Pyramiding genes for clubroot resistance in brassica vegetable crops

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Pyramiding Genes for Clubroot Resistance in *Brassica* Crops

By

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The purpose of the report

At present, Clubroot (*Plamodiophora brassicae*) is a serious disease of *Brassica* vegetables worldwide. While progress has been made to control this disease via farming practices and the introduction of tolerant varieties, the level of resistance of *Brassica* vegetables, especially Chinese Cabbage to this disease remains low. Further, the genetic mechanisms controlling the resistance of certain vegetable Brassicas to Clubroot is only partially understood. The present study was initiated with the objective of combining Clubroot resistance genes from a variety of sources to improve the resistance of *Brassica* vegetables to this disease. This report summarises the outcomes of this investigation particularly on the discovery of genes responsible for the resistance/tolerance of *Brassica* vegetables to Clubroot.

CONTENTS

MEDIA SUMMARY

Clubroot, caused by the pathogen *Plasmodiophora brassicae* is one of the most serious diseases of vegetable Brassicas worldwide. Although resistant *Brassica* varieties are available, and classical inheritance studies have been performed, little is known of the genes responsible for such resistance. The aims of this study were to firstly study the gene expression of a number of resistant and susceptible varieties of Brassica, and secondly, use such information for marker-assisted selection for improved Clubroot resistance in vegetable Brassicas, especially Chinese Cabbage.

A hydroponic testing system was developed for the gene expression studies, as it provided a good system for obtaining root tissue free from soil contaminants. Two resistant, and one susceptible variety of *Brassica* were grown in a glasshouse in hydroponics for 4 weeks prior to inoculation with Clubroot spores. These hydroponic experiments were replicated four times over the period of a year. A small 'boutique' microarray with 75 defence-related gene sequences was constructed to determine a suitable time-point for harvesting root tissues after inoculation. Root tissues from the three varieties (mentioned above) were harvested at 24, 48 and 78 hours after inoculation (hai). The messenger RNA extracted from them were then reacted with the microarray. Analyses performed determined that the best time-point was 48 hai, which was therefore chosen for the Arabidopsis ATH1 studies.

The ATH1 studies did not provide strong evidence to support the hypothesis that the resistance to Clubroot displayed by the resistant varieties was the product of active defence mechanisms such as hypersensitivity. There was also some evidence to support the hypothesis that resistance may be due to the presence of 'pre-made' defence compounds in the roots, though further confirmation will have to be performed.

This study was able to provide new insights on the nature of resistance of vegetable Brassicas to Clubroot. However, further research is necessary to clarify the nature of resistance, due to the small number of varieties used, and the continuing uncertainty over the importance of the 'pre-made' defence compounds to the resistance response.

TECHNICAL SUMMARY

Clubroot, caused by the pathogen *Plasmodiophora brassicae* is one of the most serious diseases of vegetable Brassicas worldwide. Although resistant *Brassica* varieties are available, and classical inheritance studies have been performed, little is known of the genes responsible for such resistance. The aims of this study were to firstly study the gene expression of a number of resistant and susceptible varieties of Brassica, and secondly, use such information for marker-assisted selection for improved Clubroot resistance in vegetable Brassicas, especially Chinese Cabbage.

A hydroponic testing system was developed for the gene expression studies. This was necessitated by the initial difficulty of cleaning soil and other growing media from harvested infected root tissues. These contaminants were found to be detrimental to the mRNA extraction process, and also inhibited downstream processing of the mRNA, e.g. reverse transcription. Plants from 'Granaat' (susceptible), 'Tahono' (resistant) and ECD04 (resistant) were grown in a glasshouse in hydroponics for 4 weeks prior to inoculation with spores from a virulent isolate of *P. brassicae*. These hydroponic experiments were replicated four times over the period of a year. This design provided four biological replicates, each of which was exposed to a different daylength. The glasshouse temperature was kept constant at 22-25^{o}C throughout.

A small spotted Oligoarray, consisting of 75 defence-related cDNA sequences sourced from GenBank® was constructed to determine a suitable time-point for harvesting root tissues after inoculation. These experiments were essential due to budgetary constraints and the high cost of the Affymetrix® Arabidopsis ATH1 Genechip, as only one time-point could be investigated in these experiments. Root tissues from the three varieties (mentioned above) were harvested at 24, 48 and 78 hours after inoculation (hai) from each of the biological replicates. The extracted mRNA from these samples were subsequently hybridized on the Oligoarray, and analyses were performed to determine the best time-point. The results from these studies indicated that of the time-points used, 48 hai appeared to provide the best discrimination between the resistant, and susceptible varieties, and was therefore chosen for the Arabidopsis ATH1 studies.

The ATH1 studies did not provide strong evidence to support the hypothesis that the resistance to Clubroot displayed by Tahono and EC04 was the product of active defence mechanisms such as hypersensitivity, however, there was some evidence to suggest that the susceptibility observed in Granaat may be due to the repression of a class of transcription factors involved in the hydrogen peroxide signaling pathway (e.g. WRKY75, C_2H_2 Zinc-finger protein). There was also evidence to support the hypothesis that resistance may be due to the constitutive expression of myrosinase, the enzyme responsible for glucosinolate breakdown, and chitinase. However, subsequent enzyme assays did not corroborate the gene expression data.

This study was able to provide new insights on the nature of resistance of vegetable Brassicas to Clubroot. However, further research is necessary to clarify the nature of resistance, due to the small number of genotypes employed, and the continuing uncertainty over the importance of constitutive expression to the resistance response.

INTRODUCTION

Clubroot, caused by the soil-borne obligate biotroph *Plasmodiophora brassicae* Woronin **(Figure 1)**, is one of the most serious diseases of Brassica crops worldwide. In Australia, it is responsible for losses of at least 10 % in crucifer yield (Faggian et al., 1999), causing more than AUD$ 16 million in lost profits. Agricultural practices such as the application of lime to increase soil pH or control of the disease with agrochemicals can reduce the damage to crops but their effects are often insufficient to keep the plant healthy (Kuginuki et al., 1999). Moreover, the cost and practicality of current control measures can 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 resistance in *Brassica oleracea,* there have not been many successful breeding programs for resistance. The reason for such limitation is that Clubroot resistance in *B. oleracea* is often incomplete, hardly ever expressed at high level and is usually present in varieties that are unsuitable for production purposes (Crute et al., 1983). However, the main difficulties of breeding CR Brassica lines are the lack of information on the complex nature and precise genetic control of Clubroot resistance (Rocherieux et al., 2004). The distribution and mixed infection of multiple pathogenic races in single field is another setback (Buczacki et al., 1975).

Figure 1. Gall formation in Clubroot infected broccoli (*Brassica oleracea* var. italica)

Previously, Yoshikawa (1983) was able to develop a few resistant lines of Chinese cabbage using Clubroot-resistant (CR) lines in European fodder turnips. Recently, there have been reports of CR break-down since resistance in most European fodder turnips is controlled by a single dominant gene with some minor genes (Hirai et al., 2004). Therefore, the identification of a complete set of resistance genes and their linkage markers will provide valuable tools for the establishment of a successful CR breeding system. Pyramiding of disease resistance genes using DNA markers is one of the most promising fields in marker-assisted breeding (Huang et al., 1997). The breeding of phenotypically similar cultivars will not be easy. However, this is an ideal strategy to overcome the decay of CR for the long-term sustainability of the Brassica industry.

Crute et al. (1983) reported resistance against Clubroot in different cruciferous species, namely the commonly cultivated *Brassica napus*, *Brassica rapa* and *Brassica oleracea*. Other studies suggest that Clubroot resistance is under polygenic control and involves recessive (Voorrips and Visser, 1993) and dominant alleles (Grandclement et al., 1996). Fuchs and Sacristan (1996) have indicated that since a dominant allele of a single nuclear gene controlled Clubroot resistance in *Arabidopsis thaliana*, a single locus in *B. rapa* and *B. napus* was adequate to convey Clubroot resistance against. However, 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 resistant to race 2 of *Plasmodiophora brassicae* and the need of additional genetic element(s) to exhibit complete resistance. These results therefore suggest that Clubroot resistance involves a complex polygenic mechanism among *B. rapa* and other crucifers.

Suwabe et al (2003) have identified two loci namely *Crr1* and *Crr2* for Clubroot resistance in *B. rapa* that exist on different region of chromosomes or on different chromosomes. When both loci were homozygous, Clubroot resistance was stronger when compared to heterozygous loci. These researchers therefore suggested that Clubroot resistance in *B. rapa* is under oligogenic control and the cooperation of both loci is necessary to generate resistance in *B. rapa*. However, 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 (CR) marker. Soon after, Hirai et al. (2004) identified a third novel dominant CR locus named as *Crr3* in *B. rapa* through the use of sequence tagged-site (STS) markers developed from RAPD markers. Although the precise map position of Crr3 is unknown, this locus has been shown to be independent of the previously found CR loci *Crr1* and *Crr2*. Previously, Matsumoto et al. (1998) reported a CR locus, *Cr-A* in fodder turnip (Buczacki et al.'s (1975) ECD02) as a source of resistance. It is not known whether *Cr-A* matches to any of *Crr1, Crr2* and *Crr3* or is another independent CR locus. This therefore suggests the need for common linkage markers for the precise relationship of the identified CR loci.

As reviewed by Hirai et al. (2004), the occurrence of the three CR loci in *B. rapa* (*Crr1, Crr2* and *Crr3*) is not surprising since previous studies of CR in turnips did suggest the presence of three independent CR genes. As for *B. oleracea*, another diploid species whose genome size and structure are identical to *B. rapa*, several researchers could identified more than two CR loci. Since Fuchs and Sacristan (1996) have identified one CR locus in *Arabidopsis thaliana*, Hirai et al. (2004) suggested that these CR loci identified in all previous studies may be derived by duplication. 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. Therefore, whether the CR loci found in crucifer are homologous has yet to be determined.

Originally, it was thought that quantitative trait loci (QTL) analysis in *B. oleracea* possessed a limited number of genetic factors involved in resistance. Moriguchi et al (1999) was able to identify three QTLs from naturally infected crops in the fields and the most effective QTL explained 30 % of the total phenotypic variation. When other experiments were performed under controlled conditions using field isolates, only 1 to 2 QTLs were involved in Clubroot resistance (Figdore et al., 1993; Voorrips et al., 1997). However using a genetic map constructed by random fragment length polymorphism (RFLP), random and specific PCR-based markers, Rocherieux et al., (2004) have identified a total of nine CR–related genomic regions. These were involved in isolate-specific and broad-specific resistance in the control of Clubroot in *Brassica oleracea*. Of the nine QTLs identified, one was involved against all the isolates while the others were specific to 1, 2 or 3 isolates and depending on the isolates; the degree of the QTL effect was variable. It is suggested that once major resistance genes are defeated by a pathogen strain, these genes may still possess some residual effect. However, the accumulation of these residual effects can give rise to quantitative resistance. Since most studies performed on *B. oleracea* have identified non-specific resistance, it is possible that several isolate-specific genes with quantitative effect control this form of resistance (Crute et al., 1983). Other studies have supported this argument as reviewed by Rocherieux et al. (2004).

Since no highly resistant varieties of Chinese cabbage was available, Yoshikawa (1981) bred Clubroot resistant (CR) lines of Chinese cabbage by introducing a resistant gene from a CR European turnip. Subsequently, more than 50 CR F1 hybrid (F1) cultivars of Chinese cabbage have been released in Japan. However, there have not been many successful breeding programs for resistance resulting in the CR Chinese cabbage 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).

The differences in the pathogenicity of *P. brassicae* isolates were determined by the extensive use of the differential series by Buczacki et al. (1975) in Europe and North America. Many studies have recognised that considerable differences in pathogenicity exists between field populations and even within field isolates (Buczacki et al., 1975)**.** The results generated from these tests therefore suggested a complex nature of the interaction of the *P. brassicae* populations and *B. oleracea* resistance genes, hence another reason for the inefficient deployment of CR varieties. 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. Therefore 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). 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).

However, 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 therefore, a clearer picture of the mechanism involve can be obtained (Rocherieux et al., 2004). Piao et al. (2004) have encountered different results when single spore isolates (SSI) and contaminated soil were tested. They reported that there was a high resistance in the plant hosts when SSI was used while those inoculated with field isolates were either high or intermediately resistant. Hence, the use of SSI is a pre-requisite for the better understanding of the complex interaction occurring during an infection and for the accurate scoring of Clubroot resistance. However, the routine examination of the virulence of a large number of genetically uniform SSI collections may not be feasible due to the time required for their isolation and characterisation and the variable success of the SSI method.

For the successful establishment of a CR breeding system, the identification of a complete system and information on the complex plant-pathogen relationship are required (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 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. In terms of Clubroot resistance, DNA markers should be suitable for use in MAS programs in Chinese cabbage since the need for inoculation and detection of symptoms is removed and hence, avoiding the genetic variation among races of *P. brassicae* (Piao et al., 2004).

Several research groups have developed DNA markers linked to Clubroot resistance loci in Brassica crops. For example, a number of RAPD and RFLP 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 amplified fragment linked polymorphism (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). However, complexity and high cost of this technique make the AFLP technique unsuitable for high-throughput selection such as in MAS. On the other hand, a PCR-based marker is much simpler and affordable. Therefore, 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 a sequence characterised amplified region (SCAR) markers. Recently, micro-satellites or simple sequence repeats (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 more preferable as DNA markers than RFLPs, AFLPs and RAPDs since they are inherited co-dominantly and can be analysed in a PCR-based system (Morgante and Olivieri, 1993).

For several qualitative traits in Brassica breeding programs in general, MAS strategies were developed by traditional mapping approaches. However for quantitative traits, the mapping of QTL is often not sufficient in the development of efficient DNA markers for the identification of genes of interest. These markers derived from QTL are not necessary transferable to other material and the genetic distance between the markers and the QTL are usually physically

very large (Snowdon and Friedt, 2004). Hence, MAS for quantitative traits has not been successfully achieved to date. Single nucleotide polymorphisms (SNPs) offer great potential due to their high abundance and the possibility for an extremely fine genetic mapping. 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. SNPs are originated form single-based substitutions in the DNA sequences and are the most common form of DNA polymorphism in most organisms. However, SNPs cannot be identified through gel electrophoresis and detection protocols involve target sequence PCR amplification with the help of fluorescent labelling technologies such as microarrays and/or enzymatic assays (Snowdon and Friedt, 2004).

A novel approach by Snowdon and Friedt (2004) in the high-throughput detection of SNPs involved the use of matrix-assisted laser desorption/ionisation time of flight mass spectrometry (MALDI-TOF MS). Although, these techniques and equipment are beyond the scope of most plant breeders, it is expected that SNP markers will play a major role in Brassica breeding in the coming years when such technologies become more available and cost-effective. However, a financially more accessible technique for the detection of SNPs is through microarrays. This technique has become one of the most extensively used functional genomics tools, enabling researchers to simultaneously examine changes in the expression of thousands of genes (Freeman et al., 2000). For example, this technique has been used in the analysis of diseased versus normal tissues in Arabidopsis (Schenk et al., 2000) and in the study of gene regulation during strawberry fruit development (Aharoni and O'Connell, 2002). This technique is based on the immobilisation of the gene-specific sequences (probes) onto a solid matrix and the application of labelled nucleic acids (targets) from the biological samples (Holloway et al., 2002). It is a relatively new approach and as yet, there has been no report on the use of this technique in the search for Clubroot resistance in Brassica. Microarrays may explain the complex sequence of gene activation in the hosts during Clubroot infection and ultimately in the development of MAS markers.

The present study was initiated with the following objectives:

- To investigate the genetic mechanisms responsible for Clubroot resistance in vegetable Brassicas
- To develop molecular markers, specifically SNPs for marker-assisted breeding of Clubroot-resistant varieties of Chinese Cabbage and other vegetable Brassicas.

This report summarises the outcomes of this investigation particularly on the discovery of genes responsible for the resistance/tolerance of *Brassica* vegetables to Clubroot.

MATERIALS AND METHODS:

1. Gene expression profiling for *Plasmodiophora brassicae* resistance using the RMIT *Brassica* oligoarray.

1.1 Synthesis of the *Brassica* oligonucleotide probes

A list of *Brassica* and *Arabidopsis* genes mostly involved in defence & disease resistance, stress & hormone management and general housekeeping were searched from the nucleotide database GenBank®. A total of 75 cDNA gene sequences were then used to design 150 26-mer oligonucleotide probes using the Clone Manager Professional Suite® (Version 7, Scientific and Educational Software™, USA). The software would generate a list of potential probe sequences per cDNA sequence from GenBank®; however, only 2 probes were selected based on their ranks and proximity to the 3' (Probe A) and 5'(Probe B) ends **(Appendix 1)**. The selected probes had a G/C content range of 50-55 % and melting temperature range of 65-75 ºC. These oligonucleotide probes were then 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).

1.2 Printing of the RMIT *Brassica* oligoarray

The oligoarray was printed using the BioRobotics® MicroGrid II Compact printer at the RMIT University (Bundoora, VIC). The preparation and resuspending of the probes along with the post-printing procedures were performed according to the glass slide manufacturer's guide (Amersham Biosciences, 2003).

1.3 Plant Material

The Clubroot tolerant *Brassica rapa* hybrids 'Tahono' CR-1-1 and 'Leaguer' CR 1052 provided by Henderson Seed Group Pty Ltd together with the highly susceptible 'Granaat' (ECD05 from the ECD set) were used as a source of resistance/susceptibility to study the defence mechanisms in Chinese cabbage against Clubroot disease.

1.4 Resistance test using a hydroponics system

The hydroponics system:

A hydroponics system was established as a novel technique in studying the defence mechanism against Clubroot in *Brassica* vegetables. This system was based according to Coram and Pang (2007) and is illustrated in **Figure 2**. Each hydroponics tank was able to accommodate a maximum of 24 seedlings and to generate enough root tissue; two tanks were used per *Brassica* line, i.e. the control tank and the treatment tank (one biological replicate)**.**

Maintenance of the hydroponics system:

The hydroponics 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. Maintenance of the hydroponics system involved refilling the tanks up to 6 L with distilled water every 2-3 days. However 3 days before inoculation of the treatment seedlings with isolate S, the hydroponics 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™ garden insecticide spray aerosol (Yates™, NSW) was applied once about 2 weeks after germination to control/treat insect attacks.

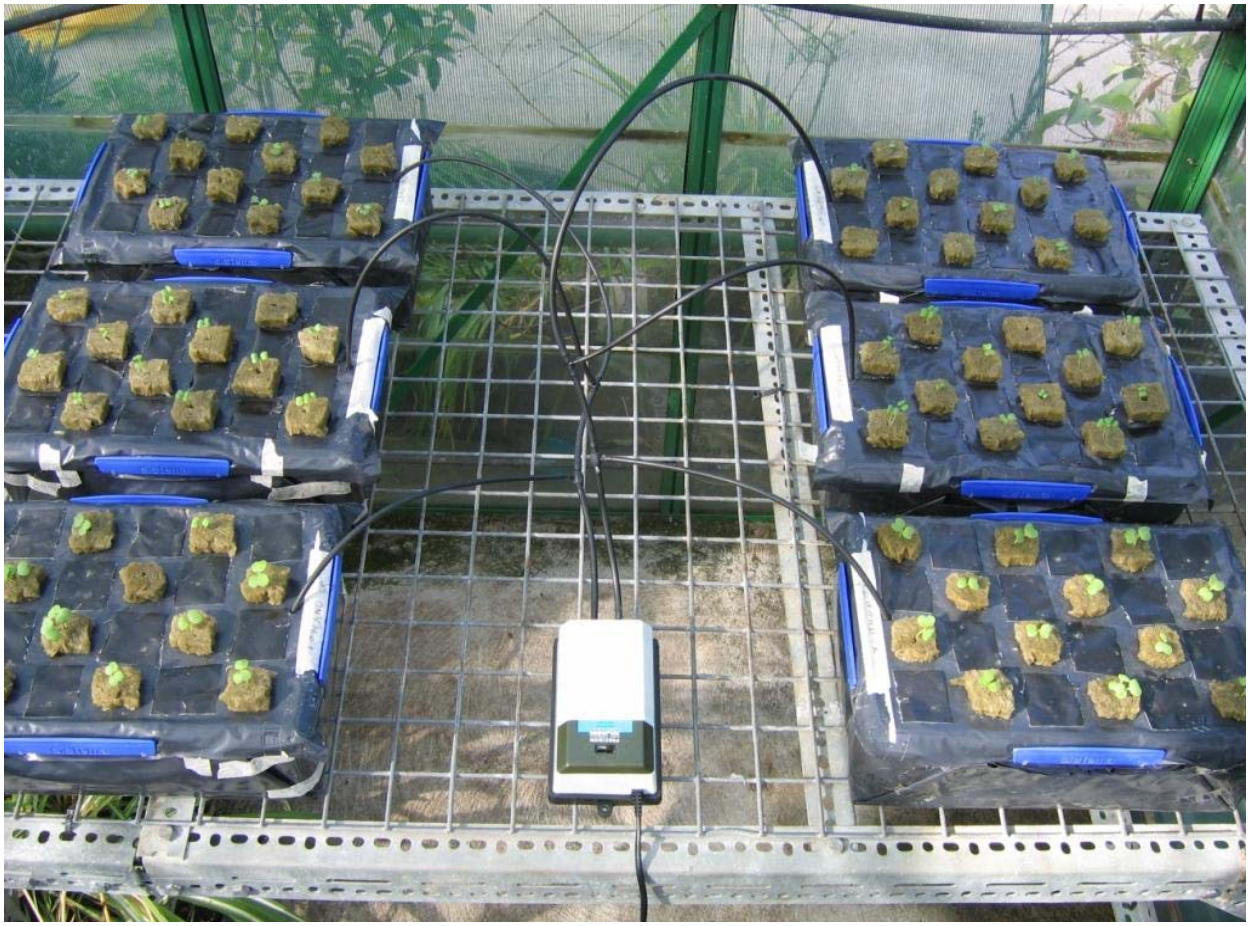


Figure 2. The setup of the hydroponics system with control (left) and treatment (right) tanks using the Clubroot tolerant Chinese cabbages 'Tahono' and 'Leaguer' and the susceptible 'Granaat'.

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 2.5×10^9 spores per mL of Clubroot isolate S. In contrast, 1 mL of MilliQ water was applied to each seedling of the control hydroponics tanks. Root collection was performed at 1, 2 and 3 days after inoculation using at least three plants for each control and treatment *Brassica* line. These plants were pooled respectively and rinsed in cold tap water before being frozen in liquid Nitrogen and stored at – 80°C. The remaining plants were allowed to grow in their respective hydroponics tanks and were examined 4 and 8 weeks after inoculation to verify for the presence of Clubroot infection.

1.5 Preparation of total RNA

The total RNA was extracted and purified according to Qiagen™ RNeasy® Plant Mini Kit (Qiagen, 2006). The concentration of total RNA was estimated by measuring the absorbance at 260 nm (A_{260}) in an Eppendorf® BioPhotometer while the integrity and size distribution of RNA was estimated by agarose gel electrophoresis.

1.6 Preparation of fluorescent labelled-cDNA targets

Fluorescence labelled-cDNA targets were prepared based on the Australian Genome Research Facility (AGRF) microarray protocols and recent publications (Coram and Pang, 2006; Mantri *et al.*, 2007) . In brief, it involved reverse transcription of the total RNA, labeling of the

cDNA with fluorescent dyes, purification of the labeled-cDNA and hybridization & washing of the RMIT *Brassica* oligoarray.

1.7 Analysis of the RMIT *Brassica* Oligoarray

The analysis of the oligoarray was performed according to recent publications (Coram and Pang, 2006; Mantri *et al.*, 2007). In brief, it involved scanning of the arrays using the Affymetrix® 428™ Array Scanner with the Affymetrix® Jaguar™ software (v2.0, Santa Clara, CA), quantification of the spot intensities using the BioDiscovery ImaGene™ software (v5.5, Marina Del Rey, CA) **(Figure 3)** and statistical analysis using the BioDiscovery GeneSight® software (v4.1.3, Marina Del Ray, CA).

1.8 Validation of the microarray data by quantitative real-time PCR (qRT-PCR)

The microarray expression results were validated by quantitative real-time PCR (qRT-PCR) on a set of genes from the list of differentially expressed cDNA. This set was chosen to represent different defence responses and expression values (up/down regulation). The primers were designed using the Clone Manager Professional Suite (Version 7, Scientific and Educational Software™, USA). The relative standard curve method was used with the actin gene as a reference. For each *Brassica* line/time-point, 5 μg of total RNA from one of the biological replicate was reverse transcribed into cDNA using oligo$(dT)_{15}$ primer (Roche™) and the Superscript II Reverse transcriptase Kit (Invitrogen™). The resulting cDNA samples were purified using the Qiagen™ Qiaquick PCR purification kit and used as template in the qRT-PCR. The amplification and standard curves generated by the iCyclerIQ™ Multi-colour Real Time PCR detection system (Bio-Rad, Hercules, CA) were used in data analysis while the melting curves showed the presence of single amplicons.

(a) (b) (c)

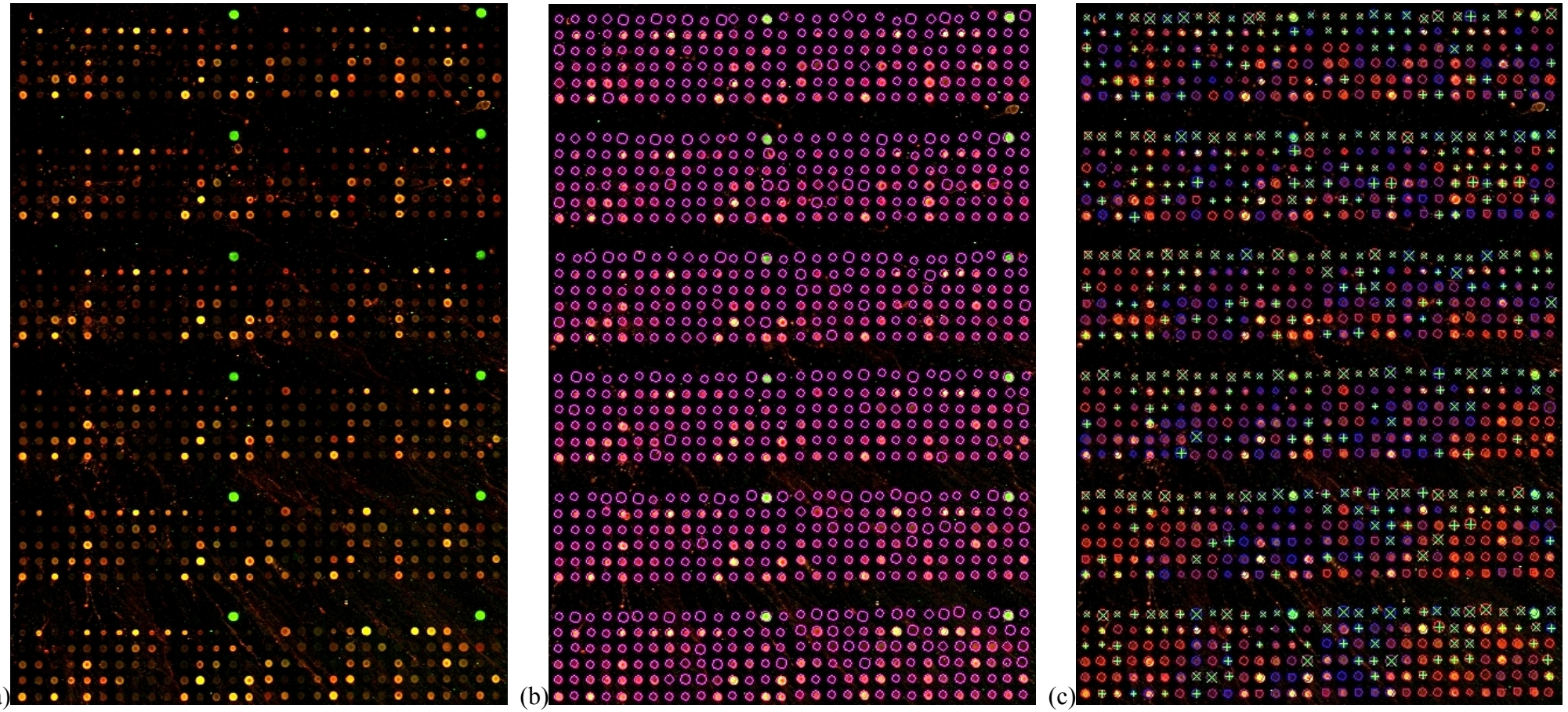


Figure 3. Analysis of the scan images using the Affymetrix™ ImaGene® software: (a) The composite image of the control and treatment 'Tahono' samples 48 h after infection, (b) Positioning of the grid onto each sub-grid of the array (6 technical replicates) before quantification of the spot intensities and (c) Flagging to screen out low quality/intensity spots.

2. Gene expression profiling for *Plasmodiophora brassicae* resistance using the Affymetrix® *Arabidopsis* ATH1 Genome Array

2.1 Preparation of total RNA

The total RNA from the Clubroot tolerant 'Tahono' and susceptible 'Granaat' 48 h after inoculation were extracted as in **Section 1.5.** In addition, another genotype 'ECD04' from the ECD set was included since previous tests indicated it could actually be Clubroot resistant. These total RNA samples were sent to the Australian Genome research Facility (AGRF, VIC, Australia) and were quality ascertained using the Agilent Bioanalyser 2100 according to the manufacturer's guidelines (Agilent Technologies, 2005).

2.2 Affymetrix® *Arabidopsis* ATH1 array processing

The Affymetrix® *Arabidopsis* ATH1 Genome Array was processed according to the manufacturer's instructions (Affymetrix, 2004a) and was performed by the AGRF personnel as a paid service. In brief, it involved the addition of Poly-A RNA controls to the total RNA, synthesis & cleanup of the double-stranded cDNA, synthesis & fragmentation of the biotin-labeled RNA and finally, hybridization onto the Affymetrix® *Arabidopsis* ATH1 array. The arrays were then washed and stained with SAPE as illustrated in **Figure 4**

4.3 Data analysis

Using the Affymetrix GeneChip® Scanner 3000 operated by the Affymetrix GeneChip® Operating Software v1.4.0.036, the labelled-arrays were scanned and the scan images were saved as a CAB file for analysis. Before the quantification of the spot intensities (**Figure 5**), it was necessary to flag those signals derived from artefacts on the scan image and positioning of the grid for optimal spot recognition. The absolute analysis was performed on each array and scatter plots would determine whether the biological replicates data may be pooled and analysed together **(Figure 6)**. Differentially expressed genes were identified by comparative analysis using their detection call (present or absent) and signal log ratio between the control and treated samples for each *Brassica* line ('Tahono', 'Granaat' and ECD04) at 48 h after inoculation with isolate S. The parameters used in this analysis were optimised from the manufacturer's technical manuals (Affymetrix, 2004b, a, c) and previous studies (Raghavan *et al.*, 2005; Madhou *et al.*, 2006). In contrast, constitutively over-expressed genes were identified by comparing the control of either 'Tahono' or 'ECD04' vs. the control of 'Granaat'.

4.4 Validation of the Affymetrix data by qRT-PCR and enzyme assays.

Quantitative Real Time PCR:

The qRT-PCRs were performed as in **Section 1.8** using a set of genes from the list of resultant differentially expressed or constitutively over-expressed cDNA. This set was chosen to represent different defence responses and expression values.

Enzyme assays:

Initially, crude root tissue extracts for each genotype were obtained according to Sela-Buurlage *et al.* (1993) and Cota *et al.* (2007). In brief, it involved grinding the root tissues in cold lysis buffer (100mM Sodium acetate, pH 6, 1% (v/v) β-mercaptoethanol), filtration of the homogenate using miracloth (Calbiochem™) and ultrafiltration using the 10 kDa Amicon® ultra-4 centrifugal filter devices (Millipore™). The total protein concentration was determined by the Bradford (1976) method using the BioRad™ Protein Assay kit and the integrity was showed by electrophoresis on 12% polyacrylamide SDS gel.

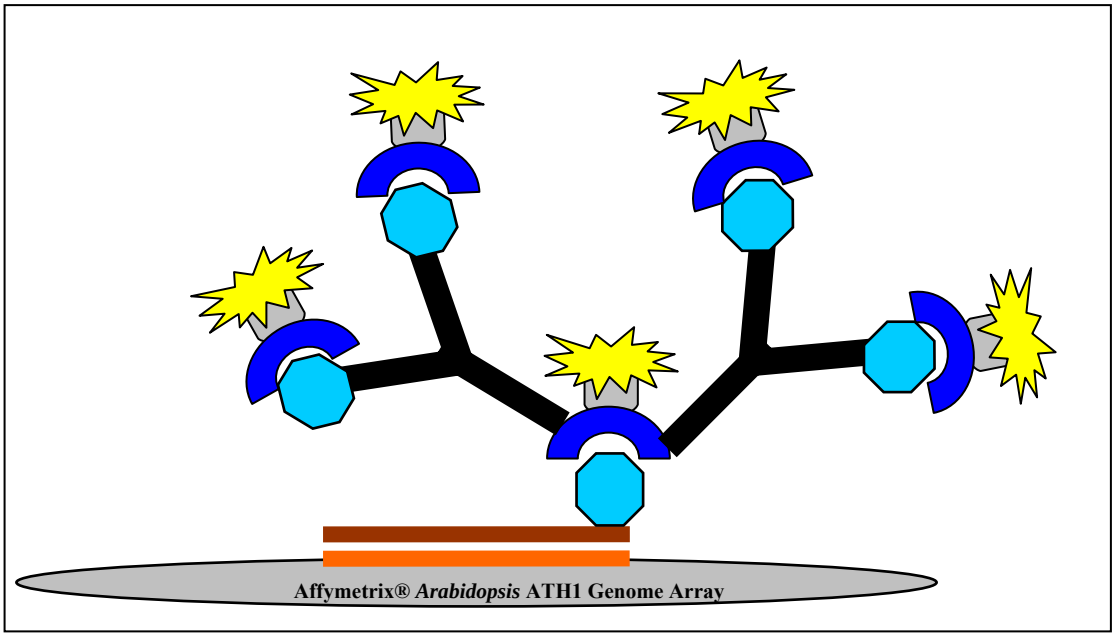


Figure 4. Schematic drawing of the principle of staining and amplification of the signal intensities (Modified from Raghavan (2004)).

Legend:

Affymetrix® oligonucleotide probe: , Biotin-labelled cRNA target: ,

SAPE: , Biotinylated anti-streptavidin antibody: , Biotin molecule: ,

Streptavidin: and Phycoerythrin:

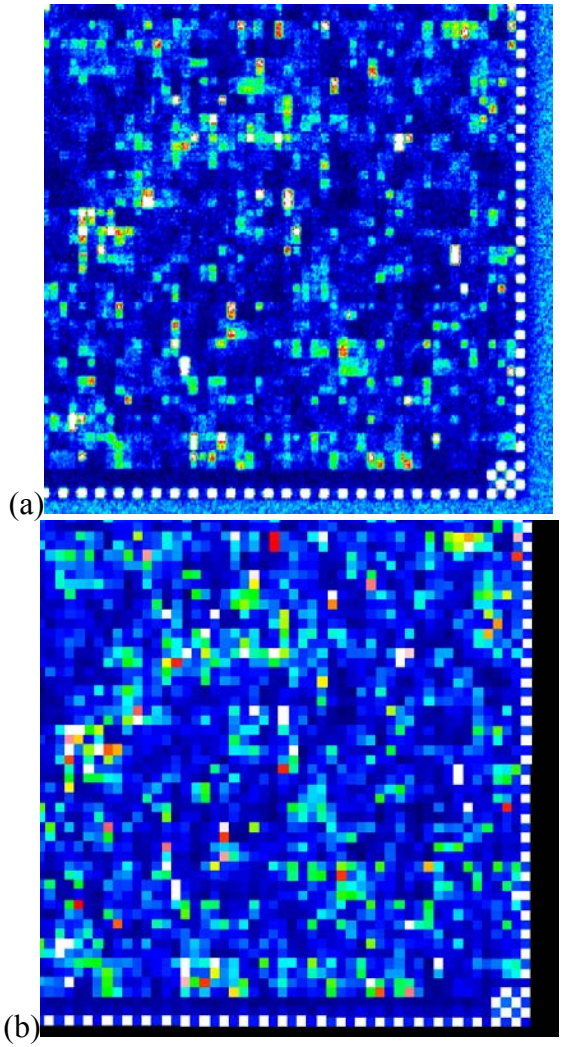


Figure 5. Quantification of the Affymetrix scan images (a) Scanned measured image of the genechip and (b) Computed averaged signal intensity image.

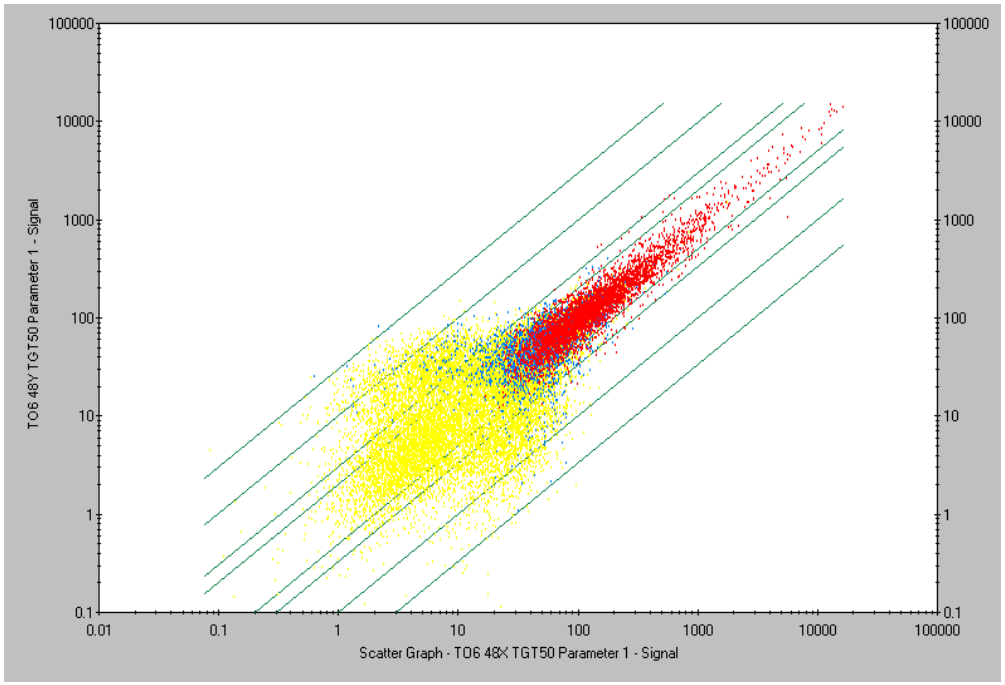


Figure 6. Scatter plot of 2 biological replicates of 'Tahono' 48 h after Clubroot inoculation. The narrower the spread of the scatter plots, the lower the variability between the biological replicate.

The total chitinase activity of each crude root tissue extract was determined by a fluorescence spectroscopic enzyme assay according to Hung *et al.* (2002) and Cota *et al.* (2007) using 4-methylumbelliferyl β-D-N,N',N'-triacetylchitotrioside hydrate [4-MU-β-$(GlcNAc)_3$] as substrate (Sigma™, Cat. No. M5639). The total myrosinase activity of each crude root tissue extract was determined according to Siemens and Mitchell-Olds (1998) and Hara *et al.* (2000) by measuring the release of glucose using sinigrin hydrate as substrate (Sigma™, Cat. No. 85440). Standard curves of the products were constructed to calculate the enzyme activity (product formed per min per mg of total protein).

RESULTS:

1.1 The hydroponics system

A hydroponics system was established as a novel technique in studying the defence mechanism against Clubroot in *Brassica* vegetables. It provided significant advantages over a soil-based system such as rapid growth resulting in abundant and 'clean' root tissues **(Figure 7)**, which are required for high purity, quantity and integrity of total RNA. In addition, this system was successfully used to infect and test the *Brassica* lines as demonstrated in **Figure 8** due to the formation of Clubroot symptoms 8 weeks after inoculation with isolate S as opposed to healthy growth of the controls. This system has been optimised in terms of the spore concentration, constituents & concentration of the hydroponics solution, timing of pesticide application and tissue collection.

(a) 

(b) 

Figure 7. The advantages of a hydroponics over a soil-based test system: (a) The rapid growth and difference in morphology of 28-days old Chinese cabbages in hydroponics solutions (X) and soil-based media (Y), and (b) The abundant growth of 'clean' root tissues in a hydroponics system essential for total RNA extraction.

Figure 8. Healthy (left) vs. diseased (right) roots caused by *Plasmodiophora brassicae* in 'Granaat' (top) and 'Leaguer' (bottom) eight weeks after inoculation with isolate S under the hydroponics system.

Due to the time and space demanding nature of these hydroponics tests, it was necessary to perform them in a staggered manner as illustrated in **Table 1**. In brief, there were 3 biological replicates performed through time (Experiment 2, 3 and one replicate of 4) as well as through space (three replicates of experiment 4) for each *Brassica* line and tissue collection at 1, 2 and 3 days after inoculation with isolate S. Moreover, the resulting Clubroot symptoms were scored to ensure a positive infection and to investigate their level of Clubroot resistance in this system.

Table 1. Symptoms[a] occurring in the *Brassica* vegetables 4 & 8 weeks after inoculation with isolate S under the hydroponics system.

Experiment	Location	Date of inoculation	Season in Victoria	Time points (day)	Biological replicate
1	Growth-room	30th Sep 2006	Early Spring	7, 14, 22 & 31	1
2	Glasshouse	7th Nov 2006	Late Spring	0.5, 1, 2 & 7	1
3	Glasshouse	21st Feb 2007	Late Summer	0.5, 1, 2 & 3	1
4	Growth-room	7th May 2007	Late Autumn	1, 2 & 3	3

Experiment	Symptoms 4 weeks after inoculation:			Symptoms 8 weeks after inoculation:		
	Tahono	Leaguer	Granaat	Tahono	Leaguer	Granaat
1	2	2	3	*NA*	*NA*	*NA*
2	0	2	3	*NA*	*NA*	*NA*
3	1	1	2	*NA*	*NA*	*NA*
4	1	3	3	3	3	3

[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 & 3 = severe clubbing.
NA, data not available.

At 4 weeks after inoculation, Tahono had less severe disease symptoms when compared to Leaguer and Granaat. However, at 8 weeks after inoculation, all of the genotypes succumbed to the infection. Moreover, it seems that infection in Experiment 3 was sub-optimal due to the lack of heavy clubbing in Granaat and therefore the data resulting from this biological replicate may affect negatively affect the analysis.

1.2 Analysis of the RMIT *Brassica* Oligoarray

For reliable analysis of the microarray data, it was essential that the construction and hybridisation of the oligoarray were optimised in terms of: printing conditions (e.g. humidity to prevent donuts spots), post-printing steps, pre-hybridisation steps and hybridisation temperature (to minimise non-specific binding) and washing steps (to reduce the background).

The scan images were then quantified and analysed to identify genes that were differentially expressed (DE) (with $\log_2$ difference $\geq$ or ≤ 0.7, i.e. those genes that showed at least a 1.6 fold change in expression). However, previous studies (Coram and Pang, 2006; Mantri et al., 2007) have used a threshold of 2 folds and further analysed their data using the False Detection Rate (FDR) method to identify reliable DE genes. This stringent analysis was not performed in this study due to limited data acquired after combining the results from the 3 biological replicates.

Those genes were tabulated for each *Brassica* line and time points as below:

Table 2. List of differentially expressed genes in 'Tahono' 48 h after inoculation with isolate S for hydroponics experiment 2, 3 and 4.

Gene ID	GenBank Accession	Putative function	cv	Log_2 Difference
BA025A	DR997831	cytokinin-binding protein	-0.4034	-1.0512
BA024B	DD182413	Clubroot resistant marker	-0.7448	-0.9118
BA037B	AY669802	IAA31	-1.3245	-0.7089
BA074A	NM123599	Ubiquitin-protein ligase	0.9325	0.8327

Table 3. List of differentially expressed genes in 'Tahono' 48 h after inoculation with isolate S for the 3 biological replicates of hydroponics experiment 4.

Gene ID	GenBank Accession	Putative function	cv	Log_2 Difference
BA032B	AI352707	Glutathione S-transferase	-0.3139	-0.9731
BA025A	DR997831	cytokinin-binding protein		-0.7568
BA034A	AI352735	Hypersensitive response	-0.52	-0.7179
BA044B	AW288083	Mannitol Stress inducible	1.2566	0.7974

Table 4. List of differentially expressed genes in 'Granaat' 48 h after inoculation with isolate S for hydroponics experiment 2, 3 and 4.

Gene ID	GenBank Accession	Putative function	cv	Log_2 Difference
BA054A	AI352935	Nitrilase I	-1.2251	-0.8156
BA034B	AI352735	Hypersensitive response gene	-0.5669	-0.5863
BA020B	AF230684	Chitinase	0.5289	0.6571
BA025A	DR997831	Cytokinin-binding protein		0.7266

Table 5. List of differentially expressed genes in 'Granaat' 48 h after inoculation with isolate S for the 3 biological replicates of hydroponics experiment 4.

Gene ID	GenBank Accession	Putative function	cv	Log_2 Difference
BA078B	H07799	Xyloglucan endo-transglycosylase	-0.5528	-0.6438
BA034A	AI352735	Hypersensitive response gene	-0.6207	-0.5218
BA074A	NM123599	Ubiquitin-protein ligase	1.5135	0.683

Table 6. List of differentially expressed genes in 'Tahono' 72 h after inoculation with isolate S for hydroponics experiment 2 and 4.

Gene ID	GenBank Accession	Putative function	cv	Log_2 Difference
BA017B	AY344061	Accelerated cell death 1		-2.0132
BA029B	AI352905	Ethylene, HEVER and SA-inducible protein	-0.7387	-0.84
BA066B	X59984	Ribosomal protein S15a	1.6341	0.3935

There was a lack of differential expression in all the *Brassica* lines at 24 hai (hours after inoculation), for Leaguer at all time points and for Granaat at 72 hai. The tables for 48 hai were constructed from both the 3 biological replicates through time and space for both Tahono and Granaat for comparison.

1.3 Validation of the microarray data by quantitative Real Time PCR (qRT-PCR)

Four genes with different expression values were selected representing different genotypes and time points. The standard curve method was used to calculate the fold-change values and was constructed using the actin gene **(Figure 9)**. **Figure 10** showed an example of amplification curves and CT values determination. The melting curve analysis showing single peak **(Figure 11)** and gel electrophoresis indicated specific amplification of single product. Most of the genes revealed similar expression pattern for microarray and qRT-PCR values for fold change **(Table 7)**.

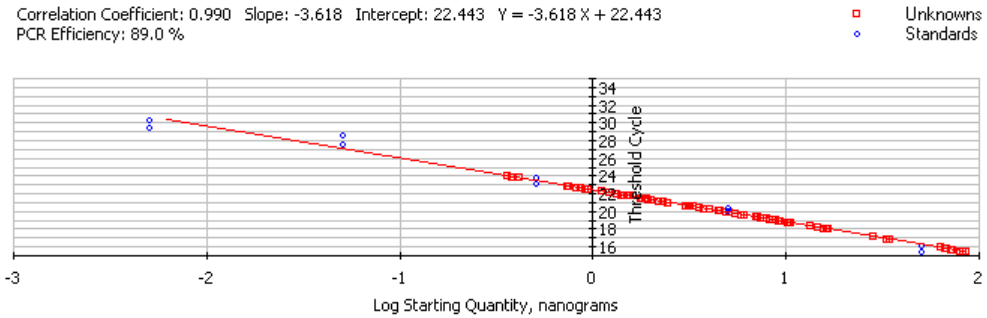


Figure 9. Example of the standard curve generated by the iCyclerIQ™ Multi-colour Real Time PCR detection system (Bio-Rad, Hercules, CA) using serial dilutions of actin target (blue circles).

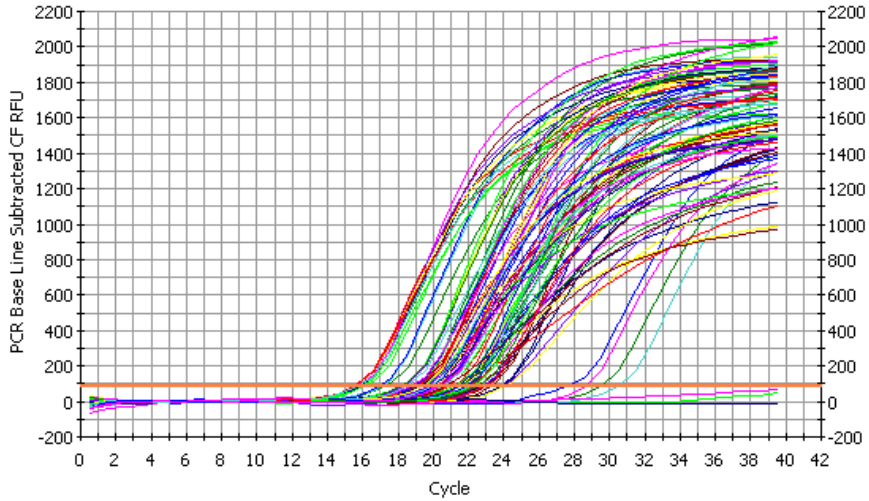


Figure 10. Example of amplification curves (coloured lines). The solid orange line represents the threshold used to calculate C_T values.

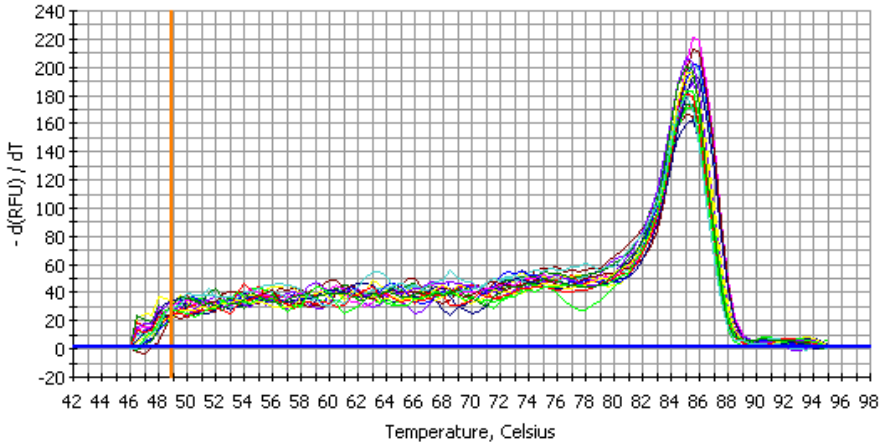


Figure 11. Example of melting curves. The solid orange line represents the threshold used to calculate C_T values. The presence of sharp single fluorescence peaks for actin target in this example indicated the presence of single amplicons.

Table 7. Expression ratios of selected transcripts assessed by microarray and qRT-PCR.

GenBank® accession number	Putative function	Granaat 48h					Tahono 48h				
		Array1		Array2		qRT-PCR	Array1		Array2		qRT-PCR
AI352707	Glutathione S-transferase	0.17	-0.17	-0.01	-0.01	0.54	0.25	-0.30	0.22	-0.97	0.34
AY156708	Xyloglucan endo-transglycosylase precursor	0.16	-0.32	0.05	-0.18	-0.17	0.35	0.28	0.10	-0.08	0.43
AY055752	Phenylalanine ammonia-lyase	0.13	*NA*,	-0.07	*NA*,	-0.02	0.05	*NA*,	0.13	*NA*,	0.79
AF230684	Chitinase	0.35	0.66	0.03	0.17	-0.19	0.25	0.48	0.26	0.3	1.90

NA, data not available due to absence of signal detection during analysis

Note:
Both array and qRT-PCR values indicate mean $\log_2$ fold change relative to untreated controls.

Values for Array1 were compiled from the biological replicates of hydroponics experiments 2, 3 and 4 while that of Array2 were derived from the 3 replicates of experiment 4. Moreover, 2 values were included per array to demonstrate the $\log_2$ fold change for probe A (left) and probe B (right).

1.4. Analysis of the Affymetrix® *Arabidopsis* ATH1 Genome array

1.4.1 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 shown in **Tables 8-10**. 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 11**. 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 clubroot-tolerant '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 'Tohano' 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 12)**. 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 11**. In brief, the only defence-related gene to be up-regulated was 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 11**) 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.

Table 8. Selection of differentially expressed genes with reliable expression in all three replicated arrays of 'Granaat' at 48 hai.

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

Table 9. Selection of differentially expressed genes with reliable expression in all three replicated arrays of 'Tahono' at 48 hai.

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

Table 10. Selection of differentially expressed genes with reliable expressions in all three replicated arrays of 'ECD04' at 48 hai.

Selection of UP-regulated genes	Increase	Selection of DOWN-regulated genes	Decrease
Total number of genes on the array	22,810	Total number of genes on the array	22,810
No. of genes called 'present' all replicates of Treatment	3,083	No. of genes called 'present' in Control	4,637
No. of genes called 'increase' in at least 2 replicates	43	No. of genes called 'decrease' in at least 2 replicates	29
No. of genes with SLR* $\geq$ 0.8 in all biological replicates	2	No. of genes with SLR* $\leq$ -0.8 in all biological replicates	2
No. of genes with a mean signal intensity $\geq$ 100 in Treatment	1	No. of genes with a signal intensity $\geq$ 100 in Control	1
% Significant Increase	0.03	% Significant Decrease	0.02

Table 11. Selected list of genes differentially expressed at 48 hai (sorted by putative function).

Probe Set ID	Locus Identifier	Mean SLR[a]			Putative function (on August 2007)	GO term (on March 2009)	Code
		GR[b]	TO[b]	ECD04[b]			
256129_at	At1g18210	-1.33	–	–	Calcium-binding protein	• Calcium ion binding	ISS
						• Unknown process	ND
252679_at	**At3g44260**	-1.20	-1.53	–	**CCR4-associated factor 1-like protein**	• Ribonuclease activity	ISS
						• Response to biotic stimulus	IEP
244950_at	cox2	–	-1.33	–	Cytochrome c oxidase subunit 2	• Unknown	ND
248964_at	At5g45340	-1.97	-1.13	–	Cytochrome P450	• Hydrolase activity	IDA
						• Abscisic acid catabolic process	TAS
247543_at	At5g61600	-1.43	-1.63	–	DNA binding protein - like DNA binding protein EREBP4	• Transcription factor activity	ISS
						• Regulation of transcription	ISS
247199_at	**At5g65210**	–	-1.00	–	**DNA binding protein TGA1a homolog**	• Calmodulin binding	ISS
						• Transcription factor activity	ISS
						• Defence response to bacterium	IMP
253088_at	**At4g36220**	–	-1.57	–	**Ferulate-5-hydroxylase (FAH1)**	• Monooxygenase activity	IDA
						• Hydroxylase activity	IMP
						• Lignin biosynthesis process	TAS
249490_s_at	At5g39110	–	1.10	–	Germin-like protein (GLP6)	• Manganese ion binding	IEA
						• Unknown process	ND
250676_at	**At5g06320**	–	-1.27	–	**Harpin-induced protein-like**	• Unknown function	ND
						• Response to bacterium	IMP
265230_s_at	At2g07707	–	-1.23	–	Hypothetical protein	• Unknown function	ND
						• Unknown process	ND
267293_at	At2g23810	–	-1.00	–	Hypothetical protein	• Unknown function	ND
						• Aging	ISS
252592_at	**At3g45640**	-1.17	–	–	**Mitogen-activated protein kinase 3**	• MAP kinase activity	ISS
						• Response to chitin	IEP
						• Response to oxidative stress	IEP
						• Camalexin biosynthetic process	IMP

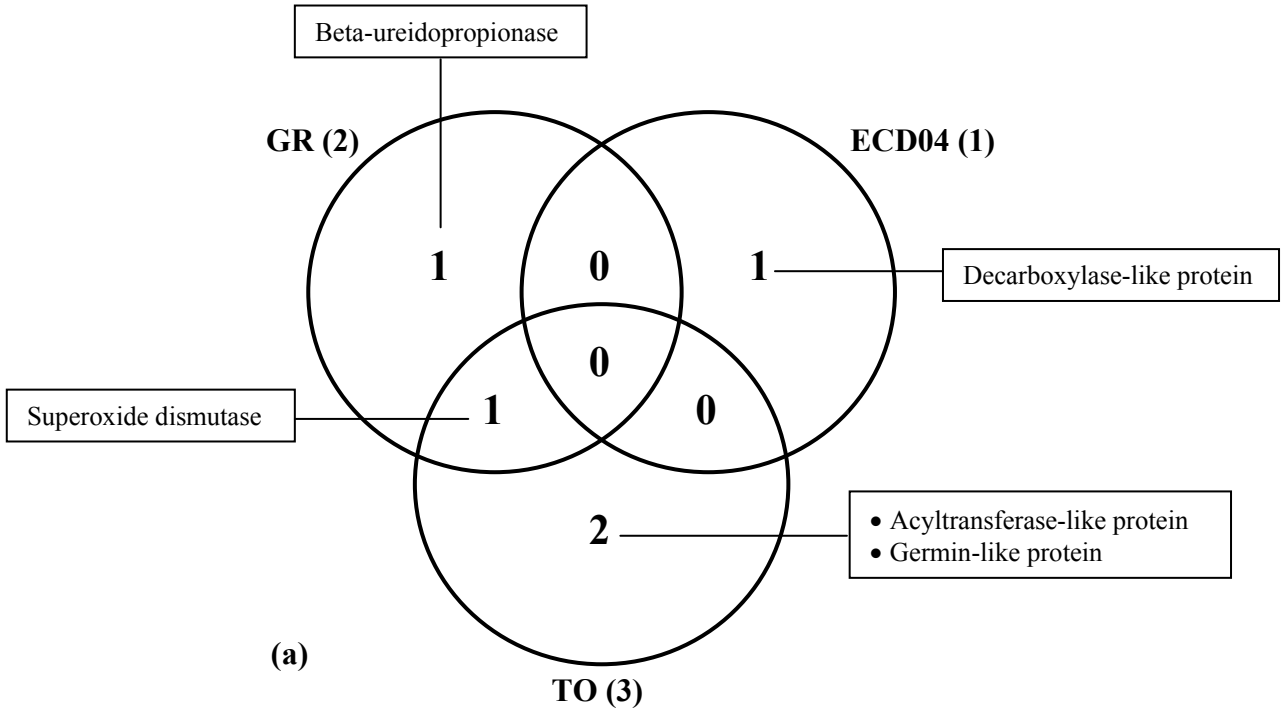
245711_at	At5g04340	-1.83	–	–	Putative C2H2 zinc finger transcription factor	• Transcription factor activity • Zinc ion binding	ISS ISS
260147_at	At1g52790	-1.07	-2.73	–	Putative oxidoreductase	• Unknown	ND
259276_at	**At3g01190**	–	-1.30	–	**Putative peroxidase**	• Peroxidase activity • Response to oxidative stress	ISS IEA
246270_at	At4g36500	–	-1.10	–	Putative protein	• Unknown	ND
251281_at	At3g61640	–	-1.73	–	Putative protein hypothetical protein	• Unknown	ND
266834_s_at	**At2g30020**	-1.87	–	–	**Putative protein phosphatase 2C**	• Protein serine/threonine phosphatise activity • Defence response to fungus	IDA IMP
250350_at	At5g12010	-1.23	–	–	Putative protein predicted proteins	• Unknown	ND
248252_at	At5g53250	–	-1.20	–	Putative protein similar to unknown protein	• Unknown	ND
248164_at	At5g54490	-1.53	–	–	Putative protein similar to unknown protein	• Calcium ion binding • Response to auxin stimulus	ISS IEP
250153_at	At5g15130	–	-1.47	–	Putative protein TMV response-related gene product	• Transcription factor activity • Regulation of transcription	ISS ISS
267028_at	**At2g38470**	-1.03	–	–	**Putative WRKY-type DNA binding protein**	• Transcription factor activity • Defence response to fungus • Camalexin biosynthesis process • Response to chitin • Defence response to bacterium	ISS IMP IMP IEP IMP
251112_s_at	At5g01320	–	–	1.13	Pyruvate decarboxylase-like protein	• Unknown	ND
264809_at	**At1g08830**	1.20	1.67	–	**Superoxidase dismutase**	• Superoxide dismutase activity • Removal of superoxide radicals • Response to oxidative stress	TAS IC IEP
247925_at	At5g57560	-1.77	–	–	Xyloglucan endotransglycosylase (TCH4) related protein	• Xyloglucan transferase activity • Plant-type cell wall organisation	IDA TAS

261892_at	**At1g80840**	-2.07	-1.47	–	**Transcription factor, putative similar to WRKY transcription factor**	• Response to salicylic acid stimulus	IEP
						• Response to chitin	IEP
						• Defence response to bacterium	IEP
						• Transcription factor activity	ISS
						• Regulation of transcription	ISS
263935_at	**At2g35930**	-1.30	-1.47	–	**Unknown protein**	• Ubiquitin-protein ligase activity	IGI
						• Response to chitin	
						• Protein ubiquitination	IEP
							IDA
249284_at	At5g41810	–	-1.10	–	Unknown protein	• Unknown function	ND
						• Unknown process	ND

[a] The mean signal log ratio was calculated by averaging the SLR from the three replicated experiments.
[b] *Brassica* lines: clubroot-susceptible 'Granaat' (GR), clubroot-tolerant '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.



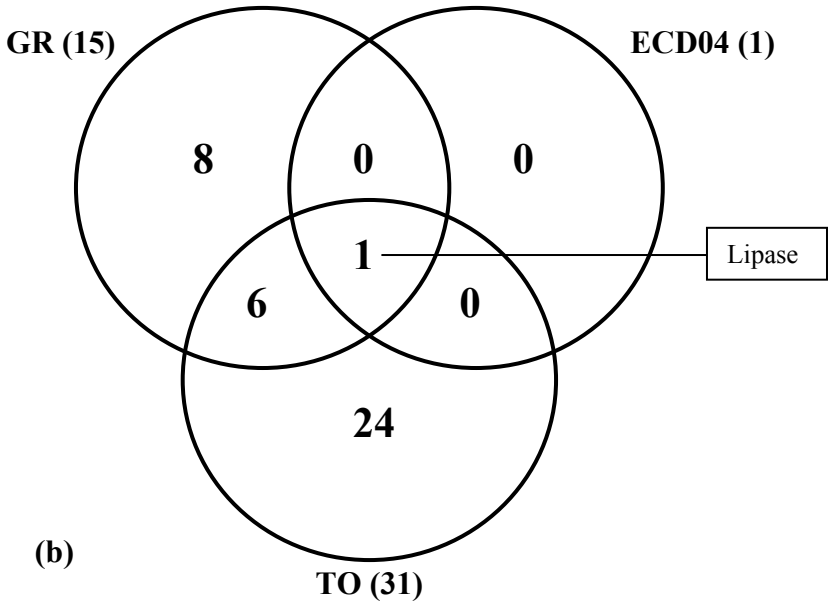


Figure 12. Regulation of the DE transcripts for each genotype (GR: 'Granaat', TO: 'Tahono' and 'ECD04') 48 hai with clubroot isolate S. Number of (a) up-regulated and (b) down-regulated transcripts are shown. Venn diagrams were generated at http://www.pangloss.com/seidel/Protocols/venn.cgi.

1.4.2. 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 12** and **13**. 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 (individual analyses not shown due to space constraints). 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 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 13)**. Selected putative 'Tahono'-specific and 'ECD04'-specific genes are illustrated in **Table 14** and **15** respectively while the commonly constitutively expressed genes in both 'Tahono' and 'ECD04' when compared to 'Granaat' are summarised in **Table 16** (only defence-related, transcription-related and responses to biotic and abiotic stresses are shown). 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 tolerant / resistant 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.

Table 12. Selection of constitutively expressed genes with reliable expressions in healthy 'Tahono' when compared to that of 'Granaat'.

Selection of genes constitutively OVER-expressed		Selection of genes constitutively UNDER-expressed	
Total number of genes on the array	22,810	Total number of genes on the array	22,810
No. of genes called 'present' in Experiment	5,114	No. of genes called 'present' in Baseline	4,981
No. of genes called 'increase'	136	No. of genes called 'decrease'	350
No. of genes with SLR* ≥ 0.8	91	No. of genes with SLR* ≤ -0.8	113
No. of genes with a signal intensity ≥ 100 in Experiment	74	No. of genes with a signal intensity ≥ 100 in Baseline	36
% Significantly greater rate	1.45	% Significantly lesser rate	0.72

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

Table 13. Selection of constitutively expressed genes with reliable expressions in healthy 'ECD04' when compared to that of 'Granaat'.

Selection of genes constitutively OVER-expressed		Selection of genes constitutively UNDER-expressed	
Total number of genes on the array	22,810	Total number of genes on the array	22,810
No. of genes called 'present' in Experiment	4,637	No. of genes called 'present' in Baseline	4,981
No. of genes called 'increase'	162	No. of genes called 'decrease'	509
No. of genes with SLR ≥ 0.8	121	No. of genes with SLR ≤ -0.8	179
No. of genes with a signal intensity ≥ 100 in Experiment	115	No. of genes with a signal intensity ≥ 100 in Baseline	90
% Significantly greater rate	2.48	% Significantly lesser rate	1.81

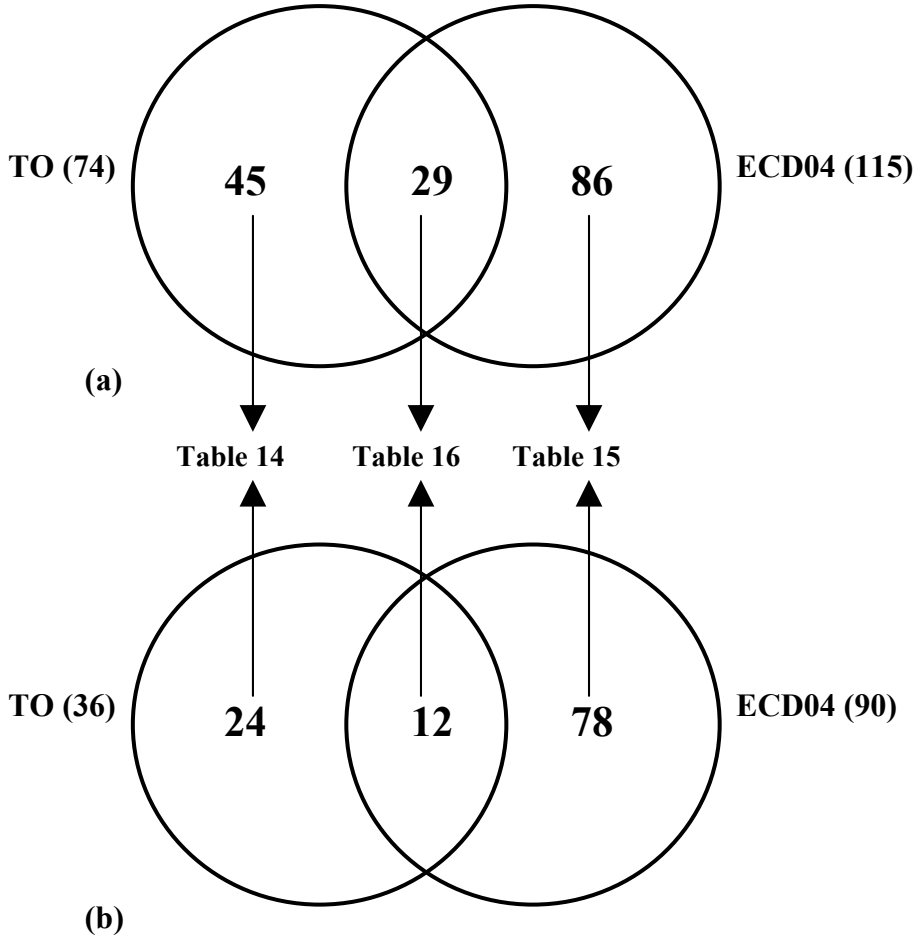


Figure 13. Regulation of the constitutively expressed transcripts (Control array 'Tahono' or ECD04 when compared to 'Granaat'). Number of genes expressed at (a) a greater rate and (b) a lesser rate are shown. Venn diagrams were generated at http://www.pangloss.com/seidel/Protocols/venn.cgi.

Table 14. Selected 'Tahono'-specific constitutively expressed genes (sorted by SLR).

Probe Set ID	Locus Identifier	SLR	Putative function (on August 2007)	GO term (on March 2009)	Code
247741_at	At5g58960	2.9	Putative predicted proteins	• Unknown	ND
				• Response to red or far red light	IMP
257946_at	At3g21710	1.7	Hypothetical protein predicted	• Unknown	ND
260552_at	At2g43430	1.5	Putative glyoxalase II	• Hydroxyacylglutathione hydrolase activity	IDA
254001_at	At4g26260	1.4	Putative protein	• Inositol oxygenase activity	IDA
250153_at	At5g15130	1.2	Putative protein TMV response-related gene product	• Transcription factor activity	ISS
				• Regulation of transcription	IEA
250580_at	At5g07440	1.2	Glutamate dehydrogenase 2	• Response to salt stress	IEP
				• Oxidoreductase activity	ISS
				• Glutamate dehydrogenase activity	IDA
251012_at	At5g02580	1.2	Putative protein	• Unknown	ND
265023_at	At1g24440	1.2	Unknown protein weak similarity to C3HC4 zinc finger	• Zinc Ion binding	IEA
253125_at	At4g36040	1.1	DnaJ-like protein DnaJ-like protein	• Heat shock protein binding	IEA
247199_at	At5g65210	1	DNA binding protein TGA1a homolog	• Transcription factor activity	ISS
				• Defence response to bacterium	IMP
				• Calmodulin binding	ISS
248000_at	At5g56190	1	WD-repeat protein-like	• Unknown	ND
258402_at	At3g15450	1	Unknown protein	• Unknown	ND
266746_s_at	At2g02930	0.9	Putative glutathione S-transferase	• Glutathione transferase activity	ISS
				• Toxin catabolic process	TAS
267461_at	At2g33830	0.9	Putative auxin-regulated protein	• Unknown	ND
247295_at	At5g64180	0.8	Putative protein similar to unknown protein	• Unknown	ND
247312_at	At5g63970	0.8	Putative protein strong similarity to unknown protein	• Zinc-ion binding	IEA
				• Unknown process	ND
250428_at	At5g10480	0.8	Putative tyrosine phosphatase-like protein	• Regulation of cell division	IMP
				• Cell differentiation	IMP
255645_at	At4g00880	0.8	Auxin-induced protein	• Response to auxin stimulus	ISS
				• Unknown function	ND
261901_at	At1g80920	0.8	J8-like protein	• Heat shock protein binding	IEA

267280_at	At2g19450	0.8	Diacylglycerol O-acyltransferase	• Diacylglycerol O-acyltransferase activity	IDA
				• Response to abscisic acid stimulus	IMP
				• Aging	IMP
246289_at	At3g56880	-0.8	Putative protein	• Unknown	ND
251222_at	At3g62580	-0.8	Putative membrane protein	• Unknown	ND
264052_at	At2g22330	-0.8	Putative cytochrome P450	• Response to wounding	IEP
				• Monooxygenase activity	IEA
				• Glucosinolate biosynthetic process	TAS
				• Camalexin biosynthetic process	TAS
				• Defence response to bacterium	IMP
				• Callose deposition in cell wall during defence response	IMP
266165_at	At2g28190	-0.8	Putative copper/zinc superoxide dismutase	• Superoxide dismutase activity	IDA
				• Response to oxidative stress	IDA
				• Removal of superoxide radicals	IC
				• Response to stress	IDA
254810_at	At4g12390	-0.9	Putative protein pectinesterase	• Pectinesterase activity	IEA
				• Unknown biological process	ND
264179_at	At1g02180	-0.9	Hypothetical protein predicted	• Unknown	ND
262832_s_at	At1g14870	-1.2	Unknown protein	• Unknown function	ND
				• Response to oxidative stress	IMP
264809_at	At1g08830	-1.3	Superoxidase dismutase	• Superoxide dismutase activity	IDA
				• Response to oxidative stress	TAS
				• Defence response to bacterium	IEP
261970_at	At1g65960	-1.4	Glutamate decarboxylase	• Calmodulin binding	TAS

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 15. Selected 'ECD04'-specific constitutively expressed genes (sorted by SLR).

Probe Set ID	Locus Identifier	SLR	Putative function (in August 2007)	GO term (performed on March 2009)	Code
248049_at	At5g56090	3.8	Putative protein contains similarity to cytochrome oxidase assembly factor	• Unknown	ND
260226_at	At1g74660	3.3	Hypothetical protein predicted	• Response to abscisic acid stimulus	IMP
				• Response to cytokinin stimulus	IMP
				• Transcription factor activity	ISS
				• Response to gibberellin stimulus	IMP
				• Response to auxin stimulus	IMP
262832_s_at	At1g14870	3.3	Unknown protein	• Response to oxidative stress	IMP
				• Unknown	ND
256647_at	At3g13610	2.8	Unknown protein contains similarity to DNA-binding protein	• Oxidoreductase activity	ISS
				• Coumarin biosynthetic process	IMP
				• Secondary metabolic process	ISS
				• Hydrogen peroxide-mediated programmed cell death	IMP
260557_at	At2g43610	1.5	Putative endochitinase	• Chitin binding	IEA
				• Chitinase activity	ISS
251370_at	At3g60450	1.3	Putative protein	• Unknown	ND
264809_at	At1g08830	1.3	Superoxidase dismutase	• Removal of superoxide radicals	IC
				• Superoxide dismutase activity	TAS
				• Response to oxidative stress	TAS

				• Defence response to bacterium	IEP
263878_s_at	At2g22040	1.2	Unknown protein	• Unknown	ND
248088_at	At5g55070	1.1	2-oxoglutarate dehydrogenase E2 subunit	• Response to oxidative stress	IDA
249882_at	At5g22890	1.1	Putative protein contains similarity to C2H2-type zinc finger protein	• Response to chitin	IEP
				• Transcription factor activity	ISS
250438_at	At5g10580	1.1	Putative protein predicted protein,	• Unknown	ND
257823_at	At3g25190	1.1	Integral membrane protein	• Unknown	ND
255263_at	At4g05160	1	4-coumarate--CoA ligase - like protein	• Jasmonic acid biosynthetic process	IDA
257375_at	At2g38640	1	Unknown protein	• Unknown	ND
262504_at	At1g21750	1	Putative protein disulfide isomerase precursor	• Regulation of programmed cell death	IMP
263924_at	At2g36530	1	Enolase (2-phospho-D-glycerate hydroylase)	• Response to abscisic acid stimulus	IEP
249717_at	At5g35730	0.9	Unknown protein	• Unknown	ND
256342_at	At1g72020	0.9	Unknown protein	• Unknown	ND
263631_at	At2g04900	0.9	Unknown protein	• Unknown	ND
263845_at	At2g37040	0.9	Phenylalanine ammonia lyase (PAL1)	• Defence response	TAS
				• Phenylalanine ammonia-lyase activity	TAS
				• Response to oxidative stress	IEP
248588_at	At5g49540	0.8	Unknown protein	• Unknown	ND
250102_at	At5g16590	0.8	Receptor-like protein kinase	• Response to symbiotic fungus	IEP
247399_at	At5g62960	-0.8	Putative protein similar to unknown protein	• Unknown	ND
250076_at	At5g16660	-0.8	Putative protein; similar to unknown protein	• Unknown	ND
250937_at	At5g03230	-0.8	Putative protein various predicted proteins	• Unknown	ND

255433_at	At4g03210	-0.8	Putative xyloglucan endotransglycosylase	• Xyloglucan:xyloglucosyl transferase activity	ISS
255602_at	At4g01026	-0.8	Expressed protein	• Unknown	ND
260238_at	At1g74520	-0.8	AtHVA22a	• Response to abscisic acid stimulus	IEP
261644_s_at	At1g27830	-0.8	Hypothetical protein	• Unknown	ND
262287_at	At1g68660	-0.8	Unknown protein	• Unknown	ND
263421_at	At2g17230	-0.8	Unknown protein	• Unknown	ND
263517_at	At2g21620	-0.8	Unknown protein	• Response to stress	ISS
				• Unknown process	ND
264181_at	At1g65350	-0.8	Ubiquitin	• Ubiquitin-dependent protein catabolic process	ISS
265005_at	At1g61667	-0.8	Expressed protein	• Unknown	ND
266815_at	At2g44900	-0.8	F-box protein family	• Ubiquitin-dependent protein catabolic process	TAS
245795_at	At1g32160	-0.9	Unknown protein	• Unknown	ND
246487_at	At5g16030	-0.9	Putative protein with poly glutamic acid stretch	• Unknown	ND
252679_at	At3g44260	-0.9	CCR4-associated factor 1-like protein	• Response to biotic stimulus	IEP
				• Ribonuclease activity	ISS
260287_at	At1g80440	-0.9	Unknown protein	• Unknown	ND
262378_at	At1g72830	-0.9	CCAAT-binding factor B subunit homolog	• Regulation of transcription	ISS
263046_at	At2g05380	-0.9	Unknown protein	• Unknown	ND
267461_at	At2g33830	-0.9	Putative auxin-regulated protein	• Unknown	ND
246270_at	At4g36500	-1	Putative protein	• Unknown	ND
263238_at	At2g16580	-1	Putative auxin-induced protein	• Response to auxin stimulus	ISS
				• Unknown function	ND

247543_at	At5g61600	-1.1	DNA binding protein - like DNA binding protein EREBP-4	• Transcription activator activity • Defence response to fungus	IEP IMP
255728_at	At1g25500	-1.1	Unknown protein	• Unknown	ND
259544_at	At1g20620	-1.1	Hypothetical protein	• Hydrogen peroxide catabolic activity	TAS
261285_at	At1g35720	-1.1	Calcium ion-dependent membrane-binding protein annexin	• Response to oxidative stress • Calcium ion binding • Response to abscisic stimulus	IGI ISS IEP
267028_at	At2g38470	-1.1	Putative WRKY-type DNA binding protein	• Defence response to fungus • Camalexin biosynthetic process • Defence response to bacterium • Transcription factor activity	IMP IMP IMP ISS
245711_at	At5g04340	-1.2	Putative c2h2 zinc finger transcription factor	• Zinc ion binding • Transcription factor	ISS ISS
251281_at	At3g61640	-1.2	Putative protein hypothetical protein	• Unknown	ND
255149_at	At4g08150	-1.2	KNAT1 homeobox-like protein	• Transcription factor activity	ISS
255412_at	At4g02980	-1.2	Auxin-binding protein 1 precursor	• Auxin binding • Positive regulation of cell division	IMP IMP
252592_at	At3g45640	-1.3	Mitogen-activated protein kinase 3	• MAP kinase activity • Signal transduction • Response to bacterium • Response to chitin • Response to oxidative stress	IC ISS IEP IEP IEP

261193_at	At1g32920	-1.3	Unknown protein	• Response to wounding	IEP
				• Unknown function	ND
247925_at	At5g57560	-1.4	TCH4 protein	• Response to auxin stimulus	IEP
				• Xyloglucan:xyloglucosyl transferase activity	IDA
				• Response to brassinosteroid stimulus	IEP
251109_at	At5g01600	-1.6	Ferritin 1 precursor	• Response to bacterium	IMP
				• Response to hydrogen peroxide	IEP
				• Response to reactive oxygen species	IGI
				• Response to bacterium	IEP
262932_at	At1g65820	-1.6	Glutathione-S-transferase	• Glutathione transferase activity	ISS
253874_at	At4g27450	-1.7	Putative stem-specific protein	• Unknown	ND
263498_at	At2g42610	-1.7	Unknown protein	• Unknown	ND
257022_at	At3g19580	-1.8	Zinc finger protein, putative similar to Cys2/His2-type zinc finger protein	• Transcription factor activity	ISS
				• Response to abscisic acid stimulus	IEP
				• Response to chitin	IEP
265481_at	At2g15960	-2	Unknown protein	• Unknown	ND
264953_at	At1g77120	-2.2	Alcohol dehydrogenase identical to alcohol dehydrogenase	• Alcohol dehydrogenase activity	ISS
				• Response to stress	IGI
265162_at	At1g30910	-2.3	Hypothetical protein predicted	• Unknown	ND
265712_s_at	At2g03330	-2.5	Unknown protein	• Unknown	ND
266834_s_at	At2g30020	-3	Putative protein phosphatase 2C	• Protein serine/threonine phosphatase activity	IEP
				• Response to fungus	IEP
					IMP

				• Response to wounding	ISS
				• Defence response to fungus	
251012_at	At5g02580	-3.5	Putative protein	• Unknown	ND

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 16. Selected genes commonly and constitutively expressed between unchallenged 30-day-old 'Tahono' and 'ECD04' (sorted by SLR of ECD04).

Probe Set ID	Locus Identifier	SLR		Putative function (on August 2007)	GO term (on March 2009)	Code
		TO	ECD04			
256674_at	At3g52360	1.2	2.8	Unknown protein	• Unknown	ND
244912_at	ccb382	2.4	2.5	Cytochrome c biogenesis ORF382 Protein sequence	• Unknown	ND
249581_at	At5g37600	1.0	1.4	Glutamate-ammonia ligase	• Glutamate-ammonia ligase activity	IDA
					• Nitrate assimilation	TAS
259276_at	**At3g01190**	1.1	1.4	Putative peroxidase	• Peroxidase activity	ISS
					• Response to oxidative stress	IEA
259525_at	At1g12560	0.9	1.3	Hypothetical protein	• Unknown function	TAS
					• Plant-type cell wall loosening	ISS
245003_at	psbC	1.4	1.3	Photosystem II (PSII) 43 KDa protein	• Unknown	ND
246880_s_at	At5g25980	1.4	1.3	Myrosinase	• Thioglucosidase activity	IMP
					• Glucosinolate catabolic process	NAS
245015_at	rbcL	1.0	1.2	Large subunit of riblose-1,5-bisphosphate carboxylase/oxygenase	• Unknown	ND
244937_at	ndhH	1.1	1.2	NADH dehydrogenase 49KDa protein	• Unknown	ND
252927_at	**At4g39090**	1.4	1.2	Cysteine proteinase RD19A identical to thiol protease	• Defence response to bacterium	IMP
					• Response to salt stress	IEP
					• Response to osmotic stress	IGI
262537_s_at	At1g17280	1.5	1.2	Putative ubiquitin-conjugating enzyme	• Ubiquitin-protein ligase activity	ISS

					• Ubiquitin-dependent protein catabolic process	IDA
259723_at	At1g60960	1.0	1.1	Putative iron-regulated transporter	• Cation transport	ISS
					• Response to nematode	IEP
244959_s_at	orf107c	1.3	1.1	Hypothetical protein	• Unknown	ND
245139_at	At2g45430	0.9	1.0	Putative AT-hook DNA-binding protein	• Unknown	ND
265435_s_at	At2g21020	0.9	1.0	Putative major intrinsic (channel) protein	• Unknown	ND
244939_at	rps12.1	1.2	1.0	Ribosomal protein S12	• Unknown	ND
261815_at	At1g08325	1.1	0.9	Leucine zipper protein	• Unknown	ND
244940_at	rps12.2	0.9	0.8	Ribosomal protein S12	• Unknown	ND
245016_at	accD	1.0	0.8	Carboxytransferase beta subunit	• Unknown	ND
253088_at	**At4g36220**	1.1	0.8	Ferulate-5-hydroxylase (FAH1)	• Monooxygenase activity	IDA
					• Lignin biosynthesis process	IMP
257339_s_at	mitochondria	1.2	0.8	ATP synthase subunit 9	• Unknown	ND
265230_s_at	At2g07707	1.4	0.8	Hypothetical protein	• Unknown	ND
249384_at	At5g39890	-0.9	-0.8	Putative protein hypothetical protein	• Unknown	ND
262502_at	At1g21600	-0.8	-0.9	Unknown protein similar to hypothetical protein	• Unknown function	ND
					• Positive regulation of transcription	IMP
245226_at	At3g29970	-2.4	-1.0	Unknown protein	• Unknown	ND
248164_at	At5g54490	-0.8	-1.0	Putative protein similar to unknown protein	• Calcium ion binding	ISS
					• Response to auxin stimulus	IEP
257784_at	At3g26970	-1.2	-1.1	Geranylgeranylated protein	• Unknown	ND

248964_at	At5g45340	-1.4	-1.3	Cytochrome P450	• Hydrolase activity	IDA
					• Abscisic acid catabolic process	TAS
251192_at	At3g62720	-1.0	-1.7	α-galactosyltransferase-like protein	• Xyloglucan transferase activity	IDA
					• Xyloglucan biosynthetic process	IGI
261892_at	**At1g80840**	-1.3	-2.1	Transcription factor, putative similar to WRKY transcription factor	• Transcription factor activity	ISS
					• Response to salicylic acid stimulus	IEP
					• Response to chitin	IEP
					• Defence response to bacterium	IEP
					• Defence response to fungus	IEP

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

1.5 Validation of microarray data by quantitative real time PCR

Four genes with different expression values were selected representing different genotypes and time points. The standard curve method was used to calculate the fold-change values and was constructed using the actin gene **(Figure 9)**. Most of the genes revealed similar expression pattern for microarray and qRT-PCR values for fold change **(Table 17)**. This confirmed the reliability of the Affymetrix data. However, the fold change values obtained through qRT-PCR were generally more exaggerated than their corresponding Affymetrix values. Similar observations were observed in other microarray studies (Coram and Pang, 2006; Mantri et al., 2007).

1.6. Validation of microarray data by enzyme assays

The protein concentrations of the crude root tissue extracts were determined using the Bradford protein assay **(Table 18)** while the integrity and size distribution were determined by SDS-PAGE (**Figure 14)**. The activity of constitutively-produced chitinase and myrosinase enzymes for Tahono, ECD04 and Granaat were tabulated (**Tables 19-20**) and their activity ratio determined and compared to their transcript ratio. It may be observed that there was a poor correspondence between the Activity and Transcript ratios (**Table 21**), indicating that the retention rates of these proteins may possibly be different in each variety. The variety 'Granaat' appeared to possess the highest base retention rate, although whether these difference between the varieties is significant is open for interpretation.

Table 17. Expression ratios of selected transcripts assessed by Affymetrix and qRT-PCR.

GenBank® accession number	Locus Identifier	Probe ID	Putative function	Granaat 48h		Tahono 48h		ECD04 48h	
				Affy	qRT-PCR	Affy	qRT-PCR	Affy	qRT-PCR
AI352707	At2g02930	266746_s_at	Glutathione S-transferase	0.5	0.54	-0.50	0.34	-0.27	17.18
AY156708	At2g06850	266215_at	Xyloglucan endo-transglycosylase precursor	0.0	-0.17	0.03	0.43	-0.47	-0.83
	At1g30370		Lipase	-1.43	-1.66	-1.53	-3.01	-1.30	-3.92
AY055752	At2g37040	263845_at	Phenylalanine ammonia-lyase	0.3	-0.02	0.37	0.79	0.17	0.45
AF230684	At2g43590	260560_at	Chitinase	*NA*	-0.19	*NA*	1.90	*NA*	5.20
	At1g08830		Superoxidase dismutase	1.20	1.46	1.67	2.64	0.67	2.54

NA, data not available due to absence of signal detection during analysis

Note:
Both Affymetrix and qRT-PCR values indicate mean $\log_2$ fold change relative to untreated controls.

Table 18. Protein concentration of the crude root tissue extract using the Bradford protein assay.

Sample	Abs (avg)	Abs (StDev)	Abs (avg - blank)	Protein Conc. (µg/mL)[a]
P1	0.755	0.024	0.569	2000
P2	0.574	0.005	0.388	1000
P3	0.395	0.007	0.209	500
P4	0.231	0.012	0.045	100
P5	0.204	0.002	0.019	50
P6 (blank)	0.186	0.002	0.000	0
Tahono	1.021	0.041	0.835	6260
ECD04	0.759	0.049	0.573	4297
Granaat	1.071	0.010	0.885	6636
Leaguer	1.081	0.033	0.895	6711
TO×LE	1.100	0.031	0.914	6856
TO×ECD05	1.033	0.047	0.847	6354

[a] Dilution of crude root tissue extract taken into consideration

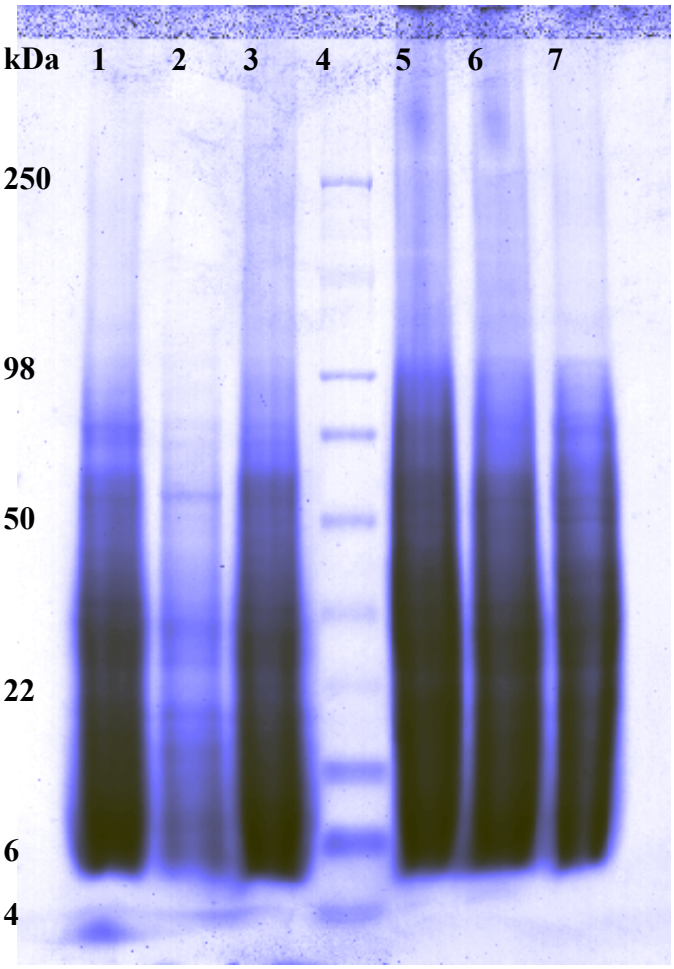


Figure 14. Integrity and size distribution of the concentrated crude root tissue extracts by SDS-PAGE. Well 1: Tahono, 2: ECD04, 3: Granaat, 4: Protein ladder, 5: Leaguer, 6: TO×LE and 7: TO×ECD05.

Note:

TO×LE - F_1 genotype generated by a cross between 'Tahono' and 'Leaguer'.

TO×ECD05 - F_1 genotype generated by a cross between 'Tahono' and 'Granaat'.

Table 19. Chitinase activity.

Sample	4-MU released (nmole)	Total protein in assay (mg)	Chitinase activity (U)[a]
TO	375	0.16	234
ECD04	176	0.11	160
ECD05	857	0.17	504
LE	1130	0.17	665
TO×LE	454	0.17	267
TO×ECD05	653	0.16	408

[a] 1 unit of chitinase activity defined as 1 nmole of 4-methylumbelliferone (Mr = 176.17) released per min per mg of total protein in current assay conditions.

Table 20. Myrosinase activity.

Sample	Sinigrin[a] broken down (nmole)	Total protein in assay (mg)	Myrosinase activity (U)[b]
TO	43	0.16	0.19
ECD04	40	0.11	0.26
GR	325	0.17	1.36
LE	513	0.17	2.13
TO×LE	720	0.17	2.92
TO×ECD05	508	0.16	2.22

[a] 1 mole of glucose released from 1 mole of sinigrin (Mr Glucose = 180.16), [b] 1 unit of myrosinase activity defined as 1 nmol of sinigrin broken down per min per mg of total protein in current assay conditions.

Table 21. Comparison between the Activity and Transcript Ratios of Myrosinase and Chitinase.

Assay	Sample	Activity (U)	Activity ratio[a]	Transcript ratio[b]
Myrosinase	Tahono	0.19	0.14	1.4
	ECD04	0.26	0.19	1.3
	Granaat	1.36	1	1
Chitinase	Tahono	234	0.46	*NA*
	ECD04	160	0.32	*NA*
	Granaat	504	1	1

[a] Activity ratio was calculated by dividing the enzyme activity of each *Brassica* line with that of Granaat.
[b] Transcript ratio was the expected level of constitutively expressed gene from the Affymetrix data.
NA, data not available due to absence of signal detection during analysis

DISCUSSION:

1.1 The hydroponics system

A hydroponics system has previously been used to study gene expressions in plants after being sprayed with defence signalling compounds (Coram and Pang, 2007). This system minimised environmental effects and was conducted in a reference design, where samples from untreated controls acted as references against post-treatment samples. In addition, this technique was suitable for the study of Clubroot disease due to successful infection in the treated plants, which resulted in the extraction of high quantity and quality total RNA representative to the phenotype of the *Brassica* lines.

The scoring of the symptoms 4 weeks after inoculation **(Table 1)** indicated that Tahono was less severely infected than Granaat and would support previous observations concerning the higher degree of resistance in Tahono. However 8 weeks after inoculation, all of the *Brassica* genotypes have succumbed to Clubroot disease, possibly due to the severe conditions of the hydroponics system. Nonetheless, this suggested that the mode of resistance in Tahono was actually a reduction in the rate and degree of infection or colonisation by the pathogen. These results would characterise Tahono as a tolerant genotype and the mechanism causing this delayed onset of symptoms is possibly polygenic and quantitative in nature (Keane and Brown, 1997). These results support the earlier findings by Moriguchi et al (1999) and Rocherieux et al. (2004), where a large number of QTL loci were mapped for resistance to Clubroot. Hence, the horizontal resistance of Tahono might possibly operate against a large range of Clubroot pathotypes but is often avoided by breeders because it is difficult to detect and manipulate. Moreover, these observations on the phenotype of these *Brassica* lines are early indication that dominant R genes responsible for vertical resistance might not be involved in the current tests.

1.2 Analysis of the RMIT *Brassica* Oligoarray

The onset of resistance in *Brassica* vegetables against Clubroot is not fully understood and hence, the timing to extract total RNA representing defence responses is vague. To avoid the costly and inefficient use of Affymetrix™ chips in future experiments, the construction of a 'boutique' oligonucleotide array (oligoarray) and its use to investigate defence-related gene expressions in 'Tahono, 'Leaguer' & 'Granaat' after inoculation with Clubroot isolate S was a viable option. Initially, root tissue collection was performed at 7, 14 and 21 dai (days after inoculation) and these timing corresponded to primary, secondary infection of *P. brassicae* and first appearance of galls respectively (Ingram and Tommerup, 1972; Devos et al., 2005). However, collection of root tissue was later changed to 1, 2 and 3 dai since previous studies indicated that most defence responses occurs within 72 h after infection (Coram and Pang, 2006) and as early as a few hours after pathogen contact (Hammond-Kosack and Jones, 1996). The previous time points would be useful in the investigation of symptoms formation after the genotypes have succumbed to Clubroot disease, but these were not the aims of this project.

For the proper interpretation of the microarray results, some understandings of the defence responses in plants are required. Disease resistance may be categorised as either passive (barriers such as cuticle and cell wall) or active mechanisms (activated upon pathogen challenge) (Guest and Brown, 1997). The use of microarray may be used to investigate those active responses that can occur within minutes (e.g. membrane depolarisation, reactive oxygen species (ROS) generation), within hours (e.g. oxidative burst, hypersensitive responses (HR), programmed cell death (PCD) and salicylic acid (SA) accumulation) and within days (e.g. accumulation of PR-related proteins and systemic acquired resistance (SAR)).

A list of genes covering a range of active defence responses were investigated using the RMIT *Brassica* oligoarray and to identify the most appropriate time point for Affymetrix study. In general, the use of the oligoarray had limited success: the inability to detect any defence-related response at 24 h after inoculation (hai) with Clubroot isolate S in all genotypes and the limited results at 72 hai. This might be attributed to the relatively small number of defence genes used to construct the oligoarray and therefore, it was likely that a clear and concise picture of the mechanisms of Clubroot resistance/tolerance in those genotypes could not be determined. In addition, the limited lists of differentially expressed (DE) genes at 48 hai indicated that environmental factors (season or daylength) were significantly affecting the test system and hence, explained the difficulties in identifying common DE genes in the biological replicates. This observation was supported when DE genes in Tahono **(Table 4)** identified by analysing the 3 biological replicates performed through time differed to those from the 3 biological replicates performed through space **(Table 5)**. Nonetheless, these findings may be of interest to *Brassica* vegetable farmers since those DE genes (or lack of) along with their respective phenotypic observations indicated that the timing of crop cultivation during which Clubroot is less infective due to environmental conditions may be used to their benefit.

Several studies have been performed to investigate the defence mechanism against *Plasmodiophora brassicae* in *Brassica* vegetables and Clubroot resistance has been found in *B. rapa* (A genome), *B. napus* (AC genome) and *B. oleracea* (C genome). In brief, these resistance genes were categorised into two groups: qualitative, dominant resistance (vertical resistance against specific Clubroot pathotypes) (Crute *et al.*, 1980; James and Williams, 1980) and quantitative, recessive resistance (horizontal resistance against a wide range of Clubroot pathotypes) (Chiang and Crete, 1970; Grandclement and Thomas, 1996; Voorrips, 1996). However, the gene expression profiling of Tahono and Granaat at 48 hai in the current study indicated a lack or limited active defence response to Clubroot infection and as indicated earlier, the mode of resistance in these genotypes might actually be of a quantitative nature and therefore, significant up-regulation of dominant defence-related genes should not be expected. However, the differential expression of genes from root tissue collected at 48 hai indicated this time point was the most appropriate timing for further investigation using the Affymetrix technology.

1.3 Analysis of the Affymetrix® *Arabidopsis* ATH1 Genome array

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 clubroot-tolerant 'Tahono' and especially for the resistant 'ECD04'. It was postulated that 'ECD04' (and possibly 'Tahono') possess a few dominant resistant genes; 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 tolerant 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 (Ludwig-Müller 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 / tolerant genotypes was the WRKY transcription factor (At1g80840) that is a putative negative regulator of defence genes (Eulgem, 2005; Journot-Catalino *et al.*, 2006).

Resistance vs tolerance:

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.

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_2O_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_2O_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_2O_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 15)** 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.

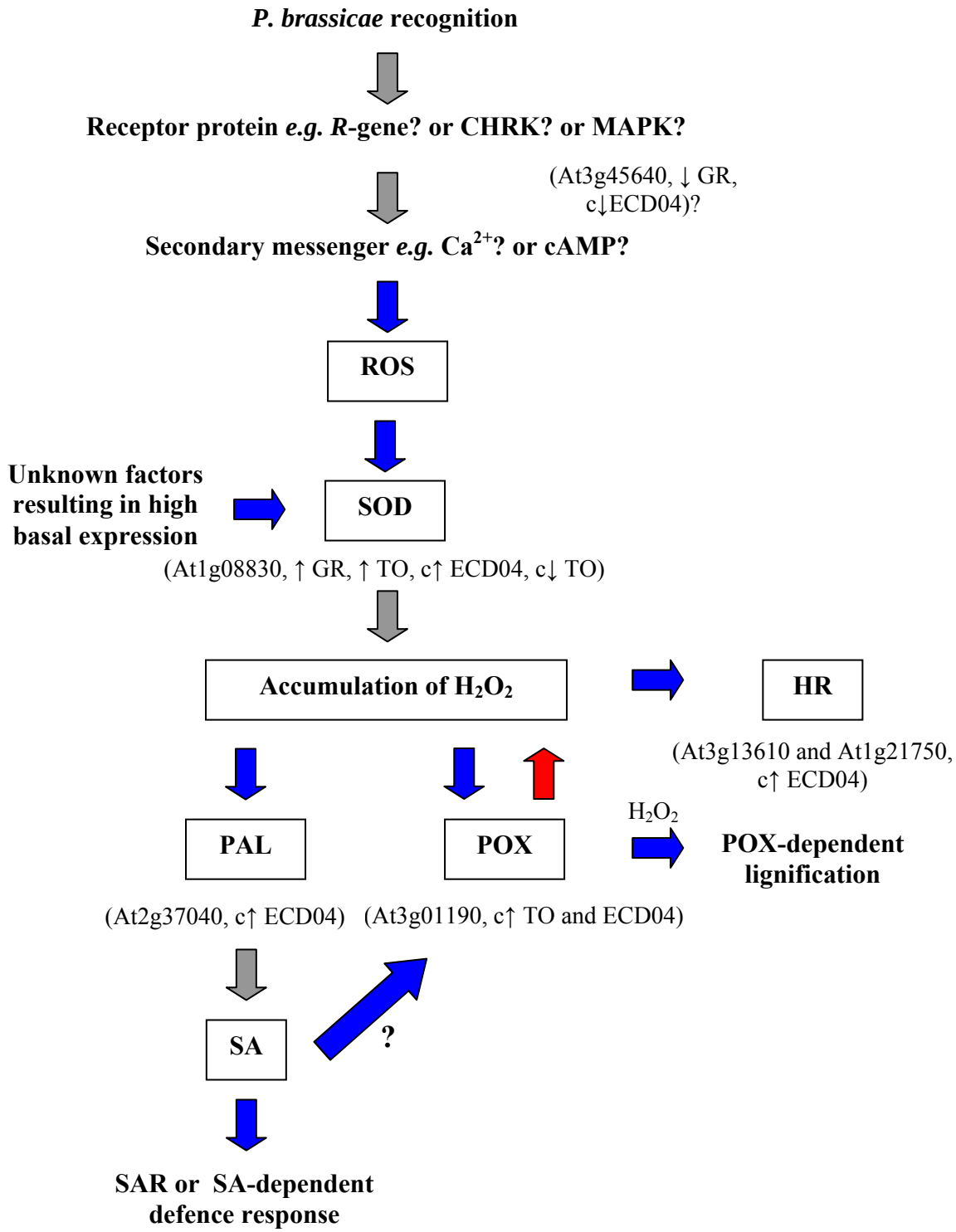


Figure 15. A hypothetical molecular cascade involving the biosynthesis of salicylic acid (SA) *via* ROS accumulation and PAL, resulting in the activation of SA-dependent defence response in *Brassica* against clubroot infection.

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 *as1*-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' **(Section 1.3)** 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 16)** 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.

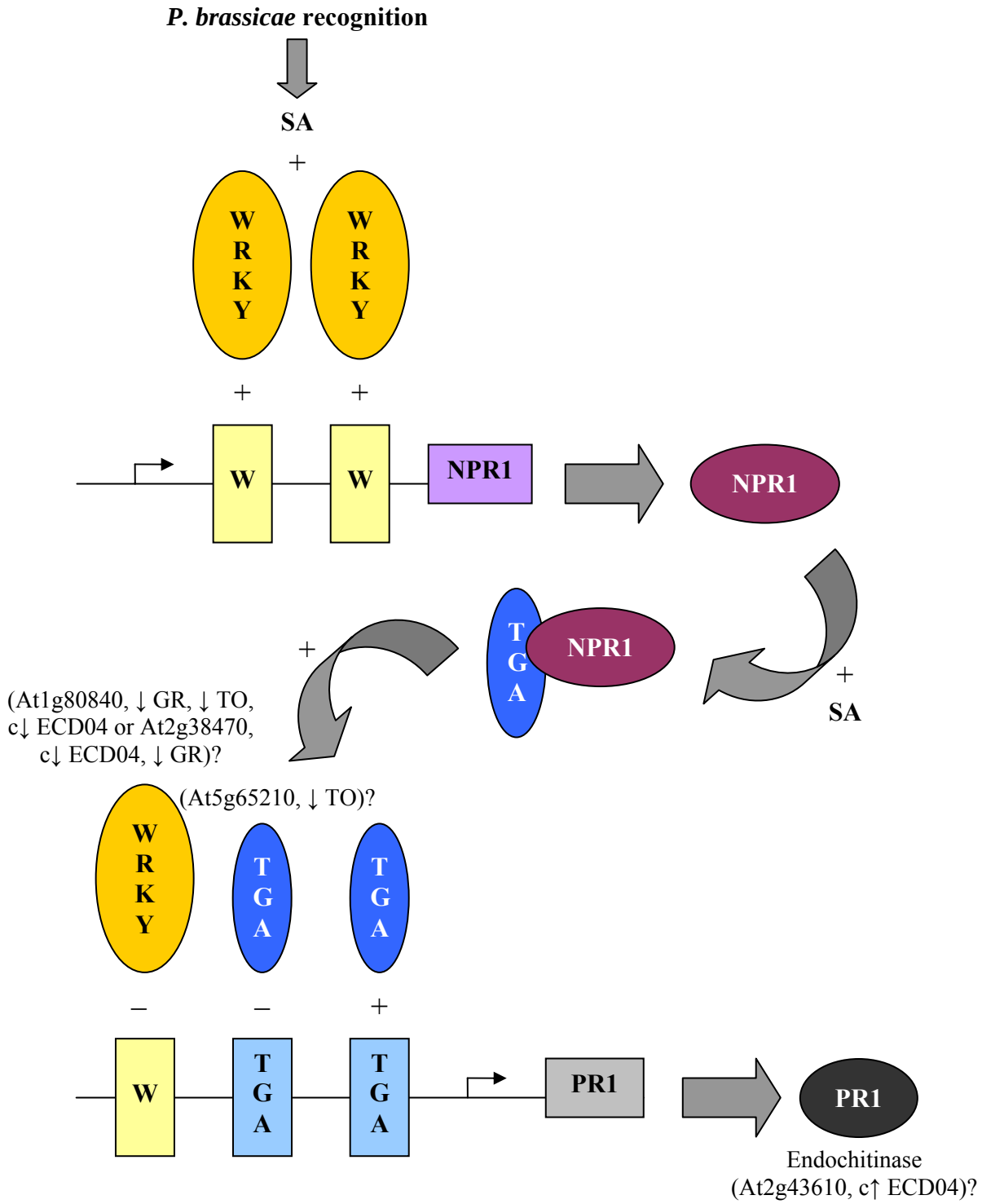


Figure 16. A hypothetical molecular cascade relaying salicylic acid (SA)-dependent signals to PR1 (and possibly endochitinase) *via* NPR1, WRKY and TGA factors, in *Brassica* against clubroot disease.
Causal interactions are indicated by grey arrows, c↑ and c↓ represent constitutive over- and under-expression 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 strong evidence that the high basal expression of lignin biosynthesis enzymes, possibly *via* ROS signalling, was partly responsible for clubroot resistance 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 **(Section 1.3)**. 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_2O_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 clubroot-tolerant 'Tahono' and resistant 'ECD04' is included in **Figure 16** 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 clubroot-tolerant / 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 clubroot-tolerant 'Tahono' and resistant 'ECD04', is demonstrated in **Figure 17**; 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 clubroot-tolerant '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.

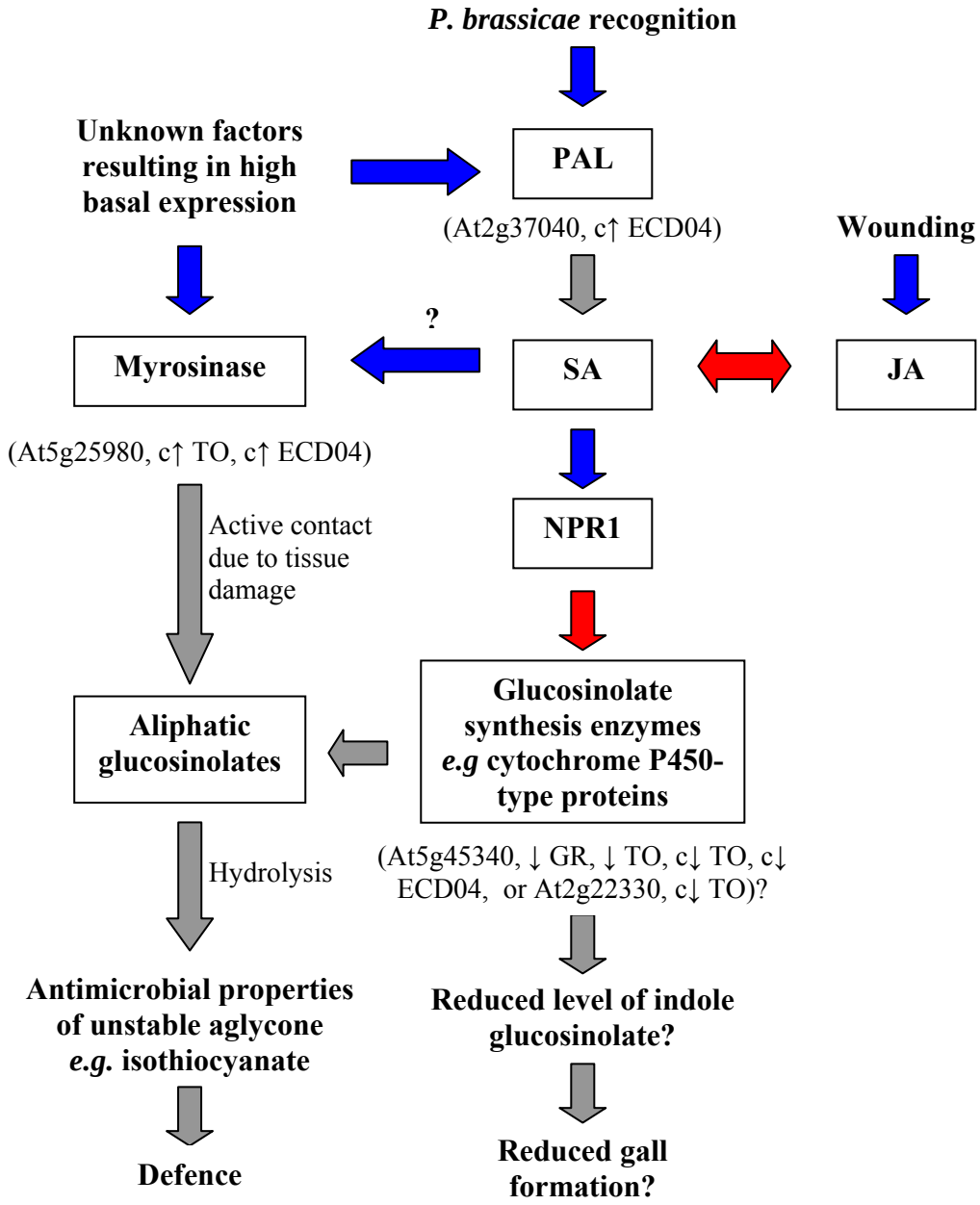


Figure 17. A hypothetical molecular cascade relaying salicylic acid (SA)-dependent signals to glucosinolate *via* NPR1 against the development of clubroot symptoms.

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.

TECHNOLOGY TRANSFER

The information obtained from this project was communicated in a number ways. Firstly, Henderson Seeds were updated on the progress of the project as necessary, especially when a major milestone was completed. Secondly, the Ph.D. student on this project, Mr. Stephan Kong presented his results as a poster, and a refereed conference paper at the 13th Australasian Plant Breeding Conference, held in Christchurch, New Zealand in 2006. This conference was attended by several hundred participants from NZ, Australia and other countries. Finally, electronic copies of the Ph.D. thesis will be available on request either from Associate-Professor Eddie Pang (eddie.pang@rmit.edu.au) or Horticulture Australia.

RECOMMENDATIONS

The outcomes from this project have improved our understanding of the genetic mechanisms controlling resistance of *Brassica* to Clubroot. The results indicated that the susceptibility of 'Granaat' to Clubroot may be due to the suppression of gene expression by the pathogen, and that constitutive expression of certain classes of defence genes may play a role in resistance. However, more research is required to determine whether active defence mechanisms, such as hypersensitivity, indicative of R gene involvement, are found in other Brassicas not included in this study.

One of the main aims of this study was to develop SNPs for the genes responsible for resistance, and to use them for marker-assisted breeding. It was hoped that this project would identify a number of different genes from several resistant varieties, and using the SNPs, to combine (pyramid) them into a single variety. Unfortunately, this aim could not be achieved in the lifetime of the project. This was due to several difficulties experienced during the project, such as the cancellation of the Brassica Genome Array by Affymetrix, which caused considerable delay, and the ongoing uncertainty as to which of the differentially, and constitutively-expressed genes are critically important for the resistance response.

My recommendation therefore, is to continue research on the constitutively-expressed genes in a number of *Brassica* genotypes, specifically those used in the European Clubroot Differential (ECD) set to ascertain whether there are strong correlations between constitutive expression, gene copy number, allelic forms and increased resistance. If, and only when, such correlations are established, would it be logical to produce SNPs for these genes. In the meantime, mapping populations from crosses between Tohano X ECD04 are being produced in the summer of 2009. F2 populations are being generated from F1 plants, and it is anticipated that they will be ready by the middle of 2010. Concurrently, the most promising candidate genes for SNP development will be identified from the current set, and mapping of these genes should be possible from 2010-2012. A PhD/Masters student will be sought to continue this work.

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Appendix 1:

Table A1. The list of synthesised oligonucleotide probes used in the construction of the RMIT *Brassica* oligoarray.

Probe ID	Function of the cDNA sequence	GenBank® Accession	Organism	Oligonucleotide sequence[a] (5' → 3')	
				Probe A	Probe B
BA001	Vacuolar ATP synthase b subunit	H07629	*Brassica napus*	CgTggTCAggTTCTggAAgTTgATgg	gATATTgATCTgCgACAATgggAgCg
BA002	Abscisic acid-insensitive protein	DQ446612	*Arabidopsis thaliana*	AATggTgggACCTCTATgTTATgCCC	TAATCCTCAATCCgATTCCACCACCg
BA003	Abscisic acid-responsive protein	DQ446602	*Arabidopsis thaliana*	AgCAAACAAgCAgAgggAggAAgTgg	CgCTCCAAgACCgCTgAACAAATCAC
BA004	Abscisic acid signal transduction	BD442751	*Arabidopsis thaliana*	gCCTTgTAAATgCCgTgAgATAgCCA	CgATgTggTCTTTgTAgTCggAggAA
BA005	Actin	AF111812	*Brassica napus*	gACAATggAACTggAATggTgAAggC	gATgCTTgTgATgATgCTCTggTCCT
BA006	ACC synthase	X82273	*Brassica oleracea*	gCAgAgAAgCAAgACCAgAACCTACT	CATAACCAATCCTTCCAACCCgCTCg
BA007	Acyl-CoA synthetase	X94624	*Brassica napus*	gCATTgTTACCCTTATCgCTggAgTg	TgAATCCTTCCTAATCgCAgTCgCCA
BA008	auxin-induced IAA	U53672	*Arabidopsis thaliana*	ggCTCACAATggCgTAATCTTCAggT	CAAACTCCgACTCTTTCCTCATCggT
BA009	1-aminocyclopropane-1-carboxylate synthase	U23482	*Arabidopsis thaliana*	CgTgTAAgCAAACAgTggACTAACCC	ggATAgTAggTATTgTgTCTgggAgg
BA010	Nitrogenous group transferase	NM118984	*Arabidopsis thaliana*	CCACAACAgCAACCTCgTTCTTCCTT	CAgggATggTACAgTCCATTCAACAg
BA011	Auxin repressed protein	AF458410	*Brassica oleracea*	CATCAAAggTgTAggAgAAgggAgCA	CCgTTTgATTTCCACCATCTgggTTg
BA012	Auxin response factor 2	AJ716227	*Brassica napus*	CgCATAAgggAAggCTCATCTAAggC	gggAACTCTATgCTgTAggAACgAgg
BA013	Auxin-induced protein	H07824	*Brassica napus*	TTTgggAACATggTACAgTCCggTCg	CggTCTCATCCACAACAACAAACCgA
BA014	Caffeoyl-CoA 3-O-methyltransferase (lignin)	AY821735	*Brassica napus*	gAAAgTTggAggAgTgATTggCTACg	CAgCAgACgAAggACAgTTTCTgAAC
BA015	Calmodulin	H07677	*Brassica napus*	gCTTCgTCCTCTgTggTAgTgATgAT	gTgTCTCCACCgTCTCTTACCTTCCT
BA016	Catalase	U68219	*Brassica napus*	ATCgTCCgTTTCTCCACTgTCgTTCA	gAgATgggTTgAgATACTgTCAgAgC
BA017	Accelerated cell death 1	AY344061	*Arabidopsis thaliana*	ACCAACCTCTCCCTTCCACTgTCTTA	AgCCAAACCATTgCCgTTCAAggTgg
BA018	Radical-induced cell death	AY578790	*Arabidopsis thaliana*	gAAACTgCgggTgATTgTAggAgATg	gAggAACTTTATCTTgCTgACggCTg
BA019	Defender against cell death	AK119013	*Arabidopsis*	TTgCTgTgCCTgTgTTggTCAgTCTT	TCTCgTgTATCgggACAgCggTTCTT

	protein		*thaliana*		
BA020	Chitinase	AF230684	*Brassica rapa*	CgTgACTACTgCgACgAgAACAACAg	TgAgTAgCAACCCAACTgTCgCTTTC
BA021	Chitinase	X61488	*Brassica napus*	TCAATggAATggAgTgTAACggTggg	gTggCATTgTTgggTCTTAgTgTTCC
BA024	Clubroot resistant marker	DD182413	*Brassica napus*	TgATTCCTCCgATgTCTggTTgCgAT	gTgCCTTCCgTTACTTTCgCTCAgAT
BA025	Cytokinin-binding protein	DR997831	*Malus domestca*	gCTTgCTTgAAgAACTgCgAgTAgCC	AgACACCgACTCACTCCAAgTTgAAC
BA026	DNA-damage resistance protein	AI352734	*Brassica napus*	CTACCgTCgCAATCCCATCCTTCACT	TACgAAgTTgCTTTgACTgACCTggg
BA027	AP2/EREBP transcription factor	DQ370141	*Brassica napus*	CTTCCgACTCAATCAgAgACTCCAAg	gTATgATggATTCAgggTTTgCTCCg
BA028	Ethylene-induced stress protein	AY460110	*Brassica rapa*	gggATgCTATTgAAgAgATgAACggg	AgAggACgTTCTCCCAATTCggCgAA
BA029	Ethylene, HEVER and SA-inducible protein	AI352905	*Brassica napus*	AAAggTgAggCTgggACTgggAACAT	TCgTgggTTCCggTATCTTCAAgAgC
BA030	Beta-glucosidase	X82577	*Brassica napus*	TTTgCTCgTAACTCTTgTCggCTCTC	CgTAgACCgTgTgCTTgACTTCATCA
BA031	Beta-1, 3-glucanase	AY836001	*Brassica rapa*	gCTggACAAATCggAgTATgCTTCgg	gTTCgATgAgAACgggAAgCAgACgT
BA032	Glutathione S-transferase	AI352707	*Brassica napus*	TgTCAACgAgTgggTggCTgAgATCA	CAgCTTCCCAgAAgATCCTTCAgTgA
BA033	Heat shock protein 90	AK222102	*Arabidopsis thaliana*	CCAACACTTTCgggAgCAggATTCAC	gAAggTTATCgTCTCTgACCgTgTTg
BA034	Hypersensitive response gene	AI352735	*Brassica napus*	CgAggTCAgAggATACTACggAgATg	gTTTCAgTTTCAgCggACCAAgTgTg
BA035	IAA-amino acid hydrolase 6	DQ233253	*Brassica rapa*	gCAATCTACCCgCCAACCACAAACgA	TgTCggCTATggACCCAAgTCTgTTC
BA036	IAA-amino acid hydrolase 2	DQ233252	*Brassica rapa*	ATgCTgCCATCCCgCAACATACAgTA	gACCAAACggTAAAgAACCACTCCCA
BA037	IAA31	AY669802	*Arabidopsis thaliana*	gATggAgATTggATgATggTCggAgA	AACTCgACCTTTgCgTATTCTCAggC
BA038	Isochorismate synthase	AF078080	*Arabidopsis thaliana*	TCTgATTCgTgCCTATggTggTATgC	AgTggAgACAAggACTATgACTgCTg
BA039	Isochorismate synthase	NM202414	*Arabidopsis thaliana*	TCTgATTCgTgCCTATggTggTATgC	AgTggAgACAAggACTATgACTgCTg
BA040	Isopentenyltransferase	AB186135	*Brassica perkinensis*	AAAgTCgTCTTCgTgATgggAgCCAC	gTgTgACATATAgTACCTCgggAACC
BA041	Isopentenyltransferase	AB186133	*Brassica perkinensis*	ATgCgACTCCggTTATCACAAggCgT	gACTACTACCATgAgTgATgAgTggg
BA042	Jasmonic acid glucosyltransferase	DQ158907	*Arabidopsis thaliana*	ACAAggTCACATAAACCCTCTCCTCC	CgATTAgCAggTgACAAAgACTACgg
BA043	Jasmonate inducible protein	Y11482	*Brassica napus*	TAgATCCATCgAggTggAATACgAgg	AgAgACAAAggAAggACCTgCCCATg
BA044	Mannitol Stress inducible	AW288083	*Brassica juncea*	gTATCTTgCgACCgTggAggCATCTA	CgAAAggTCgTgTggTTTgTTgTTgC
BA045	Metallothionein I	H07628	*Brassica napus*	AgAgggTgTCgCTgAgAACgATgCTA	ggCgATgAAgAACCAgTACgAggCTT

BA046	MAP (mitogen-activated protein) kinase	D14713	*Arabidopsis thaliana*	ATgCCTATCAAgCCTATTggTCgTgg	CTCTgAAgCACTCCAgCATCCATACA
BA047	mRNA expressed during secondary infection	AJ605576	*Plasmodiophora Brassicae*	gCACgCATTCTACACgATACAgTTgC	TCgggCAAgACCAAgTCggTCATCAA
BA048	mRNA expressed in host plant	AB009880	*Plasmodiophora Brassicae*	gCTCTTCCAgTTgCTgTTTCAAgTCg	CgAgggATgTATCAAACgCgggAATg
BA049	Myrosinase, thioglucoside glucohydrolase	Z21978	*Brassica napus*	TAgCCAgTAgCATTgAgTTCgCCCAT	CCAgTgAAAgAgggTAACgAAAggCg
BA050	Myrosinase	X79080	*Brassica napus*	ACCACAgACTCATAgATggCCTCATC	CACTgAAgCAgAAgCCAgACTTgTTg
BA051	Myrosinase-binding protein	U59443	*Brassica napus*	AgAACggACAgACAAAggAAggACCC	CTggTTACTACCgAACgACTTTCTCC
BA052	Myrosinase	X78285	*Brassica napus*	TCATTCCATTggTCCACTgTTCgAgg	gTTCgAggCAAACAgTgACgAAACCA
BA053	Nitrilase	H07604	*Brassica napus*	ggTTCCTggACCTgAAgTggATAAgC	AggCgTATATCggTggCTATCCTCgT
BA054	Nitrilase I	AI352935	*Brassica napus*	AACTCggTgCTgCTATTTgCTgggAA	ggATCAACCATCCCTgTCTATgACAC
BA055	Pathogenesis related protein, PVPR3	AI352768	*Brassica napus*	gTTCAgATgCCgATgCggTgACCTTT	gAAATgTTTCAACgCTTCCATCgCCg
BA056	Pathogenesis related protein	AF528177	*Brassica rapa*	TCACAACCAAgCACgACAggCAgTAg	AgCTCTTgTTCATCCCTCgAAAgCTC
BA057	Pathogenesis-related protein, CXc750	AI352712	*Brassica napus*	CTCCTCCTTCTCgTTCTTgTgTTTCC	CCCACTCCgggAATgAATggTTATgA
BA058	Pectin methylesterase inhibitor	DQ116449	*Brassica oleracea*	CggCTgTgTCTgACTACggAgTATgT	CggTTCAgAATgTAgCggTTgACCTg
BA059	Phenylalanine ammonia-lyase	AY055752	*Brassica rapa*	TATggAgAgTATgggCAAAggAACCg	ACCAACATCACTCCTTCCCTCCCTCT
BA060	Phenylalanine ammonia-lyase	DQ167187	*Brassica rapa*	CCCggTgACTAgCCATgTTCAATCAg	gAAAgTTCTCACCACTggAgTCAACg
BA061	Phenylalanine ammonia-lyase	AA960723	*Brassica napus*	ATCAgCAgAgCAACACAACCAAgACg	CgAgAAACAAAgCCATTCACggTggT
BA063	Polygalacturonase inhibitor protein	AA960715	*Brassica napus*	TCTCAggTgTTgTCCCAgCgAgTTTg	gAATgggAAggTgTTCAATgCAACCC
BA065	Resistance-like protein, RGA-1	AF107545	*Brassica napus*	gTCAACgAATgAAgCgTgggAACTgT	gggAAgACgACCCTCTTAgCTCgTAT
BA066	Ribosomal protein S15a	X59984	*Brassica napus*	TCCTTCCCgACAgTTTggCTACATTg	TCAgTgTgCTCAACgATgCTgTgAAg
BA069	RPM1 interacting protein 4	NM113411	*Arabidopsis thaliana*	CCAgAACCAAACCTgAgCAAgTTgAC	CTTCAAgAACgCCgACTCATCAAAgC
BA070	SGT1-like protein	AJ620883	*Brassica oleracea*	AgAggCgTTCTTAgATgACgACTTCg	TTTgCAgAgTCCAATgggACggTgCT
BA071	Superoxide dismutase	AF540558	*Brassica juncea*	CTATTgTCggAAgggCTgTTgTTgTC	AAggAgACggTgTgACCACTgTgACT
BA072	Cu/Zn superoxide dismutase	AF071112	*Brassica perkinensis*	CAggAAggAgATggTgCCACAACTgT	ATgACCTggCTACTTTgTggTTggTg
BA073	Ubiquitin conjugating enzyme	CB331875	*Brassica napus*	CTTCAgACAgTCCTTACgCTggTggT	TCAACTgCACggAgCTggACTCAgAA

BA074	Ubiquitin-protein ligase	NM123599	*Arabidopsis thaliana*	ATgAggTTgTggATgCggTggAgATT	gCTACCgACATTCTCTACCAACCCgT
BA075	Ubiquitin-protein ligase	BE038411	*Arabidopsis thaliana*	AgTgCTggCCCAgTTgCTgAAgACAT	TgTgTCCTTTgATCCCTCACAgACTC
BA076	Xyloglucan endotransglycosylase/hydrolase	AY834281	*Brassica rapa*	gAgTTCTTAggCAACACgACgggTgA	ATgCggCgAggTTTCCTgTTACTCCT
BA077	Xyloglucan endotransglycosylase precursor	AY156708	*Brassica oleracea*	CTCCgTCCTCTggAATCTCTACCAAA	TATCgTCgCCTCAAATgggTCCgCAT
BA078	Xyloglucan endo-transglycosylase	H07799	*Brassica napus*	gCCgTgggAAgATACTCAACAACTgg	gTAACTCAgCAggAACCgTCACAACT
BA079	Zinc-finger homologue	AI352966	*Brassica napus*	ggCgAAATCACACgAAgTTTACCgAg	ggTgAATTggAACCCAgAggCTTAAC
BA080	Zeatin O-glucosyltransferase 1	AY573820	*Arabidopsis thaliana*	CAACggCAggATgTgTAAggATAAgC	CTgTCCTTCCggTgAACCCgATTCTT
BA081	Zeatin O-glucosyltransferase 3	AY573822	*Arabidopsis thaliana*	TCTCTgCTTTgTCggCTCCTACCTTg	CTAAgTTgATgggCAAgCCAgACTCA

[a] Oligonucleotide modification: 5'-amine-modified Cy5 dye coupled with 10 deoxythymidines

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