2 Materials and Methods

#### 4.2.1 Synthesis and validation of the *Brassica* oligonucleotide probes

A list of *Arabidopsis* and *Brassica* genes mostly involved in defence, stress, hormone management and general housekeeping was compiled from searches performed on GenBank® ‘nr’ and ‘ESTs’ databases. A total of 75 cDNA gene sequences was used to design 150 26-mer oligonucleotide probes using the Clone Manager Professional Suite® (Version 7, Scientific and Educational Software™, USA). The software generated a list of potential probe sequences for each cDNA sequence from GenBank®; only two of these were selected per gene, based on their ranks and proximity to the 5’ (Probe A) and 3’(Probe B) ends (Table 4.1). The probes selected had a G/C content range of 50-55% and melting temperature range of 65-75°C. In addition, great care was taken in designing the orientation of the oligonucleotide probe sequences. Both the cDNA sequence from GenBank® and its corresponding oligonucleotide probes were in the same direction as its mRNA sequence to allow hybridisation with its complementary fluorescent-labelled cDNA target. The oligonucleotide probes were synthesised commercially by Operon Biotechnology Inc. (Germany) (Scale: 50 nmole per probe, Purification: Salt-free, Modification: Amino-C6 with 10 Thymine nucleotides linker at the 5’ end). The functional groupings of these genes are illustrated in Figure 4.1.
Table 4.1. The list of synthesised oligonucleotide probes used in the construction of the RMIT Brassica oligoarray (sorted by probe ID).

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<tr>
<th>Probe ID</th>
<th>Putative Function</th>
<th>GenBank® Accession</th>
<th>Organism</th>
<th>Group</th>
<th>Oligonucleotide sequence(^{a}(5' \rightarrow 3'))</th>
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<tr>
<td>BA001</td>
<td>Vacuolar ATP synthase b subunit</td>
<td>H07629</td>
<td>B. napus</td>
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<td>CgTggTCAggTTCTggAAATgTgATgg gATATTgATCTgCgACAATggAgCg</td>
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<td>BA002</td>
<td>Abscisic acid-insensitive protein</td>
<td>DQ446612</td>
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<td>DQ446602</td>
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<td>BA004</td>
<td>Abscisic acid signal transduction</td>
<td>BD442751</td>
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<td>BA006</td>
<td>Acyl-CoA synthase</td>
<td>X82273</td>
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<td>AF458410</td>
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<td>BA012</td>
<td>Auxin-response factor</td>
<td>AJ716227</td>
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<td>H07824</td>
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<td>AY821735</td>
<td>B. napus</td>
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<td>BA017</td>
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<td>YA344061</td>
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<td>A. thaliana</td>
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<td>AK119013</td>
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<td>Clubroot-resistant marker</td>
<td>DD182413</td>
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<td>Cytokinin-binding protein</td>
<td>DR997831</td>
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<td>BA026</td>
<td>DNA-damage resistance protein</td>
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<td>Ethylene, HEVER and SA-inducible protein</td>
<td>AI352905</td>
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<td>B. rapa</td>
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<td>Heat shock protein 90</td>
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<td>BA037</td>
<td>Aux/IAA family protein (IAA31)</td>
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<td>BA038</td>
<td>Isochorismate synthase</td>
<td>AF078080</td>
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<td>BA044</td>
<td>Mannitol Stress inducible</td>
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<td>BA045</td>
<td>Metallothionein I</td>
<td>H07628</td>
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<td>BA046</td>
<td>Mitogen-activated protein kinase</td>
<td>D14713</td>
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<td>BA047</td>
<td>mRNA expressed during secondary infection</td>
<td>AJ605576</td>
<td>P. brassicae</td>
<td>2 gCAGAGcTATTCCACAgATTACAgTg</td>
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101
BA048 mRNA expressed in host plant  AB009880  P. brassicae  2  gCTCTTCCAaGTTgCTgTTTCAAgTCg  CgAaggATgTAGTCAACaCggAATg
BA049 Myrosinase  Z21978  B. napus  1  TAgCCAgTAgCATgAgTTgCACCATTg  CCAAgTAAaAaAgAggTAACgAAaggCg
BA050 Myrosinase  X79080  B. napus  1  ACCACAgACTCATAgATggCCTCCTAC  CACTgAaAgCaaACaCgAgCTgTTg
BA051 Myrosinase-binding protein  U59443  B. napus  1  AgAaAggCAgACAAggAAaggACCg  CTggTTACTACCGAAAgCCTTTCTCC
BA052 Myrosinase  X78285  B. napus  1  TCAATTCCATgTgTCCACAgAgg  gTTgCaggCAACAgAgTCACgAAACC
BA053 Nitrilase  H07604  B. napus  4  gTCCTAggACCTgAAGTggTAAgCTC  AggCgTATACgAggCCTACTCCgT
BA054 Nitrilase  AI352935  B. napus  4  AACTCggTgTCTATTgCTgTTgAA  ggATCAACCACCTCCTgTCTgTACgAC
BA055 Pathogenesis-related protein  AI352768  B. napus  1  gTCAAgATgTAGgCggtACCCTTT  gAAAgTTTTCAACGCCTCATCgCg
BA056 Pathogenesis-related protein  AF528177  B. rapa  1  TCACAACCACgCACgACAgAggTAg  AggCTgTTgTgTCAACCCTCgAAAgTCC
BA057 Pathogenesis-related protein  AI352712  B. rapa  1  CTCTCTCTCTCTgTCTgTTTCCCT  CCACCTCCggAAATgTgTTgTACg
BA058 Pectin methylesterase inhibitor  DQ116449  B. oleracea  1  CggCTgTgTTgTCCCAgCgAgTTTg  CgAggTTTgATgCgAgTgGgTg
BA059 Phenylalanine ammonia-lyase  AY055752  B. rapa  3  gTCAgATgATgAgCggAAAgAACg  ACCACATCACTCCTCCTCCCTCCT
BA060 Phenylalanine ammonia-lyase  DQ167187  B. rapa  3  CCCggTgACTgACAggTCCCTCAACAg  gAAAgTTgTgTCCACAggCAACg
BA061 Phenylalanine ammonia-lyase  AA960723  B. rapa  3  ATCAgCAgAgCgACAAgCACgAgCg  CgAggAAGAgCgCCACgTgTgAg
BA063 Polygalacturonase inhibitor protein  AA960715  B. napus  5  gTCAgAggGAggTTgTCCCTCAACAggAgTCAgAg  gAAAgTgTgTCCACAggCAACg
BA065 Resistance-like protein, RGA-1  AF107545  B. napus  2  gTCAAgAggGAggTTgTCCCTCAACAggAgTCAgAg  gAAAgTgTgTCCACAggCAACg
BA066 Ribosomal protein S15a  X59984  B. napus  5  TCAgAggGAggTTgTCCCTCAACAggAgTCAgAg  gAAAgTgTgTCCACAggCAACg
BA069 RPM1 interacting protein  NM113411  A. thaliana  2  gTCAAgAggGAggTTgTCCCTCAACAggAgTCAgAg  gAAAgTgTgTCCACAggCAACg
BA070 SG1-like protein  AJ620883  B. oleracea  2  TCAgAggGAggTTgTCCCTCAACAggAgTCAgAg  gAAAgTgTgTCCACAggCAACg
BA071 Superoxide dismutase  AF540558  B. juncea  2  TCAgAggGAggTTgTCCCTCAACAggAgTCAgAg  gAAAgTgTgTCCACAggCAACg
BA072 Cu/Zn superoxide dismutase  AF071112  B. rapa  2  gTCAAgAggGAggTTgTCCCTCAACAggAgTCAgAg  gAAAgTgTgTCCACAggCAACg
BA073 Ubiquitin-conjugating enzyme  CB331875  B. napus  5  TCAgAggGAggTTgTCCCTCAACAggAgTCAgAg  gAAAgTgTgTCCACAggCAACg
BA074 Ubiquitin-protein ligase  NM123599  A. thaliana  5  gTCAAgAggGAggTTgTCCCTCAACAggAgTCAgAg  gAAAgTgTgTCCACAggCAACg
BA075 Ubiquitin-protein ligase  BE038411  A. thaliana  5  gTCAAgAggGAggTTgTCCCTCAACAggAgTCAgAg  gAAAgTgTgTCCACAggCAACg
BA076 Xyloglucan endotransglycosylase  AY834281  B. rapa  5  gTCAAgAggGAggTTgTCCCTCAACAggAgTCAgAg  gAAAgTgTgTCCACAggCAACg
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**a** Functional groups involved in:
1. Structural barriers (*e.g.* lignification), enzymatic and chemical defences (*e.g.* pathogenesis-related proteins or antimicrobial secondary metabolites).
2. Gene-for-gene resistance resulting in the synthesis of reactive oxygen species (ROS), hypersensitive response (HR) and programmed cell death.
3. Systemic acquired resistance (SAR) (*e.g.* induction of salicylic acid (SA) and SA-inducible defence responses).
4. Phytohormone biosynthesis (*e.g.* production of auxin, cytokinin, ethylene and abscisic acid).
5. Other functions (*e.g.* cell wall modification, energy and protein turn-over).

**b** Oligonucleotide modification: 5’-amine-modified Cy5 dye coupled with 10 deoxythymidines.
Figure 4.1. Functional grouping of the 75 Arabidopsis-/Brassica-derived genes on the RMIT Brassica oligoarray.

Functional groups involved in:
1. Structural barriers (e.g. lignification), enzymatic and chemical defences (e.g. pathogenesis-related proteins or antimicrobial secondary metabolites).
2. Gene-for-gene resistance resulting in the synthesis of reactive oxygen species (ROS), hypersensitive response (HR) and programmed cell death.
3. Systemic acquired resistance (SAR) (e.g. induction of salicylic acid (SA) and SA-inducible defence responses).
4. Phytohormone biosynthesis (e.g. production of auxin, cytokinin, ethylene and abscisic acid).
5. Other functions (e.g. cell wall modification, energy and protein turn-over).
The National Centre for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST) was used to identify and deduce the putative functions of these probes. The BLASTN program is a WWW-based tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) optimised to find regions of local similarity between nucleotide sequences. The program was used to compare nucleotide sequences with those in sequence databases (‘nr’ database, non-redundant and/or ‘EST’ database, expressed sequence tag). According to the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastDocs), the ‘nr’ database includes all GenBank, EMBL, DDBJ and PDB sequences (but not EST, STS, GSS, or phase 0, 1 or 2 HTGS sequences) while the ‘EST’ database includes GenBank, EMBL and DDBJ sequences from the EST division. The BLASTN program also calculated the statistical significance of matches in terms of bit scores and E-values (expect values). The bit score gave an indication of how good the alignment is: the higher the score, the better the alignment. By contrast, E-values indicated the probability that a hit occurred at random and may indicate significant homology between the probe sequences and the nucleotide database, i.e. the smaller the E-value, the less likely that the resulting putative function of the query sequences occurred by chance (Altschul et al., 1990; Altschul et al., 1997). The validated probe sets were printed on the array according to Minimum Information about a Microarray Experiment (MIAME) guidelines (Brazma et al., 2001).

4.2.2 Printing of the RMIT Brassica oligoarray

4.2.2.1 Preparation of the 384-well plate

The custom-synthesised oligonucleotide probes were resuspended into 100 µM solutions with autoclaved DEPC (diethylpyrocarbonate)-treated water and 6 µL of each 100 µM probe was transferred to a V-bottom polypropylene 384-well plate (Corning®). Each well was made up to 24 µL to a final concentration of 50 mM sodium phosphate buffer (pH 8.5) using the 6 × printing buffer (Appendix 2), the preferred buffer for oligonucleotide probes according to
Amersham Biosciences (2003). The oligonucleotide probe had a final concentration of 25 µM. There were 28 ‘buffer’ wells filled with 24 µL of 50 mM sodium phosphate buffer (pH 8.5) and 2 ‘printing control’ wells consisting of 2 µL of a 100 µM 5’-amine-modified Cy5 dye coupled with 10 deoxythymidines (aaCy5-10T) (GeneWorks®) made up to 24 µL with a final concentration of 50 mM sodium phosphate buffer (pH 8.5). The plate was sealed with an adhesive sealing sheet to protect the samples and to reduce evaporation of the buffer during storage at 4°C in the dark.

4.2.2.2 Configuration of the BioRobotics® microarray printer

Before printing the RMIT Brassica oligoarrays, the BioRobotics® MicroGrid II Compact printer (Figure 4.2) had to be configured using the Total Array System (TAS) Application Suite (version 2.6.0.1). The parameters used to print five slides may be found in Appendix 3. Each glass slide or RMIT Brassica oligoarray was composed of 12 sub-grids and each sub-grid was printed from 180 samples (150 custom-synthesised oligonucleotide probes and 30 controls) from the 384-well plate in a 30 spots × 6 spots format. The distance between centres of the circular spots (pitch distance) within each sub-grid was set to 295 µm. It was intended to perform two hybridisation experiments per slide and each hybridisation reaction was to be tested with six sub-grids, i.e. six technical replicates per hybridisation experiment.

4.2.2.3 Printing and post-printing procedures

The printing of the oligoarrays (Figure 4.3) was performed using the BioRobotics® MicroGrid II Compact printer at the RMIT University (Bundoora, VIC). First, the sealed 384-well plate was heated in an oven at 45°C for 20 min to dissolve any precipitated salt, followed by centrifugation at 1,238 × g for 10 min at room temperature to remove air bubbles trapped inside the wells. Meanwhile, the printing pins (BioRobotics Microspot 2500 pin, Cat. No. BR14811) were sonicated at high power for 15 min and checked at ×40 magnification to
Figure 4.2. The BioRobotics® MicroGrid II Compact printer (Reproduced from BioRobotics (2003)).

Figure 4.3. The microarray printer consisted of the sample plate (source area), the glass slides (destination areas ⭐️), the pin tool (⭐️) and the wash stations (⭐️) (Reproduced from BioRobotics (2003)).
ensure their channels were clean and not obstructed by dust or salt particles. The MicroGrid II Compact printer consisted of the samples/plate (biobank or source area), the glass slides (destination or target area), the pin tool and the wash stations, all controlled automatically by the TAS software. After the 384-well plate was immobilised in the biobank, the sonicated pins were inserted in the pin tool, the wash stations were filled with double-distilled water and the micro-spotting procedures were initiated at 50% humidity at room temperature. The pin tool reached the wells in the plates to draw nanoliters of the solution inside the pins and was directed to the target area where the contents were deposited onto the ‘pre-print’ glass slides. Once the printed spots were consistent in size, shape and volume, the pin tool printed onto the 25 mm × 75 mm × 1 mm CodeLink™ Activated slides (Amersham Biosciences®). The pins were cleaned and dried in the wash stations before resuming micro-spotting using samples from other wells.

The CodeLink™ slides have a hydrophilic polymer coating containing N-hydroxysuccinimide (NHS) ester reactive groups designed to couple with amine-modified nucleic acids in a humid environment at pH 8 – 9. Following the manufacturer’s instructions (Amersham Biosciences, 2003), the RMIT Brassica oligoarrays were placed in a slide storage box after printing and were incubated overnight (16-18 h) in an enclosed chamber saturated with NaCl (~75% humidity) at room temperature in the dark. These slides were treated in pre-warmed blocking solution (50 mM ethanolamine, 0.1 M Tris, pH 9.0) at 50°C for 30 min to block any residual reactive groups. The blocked-slides were rinsed twice quickly with autoclaved MilliQ water and washed with pre-warmed post-coupling solution (4 × SSC, 0.1% SDS) at 50°C for 30 min on a Ratek™ platform mixer at 40 rpm. The slides were again rinsed twice with autoclaved MilliQ water and blown dry with an air gun as soon as possible. To maintain the quality of the oligoarrays, they were stored in dust-free desiccators with activated silica gel as desiccant at room temperature in the dark until used. All solvents or solutions used in this section were
filter-sterilised using a sterile single-use 0.2 μm filter (Minisart®) to remove dust and salt particles that may interfere with printing and hybridisation and were prepared as described in Appendix 2.

For reliable analysis of the microarray data, it was essential that the construction and pre-hybridisation of the oligoarrays were optimised. Variations in experiments may occur at any stage of expression analysis, from slide printing through hybridisation to data acquisition. Hence, the orientation and shape of the spots in newly printed oligoarrays were inspected visually and by using the array scanner. This was made possible by the inclusion of the ‘printing controls’ aaCy5-10T at different locations on the array. In addition, the printing conditions, post-printing steps, pre-hybridisation steps and hybridisation temperature were standardised to prevent formation of donut spots, minimise non-specific binding and to reduce background that may interfere with analysis.

### 4.2.3 Plant material: growth, inoculation and collection

The partially-resistant F₁ *B. rapa* hybrids H06 (‘Tahono’ CR-1-1) and H09 (‘Leaguer’ CR 1052) provided by the Henderson Seed Group Pty Ltd (Table 3.1, propagated by self-fertilisation in Section 3.2.2) together with the highly susceptible host ECD05 (‘Granaat’) from the ECD set (Table 2.1) were used as sources of resistance and susceptibility respectively to study the defence mechanisms in Chinese cabbage against clubroot. Clubroot isolate S (prepared according to Section 3.2.3, ECD code: 16/02/00), which was particularly aggressive towards *B. rapa* varieties, was the source of infection.

#### 4.2.3.1 Using a soil-based system

Each *Brassica* line was sown in 10-cm diameter pots filled with autoclaved potting mix (prepared according to Section 2.2.2) at 4 seeds per pot and 8 pots per clubroot isolate, *i.e.* 4
pots each for the uninfected control and the infected treatment. Twenty-eight days after germination, each ‘treatment’ seedling was inoculated in the morning by pipetting 1 mL of \(2 \times 10^7\) spores per mL clubroot isolate S onto the base of the stem and all inoculated pots were placed inside a 65 L black plastic crate. For control pots, 1 mL of MilliQ water was applied to each seedling before being placed inside a separate plastic crate. After inoculation, humid and water-logged conditions inside the plastic crates were provided to promote clubroot infection and gall formation. Roots were collected 7, 14 and 22 days after inoculation (dai) using 1 control and 1 treatment pot at each time point for each \(Brassica\) line. This involved gently removing the potting mix from the \(Brassica\) roots for each pot, followed by a quick wash in cold tap water before freezing in liquid nitrogen and storage at -80°C. The remaining pots were examined 8 weeks after inoculation to check for the presence of clubroot infection.

The potted plants were grown in a glasshouse with a temperature range of 20 ± 3°C, a day-length of 18 h and were watered moderately about 4-5 times per week (during Summer). As well as good cultural practices, the Bayer Confidor™ systemic insecticide spray aerosol (Yates™, NSW) was applied once about 2 weeks after germination to control/treat insect attacks.

4.2.3.2 Using a hydroponic system

Due to complications that occurred from using the soil-based system (e.g. insufficient and/or poor root material), a hydroponic system was established as a novel technique in studying the defence mechanism against clubroot in \(Brassica\) vegetables. This system offered significant advantages of uniformity during total RNA extraction and downstream applications. In addition, preliminary analysis from the soil-based samples suggested that earlier time-points for root tissue collection were needed. This system was optimised in terms of the clubroot spore concentration (simulating heavily infected \(Brassica\) fields in Victoria), constituents and
concentration of the hydroponic solution (to promote healthy growth of the *Brassica* hosts),
timing of pesticide application and tissue collection (to have a representative response in the
hosts caused solely by clubroot infection).

**Preparation of the hydroponic system:**
The hydroponic tanks were constructed from 35 cm × 23 cm × 10 cm Klip IT™ 7 L plastic
tubs (Sistema®) by perforating their lids with 35 mm-diameter holes (6 holes × 4 holes
format) (Figure 4.4). To prevent algal growth in the hydroponic solution, the exteriors of the
hydroponic tanks were covered with opaque black plastic sheet. Up to six hydroponic tanks
were aerated with an aquarium air pump (Aqua One™, SR 9500 model) which was set to a
high air output of 9,500 mL/min. Each hydroponic tank was filled up to 6 L with a modified
Hoagland nutrient solution (Table 4.2) (pH 5.5). The hydroponic systems were installed in
environmentally-controlled glasshouses/growth-rooms with a temperature range of 22 ± 3°C,
a humidity range of 70-90% and 18 h photoperiod (250 W halogen bulbs about two meters
above the plants at an intensity of ~270 µmoles m⁻² h⁻¹).

**Preparation of the *Brassica* lines:**
The *Brassica* seeds were treated in 5 mL of 5 g/L of Mancozeb Plus® contact fungicide
(Yates™, NSW) for 60 min and surface-sterilised by a 5 min wash in bleach (4% active
chlorine), followed by a 5 min wash in 70% ethanol and finally five washes of 2 min each in
autoclaved MilliQ water. Each seed was sown in a damp 35 mm × 35 mm × 40 mm rockwool
seedling plug and left in the dark to germinate at room temperature. Within 12 days after
sowing, the seedlings were transferred to the hydroponic tanks by fitting the rockwool plugs
in the perforated lids. Each hydroponic tank accommodated a maximum of 24 seedlings. To
generate enough root tissue, two tanks were used per *Brassica* line, *i.e.* the control tank and
Figure 4.4. The setup of the hydroponic system with control (left) and treatment (right) tanks using the clubroot-resistant Chinese cabbages ‘Tahono’ (H06) and ‘Leaguer’ (H09) and the susceptible ‘Granaat’ (ECD05).

Table 4.2. The composition of the modified Hoagland nutrient solution used in the hydroponic system (Reproduced from Taiz and Zeiger (2002)).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Molecular weight</th>
<th>Concentration of stock solution</th>
<th>Concentration of stock solution</th>
<th>Volume of stock solution per litre of final solution</th>
<th>Element</th>
<th>Final concentration of element</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>g mol⁻¹</td>
<td>mM</td>
<td>g L⁻¹</td>
<td>mL</td>
<td>µM</td>
<td>mg L⁻¹</td>
</tr>
<tr>
<td><strong>Macronutrients</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KNO₃</td>
<td>101.10</td>
<td>1000</td>
<td>101.10</td>
<td>6.0</td>
<td>N</td>
<td>16000</td>
</tr>
<tr>
<td>Ca(NO₃)₂·4H₂O</td>
<td>236.16</td>
<td>1000</td>
<td>236.16</td>
<td>4.0</td>
<td>K</td>
<td>6000</td>
</tr>
<tr>
<td>NH₄H₂PO₄</td>
<td>115.08</td>
<td>1000</td>
<td>115.08</td>
<td>2.0</td>
<td>Ca</td>
<td>4000</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>246.48</td>
<td>1000</td>
<td>246.48</td>
<td>1.0</td>
<td>P</td>
<td>2000</td>
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<td></td>
<td></td>
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<td></td>
<td></td>
<td>S</td>
<td>1000</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Mg</td>
<td>1000</td>
</tr>
<tr>
<td><strong>Micronutrients</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>KCl</td>
<td>74.55</td>
<td>25</td>
<td>1.864</td>
<td></td>
<td>Cl</td>
<td>50</td>
</tr>
<tr>
<td>H₂BO₃</td>
<td>61.83</td>
<td>12.5</td>
<td>0.773</td>
<td></td>
<td>B</td>
<td>25</td>
</tr>
<tr>
<td>MnSO₄·H₂O</td>
<td>169.01</td>
<td>1.0</td>
<td>0.169</td>
<td>2.0</td>
<td>Mn</td>
<td>2.0</td>
</tr>
<tr>
<td>ZnSO₄·7H₂O</td>
<td>287.54</td>
<td>1.0</td>
<td>0.288</td>
<td></td>
<td>Zn</td>
<td>2.0</td>
</tr>
<tr>
<td>CuSO₄·5H₂O</td>
<td>249.68</td>
<td>0.25</td>
<td>0.062</td>
<td></td>
<td>Cu</td>
<td>0.5</td>
</tr>
<tr>
<td>H₂MoO₄ (85% MoO₃)</td>
<td>161.97</td>
<td>0.25</td>
<td>0.040</td>
<td></td>
<td>Mo</td>
<td>0.5</td>
</tr>
<tr>
<td>NaFeDTPA (10% Fe)</td>
<td>468.20</td>
<td>64</td>
<td>30.0</td>
<td>0.3</td>
<td>Fe</td>
<td>19.2</td>
</tr>
</tbody>
</table>
the treatment tank (one biological replicate to isolate RNA independently from replicate treatment tank).

Due to the time and space constraints, it was necessary to perform these tests in a staggered manner as illustrated in Table 4.3. The soil-based and a hydroponic system were set up simultaneously (preliminary experiment) with the aim of evaluating this novel technique in studying clubroot infection and resistance. The initial observations were used to optimise future experiments. Subsequently, three biological replicates were performed through time (experiments 1, 2 and one replicate of 3) as well as through space (three replicates of experiment 3) for each *Brassica* line (Figure 4.5).

Maintenance of the hydroponic system involved refilling the tanks to 6 L with distilled water every 2-3 days. Three days before inoculation of the treatment seedlings with isolate S, the hydroponic solution was changed and the tanks adjusted to 6 L twice a day until the last day of root tissue collection. As well as good cultural practices, the Bayer Confidor™ systemic insecticide spray aerosol (Yates™, NSW) was applied once about 2 weeks after germination to control/treat insect attacks.

**Inoculation of the Brassica lines and root tissue collection:**

Twenty-eight days after germination, each ‘treatment’ seedling was inoculated in the morning by pipetting 1 mL of $2.5 \times 10^9$ spores per mL of clubroot isolate S onto the base of their stems. By contrast, 1 mL of MilliQ water was applied to each seedling in the control hydroponic tanks. Roots were collected initially at 7, 14 and 22 dai (preliminary experiment only) but this was later changed to 0.5, 1, 2 and 3 dai using at least 3 plants for each control and treatment *Brassica* line (Table 4.3). These plants were pooled per treatment; only 6-8 cm of the upper parts of their root systems were collected and rapidly rinsed in cold tap water before being
Table 4.3. Details of the hydroponic experiments performed.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Location</th>
<th>Date of inoculation</th>
<th>Season in Victoria</th>
<th>Time points (days)</th>
<th>Biological replicate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Glasshouse</td>
<td>7-Nov-06</td>
<td>Late Spring</td>
<td>0.5, 1, 2 and 7</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Glasshouse</td>
<td>21-Feb-07</td>
<td>Late Summer</td>
<td>0.5, 1, 2 and 3</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>Growth-room</td>
<td>7-May-07</td>
<td>NA</td>
<td>1, 2 and 3</td>
<td>3</td>
</tr>
</tbody>
</table>

Figure 4.5. Replication in experimental design. The flow chart shows the procedures undertaken to minimise technical and biological variations in the gene expression profiles of control (green) and treated (red) samples. TO, ‘Tahono’; LE, ‘Leaguer’ and GR, ‘Granaat’.
frozen in liquid nitrogen and stored at –80°C. The remaining plants were allowed to grow in their respective hydroponic tanks and the resulting clubroot symptoms scored 4 and 8 weeks after inoculation using a scale as in Figure 2.3 to grade infection and their level of clubroot resistance in this system.

4.2.4 Preparation of total RNA

4.2.4.1 Extraction of total RNA

The total RNA from frozen Brassica roots was extracted using the Qiagen™ RNeasy® Plant Mini Kit. This technology is based on the selective binding properties of a silica-based membrane, allowing the isolation of up to 100 µg of total RNA longer than 200 bases per column. The pooled root tissues were weighed and ground to a fine powder in liquid nitrogen using autoclaved DEPC-treated mortar and pestles. For every 100 mg of root tissue, 500 µL of buffer RLT (10 µL β-mercaptoethanol added per 1 mL buffer RLT) was added in a sterile 10 mL polypropylene tube and shaken gently by hand. The remaining procedures were carried out according to the RNeasy® Mini Handbook (Qiagen, 2006b) except that each RNeasy® column was loaded and centrifuged with 800 µL of the shredded lysate twice. Also, the on-column DNase digestion using the Qiagen™ RNase-Free DNase set was performed to minimise the risks of DNA contamination in the total RNA samples. The sample was eluted using 50 µL of RNase-free water twice to maximise the yield without compromising the concentration.

4.2.4.2 Quantification and quality control of extracted total RNA

The concentration of total RNA was estimated by measuring the absorbance at 260 nm (A_{260}) in an Eppendorf® BioPhotometer. An absorbance of one unit at 260 nm corresponded to 44 µg RNA per mL. The ratio of the readings at 260 nm and 280 nm (A_{260}/A_{280}) was used to determine the purity of the samples, as pure RNA has a ratio of 1.9 – 2.1 (Qiagen, 2006b).
Once the spectrophotometer was calibrated using a 1 mL quartz cuvette filled with 495 µL of RNase-free water as reference, 5 µL of the RNA sample was added to the same cuvette and mixed gently before the absorbance was measured at the desired wavelengths. The integrity and size distribution of RNA was estimated by agarose gel electrophoresis. One µL of 5 x GelPilot loading dye was added to 5 µL of each RNA sample. The loading dye (supplied within the Qiagen™ QIAquick® PCR Purification Kit) contained three marker dyes (bromophenol blue, xylene cyanol, and orange G) that facilitated the estimation of RNA migration distance and optimisation of agarose gel run time. The RNA samples and 6 µL (0.5 µg) of GeneRuler™ 1 kb DNA ladder (Fermentas®) was loaded into wells of a 1.5% agarose gel in 1 × TBE (Tris-borate EDTA) buffer and run at 10 V/cm for 60 min. The gel was post-stained with 50 ng/mL ethidium bromide in 1 × TAE (Tris acetic acid EDTA) buffer for 5 min, destained in running water for about 20 min and scanned using a BioRad® Gel Doc system on a UV-illuminator. The 6 µL (0.5 µg) of the GeneRuler™ 1 kb DNA Ladder run along with the samples on the gel displays a 3000 bp reference band which contains around 133 ng of DNA/RNA. The concentration of total RNA was determined by comparing the brightness of the RNA samples to that of the reference band.

4.2.5 Preparation of fluorescent labelled-cDNA targets

4.2.5.1 Reverse transcription and hydrolysis of total RNA

Fluorescence labelled-cDNA targets were prepared according to the Australian Genome Research Facility (AGRF) microarray protocols. Total RNA samples for each Brassica line and time-points (extracted and purified earlier in Sections 4.2.4) were aliquoted into 50 µg portions and dried in a vacuum desiccators overnight at room temperature. Ten µL of 0.5 µg/µL p(dT)15 primer (Roche™) was added to the dried RNA samples and made up to 20 µL with autoclaved diethylpyrocarbonate (DEPC)-treated water before being transferred into 200 µL PCR tubes. The mixture was heated for 10 min at 70°C in a thermal cycler and quickly
chilled on ice for 10 min. The master mix for one first-strand cDNA synthesis reaction was prepared as follows: 6 µL of 5 × first-strand buffer (Invitrogen™), 3 µL of 0.1 M dithiothreitol (DTT) (Invitrogen™), 0.6 µL of 50 × aa-dUTP/dNTPs (Appendix 2) and 0.75 µL of 200 U/µL Superscript II Reverse Transcriptase (Invitrogen™). The master mix was added to each RNA/primer PCR tube on ice, mixed gently by pipetting to avoid frothing and incubated for 2 h at 42°C in a thermal cycler. The incorporation of the chemically reactive nucleotide analogue (amino allyl dUTP) allowed the coupling with CyDye™ fluorescent dyes (Amersham™) via the post-labelling route for use in microarray labelling.

After the completion of the cDNA synthesis, the RNA templates were hydrolysed by adding 10 µL of 1 M NaOH and 10 µL of 0.5 M ethylenediaminetetraacetic acid (EDTA) (pH 7.0) to each tube and by incubating for 15 min at 65°C. Each reaction was neutralised with 25 µL of 1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.0) and stored at -20°C.

### 4.2.5.2 Purification and labelling of cDNA targets

Prior to coupling with the CyDye™ post-labelling reactive dye pack (Amersham™), all traces of Tris or other primary amines were removed from the reverse transcription (RT) reactions to prevent the monofunctional NHS-ester CyDyes™ from coupling to free amine groups in solution. This purification step was performed using the Qiagen™ QIAquick® PCR Purification Kit according to modified manufacturer’s instructions (Qiagen, 2006a). The content of each RT reaction tube and 375 µL of buffer PBI were combined into a 1.5 mL reaction tube and briefly vortexed before being transferred to a QIAquick® column. These columns were centrifuged for 1 min at 13,000 × g and, after discarding the flow-through, 700 µL of buffer PE was added to each column. The columns were centrifuged again and the flow-through was discarded. Washing with 700 µL of buffer PE was repeated and finally, the
columns were dried by centrifugation at 13,000 × g for another minute. Cy5™ and Cy3™ reactive dyes (Amersham™) were resuspended in 30 µL of freshly prepared 0.1 M sodium bicarbonate (pH 9.0) and 15 µL of each was applied directly onto the membrane of the corresponding QIAquick® column. The column was incubated in the dark for 1.5 h at room temperature, resulting in the coupling of the CyDye™ fluorescent dyes with the amino allyl dUTP nucleotide analogue incorporated during cDNA synthesis.

The labelled cDNA target was eluted with 80 µL of sterile MilliQ water by centrifugation for 1 min at 13,000 × g. Once more, the eluate was purified using the Qiagen™ QIAquick® PCR Purification Kit. To each eluate, 400 µL of buffer PBI was added and gently vortexed. The Cy3-labelled sample was first applied to a new QIAquick® column and centrifuged for 1 min at 13,000 × g. Its corresponding Cy5-labelled sample was added to the column, centrifuged for 1 min at 13,000 × g and the flow-through discarded. Washing steps were performed as described earlier. Thirty µL of sterile MilliQ water was applied twice onto the QIAquick® column to elute the pooled CyDye™-labelled cDNA targets. The eluate was transferred to a 200 µL PCR tube and evaporated in a thermal cycler at 70°C in the dark till about 5-10 µL was left.

4.2.6 Hybridisation and washing of the RMIT Brassica oligoarray

The dried eluate was resuspended in 12 µL of freshly prepared 2 × hybridisation buffer (80 µL of 20 × SSC and 4 µL of 10% SDS, made up to 200 µL with sterile MilliQ water) with 2 µL of 10 mg/mL salmon sperm DNA (Sigma™) and made up to 24 µL with sterile MilliQ water. The mixture was incubated at 100°C for 2 min in a thermal cycler and centrifuged briefly at 13,000 × g. A 22×25 mm lifter coverslip (Grale Scientific™) was positioned onto the RMIT Brassica oligoarray (printed in Section 4.2.2) and the hot CyDye™-labelled cDNA targets were applied directly at the elevated edges of the coverslip. Great care was taken to
avoid the trapping of air bubbles and for the labelled targets to spread evenly beneath the coverslip by capillarity. The oligoarray slide was positioned horizontally with the printed side up and was enclosed in a hybridisation chamber (Corning®) after filling the water reservoirs to maintain humidity inside. The sealed chamber was wrapped in aluminium foil and incubated in a water bath at 55°C overnight (16-18 h).

The coverslip was removed by immersing the oligoarray slide in 4× SSC solution at room temperature and the slide washed in a staining dish with a series of wash buffers on a Ratek™ platform mixer at 40 rpm according to instructions from Amersham Biosciences (2003). The oligoarray was rinsed twice with pre-warmed 2× SSC / 0.1% SDS solution for 5 min at 55°C, followed by 1 min in 0.2× SSC solution and 1 min in 0.1× SSC solution, both at room temperature, and was immediately dried with an air gun and kept in the dark at room temperature until scanning.

4.2.7 Analysis of the RMIT Brassica Oligoarray

4.2.7.1 Scanning with Affymetrix® Jaguar™

Arrays were scanned in an Affymetrix® 428™ Array Scanner using Affymetrix® Jaguar™ software (v2.0, Santa Clara, CA). The 428 Scanner is an epifluorescence confocal microscope that uses a green and red laser to excite fluorophores at 532 nm (Cy3) and 635 nm (Cy5) respectively. During scanning, fluorescent emissions were captured by photomultiplier tubes in the scanner and were converted into numeric values. These digital intensity values were collected from discrete areas (called pixels) on the array surface and were saved to an image data file (.tiff) on the computer workstation. The scanner provided 10 µm scanning resolution, i.e. the scanner acquired data per 10 x 10 µm area (1 pixel) at this resolution (Affymetrix, 2001). The slide was loaded onto the scanner stage with the printed side facing up and every grid on the array was scanned separately at a gain of 60 decibels with an average of three
lines. The gain setting controlled the signal strength from the scanner, while the average line setting determined the number of times a pixel was scanned and digital intensity value, displayed as means.

4.2.7.2 Quantification of spot intensities with BioDiscovery ImaGene™

The BioDiscovery ImaGene™ software (v5.5, Marina Del Rey, CA) was used for microarray image analysis to quantify the relative expression levels within a microarray scan according to the user manual (BioDiscovery, 2002). In brief, both the Cy3 and Cy5 images produced by the Jaguar™ software were opened using the Imagene™ software and superimposed to produce a composite image (Figure 4.6a). The minimum and maximum spot diameters were determined with the ‘ruler’ tool before a rectangular grid of 30 spots × 6 spots was positioned onto each sub-grid using the ‘automatically place grid’ tool (Figure 4.6b). For optimal spot recognition, it was sometimes necessary to manually re-align/re-adjust the grid position/spot diameter by visual inspection of the array spots. To readily interpret the information in microarray experiments, each probe was given a unique identification code (Table 4.1) by loading into a gene ID file, generated in Microsoft® Excel. Eventually, the signal intensity for each array spot was quantified using the fixed circle method by measuring the mean pixels within the spot circle, while the local background was determined by a three-pixel diameter ring that began three pixels outside the spot circle. This generated two Imagene™ data (.txt) files with prefixes ‘0_’ and ‘1_’ from the Cy3 and Cy5 channels respectively.

During quantification, auto-segmentation was used to partition the scan image into regions as spots or background. This view showed which pixels were valued as signal or were to be ignored as background in the quantification process. Once the segmentation was complete, dubious spots were identified and flagged by various types of automated and manual flagging (Figure 4.6c).
Figure 4.6. Analysis of the scan images using the Affymetrix™ ImaGene® software: (a) The composite image of the control and treatment samples, (b) Positioning of the grid onto each sub-grid of the array (six technical replicates) before quantification of the spot intensities and (c) flagged spots (+ and ×) to screen those with low quality/intensity. (Source: ‘Tahono’ labelled-targets).
Under the ‘quality flags’ tool, options selected for automatic flagging included:

1. Empty spots: Lowly expressed or missing spots were flagged based on the sensitivity threshold \( R < 4 \), where \( R = \frac{(\text{signal mean} - \text{background mean}) \times \text{standard deviation}}{1} \).

   The \( R \) threshold was adjusted until all negative control spots were flagged as ‘empty’.

2. Negative spots: Spots with signal mean lower than background mean were flagged.

3. Poor spots: Four criteria were used, including background contamination (confidence level set to 0.9995), ignored pixels percentage (set to >25%), open perimeter percentage (set to >25%) and offset from expected position (set to >60%).

   Automatic multichannel flagging was set to flag a spot in both channels if it was ‘poor’ in one channel and ‘empty’ or ‘negative’ in both channels. Spots with mean signal intensity less than twice the local background were manually flagged by opening each Imagene™ data (.txt) file using Microsoft® Excel, flagging the required spots and saving the data file as text delimited (as required for subsequent analysis).

4.2.7.3 Statistical analysis with BioDiscovery GeneSight™

   The BioDiscovery GeneSight® software (v4.1.3, Marina Del Ray, CA) is a data mining, visualisation and reporting tool that was used to analyze the gene expression data generated by microarray technology.

   Configuration for differential expression analysis:

   Using the dataset builder, the Imagene™ data were loaded both as single experiments (control and treatment data for each biological replicate) and experimental groups (the control and treatment data of all the biological replicates) so that replicate data may be combined. The data set was organised into ratio data for Cy3 and Cy5 for each Chinese cabbage line and time
point. The ‘data preparation’ tool was used to perform a series of specific data transformations:

1. Local background correction: The average background intensity around each spot was subtracted from the spot signal intensity. This was the most accurate way of background correction since it allowed for variations in background intensity over the array.

2. Omit flagged spots: Low quality spots with flag values of 1, 2 and 3 were omitted from analysis to ensure only high quality spots remained.

3. Log transformation: Each signal intensity value was log-transformed with a base of 2 (no shift) to improve subsequent transformation steps.

4. Normalisation: Global normalisation using the LOcally WEighted polynomial regreSSion (LOWESS) was used (linear fitness, smoothing parameter of 0.2) to divide the data into number of overlapping intervals and to fit a polynomial function.

5. Difference: By subtracting the log-transformed signal intensities of the control from those of the treatment, a gene up-regulated by a factor of two in a treated sample had a value of 1.0 and a gene down-regulated by a factor of 2 had a value of -1.

6. Combine replicates: Data for replicate spots were combined by averaging the signal intensities of replicated spots to produce a single value with a coefficient of variation (CV).

Configuration for constitutive expression analysis:

Due to the small number of consistently differentially expressed genes that were eventually observed in this study, the constitutive expression of defence-related cDNAs was investigated. Only the Imagene™ data from the uninfected controls were loaded so that replicate data may be combined. The data set was organised into ratio data for partially-resistant and susceptible Chinese cabbage varieties (‘Tahono’ or ‘Leaguer’ controls vs ‘Granaat’ controls). Similarly, the ‘data preparation’ tool was used to perform a series of specific data transformations as
above, with some modifications. The ‘log transformation’ was not performed so that the mean normalised absolute background and spot signal intensities may be analysed. The ratios were calculated manually from the signal intensities for each probe using Microsoft Excel and log_2 transformed for easier interpretation.

4.2.7.4 Identification of differentially expressed cDNAs

The identification of differentially expressed genes in any microarray study may be divided into two aspects: ranking and selection. Ranking requires specification of a statistic or measure which provides evidence for differential expression on a per gene basis while selection requires specification of a procedure (e.g. stipulation of a critical value) for arbitrating what constitutes ‘significant’ differentially expression (Yang et al., 2005).

The gene ranking method used in this analysis was based on the fold change (FC) (i.e. ratio) in mean expression between the treated and control samples. Firstly, the inherent noise and sensitivity of this microarray system were determined by a self-self hybridisation, i.e. using the same total RNA sample for both Cy3 and Cy5 labelling in a microarray experiment. A less-stringent FC cut-off value of 1.8-fold was employed as opposed to the usual 2-fold (Coram and Pang, 2006; Mantri et al., 2007). The expression datasets (generated from GeneSight™) were used to determine the 95% confidence intervals for mean expression ratio for each array feature. Those cDNAs for which confidence interval extended beyond the determined FC cut-off value were identified as differentially expressed. While fold-difference is a useful measurement to assess change, it can be misleading if one of the genes has a transcript level below detection or above saturation, resulting in an over- or under-estimation of fold-change, respectively (Clarke and Zhu, 2006). The 1.8-fold cut-off was supported by proper experimental replications to minimise variation and to enable statistical analysis (Figure 4.5), i.e. n = 6 (where n represented the number of data points for each microarray
element) for individual dataset analysis or \( n = 18 \) (three biological replicates analysed) for combined dataset analysis. Subsequently, the equality of variance between the Cy3 and Cy5 channel means was calculated using the F distribution prior to Student’s \( t \)-statistic \((P<0.05)\). The latter compared gene-specific variation across arrays (Yang et al., 2005), a method commonly used to assess differential expression in microarray studies (Dudoit et al., 2002; Coram and Pang, 2007; Mantri et al., 2007). Lastly, it was intended to confirm those differentially expressed genes as ‘significant’ by a selection method known as the False Detection Rate (FDR), defined as the probability for a differentially expressed gene to be falsely positive (Aubert et al., 2004).

In brief, differentially expressed transcripts were initially identified by their 95% confidence interval for mean expression ratio that extended beyond the determined FC cut-off value of 1.8 and eventually, that passed the Student’s \( t \)-test \((P<0.05)\) and FDR correction (Refer to Appendix 5 for the ranking and selection method employed to identify differentially expressed genes). The list of differentially expressed genes was tabulated and genes analysed in terms of their putative function to determine the pathways involved in defence response after the inoculation with clubroot isolate S in the three \textit{Brassica} genotypes.

### 4.2.7.5 Identification of constitutively expressed cDNAs

Firstly, a 3-fold background cut-off value was calculated by multiplying the upper 95% confidence limit of the mean normalised background intensity (from all control expression datasets) by three. Those cDNAs with mean normalised absolute expressions beyond the determined background cut-off value were identified as constitutively expressed, \( i.e. \) those genes whose signal intensities were three times above that of their background intensities. Subsequently, the gene ranking method used in this analysis was based on the fold change (FC) in mean expression between the partially-resistant and susceptible control samples.
Similarly, a FC cut-off value of 1.8 fold was applied, supported by proper experimental replications to minimise variations and to enable proper statistical analysis, i.e. \( n = 18 \) (using control datasets at 48 hai from three biological replicates performed through time only). Only those genes with significant difference in their constitutive expressions between the partially-resistant varieties ‘Tahono’ or ‘Leaguer’ versus the susceptible ‘Granaat’ were selected and tabulated. This involved calculating the equality of variance using the F distribution, prior to Student’s \( t \)-statistics \( (P<0.05) \) and FDR correction as in Appendix 5.

Several genes from the resulting list of significantly constitutively expressed cDNAs were selected to investigate seasonal effects on transcriptional changes in this hydroponic system. Their mean normalised absolute signal intensities in control plants of the three biological replicates were plotted to demonstrate possible links between time / date of inoculation and the ability to produce an effective defence response, i.e. to identify patterns between season and plant growth (vigour and maturity / maturity).

### 4.2.8 Validation of the microarray data by quantitative real-time PCR

The microarray expression results were validated by quantitative real-time PCR (qRT-PCR) on a set of genes (targets) from the list of resultant differentially expressed cDNA (Table 4.4). This set was chosen to represent different defence responses and expression values (up/down regulation). Their primers were designed using the Clone Manager Professional Suite (Version 7, Scientific and Educational Software™, USA) and possessed a GC content range of 50-60\%, melting temperature range of 50-80ºC, annealing temperature of 55ºC and primer length of 20-25 nucleotides. Expected amplicon sizes were 70-250 bp. The relative standard curve method was used to determine the relative expression level for each of the selected genes. This was made possible by the ubiquitous actin gene both as a target to construct the
Table 4.4. The qRT-PCR primer sequences for validation of microarray data.

<table>
<thead>
<tr>
<th>Putative function</th>
<th>Abbreviation</th>
<th>GenBank® accession number</th>
<th>Nucleotide sequence (5' → 3')</th>
<th>Expected amplicon length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td>Actin</td>
<td>AF111812</td>
<td>F (CTCTTTCCTCACGCTATCCTC) R (CGTCAGGTAGCTCGTAGTTC)</td>
<td>225</td>
</tr>
<tr>
<td>Glutathione S-transferase</td>
<td>GST</td>
<td>AI352707</td>
<td>F (GTGGCTGAGATCAACAGAG) R (TACTGCGACTGAAGCAGAAG)</td>
<td>82</td>
</tr>
<tr>
<td>Xyloglucan endo-transglycosylase</td>
<td>XTH</td>
<td>AY156708</td>
<td>F (GTCCGCATGAAATGGACCATCTAC) R (CCTTCTACATTCCGCTGGCATAAC)</td>
<td>78</td>
</tr>
<tr>
<td>Phenylalanine ammonia-lyase</td>
<td>PAL</td>
<td>AY055752</td>
<td>F (AGAACGGTGTCGCTTTCAG) R (TGTCGGCAGTGTCGGGTAATG)</td>
<td>101</td>
</tr>
<tr>
<td>Chitinase</td>
<td>CHT</td>
<td>AF230684</td>
<td>F (TACTTCGGTCGCTGCTGATTC) R (AGCGACAGTTGGGTTGCTACTC)</td>
<td>120</td>
</tr>
</tbody>
</table>
standard curve and as the reference/endogenous control to normalise the quantification of the set of target genes.

Five µg of total RNA (extracted from ‘Granaat’ and ‘Tahono’ roots 48 hai with isolate S from hydroponic experiment 3) was reverse-transcribed into cDNA using 0.5 µg of oligo(dT)\textsubscript{15} primer (Roche\textsuperscript{TM}), 1 µL of 10 mM of each dNTP and the Superscript II Reverse transcriptase Kit (Invitrogen\textsuperscript{TM}) per 25 µL reverse transcription reaction. After 1 h incubation at 42°C in a thermal cycler, the reactions were stopped by heating at 70°C for 15 min. The resulting cDNA samples were purified using a Qiagen\textsuperscript{TM} Qiaquick PCR purification kit (Qiagen, 2006a) and diluted to 250 µL in autoclaved DEPC-treated water. Prior to amplification, five 10-fold dilutions of an untreated ‘Granaat’ cDNA stock of known concentration were accurately prepared for the standard curve. Aliquots of these dilutions were organised for the entire qRT-PCR study so that the relative quantities of the target genes, generated from the standard curve, were compared across reaction plates. Triplicate 25 µL qRT-PCR reactions (duplicate for the standard curve dilutions) were performed using the Bio-Rad IQ\textsuperscript{TM} SYBR\textsuperscript{®} Green Supermix, 0.4 µM of forward and reverse primers and 2.5 µL of cDNA template in an optically approved iCycler iQ\textsuperscript{TM} 96-well PCR plate (Bio-Rad, Cat. No. 2239441) and sealed with Microseal\textsuperscript{®} ‘B’ Film PCR adhesive seal (Bio-Rad, Cat. No. MSB1001). A multichannel pipette distributed the PCR master mix to minimise pipetting errors. The PCRs were performed in a Bio-Rad iCycler iQ\textsuperscript{TM} Multi-Color Real-Time PCR detection system with initial denaturation step at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 45 s, annealing at 55°C for 45 s and extension at 72°C for 1 min. Melting curve analysis, by applying decreasing temperature from 95°C to 45°C (0.5°C/10 s), and gel electrophoresis of the final product were used to confirm single amplicons. Duplicate negative control reactions using total RNA were run with the main reactions to confirm absence of genomic DNA.
Prior to quantification, the PCR baseline subtraction curve fit analysis mode was selected in the Bio-Rad iCycler iQ™ optical system software (version 3.1) to characterise and correct for drift in the background fluorescence over the course of the qPCR run. The threshold value was adjusted manually along the exponential phase of the amplification curves (viewed in log scale) until the slope of the standard curve or PCR efficiency was closest to -3.4 or 100%. The standard curve, constructed from the $C_T$ (cycle threshold) values of actin in the five 10-fold dilutions, was assessed and outliers were excluded to achieve a correlation coefficient closest to 1 (while maintaining a PCR efficiency $\leq 100\%$). The average amount of the target genes using their replicated $C_T$ values (outliers were excluded) was determined for each experimental sample from the standard curve. The average target amount was divided by its respective average actin amount to obtain an average normalised target value. Finally, the average normalised ‘treated’ target value was divided by its average normalised ‘untreated’ target value to generate the relative expression level (Applied Biosystems, 2005). The qRT-PCR data was compared to the microarray data resulting from the same total RNA sample. Additional references on the application of qRT-PCR may be obtained online at www.appliedbiosystems.com/support/apptech.

4.3 Results

4.3.1 Validation of the oligonucleotide probe sequences

The BLASTN results indicating the putative function, bit scores and E-values of the query probe sequences are tabulated in Appendix 4. The greatest and most common bit score was 52 while the least and most common E-value was $3e^{-05}$. Since the bit score depends on the length and database size (Altschul et al., 1990), these values may not indicate the reliability of the resulting putative functions and were disregarded. Probes (BA024A, BA024B, BA025B and BA080B) with high E-values (>0.05) in both ‘nr’ and ‘EST’ databases did not represent the GenBank sequences (and putative functions) from which they were designed. These
probes may not hybridise with their corresponding fluorescently-labelled cDNA targets and hence, were flagged during analysis of the oligoarray scan images.

### 4.3.2 The hydroponic test system

The standardised system of hydroponic plant culture, infection treatment with isolate S and replications provided significant advantages over a soil-based system. Rapid growth (Figure 4.7a) resulting in abundant and ‘clean’ root tissues (Figure 4.7b), are required for high purity, quantity and integrity of total RNA and downstream applications. The hydroponic system was used successfully in the infection and testing of the Brassica lines with clubroot isolate S as demonstrated in Figure 4.8, due to the formation of clubroot symptoms 8 weeks after inoculation as opposed to healthy growth of the controls.

The manifested symptoms in the Brassica hosts in the hydroponic system were scored and are illustrated in Table 4.5. Four weeks after inoculation (with the exception of experiment 2), the Chinese cabbage ‘Tahono’ had less severe disease symptoms than ‘Leaguer’ and ‘Granaat’. Eight weeks after inoculation, all of the treated genotypes had severe clubroot symptoms while the controls remained healthy. Dissimilar symptoms obtained for the biological replicates performed through time may be attributed to the differing date/season of inoculation with isolate S. All biological replicates were included in the analysis due to the presence of clubroot infection in the highly susceptible ‘Granaat’.

### 4.3.3 Quantification and quality control of extracted total RNA

The abundant growth of root tissues using the hydroponic system allowed significantly more total RNA to be extracted compared with roots harvested from the soil-based system. Using the Qiagen™ RNeasy® Plant Mini kit, the purity of the total RNA samples was acceptable as indicated by $A_{260/280}$ ratios between 1.7 and 2.0 (data not shown). The integrity of the total
Figure 4.7. The advantages of a hydroponic over a soil-based test system: (a) rapid growth and difference in morphology of 28-day-old Chinese cabbages in hydroponic solutions (X) and soil-based media (Y), and (b) abundant growth of ‘clean’ root tissues, essential for total RNA extraction, in a hydroponic system.
Figure 4.8. Healthy (left) vs diseased (right) roots caused by *Plasmodiophora brassicae* in ‘Granaat’ (top) and ‘Leaguer’ (bottom) eight weeks after inoculation with isolate S in the hydroponic system. Diameter of the perforated holes in the hydroponic lids was 35 mm.
Table 4.5. Symptoms\textsuperscript{a} manifesting in the *Brassica* vegetables 4 and 8 weeks after inoculation with isolate S in the hydroponic system.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Symptoms 4 weeks after inoculation</th>
<th>Symptoms 8 weeks after inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>Tahono</em>\textsuperscript{b}, <em>Leaguer</em>\textsuperscript{b}, <em>Granaat</em>\textsuperscript{c}</td>
<td><em>Tahono</em></td>
</tr>
<tr>
<td>1</td>
<td>0 2 3</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>1 1 2</td>
<td>NA</td>
</tr>
<tr>
<td>3</td>
<td>1 3 3</td>
<td>3   3</td>
</tr>
</tbody>
</table>

\textsuperscript{a} The 4-grade scale was used to assess the clubroot symptoms: 0 = no visible clubbing, 1 = small galls confined to lateral roots, 2 = moderate swellings on both lateral and/or tap root and 3 = severe clubbing.

\textsuperscript{b} Partially-resistant varieties.

\textsuperscript{c} Clubroot-susceptible variety.

NA, data not available.

Figure 4.9. Example of good quality total RNA extracted from Chinese cabbage root tissues, run on 1.5% agarose gel and stained with 50 ng/mL ethidium bromide.
RNA, determined by gel electrophoresis (Figure 4.9), was also integrity of the total RNA, determined by gel electrophoresis (Figure 4.9), was also satisfactory according to Qiagen (2006b) since the ribosomal RNA (rRNA) bands appeared sharp/not degraded and the 25S rRNA bands (~ 1500 bp) were stained at least twice the intensity of the 18S bands (~ 1000 bp). Those high quality total RNA samples were used within 3 months of extraction.

4.3.4 Analysis of the ‘boutique’ RMIT Brassica oligoarray

Identification of differentially expressed cDNAs:

The fold change (FC) cut-off value of 1.8-fold used in the gene ranking method was determined from the self-self hybridisation result. The latter yielded a 99% confidence distribution (Figure 4.10) in which 89% of the signals fell within 1.5 FC and 97.6% were within 1.8 FC. These 1.8 FC cut-offs translated into up-regulated transcripts having a log₂ difference ≥ 0.848, and down-regulated transcripts ≤ -0.848. Due to the limited numbers of differentially expressed cDNAs, the False Detection Rate (FDR) multiple testing correction was not performed.

The transcript level of each cDNA was first calculated at the average intensity of the biological replicates (i.e. at n = 18) performed in time or space and those that passed the Student’s t-test (P<0.05) are tabulated in Table 4.6. No differential expression was observed at 24 and 72 hai in all three genotypes. Only three (2%) out of the 150 unique microarray elements (probes) were consistently differentially expressed in all biological replicates and all of them occurred in ‘Tahono’ at 48 hai. The lack of any consistent expression within biological replicates performed in space was unexpected. Even if the 95% confidence intervals for their log₂ difference values were beyond the FC cut-off log₂ value of 0.848, their average log₂ difference values (closer to zero) were a concern.
Figure 4.10. The scatter plot for the self-self hybridisation generated by SPSS software (v15.0.1), where 97.6% of signals were within the green lines. Red line indicates equivalent signal intensities between Cy3- and Cy5-labelled targets while the green lines indicate FC cut-off values of 1.8-fold.
Table 4.6. List of genes consistently differentially expressed 24, 48 or 72 hours after inoculation (hai) with clubroot isolate S in all biological replicates (n = 18) performed in time or space (sorted by putative function).

<table>
<thead>
<tr>
<th>Variety&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Time (hai)</th>
<th>Replicate type&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Probe ID&lt;sup&gt;c&lt;/sup&gt;</th>
<th>GenBank Accession No.</th>
<th>Putative Function</th>
<th>log&lt;sub&gt;2&lt;/sub&gt; difference&lt;sup&gt;d&lt;/sup&gt;</th>
<th>P value&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>TO</td>
<td>48</td>
<td>T</td>
<td>BA037B</td>
<td>AY669802</td>
<td>Aux/IAA family protein (IAA31)</td>
<td>-0.709</td>
<td>0.047</td>
</tr>
<tr>
<td>TO</td>
<td>48</td>
<td>T</td>
<td>BA074A</td>
<td>NM123599</td>
<td>Ubiquitin-protein ligase</td>
<td>0.833</td>
<td>0.001</td>
</tr>
<tr>
<td>TO</td>
<td>48</td>
<td>T</td>
<td>BA078A</td>
<td>H07799</td>
<td>Xyloglucan endotransglycosylase</td>
<td>0.566</td>
<td>0.001</td>
</tr>
</tbody>
</table>

<sup>a</sup> Chinese cabbage variety: Clubroot-susceptible ‘Granaat’ (GR), partially-resistant ‘Leaguer’ (LE) and partially-resistant ‘Tahono’ (TO).

<sup>b</sup> Replicate type indicated if the biological replicated used in analysis were performed in time (T) or space (S).

<sup>c</sup> Refer to Table 4.1 for details of the oligonucleotide probes.

<sup>d</sup> Only probes with average log<sub>2</sub> difference value whose 95% confidence interval extended beyond the FC cut-off log<sub>2</sub> value of 0.848, were tabulated.

<sup>e</sup> Only significant probes that passed the Student’s t-test (P<0.05) were tabulated.
As transcriptional changes caused by inoculation of isolate S were only prominent at 48 hai, this was used as the most appropriate time for further analysis. Only 23 (15.3%) out of the 150 probes were differentially expressed in at least one genotype at 48 hai in individual experiments \( (i.e. \text{ at } n = 6) \) (Table 4.7). The relationship and co-regulation of observed differentially expressed transcripts within each genotype at 48 hai for individual experiments are summarised in Figure 4.11. The key observations are the co-induction of a pathogenesis-related (PR) protein: chitinase (AF230684 or X61488) in all genotypes and that up-regulation was most prominent at 48 hai, at which ‘Tahono’ altered the expression of more transcripts (8 genes) than ‘Leaguer’ (1 gene). Despite the differing level of clubroot resistance of ‘Granaat’ and ‘Tahono’ (reported in Chapter 3), these two lines unexpectedly had the greatest number of co-induced transcripts and there was a low number of ‘Tahono’-specific up-regulated transcripts.

Overall, upon inoculation with isolate S, there was limited differential expression in all three genotypes on the RMIT \textit{Brassica} oligoarray, which was constructed mostly from defence-related genes. In addition, the inability to observe consistent expression in the combined datasets when differential expression was detected in individual experiments (or vice versa), was a concern.

\textbf{Identification of constitutively expressed cDNAs:}

Since differential expression was most prominent at 48 hai, the control samples were used to investigate constitutive expression in all three genotypes. Similarly, the fold change (FC) cut-off value of 1.8-fold was used in the gene ranking method and translated into constitutively over-expressed transcripts having a log\(_2\) ratio \( \geq 0.848 \), and under-expressed transcripts \( \leq -0.848 \). Also, the False Detection Rate (FDR) multiple testing correction was not performed due to the limited numbers of constitutively expressed cDNAs.
Table 4.7. List of genes differentially expressed 48 hai with isolate S in individual experiments (n = 6) (sorted by putative function).

<table>
<thead>
<tr>
<th>Variety</th>
<th>Experiment</th>
<th>Probe ID</th>
<th>GenBank Accession No.</th>
<th>Putative Function</th>
<th>log$_2$ difference</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GR</td>
<td>1</td>
<td>BA002B</td>
<td>DQ446612</td>
<td>Abscisic acid-insensitive protein</td>
<td>-0.914</td>
<td>0.001</td>
</tr>
<tr>
<td>GR</td>
<td>1</td>
<td>BA003A</td>
<td>DQ446602</td>
<td>Abscisic acid-responsive protein</td>
<td>0.661</td>
<td>0.024</td>
</tr>
<tr>
<td>GR</td>
<td>2</td>
<td>BA011A</td>
<td>AF458410</td>
<td>Auxin-repressed protein</td>
<td>0.563</td>
<td>0.029</td>
</tr>
<tr>
<td>TO</td>
<td>2</td>
<td>BA011B</td>
<td>AF458410</td>
<td>Auxin-repressed protein</td>
<td>0.777</td>
<td>0.042</td>
</tr>
<tr>
<td>TO</td>
<td>3a</td>
<td>BA011A</td>
<td>AF458410</td>
<td>Auxin-repressed protein</td>
<td>0.963</td>
<td>0.002</td>
</tr>
<tr>
<td>GR</td>
<td>2</td>
<td>BA008B</td>
<td>U53672</td>
<td>Auxin-induced IAA22</td>
<td>-0.982</td>
<td>0.001</td>
</tr>
<tr>
<td>GR</td>
<td>2</td>
<td>BA031B</td>
<td>AY836001</td>
<td>3-glucanase</td>
<td>-1.377</td>
<td>0.035</td>
</tr>
<tr>
<td>TO</td>
<td>3a</td>
<td>BA014A</td>
<td>AY821735</td>
<td>Caffeoyl-CoA 3-O-methyltransferase</td>
<td>0.681</td>
<td>0.023</td>
</tr>
<tr>
<td>GR</td>
<td>1</td>
<td>BA020A</td>
<td>AF230684</td>
<td>Chitinase</td>
<td>0.667</td>
<td>0.015</td>
</tr>
<tr>
<td>GR</td>
<td>1</td>
<td>BA021A</td>
<td>X61488</td>
<td>Chitinase</td>
<td>0.923</td>
<td>0.035</td>
</tr>
<tr>
<td>LE</td>
<td>3a</td>
<td>BA021B</td>
<td>X61488</td>
<td>Chitinase</td>
<td>0.914</td>
<td>0.029</td>
</tr>
<tr>
<td>TO</td>
<td>3a</td>
<td>BA020B</td>
<td>AF230684</td>
<td>Chitinase</td>
<td>0.727</td>
<td>0.005</td>
</tr>
<tr>
<td>GR</td>
<td>1</td>
<td>BA029A</td>
<td>AI352905</td>
<td>Ethylene, HEVER and SA-inducible protein</td>
<td>0.736</td>
<td>0.007</td>
</tr>
<tr>
<td>GR</td>
<td>2</td>
<td>BA029A</td>
<td>AI352905</td>
<td>Ethylene, HEVER and SA-inducible protein</td>
<td>0.714</td>
<td>0.006</td>
</tr>
<tr>
<td>TO</td>
<td>1</td>
<td>BA029A</td>
<td>AI352905</td>
<td>Ethylene, HEVER and SA-inducible protein</td>
<td>0.772</td>
<td>0.046</td>
</tr>
<tr>
<td>TO</td>
<td>3a</td>
<td>BA029A</td>
<td>AI352905</td>
<td>Ethylene, HEVER and SA-inducible protein</td>
<td>0.991</td>
<td>0.015</td>
</tr>
<tr>
<td>TO</td>
<td>3a</td>
<td>BA032B</td>
<td>AI352707</td>
<td>Glutathione S-transferase</td>
<td>-0.926</td>
<td>0.002</td>
</tr>
<tr>
<td>TO</td>
<td>3c</td>
<td>BA032B</td>
<td>AI352707</td>
<td>Glutathione S-transferase</td>
<td>-1.217</td>
<td>0.026</td>
</tr>
<tr>
<td>TO</td>
<td>3a</td>
<td>BA044B</td>
<td>AW288083</td>
<td>Mannitol stress-inducible protein</td>
<td>1.792</td>
<td>0.002</td>
</tr>
<tr>
<td>Genotype</td>
<td>Replicate</td>
<td>Accession</td>
<td>Gene Name</td>
<td>log2 Fold Change</td>
<td>p-value</td>
<td></td>
</tr>
<tr>
<td>----------</td>
<td>-----------</td>
<td>-----------</td>
<td>-----------------------------------------------</td>
<td>-----------------</td>
<td>---------</td>
<td></td>
</tr>
<tr>
<td>TO</td>
<td>1</td>
<td>BA045A</td>
<td>H07628 Metallothionein I</td>
<td>-0.724</td>
<td>0.007</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>BA073B</td>
<td>CB331875 Ubiquitin-conjugating enzyme</td>
<td>0.819</td>
<td>0.002</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>BA073A</td>
<td>CB331875 Ubiquitin-conjugating enzyme</td>
<td>0.459</td>
<td>0.026</td>
<td></td>
</tr>
<tr>
<td>GR</td>
<td>1</td>
<td>BA074A</td>
<td>NM123599 Ubiquitin-protein ligase</td>
<td>0.926</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3a</td>
<td>BA074A</td>
<td>NM123599 Ubiquitin-protein ligase</td>
<td>2.074</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>TO</td>
<td>1</td>
<td>BA074A</td>
<td>NM123599 Ubiquitin-protein ligase</td>
<td>0.848</td>
<td>0.019</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>BA074A</td>
<td>NM123599 Ubiquitin-protein ligase</td>
<td>1.574</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3a</td>
<td>BA074A</td>
<td>NM123599 Ubiquitin-protein ligase</td>
<td>1.156</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>LE</td>
<td>3a</td>
<td>BA001A</td>
<td>H07629 Vacuolar ATP synthase b subunit</td>
<td>-0.540</td>
<td>0.023</td>
<td></td>
</tr>
<tr>
<td>TO</td>
<td>2</td>
<td>BA001A</td>
<td>H07629 Vacuolar ATP synthase b subunit</td>
<td>-0.814</td>
<td>0.035</td>
<td></td>
</tr>
<tr>
<td>TO</td>
<td>3a</td>
<td>BA078A</td>
<td>H07799 Xyloglucan endotransglycosylase</td>
<td>1.301</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>GR</td>
<td>1</td>
<td>BA077B</td>
<td>AY156708 Xyloglucan endotransglycosylase precursor</td>
<td>0.853</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>TO</td>
<td>2</td>
<td>BA077A</td>
<td>AY156708 Xyloglucan endotransglycosylase precursor</td>
<td>0.777</td>
<td>0.019</td>
<td></td>
</tr>
<tr>
<td>GR</td>
<td>1</td>
<td>BA076B</td>
<td>AY834281 Xyloglucan endotransglycosylase</td>
<td>0.951</td>
<td>0.002</td>
<td></td>
</tr>
</tbody>
</table>

a Chinese cabbage varieties: Clubroot-susceptible ‘Granaat’ (GR), partially-resistant ‘Leaguer’ (LE) and partially-resistant ‘Tahono’ (TO).

b Refer to Table 4.5 for details of the hydroponic. The three biological replicates performed in Experiment 3 are referred as ‘3a’, ‘3b’ and ‘3c’.

c Refer to Table 4.1 for details of the oligonucleotide probes.

d Only probes with log2 difference value whose 95% confidence interval extended beyond the FC cut-off log2 value of 0.848, were tabulated.

e Only probes that passed the Student’s t-test (P<0.05) were tabulated.

Co-induced transcripts in all genotypes or between ‘Granaat’ and ‘Tahono’ were highlighted in teal and black respectively.
Co-repressed transcripts between ‘Leaguer’ and ‘Tahono’ were highlighted in blue.
‘Granaat’- and ‘Tahono’-specific up-regulated transcripts were highlighted in red and pink respectively.
‘Granaat’- and ‘Tahono’-specific down-regulated transcripts were highlighted in green and yellow respectively.
Figure 4.11. Regulation of the differentially expressed transcripts for each genotype (GR: Granaat, LE: Leaguer and TO: Tahono) 48 hours after inoculation with isolate S from individual experiments. The number of (a) up-regulated and (b) down-regulated transcripts is shown. Venn diagrams were generated at http://www.pangloss.com/seidel/Protocols/venn.cgi. Transcripts were classified using their putative functions. Colour coding similar to Table 4.7.
The selection process is summarised in **Table 4.8** and those significant constitutively expressed probes that passed the Student’s *t*-test (*P*<0.05) are tabulated in **Table 4.9**. Only 8 (5.3%) and 21 (14%) of the 150 oligonucleotide probes were constitutively over-expressed in ‘Tahono’ and ‘Leaguer’ respectively when compared to ‘Granaat’. A key observation was the constitutive over-expression of the PR-protein: chitinase (AF230684 or X61488) in ‘Tahono’. By ‘Leaguer’, over-expression of a lignin biosynthesis enzyme (*e.g.* caffeoyl-CoA 3-O-methyltransferase, AY821735) and possibly in reduced synthesis of the auxin phytohormone (*e.g.* auxin-repressed protein, AF458410 and Aux/IAA family protein, AY669802) were observed. In addition, the over-expression of an ethylene, HEVER and SA-inducible protein (AI352905) as well as glutathione S-transferase (AI352707) indicated that the control samples of ‘Leaguer’ were under stress. Finally, no constitutively co-expressed probe between the two partially-resistant Chinese cabbages was detected.

**Patterns in the biological replicates:**

Since the *Brassica* hosts were grown under environmentally-controlled conditions, uniform levels of gene expression were expected between the biological replicates. As the seasons progressed from Spring (increasing temperature and photoperiod) towards Autumn (decreasing temperature and photoperiod), there was a significant decrease in the constitutive expression level of some defence- and hormone-related genes in control plants (**Figure 4.12**). The expression levels of these genes were generally greater in the clubroot-resistant/partially-resistant ‘Tahono’ when compared to that of the susceptible ‘Granaat’.

### 4.3.5 Validation of the microarray data by quantitative real-time PCR

qRT-PCR was performed to validate the RMIT oligoarray data using the relative standard curve method. The *C*<sub>T</sub> values for the target genes and actin were determined from reliable log-transformed amplification curves (no sigmoid curve detected) using the user-defined
Table 4.8. Selection of constitutively expressed genes with reliable expressions in healthy 30 days old ‘Tahono’ or ‘Leaguer’ Chinese cabbages when compared to that of ‘Granaat’.

<table>
<thead>
<tr>
<th>Selection of probes constitutively expressed in:</th>
<th>Tahono</th>
<th>Leaguer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total No. of probes</td>
<td>150</td>
<td>150</td>
</tr>
<tr>
<td>No. of probes with signal intensity $\geq 3\times$ background intensity</td>
<td>84</td>
<td>87</td>
</tr>
<tr>
<td>No. of probes with SLR* $\geq 0.848$ or $\leq -0.848$</td>
<td>13</td>
<td>24</td>
</tr>
<tr>
<td>No. of probes that passed the Student’s $t$-test ($P&lt;0.05$)</td>
<td>8</td>
<td>21</td>
</tr>
<tr>
<td>% Significant constitutively expressed probes</td>
<td>5.3</td>
<td>14</td>
</tr>
</tbody>
</table>

SLR*: Signal Log Ratio of $\pm 0.848$ was equivalent to 1.8-fold over-/under-expression.
Table 4.9. List of genes expressed constitutively between healthy 30 days old ‘Tahono’ or ‘Leaguer’ Chinese cabbages when compared to that of ‘Granaat’ from all biological replicates performed through time only (sorted by putative function).

<table>
<thead>
<tr>
<th>Variety</th>
<th>Probe ID</th>
<th>GenBank Accession No.</th>
<th>Putative Function</th>
<th>log$_2$ ratio</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LE</td>
<td>BA002A</td>
<td>DQ446612</td>
<td>Abscisic acid-insensitive protein</td>
<td>-2.106</td>
<td>0.001</td>
</tr>
<tr>
<td>LE</td>
<td>BA006A</td>
<td>X82273</td>
<td>Acyl-CoA synthase</td>
<td>-2.247</td>
<td>0.001</td>
</tr>
<tr>
<td>LE</td>
<td>BA011A</td>
<td>AF458410</td>
<td>Auxin-repressed protein</td>
<td>2.615</td>
<td>0.030</td>
</tr>
<tr>
<td>LE</td>
<td>BA012B</td>
<td>AJ716227</td>
<td>Auxin-response factor</td>
<td>1.171</td>
<td>0.039</td>
</tr>
<tr>
<td>LE</td>
<td>BA014B</td>
<td>AY821735</td>
<td>Caffeoyl-CoA 3-O-methyltransferase</td>
<td>1.132</td>
<td>0.045</td>
</tr>
<tr>
<td>LE</td>
<td>BA014A</td>
<td>AY821735</td>
<td>Caffeoyl-CoA 3-O-methyltransferase</td>
<td>2.849</td>
<td>0.025</td>
</tr>
<tr>
<td>TO</td>
<td>BA020B</td>
<td>AF230684</td>
<td>Chitinase</td>
<td>0.849</td>
<td>0.049</td>
</tr>
<tr>
<td>TO</td>
<td>BA021A</td>
<td>X61488</td>
<td>Chitinase</td>
<td>0.861</td>
<td>0.044</td>
</tr>
<tr>
<td>TO</td>
<td>BA025A</td>
<td>DR997831</td>
<td>Cytokinin-binding protein</td>
<td>-5.234</td>
<td>0.005</td>
</tr>
<tr>
<td>LE</td>
<td>BA029A</td>
<td>AI352905</td>
<td>Ethylene, HEVER and SA-inducible protein</td>
<td>2.826</td>
<td>0.034</td>
</tr>
<tr>
<td>LE</td>
<td>BA028A</td>
<td>AY460110</td>
<td>Ethylene-induced stress protein</td>
<td>-2.929</td>
<td>0.002</td>
</tr>
<tr>
<td>LE</td>
<td>BA032B</td>
<td>AI352707</td>
<td>Glutathione S-transferase</td>
<td>1.382</td>
<td>0.002</td>
</tr>
<tr>
<td>LE</td>
<td>BA032A</td>
<td>AI352707</td>
<td>Glutathione S-transferase</td>
<td>2.694</td>
<td>0.040</td>
</tr>
<tr>
<td>LE</td>
<td>BA033B</td>
<td>AK222102</td>
<td>Heat shock protein 90</td>
<td>-1.621</td>
<td>0.018</td>
</tr>
<tr>
<td>LE</td>
<td>BA034B</td>
<td>AI352735</td>
<td>Hypersensitive response gene</td>
<td>-2.108</td>
<td>0.008</td>
</tr>
<tr>
<td>LE</td>
<td>BA037A</td>
<td>AY669802</td>
<td>Aux/IAA family protein (IAA31)</td>
<td>2.946</td>
<td>0.021</td>
</tr>
<tr>
<td>LE</td>
<td>BA041B</td>
<td>AB186133</td>
<td>Isopentenyltransferase</td>
<td>1.075</td>
<td>0.001</td>
</tr>
<tr>
<td>LE</td>
<td>BA045A</td>
<td>H07628</td>
<td>Metallothionein I</td>
<td>-1.258</td>
<td>0.019</td>
</tr>
<tr>
<td>TO</td>
<td>BA052A</td>
<td>X78285</td>
<td>Myrosinase</td>
<td>-0.863</td>
<td>0.016</td>
</tr>
<tr>
<td>---</td>
<td>-----------</td>
<td>-------</td>
<td>-----------------------------------</td>
<td>--------</td>
<td>-------</td>
</tr>
<tr>
<td>LE</td>
<td>BA051A</td>
<td>U59443</td>
<td>Myrosinase-binding protein</td>
<td>-0.930</td>
<td>0.001</td>
</tr>
<tr>
<td>LE</td>
<td>BA055A</td>
<td>AI352768</td>
<td>Pathogenesis-related protein, PVPR3</td>
<td>-5.721</td>
<td>0.010</td>
</tr>
<tr>
<td>LE</td>
<td>BA058A</td>
<td>DQ116449</td>
<td>Pectin methylesterase inhibitor</td>
<td>-2.022</td>
<td>0.002</td>
</tr>
<tr>
<td>LE</td>
<td>BA060A</td>
<td>DQ167187</td>
<td>Phenylalanine ammonia-lyase</td>
<td>-1.71</td>
<td>0.001</td>
</tr>
<tr>
<td>LE</td>
<td>BA066B</td>
<td>X59984</td>
<td>Ribosomal protein S15a</td>
<td>1.046</td>
<td>0.006</td>
</tr>
<tr>
<td>TO</td>
<td>BA070A</td>
<td>AJ620883</td>
<td>SGT1-like protein</td>
<td>-1.032</td>
<td>0.001</td>
</tr>
<tr>
<td>TO</td>
<td>BA074A</td>
<td>NM123599</td>
<td>Ubiquitin-protein ligase</td>
<td>0.987</td>
<td>0.003</td>
</tr>
<tr>
<td>TO</td>
<td>BA078A</td>
<td>H07799</td>
<td>Xyloglucan endo-transglycosylase</td>
<td>-0.881</td>
<td>0.001</td>
</tr>
<tr>
<td>TO</td>
<td>BA077A</td>
<td>AY156708</td>
<td>Xyloglucan endotransglycosylase precursor</td>
<td>-1.035</td>
<td>0.013</td>
</tr>
<tr>
<td>LE</td>
<td>BA077A</td>
<td>AY156708</td>
<td>Xyloglucan endotransglycosylase precursor</td>
<td>0.888</td>
<td>0.001</td>
</tr>
</tbody>
</table>

*a* Partially-resistant Chinese cabbage varieties ‘Tahono’ (TO) or ‘Leaguer’ (LE) compared to susceptible ‘Granaat’ (GR).

*b* Refer to Table 4.1 for details of the oligonucleotide probes.

*c* Important constitutively over-expressed defence-related probes for discussion were **bolded**.

*d* Only probes with average log$_2$ ratio value beyond the FC cut-off log$_2$ value of 0.848, were tabulated.

*e* Only significant probes that passed the Student’s *t*-test (*P*<0.05) were tabulated.
Figure 4.12. Effects of season on transcriptional changes in control plants. Constitutively expressed genes encoding for (a) caffeoyl-CoA 3-O-methyltransferase (CCOMT), indole acetic acid-amino acid hydrolase (IAA) and isopentenyltransferase (IPT) and (b) chitinase (CHI), glutathione S-transferase (GST) and myrosinase (MYR).
threshold (Figure 4.13). These C_T values were derived from specific amplification of single product for each target gene, according to single peak/band on the melting curve analysis (Figure 4.14) and gel electrophoresis (data not shown). The target genes were then quantified using the linear standard curve (Figure 4.15) and according to online resources (www.appliedbiosystems.com/support/apptech), the latter had good PCR efficiency (slope value between -3.32 and -3.60 or PCR efficiency between 90 and 100%) and good precision (correlation coefficient, R^2 > 0.99). The reference gene chosen for normalisation of the quantified data was actin, the expression of which was not significantly affected by pathogen inoculation.

The expression log FC values of the four target genes are summarised in Table 4.10. Of a total of 14 comparisons between microarray and qRT-PCR data (excluding all absent data), only seven (50%) showed conserved direction of regulation. With the exception of chitinase and phenylalanine ammonia-lyase (PAL) in ‘Tahono’, none of the other target genes demonstrated significant differential expression (based on the FC cut-off log_2 value of 0.848, i.e. 1.8-fold). Generally, the lack of correlation between the microarray and qRT-PCR ratios was predominantly from target genes that demonstrated log FC values close to zero. The qRT-PCR data did validate the microarray data using this limited list of target genes since most of the comparisons had relatively similar magnitude.

4.4 Discussion

The aim of this chapter was achieved in that the gene expression profiles of partially-resistant and susceptible genotypes were differentiated with and without inoculation by clubroot. This was made possible by extracting ‘clean’ and representative total RNA samples from plants grown in a novel hydroponic test system. Despite the surprisingly few inducible genes that may be attributed to the ‘closed architecture’ system of the oligoarray, both differential and
Figure 4.13. Example of log-transformed amplification curves (coloured lines), generated by the iCyclerIQ™ Multi-colour Real-time PCR detection system (Bio-Rad, Hercules, CA) in this study. The solid orange line represents the threshold used to calculate $C_T$ values.

Figure 4.14. Example of melting curves generated by the iCyclerIQ™ Multi-colour Real-time PCR detection system (Bio-Rad, Hercules, CA). The presence of sharp single fluorescence peaks indicates the presence of single amplicons of ‘actin’ in this study.
Figure 4.15. Example of the standard curve generated by the iCyclerIQ™ Multi-colour Real-time PCR detection system (Bio-Rad, Hercules, CA) using serial dilutions of the actin target (blue circles) in this study.
Table 4.10. Expression log ratios of selected transcripts assessed by microarray and qRT-PCR.

<table>
<thead>
<tr>
<th>Putative function</th>
<th>Probe ID</th>
<th>GenBank® accession number</th>
<th>Granaat 48 h</th>
<th>Tahono 48 h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Array</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>Glutathione S-transferase</td>
<td>BA032</td>
<td>AI352707</td>
<td>0.10</td>
<td>-0.08</td>
</tr>
<tr>
<td>Xyloglucan endo-transglycosylase precursor</td>
<td>BA077</td>
<td>AY156708</td>
<td>0.01</td>
<td>-0.58</td>
</tr>
<tr>
<td>Phenylalanine ammonia-lyase</td>
<td>BA059</td>
<td>AY055752</td>
<td>0.01</td>
<td>NA</td>
</tr>
<tr>
<td>Chitinase</td>
<td>BA021</td>
<td>AF230684</td>
<td>0.07</td>
<td>0.06</td>
</tr>
</tbody>
</table>

NA, absence of valid data and A or B, probe A or B designed from 3’ or 5’ end of the same cDNA sequence respectively. Array values indicated log₂ fold change (FC) difference relative to untreated controls and qRT-PCR values indicated log₂ ratio of normalised test relative to normalised calibrator.
constitutive expression of defence-related genes were correlated to resistance/partial-resistance against *P. brassicae*. The key defence genes reported were the production of a pathogenesis-related protein (chitinase) and lignification of the roots in the partially-resistant plants. Their expressions prominently at 48 hai, identified the most responsive time for further microarray experiments.

### 4.4.1 Analysis of the hydroponic test system

A hydroponic system was established as a novel technique for studying the defence responses against clubroot in *Brassica* vegetables due to complications occurring with the soil-based system. Gel electrophoresis and spectrophotometric analysis indicated that the hydroponic system provided an abundant and ‘clean’ source of total RNA extracted from root tissues. These are important considerations since the success of an expression analysis relies on the quality of the total RNA. Contaminants such as salts, polysaccharides, DNA, proteins or lipids may interfere, causing inefficient labelling and high background during hybridisation (Bowtell and Sambrook, 2003). Since plant tissues are usually full of polysaccharides and other compounds, RNA of the quality required to make microarray analysis is more difficult to extract from plant tissues than animal tissues. In addition, experiments involving the extraction of nucleic acids or proteins from soil-contaminated samples have always been problematic. This is because soil is a complex medium with an extensive ecosystem of micro-organisms (Torsvik and Ovreas, 2002) living in a mixture of rocks, minerals and dead and decaying plants and animals, this has made downstream applications such as PCR difficult and unreliable due to the presence of inhibitory substances (Juen and Traugott, 2006). The current test system avoided these concerns and with further optimisation may become a standard method employed in microarray experiments involving soil-borne diseases.
This novel hydroponic system offered an efficient and rapid means for root tissue collection. This was not possible with the soil-based systems since much of the delicate root system was either damaged or lost in the attempt to clean them in sterile water within a reasonable processing time prior to freezing at –80°C. If the total RNA is partly degraded, labelling may be biased to those sequences that are more resistant to RNase attacks. This may alter the relative proportion of the two RNA populations under study by hybridisation on the DNA microarray (Monte and Somerville, 2003). Despite these concerns, numerous clubroot studies have used soil-based systems in their infection strategy. These researchers were investigating clubroot symptoms formation (Ludwig-Müller, 2004; Devos et al., 2005; Siemens et al., 2006) or changes in protein levels during infection (Cao et al., 2008), which are much easier to study because they are less susceptible to degradation. Ultimately, this study demonstrated the practicalities of a hydroponic over a soil-based system.

This novel hydroponic system simulated Victorian field conditions and was appropriate in the study of clubroot defence. It resulted in the successful infection of the *Brassica* hosts by following the guidelines of soil-based clubroot studies (Buczacki et al., 1975; Voorrips and Kanne, 1997; Kuginuki et al., 1999) and was indicated by significant clubbing of ‘Granaat’ with Victorian isolate S. This system, performed in glasshouses or growth-rooms, was designed to minimise or control most of the environmental factors that may interfere with defence responses in plants. Other conditions to simulate medium or high risk level of clubroot in Victorian *Brassica* farms such as host sowing time, sufficient nutrient availability, pH of the media (pH<6) and high humidity/water-logging, were followed as advised by Dr Caroline Donald (clubroot factsheets, www.vgavic.org.au/pdf). The data obtained from this hydroponic study should correlate with field conditions/studies.
The lack of similarity in levels of symptom formation in the biological replicates may be because plants were inoculated at different dates / seasons. Experiment 1 was performed in late Spring in glasshouses and produced ideal host reactions, in which ‘Tahono’ was the most resistant. In contrast, clubroot infection was generally less destructive when the hosts were infected during the hot and dry days of late Summer in glasshouses, as shown by the lack of heavy clubbing in ‘Granaat’ (experiment 2). As observed in earlier chapters and as reported by Karling (1968) and Macfarlane (1952), the survival of clubroot spores was affected considerably by ‘extreme’ temperatures (6ºC < unfavourable > 27ºC). *P. brassicae* spores were more viable in growth-rooms (experiment 3) and resulted in all three genotypes succumbing to heavy clubbing 8 weeks after inoculation, i.e. current growth-room conditions favoured clubroot infection. These host reactions / symptoms supported the field observations of Dr Caroline Donald (www.vgavic.org.au/pdf) that clubroot resting spores had a medium to high risk of infection during Spring. The less severe reactions in Summer contradicted with her survey that reported heavy clubbing in Victorian Summer. The superior results in the environmentally-controlled growth-rooms support the conclusions of Voorrips (1996) using a phytotron. This suggests that these controlled conditions should be used in functional genomics studies.

**Resistance to clubroot:**

Challenged cultivars varied as expected in severity of symptoms, suggesting that ‘Tahono’ was the most resistant and that both it and ‘Leaguer’ may be used as sources of clubroot resistance for future experiments. The defensive response in ‘Tahono’ and ‘Leaguer’ is probably a form of horizontal resistance, rather than true vertical resistance. This is because their responses were merely manifested as a reduction in the degree of infection assessed 4 weeks after inoculation compared to that of ‘Granaat’, indicating horizontal or non-pathotype specific resistance. By definition, this mode of resistance should provide low to moderate
levels of defence against a wide range of pathotypes and is normally less likely to break down easily when challenged to new virulent clubroot pathotypes (Keane and Brown, 1997). Since this form of defence is normally inherited additively in plants, this may indicate the presence of polygenic resistance (Simons, 1972; Keane and Brown, 1997) and thus supporting the conclusion of Suwabe et al. (2003), that B. rapa has at least one QTL for clubroot resistance. Such genotypes are often avoided for breeding purposes because it is difficult to detect and manipulate partial-resistance traits. This study is relatively unusual in using horizontal rather than vertical resistance to investigate transcriptional changes.

4.4.2 Analysis of the ‘boutique’ RMIT Brassica oligoarray

The current Brassica oligoarray was a cost-effective and valid tool to investigate transcriptional changes in Brassica hosts upon inoculation with clubroot spores. The list of genes selected for the construction of this array briefly covered the three major plant defence pathways. It involved pathways that function to limit the spread of virulent pathogens (Group 1, 21% of array features, Figure 4.1); the gene-for-gene resistance pathway, which functions during responses to avirulent pathogens (Group 2, 24%) and the systemic acquired resistance (SAR) pathway, which leads to strong resistance against a variety of pathogens (Group 3, 8%) (Glazebrook et al., 1997; Guest and Brown, 1997; Siemens and Mitchell-Olds, 1998; Zhao et al., 2005; Vidhyasekaran, 2007). Additionally, the possible involvement of phytohormones at the very early stages of clubroot infection (Ludwig-Müller, 2004; Ando et al., 2005; Devos et al., 2005; Siemens et al., 2006) was investigated (Group 4, 30%). Lastly, the remaining array features examined other functions such as changes in cell wall morphology, energy and protein turn over (Group 5, 17%). This biased representation of putative defence-associated and regulatory genes on the RMIT Brassica oligoarray was a viable option in determining the most responsive time for tissue collection to avoid the costly and inefficient use of Affymetrix™ chips in future experiments.
4.4.2.1 Patterns in the transcriptional changes

Plant hosts:

The vigour and maturity of the *Brassica* hosts played a major role in determining the level of clubroot resistance (or severity of gall formation). The constitutive expression of several defence-related and hormone genes in the control plants demonstrated the effects of season on their vigour/maturity and ability to produce an effective defence response. For example, lignification and auxin/cytokinin hormones perform important roles in normal plant growth and development (Whetten and Sederoff, 1995; Gaspar *et al.*, 1996; Rogers and Campbell, 2004). The reduction in their constitutive expression from glasshouse to growth-room conditions generally corresponded to an increased in the severity of clubroot symptoms, as reported in Table 4.5. This indicated that plants grown under the hydroponic system in late Spring (increasing temperature and photoperiod) had a higher vigour/maturity compared to those grown in growth-rooms under constant temperature and artificial (and probably inadequate) photoperiod. Rubio-Covarrubias *et al.* (2005) expected that a resistant cultivar would express increased resistance as a consequence of its greater maturity under the influence of higher temperature and longer photoperiod. Other studies have supported the hypothesis that plant age affects the level of resistance to pathogens (Hare, 1966; Collins *et al.*, 1999; Rubio-Covarrubias *et al.*, 2005). Collins *et al.* (1999) reported that potatoes challenged with the late blight pathogen (*Phytophthora infestans*) pass through phases of increased susceptibility, firstly when very young (low maturity) and secondly around the time of flowering (low vigour), separated by a period of increased resistance (increasing maturity and vigour). It is unclear if this pattern of resistance/susceptibility applies to a clubroot/*Brassica* patho-system. To investigate effective and representative defence responses in the *Brassica* hosts against viable clubroot spores, future glasshouse experiments should ideally be performed in late Spring, *i.e.* under increasing temperature and photoperiod.
Seasonal effects:

There was a high degree of variation in the combined microarray data of the biological replicates due to seasonal effects. This was indicated by the consistent differential expression of only 2% of array features and the clearly non-uniform patterns of some constitutively expressed genes in the control plants across the experiments. When the data were analysed as individual experiments, there was major differential expression at 48 hai, at which 15.3% of array features were differentially expressed in at least one genotype. This demonstrated similar levels of differential expression to other functional genomics studies. For example, similar studies using microarrays reported up to 14.3% differentially expressed genes in *A. thaliana* to *P. brassicae* (investigating gall formation) (Siemens *et al.*, 2006), 13.6% differentially expressed genes in chickpea to *Ascochyta rabiei* (Coram and Pang, 2006) and up to 29.7% differentially expressed genes in *A. thaliana* to *Alteraria brassicicola* (Schenk *et al.*, 2000). Other possible sources of variation and the shortcomings of this ‘boutique’ oligoarray, along with the results of the qRT-PCR will be further discussed in Section 4.4.3.

Timing of defence response:

There is strong evidence that recognition of clubroot spores occurred at 48 hai in the *Brassica* hosts using the hydroponic system. This was demonstrated by a high number of differential expressions at this time, as opposed to at 24 and 72 hai. This may be because about 2.5%, 8% and 18% of clubroot spores germinated at 12 h, 48 h and 4 days in Chinese cabbage root extracts (Friberg *et al.*, 2005). Tommerup and Ingram (1971) detected the formation of primary plasmodia in root hair cells within a day after spore germination. The lack of transcriptional changes at 24 hai in the current study may reflect insufficient recognition of the pathogen by all three Chinese cabbage genotypes. This may also demonstrate that there are no strong hypersensitive responses leading to programmed cell death, which usually occurs within 12 to 24 hours of pathogen contact or attempted penetration in general (Brown,
1997; Agrios, 2005). The major responses at 48 hai may correlate with the time of penetration from adequate amounts of germinated spores, resulting in signalling cascades that changed phytohormone levels, increased protein break-down, cell wall modification and/or lignification and synthesis of a PR protein (discussed later). The relative lack of response at 72 hai may indicate the limitations of this ‘boutique’ oligoarray since it was based on a ‘closed architecture’ system, i.e. the results were restricted to the number of transcripts and associated genes present on the array. These results have improved the understanding of the timing of clubroot resistance / reaction to infection, where 48 hai represented at least one time point for clubroot recognition in the Chinese cabbage hosts grown in the hydroponic system.

**Type of regulation:**

Most of the differentially expressed cDNAs at 48 hai were up-regulated in all three genotypes under study. This may be attributed to the biased representation of putative defence-associated and regulatory genes on the RMIT *Brassica* oligoarray. These genes were selected on the hypothesis that upon recognition of the attacking pathogen, defence mechanisms are activated for the invasion to remain localised (Coram *et al.*, 2007; Vidhyasekaran, 2007), i.e. it was believed that the resistance mechanisms in Chinese cabbages depended on induced responses. Since ‘Tahono’ was the most resistant genotype to *P. brassicae*, it was reasonable to infer that observed transcriptional responses of ‘Tahono’ might involve potentially effective genes for clubroot resistance/partial resistance, whereas the genes for ‘Granaat’ might be ineffective. The detection of only two ‘Tahono’-specific up-regulated transcripts suggested that the hypothesis was not fully supported. As observed earlier from the hydroponic test system, the mode of defence is more accurately a form of partial-resistance because all genotypes became clubbed and so there would be only a limited number of significantly up-regulated differential transcripts against clubroot. In addition, both the timing and magnitude of an inducible defence response determine the success of an appropriate resistance (Hammond-Kosack and
Jones, 1996). Since there were no or only limited differences in the timing and magnitude of the transcriptional changes between the genotypes, this suggested that other forms of defence mechanism needed to be investigated, such as the constitutively greater expression of defence-related genes in partially-resistant genotypes.

4.4.2.2 Differential and constitutive expressions occurring at 48 hai

Hypotheses regarding the nature of the defensive responses may be formulated from differential expression at 48 hai. Genes potentially involved in mechanisms for resistance and/or partial-resistance against clubroot disease were: (a) the synthesis of PR proteins (chitinase and glucanase), (b) increased protein break-down (ubiquitin proteolytic pathways), (c) enhanced physical barriers (e.g. lignification and other cell wall modifications) and (d) changes in phytohormone levels (e.g. auxin and abscisic acid). Constitutively expressed genes in uninoculated partially-resistant varieties had greater levels of expression and improved passive defences (e.g. chitinase and lignification).

Expression of defence-related transcripts:

Chitinase:

The PR protein chitinase (AF230684 and/or X61488) exhibited an early and quick defence mechanism against clubroot spores. This was illustrated by its co-induction in all three genotypes at 48 hai and its constitutive over-expression in 30-day-old uninoculated ‘Tahono’ when compared to ‘Granaat’. This PR protein possesses antifungal activity and degrades fungal cell wall structural polysaccharides or alters the fungal cell wall architecture (Fritig et al., 1998) as well as releasing elicitors for the initiation of inducible defence responses (Zhu et al., 1994; Grison et al., 1996). Ludwig-Müller et al. (1994) reported the induction of this enzyme in both susceptible and partially-resistant Chinese cabbages to clubroot, but with twice the magnitude of induction in the partially-resistant ones. Grison et al. (1996) indicated
that a transgenic oilseed rape (*B. napus*) had greater resistance to multiple fungal pathogens when transformed for increased constitutive over-expression of chitinase.

Ludwig-Müller *et al.* (1994) however reported that chitinase alone may not digest intact clubroot resting spores, even if their cell walls contain about 25% chitin (Moxham and Buczacki, 1983). Zhu *et al.* (1994) indicated that the increased constitutive over-expression of chitinases and glucanases led to a synergistic increase in the level of disease control in transgenic tobacco. Two or more PR proteins are required for an efficient constitutive non-specific resistance against a wide range of pathogens (Zhu *et al*., 1994; Hammond-Kosack and Jones, 1996). The down-regulation of the fungal cell wall-degrading enzyme: β-1,3-glucanase (AY836001) in ‘Granaat’ may also be indicative of its susceptibility to clubroot infection. No consistently up-regulated or constitutively expressed cDNAs was however seen between the partially-resistant varieties ‘Tahono’ and ‘Leaguer’. This indicated that the defence mechanisms in these two lines may be different. Since the microarray elements on the current oligoarray were limited in the range of defence pathways included, the mechanisms that make ‘Tahono’ more partially-resistant to clubroot disease than ‘Leaguer’ may not be fully understood from this study. The greater constitutive and inducible chitinase levels in ‘Tahono’ may partly explain its greater partial-resistance than ‘Leaguer’ or ‘Granaat’. Further investigation to determine the sequences of gene activation and information on the chitinase promoter regions may improve our understanding of non-specific defence responses.

*Salicylic acid:*

The production of salicylic acid (SA), leading to SA-mediated signalling in a specific ‘gene-for-gene’ resistance response, may be involved in clubroot resistance. This was indicated by the co-induction of an ethylene, HEVER and SA-inducible protein (AI352905) in ‘Granaat’ and ‘Tahono’ and its constitutive over-expression in ‘Leaguer’ when compared to ‘Granaat’.
This supported the existence of dominant clubroot resistance genes in *Brassica rapa* as reported by several studies (Hirai *et al.*, 2004; Piao *et al.*, 2004; Suwabe *et al.*, 2006) but was not in common with the more partially-resistant ‘Tahono’. The possible induction of the plant systemic acquired resistance (SAR) was insufficient to prevent infection, since galls formed after 4 weeks in all tested genotypes. This may indicate the breakdown of clubroot resistance conferred by a single dominant gene, as reported by Hirai *et al.* (2004) or most likely, a relative lack of SA-inducible defence genes in these varieties. By understanding the cause of the susceptible nature of the *Brassica* crops, breeding strategies may be devised to introgress the defence genes of interest from sources of resistance.

**Ubiquitin:**

There was evidence of increased protein degradation through the ubiquitin proteolytic pathways upon infection. Ubiquitin-conjugating enzyme (CB331875) and ubiquitin-protein ligase (NM123599) co-induced in ‘Granaat’ and ‘Tahono’, are involved in the ubiquitination of target proteins for subsequent degradation by the 26S proteasome. Delauré *et al.* (2008) indicated that ubiquitin ligases and the related protein breakdown play important roles in the signal transduction pathways leading to disease resistance in plants. Also, Ramonell *et al.* (2005) demonstrated that a putative RING-type ubiquitin ligase is involved in the initial signalling responses to chitin, leading to plant defence. The induction of these genes alone did not significantly increase the resistance of ‘Granaat’ to clubroot. Hence, the cumulative effects of several defence-related genes (as well as the induction of other transcripts not covered by this ‘boutique’ oligoarray) may explain the better resistance of ‘Tahono’.

**Changes in cell wall composition and structure:**

Parasitism may be avoided by preventing the entry of *P. brassicae* into the plant cell with the help of a physical barrier or lignification. Caffeoyl-CoA 3-O-methyltransferase (AY821735)
is involved in lignin biosynthesis (Anterola and Lewis, 2002; Do et al., 2007) and its up-regulation in ‘Tahono’ at 48 hai and constitutive over-expression in 30-day-old healthy ‘Leaguer’ when compared to ‘Granaat’ indicated the fortification of root cell walls. Cao et al. (2008) indicated that the down-regulation of a putative caffeoyl-CoA O-methyltransferase in canola at 48 hai resulted in a decrease in lignin biosynthesis and demonstrated host susceptibility to \( P. \text{brassicae} \). The most compelling evidence for the role of lignification in resistance has been reported by Moerschbacher et al. (1990) for the \( R \)-gene-mediated incompatible interaction between wheat and rust. The elevation of lignin content to render cell walls more impermeable may be a slow process, as described by Whetten and Sederoff (1995) and this inducible non-specific form of defence was insufficient to avoid infection in ‘Tahono’. By contrast, the constitutive lignification occurring in ‘Leaguer’ may explain its greater partial-resistance to clubroot disease when compared to ‘Granaat’ and this differing defence mechanism may be used advantageously in breeding strategies. For example, by combining the inducible as well as the constitutive natures of caffeoyl-CoA 3-O-methyltransferase and chitinase of ‘Tahono’ and ‘Leaguer’, the resulting hybrids may constitutively over-express and up-regulate both forms of defence in the presence of clubroot spores. This illustrates the value of pyramiding different defence pathways to achieve durable resistance against a wide range of pathogens.

Other cell-wall modifying proteins though co-induced in both ‘Granaat’ and ‘Tahono’, were xyloglucan endotransglycosylase/hydrolase (XTH) (AY834281 and/or H07799) and its precursor (AY156708). The high activity of XTH has been correlated with morphological changes such as cell division and cell elongation in plant organs (Verbelen et al., 2001). Although not a defence response, the up-regulation of XTH, resulting in the loosening of cell walls during gall formation has been reported in the early stages of clubroot infection (Devos
et al., 2005). This up-regulation may indicate an efficient and fast mechanism for P. brassicae in taking control of the host cells as early as 48 hai.

Changes in phytohormone levels:

Little is known about changes in phytohormone levels in the host cells at the very early stages of clubroot invasion and disease development. This study provided some evidence of increasing auxin level in infected hosts at 48 hai. This was indicated by the up-regulation of an auxin-repressed protein (AF458410) in both ‘Tahono’ and ‘Granaat’. Kim et al. (2007) reported that genes encoding for auxin-repressed protein have been identified in various plant species and may be induced, paradoxically, by increasing auxin concentration. This supported other clubroot studies that indicated a greater level of auxin in infected Chinese cabbages (Ando et al., 2005), possibly by the induction of IAA-amidohydrolase-like genes (Br-IAR23 and Br-ILL6) identified by RT-PCR in B. rapa (Schuller and Ludwig-Müller, 2006) or released through the indole glucosinolate pathway (Ludwig-Müller et al., 2009). The co-induction of auxin and XTH in infected plants correlated with the clubroot studies of Devos et al. (2005) to increase cell division and elongation during gall formation.

A possible increase in abscisic acid content was also observed in infected roots, due to the up-regulation of an abscisic acid (ABA)-responsive protein (DQ446602) in ‘Granaat’. This hormone plays an important role during many phases of the plant life cycle, including seed development and dormancy (Seo and Koshiba, 2002). Devos et al. (2005) reported an increase in ABA content in infected roots but at much later times (6, 13 and 24 dai). The role of this hormone in the clubroot/Brassica patho-system remains unclear. This demonstrates the need to investigate hormonal changes at a very early stage of clubroot infection, during which transcriptional changes for defence mechanisms of the host are taking place. The mechanisms behind the regulation of phytohormones and other physiological changes may also provide a
better understanding of the life cycle of *P. brassicae* and symptoms formation in susceptible hosts.

### 4.4.3 The shortcomings of this study

The current oligonucleotide array had many deficiencies despite its advantages over cDNA arrays in terms of simpler microarray preparation, increased specificity, avoidance of mis-annotated clones and the potential to detect splice variants (Kane *et al*., 2000). Issues encountered included large variations within and between replicates and limitations on the number and quality of the probe sets. The ‘boutique’ oligoarray was never intended for the elucidation of the mechanism of clubroot resistance, but for the determination of the optimum time(s) for tissue collection. The current study achieved this aim and identified 48 hai as the time of greatest differential expression to investigate defence responses when the *Brassica* hosts were grown in the hydroponic system with clubroot isolates. The factors that would require optimisation for an improved RMIT *Brassica* oligoarray are discussed below.

#### 4.4.3.1 The limitations of the RMIT *Brassica* oligoarray

**Variations within and between replicate:**

There was much variation due to seasonal effects on the biological replicates, as discussed earlier. The technical replicates of most array features in all experiments did not have a coefficient of variation (CV) < 0.15 (data not shown), as required by Clarke and Zhu (2006) for a good microarray experiment. Thomassen *et al*., (2006) reported that sources of systemic biases are introduced through every step in microarray processing: within-slide variation due to intensity bias across the slide and spotting effects caused by different tip performance and dust, between-array variations originating from printing variation, slide batch-to-batch variation and labelling of cDNA in different tubes and different days. It is believed that the replication in experimental design based on similar studies (Coram and Pang, 2006; Mantri *et al*., 2007) was adequate to minimise the genetic variable between hosts. More technical
replicates and repetitions of microarray experiments (including dye swap) as well as greater care during procedures to minimise systemic variations, may minimise those microarray variations, but may not be justified due to the limitation of RNA, time and cost.

**Inadequacy of the oligoarray probe set:**

Probably the most significant factors to contribute to the reliability as well as the limitations of the current array were the number, identity and quality of the probe sets. The methodology for probe set design was based on previous oligoarray publications (Bodrossy and Sessitsch, 2004; Draghici et al., 2006; Thomassen et al., 2006). Clarke and Zhu (2006) suggested that for arrays using short oligonucleotides as probes, a minimum set of 9 to 11 independent probes is necessary to accurately measure the transcript abundance without significant deterioration in performance. The design of only two probes per transcript in the current study was possibly insufficient. Other studies have reported the use of custom-made oligoarrays and it is common to apply a single or a maximum of two well-chosen 40-80-mer probes rather than a set of shorter probes (Kane et al., 2000; Bodrossy, 2003). Although short (20-25 base) oligonucleotide probes discriminate most between related sequences, they often hybridise poorly (Lockhart et al., 1996). The hybridisation yield of shorter probes was improved by including spacers (in this study, the probes were modified with 10 deoxythymidines) to move them away from the surface and closer to the hybridisation cocktail, as indicated by Hughes et al. (2001). The use of longer probes, coupled with a spacer, may improve the quality and reliability of the oligoarray.

Cross-hybridisation has also been reported by Draghici et al. (2006) as another source of inconsistency in microarray measurement, due to the poorly understood relationship between probe sequences, target concentration and probe intensity. Other researchers have studied the specificity limit of short oligonucleotides and have shown that greater than 66% identity may
lead to cross-hybridisation (Rouillard et al., 2003). The kinetics of hybridisation is further complicated by the incorporation of modified nucleotides into the target transcripts during the labelling process. Cross-hybridisation was minimised by validating the specificity and accuracy of the probe sequences using the NCBI BLASTN program. Removing or redesigning microarray probes prone to cross-hybridisation may be a reasonable strategy but prevents the comparison of data between different generations of arrays.

Finally, the unavoidable weakness of the current ‘boutique’ oligoarray is that only the genes that encode cDNAs included on the array or especially those with abundant transcript levels are assessed, i.e. it was a ‘closed architecture’ system. It is entirely possible that the partially-resistant and susceptible genotypes can differ greatly but the differences will not be detected unless those probes are present on the microarray. The rather small number of cDNA on the *Brassica* RMIT oligoarrays limits their power to detect potentially important and rare transcripts (Bowtell and Sambrook, 2003; Schena, 2003). This was demonstrated by the limited number of ‘Tahono’-specific transcripts despite its greater level of clubroot partial-resistance than the susceptible ‘Granaat’. The possibly different mechanisms for clubroot partial-resistance between ‘Tahono’ and ‘Leaguer’ could not be fully understood in this study.

To overcome this without the need to construct very large microarrays, Matsumura et al. (2003) developed the Super Serial Analysis of Gene Expression (SuperSAGE) technique. In brief, this technique is an improvement on SAGE by generating longer 26-bp gene tags that can be more accurately annotated (Velculescu et al., 1995). This process is laborious and expensive but the combination of SuperSAGE and microarrays should enable the development of a more efficient functional genomics tool to identify defence-related genes of varying transcript abundance.
4.4.3.2 The limitations of the qRT-PCR analysis

Due to the error-prone nature of high-throughput technology, the microarray data was experimentally validated by an independent method: quantitative real-time PCR (qRT-PCR). Most of the comparisons showed values of similar magnitude or greater, as commonly reported in other similar studies (Coram and Pang, 2006; Mantri et al., 2007). The significant up-regulation of chitinase in ‘Tahono’ at 48 hai validated the hypothetical defence model (based on individual microarray datasets) involving the induction of this PR protein against clubroot spores. Despite the good PCR efficiencies and precision of the standard curves generated, 50% of the qRT-PCR data showed contradictory results to the microarray data. It is doubtful that these ambiguities arose from PCR inhibitors, due to the use of high quality total RNA, or were consequences of inaccurate pipetting (Dallas et al., 2005). Such contradictory comparisons, albeit in smaller number, have been reported in other similar studies (Coram and Pang, 2006; Mantri et al., 2007) and may be attributed to their ratios close to zero. The lack of or limited significant differential expression in this qRT-PCR study may partly be attributed to the selection of genes, which were not induced or repressed on the microarray data.

The correlation between the results from these two methods was affected due to the sequences selected for the probes and primers, i.e. the high variation between probes A and B occurred since they were constructed from different positions on their respective GenBank sequences. When testing for genes with moderate transcripts levels, microarray probes and qRT-PCR primers from the same region generally are in greater agreement (Etienne et al., 2004). Genes that showed poor transcriptional correlation may be explained by having different levels of detection, different subsets of alternative transcripts being recognised or probe sequence annotation errors (Dallas et al., 2005). Overall, the qRT-PCR data did support the microarray data to a certain extent and the significant up-regulation of PAL and chitinase were valuable
information in modelling a hypothetical inducible defence response to clubroot spores in a partially-resistant genotype.

4.5 Summary

The large-scale profiling of the transcriptional changes to the early stages of *P. brassicae* infection in *Brassica* crops has not previously been documented. Hence, a cost-effective *Brassica* oligoarray of 150 *Arabidopsis*-/*Brassica*-derived features was constructed using nucleotide sequences from GenBank®. This array, with a biased representation of defence-associated and regulatory genes, was used to investigate the gene expression of the clubroot-resistant/partially-resistant Chinese cabbages ‘Tahono’ and ‘Leaguer’ and the susceptible ‘Granaat’ when challenged with aggressive clubroot isolate S. A novel hydroponic test system (performed in glasshouses or growth-rooms) was established to study the transcriptional changes occurring in the hosts’ roots. This was due to complications occurring with a soil-based system and to minimise environmental effects, which might interfere with plant defence responses. This system simulated Victorian field conditions with high risk of clubroot and provided an abundant and ‘clean’ source of total RNA as well as an efficient and rapid method for root tissue collection. This technique successfully infected the *Brassica* hosts, which displayed the expected gall severity based on previous resistance tests in soil *(Chapters 2 and 3)*. The degree of clubbing was, however, variable amongst the biological replicates and the hydroponic system was more favourable to clubroot development. There was also an effect of season in that the plants had greater vigour and maturity when in days with increasing temperature and photoperiod (*i.e.* in Spring than in late Summer). For future clubroot studies, replicated hydroponic experiments should preferably be used for reproducible defence responses in the hosts. Their symptom formation indicated that ‘Leaguer’ and especially ‘Tahono’ reduced but did not eliminate clubroot development. Their defensive responses are a form of horizontal resistance, rather than vertical resistance.
The microarray data, validated by qRT-PCR, indicated a relatively few number of constitutively and differentially expressed genes in response to pathogen attack, prominently at 48 hai as opposed to 24 and 72 hai. The lack of transcriptional changes at 24 hai demonstrated that there is no strong hypersensitive response in those genotypes. The major responses at 48 hai may correlate with timing of penetration from adequate amounts of germinated clubroot spores. The key observations at 48 hai were the constitutive over-expression (when compared to ‘Granaat’) and induction of a PR protein (chitinase) as well as the up-regulation of a lignin biosynthesis enzyme (caffeoyl-CoA 3-O-methyltransferase) in ‘Tahono’. By contrast, ‘Leaguer’ exhibited inducible chitinase levels and constitutive over-expression of caffeoyl-CoA 3-O-methyltransferase when compared to ‘Granaat’. The differing modes of resistance from these partially-resistant varieties illustrated the basis for pyramiding these defence pathways, i.e. by introgressing these defence-related genes into an elite cultivar, durable resistance may be achieved against a wide range of pathogens. This study also reported increased protein break-down (through the ubiquitin proteolytic pathways), cell wall modification (during cell elongation and division) and changes in phytohormone levels (auxin and abscisic acid) at 48 hai but may not be related to resistance. The role of these proteins remains unclear but demonstrated the need to investigate further the transcriptional changes in the *Brassica* hosts at a very early stage of clubroot infection.

Despite the advantages of the current oligoarray, it had many shortcomings such as high variation in the signal intensities and limited power to detect potentially important and rare transcripts. Hence, the mechanisms that make ‘Tahono’ more partially-resistant to clubroot disease than ‘Leaguer’ were not fully understood from the oligoarray. These issues may be addressed by an improved probe design, the construction of a bigger microarray and/or by complementing this study using other techniques such as SAGE. The ‘boutique’ oligoarray
was however never intended to elucidate the mechanism(s) of clubroot resistance, but to screen and optimise times for tissue collection. This study achieved this aim and identified 48 hai as the most responsive time to investigate defence responses in future experiments.
Chapter 5

Gene expression profiling for clubroot resistance using the Affymetrix® Arabidopsis ATH1 genome array

5.1 Introduction

The importance of identifying disease resistance genes for the development of molecular markers in breeding strategies was explained in previous chapters. This has been possible by investigating the transcriptional changes in ‘challenged’ plant hosts using large-scale gene expression profiling, such as microarray technology. The limitations of the ‘boutique’ Brassica oligoarray were however outlined in Chapter 4. In this chapter, the use of a more sophisticated microarray platform such as the Affymetrix Arabidopsis ATH1 genechip, would provide a more detailed analysis on the defence mechanisms in the Brassica vegetables against clubroot disease and hence identify more disease resistance genes.

The Affymetrix Arabidopsis ATH1 genome array was designed in collaboration with The Institute for Genome Research (TIGR) and contains more than 22,500 probe sets representing approximately 24,000 gene sequences on a single array (Affymetrix, 2004a). This genechip is a versatile and powerful tool for the analysis of gene expression in Arabidopsis thaliana, the most commonly studied plant model organism due to its suitability for molecular and genetics experiments. A. thaliana was the first plant genome to be completely sequenced and remains the most informative eukaryotic genome to date due to the extensive work being done to unravel its functions (Arabidopsis Genome Initiative, 2000). The close phylogenetic relationship between the genera of Brassica and Arabidopsis, on the basis of DNA sequences (Koch et al., 2001; Paterson et al., 2001), makes Brassica vegetables a clear potential beneficiary of the Arabidopsis genechip and related functional genomics studies.
Many studies have reported that gene syntheny, gene content, gene order and homology at both the nucleic acid and amino acid sequence level are closely related between Brassica and Arabidopsis (Quiros et al., 2001; Hall et al., 2002). However, several issues remain to be addressed regarding the application of this technique in Brassica using arrays designed for Arabidopsis genome. Due to significant divergence between these two members of the family Brassicaceae, sequence distance values of up to 22.9% have been reported for nuclear coding DNA sequences (Koch et al., 2001). Moreover, an approximately 50% lower hybridisation signal intensity for Brassica compared to Arabidopsis targets has been demonstrated (Hudson et al., 2007) and may present a barrier in applying Arabidopsis microarrays to Brassica. Cross-species hybridisation are however necessary exploratory experiments and all potential responses have to be confirmed by a second technique such as RNA gel blot analysis or qRT-PCR using Brassica-specific probes or primers (Hudson et al., 2007). The Affymetrix Arabidopsis genechip was and is the most advanced microarray platform in existence for Brassica functional genomics studies, until the release of the Affymetrix Brassica counterpart promised for 2007 and originally intended for use in this project. Because of the delay in its production, it was decided to use the Arabidopsis genechip.

The aims of the experiments described in this chapter were:

1. **To profile the gene expression for clubroot resistance using the Affymetrix Arabidopsis ATH1 genome array.** The detailed and thorough investigation of defence-related genes in the clubroot-susceptible ‘Granaat’, partially-resistant ‘Tahono’ and a putative clubroot-resistant B. rapa line ‘ECD04’ at 48 hai with clubroot isolate S, was expected to improve the understanding of their defence mechanisms.

2. **To postulate possible defence pathways by the Brassica genotypes under study.** The speed, coordination and magnitude of detection, signal transduction and activation of
genes are critical for an effective disease resistance. Identification of possible weaknesses (in susceptible plants) or strengths (in partially-resistant or resistant plants) in their defence mechanisms may provide vital information for breeding strategies.

5.2 Materials and Methods

5.2.1 Plant materials and spore isolates

The root tissues used in this chapter were collected at 48 hai and were generated from hydroponic experiment 3 (Table 4.3). In brief, the partially-resistant F$_1$ B. *rapa* hybrid H06 (‘Tahono’ CR-1-1, Table 3.1) provided by Henderson Seed Group Pty Ltd, together with the highly susceptible host ECD05 (‘Granaat’, Table 2.1) from the ECD set, were used as sources of partial-resistance / susceptibility to clubroot isolates. Additionally, a putative clubroot-resistant *B. rapa* genotype (ECD04, Table 2.1) from the ECD set was included in the analysis and its root tissues were collected at 48 hai from another hydroponic system (experiment 4 set up as in Section 4.2.3.1 and Table 5.1). From previous challenge tests, ‘Granaat’, ‘Tahono’ and ECD04 had disease indices of 69, 5 and 0 respectively against clubroot isolate S (Appendix 1). In this study, each genotype were grown in three independent hydroponic systems (three biological replicates performed across space, Figure 4.5) while clubroot isolate S (ECD code: 16/02/00, prepared according to Section 3.2.3) was the source of infection.

Table 5.1. Details of the hydroponic experiments performed.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Location</th>
<th>Date of inoculation</th>
<th>Time (days)</th>
<th>Biological replicates</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Growth-room</td>
<td>7-May-07</td>
<td>1, 2 &amp; 3</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>Growth-room</td>
<td>3-Aug-07</td>
<td>1, 2 &amp; 3</td>
<td>3</td>
</tr>
</tbody>
</table>
5.2.2 Preparation and quality control of total RNA

The total RNA from the frozen *Brassica* roots was extracted using the Qiagen™ RNeasy® Plant Mini Kit and assessed in a spectrophotometer and by gel electrophoresis as in Section 4.2.4. These total RNA samples were packed in dry ice and sent to the Australian Genome Research Facility (AGRF, VIC, Australia). All work on these total RNA samples was performed by AGRF staff as a paid service and raw Affymetrix data delivered for analysis. The methods used are outlines briefly below.

5.2.3 Affymetrix® *Arabidopsis* ATH1 array processing

The total RNA samples were initially quality tested using the Agilent Bioanalyser 2100 according to the manufacturer’s guidelines (Agilent Technologies, 2005). The Affymetrix® *Arabidopsis* ATH1 Genome Array was processed according to the manufacturer’s instructions (Affymetrix, 2004b). These steps are outlined in Figure 5.1 and briefly explained in the following sections.

5.2.3.1 Preparation of Poly-A RNA Controls

Prior to the synthesis and labelling of the cDNA targets, the extracted total RNA was spiked with a determined amount of Poly-A RNA controls based on the quantity of total RNA to be used onto the genechip. The poly-A RNA spike-in controls consisted of transcripts for the *lys*, *phe*, *thr* and *dap* genes from *B. subtilis* and were prepared to final concentration according to Table 5.2 using the GeneChip® Poly-A RNA Control Kit (Millenium Sciences, Cat. # 900433). This kit was designed specifically to provide positive controls that would be amplified and labelled together with the total RNA samples to monitor the entire *Brassica* target labelling process independently from the quantity of the starting RNA.
Figure 5.1. Outline of one-cycle target labelling assays for expression analysis (reproduced from Affymetrix (2004b)).
Table 5.2. Final concentration of Poly-A RNA controls in samples (reproduced from Affymetrix (2004b)).

<table>
<thead>
<tr>
<th>Poly-A RNA spike</th>
<th>Final concentration (Ratio of copy number)</th>
</tr>
</thead>
<tbody>
<tr>
<td>lys</td>
<td>1 : 100,000</td>
</tr>
<tr>
<td>phe</td>
<td>1 : 50,000</td>
</tr>
<tr>
<td>thr</td>
<td>1 : 25,000</td>
</tr>
<tr>
<td>dap</td>
<td>1 : 6,667</td>
</tr>
</tbody>
</table>

5.2.3.2 Double-stranded cDNA synthesis and cleanup

About 7 µg of each *Brassica* Total RNA with poly-A RNA spike-in controls added, was used to synthesise single-stranded cDNA before being converted into double-stranded cDNA. All the necessary reagents for these reactions came from the GeneChip® One-Cycle cDNA Synthesis Kit (Millenium Sciences, Cat # 900431) and were performed in a thermal cycler. The subsequent double-stranded cDNA was cleaned using the GeneChip® Sample Cleanup Module (Millenium Science, Cat. # 900371). This kit contained spin columns for both cDNA and cRNA cleanup procedures and optimised elution volume compatible with the assay flow, eliminating the need for concentrating samples.

5.2.3.3 Synthesis and fragmentation of biotin-labelled cRNA

The purified double-stranded cDNA was used as a template in the *in vitro* transcription (IVT) step and synthesis of biotin-labelled complementary RNA (cRNA). The incorporation of biotin-labelled ribonucleotide was achieved by using the GeneChip® IVT Labelling Kit (Millenium Sciences, Cat. # 900449). The cRNA was then purified using the GeneChip® Sample Cleanup Module since it was necessary to remove incorporated NTPs so that the concentration and purity of the cRNA could be accurately determined by the Agilent Bioanalyzer 2100. A total of 20 µg biotin-labelled cRNA was fragmented into 35 – 200 bp fragments by metal-induced hydrolysis using the fragmentation buffer from the GeneChip® Sample Cleanup Module. This step was required to maintain a sufficiently concentrated small
cRNA volume and is critical in obtaining optimal assay sensitivity. Additionally, the quality and quantity of the fragmented cRNA was checked using the Bioanalyzer 2100 before proceeding with hybridisation onto the Affymetrix® array.

5.2.3.4 Hybridisation of the Affymetrix® Arabidopsis Genome Array

Three hundred µL of the hybridisation cocktail was prepared as in Table 5.3 for a standard format array and included the alignment control (oligonucleotide B2) and spike controls (bioB, bioC, bioD and cre). Only 200 µL of the cocktail was loaded onto the Affymetrix® GeneChip, followed by incubation at 45°C for 16 h in an oven with a rotating wheel at 60 rpm.

Table 5.3. Hybridisation cocktail for a single Arabidopsis ATH1 Genome Array (reproduced from Affymetrix (2004b)).

<table>
<thead>
<tr>
<th>Component</th>
<th>Standard format array</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fragmented cRNA</td>
<td>15 µg</td>
<td>0.05 µg/µL</td>
</tr>
<tr>
<td>3 nM of control oligonucleotide B2</td>
<td>5 µL</td>
<td>50 pM</td>
</tr>
<tr>
<td>20 × eukaryotic hybridisation controls (bioB, bioC, bioD and cre)</td>
<td>15 µL</td>
<td>1.5, 5, 25 and 100 pM respectively</td>
</tr>
<tr>
<td>10 mg/mL of herring sperm DNA</td>
<td>3 µL</td>
<td>0.1 mg/mL</td>
</tr>
<tr>
<td>50 mg/mL of BSA</td>
<td>3 µL</td>
<td>0.5 mg/mL</td>
</tr>
<tr>
<td>2 × hybridisation buffer</td>
<td>150 µL</td>
<td>1 ×&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>100% DMSO</td>
<td>21 µL</td>
<td>7%</td>
</tr>
<tr>
<td>Molecular Biology grade water</td>
<td>To final volume of 300 µL</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> 1 × hybridisation buffer: 100 mM MES (Appendix 2), 1 M NaCl, 20 mM EDTA and 0.01% Tween-20.

5.2.3.5 Washing, staining and scanning of the array

After 16 h of hybridisation, the hybridisation cocktail was removed from the probe array and the probe array was loaded on the Affymetrix® GeneChip® Fluidics Station 450. The station was operated by the Affymetrix GeneChip® Operating Software v1.4.0.036 using the
appropriate script to perform a series of steps to wash and stain the array. In brief, the biotin-labelled cRNA targets hybridised to complementary Affymetrix probe sequences on the array were first coupled to streptavidin phycoerythrin (SAPE). The detection of the hybridised cRNA targets was therefore possible since the phycoerythrin moiety in SAPE has fluorescence properties. However, to amplify the signals generated, a biotinylated anti-streptavidin antibody was included to the staining procedure. The binding of the antibody to the streptavidin moiety resulted in an increase in biotin molecules that attracted more SAPE and amplified the signal (Figure 5.2). Upon completion of a final wash, the stained array was scanned using the Affymetrix GeneChip® Scanner 3000 operated by the Affymetrix GeneChip® Operating Software v1.4.0.036. Only one scan was required using a preset pixel resolution (3 µm) and wavelength (570 nm) on the scanner. The scanner converted the fluorescent signals on the Genechip into a DAT file (scanned measured image of the genechip) that was used to generate the CEL file (computed averaged signal intensity images) and the analysis results as the CHP file. The DAT files for each total RNA samples were received as a compressed CAB file from the AGRF staff and are included in the attached DVD.

5.2.4 Image analysis

After registering on the Affymetrix website (www.affymetrix.com), the Affymetrix GeneChip® Operating Software and the Arabidopsis ATH1 library file were downloaded (performed in August 2007) and installed. The CAB files for each total RNA sample were loaded using the Data Transfer Tool to re-generate the DAT and CEL files. The first step prior to running an absolute analysis (creating the CHP file) was to place a grid over the DAT file to verify the alignment of the probe cells in the array. The hybridisation of the oligonucleotide B2 along the border with alternating intensities and corners with checkboard pattern was also examined for proper alignment and positive hybridisation. The DAT file was also assessed
Figure 5.2. Schematic drawing of the principle of staining and amplification (Based on Raghavan (2004)).

Legend:
Affymetrix® oligonucleotide probe: ; biotin-labelled cRNA target: ;
SAPE: , biotinylated anti-streptavidin antibody: ; biotin molecule: ;
streptavidin: and phycoerythrin: .
visually for the possible presence of artefacts such as scratches or deposits caused by non-uniform spread of the hybridisation cocktail and improper drying, washing or staining of the array. Those probe cells affected by artefacts or that were outliers were masked and were excluded from further analysis.

5.2.5 Absolute analysis (Single array analysis)

**Quantification of the Affymetrix probe sets:**

After the inspection of the image, absolute or single array analysis was performed for each genechip using the Affymetrix GeneChip Operating Software. This analysis generated a detection \( p \)-value, a detection call, and a signal intensity value for all probe sets according to the default parameter settings (Appendix 6).

Detection calls defined the probe sets as detected (present) or not detected (absent) based on their detection \( p \)-value calculated from a discrimination score (R). The R score was calculated for each probe pair using the following equation:

\[
R = \frac{PM - MM}{PM + MM}
\]

where PM was the perfect match intensity and MM was the mismatch intensity. Probe pairs with R scores higher than the user-definable threshold ‘Tu’ indicated the presence of the transcript while a lower value indicated the absence of the transcript. In addition, the overall scoring results of a probe set were summarised as a detection \( p \)-value that was generated by a One-sided Wilcoxon’s Signed Rank test. When the R scores were close to 1.0 for the majority of the probe pairs, the calculated detection \( p \)-value was less, *i.e.* a lesser \( p \)-value indicated that the probability of error in the detection calls was smaller. In contrast, an R score of zero (when PM = MM) or negative value (when MM > PM) resulted in a greater and less significant detection \( p \)-values. Moreover, the user-modifiable detection \( p \)-value cut offs, Alpha 1 (\( \alpha_1 \)) and Alpha 2 (\( \alpha_2 \)), were used to fine-tune the detection calls by increasing or decreasing levels of stringency (Affymetrix, 2004c).
The fluorescence intensity of each cell was quantified and the signal value for each probe set represented the relative level of expression of a transcript. This value was calculated using the One-Step Tukey’s Biweight Estimate and yielded robust weighted means that were relatively insensitive to outliers. The overall signal of a probe set relied on the intensity of each probe pair. The mismatch intensity was used to estimate stray signal while the real signal was estimated by taking the log of the Perfect Match intensity after subtracting the stray signal estimate (Affymetrix, 2004c).

Quality control of the Affymetrix ATH1 Genome Array:

The absolute analysis generated the CHP file as well as a report for each array. The report contained information that was used to assess the reliability of the total RNA samples and array hybridisation. These factors, involved in quality control of the Affymetrix genechip, are defined below, according to Affymetrix (2004c):

- **Noise (Raw Q):** Noise resulted from small variations in the signal values when the scanner scanned the arrays and it determined the degree of pixel-to-pixel variation among the probe cells.

- **Scale factor:** The scale factor provided a measure of the brightness of the array and non-biological factors such as amount and quality of the cRNA or SAPE that may contribute to the overall variability in hybridisation intensities. In order to compare data from multiple arrays, it was essential that the intensity of the arrays was brought to the same level, *i.e.* to scale all arrays to one target intensity. An arbitrary target value of 50 was selected and the average intensity of all genes was scaled (minus the highest and lowest 2% signal values) on each array within a data set to that number.

- **Average background:** The background value was a measure of the signal intensity caused by auto-fluorescence of the array surface and non-specific binding of the target or the stain molecule: streptavidin phycoerythrin (SAPE).
• Housekeeping controls: The 3’/5’ ratios of those endogenous control genes gives an indication of the integrity of the RNA samples, efficiency of first-strand cDNA synthesis and *in vitro* transcription of cRNA. This is because reverse transcriptase synthesised cDNA starting from the 3’ end of a mRNA and ending at the 5’ end.

• Spike controls: The addition of exogenous genes at staggered concentrations served as controls for hybridisation. Their 3’/5’ ratios are not as informative since they do not relate to the quality of the samples and data.

**Reproducibility of the Affymetrix experiments:**

The reproducibility of the replicated Affymetrix experiments was also inspected. All three CHP files for the ‘treatment’ arrays for each genotype were simultaneously loaded onto the Affymetrix GeneChip® Operating Software and the ‘Scatter Graph’ tool was used to construct scatter plots of the signal values. This permitted the visual inspection for major deviations between replicated array data.

To further evaluate the reproducibility of the microarray experiments, the variation coefficient (VC) was calculated for all genes called ‘Present’ or ‘Marginally Present’ in all three replicated arrays for each genotype. This type of analysis has been used in whole genome transcript analysis studies (Müssig *et al.*, 2002; Raghavan, 2004), where a VC of < 50% represented low variability between replicated arrays. The distribution of VC was displayed in a histogram and constructed according to Appendix 7.

**5.2.6 Comparative analysis (experiment versus baseline arrays)**

Once the arrays had been globally scaled at TGT (target value) of 50 and the reliability and reproducibility of the scan images had been checked, comparative analysis was performed. This involved the comparison of two samples in order to detect and quantify changes in gene
expression, in which one of the three ‘treatment’ arrays was designated as the experiment and the corresponding ‘untreated’ control array as the baseline for each genotype. In addition to investigating constitutively expressed genes, the ‘Tahono’ and the ‘ECD04’ control arrays were each compared to the ‘Granaat’ control array. These comparative analyses (CHP files) generated change \( p \)-values, change calls and signal log ratios for all probe sets according to the default parameter settings (Appendix 6).

Change calls defined the probe sets as ‘increase’, ‘decrease’ or ‘no change’ based on their change \( p \)-values. As in the single array analysis, the change \( p \)-value, which indicated the likelihood of significant change and direction, was computed by the Wilcoxon’s Signed Rank test. The change \( p \)-value was then categorised by user-defined cut-offs (Gamma 1, \( \gamma_1 \) and Gamma 2, \( \gamma_2 \)) to generate discrete change calls (Affymetrix, 2004c).

The signal log ratio for each gene was calculated to estimate the magnitude and direction of change of a transcript when two arrays were compared. This signal log ratio was computed using the One-Step Tukey’s Biweight method by taking a mean of the log ratios of probe pair intensities across the two arrays (Affymetrix, 2004c). The log scale used was to the base 2 and therefore, a signal log ratio of 1.0 indicated a 2-fold increase while -1.0 demonstrated a 2-fold decrease.

5.2.7 Data mining
Data mining was performed to select those genes that were significantly up-/down-regulated in all three replicated arrays for each genotype or constitutively over-/under-expressed between control arrays. For differential expression analysis, the CHP files generated from the three replicated comparative analyses of each genotype were loaded onto the Affymetrix GeneChip® Operating software and, using Microsoft Excel, steps involved in the selection of
genes were performed as in Table 5.4. In contrast, the selection of constitutively expressed genes only involved individual CHP file from the comparative analysis between the controls, followed by steps in Table 5.5. Finally, those selected genes, represented on the genechip as ‘Probe Set ID’ or array element by Affymetrix, were extracted and saved as a text-delimited file (TXT).

A number of queries was investigated using these TXT files. Venn diagrams were drawn to illustrate the results and additional up-to-date information concerning these selected Probe Set IDs was collected from the WWW-based Affymetrix NetAffx™ Analysis Centre (www.affymetrix.com/analysis/index.affx). The latter enables researchers to correlate their genechip array results with array content information such as probe sequences and gene annotation information from other public databases. For example, the Affymetrix Probe Set ID ‘247741_at’ had a representative Public ID (locus identifier) ‘At5g58960’, ENTREZ gene ID ‘836013’, Unigene ID ‘29251’ and RefSeq Transcript IDs: ‘NM1037023’, ‘NM125286’ and ‘NM180886’.

The locus identifiers for relevant gene lists were extracted from the NetAffx™ Analysis Centre and saved as another text-delimited file (TXT). These TXT files were then uploaded onto the GO (gene ontology) annotation search tool (www.arabidopsis.org/tools/index.jsp) of the TAIR (The Arabidopsis Information Resource) database to classify the locus identifiers into their respective functional categories. The relevant gene lists were annotated to molecular and biological functions and the frequency of the GO terms illustrated in pie-charts. The GO molecular function defined the action characteristic of a gene product while the GO biological process indicated the phenomenon marked by changes that lead to a particular result, possibly mediated by one or more gene products (Ashburner and Lewis, 2002). The major gene lists that were investigated were significantly differentiated or constitutively expressed genes for
Table 5.4. Conditions for selection of differentially expressed genes (treatments vs control for each genotype).

<table>
<thead>
<tr>
<th>Selection of UP-regulated genes</th>
<th>Selection of DOWN-regulated genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detection call ‘Present’ in all replicates of Treatment</td>
<td>Detection call ‘Present’ in Control</td>
</tr>
<tr>
<td>Change call ‘Increase’ in at least 2 replicates</td>
<td>Change call ‘Decrease’ in at least 2 replicates</td>
</tr>
<tr>
<td>SLR* ≥ 0.8 in all biological replicates</td>
<td>SLR* ≤ -0.8 in all biological replicates</td>
</tr>
<tr>
<td>Mean signal value ≥ 100 in Treatment</td>
<td>Mean signal value ≥ 100 in Control</td>
</tr>
<tr>
<td>Significant up-regulation</td>
<td>Significant down-regulation</td>
</tr>
</tbody>
</table>

SLR*: Signal Log Ratio of ± 0.8 was equivalent to 1.75-fold increase/decrease.

Table 5.5. Conditions for selection of constitutively over-/under-expressed genes (‘Tahono’ control or ECD04 control (experiment) vs ‘Granaat’ control (baseline)).

<table>
<thead>
<tr>
<th>Selection of constitutively OVER-expressed genes</th>
<th>Selection of constitutive UNDER-expressed genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Detection call ‘Present’ in experiment</td>
<td>Detection call ‘Present’ in baseline</td>
</tr>
<tr>
<td>Change call ‘Increase’ in experiment</td>
<td>Change call ‘Decrease’ in experiment</td>
</tr>
<tr>
<td>SLR ≥ 0.8 in experiment</td>
<td>SLR ≤ -0.8 in experiment</td>
</tr>
<tr>
<td>Signal value ≥ 100 in experiment</td>
<td>Signal value ≥ 100 in baseline</td>
</tr>
<tr>
<td>Significant over-expression</td>
<td>Significant under-expression</td>
</tr>
</tbody>
</table>
each genotype and those commonly differentiated or constitutively expressed genes in all genotypes.

5.2.8 Validation of microarray data by quantitative real-time PCR

Validation of the Affymetrix data was performed by quantitative real-time PCR using the relative standard curve method as in Section 4.2.8. Modifications in the protocol are described below.

Validation of differential expression analysis:

Along with the five sets of qRT-PCR primers used earlier in Chapter 4 (Table 4.4), two additional sets of primers (Table 5.6) were included. This experiment was performed according to Section 4.2.8 for all three genotypes and, similarly, involved the use of actin as a target to construct the standard curve and as the reference/endogenous control to normalise the quantification of the set of target genes. The previously used primers were designed from GenBank nucleotide sequences and hence, their equivalent Affymetrix probe set ID (and locus identifier) had to be identified to compare the Affymetrix and qRT-PCR data. Using the Affymetrix NetAffx™ BLAST tool (www.affymetrix.com/analysis/index.affx) and the primer sequences to search for homologous gene sequences within the ATH1 Arabidopsis targets.

Validation of constitutive expression analysis:

The constitutive expression analysis of the Affymetrix data was validated by four new set of qRT-PCR primers (Table 5.7), along with the actin primer. In this study, only the total RNA from the control plants was used for each genotype while the remaining steps were performed according to Section 4.2.8. Since these primers were designed from selected Affymetrix gene sequences, the NetAffx™ BLAST tool was not used to identify their locus identifiers.
Table 5.6. The additional qRT-PCR primer sequences for validation of the differential expression analysis of the Affymetrix data.

<table>
<thead>
<tr>
<th>Putative function</th>
<th>Abbreviation</th>
<th>Probe ID</th>
<th>Locus Identifier</th>
<th>Nucleotide sequence (5’ → 3’)</th>
<th>Expected amplicon length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipase</td>
<td>LIP</td>
<td>256306_at</td>
<td>At1g30370</td>
<td>F (GCGCTGGCGCTTATGAACGCTTAC) R (TTACCTACCCTCGGCGCACCACAAAC)</td>
<td>98</td>
</tr>
<tr>
<td>Superoxide dismutase</td>
<td>SOD</td>
<td>264809_at</td>
<td>At1g08830</td>
<td>F (ACTGCCACCTTCACAATACAC) R (ATGGACAAACACAGCCCTAC)</td>
<td>81</td>
</tr>
</tbody>
</table>

Table 5.7. The qRT-PCR primer sequences for validation of the constitutive expression analysis of the Affymetrix data.

<table>
<thead>
<tr>
<th>Putative function</th>
<th>Abbreviation</th>
<th>Probe ID</th>
<th>Locus Identifier</th>
<th>Nucleotide sequence (5’ → 3’)</th>
<th>Expected amplicon length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WRKY transcription factor</td>
<td>TRF</td>
<td>261892_at</td>
<td>At5g01320</td>
<td>F (GGCTTAACCCGCCACATCTC) R (CGGCACAGTCAGGTACTT)</td>
<td>81</td>
</tr>
<tr>
<td>Polygalacturonase</td>
<td>PGAL</td>
<td>261834_at</td>
<td>At1g10640</td>
<td>F (GATGCTTTAGGTGTCGAGAC) R (GGGCTTCCAAATCTTCAAC)</td>
<td>81</td>
</tr>
<tr>
<td>Leucine zipper protein</td>
<td>LZP</td>
<td>261815_at</td>
<td>At1g08325</td>
<td>F (TACTCTCCCGCATCTCCAGTC) R (GTGAACGCTCCTGCTCAAG)</td>
<td>121</td>
</tr>
<tr>
<td>Ferulate-5-hydroxylase</td>
<td>FAH</td>
<td>253088_at</td>
<td>At4g36220</td>
<td>F (GCGACCCAAACCTTTGGACTGAC) R (ACGTCTACGACCCAGACAGCAAC)</td>
<td>123</td>
</tr>
</tbody>
</table>
5.3 Results

5.3.1 The hydroponic test system

The manifested symptoms in the *Brassica* hosts in the hydroponic system were scored and are illustrated in Table 5.8. The fodder turnip ‘ECD04’ had no clubbing throughout the experiment while the Chinese cabbage ‘Granaat’ was clearly susceptible from 4 weeks after inoculation. As reported earlier, the Chinese cabbage ‘Tahono’ from hydroponic experiment 3 demonstrated a reduction in the degree of infection 4 weeks after inoculation, but eventually succumbed to heavy clubbing after 8 weeks. Nonetheless, all biological replicates were included in the analysis due to the presence of clubroot infection in the highly susceptible ‘Granaat’.

5.3.2 Total RNA analysis

Spectrophotometry and gel electrophoresis of the 12 total RNA samples (three ‘challenged’ and one control samples for each genotype) indicated adequate concentration (>0.5 µg/µL, data not shown), good purity (A260/280 ratio ranging between 1.7 and 2.0, data not shown) and integrity (clear ribosomal bands with the 25S rRNA band intensity approximately twice that of the S18 rRNA, Figure 4.9) (Qiagen, 2006). The total RNA profiles from the Agilent Bioanalyser 2100 also confirmed that the total RNA samples were fit for hybridisation onto the Affymetrix *Arabidopsis* ATH1 genechip. These profile data are included in the attached DVD as Adobe (PDF) files.

5.3.3 Absolute analysis of the Affymetrix *Arabidopsis* genechip

Image analysis:

The scanned images of every array were not significantly affected by artefacts or outliers since fewer than 500 (out of about 247,500) probe cells were masked per array (Figure 5.3a and 5.3b). Moreover, positive hybridisation of the B2 oligonucleotide along the border of the
Table 5.8. Symptoms\textsuperscript{a} manifesting in the *Brassica* vegetables 4 and 8 weeks after inoculation with clubroot isolate S in the hydroponic system.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Symptoms 4 weeks after inoculation</th>
<th>Symptoms 8 weeks after inoculation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ECD04\textsuperscript{b}</td>
<td>Tahono\textsuperscript{c}</td>
</tr>
<tr>
<td>3\textsuperscript{e}</td>
<td>–</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>–</td>
</tr>
</tbody>
</table>

\textsuperscript{a} The 4-grade scale was used to assess the clubroot symptoms: 0 = no visible clubbing, 1 = small galls confined to lateral roots, 2 = moderate swellings on both lateral and/or tap root and 3 = severe clubbing.

\textsuperscript{b} Clubroot-resistant fodder turnip variety.

\textsuperscript{c} Partially-resistant Chinese cabbage variety.

\textsuperscript{d} Clubroot-susceptible Chinese cabbage variety.

\textsuperscript{e} Results reproduced from previous chapter for easy comparison.

\(-\), test not performed.
Figure 5.3. Image analysis (a) Detection of artefacts and (b) Masking of probe cells affected by artefacts, followed by quantification of the (c) Scanned measured image and (d) Computed averaged signal intensity image. The alternating intensities and checkboard pattern along the border indicated positive hybridisation of the cocktail.
array and presence of the checkboard pattern at each corner (Figure 5.3c and 5.3d) indicated efficient hybridisation of the targets to the probes.

Quality control of the Affymetrix ATH1 Genome Arrays:
Reports generated from the CHP files for each absolute analysis were examined to assess the quality and reliability of the scan images. These reports are summarised in Table 5.9 for all arrays. In brief, the noise (Raw Q) values were comparable within each genotype, all scale factors were <3-fold when scaled to an arbitrary target intensity value (TGT) of 50, all average background were within the recommended range of 20 to 100, all endogenous housekeeping control genes (with the exception of actin) had 3'/5’ ratios <3 and expected staggered intensities of spike-in exogenous controls were observed in all arrays.

Reproducibility of the Affymetrix experiments:
Scatter plots of the signal values between replicated arrays for each genotype showed that the majority of the significantly expressed genes (red dots) were aligning along the central line within the 2-fold reference lines (Figure 5.4). Therefore, most genes generated less than 2-fold variation in signal intensity between two independent hybridisations. In contrast, the variation coefficient (VC) histogram (Figure 5.5) illustrated that 98.5% (of 3,668 genes that were called ‘Present’ or ‘Marginally Present’ in all three biological replicates), 98.7% (of 3,780 genes) and 99.2% (of 3,458 genes) for ‘Granaat’, ‘Tahono’ and ECD04 respectively, had variation coefficients <50%.

5.3.4 Comparative analysis of the Affymetrix Arabidopsis ATH1 genechip

5.3.4.1 Analysis of differentially regulated genes
The genes of interest were identified by a selection process, involving detection $p$-value and detection call, change $p$-value and change call and the signal log ratio for all probe sets as
Table 5.9. Summary report of the absolute analyses.

<table>
<thead>
<tr>
<th></th>
<th>Granaat</th>
<th>Tahono</th>
<th>ECD04</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Array 1</td>
<td>Array 2</td>
</tr>
<tr>
<td>Noise (RawQ)</td>
<td>2.98</td>
<td>3.25</td>
<td>2.70</td>
</tr>
<tr>
<td>Scale factor @ TGT50</td>
<td>1.55</td>
<td>1.45</td>
<td>1.95</td>
</tr>
<tr>
<td>Average background</td>
<td>74.32</td>
<td>87.92</td>
<td>65.20</td>
</tr>
<tr>
<td>% present</td>
<td>21.8</td>
<td>21.6</td>
<td>21.4</td>
</tr>
<tr>
<td>% absent</td>
<td>76.0</td>
<td>76.3</td>
<td>76.2</td>
</tr>
</tbody>
</table>

Housekeeping controls

<table>
<thead>
<tr>
<th></th>
<th>Signal ratios (3'/5') of endogenous control genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATHAL-ACTIN</td>
<td>5.49</td>
</tr>
<tr>
<td>ATHAL-GAPDH</td>
<td>0.06</td>
</tr>
<tr>
<td>ATHAL-UBQ</td>
<td>2.38</td>
</tr>
</tbody>
</table>

Spike controls

<table>
<thead>
<tr>
<th></th>
<th>Signal values (all) of exogenous control genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>bioB</td>
<td>114.41</td>
</tr>
<tr>
<td>bioC</td>
<td>369.72</td>
</tr>
<tr>
<td>bioD</td>
<td>989.77</td>
</tr>
<tr>
<td>cre</td>
<td>6096.68</td>
</tr>
<tr>
<td>lys</td>
<td>77.81</td>
</tr>
<tr>
<td>phe</td>
<td>72.81</td>
</tr>
<tr>
<td>thr</td>
<td>115.81</td>
</tr>
<tr>
<td>dap</td>
<td>455.30</td>
</tr>
</tbody>
</table>

Noise (RawQ), degree of pixel-to-pixel variation among the probe cells; TGT, target value; ATHAL, Arabidopsis thaliana; ACTIN, actin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; UBQ, ubiquitin; bioB, Escherichia coli biotin synthase; bioC, E. coli bioC protein; bioD, E. coli dethiobiotin; cre, P1 Bacteriophage cre recombinase protein; lys, Bacillus subtilis lysine; phe, B. subtilis phenylalanine; thr, B. subtilis threonine and dap, B. subtilis diaminopropionic.
Scatter plots of the three replicated experiments of ‘Granaat’:

(a)  
(b)  
(c)  

Scatter plots of the three replicated experiments of ‘Tahono’:

(d)  
(e)  
(f)  

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Scatter plots of the three replicated experiments of ECD04:

![Scatter plots](image)

(g)         (h)            (i)

Figure 5.4. Comparison of scatter plots from biological replicates of the three *Brassica rapa* genotypes.

Scatter plots illustrated the alignment of the signals along a central line within the low fold change, thus highlighting the reproducibility of the microarray experiments. Each gene is represented by one dot. The signal value of each gene in one experiment was given on the x-axis while that of the same gene in the other experiment was provided on the y-axis. The diagonal lines showed fold changes by factor of 2, 3, 10 and 30 between the two independent hybridisation for visual reference. Significantly expressed genes with detection calls: ‘Present’ or ‘Marginally Present’ in the two samples were showed as red and blue dots respectively while those ‘Absent’ genes with insignificant levels of expression were indicated as yellow dots.

Scatter plots of signal values of the replicated arrays (X, Y and Z) for:
(a), (b) and (c) the clubroot-susceptible Chinese cabbage ‘Granaat’ (GR);
(d), (e) and (f) the partially-resistant Chinese cabbage ‘Tahono’ (TO) and
(g), (h) and (i) the clubroot-resistant fodder turnip ‘ECD04’.

Note:
‘TGT50’: Signal values were scaled at target value of 50.
‘Default’: Default parameters used to quantify signal values (Appendix 6).
The histogram represents variation coefficient (VC) of genes called ‘Present’ or ‘Marginally Present’ in all three independently-labelled samples (3 biological replicates), that were hybridised onto three separate arrays.

The variation coefficients (in %) were calculated using the formulae: \( VC = \frac{\text{standard deviation}}{\text{mean signal}} \times 100 \), where a VC of < 50% represented low variability between replicated arrays (Müssig et al., 2002).

(a) Granaat: 3,668 genes were called ‘Present’ or ‘Marginally Present’ in all three ‘treatment’ arrays and 98.5% of the genes had a VC < 50%.

(b) Tahono: 3,780 genes were called ‘Present’ or ‘Marginally Present’ in all three ‘treatment’ arrays and 98.7% of the genes had a VC < 50%.

(c) ECD04: 3,458 genes were called ‘Present’ or ‘Marginally Present’ in all three ‘treatment’ arrays and 99.2% of the genes had a VC < 50%.
shown in Tables 5.10, 5.11 and 5.12. In brief, a total of 3,255 (14.3% of genechip), 3,355 (14.7%) and 3,083 genes (13.5%) were called ‘Present’ in all three treatment arrays as opposed to 4,981 (21.8%), 5,114 (22.4%) and 4,637 genes (20.3%) in the single control arrays for ‘Granaat’, ‘Tahono’ and ‘ECD04’ respectively. The fold change cut-off value of 1.75-fold was used and selected genes (only defence-related, responses to abiotic and biotic stress, transcription-related and unknowns) that were significantly up-/down-regulated upon inoculation with clubroot isolate S were summarised in Table 5.13 (Refer to Appendix 8 for full gene lists of individual analysis). Of all the genes called ‘Present’, only 17 (0.36%), 34 (0.70%) and 2 (0.05%) were differentially expressed in ‘Granaat’, ‘Tahono’ and ECD04 respectively. This relatively low number of differentiated genes in the susceptible ‘Granaat’ and partially-resistant ‘Tahono’ has been reported in the previous chapter. However, the differential expression of only two genes in the clubroot-resistant ‘ECD04’ was unexpected. Otherwise, down-regulation was most prominent, due to a greater % significant decrease in all three genotypes, which contradicted the observed direction and trend of expression at 48 hai in both ‘Granaat’ and ‘Tahono’ when the RMIT Brassica oligoarray was used.

Venn diagrams were constructed to observe the relationship and co-regulation of these significantly differentiated genes at 48 hai (Figure 5.6). The key observations are the co-repression of a putative lipase (At1g30370) in all three genotypes and the lack of any other gene co-regulation between the Chinese cabbage ‘Tahono’ and fodder turnip ‘ECD04’. In contrast, there was a total of eight co-regulated genes between the two Chinese cabbage varieties ‘Granaat’ and ‘Tahono’. These relationships correlated with that of the RMIT Brassica oligoarray, i.e. ‘Granaat’ and ‘Tahono’ may have more conserved defence mechanisms than ‘ECD04’ and ‘Tahono’. The functional classification of these genes was then conducted by annotation for GO molecular functions and GO biological processes and included in Table 5.13. In brief, the only defence-related gene to be up-regulated was
Table 5.10. Selection of differentially expressed genes with reliable expression in all three replicated arrays of ‘Granaat’ at 48 hai.

<table>
<thead>
<tr>
<th>Selection of UP-regulated genes</th>
<th>Increase</th>
<th>Selection of DOWN-regulated genes</th>
<th>Decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of genes on the array</td>
<td>22,810</td>
<td>Total number of genes on the array</td>
<td>22,810</td>
</tr>
<tr>
<td>No. of genes called ‘present’ in all replicates of Treatment</td>
<td>3,255</td>
<td>No. of genes called ‘present’ in Control</td>
<td>4,981</td>
</tr>
<tr>
<td>No. of genes called ‘increase’ in at least 2 replicates</td>
<td>69</td>
<td>No. of genes called ‘decrease’ in at least 2 replicates</td>
<td>98</td>
</tr>
<tr>
<td>No. of genes with SLR ≥ 0.8 in all biological replicates</td>
<td>4</td>
<td>No. of genes with SLR ≤ -0.8 in all biological replicates</td>
<td>24</td>
</tr>
<tr>
<td>No. of genes with a mean signal intensity ≥ 100 in Treatment</td>
<td>2</td>
<td>No. of genes with a mean signal intensity ≥ 100 in Control</td>
<td>15</td>
</tr>
<tr>
<td>% Significant Increase</td>
<td>0.09</td>
<td>% Significant Decrease</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Table 5.11. Selection of differentially expressed genes with reliable expression in all three replicated arrays of ‘Tahono’ at 48 hai.

<table>
<thead>
<tr>
<th>Selection of UP-regulated genes</th>
<th>Increase</th>
<th>Selection of DOWN-regulated genes</th>
<th>Decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of genes on the array</td>
<td>22,810</td>
<td>Total number of genes on the array</td>
<td>22,810</td>
</tr>
<tr>
<td>No. of genes called ‘present’ in all replicates of Treatment</td>
<td>3,355</td>
<td>No. of genes called ‘present’ in Control</td>
<td>5,114</td>
</tr>
<tr>
<td>No. of genes called ‘increase’ in at least 2 replicates</td>
<td>101</td>
<td>No. of genes called ‘decrease’ in at least 2 replicates</td>
<td>113</td>
</tr>
<tr>
<td>No. of genes with SLR ≥ 0.8 in all biological replicates</td>
<td>7</td>
<td>No. of genes with SLR ≤ -0.8 in all biological replicates</td>
<td>35</td>
</tr>
<tr>
<td>No. of genes with a mean signal intensity ≥ 100 in Treatment</td>
<td>3</td>
<td>No. of genes with a mean signal intensity ≥ 100 in Control</td>
<td>31</td>
</tr>
<tr>
<td>% Significant Increase</td>
<td>0.09</td>
<td>% Significant Decrease</td>
<td>0.61</td>
</tr>
</tbody>
</table>
Table 5.12. Selection of differentially expressed genes with reliable expressions in all three replicated arrays of ‘ECD04’ at 48 hai.

<table>
<thead>
<tr>
<th>Selection of UP-regulated genes</th>
<th>Increase</th>
<th>Selection of DOWN-regulated genes</th>
<th>Decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of genes on the array</td>
<td>22,810</td>
<td>Total number of genes on the array</td>
<td>22,810</td>
</tr>
<tr>
<td>No. of genes called ‘present’ all replicates of Treatment</td>
<td>3,083</td>
<td>No. of genes called ‘present’ in Control</td>
<td>4,637</td>
</tr>
<tr>
<td>No. of genes called ‘increase’ in at least 2 replicates</td>
<td>43</td>
<td>No. of genes called ‘decrease’ in at least 2 replicates</td>
<td>29</td>
</tr>
<tr>
<td>No. of genes with SLR* ≥ 0.8 in all biological replicates</td>
<td>2</td>
<td>No. of genes with SLR* ≤ -0.8 in all biological replicates</td>
<td>2</td>
</tr>
<tr>
<td>No. of genes with a mean signal intensity ≥ 100 in Treatment</td>
<td>1</td>
<td>No. of genes with a signal intensity ≥ 100 in Control</td>
<td>1</td>
</tr>
<tr>
<td>% Significant Increase</td>
<td>0.03</td>
<td>% Significant Decrease</td>
<td>0.02</td>
</tr>
</tbody>
</table>
Table 5.13. Selected list of genes differentially expressed at 48 hai (sorted by putative function).

<table>
<thead>
<tr>
<th>Probe Set ID</th>
<th>Locus Identifier</th>
<th>Mean SLR^\text{a}</th>
<th>Putative function (on August 2007)</th>
<th>GO term (on March 2009)</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>256129_at</td>
<td>At1g18210</td>
<td>-1.33</td>
<td>Calcium-binding protein</td>
<td>Calcium ion binding</td>
<td>ISS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Unknown process</td>
<td>ND</td>
</tr>
<tr>
<td>252679_at</td>
<td>At3g44260</td>
<td>-1.20</td>
<td>CCR4-associated factor 1-like protein</td>
<td>Ribonuclease activity</td>
<td>ISS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-1.53</td>
<td></td>
<td>• Response to biotic stimulus</td>
<td>IEP</td>
</tr>
<tr>
<td>244950_at</td>
<td>cox2</td>
<td>–</td>
<td>Cytochrome c oxidase subunit 2</td>
<td>• Unknown</td>
<td>ND</td>
</tr>
<tr>
<td>248964_at</td>
<td>At5g45340</td>
<td>-1.97</td>
<td>Cytochrome P450</td>
<td>• Hydrolase activity</td>
<td>IDA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-1.13</td>
<td></td>
<td>• Abscisic acid catabolic process</td>
<td>TAS</td>
</tr>
<tr>
<td>247543_at</td>
<td>At5g61600</td>
<td>-1.43</td>
<td>DNA binding protein - like DNA binding protein EREBP4</td>
<td>• Transcription factor activity</td>
<td>ISS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-1.63</td>
<td></td>
<td>• Regulation of transcription</td>
<td>ISS</td>
</tr>
<tr>
<td>247199_at</td>
<td>At5g65210</td>
<td>–</td>
<td>DNA binding protein TGA1a homolog</td>
<td>• Calmodulin binding</td>
<td>ISS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-1.00</td>
<td></td>
<td>• Transcription factor activity</td>
<td>ISS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Defence response to bacterium</td>
<td>• Monooxygenase activity</td>
<td>IDA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>• Lignin biosynthesis process</td>
<td>• Hydroxylase activity</td>
<td>IMP</td>
</tr>
<tr>
<td>253088_at</td>
<td>At4g36220</td>
<td>–</td>
<td>Ferulate-5-hydroxylase (FAH1)</td>
<td>• Manganese ion binding</td>
<td>IEA</td>
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<tr>
<td></td>
<td></td>
<td>-1.57</td>
<td></td>
<td>• Unknown process</td>
<td>ND</td>
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<tr>
<td>249490_s_at</td>
<td>At5g39110</td>
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<td>Germin-like protein (GLP6)</td>
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<tr>
<td>Accession</td>
<td>Gene ID</td>
<td>Log2 Fold Change</td>
<td>Symbol</td>
<td>Description</td>
<td>Functions</td>
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<tr>
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<td>---------</td>
<td>------------------</td>
<td>--------</td>
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<td>---------------------------------------------------------------------------</td>
</tr>
<tr>
<td>250676_at</td>
<td>At5g06320</td>
<td>-1.27</td>
<td></td>
<td>Harpin-induced protein-like</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Response to bacterium</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Unknown function</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>• Unknown process</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Unknown function</td>
</tr>
<tr>
<td>265230_s_at</td>
<td>At2g07707</td>
<td>-1.23</td>
<td></td>
<td>Hypothetical protein</td>
<td>• Unknown function</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Unknown process</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Unknown function</td>
</tr>
<tr>
<td>267293_at</td>
<td>At2g23810</td>
<td>-1.00</td>
<td></td>
<td>Hypothetical protein</td>
<td>• Aging</td>
</tr>
<tr>
<td>252592_at</td>
<td>At3g45640</td>
<td>-1.17</td>
<td></td>
<td>Mitogen-activated protein kinase 3</td>
<td>• MAP kinase activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Response to chitin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Response to oxidative stress</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Camalexin biosynthetic process</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Transcription factor activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Zinc ion binding</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Unknown</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Peroxidase activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Response to oxidative stress</td>
</tr>
<tr>
<td>245711_at</td>
<td>At5g04340</td>
<td>-1.83</td>
<td></td>
<td>Putative C2H2 zinc finger transcription factor</td>
<td>• Unknown</td>
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<tr>
<td>260147_at</td>
<td>At1g52790</td>
<td>-1.07 -2.73</td>
<td></td>
<td>Putative oxidoreductase</td>
<td>• Unknown</td>
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<tr>
<td>259276_at</td>
<td>At3g01190</td>
<td>-1.30</td>
<td></td>
<td>Putative peroxidase</td>
<td>• Unknown</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Peroxidase activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Response to oxidative stress</td>
</tr>
<tr>
<td>246270_at</td>
<td>At4g36500</td>
<td>-1.10</td>
<td></td>
<td>Putative protein</td>
<td>• Unknown</td>
</tr>
<tr>
<td>251281_at</td>
<td>At3g61640</td>
<td>-1.73</td>
<td></td>
<td>Putative protein hypothetical protein</td>
<td>• Unknown</td>
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<tr>
<td>266834_s_at</td>
<td>At2g30020</td>
<td>-1.87</td>
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<td>Putative protein phosphatase 2C</td>
<td>• Protein serine/threonine phosphatase activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Defence response to fungus</td>
</tr>
<tr>
<td>Gene ID</td>
<td>Accession</td>
<td>Log2 Fold Change</td>
<td>p-value</td>
<td>Function</td>
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<td>--------------------------------------------------------------------------</td>
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</tr>
<tr>
<td>250350_at</td>
<td>At5g12010</td>
<td>-1.23</td>
<td>–</td>
<td>Putative protein predicted proteins</td>
<td></td>
</tr>
<tr>
<td>248252_at</td>
<td>At5g53250</td>
<td>–</td>
<td>-1.20</td>
<td>Putative protein similar to unknown protein</td>
<td></td>
</tr>
<tr>
<td>248164_at</td>
<td>At5g54490</td>
<td>-1.53</td>
<td>–</td>
<td>Putative protein similar to unknown protein</td>
<td></td>
</tr>
<tr>
<td>250153_at</td>
<td>At5g15130</td>
<td>–</td>
<td>-1.47</td>
<td>Putative protein TMV response-related gene product</td>
<td></td>
</tr>
<tr>
<td>267028_at</td>
<td>At2g38470</td>
<td>-1.03</td>
<td>–</td>
<td>Putative WRKY-type DNA binding protein</td>
<td></td>
</tr>
<tr>
<td>251112_s_at</td>
<td>At5g01320</td>
<td>–</td>
<td>–</td>
<td>Pyruvate decarboxylase-like protein</td>
<td></td>
</tr>
<tr>
<td>264809_at</td>
<td>At1g08830</td>
<td>1.20</td>
<td>1.67</td>
<td>Superoxidase dismutase</td>
<td></td>
</tr>
<tr>
<td>247925_at</td>
<td>At5g57560</td>
<td>-1.77</td>
<td>–</td>
<td>Xyloglucan endotransglycosylase (TCH4) related protein</td>
<td></td>
</tr>
<tr>
<td>Gene ID</td>
<td>Accession</td>
<td>SLR 1</td>
<td>SLR 2</td>
<td>SLR 3</td>
<td>Description</td>
</tr>
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</tr>
<tr>
<td>261892_at</td>
<td>At1g80840</td>
<td>-2.07</td>
<td>-1.47</td>
<td>–</td>
<td>Transcription factor, putative similar to WRKY transcription factor</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Response to salicylic acid stimulus</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
<td>• Response to chitin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Defence response to bacterium</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Transcription factor activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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<td></td>
<td>• Regulation of transcription</td>
</tr>
<tr>
<td>263935_at</td>
<td>At2g35930</td>
<td>-1.30</td>
<td>-1.47</td>
<td>–</td>
<td>Unknown protein</td>
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<td></td>
<td>• Ubiquitin-protein ligase activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Response to chitin</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Protein ubiquitination</td>
</tr>
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<td>249284_at</td>
<td>At5g41810</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Unknown protein</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Unknown function</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Unknown process</td>
</tr>
</tbody>
</table>

\(^a\) The mean signal log ratio was calculated by averaging the SLR from the three replicated experiments.

\(^b\) *Brassica* lines: clubroot-susceptible ‘Granaat’ (GR), partially-resistant ‘Tahono’ (TO) and clubroot-resistant ‘ECD04’.

– Gene was not significantly expressed, using a threshold log\(_2\) ratio of 0.8 (1.75-fold change).

Putative defence-related or genes responding to chitin, fungus, bacterium, biotic stress or oxidative stress, which were paradoxically down-regulated, are **bolded**.

**Code abbreviations:**

IDA, inferred from direct assay; IEA, inferred from electronic annotation; IEP, inferred from expression pattern; IGI, inferred from genetic interaction; IMP, inferred from mutant phenotype; IPI, inferred from physical interaction; ISS, inferred from sequence or structural similarity; NAS, non-traceable author statement; ND, no biological data available; TAS, traceable author statement and NR, not recorded.
Figure 5.6. Regulation of the DE transcripts for each genotype (GR: ‘Granaat’, TO: ‘Tahono’ and ‘ECD04’) 48 hai with clubroot isolate S. Number of (a) up-regulated and (b) down-regulated transcripts are shown. Venn diagrams were generated at http://www.pangloss.com/seidel/Protocols/venn.cgi.

Note: These figures were constructed using full gene lists from Appendix 8.
superoxide dismutase (At1g08830) and the lack of induced genes such as chitinase (AF230684 or X61488) from the previous chapter was unexpected. Furthermore, the current study (although performed in growth rooms) paradoxically indicated the down-regulation of a lignin-biosynthesis enzyme: ferulate-5-hydroxylase (At4g36220) and other genes that may be involved in response to chitin, fungi, bacteria, biotic stress or oxidative stress (bolded in Table 5.13) such as a CCR4-associated factor-like protein (At3g44260), DNA binding protein (At5g65210), hairpin-induced protein-like (At5g06320), mitogen-activated protein kinase (At3g45640), peroxidase (At3g01190), protein phosphatase (At2g30020), WRKY-type DNA binding protein (At2g38470), WRKY transcription factor (At1g80840) and unknown protein (At2g35930). There was a total of 16 genes (mostly down-regulated) with unknown processes that may be of interest in the investigation of clubroot resistance or susceptibility.

5.3.4.2 Analysis of constitutively expressed genes

Due to the limited number of differentially regulated genes, constitutive gene expression was investigated and the selection process performed as in Tables 5.14 and 5.15. In brief, a total of 5,114 (22.4% of genechip) and 4,637 (20.3%) genes were called ‘Present’ in the experimental arrays as opposed to 4,981 (21.8%) and 4,981 (21.8%) in the baseline arrays for ‘Tahono’ and ‘ECD04’ respectively when compared to ‘Granaat’. Similarly, a fold change cut-off value of 1.75-fold was used to select those genes that were expressed at a greater/lesser rate in healthy untreated plants and individual analyses are summarised in Appendix 9. Of all the genes called ‘Present’, 110 (2.17%) and 205 (4.29%) were constitutively expressed in ‘Tahono’ and ‘ECD04’ when compared to ‘Granaat’ respectively. The key observation was that constitutive over-expression was most prominent in 30-day-old healthy untreated plants, in which ‘ECD04’ expressed more transcripts (115 genes) than ‘Tahono’ (74 genes). Both the Affymetrix Arabidopsis genechip and the RMIT Brassica oligoarray studies indicated the involvement of constitutive gene expression for clubroot
Table 5.14. Selection of constitutively expressed genes with reliable expressions in healthy ‘Tahono’ when compared to that of ‘Granaat’.

<table>
<thead>
<tr>
<th>Selection of genes constitutively OVER-expressed</th>
<th>Selection of genes constitutively UNDER-expressed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of genes on the array</td>
<td>22,810</td>
</tr>
<tr>
<td>No. of genes called ‘present’ in Experiment</td>
<td>5,114</td>
</tr>
<tr>
<td>No. of genes called ‘increase’</td>
<td>136</td>
</tr>
<tr>
<td>No. of genes with SLR* ≥ 0.8</td>
<td>91</td>
</tr>
<tr>
<td>No. of genes with a signal intensity ≥ 100 in Experiment</td>
<td>74</td>
</tr>
<tr>
<td>% Significantly greater rate</td>
<td>1.45</td>
</tr>
</tbody>
</table>

*SLR means Signal Log Ratio, whereby a value of 0.8 indicate a 1.75-fold change

Table 5.15. Selection of constitutively expressed genes with reliable expressions in healthy ‘ECD04’ when compared to that of ‘Granaat’.

<table>
<thead>
<tr>
<th>Selection of genes constitutively OVER-expressed</th>
<th>Selection of genes constitutively UNDER-expressed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of genes on the array</td>
<td>22,810</td>
</tr>
<tr>
<td>No. of genes called ‘present’ in Experiment</td>
<td>4,637</td>
</tr>
<tr>
<td>No. of genes called ‘increase’</td>
<td>162</td>
</tr>
<tr>
<td>No. of genes with SLR ≥ 0.8</td>
<td>121</td>
</tr>
<tr>
<td>No. of genes with a signal intensity ≥ 100 in Experiment</td>
<td>115</td>
</tr>
<tr>
<td>% Significantly greater rate</td>
<td>2.48</td>
</tr>
</tbody>
</table>
resistance and as expected, the former was able to provide a more thorough and detailed list of genes involved, though for *Arabidopsis* and not *Brassica*.

Venn diagrams were constructed to observe the relationship and co-regulation of these constitutively expressed genes in 30-days-old untreated plants (Figure 5.7). Selected putative ‘Tahono’-specific and ‘ECD04’-specific genes are illustrated in Table 5.16 and 5.17 respectively while the commonly constitutively expressed genes in both ‘Tahono’ and ‘ECD04’ when compared to ‘Granaat’ are summarised in Table 5.18 (only defence-related, transcription-related and responses to biotic and abiotic stresses are shown; refer to Appendix 9 for full gene lists of individual analyses). In brief, there were only two constitutively over-expressed ‘Tahono’-specific genes: glutathione-S-transferase (At2g02930) and DNA binding TGA-like protein (At5g65210) that were defence-related. Additionally, the constitutive under-expression of putative superoxide dismutases (At2g28190 and At1g08830) may indicate that the ‘Granaat’ controls were unexpectedly under oxidative stress. Similarly, the ‘ECD04’ controls demonstrated oxidative stress-related constitutive over-expression as indicated by superoxidase dismutase (At1g08830), 2-oxoglutarate dehydrogenase subunit (At5g55070), putative disulfide isomerase precursor (At1g21750), phenylalanine ammonia lyase (At2g37040) and unknown proteins (At3g13610 and At1g14870). In contrast to ‘Tahono’, there was a greater number of constitutively over-expressed ECD04-specific (defence-related or chitin-responsive) genes such as endochitinase (At2g43610), putative C2H2-type zinc finger protein (At5g22890) and a receptor-like protein kinase (At5g16590). Defence-related genes commonly constitutively over-expressed in both ‘Tahono’ and ‘ECD04’ were myrosinase (At5g25980) and the lignin biosynthesis enzyme, ferulate-5-hydroxylase (At4g36220). Additionally, the under-expression of a WRKY transcription factor may indicate important control of defence responses in both partially-resistant / resistant
Figure 5.7. Regulation of the constitutively expressed transcripts (Control array ‘Tahono’ or ECD04 when compared to ‘Granaat’). Number of genes expressed at (a) a greater rate and (b) a lesser rate are shown. Venn diagrams were generated at http://www.pangloss.com/seidel/Protocols/venn.cgi.

Note: These figures were constructed using full gene lists from Appendix 9.
Table 5.16. Selected ‘Tahono’-specific constitutively expressed genes (sorted by SLR).

<table>
<thead>
<tr>
<th>Probe Set ID</th>
<th>Locus Identifier</th>
<th>SLR</th>
<th>Putative function (on August 2007)</th>
<th>GO term (on March 2009)</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>247741_at</td>
<td>At5g58960</td>
<td>2.9</td>
<td>Putative predicted proteins</td>
<td>• Unknown</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Response to red or far red light</td>
<td>IMP</td>
</tr>
<tr>
<td>257946_at</td>
<td>At3g21710</td>
<td>1.7</td>
<td>Hypothetical protein predicted</td>
<td>• Unknown</td>
<td>ND</td>
</tr>
<tr>
<td>260552_at</td>
<td>At2g43430</td>
<td>1.5</td>
<td>Putative glyoxalase II</td>
<td>• Hydroxyacylglutathione hydrolase activity</td>
<td>IDA</td>
</tr>
<tr>
<td>254001_at</td>
<td>At4g26260</td>
<td>1.4</td>
<td>Putative protein</td>
<td>• Inositol oxygenase activity</td>
<td>IDA</td>
</tr>
<tr>
<td>250153_at</td>
<td>At5g15130</td>
<td>1.2</td>
<td>Putative protein TMV response-related gene product</td>
<td>• Transcription factor activity</td>
<td>ISS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Regulation of transcription</td>
<td>IEP</td>
</tr>
<tr>
<td>250580_at</td>
<td>At5g07440</td>
<td>1.2</td>
<td>Glutamate dehydrogenase 2</td>
<td>• Response to salt stress</td>
<td>ISS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Oxidoreductase activity</td>
<td>IDA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Glutamate dehydrogenase activity</td>
<td></td>
</tr>
<tr>
<td>251012_at</td>
<td>At5g02580</td>
<td>1.2</td>
<td>Putative protein</td>
<td>• Unknown</td>
<td>ND</td>
</tr>
<tr>
<td>265023_at</td>
<td>At1g24440</td>
<td>1.2</td>
<td>Unknown protein weak similarity to C3HC4 zinc finger</td>
<td>• Zinc Ion binding</td>
<td>IEP</td>
</tr>
<tr>
<td>253125_at</td>
<td>At4g36040</td>
<td>1.1</td>
<td>DNAJ-like protein DNAJ-like protein</td>
<td>• Heat shock protein binding</td>
<td>IEP</td>
</tr>
<tr>
<td>247199_at</td>
<td>At5g65210</td>
<td>1</td>
<td>DNA binding protein TGA1a homolog</td>
<td>• Transcription factor activity</td>
<td>ISS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Defence response to bacterium</td>
<td>IMP</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Calmodulin binding</td>
<td>ISS</td>
</tr>
<tr>
<td>248000_at</td>
<td>At5g56190</td>
<td>1</td>
<td>WD-repeat protein-like</td>
<td>• Unknown</td>
<td>ND</td>
</tr>
<tr>
<td>Gene ID</td>
<td>Accession</td>
<td>Log2Ratio</td>
<td>Description</td>
<td>Functions</td>
<td></td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
<td>-----------</td>
<td>--------------------------------------------------</td>
<td>---------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>258402_at</td>
<td>At3g15450</td>
<td>1</td>
<td>Unknown protein</td>
<td>• Unknown</td>
<td></td>
</tr>
<tr>
<td>266746_s_at</td>
<td>At2g02930</td>
<td>0.9</td>
<td>Putative glutathione S-transferase</td>
<td>• Glutathione transferase activity • Toxin catabolic process</td>
<td></td>
</tr>
<tr>
<td>267461_at</td>
<td>At2g33830</td>
<td>0.9</td>
<td>Putative auxin-regulated protein</td>
<td>• Unknown</td>
<td></td>
</tr>
<tr>
<td>247295_at</td>
<td>At5g64180</td>
<td>0.8</td>
<td>Putative protein similar to unknown protein</td>
<td>• Unknown</td>
<td></td>
</tr>
<tr>
<td>247312_at</td>
<td>At5g63970</td>
<td>0.8</td>
<td>Putative protein strong similarity to unknown protein</td>
<td>• Zinc-ion binding • Unknown process</td>
<td></td>
</tr>
<tr>
<td>250428_at</td>
<td>At5g10480</td>
<td>0.8</td>
<td>Putative tyrosine phosphatase-like protein</td>
<td>• Regulation of cell division • Cell differentiation</td>
<td></td>
</tr>
<tr>
<td>255645_at</td>
<td>At4g00880</td>
<td>0.8</td>
<td>Auxin-induced protein</td>
<td>• Response to auxin stimulus • Unknown function</td>
<td></td>
</tr>
<tr>
<td>261901_at</td>
<td>At1g80920</td>
<td>0.8</td>
<td>J8-like protein</td>
<td>• Heat shock protein binding</td>
<td></td>
</tr>
<tr>
<td>267280_at</td>
<td>At2g19450</td>
<td>0.8</td>
<td>Diacylglycerol O-acyltransferase</td>
<td>• Diacylglycerol O-acyltransferase activity • Response to abscisic acid stimulus • Aging</td>
<td></td>
</tr>
<tr>
<td>246289_at</td>
<td>At3g56880</td>
<td>-0.8</td>
<td>Putative protein</td>
<td>• Unknown</td>
<td></td>
</tr>
<tr>
<td>251222_at</td>
<td>At3g62580</td>
<td>-0.8</td>
<td>Putative membrane protein</td>
<td>• Unknown</td>
<td></td>
</tr>
<tr>
<td>264052_at</td>
<td>At2g22330</td>
<td>-0.8</td>
<td>Putative cytochrome P450</td>
<td>• Response to wounding • Monooxygenase activity • Glucosinolate biosynthetic process</td>
<td></td>
</tr>
<tr>
<td>Accession</td>
<td>Gene ID</td>
<td>Log2 FC</td>
<td>Description</td>
<td>Function</td>
<td></td>
</tr>
<tr>
<td>-----------</td>
<td>-------------</td>
<td>----------</td>
<td>------------------------------------------</td>
<td>-----------------------------------------------</td>
<td></td>
</tr>
</tbody>
</table>
| 266165_at | At2g28190   | -0.8     | Putative copper/zinc superoxide dismutase | • Camalexin biosynthetic process
|           |             |          |                                          | • Defence response to bacterium
|           |             |          |                                          | • Callose deposition in cell wall during defence response |
| 254810_at | At4g12390   | -0.9     | Putative protein pectinesterase          | • Superoxide dismutase activity
|           |             |          |                                          | • Response to oxidative stress
|           |             |          |                                          | • Removal of superoxide radicals
|           |             |          |                                          | • Response to stress |
| 264179_at | At1g02180   | -0.9     | Hypothetical protein predicted           | • Pectinesterase activity
|           |             |          |                                          | • Unknown biological process
| 262832_s_at | At1g14870 | -1.2     | Unknown protein                          | • Unknown
|           |             |          |                                          | • Unknown function
|           |             |          |                                          | • Response to oxidative stress |
| 264809_at | At1g08830   | -1.3     | Superoxidase dismutase                   | • Superoxide dismutase activity
|           |             |          |                                          | • Response to oxidative stress
|           |             |          |                                          | • Defence response to bacterium |
| 261970_at | At1g65960   | -1.4     | Glutamate decarboxylase                  | • Calmodulin binding |

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Note:
Refer to Appendix 9 for full gene lists of individual analysis.

Code abbreviations:
IDA, inferred from direct assay; IEA, inferred from electronic annotation; IEP, inferred from expression pattern; IGI, inferred from genetic interaction; IMP, inferred from mutant phenotype; IPI, inferred from physical interaction; ISS, inferred from sequence or structural similarity; NAS, non-traceable author statement; ND, no biological data available; TAS, traceable author statement and NR, not recorded.
Table 5.17. Selected ‘ECD04’-specific constitutively expressed genes (sorted by SLR).

<table>
<thead>
<tr>
<th>Probe Set ID</th>
<th>Locus Identifier</th>
<th>SLR</th>
<th>Putative function (in August 2007)</th>
<th>GO term (performed on March 2009)</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>248049_at</td>
<td>At5g56090</td>
<td>3.8</td>
<td>Putative protein contains similarity to cytochrome oxidase assembly factor</td>
<td>• Unknown</td>
<td>ND</td>
</tr>
<tr>
<td>260226_at</td>
<td>At1g74660</td>
<td>3.3</td>
<td>Hypothetical protein predicted</td>
<td>• Response to abscisic acid stimulus</td>
<td>IMP</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Response to cytokinin stimulus</td>
<td>IMP</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Transmission factor activity</td>
<td>ISS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Response to gibberellin stimulus</td>
<td>IMP</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Response to auxin stimulus</td>
<td>IMP</td>
</tr>
<tr>
<td>262832_s_at</td>
<td>At1g14870</td>
<td>3.3</td>
<td>Unknown protein</td>
<td>• Response to oxidative stress</td>
<td>IMP</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Unknown</td>
<td>ND</td>
</tr>
<tr>
<td>256647_at</td>
<td>At3g13610</td>
<td>2.8</td>
<td>Unknown protein contains similarity to DNA-binding protein</td>
<td>• Oxidoreductase activity</td>
<td>ISS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Coumarin biosynthetic process</td>
<td>IMP</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Secondary metabolic process</td>
<td>ISS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Hydrogen peroxide-mediated programmed cell death</td>
<td>IMP</td>
</tr>
<tr>
<td>260557_at</td>
<td>At2g43610</td>
<td>1.5</td>
<td>Putative endochitinase</td>
<td>• Chitin binding</td>
<td>IEA</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>• Chitinase activity</td>
<td>ISS</td>
</tr>
<tr>
<td>251370_at</td>
<td>At3g60450</td>
<td>1.3</td>
<td>Putative protein</td>
<td>• Unknown</td>
<td>ND</td>
</tr>
<tr>
<td>264809_at</td>
<td>At1g08830</td>
<td>1.3</td>
<td>Superoxidase dismutase</td>
<td>• Removal of superoxide radicals</td>
<td>IC</td>
</tr>
<tr>
<td>Accession</td>
<td>Genbank Code</td>
<td>Log2 Fold Change</td>
<td>Description</td>
<td></td>
<td></td>
</tr>
<tr>
<td>-----------</td>
<td>--------------</td>
<td>------------------</td>
<td>-------------</td>
<td></td>
<td></td>
</tr>
<tr>
<td>263878_s_at</td>
<td>At2g22040</td>
<td>1.2</td>
<td>Unknown protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>248088_at</td>
<td>At5g55070</td>
<td>1.1</td>
<td>2-oxoglutarate dehydrogenase E2 subunit</td>
<td></td>
<td></td>
</tr>
<tr>
<td>249882_at</td>
<td>At5g22890</td>
<td>1.1</td>
<td>Putative protein contains similarity to C2H2-type zinc finger protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>250438_at</td>
<td>At5g10580</td>
<td>1.1</td>
<td>Putative protein predicted protein,</td>
<td></td>
<td></td>
</tr>
<tr>
<td>257823_at</td>
<td>At3g25190</td>
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<td>Integral membrane protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>255263_at</td>
<td>At4g05160</td>
<td>1</td>
<td>4-coumarate--CoA ligase - like protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>257375_at</td>
<td>At2g38640</td>
<td>1</td>
<td>Unknown protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>262504_at</td>
<td>At1g21750</td>
<td>1</td>
<td>Putative protein disulfide isomerase precursor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>263924_at</td>
<td>At2g36530</td>
<td>1</td>
<td>Enolase (2-phospho-D-glycerate hydrolyase)</td>
<td></td>
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</tr>
<tr>
<td>249717_at</td>
<td>At5g35730</td>
<td>0.9</td>
<td>Unknown protein</td>
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</tr>
<tr>
<td>256342_at</td>
<td>At1g72020</td>
<td>0.9</td>
<td>Unknown protein</td>
<td></td>
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</tr>
<tr>
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<td>Unknown protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>263845_at</td>
<td>At2g37040</td>
<td>0.9</td>
<td>Phenylalanine ammonia lyase (PAL1)</td>
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</tr>
<tr>
<td>248588_at</td>
<td>At5g49540</td>
<td>0.8</td>
<td>Unknown protein</td>
<td></td>
<td></td>
</tr>
<tr>
<td>250102_at</td>
<td>At5g16590</td>
<td>0.8</td>
<td>Receptor-like protein kinase</td>
<td></td>
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</tr>
</tbody>
</table>

- **Superoxide dismutase activity**  
- **Response to oxidative stress**  
- **Defence response to bacterium**  
- **Unknown**  
- **Response to oxidative stress**  
- **Response to chitin**  
- **Transcription factor activity**  
- **Jasmonic acid biosynthetic process**  
- **Regulation of programmed cell death**  
- **Response to abscisic acid stimulus**  
- **Defence response**  
- **Phenylalanine ammonia-lyase activity**  
- **Response to oxidative stress**  
- **Unknown**  
- **Response to symbiotic fungus**  

**TAS**  
**IEP**  
**ND**  
**IDA**  
**ISS**
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**Note:**
Refer to Appendix 9 for full gene lists of individual analysis.

**Code abbreviations:**
IDA, inferred from direct assay; IEA, inferred from electronic annotation; IEP, inferred from expression pattern; IGI, inferred from genetic interaction; IMP, inferred from mutant phenotype; IPI, inferred from physical interaction; ISS, inferred from sequence or structural similarity; NAS, non-traceable author statement; ND, no biological data available; TAS, traceable author statement and NR, not recorded.
Table 5.18. Selected genes commonly and constitutively expressed between unchallenged 30-day-old ‘Tahono’ and ‘ECD04’ (sorted by SLR of ECD04).

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<th>GO term (on March 2009)</th>
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- **Response to salt stress**
- **Response to osmotic stress**
- **Ubiquitin-protein ligase activity**
- **Ubiquitin-dependent protein catabolic process**
- **Cation transport**
- **Response to nematode**
- **Unknown**
- **Monooxygenase activity**
- **Lignin biosynthesis process**
- **Positive regulation of transcription**
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</table>

Putative defence-related or genes responding to chitin, fungus, bacterium, biotic stress or oxidative stress, were **bolded**.

**Note:**
Refer to Appendix 9 for full gene lists of individual analysis.

**Code abbreviations:**
- **IDA**, inferred from direct assay
- **IEA**, inferred from electronic annotation
- **IEP**, inferred from expression pattern
- **IGI**, inferred from genetic interaction
- **IMP**, inferred from mutant phenotype
- **IPI**, inferred from physical interaction
- **ISS**, inferred from sequence or structural similarity
- **NAS**, non-traceable author statement
- **ND**, no biological data available
- **TAS**, traceable author statement
- **NR**, not recorded
genotypes. Finally, there was a total of 66 genes (13 ‘Tahono’-specific, 36 ‘ECD04’-specific and 17 genes common in both genotypes) with unknown functions that may be of interest in future clubroot studies.

The frequency of the gene ontology (GO) annotations was also shown as pie charts. Of particular interest was the large number of genes constitutively over-expressed with unknown molecular functions (18.4% and 11.9%) (Figure 5.8a, c) and involved in response to stress and abiotic or biotic stimulus (19.3% and 14.8%) (Figure 5.8b, d) in ‘Tahono’ and ‘ECD04’ when compared to ‘Granaat’ respectively. Moreover, there were (unexpectedly) an even greater number of genes constitutively under-expressed with unknown molecular functions (21.6% and 23.5%) (Figure 5.9a, c) and related to responses to stress and abiotic or abiotic stimuli (21.6% and 26.9%) (Figure 5.9b, d) in ‘Tahono’ and ‘ECD04’ respectively when compared to ‘Granaat’.

Of minor interest, there were major differences (> 3-fold) in genes constitutively over-expressed for protein binding (9.2% and 1.4%), structural molecular activity (1.3% and 14.0%) and receptor binding / activity (0.0% and 0.7%) (Figure 5.8a, c) and involved in cell organisation / biogenesis (1.4% and 7.6%) and signal transduction (0.0% and 0.7%) (Figure 5.8b, d) in ‘Tahono’ and ‘ECD04’ respectively. There were also major differences in genes constitutively under-expressed with structural molecular activity (2.7% and 0.9%), transcription factor activity (2.7% and 7.8%), kinase activity (0.0% and 6.1%) and DNA / RNA / nucleic acid / nucleotide binding (0.0% and 7.0%) (Figure 5.9a, c) and related to protein metabolism (1.4% and 4.7%), transport / electron transport (0.0% and 5.2%), developmental processes (0.0% and 2.8%) and signal transduction (0.0% and 1.9%) (Figure 5.9b, d) in ‘Tahono’ and ‘ECD04’ respectively.
Pie charts for constitutively OVER-expressed genes:

**Functional Categorization by annotation for: GO Molecular Function**

- unknown molecular functions: 18.42% (raw value = 14)
- transporter activity: 15.78% (raw value = 12)
- other enzyme activity: 14.47% (raw value = 11)
- hydrolase activity: 13.15% (raw value = 10)
- protein binding: 9.21% (raw value = 7)
- other binding: 7.89% (raw value = 6)
- transferase activity: 7.89% (raw value = 6)
- kinase activity: 5.26% (raw value = 4)
- transcription factor activity: 2.63% (raw value = 2)
- DNA or RNA binding: 1.31% (raw value = 1)
- nucleotide binding: 1.31% (raw value = 1)
- other molecular functions: 1.31% (raw value = 1)
- structural molecule activity: 1.31% (raw value = 1)

**Functional Categorization by annotation for: GO Biological Process**

- other metabolic processes: 24.26% (raw value = 34)
- other cellular processes: 22.05% (raw value = 32)
- response to abiotic or biotic stimulus: 10.71% (raw value = 15)
- response to stress: 8.67% (raw value = 12)
- transport: 7.89% (raw value = 11)
- unknown biological processes: 7.89% (raw value = 11)
- protein metabolism: 7.14% (raw value = 10)
- other biological processes: 3.68% (raw value = 5)
- developmental processes: 2.63% (raw value = 4)
- electron transport or energy pathways: 2.14% (raw value = 3)
- cell organization and biogenesis: 1.43% (raw value = 2)
- transcription: 0.71% (raw value = 1)

Figure 5.8. Functional classification by annotation for: (a, c) GO molecular functions and (b, d) GO biological processes. The pie charts represent the frequency of GO terms in the list of genes that were constitutively OVER-expressed in 30-day-old untreated: (a, b) ‘Tahono’ and (c, d) ECD04 when compared to that of ‘Granaat’. Classification performed in June 2008.
Figure 5.9. Functional classification by annotation for: (a, c) GO molecular functions and (b, d) GO biological processes. The pie charts represent the frequency of GO terms in the list of genes that were constitutively UNDER-expressed in 30-day-old untreated: (a, b) ‘Tahono’ and (c, d) ECD04 when compared to that of ‘Granaat’. Classification performed in June 2008.
5.3.5 Validation of microarray data by quantitative real-time PCR

Similarly to the results of Section 4.3.4, the current qRT-PCR data was generated from reliable log-transformed amplification curves (no sigmoid curve detected, data not shown), that were derived from single specific amplicons (single peaks/bands on the melting curve analysis and gel electrophoresis, data not shown). The linear standard curve had good precision (correlation coefficients, $R^2 > 0.99$) but lower PCR efficiency of 82.5%.

The expression log FC values of the differentially expressed and constitutively expressed target genes are summarised in Table 5.19 and 5.20 respectively. Of a total of 26 comparisons between Affymetrix data and qRT-PCR data, 20 (77%) showed conserved direction of expression as well as relatively similar magnitude. The Affymetrix platform did not detect the up-regulation of the chitinase gene (AF230684) and contradicted both the qRT-PCR here and in Chapter 4 and RMIT Brassica oligoarray data in Chapter 4. Of minor interest, there was a strong up-regulation of GST (AI352707) in ‘ECD04’ and a consistent down-regulation of lipase (At1g30370) in all three genotypes at 48 hai.

5.4 Discussion

The first aim of this chapter was achieved in that the gene expression profiles of resistant, partially-resistant and susceptible plants were differentiated with and without challenge by clubroot. The surprisingly few inducible genes in the hosts’ root hairs suggested the lack of dominant genes, their suppression by P. brassicae in these genotypes or possibly due to a dilution effect when total root mass were used. By contrast, there was more differentiation in constitutively expressed genes, suggesting that clubroot resistance or partial-resistance is derived from the high basal levels of defence-related genes such as PAL, chitinase and myrosinase. This situation contrasts with most other pathogen-plant systems examined so far.
Table 5.19. Expression log ratios of differentially expressed transcripts assessed by Affymetrix and qRT-PCR.

<table>
<thead>
<tr>
<th>Putative function</th>
<th>GenBank® accession number</th>
<th>Locus Identifier</th>
<th>Probe ID</th>
<th>Granaat 48 hai</th>
<th>Tahono 48 hai</th>
<th>ECD04 48 hai</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Affy</td>
<td>qRT-PCR</td>
<td>Affy</td>
</tr>
<tr>
<td>Glutathione S-transferase</td>
<td>AI352707</td>
<td>At1g02940</td>
<td>262103_at</td>
<td>-0.70</td>
<td>0.51</td>
<td>0.30</td>
</tr>
<tr>
<td>Xyloglucan endo-transglycosylase precursor</td>
<td>AY156708</td>
<td>At2g06850</td>
<td>266215_at</td>
<td>0.00</td>
<td>-0.12</td>
<td>0.03</td>
</tr>
<tr>
<td>Lipase</td>
<td></td>
<td>At1g30370</td>
<td>256306_at</td>
<td>-1.43</td>
<td>-1.84</td>
<td>-1.53</td>
</tr>
<tr>
<td>Phenylalanine ammonia-lyase</td>
<td>AY055752</td>
<td>At2g37040</td>
<td>263845_at</td>
<td>0.30</td>
<td>-0.14</td>
<td>0.37</td>
</tr>
<tr>
<td>Chitinase</td>
<td>AF230684</td>
<td>At2g43590</td>
<td>260560_at</td>
<td>-0.10</td>
<td>-0.16</td>
<td>0.73</td>
</tr>
<tr>
<td>Superoxidase dismutase</td>
<td></td>
<td>At1g08830</td>
<td>264809_at</td>
<td>1.20</td>
<td>1.49</td>
<td>1.67</td>
</tr>
</tbody>
</table>

Array values indicate average log₂ fold change relative to untreated controls and qRT-PCR values indicate log₂ ratio of normalised test relative to normalised calibrator.
Table 5.20. Expression log ratios of constitutively expressed transcripts assessed by Affymetrix and qRT-PCR.

<table>
<thead>
<tr>
<th>Putative function</th>
<th>Locus Identifier</th>
<th>Probe ID</th>
<th>Tahono 48h</th>
<th>ECD04 48h</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Affy</td>
<td>qRT-PCR</td>
</tr>
<tr>
<td>WRKY transcription factor</td>
<td>At5g01320</td>
<td>261892_at</td>
<td>-1.3</td>
<td>-1.0</td>
</tr>
<tr>
<td>Polygalacturonase</td>
<td>At1g10640</td>
<td>261834_at</td>
<td>-1.5</td>
<td>-3.2</td>
</tr>
<tr>
<td>Leucine zipper protein</td>
<td>At1g08325</td>
<td>261815_at</td>
<td>1.1</td>
<td>-0.9</td>
</tr>
<tr>
<td>Ferulate-5- hydroxylase</td>
<td>At4g36220</td>
<td>253088_at</td>
<td>1.1</td>
<td>-0.3</td>
</tr>
</tbody>
</table>

Array values indicate average log₂ fold change relative to untreated controls of ‘Granaat’ and qRT-PCR values indicate log₂ ratio of normalised test relative to normalised calibrator.
(Siemens et al., 2006; Coram et al., 2007) but does agree with the fewer results from the Brassica oligoarray and qRT-PCR in Chapter 4. The achievement of this aim made possible the postulation of defence pathways in these Brassica genotypes, though a major disadvantage was the mismatch between Arabidopsis and Brassica genes, in particular chitinase and so aim 2 can only be achieved in part.

5.4.1 Hydroponic test system: possible source of clubroot resistance

There is strong evidence that the Brassica rapa genotype ‘ECD04’ (fodder turnip) is highly resistant to clubroot disease as opposed to the partially-resistant Chinese cabbage ‘Tahono’ and hence, may be a potential source of resistance genes for Brassica breeding. This was indicated by the lack of galls at 8 weeks after inoculation with the virulent clubroot isolate S while both ‘Granaat’ and ‘Tahono’ manifested heavy clubbing. As discussed in Chapter 2, European fodder turnip has been used as a source of resistance to breed new clubroot-resistant Brassica varieties (Yoshikawa, 1983), but recent reports indicated their breakdown with emerging clubroot pathotypes (Hirai et al., 2004). Previous resistance tests (Table 2.2) showed the absence of clubbing in ‘ECD04’ against a wide range of Victorian isolates and hence, it is very unlikely that these observations occurred due to disease avoidance. Therefore, this indicated that ‘ECD04’ may carry more than one or all of the three reported dominant, race-specific resistance genes of Brassica rapa (Matsumoto et al., 1998; Hirai et al., 2004; Piao et al., 2004). This makes ‘ECD04’ an ideal candidate in Brassica breeding for Victorian conditions. The introgression of resistance genes into Chinese cabbage varieties may be difficult due to its differing agricultural properties, but may be overcome by backcross or pedigree methods of breeding (Allard, 1960; Moreno-Gonzalez and Cubero, 1993). The latter may be enhanced through the use of molecular markers linked to these defence-related genes. Therefore, identification of these genes should provide vital information towards the aims of this study. The gene expression profiles of ‘ECD04’ may involve the transcription of
a greater number of important defence-related genes against pathogen invasion when compared to ‘Granaat’ and even to ‘Tahono’.

### 5.4.2 Absolute analysis of the Affymetrix *Arabidopsis* genechip

The Affymetrix *Arabidopsis* genechip offers many advantages over spotted cDNA or oligonucleotide arrays. The optimised / standardised fabrication process, labelling protocol and data-processing techniques used in Affymetrix technology ensured data quality, statistically sound results and make data mining across a normalised database feasible (Zhu, 2003). Additionally, the use of probe sets (involving perfect and mismatches) to represent one gene and the inclusion of a wide range of endogenous and exogenous controls to quality ascertain the data, allowed for a more robust microarray experiment (Affymetrix, 2004c, a, b). Prior to comparative analyses, the reliability, quality and reproducibility of the absolute analyses in this study are discussed below.

#### 5.4.2.1 Quality control of the Affymetrix data

All labelled targets were synthesised successfully from good quality RNA samples. This was indicated by the endogenous housekeeping controls ‘GAPDH’ and ‘UBQ’ having 3’/5’ ratios of less than three in all arrays. However, the ratios of the ‘actin’ gene, ranging from 2.17 up to 8.80, suggests a loss of array sensitivity due to degraded total RNA samples or poor assay quality. This contradicted the RNA profiles generated earlier by spectrophotometry, gel electrophoresis and the RNA bioanalyser. There is no single threshold cut-off to assess sample quality for all of the diverse organisms and tissues using these 3’/5’ ratios. This is due to the presence of different isoforms of these housekeeping genes and their different expression patterns (Affymetrix, 2004b) and therefore the current cut-off ratio may not be applicable to all situation. Additionally, high actin 3’/5’ ratio has been reported in other Affymetrix studies and the 3’-bias for this control gene due to probe design is suspected.
All total RNA samples used in this study were considered fit for hybridisation.

All genechip hybridisations were performed effectively without any significant loss to sensitivity and accuracy. This was indicated by alternating intensities and the checkboard pattern along the border of the genechip, expected staggered signal intensities for the exogenous spike-in controls, scale factors of less than 3-fold, comparable noise (Raw Q) values and average background values between 20 and 100 in all arrays. The scaling method, commonly applied to oligonucleotide arrays (Affymetrix, 2004b), was used to overcome the effect of noise in the data. This resulted in insignificant pixel-to-pixel variations (noise) so that the arrays could be compared more accurately. The low backgrounds may indicate low signal to noise ratios and allowed the detection of transcripts at very low levels (Affymetrix, 2004c), i.e. loss of sensitivity due to high background was avoided. None of the arrays was discarded due to poor hybridisation or quality in this study.

All replicated arrays had high levels of reproducibility. This was indicated by the scale factors being less than 3-fold, linear scatter plots mostly within the 2-fold reference lines and ≥ 98.5% of genes (called ‘present’ or ‘marginally present’ in all three biological replicates) with variation coefficients of less than 50% between replicated arrays. These methods are commonly used to assess the biological and technical inconsistencies between replicated arrays (Zhu and Wang, 2000; Müssig et al., 2002; Affymetrix, 2004b; Raghavan, 2004) due to the inherent variability of microarray experiments. These results may be attributed to the benefits of the hydroponic system during root collection and handling (explained in Chapter 4). Furthermore, biological replicates performed through space in environmentally-controlled growth-rooms and the pooling of roots from individual plants are necessary to eliminate
differences that arise between biological samples (Zhu and Wang, 2000). None of the arrays was discarded due to poor reproducibility and could be used in comparative analyses.

The independent qRT-PCR method successfully validated the usually error-prone Affymetrix data. This was demonstrated by a 77% correlation between the two datasets. As discussed in the previous chapter, the contradictory comparisons have been reported in other similar studies (Coram and Pang, 2006; Mantri et al., 2007) and was attributed to their ratios being close to zero, i.e. those target genes that were not significantly expressed (based on the fold change cut-off $\log_2$ value of 0.848 or 1.75-fold change). Most of the comparisons showed relatively similar magnitude, if not exaggerated qRT-PCR values as reported by the above authors. The results indicated that the data generated from the Affymetrix Arabidopsis ATH1 genechip generally had better association with their qRT-PCR data than the RMIT Brassica oligoarray. The contradictory results of the Brassica-specific chitinase gene (AF230684) were of particular interest and are discussed in the next section. The Affymetrix data was confidently used in the exploratory analysis of differential and constitutive gene expressions.

5.4.3 Comparative analysis of the Affymetrix Arabidopsis genechip

Although not a perfect tool, the Affymetrix Arabidopsis genechip was valid for investigating transcriptional changes in the Brassica hosts upon challenge with clubroot spores. Discoveries have been made in Brassica (Hammond et al., 2005; Hudson et al., 2007) and in other plant species such as Cardamine kokaiensis (Brassicaceae herb) (Shin-Ichi et al., 2008) using the Affymetrix Arabidopsis genechip as an exploratory tool. Used in this context, the microarray may guide the design of lower-throughput experiments to find homologous genes that rapidly or strongly change in expression, e.g. gel blots or qRT-PCR (Hudson et al., 2007). Additionally, these genes may provide biomarkers for cellular responses and may offer vital insights into signal transduction and developmental mechanisms. Ultimately, such
information may be used in the development of molecular markers linked to disease resistance for marker-assisted selection in plant breeding (Collard et al., 2005). Therefore, cross-species exploratory microarray hybridisation is a potentially useful technique for applying model genomics in related plant species for which few functional genomics are available (Zhu et al., 2001b), at least until the whole genome sequencing of Brassica.

5.4.3.1 Patterns in the transcriptional changes

Hybridisation efficiency:
In this study, the hybridisation of the labelled Brassica samples onto the Affymetrix Arabidopsis genechip was less efficient and the limitations of using a cross-species platform were noticeable. This was indicated by the low 19 to 23% of genes called ‘present’, of which only 0.2% (53 out of 22,810 genes) and 1.4% (315 genes) were significant, as expressed in the current differential and constitutive analyses respectively. In contrast, studies using labelled Arabidopsis samples on the Arabidopsis genechip reported between 57 and 69% of genes called ‘present’, in which about 1.0% (between 204 to 233 genes) were significantly differentiated (Zhu and Wang, 2000; Raghavan et al., 2005; Madhou et al., 2006). This reduced hybridisation efficiency was expected as demonstrated by a similar experiment on a Brassicaceae herb using the Arabidopsis genechip, in which 28 to 36% of genes were called ‘present’ (Dr Shin-Ichi, pers. comm.) and 0.3% (69 genes) were differentially expressed (Shin-Ichi et al., 2008). This may be explained by the sequence divergence between Brassica RNA samples and the Arabidopsis sequences on the chip, causing high mismatches between most of the oligonucleotide probes and the target RNA (Koch et al., 2001). Therefore, the assumption that a ‘mismatch’ probe hybridisation value can be safely subtracted from a ‘perfect match’ probe hybridisation value may not hold true. This may result in transcripts called ‘absent’ since the mismatch hybridisation levels may be comparable to those of the ‘match’ (Chismar et al., 2002). To resolve this, Zhu et al. (2001a) used a chip design without
mismatch probes while Hammond et al. (2005) and Hudson et al. (2007) masked the mismatch Affymetrix data. These were not performed here, as it would require custom-made microarray platforms and data-mining / analytical software. Changing the statistical algorithms’ parameters was also discouraged by Affymetrix (‘Fine tuning your data analysis’ technical manual, www.biocompare.com) and other scientists (Dr Müller and Dr Magnino, pers. comm., 4th Annual Integrated Sciences QPCR User Meeting held in 2007). Instead, a less-stringent cut-off value of 1.75-fold change was used in this study, a method followed by Shin-Ichi et al. (2008) (cut-off value of 1.5-fold change applied). This resulted in the statistically sound discovery of highly expressed transcripts and by data processing and global scaling of the microarray data, the detection of genes with lower signal intensities.

**Differential expression:**

Down-regulation was most prominent at 48 hai and the limited number of differentially expressed genes was attributed to a cross-species microarray platform. This was demonstrated by a total of 47 repressed genes as opposed to only 6 induced genes in all three genotypes. These results contradicted with the mostly up-regulated profiles of the RMIT *Brassica* oligoarray data. This may be due to the biased representation of putative defence-associated and regulatory genes of the oligoarray. The very low number of up-regulated genes using the Affymetrix technology was unexpected for the partially-resistant ‘Tahono’ and especially for the resistant ‘ECD04’. It was postulated that ‘ECD04’ (and possibly ‘Tahono’) possess a few dominant resistant genes (from Section 5.4.1); these *R* (resistance) genes would allow recognition of distinct races of *P. brassicae* and trigger defence responses in their roots (Matsumoto et al., 1998; Hirai et al., 2004; Piao et al., 2004). Such defence reactions would include programmed cell death (hypersensitive reaction, HR), modifications of cell walls as well as production of antimicrobial proteins, metabolites and pathogenesis-related (*PR*)
proteins (Eulgem, 2005; Coram and Pang, 2007; Vidhyasekaran, 2007). This hypothesis is not supported using these *Brassica* genotypes, as noted in the previous chapter.

The lack of previously identified differentially expressed genes such as the *Brassica*-specific chitinase (AF230684) in the Affymetrix ‘Granaat’ or ‘Tahono’ data, was another concern when its up-regulation was observed in their qRT-PCR data. This may be explained by the presence of different chitinase coding regions and isoforms in *Arabidopsis* than in *Brassica* (Kasprzewska, 2003). Sequence polymorphisms with the target organism have probably reduced the quality of information available from experiments using genechips designed for a model species (*Arabidopsis*) to monitor the transcriptome of a closely related species (*Brassica*). The approach used by Hammond *et al.* (2005) and Hudson *et al.* (2007), of masking the mismatched Affymetrix data or by selecting for homologous *B. oleracea*-specific sequences on the *Arabidopsis* genechip prior to analysis, may have overcome these problems. Hence, the construction of a *B. rapa*-specific ‘masking file’ may provide new analytical possibilities in future clubroot studies for Chinese cabbage using the cross-species Affymetrix *Arabidopsis* ATH1 genechip.

**Constitutive expression:**

Constitutive over-expression was prominent in 30-day-old untreated resistant and partially-resistant plants and may play an important role in the defence mechanism against clubroot infection. This was illustrated by 189 genes constitutively over-expressed as opposed to 126 genes constitutively under-expressed in ‘Tahono’ and ‘ECD04’ vs ‘Granaat’ untreated controls. The large proportion of these genes involved in responses to stress and abiotic or biotic stimulus from the gene ontology (GO) pie charts also supported this conclusion. As discussed in earlier chapters, the constitutive expression of defence-related genes provided an effective non-specific form of defence against a wide range of pathogens (Zhu *et al.*, 1994;
Hammond-Kosack and Jones, 1996; Keane and Brown, 1997; Vidhyasekaran, 2007). These genes in particular were much greater in ‘Tahono’ and ‘ECD04’ than ‘Granaat’: myrosinase (At5g25980), which is involved in the breakdown of glucosinolates into antimicrobial by-products (LudwigMüller et al., 1997; Hara et al., 2000)), ferulate-5-hydroxylase (At4g36220), involved in lignin biosynthesis (Humphreys et al., 1999) and peroxidase (At3g01190), responsible for the scavenging of ROS (Kawano, 2003) and lignin biosynthesis (Vidhyasekaran, 2007). The gene that was lesser in the resistant / partially-resistant genotypes was the WRKY transcription factor (At1g80840) that is a putative negative regulator of defence genes (Eulgem, 2005; Journot-Catalino et al., 2006). The functions and regulation of these genes and possible transcriptional network in clubroot defence are discussed in more detail in Section 5.4.3.2.

Resistance vs partial-resistance:

There are some evidences that the differing level of clubroot resistance between ‘Tahono’ and ‘ECD04’ may be attributed to genotype-specific constitutively expressed genes.’ The greater clubroot resistance of ‘ECD04’ than ‘Tahono’ may be explained by the greater basal levels of endochitinase (At2g43610), which is involved in chitin degradation (Grison et al., 1996; Cota et al., 2007)), 4-coumarate-CoA ligase-like protein (At4g05160), in lignin biosynthesis (Heath et al., 2002), superoxidase dismutase (At1g08830) in ROS scavenging (Hammond-Kosack and Jones, 1996) and phenylalanine ammonia lyase (At2g37040), in salicylic acid synthesis (Vidhyasekaran, 2007) as well as lesser basal level of another WRKY transcription factor (At2g38470), putative negative regulators of defence genes (Eulgem, 2005; Journot-Catalino et al., 2006). Of particular interest were the major differences in genes constitutively expressed in the GO pie charts for receptor / DNA / RNA / nucleic acid / nucleotide binding and signal transduction between ‘Tahono’ and ‘ECD04’. This is because several types of transcription factors have been implicated in disease resistance. Some are functionally linked
to each other and to signal transducers, revealing regulatory circuits within a complex transcriptional network (Eulgem, 2005). The functions and regulation of these genes and hypothetical pathways in clubroot defence may explain for ECD04’s greater resistance to clubroot and are discussed in more detail in the next section.

5.4.3.2 Defence pathways against clubroot disease

Plant immune responses involve a multitude of physiological reactions that are induced by pathogen recognition. Upon detection, the signal transduction and activation of defence-related genes soon follow. Such defence reactions include programmed cell death (hypersensitive response, HR) and modifications of cell walls as well as the production of antimicrobial proteins, metabolites and pathogenesis-related proteins (PR) (Eulgem, 2005; Coram and Pang, 2007; Vidhyasekaran, 2007). This knowledge of gene expression is being extended significantly by large scale-gene expression profiling, such as microarray technology. In this study, the differential and constitutive transcriptional changes or patterns have identified novel regulatory systems and supported previously reported roles of defence genes against clubroot disease in the Brassica genotypes. The activation of the defence transcriptome is a complex multidimensional process involving a large number of genes defined by spatial and temporal patterns (Schmelzer et al., 1989). The regulatory pathways identified in this study are postulated in the steps below.

Recognition and signal transduction of pathogen elicitors:

Chitin-receptor

The first step in a quick and effective defence response is the recognition of the pathogen by the plant. In this study, there was insufficient evidence to identify the type of receptor proteins involved in the recognition of P. brassicae. The hypothesis was that the clubroot-resistant ‘ECD04’ line might possess a few dominant genes and hence their resistance (R) genes would
allow recognition of distinct races of *P. brassicae* (Matsumoto *et al.*, 1998; Hirai *et al.*, 2004; Piao *et al.*, 2004) but could not be confirmed. There was evidence of hypersensitive responses in this study (discussed later), that may be the outcome of recognition by ligand / receptor interactions specified by paired plant resistance (*R*) and pathogen avirulence (*avr*) genes (Lamb and Dixon, 1997) from the constitutive over-expression of an endochitinase (*At2g43610*) in ‘ECD04’. Since the cell wall of *P. brassicae* has 25% chitin (Moxham and Buczacki, 1983), this indicated that chitooligosaccharide elicitors may trigger *Brassica* defence responses against clubroot invasion. A report suggested that a putative chitinase-related receptor-like kinase (CHRK) linked to a serine / threonine kinase domain (Kasprzewska, 2003), may be a potential receptor protein in this clubroot / *Brassica* pathosystem.

**MAPK**

The down-regulation of a mitogen-activated protein kinase (MAPK, *At3g45640*) in challenged ‘Granaat’ may suggest a reduced ability to relay a strong intracellular signal and may explain its high susceptibility to clubroot. The MAPK cascade forms an important component in the signalling mechanism that transduces extracellular signals into a wide range of intracellular responses (Vidhyasekaran, 2007). Activation of MAPKs by elicitors from different plant pathogens in various plant species has been reported while loss-of-function studies of MAPKs revealed less disease resistance (Zhang and Klessig, 2001; Bent and Mackey, 2007). Due to the constitutive under-expression of this same protein in the clubroot-resistant ‘ECD04’, this source of susceptibility may not hold true. More research is needed to identify these receptor proteins to *P. brassicae* since their genes would permit specific and strong defence responses against *P. brassicae*. 
Regulation of reactive oxygen species, salicylic acid and hypersensitive response:

There is some evidence that defence responses against clubroot disease begin with an oxidative burst followed by the accumulation of reactive oxygen species (ROS) in the roots of the *Brassica* genotypes, especially ‘ECD04’. This was indicated by the up-regulation of superoxide dismutase (SOD, At1g08830) in challenged ‘Granaat’ and ‘Tahono’, constitutive over-expression of peroxidase (At3g01190) in both untreated ‘Tahono’ and ‘ECD04’ and superoxide dismutase (At1g08830) in untreated ‘ECD04’ only. The oxidative burst is the fastest active defence response induced by pathogens in resistant interactions and results in the rapid and transient production of ROS such as H$_2$O$_2$, which is produced and scavenged by SOD and peroxidase respectively (Hammond-Kosack and Jones, 1996; Vidhyasekaran, 2007). The constitutive nature of SOD was unexpected and indicated that the ‘ECD04’ untreated plant controls may be under some form of stress. Activation of oxidative burst and accumulation of ROS appear to be a central component of a highly amplified and integrated signalling system in response to *P. brassicae* recognition. The down-stream signalling of ROS on the transcription of defence-related proteins is discussed below.

The Affymetrix study indicated that the accumulation of ROS, most probably H$_2$O$_2$, may have resulted in the synthesis of salicylic acid (SA) in the *Brassica* roots. The constitutive over-expression of phenylalanine ammonia lyase (PAL, At2g37040) in the ‘ECD04’ controls supported this. PAL is activated by increasing ROS level and is a key regulator of the phenylpropanoid pathway, which synthesises salicylic acid from phenylalanine (Mauch-Mani and Slusarenko, 1996). Several roles of SA have been proposed in plant defence: as directly antimicrobial, in the regulation of PR proteins and as a key role in the establishment of systemic acquired resistance (SAR) (Hammond-Kosack and Jones, 1996; Glazebrook *et al.*, 1997). Additionally, SA has been reported to inhibit or react with catalase and peroxidase to intensify oxidative stress resulting from ROS or to be converted into SA free radical for lipid
peroxidation (Hammond-Kosack and Jones, 1996; Vidhyasekaran, 2007). Lipid peroxidation may activate genes through the jasmonic acid (JA) pathway; however, there was no evidence to suggest the involvement of JA in this study, especially since SA and JA are antagonistic mechanisms (Glazebrook et al., 2003). Nevertheless, the constitutively expressed elevated levels of SA in several Arabidopsis mutants correlated with constitutively high PR gene expression and hence, with increased disease resistance (Ryals et al., 1996). Therefore, this may also explain greater resistance of ‘ED04’ against P. brassicae.

The results of this study suggest that the elevated level of ROS may have initiated a hypersensitive response (HR) or programmed cell death (PCD) in the roots of ‘ECD04’. The unexpected constitutive over-expression of a putative protein involved in H\textsubscript{2}O\textsubscript{2}-mediated PCD (At3g13610) and protein disulfide isomerise precursor involved in the regulation of PCD (At1g21750) suggested that the untreated roots were undergoing oxidative stress. HR plays a role in disease resistance and PCD deprives the obligate biotrophic pathogen of access to further nutrients and may even be lethal to the germinating spores (Hammond-Kosack and Jones, 1996). Moreover, the disintegration of the cell components may initiate the myrosinase-glucosinolate defence system in Brassica (discussed later) as well as the induction of local and systemic resistance (Heath, 2000). The reasons for the constitutive over-expression of HR-related genes in untreated ‘ECD04’ remain elusive. It is possible that the hyper-responsive nature of this genotype may be responsible for its greater resistance to pathogens as opposed to ‘Tahono’ and ‘Granaat’.

A hypothetical molecular cascade was constructed (Figure 5.10) to illustrate possible downstream effects of an oxidative burst in Brassica roots in response to P. brassicae from the results of this study so far.
**P. brassicae** recognition

- Receptor protein *e.g.* R-gene? or CHRK? or MAPK?
  - (At3g45640, ↓ GR, c↓ ECD04)?
- Secondary messenger *e.g.* Ca^{2+}? or cAMP?
  - ROS
  - SOD
  - Unknown factors resulting in high basal expression
  - (At1g08830, ↑ GR, ↑ TO, c↑ ECD04, c↓ TO)
  - Accumulation of H_{2}O_{2}
  - PAL
  - POX
  - H_{2}O_{2}
  - POX-dependent lignification
  - (At3g13610 and At1g21750, c↑ ECD04)
  - (At2g37040, c↑ ECD04) (At3g01190, c↑ TO and ECD04)
  - (At3g01190, c↑ TO and ECD04)
  - SA
  - SAR or SA-dependent defence response

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**Figure 5.10.** A hypothetical molecular cascade involving the biosynthesis of salicylic acid (SA) via ROS accumulation and PAL, resulting in the activation of SA-dependent defence response in *Brassica* against clubroot infection.

Grey arrows represent casual interaction, blue arrows represent activating mechanisms, red arrows represent repressing mechanisms, c↑ and c↓ represent constitutive over- and under-expression and finally, ↑ and ↓ indicate up- and down-regulation. CHRK, chitinase-related receptor-like kinase; MAPK, mitogen activated protein kinase; cAMP, cyclic AMP; ROS, reactive oxygen species; HR, hypersensitive response; SOD, superoxide dismutase; PAL, phenylalanine ammonia lyase and POX, peroxidase.
Regulation of defence-related transcription factors and pathogenesis-related (PR) protein:

**WRKY and TGA transcription factors**

Members of the transcription factor families such as WRKY (At1g80840 and At2g38470) and TGA (At5g65210), may be involved in responses to clubroot infection in the *Brassica* genotypes and may play major roles in transcriptional reprogramming during various immune responses. The expression of a large number of genes encoding for transcription factors has been reported by Cheong *et al.* (2002). These bind to conserved promoter elements (such as W boxes for WRKY and TGA boxes for TGA factors) in upstream regions of defence-related genes to regulate their expression (Eulgem, 2005). The up-regulation of *Arabidopsis* WRKY genes by chitin or treatment with defence elicitors has been reported and their accumulation appears to be a general characteristics of plant defence events (Jinrong *et al.*, 2004). Similarly, the TGA factors, which interact with the positive regulator NPR1 (non-expresser of pathogenesis-related protein), have important roles in the regulation and induction of SA-dependent transcriptional programming and systemic acquired resistance (SAR) (Zhang *et al.*, 2003). Members of this subfamily of basic leucine zipper (bZIP) transcription factors were originally identified by their ability to bind to the *asl*-like elements, a class of general stress-responsive *cis*-elements (Jakoby *et al.*, 2002; Eulgem, 2005). Hence, the lack of induced WRKY or TGA genes in this study contradicted these reports. However, at least one member of the WRKY family can act as a transcriptional repressor and additional W boxes were negatively regulated (Journot-Catalino *et al.*, 2006). Results from TGA knock-out mutants have implicated TGAs in PR repression in basal resistance (Zhang *et al.*, 2003). A general mechanism of NPR1-dependent (and/or SA-dependent) defence gene activation may involve de-repression via WRKY and TGA factors combined with activation of TGA and other types of transcription factor (Eulgem, 2005). A possible role of these transcription factors in clubroot defence is further discussed in the next paragraph, involving the regulation of the pathogen-related protein (PR) observed in this study.
**Pathogenesis-related protein**

The *PR* protein endochitinase (At2g43610), up-regulated in ‘Granaat’ and ‘Tahono’ (Chapter 4) and constitutively over-expressed in ‘ECD04’ when compared to ‘Granaat’ (current chapter), may be regulated via NPR1, WRKY and TGA transcription factors. The latter are commonly used by SAR, R-gene mediated resistance or basal defences (Eulgem, 2005). The role of chitinases has been discussed earlier and they are induced by an increase in endogenous salicylic acid and jasmonic acid content in plants (Kasprzewska, 2003). The elevated SA levels (possibly induced by increasing ROS due to elevated SOD activity), may have caused an increased in NPR1 transcription via the positive regulators WRKY factors. The NPR1 would then couple with TGA factors prior to binding to positive and negative cis-elements (TGA boxes) to activate or repress *PR* transcription respectively (Zhang et al., 2003; Eulgem, 2005). The involvement of SA and NPR1 were not evident in this study. The constitutive under-expression of the negative regulator WRKY factors may, however, have contributed to the constitutive over-expression of endochitinase in ‘ECD04’ controls. The down-regulation of a putative TGA factor (possibly a negative regulator, At5g65210) in challenged ‘Tahono’, may also explain the up-regulation of the *Brassica*-specific chitinase in the *Brassica* oligoarray results. Acidic endochitinases, induced by elevated SA levels, are usually secreted to the apoplast and are involved in the early stage of defence against clubroot (Mami et al., 2000; Kasprzewska, 2003). The increase in apoplastic chitinase content intensifies the production of elicitor molecules and indirectly enhances the infection signalling (Kasprzewska, 2003). The mechanisms in the regulation of this *PR* protein offered an efficient means of defence, especially in ‘ECD04’ as well as indicating important genes / biomarkers for the development of molecular markers. A hypothetical molecular cascade was constructed (Figure 5.11) to link the constitutive accumulation of SA and its effect on the constitutive production of PR proteins (possibly endochitinase) in ‘ECD04’, which was effective against *P. brassicae* infection in this study.
Figure 5.11. A hypothetical molecular cascade relaying salicylic acid (SA)-dependent signals to PR1 (and possibly endochitinase) via NPR1, WRKY and TGA factors, in *Brassica* against clubroot disease.

Causal interactions are indicated by grey arrows, c↑ and c↓ represent constitutive over- and under-expression and finally, ↑ and ↓ indicate up- and down-regulation. Activating mechanisms are marked by ‘+’ and repressing mechanisms are marked by ‘−’. Coding region of genes is represented by squares, cis-elements by upright rectangles and transcription factors as well as NPR1 by ovals. Transcription start sites of genes are marked by black arrows. Adapted from Eulgem (2005).
Regulation of lignin biosynthesis:

Lignin is an important factor in plant defence responses because it represents an undegradable mechanical barrier to most pathogens. There is a strong correlation between the high basal expression of lignin biosynthesis enzymes and clubroot resistance, possibly via ROS signalling in this study. This was demonstrated by the constitutive over-expression of ferulate-5-hydroxylase (F5H, At4g36220) and a putative peroxidase (At3g01190) in both ‘Tahono’ and ‘ECD04’ when compared to ‘Granaat’ controls. This was supported by the similar expression of Caffeoyl-CoA 3-O-methyltransferase (AY821735) in ‘Tahono’ from Chapter 4. F5H is one of many enzymes to produce phenolic precursors of lignin through the phenylpropanoid pathway (Humphreys et al., 1999). These precursors may then be used to strengthen cell walls by a peroxidase-catalysed polymerisation reactions using H$_2$O$_2$ (Kawano, 2003; Kawasaki et al., 2006). Moreover, these lignin precursors and the free radicals produced during polymerisation in the cell wall may affect pathogen membrane plasticity and inactivate pathogen enzymes, toxins or elicitors (Hammond-Kosack and Jones, 1996). Of particular interest is the dual functionality of peroxidase as a ROS scavenger and in the catalysis of ROS (Kawano, 2003). Mori et al. (2001) reported that SA or chitosaccharide elicitors induce the production of ROS in an apoplastic peroxidase-dependent manner. The resultant ROS stimulates the opening of Ca$^{2+}$ channels and the influx of Ca$^{2+}$ ions that follows, possibly inducing the Ca$^{2+}$-dependent defence responses inside the cell. The hypothetical cascade involving the high basal level of lignification in the partially-resistant ‘Tahono’ and resistant ‘ECD04’ is included in Figure 5.10 along with the mechanisms that may control the constitutive over-expression of SA.
Regulation of myrosinase and glucosinolate content:

Myrosinase

The myrosinase-glucosinolate system is considered to be a defence system in *Brassicaceae* species against insects and possibly also against pathogens. There is some evidence that the high basal level of myrosinase in the roots of the *Brassica* hosts protects against clubroot invasion. This was highlighted by the constitutive over-expression of myrosinase (At5g25980) in both the untreated ‘Tahono’ and ‘ECD04’ when compared to ‘Granaat’ controls. This system is activated by tissue damage caused by wounding or pathogen attacks, in which the myrosinase enzyme catalyses the hydrolysis of the thioglucoside linkage in glucosinolates (Taipalensuu *et al.*, 1997). This leads to the release of a glucose and an unstable aglycone, which can spontaneously rearrange into various end products such as isothiocyanates, nitrile and thiocyanate. Due to the general toxicity and volatility of these by-products, they possess potent antimicrobial properties and play important roles in plant-pathogen interactions (Hara *et al.*, 2000; Yan and Chen, 2007). The major myrosinase-containing organ in *B. napus* is the root system, which displayed 10- to 100-fold greater myrosinase activity than the stem or leaf (Hara *et al.*, 2000). Therefore, a constitutively high myrosinase in the roots level may involve the quick turn-over of these secondary plant metabolites during a defence response against soil-borne pathogens. The results in this study supported those of Siemens and Mitchell-Olds (1998) who reported the potential benefit of increased pest resistance by a high basal level of myrosinase. The cost of maintaining high myrosinase production was associated, however, with a significant decrease in seed production. This has important implications for the constitutive over-expression of defence-related genes against clubroot and studying the costs of defence may provide more important information about alternative functions of these systems.
Glucosinolates

In this study, there is some evidence that partially-resistant / resistant Brassica varieties had a lower basal level of glucosinolates or maybe specific glucosinolate(s), possibly due to elevated level of salicylic acid (SA). This was demonstrated by the constitutive under-expression of cytochrome P450-type proteins (At5g45340 or At2g22330) in both untreated ‘Tahono’ and ‘ECD04’ and its down-regulation (At5g45340) in ‘Tahono’. Five cytochrome P450-type gene products catalyse the conversion of phenylalanine, tryptophan or short-chain and long-chain elongated methionine substrates into glucosinolate precursors (Yan and Chen, 2007). The total glucosinolate content in roots of two susceptible Chinese cabbage varieties was greater throughout the experimental period than in roots of two resistant varieties when challenged with P. brassicae spores (Ludwig-Müller et al., 1997). Additionally, the development of more severe clubroot symptoms may be correlated with higher glucosinolate content (Ludwig-Müller, 2009). This may be due to a relationship between enhanced auxin levels in infected roots and indole glucosinolate degradation, suggesting plants with lower concentration of indole glucosinolates may show reduced symptoms (Ludwig-Müller et al., 1999). However, there are conflicting reports showing positive correlation between (aliphatic) glucosinolate levels and resistance to pathogens in seed rape (B. napus) as well as inducible glucosinolate levels with no change in myrosinase levels to stem rot disease (Sclerotinia sclerotiorum) (Siemens and Mitchell-Olds, 1998; Li et al., 1999).

The mutually antagonistic jasmonic acid (JA) and salicylic acid (SA) signalling pathways may be involved in the regulation of glucosinolate levels, in which increased SA signalling represses glucosinolate synthesis (Yan and Chen, 2007). Of particular interest is that insect feeding induced glucosinolate biosynthesis requires the functions of regulatory proteins NPR1 and ETR1 (ethylene receptor 1) (Mewis et al., 2005). NPR1 appears to be a point of intersection of multiple signalling pathways, i.e. the SA-dependent regulation of glucosinolate
synthesis and the pathogenesis-related protein endochitinase. There is increasing evidence that *Brassicaceae* specialists-insects and possibly pathogens, may be more responsive to particular glucosinolates (Rask *et al.*, 2000). Therefore, the composition of plant glucosinolate profiles, despite more than 100 glucosinolate substrates and several myrosinase forms being reported (Bones and Rossiter, 1996), may provide essential information for the modification of plants to obtain the optimal combination of myrosinases and glucosinolates.

The hypothetical cascade involving the high basal level of myrosinase and possibly reduced level of glucosinolate in the partially-resistant ‘Tahono’ and resistant ‘ECD04’, is demonstrated in **Figure 5.12**; this may result in defence or reduced clubroot symptoms.

**Transcription-related and unknown proteins:**

From the constitutive expression analyses, there was a large number of transcription-related and unknown genes that may be of interest in future clubroot studies. This was indicated by up to 40.0% and 35.4% of constitutively expressed genes with unknown function from the ‘Tahono’ and ‘ECD04’ GO pie charts respectively. The significantly greater number of genes involved in DNA / RNA / nucleic acid / nucleotide binding in the clubroot-resistant ‘ECD04’ may potentially be involved in defence pathways, but are not conserved with the partially-resistant ‘Tahono’. Alternatively, these genes may be involved in other metabolic pathways that may be related to the differing physiological properties between the Chinese cabbages and turnips. As more loss-of-function studies in *Arabidopsis* or *Brassica* are published, these unknown or transcription-related genes may reveal interesting new defence mechanisms against clubroot disease. These may provide novel biomarkers for the development of molecular markers in the breeding of clubroot-resistant *Brassica* crops.
Figure 5.12. A hypothetical molecular cascade relaying salicylic acid (SA)-dependent signals to glucosinolate via NPR1 against the development of clubroot symptoms.

Grey arrows indicate casual interactions, blue arrows are activating mechanisms, red arrows are repressing mechanisms, c↑ and c↓ represent constitutive over- and under-expression and finally, ↑ and ↓ indicate up- and down-regulation. PAL, phenylalanine ammonia lyase; SA, salicylic acid; JA, jasmonic acid and NPR1, non-expresser of pathogenesis-related genes 1.
5.5 Summary

The identification of defence-related genes is critical in the development of molecular markers for marker-assisted selection in *Brassica* breeding strategies against clubroot disease. This was made possible by investigating the transcriptional changes in ‘challenged’ genotypes using large-scale gene expression profiling, such as microarray technology. The limitations of the ‘boutique’ *Brassica* oligoarray were apparent in Chapter 4. Therefore, a more sophisticated microarray platform, such as the Affymetrix *Arabidopsis* ATH1 genechip, was used. The latter offers many advantages over the spotted oligonucleotide array such as optimised / standardised fabrication process, labelling protocol, built-in controls and data-processing techniques to ensure data quality, statistically sound results and data mining across a normalised database. This cross-species platform was a valid exploratory tool and makes *Brassica* vegetables a clear beneficiary of *Arabidopsis* functional genomics studies due to the close phylogenetic relationship between these two genera based on DNA sequences.

In this study, the hydroponic test systems (three biological replicates per genotype) were set up inside growth-rooms. The 30-day-old ‘untreated’ roots and ‘challenged’ root tissues at 48 hai with aggressive clubroot isolate S were collected from the partially-resistant Chinese cabbage ‘Tahono’ and susceptible Chinese cabbage ‘Granaat’. Additionally, a clubroot-resistant turnip ‘ECD04’ was included in this experiment. This *B. rapa* species (disease index, DI of zero from previous resistance tests using several Victorian clubroot isolates) had no visible gall formation at 8 weeks after inoculation while both ‘Granaat’ (DI = 69) and ‘Tahono’ (DI = 17) manifested heavy clubbing. This indicated that ‘ECD04’ may carry at least one dominant resistance gene and is possible candidate in *Brassica* breeding for clubroot resistance in Victorian conditions. These samples were processed and hybridised onto the Affymetrix *Arabidopsis* genechip by experienced AGRF staff as a paid service.
The resulting Affymetrix genechips were labelled effectively from good quality RNA samples without any significant loss to sensitivity and accuracy, according to their absolute analysis summary reports. All replicated arrays had high levels of reproducibility based on scatter plots and variation coefficient histograms while the Affymetrix data were validated by qRT-PCR. The comparative analyses of the Affymetrix data indicated an unexpectedly low number of differentially expressed genes that were prominently down-regulated at 48 hai in all three genotypes. This was probably due to the limitations of using a cross-species platform, i.e. the sequence divergence between Brassica RNA samples and the Arabidopsis sequences on the chip was considerable. The construction of a B. rapa-specific ‘masking file’ may provide new analytical possibilities in future clubroot studies for Chinese cabbages using this cross-species Affymetrix genechip.

It was postulated that ‘ECD04’ (and possibly ‘Tahono’) possesses a few dominant inducible genes and these R (resistance) genes would allow recognition of specific P. brassicae races and trigger defence responses in the roots. Due the low number of inducible up-regulated genes in this study as well as in the previous chapter, this hypothesis is not supported using these Brassica genotypes. This Affymetrix study demonstrated however, that constitutive over-expression was significant in 30-day-old untreated plants and this may play an important role in the defence mechanisms against clubroot infection. Furthermore, there was some evidence that the differing levels of clubroot resistance between ‘Tahono’ and ‘ECD04’ may be attributed to genotype-specific constitutively expressed genes.

Molecular cascades providing novel insights as well as supporting previous studies on the regulatory systems of defence genes in Brassica against clubroot disease were postulated. An elevated levels of salicylic acid (SA) appears to be a key regulatory component in many constitutive defence pathways in both ‘Tahono’ and ‘ECD04’. Upon recognition of P.
*brassicae* elicitors (possibly by a putative chitinase-related receptor-like receptor linked to a serine / threonine kinase domain or a mitogen-activated protein kinase receptor (At3g45640)), this may have initiated an oxidative burst, followed by accumulation of reactive oxygen species (ROS) such as H$_2$O$_2$ in the roots. This was indicated by the up-regulation and, unexpectedly the constitutive over-expression of ROS-producing superoxide dismutase (SOD, At1g08830) and the ROS-scavenging peroxidase (At3g01190). The accumulation of ROS was responsible for initiating a hypersensitive response (HR) or programmed cell death (PCD), as indicated by over-expressing PCD-related proteins (At3g13610 and At1g21750), promoting root lignification *via* a peroxidase-dependent pathway and ferulate-5-hydroxylase (F5H, At4g36220) and finally, in triggering phenylalanine ammonia lyase (PAL, At2g37040) expression, which synthesised more SA. The reason for a high basal level of ROS remained elusive and indicated that the control plants might have been under some form of stress.

Two SA-dependent defence responses were reported in this study: firstly, the constitutive over-expression of a pathogenesis-related (PR) protein endochitinase (At2g43610), possibly *via* the NPR1, WRKY (At1g80840 or At2g38470) and TGA (At5g65210) factors. The elevated level of SA and constitutive or differential repression of negative regulators (WRKY and TGA) may explain the greater clubroot resistance in ‘ECD04’ as opposed to ‘Tahono’. Secondly, the myrosinase / glucosinolate defence system may also be regulated *via* SA signalling. It is possible that the high basal level of myrosinase (At5g25980) may allow a fast and efficient break-down of glucosinolate compounds to release antimicrobial compounds such as isothiocyanate. It is reported that the NPR1 factors are involved in the negative regulation of glucosinolate synthesis enzymes such as cytochrome P450-type proteins (At5g45340 or At2g22330) and their down-regulation may prevent the production of indole glucosinolates, resulting in reduced gall formation in partially-resistant / resistant *Brassica* varieties. Finally, there was a high number of transcription-related and unknown genes that
may be of interest in future clubroot studies. As more loss-of-function studies in *Arabidopsis* are published, these genes may reveal interesting new defence mechanisms against clubroot. Ultimately, these genes involved in defence may potentially be used in the development of molecular markers for marker-assisted selection in *Brassica* breeding strategies against clubroot disease.
Chapter 6

Summary, conclusions and future directions

6.1 Project summary

In Chapter 1, the state of knowledge about *Plasmodiophora brassicae* and its detrimental effects on Australian and world-wide *Brassica* plantations was reviewed. A key finding was that current cultural and biological control measures were prohibitive and impractical while chemical control measures were costly and usually environmentally harmful. Therefore, the breeding of resistant *Brassica* cultivars, by pyramiding clubroot-resistant genes, is an effective approach to minimise loss and for the long-term viability of the *Brassica* industry in Australia. There have however been few successful breeding programs for resistance despite the identification of several sources of clubroot resistance, since the resistance is rarely expressed at a high level. The lack of information on the complex genetic control of resistance in the hosts and the distribution and mixed infection of multiple pathogenic races in a single field, are other impediments. Therefore, the identification of the number of genes involved in clubroot resistance and their mechanisms of action are essential first steps in effective breeding strategies.

Plants have evolved a number of mechanisms involving developmental, morphological, physiological and biochemical strategies to survive against different biotic and abiotic stresses. Studies on the mechanisms of plant defence reported transcriptional activation and repression of genes, from pathogen recognition to response / adaptation. Our understanding of the speed, coordination and magnitude of these response networks may be improved by investigating the gene expression profiles in response to clubroot infection. One such approach involves the use of high-throughput microarray techniques, which has been exploited in a number of crops. The experimental design leading to the identification of
defence-related genes in selected characterised Chinese cabbage varieties using both a ‘boutique’ Brassica oligoarray and the Affymetrix Arabidopsis genechip, is summarised below.

Since modern programs for disease resistance are based on artificially induced infections, the work described in Chapter 2 was primarily to collect, identify and characterise virulent P. brassicae populations. Clubroot isolates were prepared from naturally-infected roots originating from Brassica vegetable farms in Victoria and characterised using the European Clubroot Differential (ECD) series. The latter is an internationally-accepted standard method comprising 15 differential Brassica hosts with different numbers and types of resistance genes. The virulence and aggressiveness of the clubroot isolates used for inoculation was determined by the reactions (degree of gall formation) of the plants within the ECD set. Six Victorian triplet ECD codes were identified but there was no evidence of new pathotypes when these codes were compared to previous Australian-wide clubroot surveys. Due to the more aggressive reactions on the Brassica species from which the isolates were extracted, this indicated that P. brassicae populations possess a certain degree of species specificity. These results were however insufficient to support the contention that P. brassicae is composed of several pathotypes despite the diverse reactions of the isolates. These characterised isolates were subsequently used as sources of infection to permit accurate and reproducible test systems.

Breeding programs designed to produce disease resistant varieties should firstly begin with the search and identification of plants with resistance-conferring genes. Therefore the work described in Chapter 3 was to assess the level of clubroot resistance of 19 commercially-available B. rapa hybrids (especially Chinese cabbage varieties) from Asian and Australian suppliers. Due to the lack of clubroot resistance in Chinese cabbages, 18 worldwide Brassica
landraces were also characterised to discover new sources of clubroot resistance. These resistance tests were performed using similar experimental conditions to the ECD tests but inoculated only with the clubroot isolates characterised as virulent from Chapter 2. Eight partially-resistant Chinese cabbage hybrids were identified, especially ‘Tahono’ (Disease index, DI = 5, Refer to Section 3.3.2) and ‘Leaguer’ (DI = 12). The mostly susceptible reactions (DI ≥ 33) within the (heterogeneous) landraces were however unexpected and the above two lines were therefore used as potential sources of clubroot resistance in subsequent microarray studies to investigate their defence responses.

Large-scale profiling of the transcriptional changes to the early stages of *P. brassicae* infection in *Brassica* crops has not been performed using microarray technology. Therefore in Chapter 4, a ‘boutique’ *Brassica* oligoarray of 150 *Arabidopsis* - / *Brassica*-derived features was constructed using information from nucleotide databases such as GenBank. This array, representing a biased selection of defence-associated and regulatory genes, was used to investigate the gene expression of selected *Brassica* varieties (susceptible ‘Granaat’ and partially-resistant ‘Tahono’ and ‘Leaguer’ Chinese cabbages) at 24, 48 and 72 h after inoculation (hai) with virulent clubroot isolate S (pathogen and hosts characterised in Chapter 2 and Chapter 3 respectively). A novel hydroponic test system was established successfully to study the transcriptional changes occurring in the root tissues due to complications of using a soil-based system. This hydroponic system, when performed in glasshouses/growth-rooms, could minimise environmental variables that may interfere with plant defence responses (further discussed in Section 6.2.2). This study identified 48 hai as the most responsive time to investigate defence responses. The gene expression profiling at 48 hai revealed 10, 11 and 2 differentially expressed genes upon infection in ‘Granaat’, ‘Tahono’ and ‘Leaguer’ respectively and 8 and 21 constitutively expressed genes in 30-day-old ‘Tahono’ and ‘Leaguer’ when compared to ‘Granaat’ untreated controls respectively. The key
observations were the expression of a pathogenesis-related (PR) protein (chitinase) and a lignin biosynthesis enzyme (caffeoyl-CoA 3-O-methyltransferase) in these partially-resistant varieties. Despite the ‘closed architecture’ system of the Brassica oligoarray, its construction was a viable option to avoid the costly and inefficient use of Affymetrix genechips.

The Affymetrix Arabidopsis ATH1 genechip is a more sophisticated microarray platform and provided a potentially more thorough and robust analysis of the defence mechanisms in the Brassica vegetables against clubroot disease in Chapter 5. This genechip possesses many advantages over the spotted oligoarray in terms of scale, built-in quality controls and reliability of data. The close phylogenetic relationship between the genera Brassica and Arabidopsis makes Brassica researches a clear potential beneficiary of the Arabidopsis genechip and related functional genomics studies. In this study, the susceptible ‘Granaat’ and partially-resistant ‘Tahono’ Chinese cabbages as well as a putative highly resistant ‘ECD04’ fodder turnip (DI = 0) against clubroot were grown hydroponically, challenged with clubroot isolate S and analysed on the cross-species Affymetrix genechip. The gene expression profiling at 48 hai revealed 17, 34 and 2 differentially expressed genes upon infection in ‘Granaat’, ‘Tahono’ and ‘ECD04’ respectively and 110 and 205 constitutively expressed genes in 30-day-old ‘Tahono’ and ‘ECD04’ when compared to ‘Granaat’ untreated controls respectively. Molecular cascades providing novel insights into the complex regulatory systems of defence genes in Brassica against clubroot were postulated. The key observations were the elevated levels of ROS and SA that appear to be essential regulatory components in many defence pathways in partially-resistant ‘Tahono’ and resistant ‘ECD04’. Three major SA-dependent defence responses were identified namely, the expression of a PR protein (endochitinase), lignification and regulation of the myrosinase / glucosinolate system. Ultimately, the information from both microarray studies may be used in the development of molecular markers for the marker-assisted selection of clubroot-resistant Brassica crops.
6.2 Project conclusions

6.2.1 Phenotyping of \textit{P. brassicae} and the \textit{Brassica} host

The small-scale surveys on the distribution and diversity of \textit{P. brassicae} populations as well as the possible new sources of clubroot resistance provided vital information that may be used directly by Victorian \textit{Brassica} farmers, breeders and researchers.

Firstly, no new Victorian clubroot pathotype was identified from the study described in Chapter 2 when compared to a previous survey. This was expected because the gap between these two surveys was less than 3 years, which is insufficient time for the mutation, selection and proliferation of new pathotypes (especially for soil-borne pathogens). The preventive control measures in \textit{Brassica} farms (reviewed in Section 1.3) should presently prevent the spread of current and new races of \textit{P. brassicae}. Secondly, the current Victorian \textit{P. brassicae} races had diverse virulent reactions towards cultivated \textit{Brassica} vegetables, especially to Chinese cabbages. This observation was further supported by the lack of any highly resistant commercially-available Chinese cabbages described in Chapter 3. Victorian \textit{Brassica} farmers therefore do not have access to resistant cultivars suitable for Victorian clubroot conditions and this reiterates the need to breed clubroot-resistant \textit{Brassica} vegetables. Lastly, the lack of highly resistant \textit{Brassica} landraces from the survey described in Chapter 3, was not expected and restates the difficulties in breeding for clubroot resistance. The discovery of new sources of resistance should be a priority for future \textit{Brassica} breeding programs.

In conclusion, this information will help in the selection and introduction of new resistant varieties with the ability to yield in Australian conditions, since there is no conventional breeding program to develop clubroot-resistant \textit{Brassica} crops in Australia. These elite cultivars are expected to be very effective against most of the host-specific clubroot pathotypes in Victoria, at least soon after release.
6.2.2 Effect of environment and heterogeneity on the host-pathogen interaction

Environmental variables significantly affected the results and interpretation of the data in this project. The cause of these fluctuations was due to glasshouse limitations as reported in Sections 2.3.1, 3.3.2 and 4.3.2 and therefore, future clubroot experiments should be performed in a phytotron to provide more reliable results. Seasonal changes such as the reduced temperature (as reviewed in Section 1.1) and possibly photoperiod in Winter may have suppressed the germination and invasion of clubroot spores and resulted in lack of infection or low infection rates in the hosts. These observations supported current cultural control measures of clubroot that may be beneficial to farmers, i.e. the cultivation of susceptible Brassica varieties can still be profitable when environmental conditions are unfavourable to clubroot infection. Spring seemed to be the most appropriate time to conduct clubroot experiments for microarray purposes. Hosts grown in Spring had high vigour and maturity and may be infected only by aggressive isolates, thus allowing the discovery of candidate genes in future functional genomics studies.

For research purposes, however, a reliable and reproducible test system and tissue samples (ideally from a controlled population genetic system e.g. doubled haploid or recombinant inbred lines) that are easily obtained are required. The effects of other environmental variables such as available nutrients and pH in the rhizosphere (as reviewed in Section 1.1) were minimised by the development of a novel hydroponic test system as described in Chapter 4. This system was devised for the infection of Brassica species by clubroot. This produced galls of the same size and in the same times as in pot trials but had the advantage that it made collection of infected roots immeasurably easier, quicker and more efficient. This has always been a major problem in the study of any root-borne disease and most of the gene expression literature has therefore been an infection of aerial plant parts. This was vital to being able to collect large quantities of high-quality uncontaminated total RNA, the key to
analysis of gene expression. Therefore, this hydroponic test system may potentially be used in other soil-borne pathogen-host interactions by avoiding the complications associated with soil.

The heterogeneity of the field isolates and ECD series was another concern. This may be avoided by using genetically-uniform single-spore isolates and by breeding homozygous ECD hosts (near isogenic lines, NILs) that are able to differentiate any *P. brassicae* populations. This will be ideal to reduce background genetic variations in this complex host-parasite interaction. In the current microarray experiments, biological replicates and tissue pooling were performed to minimise the genetic variables between the *Brassica* hosts. Originally the replicated hydroponic tests were conducted across time and this resulted in high variability amongst replicated data as reported in Section 4.3.4 due to the significant effects of season. Although this supported the contention that effective plant defence responses are highly dependent on favourable environmental conditions, future clubroot experimental replicates should ideally be performed simultaneously under controlled conditions so that changes in gene expression are correlated to the different defence mechanisms of the hosts rather than to environmental variation.

In conclusion, future functional genetics studies on clubroot may benefit from these information with an improved experimental design and total RNA representing more accurately defence responses in *Brassica* vegetable crops will expand our understanding of clubroot resistance.

### 6.2.3 Genetics of clubroot resistance

New insights on the genes and pathways involved in defence against clubroot were provided from the use of a ‘boutique’ *Brassica* oligoarray and the Affymetrix *Arabidopsis* genechip.
The limited number of differentially expressed defence genes from both microarray experiments was unexpected and may be due to their suppression by the pathogen upon invasion. The contention that effective defence mechanisms against clubroot involved inducible responses was therefore not fully supported using these genotypes. The suppression of the plant defensive arsenals, possibly by delivering repressor proteins through a type III secretion system into host cells (see Section 1.5.1), have been reported in other obligate parasites. Hence, current *P. brassicae* populations might have evolved virulence mechanisms to overcome the *R*-gene mediated immunity (aka gene-for-gene resistance) of most *Brassica* vegetables and this may explain the lack of highly resistant lines from the survey described in Chapter 3. The identification of dominant resistance genes (encoding for *R* proteins or PRRs) that have not been overcome by the Victorian isolates would be an easy approach for breeding purposes.

Clubroot resistance involves a combination of both inducible and constitutive expression of defence genes that may be polygenically inherited. The defensive responses in Chinese cabbages ‘Tahono’ and ‘Leaguer’ were probably a form of partial-resistance, rather than “true resistance” from the work described in Section 3.3.2 and Chapter 4. This may complicate the breeding of resistant crops, since these partially-resistant traits are difficult to detect and manipulate. Despite difficulties in discovering minor genes for clubroot resistance, breeders however have methods for improving polygenic traits (such as yield) called recurrent selection. A hypothetical model involving the defence-related genes identified in both microarray studies is illustrated in Figure 6.1. It is postulated that the recognition of the *P. brassicae* chitin cell wall by a pathogen-associated molecular pattern (PAMP)-like receptor at 48 hai, was involved in triggering the production of reactive oxygen species (ROS). The latter initiated the hypersensitive response (HR) and the production of salicylic acid (SA) in the
roots. This elevated level of SA was possibly involved in the regulation of a PR protein (endochitinase) via the NPR1, WRKY and TGA transcription factors, enhanced lignification
Figure 6.1. Hypothetical model of *Brassica* transcriptional changes derived from resistant/partially-resistant *Brassica*-clubroot interaction from the current study (green), from other clubroot studies (pink) and from studies involving other patho-systems (black).

- **Cell wall degradation**
  - Pectinase
  - Cellulase

- **Cell wall / membrane**

- **PAMP Receptors**
  - *e.g.* CHRK?

- **PR Proteins**
  - *e.g.* Chitinase $c\uparrow$
  - *e.g.* Glucanase $c\uparrow$

- **NBS / LRR / LZP?**

- **Hormones**
  - Nitrilase
  - IPT
  - IAA

- **Other Proteins**
  - C-P450 $c\downarrow$

- **Glucosinolate** $c\downarrow$

- **Auxin / Cytokinin**

- **Protein Degradation**
  - E1, E2, E3 $\uparrow$
  - Myrosinase $c\uparrow$

- **Antimicrobial Compounds**
  - *e.g.* isothiocyanate

- **Hyperplasia and Hypertrophy**
  - XTH

- **Fortifications**
  - CMT $c\uparrow$
  - FSH $c\uparrow$

- **Glucosinolate $c\downarrow$**, **Auxin $c\uparrow$**, **NBS / LRR / LZP?**

- **Unknown transcripts**

- **Oxidative Burst** (Rapid)
  - **ROS**

- **Protein Kinase Activation**
  - Ca-binding protein

- **SAR**

- **Oxidative Burst** (Delayed)
  - Peroxidase $c\uparrow$
  - Catalase
  - SOD $c\uparrow$
  - GST

- **Hypersensitive Response**
  - Programmed Cell Death $c\uparrow$

- **Unknown factor causing high basal levels**

- **Antimicrobial Compounds**
  - *e.g.* isothiocyanate

- **Hyperplasia and Hypertrophy**
  - XTH

- **Lignin**

- **Cell wall / membrane**

- **Plasmotiphora brassicae**

- **Avr**

- **Elicitors e.g. chitin**

- **'Gene-for-gene' interaction**

- **Ca$^{2+}$**

- **K$^+$**

- **GTP**

- **H$_2$O$_2$**

- **POX**

- **Phytoalexins and Flavanoids**

- **Unknown transcripts**
c↑ and c↓ representing constitutive over- and under-expression while ↑ and ↓ representing up- and down-regulation of genes. CHRK, chitin receptor-like kinase; CMT, caffeoyl-CoA 3-O-methyltransferase; C-P450, cytochrome p450-type protein; E1/E2/E3, ubiquitin proteolytic complex; F5H, ferrulate-5-hydroxylase; GST, glutathione-S-transferase; GTP, guanosine triphosphate; IAA, indole acetic acid; IPT, isopentenyltransferase; LRR, leucine-rich repeats; LZP, leucine-zipper protein; NBS, nucleotide binding sites; NPR1, non-expressor of PR1; PAL, phenylalanine ammonia lyase; PAMP, pathogen-associated molecular pattern; POX, peroxidase; PR, pathogenesis-related; ROS, reactive oxygen species; SA, salicylic acid; SAR, systemic acquired resistance; SOD, superoxide dismutase and XTH, xyloglucan endotransglucosylate hydrolase. Modified from Coram et al. (2007) to fit a Brassica model.
via a peroxidase-dependent mechanisms or by caffeoyl-CoA 3-O-methyltransferase and may have resulted in high basal levels of myrosinase and low glucosinolate content (most likely indole glucosinolate) in the roots.

In conclusion, this study documented the first report of the large-scale profiling of the transcriptional changes to the very early stages of P. brassicae infection in Brassica vegetable crops using a microarray approach. These genes are potential candidates for breeding resistant/partially-resistant crops due to their effectiveness against a wide range of pathogens.

6.2.4 Breeding strategy

Breeding programs using either conventional or transgenic approaches have mostly used single dominant resistant genes (reviewed in Section 1.5.1) to provide resistance to pathogens in many crop species. Sources of differential clubroot partial-resistance/resistance have been identified in the Chinese cabbages and fodder turnip against clubroot and these should provide a satisfactory method of developing resistant varieties due to their already marketable agronomic traits. The constitutive expression of plant defences in the host may provide an unusual strategy for Brassica breeding (rather than the use of dominant inducible resistance genes). P. brassicae may be able to suppress the R-gene mediated activation pathway; however, the transcriptional control mechanisms of constitutively expressed genes remain unaffected. Therefore in the absence of new dominant resistance genes (R or PRR genes), the selection of constitutively expressed defence genes and their promoter regions may convey resistance against clubroot. Since SA played a central key component in the regulation of many SA-defence responses, the breeding of crops with elevated levels of SA may provide effective protection against a wide range of pathogens, though, the energy cost associated with the constitutive expression of SA and downstream defences and the profitability (if any) of these crops have to be further investigated. Finally, most of the genes reported in this study
were identified from the resistant fodder turnip ‘ECD04’, suggesting that the differences in gene expression for defence may be associated with its characteristic turnip root system (as in lignified and high level of glucosinolates). Therefore, breeding for both constitutively expressed defence genes and structurally ‘tough’ roots may be another viable strategy against clubroot.

In conclusion, it is important that the development and continuous improvement of the current ‘adapted’ Brassica cultivars as well as the genetic variability available in Brassica landraces be considered for the intermediate- and long-term breeding objectives for durable resistance. This study has indicated the lack of clubroot resistance in cultivated Chinese cabbages because *P. brassica* may have overcome all of their dominant resistance genes. Therefore, the identification and selection for new dominant resistance genes (from landraces) may solve their susceptibility but subsequently, may give rise to the boom and bust cycle. This may however be avoided by the marker-assisted selection of multiple defence-related genes, *e.g.* by pyramiding the inducible and constitutively expressed candidate genes discovered in ‘Tahono’ or ‘ECD04’ into an elite cultivar or the breeding of complex polygenic clubroot resistance by recurrent selection.

### 6.3 Future directions

#### 6.3.1 Direct application of the results from this study

To extend from the results of this study, it is recommended that additional extensive surveys to be conducted, involving a wider range of isolates from different Brassica farms throughout Australia, as well as a more diverse collection of Brassica crops and landraces from around the world. In the current project, the bulking of selfed seeds from *F₁* hybrids was not sensible since they are probably segregating for resistance in *F₂*. A more reliable and abundant source of Brassica seeds along with comprehensive information on their origin may facilitate the
design and interpretation of future projects. The resulting database for sources of virulence and resistance will help in the planning of effective breeding strategies and the deployment of durable resistance.

Secondly, more in-depth expression studies involving the use of additional genotypes (preferably with true resistance) and more time-points, supplemented with simultaneous microscopic observations on the primary zoospores infecting root hair cells, may provide a better understanding of the roles of the proposed molecular cascades in conferring partial-resistance/resistance in *Brassica* vegetables. A limited number of inducible genes in hosts’ root hair were reported in both microarray platforms in this study and was possibly caused by a dilution effect using total root mass. This may be overcome by using pools of root hair microdissections prior to RNA extraction to enrich for root hair-specific gene expressions. The ‘boutique’ oligoarray could be optimised by including the reported candidate genes as well as other forms of constitutive plant defences, while the construction of a *B. rapa*-specific ‘masking’ file may provide new analytical possibilities using the Affymetrix *Arabidopsis* genechip. Alternatively, another transcriptional profiling technique known as SuperSAGE (most recent adaptation of the serial analysis of gene expression technique), may be used to investigate these defence responses.

Thirdly, it may be useful to identify the copy number and allelic forms of important candidate genes. The presence of greater copy number, identified by Southern blots or SuperSAGE technique, in either susceptible or resistant genotypes may possibly explain the lack of apparent differences in expression between partially-resistant and susceptible plants. Allelic differences, identified by sequencing and aligning the candidate genes from the susceptible and resistant genotypes, may provide an alternative explanation for the variation in clubroot resistance.
Finally, since changes in mRNA levels may not necessarily correlate with protein/enzyme activity levels, the functions of the candidate partially-resistant/resistant genes identified in the current and future functional genomics clubroot studies need to be further validated using proteomic or transgenic approaches. These include enzyme assays, gene silencing by knockout-mutants / antisense / RNAi experiments or gene over-expression using CaMV 35S promoter via Agrobacterium-mediated genetic transformation, followed by tests to determine their resulting levels of resistance.

### 6.3.2 Development of markers

The ultimate aim of this project was the development of molecular markers to assist in the selection of clubroot-resistant genotypes. The next logical step would be the discovery of polymorphisms in the candidate gene sequences, from which molecular markers such as SNPs may be developed. By sequencing the candidate genes using PCR primers and by aligning their sequences, point mutations may be discovered in the susceptible/partially-resistant/resistant genotypes. Molecular markers developed from the coding regions of candidate genes may ultimately be used as ‘perfect markers’. Markers identified in preliminary genetic mapping studies are seldom suitable for marker-assisted selection (MAS) without further testing and development. Generally, the steps necessary for the development of SNPs for use in MAS include: the verification of the co-segregation of the markers with resistance (e.g. in F$_2$ populations), field validation of markers (by testing their effectiveness in determining target phenotype in different varieties or species) and possibly marker conversion (into PCR-based markers for high-throughput testing). Once molecular markers from a range of candidate genes have been developed and adequately validated, their implementation may aid the introgression of dominant genes or quantitative trait loci (QTL) associated with clubroot resistance into an elite cultivar, i.e. pyramiding the clubroot resistance genes into an elite cultivar. It will also allow the accelerated recovery of the recurrent parent with the
desired agronomic traits, a process also known as marker-assisted backcrossing. These markers may also be used to characterise the ECD genotypes accurately and possibly to breed near isogenic lines to improve their reliability, accuracy and differential abilities.

**Concluding remarks**

The discovery of novel genes, determination of their expression profiles in response to clubroot infection and an understanding of their roles in clubroot resistance, will provide basic knowledge for establishing effective breeding strategies. Using microarray technology, the current study documented the first report of the transcriptional changes in hydroponically-grown susceptible and partially-resistant Chinese cabbages at a very early stage of clubroot invasion. The results of this project will help in the planning and design of future clubroot studies of clubroot in *Brassica* with the ultimate aim of developing durable resistance against a wide range of clubroot pathotypes.
Bibliography

Affymetrix. (2001). Affymetrix Jaguar 2.0 user’s guide. (California: Affymetrix Inc.).


BioDiscovery. (2002). Imagen 5.5 user manual. (California: BioDiscovery Inc.).


Proposals for attempted rationalisation through an international approach.
Transactions of the British Mycological Society 65, 295-303.


in the ABI-Rps2-Ck1 chromosomal segment and related regions. Genetics 157, 1321-1330.

Raa, J. (1971). Indole-3-acetic acid levels and the role of indole-3-acetic acid oxidase in the normal root and clubroot of cabbage. Physiologia Plantarum 25, 130-134.

Raghavan, C. (2004). Examination of whole genome response in Arabisopsis to auxinic herbicide 2, 4-dichlorophenoxyacetic acid. In Department of Biology/Biotechnology. (Bundoora: RMIT).


to *Phytophthora infestans* after leaf penetration in susceptible and resistant potato cultivars. American Journal of Potato Research 82, 139-146.


Siemens, J., Keller, I., Sarx, J., Kunz, S., Schüller, A., Nagel, W., Schmulling, T., 
clubroots indicate a key role for cytokinins in disease development. Molecular Plant-
Microbe Interactions 19, 480-494.

Siemens, J., Nagel, M., Ludwig-Müller, J., and Sacristan, M.D. (2002). The interaction of 
*Plasmodiophora brassicae and Arabisopsis thaliana*: Parameters for disease 
quantification and screening of mutant lines. Journal of Phytopathology-
Phytopathologische Zeitschrift 150, 592-605.

Simons, M.D. (1972). Polygenic resistance to plant disease and its use in breeding resistant 

Singh, K.B. (1993). Experiences, difficulties and prospects of disease resistance breeding in 

to *Ascochyta rabiei*. Plant Disease 65, 586-587.

Snowdon, R.J., and Friedt, W. (2004). Molecular markers in *Brassica* oilseed breeding: 

*N*-homologue LRR domain adopts a folding which explains the TMV-Cg-induced 
HR-like response in sensitive tobacco plants. Journal of Molecular Graphics and 
Modelling 26, 850-860.

Protection 19, 837-841.

Suwabe, K., Tsukazaki, H., Iketani, H., Hatakeyama, K., Fujimura, M., Nunome, T., 
resistance to clubroot (*Plasmodiophora brassicae* Woronin) in *Brassica rapa* L. 
Theoretical and Applied Genetics 107, 997-1002.

Suwabe, K., Tsukazaki, H., Iketani, H., Hatakeyama, K., Kondo, M., Fujimura, M., 
repeat-based comparative genomics between *Brassica rapa* and *Arabisopsis thaliana*: 

of fluazinam, a new pesticide, for Chinese cabbage clubroot. Annals of the 
Phytopathological Society of Japan 61, 395-398.
Taipalensuu, J., Andreasson, E., Eriksson, S., and Rask, L. (1997). Regulation of the
wound-induced myrosinase-associated protein transcript in *Brassica napus* Plants.
European Journal of Biochemistry 247, 963-971.

Associates Inc.).

parasitica*, a biotrophic fungal pathogen of crucifers. Theoretical and Applied
Genetics 88, 490-496.

Thomassen, M., Skov, V., Eiriksdottir, F., Tan, Q., Jochumsen, K., Fritzner, N.,
geno me wide oligonucleotide chip with duplicate measurement of each gene.
Biochemical and Biophysical Research Communications 344, 1111-1120.

Tinggal, S.H., and Webster, J. (1981). Technique for single spore infection by
*Plasmodiophora brassicae*. Transactions of the British Mycological Society 76, 187-
190.

in *Brassica* tissue cultures and in intact roots. New Phytologist 70, 327-&.

Torsvik, V., and Ovreas, L. (2002). Microbial diversity and function in soil: from genes to
ecosystems. Current Opinion in Microbiology 5, 240-245.

*Plasmodiophora brassicae* - an analysis by international experimentation.
Transactions of the British Mycological Society 87, 279-287.

cyanamide and liming for control of clubroot disease in cauliflower. Crop Protection
24, 798-803.

crop improvement. Trends in Plant Science 10, 621-630.

sequencing technologies and their implications for crop genetics and breeding. Trends
in Biotechnology 27, 522-530.


Verbelen, J.-P., Vissenberg, K., Kerstens, S., and Le, J.i.e. (2001). Cell expansion in the
epidermis: microtubules, cellulose orientation and wall loosening enzymes. Journal of
Plant Physiology 158, 537-543.


## Appendix 1

Table A1.1. Detailed assessment of clubroot symptoms formation using the ECD series

| Plasmoplasma brassicae isolate ID | A | B | C | D | E | F | G | H | I | J | K | L | M | N | O | P | Q | R | S | T |
| ECD 12.0.0.0 | 11.0.0 | 12.0.0 | 11.0.0 | 0.0.0 | 0.0.0 | 0.0.0 | 0.0.0 | 12.0.0 | 12.0.0 | 11.0.0 | 12.0.0 | 0.0.0 | 0.0.0 | 0.0.0 | 12.0.0 | 11.0.0 | 0.0.0 | 0.0.0 | 12.0.0 | 0.0.0 | 10.0.0 | 10.0.0 |
| 01 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ECD 12.0.0 | 12.0.0 | 12.0.0 | 12.0.0 | 11.0.0 | 0.0.0 | 0.0.0 | 0.0.0 | 12.0.0 | 12.0.0 | 11.0.0 | 12.0.0 | 0.0.0 | 0.0.0 | 0.0.0 | 12.0.0 | 11.0.0 | 0.0.0 | 0.0.0 | 12.0.0 | 0.0.0 | 12.0.0 | 12.0.0 |
| 02 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ECD 13.0.0 | 13.0.0 | 13.0.0 | 12.0.0 | 0.0.0 | 0.0.0 | 0.0.0 | 0.0.0 | 12.0.0 | 12.0.0 | 12.0.0 | 12.0.0 | 0.0.0 | 0.0.0 | 0.0.0 | 12.0.0 | 11.0.0 | 0.0.0 | 0.0.0 | 12.0.0 | 0.0.0 | 10.0.0 | 10.0.0 |
| 03 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| ECD 11.0.0 | 12.0.0 | 10.0.0 | 12.0.0 | 0.0.0 | 0.0.0 | 0.0.0 | 0.0.0 | 12.0.0 | 12.0.0 | 11.0.0 | 12.0.0 | 0.0.0 | 0.0.0 | 0.0.0 | 12.0.0 | 11.0.0 | 0.0.0 | 0.0.0 | 12.0.0 | 0.0.0 | 11.0.0 | 10.0.0 |
| 04 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Table A1.1. Detailed assessment of clubroot symptoms formation using the ECD series |

a Distribution of plants using the 4-grade scale for clubroot symptom severity (Refer to Figure 2.3), for e.g. 2.1.3.6 means there were 2, 1, 3 and 6 plants with symptom grade 0, 1, 2 and 3 respectively.

b Disease index (DI) calculated by $DI = (1n_1 + 2n_2 + 3n_3)100/3N$, where $n_1$ to $n_3$ is the number of plants in the indicated class and $N_1$ is the total number of plants tested.
Table A1.2. Detailed assessment of clubroot symptoms formation using the Henderson Seed Pty Ltd’s self-pollinated *Brassica campestris* genotypes.

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Table A1.3. Detailed assessment of clubroot symptoms formation using some commercially-available *Brassica oleracea* genotypes.

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NA, data not available due to seed unavailability or lack of germination.
Table A1.4. Detailed assessment of clubroot symptoms formation using selected worldwide *Brassica* landraces.

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NA, data not available due to seed unavailability or lack of germination.
Preparation of reagents, solutions and media for microarray:

6 × Print buffer: 300 mM sodium phosphate (pH 8.5)

The following were dissolved in 90 mL of autoclaved DEPC-treated water:

0.41 g sodium phosphate monobasic (Sigma S0751)
3.79 g sodium phosphate dibasic (Sigma S0876)

The pH was adjusted to 8.5 and solution made up to 100 mL with autoclaved DEPC-treated water. Medium was not autoclaved and stored at 4°C.

Blocking Solution: 0.1 M Tris, 50 mM ethanolamine (pH 9.0)

The following were dissolved in 900 mL of sterile MilliQ water:

6.06 g Trizma Base (Sigma T6791)
7.88 g Trizma HCl (Sigma T6666)
3 mL ethanolamine (Sigma E9508)

The pH was adjusted to 9.0 and solution made up to 1000 mL with sterile MilliQ water. Medium was not autoclaved and stored at 4°C.

20 × SSC (pH 7.0)

The following was dissolved in 800 mL of sterile MilliQ water:

175.3 g NaCl
88.2 g sodium citrate

The pH was adjusted to 7.0 and solution made up to 1000 mL with sterile MilliQ water. Medium was autoclaved and kept on the bench.
**10 % SDS (pH 7.2)**

The following was dissolved in 900 mL of sterile MilliQ water:

100 g sodium dodecyl sulphate (Sigma L4522)

The solution was heated slightly to dissolve the salt. The pH was then adjusted to 7.2 and solution made up to 1000 mL with sterile MilliQ water. Medium was not autoclaved and kept on the bench.

**50 x aa-dUTP/dNTPs**

10 µL of each 100 mM dATP, dCTP and dGTP (Invitrogen™)

4 µL of 100 mM dTTP (Invitrogen™)

6 µL of 100 mM aa-dUTP (Sigma A0410)

Solution was stored at -20°C.

**12 x MES buffer (pH 6.6)**

The following were dissolved in 800 mL of sterile MilliQ water:

64.61g MES hydrate

193.3g MES sodium salt

The pH was adjusted to 6.6 and solution made up to 1000 mL with sterile MilliQ water.

Medium was filter-sterilised using a 0.2 µm filter and stored at 4°C in the dark.
Appendix 3

Settings for BioRobotics® TAS Application Suite:

Before printing of the array, the BioRobotics® Total Array System (TAS) Application Suite software v2.6.0.1 had to be configured. The parameters to print 5 oligoarrays were set up as follows:

**Options Tab:**

- **Tool type:** Tool: 2x1 configuration, *i.e.* 2 pins were used
- **Pin refill frequency:** Spots per source visit: [(12 spots/slide × No. of slides) + 20 blotting spots] = 80 for 5 slides
  - ‘Source’ means position of wells on the 384-wells plate
  - ‘12 spots/slide’ means 1 source printed once in 12 sub-arrays per slide
  - ‘Blotting spots’ means number of spots printed on the pre-spot slides until they have a consistent size, volume and shape
- **Wash frequency:** Wash before pin refills to prevent carry-over of samples

**Source Tab:**

- **Microplate options:** Microplate type: 384-well (low profile)
  - No. of plates: 1
  - No. of samples: 180
- **Last plate:** 90/384, meaning 90 sources on a 384-well plate per pin
- **Source loading:** Hold 1 plate at a time and plates have lids
- **Source action:** Dwell

**Target Tab:**

- **Size:** 15 × 6 per pin, resulting in sub-array having a 30 × 6 format
- **Pitch:** 0.295 mm, meaning distance between centers of spots
within each sub-array is 0.295 mm

Format: standard

Adapter plate & slide layout - 7 targets, meaning 2 pre-spot slides followed by 5 real slides

Edit layout: Adaptor layout 30 vertical slides

No. of copies: fill 7 slides

Slide layout: Mirror horizontal margins

X- and Y- spacing adjusted to fit 12 grids/slide with 6 slides/hybridisation

Layout sample set #: 1

Target action -

Delay before spotting: 0.000 s

Target height: 0.248 mm

Dwell time: 0.000 s

Multiple strikes: 1

Pre-spotting: 20 spots

Pre-spot pitch: 0.700 mm

Edit soft touch -

Soft touch: Target height 0.248 mm

Soft touch distance: 1.000 mm

Speed: 4.000 mm/s

Climate: All set at 50 % for Na₃PO₄ buffer

Bath 1 and 2: Used both baths for 3 s

Action: wiggle 0.3 mm

Behavior: 0.0 mm

MWS: Used main wash station for 1 cycle

Entire wash cycle 2 times

Any other parameters that have not been mentioned were kept at their default settings.
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<th>Database*</th>
<th>BLASTn Results</th>
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Table A4.2. The BLASTn results for the oligonucleotide probe B sequences (sorted by ID).

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<th>Score E-value</th>
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<td>gil26453025</td>
<td>52.0, 3e-05</td>
</tr>
<tr>
<td>BA020B</td>
<td>TgATAgTAACCAACgATCCgTgCTgTTTCTTCC</td>
<td>EST</td>
<td>gil13182383</td>
<td>52.0, 3e-05</td>
</tr>
<tr>
<td>BA021B</td>
<td>gTggCATgTgTgTgTgCTgTTCTTTTC</td>
<td>EST</td>
<td>gil177981</td>
<td>52.0, 3e-05</td>
</tr>
<tr>
<td>BA024B</td>
<td>gTgCTCCCGTTACTCCggCTCTgACAT</td>
<td>EST</td>
<td>gil11083662</td>
<td>36.2, 3.6</td>
</tr>
<tr>
<td>BA025B</td>
<td>AgACCCgACTCTACCTCACCAgTTgAAC</td>
<td>nr</td>
<td>gil11319454</td>
<td>34.2, 6.5</td>
</tr>
<tr>
<td>BA026B</td>
<td>TACgAAgTgTgCTgTACgACCTgTgg</td>
<td>EST</td>
<td>gil54089774</td>
<td>52.0, 3e-05</td>
</tr>
<tr>
<td>BA027B</td>
<td>gTATgATgATCCgAGgTTgTgCTCCg</td>
<td>nr</td>
<td>gil89357184</td>
<td>52.0, 3e-05</td>
</tr>
</tbody>
</table>
BA028B  AgAgCgTTCTCCAATTCCggCgAA  EST  gi38490683|gb|AY460110.1|  AY460110 Brassica rapa root 5 days... 52.0 3e-05
BA029B  TgTgggTTTCCggTAATTTCAAgAgC  EST  gi409011|gb|AI352905.1|  MB73-12 PGZ204.BNlib Brassica napus... 52.0 3e-05
BA030B  CgTAaACCggTgCTgCCTTACCTCA  nr  gi757739|emb|X82577.1|BNBGL|  B. napus mRNA for beta-glucosidase 52.0 3e-05
BA031B  gTTCgATgAgACggAgAgCgACgTg  nr  gi37654854|gb|AY395720.1|  Brassica rapa subsp. chinesis beta-glucosidase 52.0 3e-05
BA032B  CAgCTTCCAggATgCTTCTAggTA  nr  gi31790104|gb|AY299481.1|  Brassica juncea glutathione S-transferase 52.0 3e-05
BA033B  gAAggTTATgCTTgTgCCTgTg  nr  gi9169891|gb|AY081302.1|  Arabidopsis thaliana heat shock protein 52.0 3e-05
BA034B  gTTTCgATTTCCggCgAACAgAgTg  EST  gi4089941|gb|AI352735.1|  MB56-1G PGZ204.BNlib Brassica napus... 52.0 3e-05
BA035B  TgTgggCTATgAgACCCAAgTCTgTTC  nr  gi81239130|gb|DQ233253.1|  Brassica rapa IAA-amino acid hydrolases 52.0 3e-05
BA036B  ggACCggAgTAAGAgACACCCTTCA  nr  gi23308284|gb|BT000543.1|  Arabidopsis thaliana putative auxins... 52.0 3e-05
BA037B  AACTcAgACCTTgAgATTTCTAggC  nr  gi42572104|ref|NM_202414.1|  Arabidopsis thaliana ICS1 (ISOCHOR... 52.0 3e-05
BA038B  AgTggAgACAAAgACTATgACTgTg  nr  gi42572104|ref|NM_202414.1|  Arabidopsis thaliana ICS1 (ISOCHOR... 52.0 3e-05
BA039B  gTggACAgACAAAgACTATgACTgTg  nr  gi74038592|dbj|AB186135.1|  Brassica rapa subsp. pekinensis B... 52.0 3e-05
BA040B  gAAgATCCTAggATTgAgAgTgg  nr  gi74038588|dbj|AB186135.1|  Brassica rapa subsp. pekinensis B... 52.0 3e-05
BA041B  CgATTAgCAAggTggACAAAgACTAggC  nr  gi42569529|ref|NM_128733.1|  Arabidopsis thaliana UDP-glycosyltrans... 52.0 3e-05
BA042B  AgAAGaAAgAGAgACTACgATCgTg  nr  gi110744033|gb|ALC189248.1|  Arabidopsis thaliana pekinensis c... 52.0 3e-05
BA043B  CgAAgATgATggTgTgTgTgTgTgC  EST  gi6694947|gb|AW288083.1|  12.1T7 Mannitol Stress inducible cD... 52.0 3e-05
BA044B  CgTggAgACCAgAgTCATgAgCTgTT  nr  gi2243129|emb|Y10850.1|BJY10850|  Brassica juncea mRNA for metal... 52.0 3e-05
BA045B  gCgATgAgACCAAggTACgAgCTT  nr  gi79317508|ref|NM_001035940.1|  Arabidopsis thaliana ATMPK1 (... 52.0 3e-05
BA046B  CTgCAaACgCTTCgCgATCCTACACA  nr  gi38153672|emb|AJ605556.1|  Plasmodiophora brassicae mRNA expr... 52.0 3e-05
BA047B  TgTggCCAgAgACCAgACgTgCCTgC  nr  gi5706361|dbj|AB000988.1|  Plasmodiophora brassicae mRNA expr... 52.0 3e-05
BA048B  CgAgAgAgATgATgATgCgGAggATg  nr  gi90656056|gb|DQ456999.1|  Brassica oleracea var. gemmifera myro... 52.0 3e-05
BA049B  CCAgATgAAgAgAggTACgAAgAgCT  nr  gi840724|emb|X79080.1|BNMYRMC|  B. napus mRNA for myrosinase MC... 52.0 3e-05
BA050B  CACTgAAgCAAgCAgAgACCTgTTg  nr  gi1656558|gb|U59443.1|BNU59443|  Brassica napus myrosinase-bindin... 52.0 3e-05
BA051B  CTgTgCTACTACCgACgACgCTCTCCTCC  nr  gi607042|emb|X78285.1|BNMYR1|  B. napus (Svalfs Karat 20516-K) mRN... 52.0 3e-05
BA052B  gTTCgAggCAACAggTgACgAAACAg  nr  gi42113953|gb|AF380304.1|AF380304|  Brassica napus nitrate-aspartate-like... 44.1 0.007
BA053B  AggCgTATgAggTgATgCTATgCTAgg  nr  gi42113953|gb|AF380304.1|AF380304|  Brassica napus nitrate-aspartate-like... 44.1 0.007
BA054B  gATCAACCACCTCgCTgATgACAC  nr  gi4089974|gb|AI352768.1|  MB58-1G PGZ204.BNlib Brassica napus... 52.0 3e-05
BA055B  gAAATgTTCTCAAggCCTTCgCTCgCCg  EST  gi48527853|gb|AY623008.1|  Brassica rapa pathogenesis-related... 52.0 3e-05
BA056B  AgCTTgAgTgTgTTATgACgAgCTC  nr  gi4089918|gb|AI352712.1|  MB43-10 PGZ204.BNlib Brassica napus... 52.0 3e-05
BA057B  CgCTTCgAggAggATgTTgATgCTg  EST  gi71034460|gb|DQ116449.1|  Brassica oleracea var. botrytis pe... 52.0 3e-05
| BA059B | ACCAACATCACTCCTCCCTCCCTCT | nr | gi|16209614|gb|AY055752.1 | Brassica rapa subsp. pekinensis ph... | 52.0 | 3e-05 |
| BA060B | gAAAgtTTCCTACCACTgAgTCAACg | nr | gi|74048978|gb|DO167187.1 | Brassica rapa phenylalanine ammonia... | 52.0 | 3e-05 |
| BA061B | CgAgAAACAAAgCCATTTCAgCCCTgTggT | nr | gi|60499706|gb|AY795080.1 | Brassica napus phenylalanine ammonia... | 40.1 | 0.11 |
| BA063B | gAATgggAAggTgTTCAATgCAACCC EST | EST | gi|3126615|gb|AA960751.1 | DH26-2-T3 PZ204.BNlib Brassica napu... | 52.0 | 3e-05 |
| BA066B | CTCAgTgTgCTCAACgATgCTgTgAag | nr | gi|2130984|emb|X59984.1 | B. napus mRNA for ribosomal pro... | 52.0 | 3e-05 |
| BA069B | gAAAgTTCTCACCACTggAgTCAACg nr | nr | gi|74048978|gb|DQ167187.1 | Brassica rapa phenylalanine ammoni... | 52.0 | 3e-05 |
| BA070B | gCTACCgACATTCTTCTACCAACCCgT nr | nr | gi|79527507|ref|NM_123599.2 | Arabidopsis thaliana ubiquitin-p... | 52.0 | 3e-05 |
| BA071B | ATgACCTggCTACTTTgTggTTggTg | nr | gi|3288849|gb|AF540558.1 | Brassica juncea clone SOD 8A super... | 52.0 | 3e-05 |
| BA072B | AAggAcgcgaggTgTgACCACTgTgACT | nr | gi|24412234|gb|AF540558.1 | Brassica juncea clone SOD 8A super... | 52.0 | 3e-05 |
| BA073B | TCAACTgCACggAgCTggACTgCAGAA EST | EST | gi|38097351|gb|CB331875.1 | 15D25C Seed cDNA library Brassica ... | 52.0 | 3e-05 |
| BA074B | gCTACCgACATTCTTCTACCAACCCgT nr | nr | gi|79527507|ref|NM_123599.2 | Arabidopsis thaliana ubiquitin-p... | 52.0 | 3e-05 |
| BA075B | TgTgTcCTTTgATCCCTCACAsgACgACTC nr | nr | gi|7932671|ref|NM_001036151.1 | Arabidopsis thaliana ubiquiti... | 52.0 | 3e-05 |
| BA076B | ATgCggCgAggTTTCCTgTTACTCCT nr | nr | gi|56480905|gb|AY384281.1 | Brassica rapa xyloglucan endotrans... | 52.0 | 3e-05 |
| BA077B | TATCgTcgcCCTTCAACATgppgCTCCgCAT | nr | gi|33753937|gb|AY156708.1 | Brassica oleracea var. botrytis yx... | 52.0 | 3e-05 |
| BA078B | gTAACTCAgCAggAACCgTCAACAACCT EST | EST | gi|872621|gb|H07799.1 | khsh166 BNL1 Brassica napus cDNA 3’si... | 52.0 | 3e-05 |
| BA079B | ggTgAAATgAgAAACCAgAtgCTTACAtACAC nr | nr | gi|4090172|gb|AI352966.1 | MB75-5H PZ204.BNlib Brassica napu... | 52.0 | 3e-05 |
| BA080B | gCTgTCCCTCCgTgAAACCCgATTTCTT EST | EST | gi|11074233|gb|BT026383.1 | Arabidopsis thaliana At2g36750 mRNA, ... | 52.0 | 3e-05 |
| BA081B | CTgTCCCTCCgTgAAACCCgATTCTT EST | EST | gi|6318044|gb|AY573822.1 | Arabidopsis thaliana zeanit O-gluc... | 52.0 | 3e-05 |

a The NCBI BLASTN program using the nr (‘non-redundant’) or EST (Expressed Sequence Tag) nucleotide databases in sequence analysis.

Note:
Shaded rows indicate oligonucleotide probes whose results were not similar to their expected putative function in both the ‘nucleotide’ and ‘EST’ databases of GenBank.
Appendix 5

**Ranking method for identification of differentially expressed cDNAs:**

The Microsoft Excel software was used for the following:

1. The GeneSight™ dataset was imported into Microsoft Excel and the fold change cut-off of 1.8 ($\log_2$ of $>0.848$ or $<-0.848$) was applied.

2. The equality of variance was determined for each array feature by comparing the sample variances (control, $s_1^2$ and treatment, $s_2^2$) using the F distribution. The F statistic ($F = s_1^2 / s_2^2$) was calculated using:

$$F = \frac{(CV_{control} \times \text{sample mean}_{control})^2}{(CV_{test} \times \text{sample mean}_{test})^2},$$

where CV was the coefficient of variation obtained from the GeneSight™ dataset.

The degrees of freedom for each variable ($n_1 - 1$, $n_2 - 1$) were determined. For example whenever each array feature had 6 technical replicates and 3 biological replicates, $n = 18$ for both control and treatment and hence, $df_{control} = 18 - 1 = 17$ and $df_{test} = 18 - 1 = 17$.

The F statistic probability was calculated using the F distribution tables ([http://downloads.hawkeslearning.com/Downloads/Docs/StatisticsTables/](http://downloads.hawkeslearning.com/Downloads/Docs/StatisticsTables/)). This was a two-tailed test and F at $P = 0.025$ for each tail was calculated to give a total $P = 0.05$. Using these parameters, the F statistic must be between 0.37 and 2.67 to assume equal variances between control and treatment means at $P = 0.05$.

Hence, for the analysis using all three biological replicates:

$$F_{0.025} (17, 17) = 2.67$$

$$F_{0.975} (17, 17) = 1 / F_{0.975} (17, 17) = 1 / 2.67 = 0.37$$
Note 1: for the analysis using only 1 biological replicate:

\[ F_{0.025} (5, 5) = 7.15 \]
\[ F_{0.975} (5, 5) = 0.14 \]

Note 2: for the analysis using only 2 biological replicates:

\[ F_{0.025} (11, 11) = 3.47 \]
\[ F_{0.975} (11, 11) = 0.29 \]

The F statistic for each array feature was calculated using the ‘FDIST’ function. The ‘IF’ function was used to determine if the F statistic probabilities are within the 0.37 and 2.67 interval. If the result was ‘TRUE’, then the variances are equal.

3. Assuming equal sample variances, the sample variances were pooled according to:

\[ s^2_p = \frac{(n_1 - 1)s_1^2 + (n_2 - 1)s_2^2}{n_1 + n_2 - 2} \]

Since both control \((n_1)\) and treatment \((n_2)\) were 18, the ‘AVERAGE’ function was used to pool the variances.

\[ \text{AVERAGE} (((\text{CV}_{\text{control}} \times \text{sample mean}_{\text{control}})^2, (\text{CV}_{\text{test}} \times \text{sample mean}_{\text{test}})^2)) \]

4. The \( t \) statistic for each sample was calculated using a two-sample \( t \) test assuming equal variances:

\[ t = \frac{\text{(sample mean}_{\text{control}} - \text{sample mean}_{\text{test}})}{\sqrt{(s^2_p * (1/n_1 + 1/n_2))}} \]

, where \( s^2_p \) was the pooled sample variances and \( n_1 \) and \( n_2 \) were the total number of array features in the control and test respectively.

Each \( t \) statistic value was converted into a positive number by squaring and then taking the square root. The \( P \) value for each \( t \) statistic was calculated using the ‘TDIST’ function where \( x = \text{sample } t \text{ statistic value}, \text{df} = 18 + 18 - 2 = 34 \) and tails = 2.
Selection method for identification of DE cDNAs:

1. For each dataset, the cDNAs were sorted in ascending order according to their $P$ value.

2. The False Detection Rate (FDR) multiple testing correction was applied as described:
   - The ranked cDNAs were numbered from 1 to $R$.
   - The arbitrary $P$ value cut-off for DE of $P<0.1$ was used.
   - The $P$ value of each cDNA was compared to a threshold that depends on the position of the gene in the list. These thresholds are $((1/R) \times \alpha)$ for the first gene, then $((2/R) \times \alpha)$ for the second gene and so on, where $R$ is the number of genes in the list and $\alpha$ is the desired significance level (0.1).
   - The observed $P$ value has to be less than the individual threshold for each cDNA to pass the threshold and be accepted as DE for e.g. $P_1 < (1/R) \times \alpha$ and $P_2 < (2/R) \times \alpha$. 
Appendix 6

Settings for Affymetrix GeneChip® Operating Software:

This software (v1.4.0.036) as well as the *Arabidopsis* ATH1 library may be downloaded from www.affymetrix.com and was setup as follows:

Expression settings:

Scaling: All probe sets with target signal of 50

Normalisation: User defined Normalisation value of 1

Baseline: None in an absolute analysis or added accordingly in a comparative analysis

<table>
<thead>
<tr>
<th>Default parameter</th>
<th>11 probe pairs/probe set, 18 µm feature size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha1</td>
<td>0.05</td>
</tr>
<tr>
<td>Alpha2</td>
<td>0.065</td>
</tr>
<tr>
<td>Tau</td>
<td>0.015</td>
</tr>
<tr>
<td>Gamma1L</td>
<td>0.0045</td>
</tr>
<tr>
<td>Gamma1H</td>
<td>0.0045</td>
</tr>
<tr>
<td>Gamma2L</td>
<td>0.006</td>
</tr>
<tr>
<td>Gamma2H</td>
<td>0.006</td>
</tr>
<tr>
<td>Perturbation</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Cell summary report:

Default

Expression report:

Probe pairs threshold: 8

Antisense probe sets

Default housekeeping controls and spike controls
Appendix 7

Reproducibility of Affymetrix arrays using the variation coefficients (VC):

- All three CHP files for the ‘treatment’ arrays for each genotype were simultaneously loaded onto the Affymetrix GeneChip® Operating Software and only the genes that were either called ‘Present’ or ‘Marginally Present’ in all three replicates were selected. The signal values of those genes were then pasted onto a Microsoft Excel spreadsheet for analysis. The mean and standard deviation (SD) of the signal values for each gene were calculated and used to generate the variation coefficient (VC) as follows:

\[
\text{VC (in \%)} = \left( \frac{\text{standard deviation}}{\text{mean signal}} \right) \times 100
\]

- The VC values for each gene were then categorised into five classes:
  1. \( \text{VC} \leq 10 \)
  2. \( 10 < \text{VC} \leq 20 \)
  3. \( 20 < \text{VC} \leq 30 \)
  4. \( 30 < \text{VC} \leq 50 \)
  5. \( \text{VC} > 50 \)

- The frequency of genes in each VC class was determined and the % of genes in a particular VC class for each genotype were calculated as follows:

\[
\% \text{ of genes in a VC class} = \left( \frac{\text{No. of genes in the VC class}}{\text{Total No. of genes called either ‘Present’ or ‘Marginally Present’ in all three ‘treatment’ arrays}} \right)
\]

- The % of genes for each VC class was used to construct the histogram.
Appendix 8

Selection of significantly differentiated genes:

Table A8.1. Selection of differentially regulated genes with reliable expressions in all three replicated arrays of ‘Granaat’ 48 hai to clubroot isolate S.

<table>
<thead>
<tr>
<th>Selection of UP-regulated genes</th>
<th>Increase</th>
<th>Selection of DOWN-regulated genes</th>
<th>Decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of genes on the array</td>
<td>22,810</td>
<td>Total number of genes on the array</td>
<td>22,810</td>
</tr>
<tr>
<td>No. of genes called ‘present’ in all replicates of Treatment</td>
<td>3,255</td>
<td>No. of genes called ‘present’ in Control</td>
<td>4,981</td>
</tr>
<tr>
<td>No. of genes called ‘increase’ in at least 2 replicates</td>
<td>69</td>
<td>No. of genes called ‘decrease’ in at least 2 replicates</td>
<td>98</td>
</tr>
<tr>
<td>No. of genes with SLR* ≥ 0.8 in all biological replicates</td>
<td>4</td>
<td>No. of genes with SLR* ≤ -0.8 in all biological replicates</td>
<td>24</td>
</tr>
<tr>
<td>No. of genes with a mean signal intensity ≥ 100 in Treatment</td>
<td>2</td>
<td>No. of genes with a mean signal intensity ≥ 100 in Control</td>
<td>15</td>
</tr>
<tr>
<td>% Significant Increase</td>
<td>0.06</td>
<td>% Significant Decrease</td>
<td>0.30</td>
</tr>
</tbody>
</table>

*SLR means Signal Log Ratio, whereby a value of 0.8 indicate a 1.75-fold change

Table A8.2. List of differentially regulated genes in ‘Granaat’ (sorted by mean SLR).

<table>
<thead>
<tr>
<th>Probe Set ID</th>
<th>Locus Identifier</th>
<th>Mean SLR*</th>
<th>Putative function (from the Affymetrix Arabidopsis library downloaded on August 2007)</th>
</tr>
</thead>
<tbody>
<tr>
<td>261892_at</td>
<td>At1g80840</td>
<td>-2.07</td>
<td>transcription factor, putative similar to WRKY transcription factor GB:BAA87058 GI:6472585 from [Nicotiana tabacum];supported by full-length cDNA: Ceres:6437.</td>
</tr>
<tr>
<td>248964_at</td>
<td>At5g45340</td>
<td>-1.97</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>266834_s_at</td>
<td>At2g30020</td>
<td>-1.87</td>
<td>putative protein phosphatase 2C</td>
</tr>
<tr>
<td>245711_at</td>
<td>At5g04340</td>
<td>-1.83</td>
<td>putative c2h2 zinc finger transcription factor</td>
</tr>
<tr>
<td>247925_at</td>
<td>At5g57560</td>
<td>-1.77</td>
<td>TCH4 protein (gb</td>
</tr>
<tr>
<td>248164_at</td>
<td>At5g54490</td>
<td>-1.53</td>
<td>putative protein similar to unknown protein (pir</td>
</tr>
<tr>
<td>247543_at</td>
<td>At5g61600</td>
<td>-1.43</td>
<td>DNA binding protein - like DNA binding protein EREBP-4, Nicotiana tabacum, PIR:T02434;supported by full-length cDNA: Ceres:92102.</td>
</tr>
<tr>
<td>256306_at</td>
<td>At1g30370</td>
<td>-1.43</td>
<td>lipase, putative contains Pfam profile: PF01764: Lipase</td>
</tr>
<tr>
<td>256129_at</td>
<td>At1g18210</td>
<td>-1.33</td>
<td>calcium-binding protein, putative similar to calcium-binding protein GI:6901652 from [Olea europaea];supported by full-length cDNA: Ceres:19462.</td>
</tr>
<tr>
<td>263935_at</td>
<td>At2g35930</td>
<td>-1.30</td>
<td>unknown protein</td>
</tr>
<tr>
<td>250350_at</td>
<td>At5g12010</td>
<td>-1.23</td>
<td>putative protein predicted proteins, Arabidopsis thaliana</td>
</tr>
<tr>
<td>252679_at</td>
<td>At3g44260</td>
<td>-1.20</td>
<td>CCR4-associated factor 1-like protein CAF1_MOUSE CCR4-ASSOCIATED FACTOR 1 – Mus musculus, SWISSPROT:CAF1_MOUSE; supported by cDNA: gi_15292828_gb_AY050848.1</td>
</tr>
<tr>
<td>252592_at</td>
<td>At3g45640</td>
<td>-1.17</td>
<td>mitogen-activated protein kinase 3; supported by cDNA: gi_14423447_gb_AF386961.1_AF386961</td>
</tr>
<tr>
<td>260147_at</td>
<td>At1g52790</td>
<td>-1.07</td>
<td>putative oxidoreductase similar to adventitious rooting related oxygenase GB:CAA12386 from [Malus domestica]</td>
</tr>
<tr>
<td>267028_at</td>
<td>At2g38470</td>
<td>-1.03</td>
<td>putative WRKY-type DNA binding protein</td>
</tr>
<tr>
<td>247275_at</td>
<td>At5g64370</td>
<td>-0.80</td>
<td>beta-ureidopropionase</td>
</tr>
<tr>
<td>264809_at</td>
<td>At1g08830</td>
<td>1.20</td>
<td>superoxide dismutase identical to GB:P24704;supported by full-length cDNA: Ceres:33493.</td>
</tr>
</tbody>
</table>

*The mean Signal Log Ratio was calculated by averaging the SLR from the three replicated experiments
Table A8.3. Selection of differentially regulated genes with reliable expressions in all three replicated arrays of ‘Tahono’ 48 hai to clubroot isolate S.

<table>
<thead>
<tr>
<th>Selection of UP-regulated genes</th>
<th>Increase</th>
<th>Selection of DOWN-regulated genes</th>
<th>Decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of genes on the array</td>
<td>22,810</td>
<td>Total number of genes on the array</td>
<td>22,810</td>
</tr>
<tr>
<td>No. of genes called ‘present’ in all replicates of Treatment</td>
<td>3,355</td>
<td>No. of genes called ‘present’ in Control</td>
<td>5,114</td>
</tr>
<tr>
<td>No. of genes called ‘increase’ in at least 2 replicates</td>
<td>101</td>
<td>No. of genes called ‘decrease’ in at least 2 replicates</td>
<td>113</td>
</tr>
<tr>
<td>No. of genes with SLR ≥ 0.8 in all biological replicates</td>
<td>7</td>
<td>No. of genes with SLR ≤ -0.8 in all biological replicates</td>
<td>35</td>
</tr>
<tr>
<td>No. of genes with a mean signal intensity ≥ 100 in Treatment</td>
<td>3</td>
<td>No. of genes with a mean signal intensity ≥ 100 in Control</td>
<td>31</td>
</tr>
<tr>
<td>% Significant Increase</td>
<td>0.09</td>
<td>% Significant Decrease</td>
<td>0.61</td>
</tr>
</tbody>
</table>

Table A8.4. List of differentially regulated genes in ‘Tahono’ (sorted by mean SLR).

<table>
<thead>
<tr>
<th>Probe Set ID</th>
<th>Locus Identifier</th>
<th>Mean SLR</th>
<th>Putative function (from the Affymetrix Arabidopsis library downloaded on August 2007)</th>
</tr>
</thead>
<tbody>
<tr>
<td>260147_at</td>
<td>At1g52790</td>
<td>-2.73</td>
<td>putative oxidoreductase similar to adventitious rooting related oxygenase GB:CAA12386 from [Malus domestica]</td>
</tr>
<tr>
<td>266184_s_at</td>
<td>At2g38940</td>
<td>-2.13</td>
<td>phosphate transporter (AtPT2) identical to GB:U62331</td>
</tr>
<tr>
<td>251281_at</td>
<td>At3g61640</td>
<td>-1.73</td>
<td>putative protein hypothetical protein At2g46330 - Arabidopsis thaliana, EMBL:AC006526; supported by full-length cDNA: Ceres:11394.</td>
</tr>
<tr>
<td>247543_at</td>
<td>At5g61600</td>
<td>-1.63</td>
<td>DNA binding protein - like DNA binding protein EREBP-4, Nicotiana tabacum, PIR:T02434; supported by full-length cDNA: Ceres:92102.</td>
</tr>
<tr>
<td>253088_at</td>
<td>At4g36220</td>
<td>-1.57</td>
<td>ferulate-5-hydroxylase (FAH1); supported by cDNA: gi_1488254_gb_U38416.1_ATU38416</td>
</tr>
<tr>
<td>252679_at</td>
<td>At3g44260</td>
<td>-1.53</td>
<td>CCR4-associated factor 1-like protein CAF1_MOUSE CCR4-ASSOCIATED FACTOR 1 - Mas musculus, SWISSPROT:CAF1_MOUSE; supported by cDNA: gi_15292828_gb_AY050848.1</td>
</tr>
<tr>
<td>256306_at</td>
<td>At1g30370</td>
<td>-1.53</td>
<td>lipase, putative contains Pfam profile: PF01764: Lipase</td>
</tr>
<tr>
<td>257339_s_at</td>
<td>At2g07741</td>
<td>-1.33</td>
<td>ATP synthase subunit 9</td>
</tr>
<tr>
<td>250153_at</td>
<td>At5g15130</td>
<td>-1.47</td>
<td>putative protein TMV response-related gene product, Nicotiana tabacum, EMBL:AB024510</td>
</tr>
<tr>
<td>261892_at</td>
<td>At1g80840</td>
<td>-1.47</td>
<td>transcription factor, putative similar to WRKY transcription factor GB:BAA87058 GI:6472585 from [Nicotiana tabacum]; supported by full-length cDNA: Ceres:6437.</td>
</tr>
<tr>
<td>263935_at</td>
<td>At2g35930</td>
<td>-1.47</td>
<td>unknown protein</td>
</tr>
<tr>
<td>244950_at</td>
<td>cox2</td>
<td>-1.33</td>
<td>cytochrome c oxidase subunit 2</td>
</tr>
<tr>
<td>266012_s_at</td>
<td>At2g07741</td>
<td>-1.33</td>
<td>predicted protein</td>
</tr>
<tr>
<td>259276_at</td>
<td>At3g01190</td>
<td>-1.30</td>
<td>putative peroxidase very similar to peroxidase GB:CAA66963 from [Arabidopsis thaliana]; supported by full-length cDNA: Ceres:37597.</td>
</tr>
<tr>
<td>250676_at</td>
<td>At5g06320</td>
<td>-1.27</td>
<td>harpin-induced protein-like; supported by cDNA: gi_9502175_gb_AF264699.1_AF264699</td>
</tr>
<tr>
<td>262133_at</td>
<td>At1g78000</td>
<td>-1.27</td>
<td>high affinity sulphate transporter, putative similar to high affinity sulphate transporter GI:1217966 from [Hordeum vulgare]; supported by cDNA: gi_14245726_dbj_AB042322.2_AB042322</td>
</tr>
<tr>
<td>260475_at</td>
<td>At1g11080</td>
<td>-1.23</td>
<td>Serine carboxypeptidase isolog</td>
</tr>
<tr>
<td>264313_at</td>
<td>At1g70410</td>
<td>-1.23</td>
<td>carbonic anhydrase, putative similar to carbonic anhydrase GI:882241 from [Flaveria linearis]; supported by full-length cDNA: Ceres: 38715</td>
</tr>
<tr>
<td>265230_s_at</td>
<td>At2g07707</td>
<td>-1.23</td>
<td>hypothetical protein</td>
</tr>
<tr>
<td>248252_at</td>
<td>At5g53250</td>
<td>-1.20</td>
<td>putative protein similar to unknown protein (emb</td>
</tr>
<tr>
<td>248790_at</td>
<td>At5g47450</td>
<td>-1.20</td>
<td>membrane channel protein-like; aquaporin (tonoplast intrinsic protein)-like</td>
</tr>
<tr>
<td>248964_at</td>
<td>At5g45340</td>
<td>-1.13</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>246270_at</td>
<td>At4g36500</td>
<td>-1.10</td>
<td>putative protein</td>
</tr>
<tr>
<td>249284_at</td>
<td>At5g41810</td>
<td>-1.10</td>
<td>unknown protein; supported by full-length cDNA: Ceres:126660.</td>
</tr>
<tr>
<td>256633_at</td>
<td>At3g28340</td>
<td>-1.10</td>
<td>unknown protein</td>
</tr>
<tr>
<td>245399_at</td>
<td>At4g17340</td>
<td>-1.03</td>
<td>membrane channel like protein; supported by full-length cDNA:</td>
</tr>
<tr>
<td>probe_set</td>
<td>gene_id</td>
<td>log2_expression</td>
<td>description</td>
</tr>
<tr>
<td>-----------</td>
<td>---------</td>
<td>----------------</td>
<td>-------------</td>
</tr>
<tr>
<td>247199_at</td>
<td>At5g65210</td>
<td>-1.00</td>
<td>DNA binding protein TGA1a homolog</td>
</tr>
<tr>
<td>256891_at</td>
<td>At3g19030</td>
<td>-1.00</td>
<td>hypothetical protein contains similarity to phosphoserine aminotransferase GB:P19689 from [Yersinia enterocolitica]; supported by full-length cDNA: Ceres:31032.</td>
</tr>
<tr>
<td>267293_at</td>
<td>At2g23810</td>
<td>-1.00</td>
<td>hypothetical protein</td>
</tr>
<tr>
<td>263404_s_at</td>
<td>At2g04100</td>
<td>-0.93</td>
<td>hypothetical protein similar to hypothetical protein GB:AAC27412</td>
</tr>
<tr>
<td>263478_at</td>
<td>At2g31880</td>
<td>-0.87</td>
<td>putative receptor-like protein kinase; supported by cDNA: gi_16648754_gb_AY058153.1_</td>
</tr>
<tr>
<td>247359_at</td>
<td>At5g63560</td>
<td>1.07</td>
<td>acyltransferase-like protein; supported by full-length cDNA: Ceres:157547.</td>
</tr>
<tr>
<td>249490_s_at</td>
<td>At5g39110</td>
<td>1.10</td>
<td>germin -like protein germin -like protein GLP6, Arabidopsis thaliana, EMBL:ATU75194</td>
</tr>
<tr>
<td>264809_at</td>
<td>At1g08830</td>
<td>1.67</td>
<td>super oxidase dismutase identical to GB:P24704; supported by full-length cDNA: Ceres:33493.</td>
</tr>
</tbody>
</table>
Table A8.5. Selection of differentially regulated genes with reliable expressions in all three replicated arrays of ‘ECD04’ 48 hai to clubroot isolate S.

<table>
<thead>
<tr>
<th>Selection of UP-regulated genes</th>
<th>Increase</th>
<th>Selection of DOWN-regulated genes</th>
<th>Decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of genes on the array</td>
<td>22,810</td>
<td>Total number of genes on the array</td>
<td>22,810</td>
</tr>
<tr>
<td>No. of genes called ‘present’ in all replicates of Treatment</td>
<td>3,083</td>
<td>No. of genes called ‘present’ in Control</td>
<td>4637</td>
</tr>
<tr>
<td>No. of genes called ‘increase’ in at least 2 replicates</td>
<td>43</td>
<td>No. of genes called ‘decrease’ in at least 2 replicates</td>
<td>29</td>
</tr>
<tr>
<td>No. of genes with SLR ≥ 0.8 in all biological replicates</td>
<td>2</td>
<td>No. of genes with SLR ≤ -0.8 in all biological replicates</td>
<td>2</td>
</tr>
<tr>
<td>No. of genes with a mean signal intensity ≥ 100 in Treatment</td>
<td>1</td>
<td>No. of genes with a signal intensity ≥ 100 in Control</td>
<td>1</td>
</tr>
<tr>
<td>% Significant Increase</td>
<td>0.03</td>
<td>% Significant Decrease</td>
<td>0.02</td>
</tr>
</tbody>
</table>

Table A8.6. List of differentially regulated genes in ‘ECD04’.

<table>
<thead>
<tr>
<th>Probe Set ID</th>
<th>Locus Identifier</th>
<th>Mean SLR</th>
<th>Putative function (from the Affymetrix <em>Arabidopsis</em> library downloaded on August 2007)</th>
</tr>
</thead>
<tbody>
<tr>
<td>256306_at</td>
<td>At1g30370</td>
<td>-1.3</td>
<td>Lipase, putative contains Pfam profile: PF01764: Lipase</td>
</tr>
<tr>
<td>251112_s_at</td>
<td>At5g01320</td>
<td>1.13</td>
<td>Pyruvate decarboxylase-like protein pyruvate decarboxylase (EC 4.1.1.1) pdc1 - <em>Arabidopsis</em> thaliana, PIR:T05315</td>
</tr>
</tbody>
</table>
Appendix 9

Selection of constitutively over-/under-expressed genes:

Table A9.1. Selection of constitutively expressed genes with reliable expressions in healthy ‘Tahono’ when compared to that of ‘Granaat’.

<table>
<thead>
<tr>
<th>Selection of genes constitutively expressed at a HIGHER rate</th>
<th>Selection of genes constitutively expressed at a LOWER rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of genes on the array</td>
<td>22,810</td>
</tr>
<tr>
<td>No. of genes called ‘present’ in Experiment</td>
<td>5,114</td>
</tr>
<tr>
<td>No. of genes called ‘increase’</td>
<td>136</td>
</tr>
<tr>
<td>No. of genes with SLR* ≥ 0.8</td>
<td>91</td>
</tr>
<tr>
<td>No. of genes with a signal intensity ≥ 100 in Experiment</td>
<td>74</td>
</tr>
<tr>
<td>% Significantly higher rate</td>
<td>1.4</td>
</tr>
</tbody>
</table>

*SLR means Signal Log Ratio, whereby a value of 0.8 indicate a 1.75-fold change

Table A9.2. List of constitutively expressed genes in ‘Tahono’ (sorted by SLR).

<table>
<thead>
<tr>
<th>Probe Set ID</th>
<th>Locus Identifier</th>
<th>SLR</th>
<th>Putative function (from the Affymetrix Arabidopsis library downloaded on August 2007)</th>
</tr>
</thead>
<tbody>
<tr>
<td>245226_at</td>
<td>At3g29970</td>
<td>-2.4</td>
<td>gene_id:K17E7.15~unknown protein</td>
</tr>
<tr>
<td>251112_s_at</td>
<td>At5g01320</td>
<td>-2.2</td>
<td>pyruvate decarboxylase-like protein pyruvate decarboxylase (EC 4.1.1.1) pdcl1 - Arabidopsis thaliana, PIR:T05315</td>
</tr>
<tr>
<td>265414_at</td>
<td>At2g16660</td>
<td>-1.9</td>
<td>nodulin-like protein</td>
</tr>
<tr>
<td>252929_at</td>
<td>At4g38970</td>
<td>-1.7</td>
<td>putative fructose-bisphosphate aldolase fructose-bisphosphate aldolase (EC 4.1.2.13)- rice, PIR2:T02057; supported by cDNA: gi_16226652_gb_AF428455.1_AF428455</td>
</tr>
<tr>
<td>258467_at</td>
<td>At3g06060</td>
<td>-1.6</td>
<td>unknown protein contains Pfam profile: PF00106 short chain dehydrogenase</td>
</tr>
<tr>
<td>261834_at</td>
<td>At1g10640</td>
<td>-1.5</td>
<td>polygalacturonase PG1, putative similar to polygalacturonase PG1 GI:5669846 from [Glycine max]; supported by cDNA: gi_14532455_gb_AY039852.1_</td>
</tr>
<tr>
<td>248964_at</td>
<td>At5g45340</td>
<td>-1.4</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>261970_at</td>
<td>At1g65960</td>
<td>-1.4</td>
<td>glutamate decarboxylase (gad), putative similar to glutamate decarboxylase (gad) GI:294111 from [Petunia hybrida]; supported by cDNA: gi_1184959_gb_U46665.1_ATU46665</td>
</tr>
<tr>
<td>257834_at</td>
<td>At3g26720</td>
<td>-1.3</td>
<td>alpha-mannosidase, putative similar to lysosomal alpha-mannosidase GB:AAC34130 [Homo sapiens] (Hum. Mol. Genet. 6 (5), 717-726 (1997)); supported by cDNA: gi_14517402_gb_AY039536.1_</td>
</tr>
<tr>
<td>261892_at</td>
<td>At1g80840</td>
<td>-1.3</td>
<td>transcription factor, putative similar to WRKY transcription factor GB:AA87058 GI:6472585 from [Nicotiana tabacum];supported by full-length cDNA; Ceres:6437,</td>
</tr>
<tr>
<td>264809_at</td>
<td>At1g08830</td>
<td>-1.3</td>
<td>superoxide dismutase identical to GB:P24704;supported by full-length cDNA; Ceres:33493,</td>
</tr>
<tr>
<td>AFFX-r2-Ec-bioC-3_at</td>
<td>AFFX-r2-Ec-bioC-3</td>
<td>-1.3</td>
<td>Escherichia coli /REF=J04423 /DEF=E coli bioC protein corresponding to nucleotides 4609-4883 of J04423 /LEN=777 (-5 and -3 represent transcript regions 5 prime and 3 prime respectively)</td>
</tr>
<tr>
<td>257784_at</td>
<td>At3g26970</td>
<td>-1.2</td>
<td>geranylgeranylated protein, putative similar to ATGP4 GB:AAAD00115 from [Arabidopsis thaliana]</td>
</tr>
<tr>
<td>262832_s_at</td>
<td>At1g14870</td>
<td>-1.2</td>
<td>unknown protein</td>
</tr>
<tr>
<td>AFFX-BioC-3_at</td>
<td>AFFX-BioC-3</td>
<td>-1.2</td>
<td>J04423 E coli bioC protein (-5 and -3 represent transcript regions 5 prime and 3 prime respectively)</td>
</tr>
<tr>
<td>AFFX-r2-Ec-bioC-5_at</td>
<td>AFFX-r2-Ec-bioC-5</td>
<td>-1.2</td>
<td>Escherichia coli /REF=J04423 /DEF=E coli bioC protein corresponding to nucleotides 4257-4573 of J04423 /LEN=777 (-5 and -3 represent transcript regions 5 prime and 3 prime respectively)</td>
</tr>
<tr>
<td>Probe ID</td>
<td>Gene ID</td>
<td>Expression</td>
<td>Description</td>
</tr>
<tr>
<td>-----------</td>
<td>---------------</td>
<td>------------</td>
<td>---------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>AFFX-BioC-5_at</td>
<td>At5g44130</td>
<td>-1</td>
<td>putative protein contains similarity to surface protein; supported by cDNA: gi_16648846_gb_AY058201.1</td>
</tr>
<tr>
<td>249037_at</td>
<td>At3g62720</td>
<td>-1</td>
<td>alpha galactosyltransferase-like protein alpha galactosyltransferase - Trigonella foenum-graecum</td>
</tr>
<tr>
<td>251192_at</td>
<td>At5g39890</td>
<td>-0.9</td>
<td>putative protein hypothetical protein F8M21.10 - Arabidopsis thaliana</td>
</tr>
<tr>
<td>264658_at</td>
<td>At1g099910</td>
<td>-1</td>
<td>putative protein similar to LG27/30-like gene GB: CAB45078</td>
</tr>
<tr>
<td>249384_at</td>
<td>At5g39890</td>
<td>-0.9</td>
<td>putative protein hypothetical protein F8M21.10 - Arabidopsis thaliana</td>
</tr>
<tr>
<td>252213_at</td>
<td>At3g50210</td>
<td>-0.9</td>
<td>flavonol synthase - like protein SRG1 protein, Arabidopsis thaliana</td>
</tr>
<tr>
<td>253497_at</td>
<td>At4g31880</td>
<td>-0.9</td>
<td>putative protein microtubule-associated protein 1B (MAP1B), Homo sapiens</td>
</tr>
<tr>
<td>254810_at</td>
<td>At4g12390</td>
<td>-0.9</td>
<td>putative protein pectinesterase - Citrus sinensis, PID: g2098711; supported by full-length cDNA:</td>
</tr>
<tr>
<td>258222_at</td>
<td>At3g15680</td>
<td>-0.9</td>
<td>putative zinc finger protein contains Pfam profile: PF00641 Zn-finger in Ran binding protein and</td>
</tr>
<tr>
<td>264179_at</td>
<td>At1g02180</td>
<td>-0.9</td>
<td>putative protein predicted by genemark.hmm</td>
</tr>
<tr>
<td>246289_at</td>
<td>At3g56880</td>
<td>-0.8</td>
<td>putative protein predicted protein At2g41010 - Arabidopsis thaliana</td>
</tr>
<tr>
<td>248164_at</td>
<td>At5g54490</td>
<td>-0.8</td>
<td>putative protein similar to unknown protein (pirlfT05752); supported by full-length cDNA:</td>
</tr>
<tr>
<td>251222_at</td>
<td>At3g62580</td>
<td>-0.8</td>
<td>putative membrane protein clone: 2-72. - Mus musculus, EMBL:AB030201; supported by cDNA:</td>
</tr>
<tr>
<td>251486_at</td>
<td>At3g59540</td>
<td>-0.8</td>
<td>60S RIBOSOMAL PROTEIN L38-like protein 60S RIBOSOMAL PROTEIN L38 - Lycopersicon esculentum, EMBL:</td>
</tr>
<tr>
<td>251821_at</td>
<td>At3g55050</td>
<td>-0.8</td>
<td>protein phosphatase 2C - like protein protein phosphatase 2C homolog, Mesembryanthemum crystallinum</td>
</tr>
<tr>
<td>255300_at</td>
<td>At4g04870</td>
<td>-0.8</td>
<td>putative phosphatidyglycerotransferase similar to CDP-diacylglycerol-glycerol-3-phosphate 3-phosphatidy</td>
</tr>
<tr>
<td>262502_at</td>
<td>At1g21600</td>
<td>-0.8</td>
<td>unknown protein similar to hypothetical protein GB: AAD41412</td>
</tr>
<tr>
<td>263096_at</td>
<td>At2g16060</td>
<td>-0.8</td>
<td>class 1 non-symbiotic hemoglobin (AHB1) identical to GP: 2581783:U949998; supported by full-length cDNA:</td>
</tr>
<tr>
<td>264052_at</td>
<td>At2g22330</td>
<td>-0.8</td>
<td>putative cytochrome P450</td>
</tr>
<tr>
<td>266165_at</td>
<td>At2g28190</td>
<td>-0.8</td>
<td>putative copper/zinc superoxide dismutase identical to GP: 3273753: AF061519; supported by full-length cDNA:</td>
</tr>
<tr>
<td>247295_at</td>
<td>At5g64180</td>
<td>0.8</td>
<td>putative protein similar to unknown protein (dbj BAA96220.1); supported by full-length cDNA:</td>
</tr>
<tr>
<td>247312_at</td>
<td>At5g63970</td>
<td>0.8</td>
<td>putative protein strong similarity to unknown protein (gbIAAF01562.1)</td>
</tr>
<tr>
<td>250428_at</td>
<td>At5g10480</td>
<td>0.8</td>
<td>putative protein phosphatase protein tyrosine phosphatase-like protein PTPLB, Mus musculus, EMBL:</td>
</tr>
<tr>
<td>252293_at</td>
<td>At3g48990</td>
<td>0.8</td>
<td>4-coumarate-CoA ligase -like protein 4-coumarate-CoA ligase enzyme, Pinus taeda, gb: AAA92669; supported by full-length cDNA: Ceres: 41541.</td>
</tr>
<tr>
<td>252872_at</td>
<td>At4g40010</td>
<td>0.8</td>
<td>putative serine/threonine protein kinase serine-threonine protein kinase TaPK3, Triticum aestivum</td>
</tr>
<tr>
<td>254050_s_at</td>
<td>At4g25670</td>
<td>0.8</td>
<td>putative protein; supported by cDNA: gi_14517537_gb_AY039604.1</td>
</tr>
<tr>
<td>255645_at</td>
<td>At4g00880</td>
<td>0.8</td>
<td>coded for by A. thaliana cDNA T43845 similar to auxin-induced protein</td>
</tr>
<tr>
<td>258285_at</td>
<td>At3g16140</td>
<td>0.8</td>
<td>photosystem I subunit VI precursor identical to GB: CAB52749 from [Arabidopsis thaliana]; supported by full-length cDNA: Ceres: 9633.</td>
</tr>
</tbody>
</table>
261901_at At1g80920 0.8 J8-like protein similar to DnaJ homologue J8 GB: AAC72399
GI:3851670 from [Arabidopsis thaliana]; supported by full-length cDNA: Ceres:4150.
262038_at At1g35580 0.8 invertase, putative similar to neutral invertase GB:76145 GI:4200165
from [Daucus carota]
262308_at At1g71010 0.8 unknown protein
266012_s_at At2g07741 0.8 predicted protein
267187_s_at At2g44160 0.8 putative methylenetetrahydrofolate reductase; supported by cDNA:
gi_15215809_gb_AY050434.1_
267280_at At2g19450 0.8 diacylglycerol O-acyltransferase; supported by cDNA:
gi_15450799_gb_AY054480.1_
244949_s_at ycf1.1 0.9 hypothetical protein
245139_at At2g45430 0.9 putative AT-hook DNA-binding protein highly similar to
hypothetical protein gi2245139:gnl:PID:e327087:Z97344
246238_at At4g36670 0.9 sugar transporter like protein
249332_at At5g40980 0.9 putative protein similar to unknown protein (gb AAF03445.1)
258221_at At3g29160 0.9 Snf1-related protein kinase KIN11 (AKIN11) identical to protein
kinase AKin11 GI:1729444 [Arabidopsis thaliana]
259525_at At1g12560 0.9 hypothetical protein
259804_at At1g72160 0.9 cytosolic factor, putative similar to GI:807956 from [Saccharomyces
cerevisiae]; supported by cDNA: gi_15081613_gb_AY048199.1_
262939_s_at At1g79530 0.9 glyceraldehyde-3-phosphate dehydrogenase, putative similar to
glyceraldehyde-3-phosphate dehydrogenase GI:1100222 from [Pinus
sylvestris]
264301_at At1g78780 0.9 hypothetical protein contains similarity to pathogen-related protein
GI:499073 from [Hordeum vulgare]; supported by full-length cDNA:
Ceres:772.
264506_at At1g09560 0.9 germin-like protein Identical to Arabidopsis germin-like protein,
gi1755178. Location of EST 180L10T7, gi906417; supported by
CDNA: gi_13265455_gb_AF324678.2_AF324678
265435_s_at At2g21020 0.9 putative major intrinsic (channel) protein
266044_s_at At2g07725 0.9 putative glutathione S-transferase; supported by full-length cDNA:
Ceres:27915.; supported by cDNA:
gi_11095995_gb_AF288181.1_AF288181
267461_at At2g33830 0.9 putative auxin-regulated protein; supported by full-length cDNA:
Ceres:1711.
245010_at ndhJ 1 NADH dehydrogenase subunit
245015_at rbcL 1 large subunit of ribbose-1,5-bisphosphate carboxylase/oxygenase
245016_at accD 1 carboxytransferase beta subunit
247199_at At5g65210 1 DNA binding protein TGA1a homolog; supported by full-length
cDNA; Ceres:31032.
247534_at At5g61580 1 pyrophosphate-dependent phosphofructo-1-kinase - like protein
pyrophosphate-dependent phosphofructo-1-kinase, Prunus
armeniaca, EMBL:U93272
248000_at At5g56190 1 WD-repeat protein-like; supported by full-length cDNA:
Ceres:109499.
249581_at At5g37600 1 glutamate--ammonia ligase; supported by cDNA:
gi_16226386_gb_AF428386.1_AF428386
258402_at At3g15450 1 unknown protein very similar to unknown protein GB:AAC39468
from [Arabidopsis thaliana]; supported by cDNA:
gi_14335087_gb_AY037223.1_
259723_at At1g60960 1 putative iron-regulated transporter similar to iron-regulated
transporter 1 GB:AAD30548 from [Lycopersicon
esculentum]; supported by full-length cDNA; Ceres:15980.
262133_at At1g78000 1 high affinity sulphate transporter, putative similar to high affinity
sulphate transporter GI:1217966 from [Hordeum vulgare]; supported by
cDNA: gi_14245726_dbj_AB042322.2_AB042322
263019_at At1g23870 1 trehalose 6-phosphate synthase, putative similar to trehalose 6-
phosphate synthase GB:CAA09463 GI:3647365 from [Yarrowia
lipolytica]
<table>
<thead>
<tr>
<th>Probe</th>
<th>Gene Symbol</th>
<th>Description</th>
<th>Accession Numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>264313_at</td>
<td>At1g70410</td>
<td>carbonic anhydrase, putative similar to carbonic anhydrase</td>
<td>GI:882241 from [Flaveria linearis]; supported by full-length cDNA: Ceres: 38715.</td>
</tr>
<tr>
<td>244937_at</td>
<td>ndhH</td>
<td>NADH dehydrogenase 49KDa protein</td>
<td></td>
</tr>
<tr>
<td>244961_at</td>
<td>ycf5</td>
<td>hypothetical protein</td>
<td></td>
</tr>
<tr>
<td>245011_at</td>
<td>psbG</td>
<td>photosystem II G protein</td>
<td></td>
</tr>
<tr>
<td>245026_at</td>
<td>atpH</td>
<td>ATPase III subunit</td>
<td></td>
</tr>
<tr>
<td>253088_at</td>
<td>At4g36220</td>
<td>ferulate-5-hydroxylase (FAH1) ; supported by cDNA:</td>
<td>gi_1488254_gb_U38416.1_ATU38416</td>
</tr>
<tr>
<td>253125_at</td>
<td>At4g36040</td>
<td>DNAJ-like protein protein</td>
<td></td>
</tr>
<tr>
<td>259276_at</td>
<td>At3g01190</td>
<td>putative peroxidase very similar to peroxidase GB:CAA66963 from</td>
<td>[Arabidopsis thaliana]; supported by full-length cDNA: Ceres: 37597.</td>
</tr>
<tr>
<td>261815_at</td>
<td>At1g08325</td>
<td>leucine zipper protein, putative similar to basic leucine zipper protein</td>
<td>GI:2865394 from [Zea mays]</td>
</tr>
<tr>
<td>244939_at</td>
<td>rps12.1</td>
<td>ribosomal protein S12 (trans-splice part 1 of 2)</td>
<td></td>
</tr>
<tr>
<td>250153_at</td>
<td>At5g15130</td>
<td>putative TMV response-related gene product, Nicotiana tabacum, EMBL:AB024510</td>
<td></td>
</tr>
<tr>
<td>250580_at</td>
<td>At5g07440</td>
<td>glutamate dehydrogenase 2 ; supported by cDNA:</td>
<td>gi_14423477_gb_AF386976.1_AF386976</td>
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<tr>
<td>251012_at</td>
<td>At5g02580</td>
<td>putative protein ; supported by full-length cDNA:</td>
<td>Ceres: 16476.</td>
</tr>
<tr>
<td>256674_at</td>
<td>At3g52360</td>
<td>unknown protein ; supported by full-length cDNA:</td>
<td>Ceres: 31357.</td>
</tr>
<tr>
<td>257339_s_at</td>
<td>mitochondria.1</td>
<td>ATP synthase subunit 9</td>
<td></td>
</tr>
<tr>
<td>265023_at</td>
<td>At1g24440</td>
<td>unknown protein weak similarity to C3HC4 zinc finger; supported by</td>
<td></td>
</tr>
<tr>
<td>244959_s_at</td>
<td>orf107c</td>
<td>hypothetical protein</td>
<td></td>
</tr>
<tr>
<td>257217_at</td>
<td>At3g14940</td>
<td>phosphoenolpyruvate carboxylase (PPC) identical to</td>
<td>gi_3264804_gb_AF071788.1_AF071788</td>
</tr>
<tr>
<td>245003_at</td>
<td>psbC</td>
<td>PSII 43 KDa protein</td>
<td></td>
</tr>
<tr>
<td>246880_s_at</td>
<td>At5g25980</td>
<td>myrosinase TGG2 ; supported by cDNA:</td>
<td>gi_13507564_gb_AF360348.1_AF360348</td>
</tr>
<tr>
<td>252927_at</td>
<td>At4g39090</td>
<td>cisteine protease RD19A identical to thiol protease SP:P43296, GI:435618</td>
<td></td>
</tr>
<tr>
<td>254001_at</td>
<td>At4g26260</td>
<td>putative protein PRE87 mRNA, Pinus radiata, AF049069</td>
<td></td>
</tr>
<tr>
<td>265230_s_at</td>
<td>At2g07707</td>
<td>hypothetical protein</td>
<td></td>
</tr>
<tr>
<td>260552_at</td>
<td>At2g43430</td>
<td>putative glyoxalase II ; supported by cDNA:</td>
<td>gi_2570339_gb_U90928.1_ATU90928</td>
</tr>
<tr>
<td>262537_s_at</td>
<td>At1g17280</td>
<td>putative ubiquitin-conjugating enzyme First 212 a.a. are 41% identical</td>
<td></td>
</tr>
<tr>
<td>260147_at</td>
<td>At1g52790</td>
<td>putative oxidoreductase similar to adventitious rooting related oxygenase</td>
<td></td>
</tr>
<tr>
<td>257946_at</td>
<td>At3g21710</td>
<td>hypothetical protein predicted by gene mark</td>
<td></td>
</tr>
<tr>
<td>266552_at</td>
<td>At2g46330</td>
<td>unknown protein ; supported by cDNA:</td>
<td>gi_15294196_gb_AF410245.1_AF410276</td>
</tr>
<tr>
<td>253079_s_at</td>
<td>At4g36190</td>
<td>putative protein F56F10.1, Caenorhabditis elegans, PATX:G1688051</td>
<td></td>
</tr>
<tr>
<td>266184_s_at</td>
<td>At2g38940</td>
<td>phosphate transporter (AtPT2) identical to GB: U62331</td>
<td></td>
</tr>
<tr>
<td>244912_at</td>
<td>ccb382</td>
<td>cytochrome c biogenesis orf382 Protein sequence is in conflict with the</td>
<td></td>
</tr>
<tr>
<td>247741_at</td>
<td>At5g58960</td>
<td>putative predicted proteins, Arabidopsis thaliana and Oryza sativa</td>
<td></td>
</tr>
</tbody>
</table>

**Note:** Common constitutively expressed genes in both ‘Tahono’ and ECD04 when compared to ‘Granaat’ were **bolded.**
Table A9.3. Selection of constitutively expressed genes with reliable expressions in healthy ‘ECD04’ when compared to that of ‘Granaat’.

<table>
<thead>
<tr>
<th>Selection of genes constitutively expressed at a HIGHER rate</th>
<th>Selection of genes constitutively expressed at a LOWER rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total number of genes on the array</td>
<td>22,810</td>
</tr>
<tr>
<td>No. of genes called ‘present’ in Experiment</td>
<td>4,637</td>
</tr>
<tr>
<td>No. of genes called ‘increase’</td>
<td>162</td>
</tr>
<tr>
<td>No. of genes with SLR* ≥ 0.8</td>
<td>121</td>
</tr>
<tr>
<td>No. of genes with a signal intensity ≥ 100 in Experiment</td>
<td>115</td>
</tr>
<tr>
<td>% Higher rate</td>
<td>2.48</td>
</tr>
<tr>
<td>Total number of genes on the array</td>
<td>22,810</td>
</tr>
<tr>
<td>No. of genes called ‘present’ in Baseline</td>
<td>4,981</td>
</tr>
<tr>
<td>No. of genes called ‘decrease’</td>
<td>509</td>
</tr>
<tr>
<td>No. of genes with SLR* ≤ -0.8</td>
<td>179</td>
</tr>
<tr>
<td>No. of genes with a signal intensity ≥ 100 in Baseline</td>
<td>90</td>
</tr>
<tr>
<td>% Lower rate</td>
<td>1.81</td>
</tr>
</tbody>
</table>

*SLR means Signal Log Ratio, whereby a value of 0.8 indicate a 1.75-fold change

Table A9.4. List of constitutively expressed genes in ‘ECD04’ (sorted by SLR).

<table>
<thead>
<tr>
<th>Probe Set ID</th>
<th>Locus Identifier</th>
<th>SLR</th>
<th>Putative function (from the Affymetrix Arabidopsis library downloaded on August 2007)</th>
</tr>
</thead>
<tbody>
<tr>
<td>251012_at</td>
<td>At5g02580</td>
<td>-3</td>
<td>putative protein; supported by full-length cDNA: Ceres:16476.</td>
</tr>
<tr>
<td>251112_s_at</td>
<td>At5g01320</td>
<td>-3</td>
<td>putative protein phosphatase 2 C</td>
</tr>
<tr>
<td>266834_s_at</td>
<td>At2g30020</td>
<td>-2</td>
<td>unknown protein</td>
</tr>
<tr>
<td>267512_s_at</td>
<td>At2g03330</td>
<td>-2.5</td>
<td>hypothetical protein predicted by genscan+</td>
</tr>
<tr>
<td>265162_at</td>
<td>At1g30910</td>
<td>-2.2</td>
<td>alcohol dehydrogenase identical to alcohol dehydrogenase GI:469467 from (Arabidopsis thaliana); supported by full-length cDNA: Ceres:4033.</td>
</tr>
<tr>
<td>264953_at</td>
<td>At1g77120</td>
<td>-2.2</td>
<td>transcription factor, putative similar to WRKY transcription factor GB:BAA87058 GI:6472585 from [Nicotiana tabacum]; supported by full-length cDNA: Ceres:6437.</td>
</tr>
<tr>
<td>261892_at</td>
<td>At1g80840</td>
<td>-2.1</td>
<td>putative protein PG1, putative similar to polygalacturonase PG1 GI:5669846 from [Glycine max]; supported by cDNA: gi_14532455_gb_AY039852.1.</td>
</tr>
<tr>
<td>261834_at</td>
<td>At1g10640</td>
<td>-2</td>
<td>putative protein stem-specific protein - Nicotiana tabacum,PID:g20037; supported by full-length cDNA: Ceres:35207.</td>
</tr>
<tr>
<td>265481_at</td>
<td>At2g15960</td>
<td>-2</td>
<td>putative protein; supported by cDNA: gi_15028290_gb_AY045948.1.</td>
</tr>
<tr>
<td>257022_at</td>
<td>At3g19580</td>
<td>-1.8</td>
<td>putative protein; supported by full-length cDNA: Ceres:35207.</td>
</tr>
<tr>
<td>266123_at</td>
<td>At2g45180</td>
<td>-1.8</td>
<td>unknown protein identical to GB:AA82643 supported by full-length cDNA: Ceres:17187.</td>
</tr>
<tr>
<td>AFFX-Athal-25SrRNA_s_at</td>
<td>AFFX-Athal-25SrRNA</td>
<td>-1.8</td>
<td>Arabidopsis thaliana /REF=X52320 /DEF=25S rRNA /LEN=4430.</td>
</tr>
<tr>
<td>251192_at</td>
<td>At3g62720</td>
<td>-1.7</td>
<td>alpha galactosyltransferase-like protein alpha galactosyltransferase - Trigonella foenum-graecum, EMBL:TF0245478; supported by cDNA: gi_15983425_gb_AF424587.1_AF424587.</td>
</tr>
<tr>
<td>253874_at</td>
<td>At4g27450</td>
<td>-1.7</td>
<td>putative protein stem-specific protein - Nicotiana tabacum,PID:g20037; supported by full-length cDNA: Ceres:35207.</td>
</tr>
<tr>
<td>263498_at</td>
<td>At2g42610</td>
<td>-1.7</td>
<td>unknown protein; supported by cDNA: gi_6691164_gb_AF218765.1_AF218765.</td>
</tr>
<tr>
<td>264371_at</td>
<td>At1g12090</td>
<td>-1.7</td>
<td>pEARLI 1-like protein may be induced when levels of Aluminum become toxic or other stresses become present in the plant; supported by full-length cDNA: Ceres:5712.</td>
</tr>
<tr>
<td>251109_at</td>
<td>At5g01600</td>
<td>-1.6</td>
<td>ferritin 1 precursor; supported by full-length cDNA: Ceres:1100.</td>
</tr>
<tr>
<td>262932_at</td>
<td>At1g65820</td>
<td>-1.6</td>
<td>glutathione-s-transferase, putative similar to GST3_HUMAN SP:O14880; supported by full-length cDNA: Ceres: 8446.</td>
</tr>
<tr>
<td>267374_at</td>
<td>At2g26230</td>
<td>-1.5</td>
<td>putative uricase subunit similar to nodulin-35; identical to GB:Y11120; supported by full-length cDNA: Ceres:38538.</td>
</tr>
<tr>
<td>245278_at</td>
<td>At4g17730</td>
<td>-1.4</td>
<td>syntaxin; supported by cDNA: gi_2149379_gb_U85036.1_ATU85036.</td>
</tr>
<tr>
<td>247925_at</td>
<td>At5g57560</td>
<td>-1.4</td>
<td>TCH4 protein (gb</td>
</tr>
<tr>
<td>Accession</td>
<td>Gene Symbol</td>
<td>Expression Change</td>
<td>Description</td>
</tr>
<tr>
<td>-----------</td>
<td>-------------</td>
<td>-------------------</td>
<td>-------------</td>
</tr>
<tr>
<td>252929_at</td>
<td>At4g38970</td>
<td>-1.4</td>
<td>putative fructose-bisphosphate aldolase fructose-bisphosphate aldolase (EC 4.1.2.13)- rice, PIR2:T02057; supported by cDNA: gi_16226652_gb_AF428455.1_AF428455</td>
</tr>
<tr>
<td>267017_at</td>
<td>At2g39150</td>
<td>-1.4</td>
<td>unknown protein</td>
</tr>
<tr>
<td>244978_at</td>
<td>rpoA</td>
<td>-1.3</td>
<td>RNA polymerase alpha subunit</td>
</tr>
<tr>
<td>247440_at</td>
<td>At5g62680</td>
<td>-1.3</td>
<td>peptide transporter</td>
</tr>
<tr>
<td>247867_at</td>
<td>At5g57630</td>
<td>-1.3</td>
<td>SNF1 related protein kinase-like protein ; supported by cDNA: gi_14334389_gb_AY034100.1_</td>
</tr>
<tr>
<td>248964_at</td>
<td>At5g45340</td>
<td>-1.3</td>
<td>cytochrome P450</td>
</tr>
<tr>
<td>252592_at</td>
<td>At3g45640</td>
<td>-1.3</td>
<td>mitogen-activated protein kinase 3 ; supported by cDNA: gi_14423447_gb_AF386961.1_AF386961</td>
</tr>
<tr>
<td>257004_s_at</td>
<td>At3g14150</td>
<td>-1.3</td>
<td>glycolate oxidase, putative similar to GB:999542 from [Spinacia oleracea] (J. Biol. Chem. 264 (6), 3624-3628 (1989)), contains Pfam profile: PF01070 FMN-dependent dehydrogenase; supported by full-length cDNA: Ceres:98839.</td>
</tr>
<tr>
<td>261193_at</td>
<td>At1g32920</td>
<td>-1.3</td>
<td>unknown protein ; supported by cDNA: gi_15450636_gb_AY052686.1_</td>
</tr>
<tr>
<td>245711_at</td>
<td>At5g04340</td>
<td>-1.2</td>
<td>putative c2h2 zinc finger transcription factor</td>
</tr>
<tr>
<td>251281_at</td>
<td>At3g61640</td>
<td>-1.2</td>
<td>putative protein hypothetical protein At2g46330 - Arabidopsis thaliana, EMBL:AC006526; supported by full-length cDNA: Ceres:11394.</td>
</tr>
<tr>
<td>255149_at</td>
<td>At4g08150</td>
<td>-1.2</td>
<td>auxin-binding protein 1 precursor ; supported by full-length cDNA: Ceres:34126.</td>
</tr>
<tr>
<td>255412_at</td>
<td>At4g02980</td>
<td>-1.2</td>
<td>DNA binding protein - like DNA binding protein EREBP-4, Nicotiana tabacum, PIR:T02434; supported by full-length cDNA: Ceres:92102.</td>
</tr>
<tr>
<td>257253_at</td>
<td>At3g24190</td>
<td>-1.2</td>
<td>unknown protein; supported by cDNA: gi_15294249_gb_AF410316.1_AF410316</td>
</tr>
<tr>
<td>267381_at</td>
<td>At2g26190</td>
<td>-1.2</td>
<td>unknown protein ; supported by cDNA: gi_16930468_gb_AF419588.1_AF419588</td>
</tr>
<tr>
<td>258338_at</td>
<td>At3g16150</td>
<td>-1.1</td>
<td>DNA binding protein - like DNA binding protein EREBP-4, Nicotiana tabacum, PIR:T02434; supported by full-length cDNA: Ceres:92102.</td>
</tr>
<tr>
<td>259544_at</td>
<td>At1g20620</td>
<td>-1.1</td>
<td>hypothetical protein ; supported by full-length cDNA: Ceres:35868.</td>
</tr>
<tr>
<td>259803_at</td>
<td>At1g72150</td>
<td>-1.1</td>
<td>cytosolic factor, putative similar to GI:807956 from [Saccharomyces cerevisiae]; supported by cDNA: gi_15028180_gb_AY045913.1_</td>
</tr>
<tr>
<td>261285_at</td>
<td>At1g35720</td>
<td>-1.1</td>
<td>Ca2+ -dependent membrane-binding protein annexin identical to GB: AAD34236 from [Arabidopsis thaliana]; supported by full-length cDNA: Ceres:21689.</td>
</tr>
<tr>
<td>267028_at</td>
<td>At2g38470</td>
<td>-1.1</td>
<td>putative WRKY-type DNA binding protein</td>
</tr>
<tr>
<td>244966_at</td>
<td>petG</td>
<td>-1</td>
<td>cytochrome b6-f complex, subunit V</td>
</tr>
<tr>
<td>245207_at</td>
<td>At5g12310</td>
<td>-1</td>
<td>RING finger-like protein similarity to predicted protein, Arabidopsis thaliana, AF361602- Contains Zinc finger, C3HC4 type (RING finger), signature AA61-70</td>
</tr>
<tr>
<td>245226_at</td>
<td>At3g29970</td>
<td>-1</td>
<td>gene_id:K17E7.15- unknown protein</td>
</tr>
<tr>
<td>246270_at</td>
<td>At4g36500</td>
<td>-1</td>
<td>putative protein</td>
</tr>
<tr>
<td>246550_at</td>
<td>At5g14920</td>
<td>-1</td>
<td>putative protein predicted protein, Arabidopsis thaliana; supported by full-length cDNA: Ceres:32599.</td>
</tr>
<tr>
<td>248164_at</td>
<td>At5g54490</td>
<td>-1</td>
<td>putative protein similar to unknown protein (pir:T05752); supported by full-length cDNA: Ceres:109272.</td>
</tr>
<tr>
<td>249928_at</td>
<td>At5g22250</td>
<td>-1</td>
<td>CCR4-associated factor-like protein</td>
</tr>
<tr>
<td>251325_s_at</td>
<td>At3g61470</td>
<td>-1</td>
<td>Lhca2 protein ; supported by full-length cDNA: Ceres:123159.</td>
</tr>
<tr>
<td>251994_at</td>
<td>At3g52890</td>
<td>-1</td>
<td>protein kinase - like protein kinase, Arabidopsis thaliana, PIR:JN0505; supported by cDNA: gi_7716429_gb_AF236104.1_AF236104</td>
</tr>
</tbody>
</table>
putative auxin-induced protein
unknown protein; supported by full-length cDNA: Ceres:14471.
glycoprotein(EPI), putative similar to glycoprotein(EPI)
GI:349436 from [Daucus carota]; supported by cDNA:
\text{gi\_14334885\_gb\_AY035116.1}\_1
putative proline-rich cell wall protein (pirlIS52985); similar to
ESTs gbAI239404, gbIR89984, and emblZ17709 similar to auxin
down regulated GB:X69640 GI:296442 from [Glycine
max]; supported by full-length cDNA: Ceres:36784.
putative ATP binding protein
unknown protein contains similarity to obtusifoliol 14-alpha-
demethylase (CYP51) GB:Y09292 GI:1707854 from [Triticum
aestivum]; supported by full-length cDNA: Ceres:29745.
putative protein similar to unknown protein (gb|AAF30309.1)
CCCR4-associated factor 1-like protein CAF1_MOUSE CCCR4-
ASSOCIATED FACTOR 1 - Mus musculus,
SWISSPROT:CAF1_MOUSE; supported by cDNA:
\text{gi\_15292828\_gb\_AY050848.1}\_1
unknown protein contains two Kelch motifs; supported by full-
length cDNA: Ceres:32885.
CCCAAT-binding factor B subunit homolog, putative similar to
CCAAT-binding factor B subunit homolog GI:1173615 from
(Brassica napus); supported by cDNA:
\text{gi\_15982863\_gb\_AY057539.1}\_1
unknown protein similar to hypothetical protein
GB:AAD41412 GI:5263310 from (Arabidopsis thaliana); supported by cDNA:
\text{gi\_13265575\_gb\_AF324715.2\_AF324715}
unknown protein: supported by cDNA:
\text{gi\_15146251\_gb\_AY049267.1}\_1
putative auxin-regulated protein; supported by full-length cDNA:
Ceres:1711.
putative protein predicted proteins, Arabidopsis thaliana and
Drosophila melanogaster
putative protein similar to unknown protein (gb AAF17656.1)
putative protein hypothetical protein F8M21.10 - Arabidopsis
thaliana, PIR:T49947; supported by full-length cDNA:
Ceres:100590.
putative protein; similar to unknown protein (gb|AAF26969.1)
putative protein various predicted proteins, Arabidopsis thaliana;
supported by cDNA: \text{gi\_13878024\_gb\_AF370275.1\_AF370275}
exonuclease RRP41; supported by cDNA:
\text{gi\_6164937\_gb\_AF191741.1\_AF191741}
putative xyloglucan endotransglycosylase; supported by full-length
CDS: Ceres:17748.
Expressed protein; supported by full-length cDNA: Ceres:36229.
NAD dependent epimerase, putative contains Pfam profile:
PFO1370 NAD dependent epimerase/dehydratase family; supported
by cDNA: \text{gi\_13877894\_gb\_AF370210.1\_AF370210}
ferridoxin precursor identical to FERREDOXIN PRECURSOR
GB:P16972 from [Arabidopsis thaliana]; supported by cDNA:
\text{gi\_13265544\_gb\_AF324706.2\_AF324706}
AtHVA22a identical to AtHVA22a GB:AF141659; homolog of
HVA22 GB:A48892 (Hordeum vulgare) - (induced by abscisic acid
(ABA), likely a regulatory protein J Biol Chem 1993 Nov
5:268(31):23652-60); supported by cDNA:
\text{gi\_4884931\_gb\_AF141659.1\_AF141659}
hypothetical protein contains similarity to cockayne syndrome
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<td>262287_at</td>
<td>At1g68660</td>
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<td>At2g17230</td>
<td>Unknown protein; supported by full-length cDNA: Ceres:641.</td>
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<td>At2g21620</td>
<td>Unknown protein; supported by full-length cDNA: Ceres:31655.</td>
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<td>264181_at</td>
<td>At1g65350</td>
<td>Ubiquitin, putative similar to ubiquitin GI:902583 from [Zea mays].</td>
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<td>At1g16676</td>
<td>Expressed protein; supported by full-length cDNA: Ceres:1860.</td>
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<td>266695_at</td>
<td>At2g19810</td>
<td>Putative CUCCH-type zinc finger protein; supported by full-length cDNA: Ceres:101255.</td>
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<tr>
<td>AFFX-r2-Bs-dap-5_at</td>
<td>AFFX-r2-Bs-dap-5</td>
<td>Bacillus subtilis /REF=L38424 /DEF=B subtilis dapB, jojF, jojG genes corresponding to nucleotides 1439-1846 of L38424 /LEN=1931 (-5, -M, -3 represent transcript regions 5 prime, Middle, and 3 prime respectively)</td>
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<td>AFFX-r2-Bs-dap-M_at</td>
<td>AFFX-r2-Bs-dap-M</td>
<td>Bacillus subtilis /REF=L38424 /DEF=B subtilis dapB, jojF, jojG genes corresponding to nucleotides 2055-2578 of L38424 /LEN=1931 (-5, -M, -3 represent transcript regions 5 prime, Middle, and 3 prime respectively)</td>
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<tr>
<td>244940_at</td>
<td>rps12.2</td>
<td>Ribosomal protein S12 (trans-splice part 2 of 2)</td>
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<td>245016_at</td>
<td>accD</td>
<td>Carboxytransferase beta subunit</td>
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<td>247382_at</td>
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<td>1,4-benzoquinone reductase-like; Trp repressor binding protein-like</td>
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<td>248512_at</td>
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<td>248588_at</td>
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<td>250102_at</td>
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<td>Receptor-like protein kinase</td>
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<td>Cytosolic triosephosphataseomerase; supported by full-length cDNA: Ceres:28516.</td>
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<td>252055_at</td>
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<td>252293_at</td>
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<td>Ferulate-5-hydroxylase (FAH1); supported by cDNA: gi_1488254_gb_U38416.1_ATU38416</td>
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<td>H+ - transporting ATPase type 2, plasma membrane; supported by cDNA: gi_14334803_gb_AY035075.1_</td>
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<td>254049_at</td>
<td>At4g25740</td>
<td>Putative ribosomal protein S10 40S ribosomal protein S10 - Lumbricus rubellus, PID:e1329701; supported by cDNA: gi_14334535_gb_AY035172.1_</td>
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<td>Expressed protein; supported by full-length cDNA: Ceres:23587.</td>
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<td>2568575_at</td>
<td>At3g147790</td>
<td>dTDP-glucose 4,6-dehydratase, putative similar to dTDP-glucose 4,6-dehydratase GB:AE000666 GI:6626257 from [Methanobacterium thermototrophicum]</td>
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<td>ATP synthase subunit 9</td>
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<td>Glutamine synthetase, putative similar to Gln synthetase GB:228456 from [Arabidopsis thaliana]; supported by full-length cDNA: Ceres:5507.</td>
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<td>259343_s_at</td>
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<td>Putative methionine synthase similar to cobalamin-independent methionine synthase GB:AAC50037 [Arabidopsis thaliana]; supported by full-length cDNA: Ceres:111720.</td>
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<tr>
<td>262837_at</td>
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<td>Dynamin, putative similar to dynamin-1 SP:P21575 [Rattus norvegicus (Rat)]; supported by full-length cDNA: Ceres:12880.</td>
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<td>60s ribosomal protein 127a. similar to 60S RIBOSOMAL PROTEIN L27A GB:P49637 GI:1710530 from [Arabidopsis thaliana]; supported by full-length cDNA: Ceres:23092.</td>
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<td>At5g35730</td>
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<td>At5g11560</td>
<td>putative protein predicted proteins, Homo sapiens, Drosophila melanogaster, Caenorhabditis elegans</td>
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<td>At3g59540</td>
<td>60S RIBOSOMAL PROTEIN L38-like protein 60S RIBOSOMAL PROTEIN L38 - Lycopersicon esculentum, EMBL:X69979; supported by cDNA: gi_13605719_gb_AF361841.1_AF361841</td>
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<td>putative protein hypothetical protein T25K17.20 - Arabidopsis thaliana,PTR2:T06005; supported by full-length cDNA: Ceres:7308.</td>
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<td>At2g72020</td>
<td>unknown protein ; supported by full-length cDNA: Ceres:2031.</td>
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<td>At3g15640</td>
<td>putative cytochrome c oxidase subunit Vb similar to cytochrome oxidase IV GB:223590 [Bos taurus]; contains Pfam profile: PF01215 cytochrome c oxidase subunit Vb; supported by full-length cDNA: Ceres:34224.</td>
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<td>At1g53310</td>
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<td>At1g08325</td>
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<td>dihydrolipoamide S-acetyltransferase, putative similar to dihydrolipoamide S-acetyltransferase GL:5669871 from [Zea mays]; supported by cDNA: gi_14161721_gb_AY033001.1_</td>
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<td>At2g04900</td>
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<td>At2g37040</td>
<td>phenylalanine ammonia lyase (PAL1) ; supported by cDNA: gi_15028192_gb_AY045919.1_</td>
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<td>At1g12110</td>
<td>putative NPK1-related protein kinase 2 similar to nitrate chloride transporter GB:Q05085 from (Arabidopsis thaliana); supported by cDNA: gi_166667_gb_L10357.1_ATCH1L1A</td>
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<td>At2g37600</td>
<td>60S ribosomal protein L36 ; supported by full-length cDNA: Ceres:23114.</td>
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<td>At1g21750</td>
<td>putative protein disulfide isomerase precursor Similar to gbZ11499 protein disulfide isomerase from Medicago sativa. ESTs gblAI099693, gblRE65226, gblAA657311, gblTF40668, gblTF42754, gblTF4005, gblTF76445, gblH36733, gblTF43168 and gblTF20649 come from this</td>
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<td>At2g21020</td>
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<td>At2g37790</td>
<td>putative alcohol dehydrogenase</td>
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AFFX-Athal-Actin_M_at  AFFX-Athal-Actin_M  1 Arabidopsis thaliana /REF=U37281.1 /DEF=actin-2 mRNA, complete cds /LEN=1637 (._5, _M, _3 represent transcript regions 5 prime, Middle, and 3 prime respectively)

244959_s_at  orf107c  1.1 hypothetical protein
248088_at  At5g55070  1.1 2-oxoglutarate dehydrogenase E2 subunit ; supported by cDNA: gi_14596218_gb_AY042897.1

249882_at  At5g22890  1.1 putative protein contains similarity to C2H2-type zinc finger protein

250438_at  At5g10580  1.1 putative protein predicted protein, Arabidopsis thaliana; supported by cDNA: gi_14326488_gb_AF385697.1_AF385697

257823_at  At3g25190  1.1 integral membrane protein, putative contains Pfam profile: PF01988 integral membrane protein; similar to nodulin-21 GB:CAA34506 [Glycine max]; supported by cDNA: gi_14030610_gb_AF375396.1_AF375396

258008_at  At3g19430  1.1 putative late embryogenesis abundant protein similar to GB:AAB01570 from [Picea glauca]

258979_at  At3g09440  1.1 heat-shock protein (At-hsc70-3) identical to (At-hsc70-3) (cytosolic Hsp70) GB:CAAN76606 [Arabidopsis thaliana]; supported by cDNA: gi_15292924_gb_AY050896.1

259723_at  At1g60960  1.1 putative iron-regulated transporter similar to iron-regulated transporter 1 GB:AAD30548 from [Lyconcipersicon esculentum]; supported by full-length cDNA: Ceres:15980.

266044_s_at  At2g07725  1.1 citrate synthase similar to GB:X17528, 10 possible frameshifts in that submission; supported by full-length cDNA: Ceres:16528.

267368_at  At2g44350  1.1 NADH dehydrogenase 49KDa protein

245015_at  ndH_rbcL  1.2 large subunit of ribose-1,5-bisphosphate carboxylase/oxygenase

247983_at  At5g56630  1.2 pyrophosphate-dependent phosphofructo-1-kinase-like protein ; supported by cDNA: gi_14532861_gb_AY040055.1

251840_at  At3g54960  1.2 protein disulfide-isomerase-like protein protein disulphide isomerase, Fasciola hepatica

252927_at  At4g39090  1.2 cysteine proteinase RD19A identical to thiol protease SP:P43296, GI:435618 from [Arabidopsis thaliana]; supported by full-length cDNA: Ceres:31732.

256674_at  At3g52360  1.2 unknown protein ; supported by full-length cDNA: Ceres:31357.

262537_s_at  At1g17280  1.2 putative ubiquitin-conjugating enzyme First 212 a.a. are 41% identical to Ubiquitin-Conjugating Enzyme E2 [Saccharomyces cerevisiae] (gi|480374). Location of ests H36180 14702 Lambda-PRL2 cDNA clone 175C6T7 (gb|H36180) and H36169 14691 Lambda-PRL2 cDNA cl

263878_s_at  At2g22040  1.2 unknown protein

266705_at  At2g19750  1.2 40S ribosomal protein S30 ; supported by cDNA: gi_16974466_gb_AY061910.1

245003_at  psbC  1.3 PSII 43 KDa protein

245355_at  At4g17390  1.3 60S ribosomal protein L15 homolog ; supported by full-length cDNA: Ceres:31538.

245939_at  At5g19760  1.3 oxoglutarate/malate translocator-like protein oxoglutarate/malate translocator - Solanum tuberosum, PIR:T07405; supported by full-length cDNA: Ceres:19510.

246880_s_at  At5g25980  1.3 myosinase TGG2 ; supported by cDNA: gi_13507564_gb_AF360348.1_AF360348

251370_at  At3g60450  1.3 putative protein prib5, Ribes nigrum, EMBL:RNI7578; supported by full-length cDNA: Ceres:15792.

251787_at  At3g55410  1.3 2-oxoglutarate dehydrogenase, E1 subunit - like protein 2-oxoglutarate dehydrogenase, E1 subunit, Arabidopsis thaliana, EMBL:ART223802

259525_at  At1g12560  1.3 hypothetical protein

261729_s_at  At1g47840  1.3 hexokinase, putative similar to hexokinase 2 GB:AAB49911 GI:1899025 from [Arabidopsis thaliana]
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<td>unknown protein proline, tyrosine, and serine-rich protein</td>
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<td>264506_at</td>
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<td>germin-like protein Identical to Arabidopsis germin-like protein, gi</td>
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<td>superoxidase dismutase identical to GB:P24704;supported by full-length cDNA: Ceres:33493.</td>
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<td>glutamate--ammonia ligase ; supported by cDNA: gi</td>
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<td>membrane channel like protein ;supported by full-length cDNA: Ceres:99796.</td>
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<td>synaptic glycoprotein SC2-like protein synaptic glycoprotein SC2 spliced variant, Homo sapiens, EMBL:AF038958;supported by full-length cDNA: Ceres:6774.</td>
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<td>260557_at</td>
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<td>1.8</td>
<td>putative methylenetetrahydrofolate reductase ; supported by cDNA: gi</td>
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<td>1.9</td>
<td>putative protein similar to unknown protein (gb</td>
</tr>
<tr>
<td>251843_x_at</td>
<td>At3g54590</td>
<td>1.9</td>
<td>extensin precursor -like protein extensin precursor, Kidney bean, PIR:T10863</td>
</tr>
<tr>
<td>265443_at</td>
<td>At2g20750</td>
<td>1.9</td>
<td>beta-expansin ;supported by full-length cDNA: Ceres:109135.</td>
</tr>
<tr>
<td>247645_at</td>
<td>At5g60530</td>
<td>2.2</td>
<td>late embryonic abundant protein - like late embryonic abundant protein EMB7, white spruce, PIR:T09288; supported by cDNA: gi</td>
</tr>
<tr>
<td>260475_at</td>
<td>At1g11080</td>
<td>2.2</td>
<td>Serine carboxypeptidase isolog</td>
</tr>
<tr>
<td>244912_at</td>
<td>ccb382</td>
<td>2.5</td>
<td>cytochrome c biogenesis orf382 Protein sequence is in conflict with the conceptual translation</td>
</tr>
<tr>
<td>256647_at</td>
<td>At3g13610</td>
<td>2.8</td>
<td>unknown protein contains similarity to DNA-binding protein zyxin GB:X99063 GI:1430882 from [Mus musculus]</td>
</tr>
<tr>
<td>260226_at</td>
<td>At1g74660</td>
<td>3.3</td>
<td>hypothetical protein predicted by genefinder;supported by full-length cDNA: Ceres:14583.</td>
</tr>
<tr>
<td>262832_s_at</td>
<td>At1g14870</td>
<td>3.3</td>
<td>unknown protein</td>
</tr>
<tr>
<td>248049_at</td>
<td>At5g56090</td>
<td>3.8</td>
<td>putative protein contains similarity to cytochrome oxidase assembly factor</td>
</tr>
</tbody>
</table>

Note: Common constitutively expressed genes in both ‘Tahono’ and ECD04 when compared to ‘Granaat’ were **bolded**.