Identification and characterisation of genes controlling the resistance response to ascochyta blight (*Ascochyta rabiei* (Pass.) Labrousse) in chickpea (*Cicer arietinum* L.)

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Declaration

I declare that this thesis contains my original work and that information from published or unpublished sources has been clearly acknowledged within the thesis. None of the work has been submitted previously in whole or part for any other academic award in any other university. The content of the thesis is the result of work carried out since the official commencement date of the research program.

Tristan Coram

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Thesis Abstract

Ascochyta blight, caused by *Ascochyta rabiei* (Pass.) Labrousse, is one of the most destructive diseases of chickpea (*Cicer arietinum* L.) worldwide. Despite the existence of highly resistant uncultivated genotypes, attempts to develop cultivars with a high level of durable resistance have been unsuccessful. Important reasons behind this obstacle were identified as the conflicting reports concerning the genetics of resistance, as well as a limited understanding concerning the genes, and pathways of gene activation, involved in an effective defence response. This study investigated the chickpea defence response to *A. rabiei* using a functional genomics approach, which has the capacity to improve the overall understanding of the coordinated defence response at a molecular level.

To enable the employment of functional genomics techniques, an existing cDNA library derived from a highly resistant uncultivated genotype was used to generate a resource of Expressed Sequence Tags (ESTs) that, after clustering, comprised 516 unigenes. The unigenes were functionally annotated resulting in the identification of 20 specific defence-related unigenes, as well as numerous transcripts with possible involvement in the coordination of defence responses. Additionally, 14 Simple Sequence Repeats (SSRs) were identified for potential use as molecular markers, including one SSR within a defence-related unigene. Importantly, comparison of the chickpea unigenes to annotated sequences of model legumes indicated that a high proportion of the chickpea transcriptome may be insufficiently homologous to model legumes, which would limit the use of their EST collections for the study of chickpea.

To explore the expression patterns of the defence-related unigenes in an *A. rabiei* resistant and susceptible genotype, the unigenes were employed as probes in the construction of

microarrays. An artificial inoculation procedure that resulted in disease progression similar to that observed in the field was developed before sampling infected genotypes over a time-course (12, 24, 48, 96 h) after *A. rabiei* infection. Extracted RNA samples were converted into fluorogenic probes and used as targets in microarray hybridisations. Resulting expression data was analysed to identify differentially expressed unigenes over the time-course. Comparison of the expression profiles from the resistant and susceptible genotype enabled the identification of three putative genes that were exclusively up-regulated in the resistant genotype, thus may be involved in an effective defence response. The results validated the microarray methodologies used to enable the construction of large-scale arrays.

Considering that an overall defence response can involve hundreds of genes, from recognition to signalling to direct involvement, the entire set of chickpea unigenes were used to construct large-scale microarrays. To supplement the chickpea probes, 156 putative defence-related grasspea (*Lathyrus sativus* L.) ESTs and 41 lentil (*Lens culinaris* Med.) Resistance Gene Analogs (RGAs) were also included as probes on the array, and enabled exploration of the potential for cross-species hybridisation. Microarray expression profiles for three chickpeas and one wild relative were generated over a time course (6, 12, 24, 48, 72 h). Hybridisation to grasspea probes was successful but all lentil probes failed, most likely due to the presence of non-coding regions in these RGA probes. A total of 97 differentially expressed ESTs were identified using a robust experimental system that included multiple replication, stringent statistical tests and confirmation of microarray data by quantitative RT-PCR. The results indicated that genes involved in the active defence response were similar to those governed by *R*-gene mediated resistance, including the production of reactive oxygen species (oxidative burst) and the hypersensitive response, down-regulation of ‘housekeeping’ gene expression, and expression of pathogenesis-related proteins. The comparison between resistant and susceptible genotypes identified certain gene expression ‘signatures’ that may be predictive of

resistance. The results confirmed histopathology studies of the chickpea defence response and provided novel insights to the molecular control of these events.

To further characterise the regulation of potential defence-related genes, the large-scale microarray (excluding failed lentil probes) was again used to study the expression profiles of the three chickpea genotypes (excluding the wild relative) after treatment with the known defence signalling compounds, ethylene (E), salicylic acid (SA), and jasmonate (JA). Treatments were administered to hydroponically cultured seedlings, and tissue was harvested at two post-treatment time-points (3 and 27 h). Stringent data quality control resulted in differential expression of 425 ESTs, and comparison between genotypes revealed the presence of a wider range of inducible defence responses in resistant genotypes. Linking the results with the previous microarray results to identify possible regulation of the important ESTs for *A. rabiei* defence indicated the presence of other pathogen-specific signalling mechanisms in addition to E, SA and JA. The lower arsenal of defence-related gene expression observed in the susceptible genotype may be a result of 'breaks' in the pathways of defence-related gene activation. The observation that resistant and susceptible genotypes possessed differing responses to the signalling compounds, and that the susceptible genotype was able to mount some defence to *A. rabiei* in the previous microarray experiments indicated that the 'breaks' may not related to pathogen recognition, but to signal transduction.

To draw together the findings of all experiments, a model was constructed for a hypothetical mechanism of chickpea resistance to *A. rabiei*. The model was synthesised based on both the evidence gathered in this study and previously documented defence mechanisms in chickpea, and identified signal transduction as a key to resistance. Enhancement of the body of knowledge regarding chickpea resistance to *A. rabiei* as a result of this study was described, as well as recommendations for further study of the candidate resistance genes.

Thesis Publications

Refereed Journals

Coram TE and Pang ECK (2005). Isolation and analysis of candidate ascochyta blight defence genes in chickpea. I. Generation and analysis of an expressed sequence tag (EST) library. *Physiological and Molecular Plant Pathology* 66:192-200.

Coram TE and Pang ECK (2005). Isolation and analysis of candidate ascochyta blight defence genes in chickpea. II. Microarray expression analysis of putative defence-related ESTs. *Physiological and Molecular Plant Pathology* 66:201-210.

Coram TE and Pang ECK (2006). Identification and expression profiling of chickpea genes differentially regulated during a resistance response to *Ascochyta rabiei*. *Plant Biotechnology Journal*. Accepted and In Press.

Manuscript in preparation

Coram TE and Pang ECK. Transcriptional profiling of chickpea genes induced by methyl jasmonate, salicylic acid, and aminocyclopropane carboxylic acid. *Plant Physiology* (Target: July 2006).

Refereed Conference

Coram TE and Pang ECK (2006). Identifying and characterising the patterns of gene expression upon infection of chickpea plants by Ascochyta blight. In: *Proceedings of the 13th Australasian Plant Breeding Conference*, Christchurch, New Zealand, 18-21 April.

Pang ECK and **Coram TE** (2005). The use of microarrays for understanding the resistance response of crop plants to pathogens. In: *Proceedings of the International Conference on Crop Security*, Malang, Indonesia, 20-22 September.

Coram TE and Pang ECK (2004). Discovering and characterising the genetic mechanism controlling the resistance response to Ascochyta blight in chickpea. In: *Proceedings of the 4th International Crop Science Congress,* Brisbane, Queensland, 26 September – 1 October.

Coram TE and Pang ECK (2002). Ascochyta blight of chickpea –searching for resistance gene analogues and characterising an enriched EST library. In: *Proceedings of the 12th Australasian Plant Breeding Conference*, Perth, Western Australia, 15-20 September pp 17-22.

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Chapter 1

Introduction: Review of literature

This review is intended to provide an overview of the current state of knowledge regarding chickpea and the fungal disease known as ascochyta blight, particularly in Australia. Firstly, the review focuses on the resistance of chickpea to the disease and covers topics including the genetics of resistance, sources of resistance, and molecular breeding. For comprehensive information on all aspects of ascochyta blight of chickpea, the reader may also refer to excellent reviews by Pande *et al.* (2005), Gan *et al.* (2006), and Millan *et al.* (2006). Secondly, this review summarises general plant-pathogen interactions, including the current state of knowledge regarding plant defence mechanisms and resistance genes. For more detailed discussions on these topics please refer to Martin *et al.* (2003) and Grant and Mansfield (1999). Thirdly, the review highlights the relatively new field of functional genomics, with emphasis on the use of microarrays for improving ascochyta blight resistance breeding in chickpea. Again, excellent reviews on microarray technology are available, including Aharoni and Vorst (2001), and Clarke and Zhu (2006). Finally, gaps in the knowledge of chickpea resistance to ascochyta blight are identified throughout the review, indicating potential opportunities for study. These opportunities lead to the development of a rationale for thesis study that may address some of the knowledge gaps.

1.1 Chickpea

1.1.1 Cultivation and uses

The commonly cultivated chickpea, *Cicer arietinum* Ladizinsky, is a self-pollinated, diploid ($2n = 2x = 16$) annual pulse crop with a relatively small genome of 740 Mb (Arumuganathan and Earle, 1991). Chickpea originated in South-Eastern Anatolia (Turkey) (Ladizinsky,

1975), and was traditionally cultivated in Asia, the Mediterranean, the Middle East, and Northern Africa. In contemporary times, chickpea has also become popular throughout the temperate regions of the world, in countries such as Mexico, Canada and Australia (Duke, 1981). Chickpea plants are usually 0.2-1 m tall with a highly nodulated root system. The crop normally matures within one month of flowering, with the cycle varying from three to six months. Chickpea is valued for its nutritious seeds, which contain 20-30% protein, ~40% carbohydrate and only 3–6% oil (Gil *et al.*, 1996). Chickpeas are mainly used for human consumption, providing an important source of protein especially for people in developing countries. Seeds are eaten whole, as dhal or flour, or the young shoots eaten as vegetables. In some countries chickpea is also used as feed for livestock. Of the cultivated chickpea, distinct cultivar groups exist; the large-seeded, ram-head shaped, cream coloured Kabuli; the small-seeded, dark coloured, angular Desi; and intermediate types (Van der Maeson, 1972; Duke, 1981; Cubrero, 1987). Desi chickpeas, accounting for approximately 85% of global chickpea production, are generally grown in the south of Asia, Iran, Ethiopia and Mexico, whilst Kabuli types are grown in the Mediterranean and Latin American regions (Anon, 2002).

Chickpea can usually be cultivated with minimal input of irrigation, fertilizers and pesticides (Singh *et al.*, 1993). In India, chickpea is grown after the harvest of cereal crops in the cool season, but in temperate regions such as the Mediterranean, the crop is grown as a winter annual (Langer and Hill, 1982). Chickpea can be produced in low fertility soils but is best suited to light loam soils and well-drained clays. Chickpeas are often used in rotation with other crops, and contribute to the maintenance of soil fertility through the fixation of atmospheric nitrogen (Singh, 1997). In Australia, chickpea is often used in rotation with wheat, where the main benefits are increased grain yield and grain protein concentration attributable to increased nitrogen supply and improved water-use efficiency (Dalal *et al.*, 1998; Marcellos *et al.*, 1998). Additional benefits of using chickpea in crop rotations are

improved sowing time and tillage practices for chickpea (Horn *et al.*, 1996a; b). In North America, chickpea is also grown in rotation with field peas and lentils (Kaiser and Muehlbauer, 1988).

1.1.2 Production of chickpea

Chickpea is the third most important pulse crop in the world behind dry bean (*Phaseolus vulgaris* L.) and pea (*Pisum sativum* L.) (Food and Agriculture Organization, 2006). The world production of chickpea in 2005 was ~9.2 million metric tonnes from ~11.2 million hectares, constituting ~15% of world pulse production from ~15% of the total global area used to grow pulses (Food and Agriculture Organization, 2006). In 2005, Australia was listed as the largest exporter and seventh largest producer (Food and Agriculture Organization, 2006). Australian chickpea production increased rapidly since its first cultivation in Queensland in 1978 and, excluding Tasmania and the Northern Territory, the crop is now grown in all states (Figure 1.1). However, the trend of Australian chickpea production declined since 1997 (Figure 1.2), due to outbreaks of ascochyta blight.

Chickpea yields have remained low compared to other pulses (world average ~0.8 metric tonnes/ha; Food and Agriculture Organization, 2006), mainly due to a series of biotic and abiotic stresses that reduce yield and yield stability (Millan *et al.*, 2006). The fungal diseases are considered the most serious, and can be either foliar or soil-borne. Foliar fungal diseases include ascochyta blight, botrytis grey mould, alternaria blight and stemphylium blight, whilst soil-borne fungal diseases include fusarium wilt, verticillium wilt, dry root rot, collar rot and wet root rot. Ascochyta blight is a highly destructive disease of chickpea, thus improving resistance to this disease is a major aim of chickpea breeders around the world (Millan *et al.*, 2006).

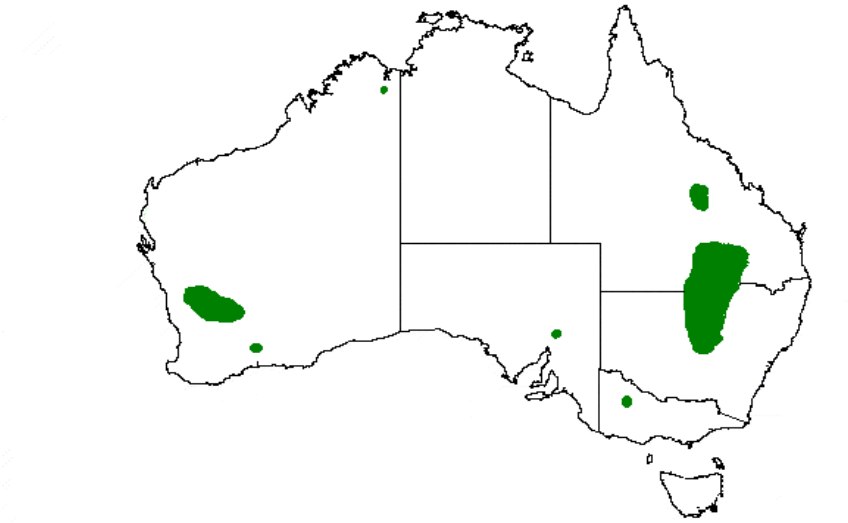

Figure 1.1 Chickpea production areas in Australia (highlighted in green) (Pulse Australia, 2001).

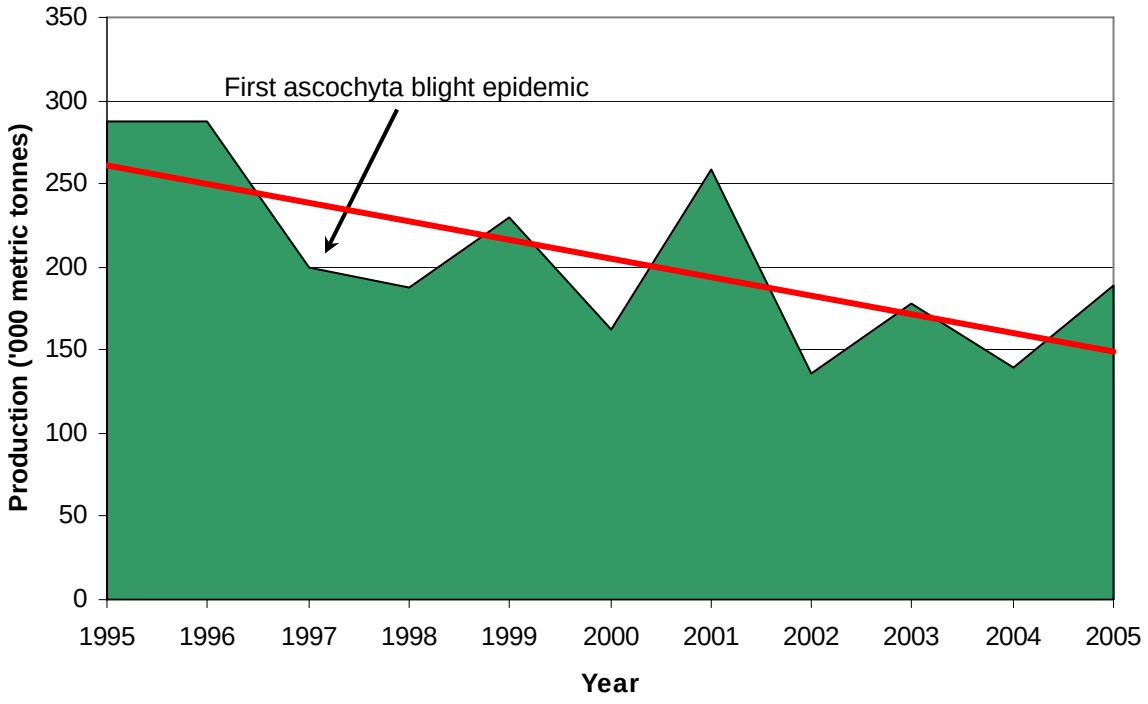

Figure 1.2 Annual Australian chickpea production since 1995, showing a linear trendline (red line) and the first ascochyta blight epidemic in 1997 (Food and Agriculture Organization, 2006).

1.2 Ascochyta blight of chickpea

1.2.1 Causal organism

Ascochyta blight of chickpea is caused by the ascomycete fungus *Ascochyta rabiei* (Pass.) Labrousse. Ascochyta blight has been recorded in most chickpea-growing areas in the world (Pande *et al.*, 2005), and has been present in Australia since at least 1991 (Khan *et al.*, 1999). Under favourable environmental conditions, where relative humidity is >60% and temperature 10-20°C, ascochyta blight has devastating destructive power and develops in epiphytotic proportions (Nasir *et al.*, 2000). Under such conditions, some researchers have demonstrated a chickpea yield loss of up to 100% (Reddy and Singh, 1990). *A. rabiei* has two stages to its life cycle; asexual (anamorph) and sexual (teleomorph) (Figure 1.3).

In Australia, the disease first appeared in South Australian evaluation trials in 1973 (Cother, 1977). Although this outbreak was eradicated, sporadic outbreaks were recorded in the 1980s and early 1990s (Khan *et al.*, 1999). The first Australian epidemic did not occur until 1998, when chickpea crops in South Australia, Victoria, New South Wales, and Queensland were severely devastated. This epidemic reduced chickpea yields of that season from an estimated 300,000 metric tonnes (Pulse Australia, 2001) to just 191,000 metric tonnes (Figure 1.2). The disease is currently the most important yield-limiting factor to chickpea cultivation in Australia, affecting up to 95% of the production area in Australia (Knight and Siddique, 2002). Chickpea yields, as well as farmer confidence in the crop, have not recovered since the first epidemic of ascochyta blight, in fact, annual production has steadily declined (Figure 1.2).

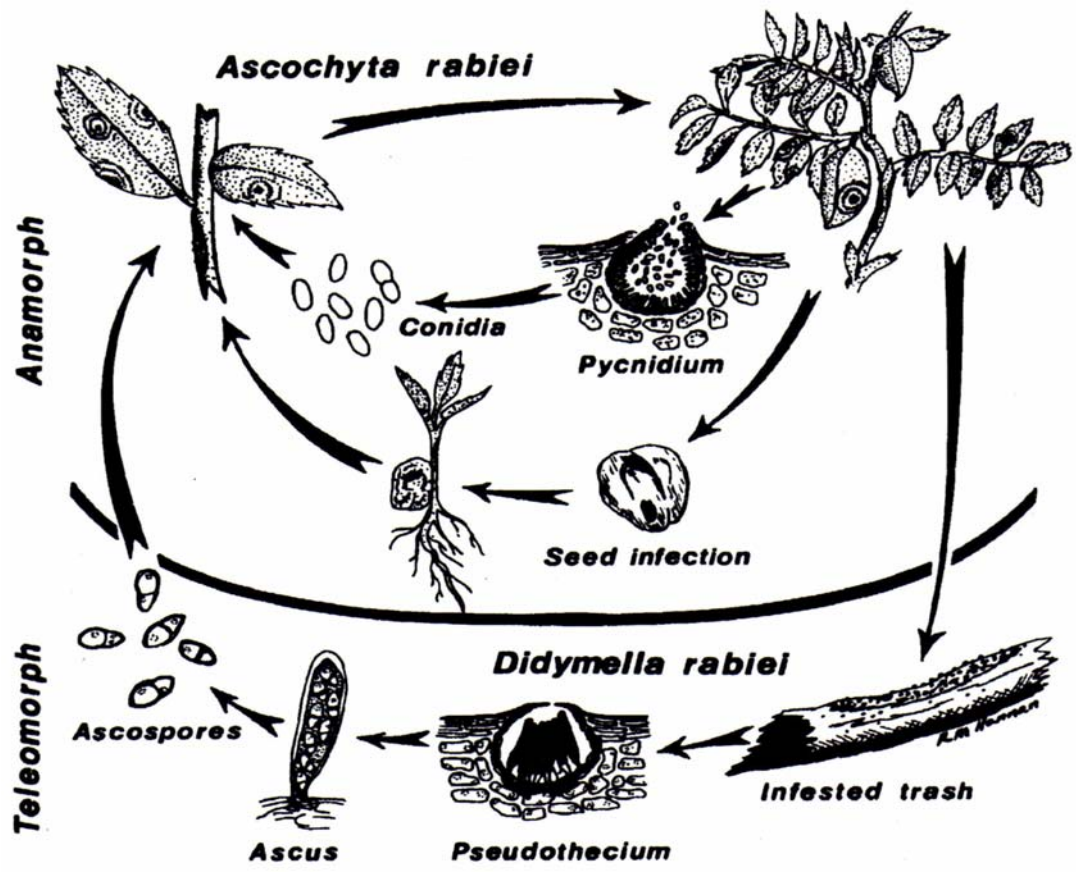

Figure 1.3 Life cycle of *Ascochyta rabiei* (Kaiser, 1997).

1.2.2 Symptoms

Ascochyta blight inflicts severe damage upon all above-ground parts of the plant at any growth stage, leading to rapid cell collapse and spread of necrotic lesions (Figures 1.4 and 1.5). Such lesions are usually round or elongated, and have pycnidia arranged in concentric circles (Nene, 1982). Additionally, stem lesions produce a girdling effect, where the portion of the plant above the stem lesion rapidly dies (Pande *et al.*, 2005). In the field, ascochyta blight initially appears as small patches of blighted plants, which can rapidly spread across entire fields under favourable environmental conditions. Air-borne conidia and ascospores infect younger leaves, whilst water-borne conidia are splash-dispersed to infect foliage on the same or nearby plants. Subsequently, symptoms spread rapidly to all aerial parts of the plant, causing rapid tissue collapse and plant death.

Figure 1.4 Necrotic lesions on a chickpea pod caused by *A. rabiei* (C. Pittock, pers. comm.).

Figure 1.5 Girdling effect of stem lesions caused *A. rabiei*.

1.2.3 Reproduction

1.2.3.1 Asexual (imperfect) stage

The asexual stage of the fungus is characterised by the production of pycnidia (fruiting bodies), which produce spores (Figure 1.3). Pycnidia appear as tiny dots in lesions produced on host plants, the pycnidium has a prominent ostiole, and spores are hyaline, oval-oblong, straight or slightly curved in shape. Spore sizes are in the range of 8.2-10.0 x 4.2-4.5 µm (Nene, 1982). Optimum growth and spore germination temperature is 20ºC (Kaiser, 1973), but temperatures <10ºC or >30ºC are unfavourable. Additionally, continuous light has been reported to both increase (Kaiser, 1973) and decrease sporulation (Chauhan and Sinha, 1973).

1.2.3.2 Sexual (perfect) stage

The sexual stage was first identified as *Mycosphaerella rabiei* but later transferred to the *Didymella* genus based on asci features, and renamed *Didymella rabiei* (Kov.) von Arx. It is a bipolar heterothallic ascomycete characterised by pseudothecia developing on chickpea crop residues that have over-wintered in the field. Pseudothecia are dark brown to black, subglobose, 120-270 µm in diameter, erupting from the host tissue and without an ostiole (Pande *et al.*, 2005). Binucleate asci are cylindrical to subclavate surrounded by paraphyses and contain eight hyaline unequally bicellular spores. Ascospores are ellipsoid to biconic with a constriction at the septum and measure 9.5-16.0 x 4.5-7.0 µm (Pande *et al.*, 2005). The sexual stage has been discovered in the USA, USSR, Greece, Syria, Spain, Bulgaria and Hungary but not in India, Pakistan or Turkey (Trapero-Casas and Kaiser, 1992).

The development of viable ascospores of the teleomorph requires the pairing of two compatible *A. rabiei* mating types, referred to as MAT1-1 and MAT1-2 (Yoder *et al.*, 1986). In Australia, only one pathotype (Nasir *et al.*, 2000) and one mating type (MAT1-1) of the fungus have been detected (Khan *et al.*, 1999). However, the teleomorph has recently been

isolated in Western Australia (Galloway and MacLeod, 2003), which implies that either both mating types are present in that area or a low level of homothallic compatibility exists in *A. rabiei*. The importance of the teleomorphic stage to disease epidemiology is still unclear. The presence of both mating types in a population does not necessarily indicate that the population is sexually reproducing, because environmental conditions may prevent mating of compatible isolates. However, the presence of the sexual stage may lead to greater pathogen virulence, since sexual reproduction creates new genotypes via recombination.

1.2.4 Pathogenic variability

There are many reports of different pathotypes of *A. rabiei*, which are identified from variations in host-pathogen interactions and breakdown of host plant resistance in some cultivars in different locations. However, there is debate whether differences are caused by the presence of different pathotypes or variation in virulence of isolates (Udupa *et al.*, 1998). Generally, *A. rabiei* pathotypes can be classified into three broad groups; pathotype I (low virulence), pathotype II (average virulence) and pathotype III (high virulence) (Udupa *et al.*, 1998). The pathotype of an *A. rabiei* isolate is determined by assaying its pathogenicity on a set of differential chickpea cultivars (Udupa *et al.*, 1998), and several different pathotypes have been identified in different geographical areas (Porta-Puglia *et al.*, 1996; Udupa *et al.*, 1998; Nasir *et al.*, 2000; Chongo and Gossen, 2001; Chen *et al.*, 2004; Chongo *et al.*, 2004). However, it is important to note that pathotype studies in different regions have used different assay procedures and cultivars, and that variability in environmental conditions may affect results (Udupa *et al.*, 1998). Subsequently, the difficulty in standardising assay procedures and disease rating scales may lead to different estimates of pathotype diversity and variability.

The use of molecular markers for DNA fingerprinting has been applied in an attempt to define differences among putative *A. rabiei* pathotypes. Combinations of Random Amplified

Polymorphic DNA (RAPD) and microsatellite markers were used to distinguish variability within and among major pathotypes of *A. rabiei* from Syria and Lebanon (Udupa *et al.*, 1998), where genetic diversity analysis revealed three pathotypes that could be resolved into several genotypes. Fischer *et al.* (1995) also used RAPD markers to assess pathogenic groups among Italian *A. rabiei* isolates, but were unable to correlate RAPD patterns and pathogenic groups. Isozyme markers have also failed to correlate to pathogenic groups in *A. rabiei* isolates from Pakistan (Hussain and Barz, 1997). A study on 37 *A. rabiei* isolates from India, three from Syria, two from Pakistan and five from the USA, utilised RAPD markers to group isolates according to their geographic origin, but again no correlation between genetic diversity and pathogenicity was detected (Santra *et al.*, 2001). For Australian isolates, Phan *et al.* (2003) used the sequence tagged microsatellite (STMS) technique to identify specific DNA fragments that may be used as isolate-specific genetic markers.

Many authors suggested that further research was required to understand the pathogenic variability of *A. rabiei*, since the durability of host resistance is related to the variability of the pathogen and its capacity to overcome resistance. A standard set of differential lines, which clearly distinguish all *A. rabiei* isolates from a broad geographical area, may help in the identification of *A. rabiei* pathotypes (Pande *et al.*, 2005).

1.2.5 Mode of infection

The infection process in both resistant and susceptible genotypes has been well studied. Spores begin to germinate on plant surfaces 12 hours post-inoculation (hpi) and germ tubes elongate and form ramifications on leaf surfaces (Pandey *et al.*, 1987). A mucilaginous substance is secreted to facilitate attachment to the host surface, and cell-wall-lytic enzymes are produced to assist host tissue penetration (Jayakumar *et al.*, 2006). Hyphal branches form appressoria, which are involved in direct cuticle penetration between two epidermal cells at

24 hpi, using mechanical forces (Kohler *et al.*, 1995). Hyphae push forward subcuticularly along the junction of epidermal cells before penetrating through the juncture of guard and subsidiary cells, even when the stoma is open (Pandey *et al.*, 1987). Hyphae have also been reported to penetrate through hydathodes (Kohler *et al.*, 1995).

In the early stages of infection following penetration, hyphae grow in parallel between epidermal and palisade parenchyma cells, spreading within the intercellular space to form dark aggregates by four days post-inoculation (dpi) (Kohler *et al.*, 1995). Epidermal cells appear intact after three dpi but macroscopic symptoms were observed at four dpi as yellow specks on the stem surface, representing necrotic epidermal cells (Pandey *et al.*, 1987). In later stages of infection (five dpi), the entire cortex and pith are completely disintegrated. The breakdown of tissue distant from invading hyphae was observed, indicating the presence of cell wall degrading enzymes. Hyphae in the cortical tissue aggregate, forming a pseudoparenchymatous mycelial mass that differentiates into pycnidia. Pycnidia, visible as black spots, protrude from the stem epidermis and conidia are produced through an ostiole by six to eight dpi. Most non-lignified tissues are destroyed by seven dpi, resulting in the girdling and collapse of some plants. *A. rabiei* is usually detected in the phloem and rarely in the xylem (Kohler *et al.*, 1995), suggesting that the phloem may provide a nutrient source or be easier to destroy. By the end of infection, almost all cells are destroyed and filled with fungal biomass, typical of the nectrotrophic mode of infection.

1.2.6 *A. rabiei* toxins and enzymes involved in infection

Extensive degradation of tissue in advance of invading hyphae indicate the involvement of toxins and cell wall degrading enzymes produced by *A. rabiei* (Pandey *et al.*, 1987; Hohl *et al.*, 1990). Culture filtrates of the pathogen were found to kill cells isolated enzymatically from plant leaflets, leading to the identification of the toxins solanapyrone A, B and C, as well

as cytochalasin D, and a proteinaceous toxin (Alam *et al.*, 1989; Chen and Strange, 1991; Jayakumar *et al.*, 2006). There is a strong correlation between the production of solanapyrones by different isolates of *A. rabiei* and their pathogenicity (Kaur, 1995). Solanapyrone C was the only detectable phytotoxin collected from chickpea cultivars varying in disease susceptibility, where lower levels were observed in resistant cultivars (Shahid and Riazuddin, 1998). The cause of the reduced levels was unknown, but the authors suggested that resistant plants might possess the ability to inactivate/degrade solanapyrone C or inhibit its synthesis.

A. rabiei degrades phytoalexins produced in chickpea plants by converting the pterocarpans into inactive forms (Tenhaken *et al.*, 1991). Other pathogenic enzymes such as cutinise and polygalacturonase were purified from culture filtrates of *A. rabiei* (Tenhaken and Barz, 1991; Tenhaken *et al.*, 1997), and may be necessary for the infection process.

1.2.7 Epidemiology

Comprehensive information on the epidemiology of the disease can be found in excellent reviews by Pande *et al.* (2005) and Gan *et al.* (2006). Briefly, *A. rabiei* survives on, or in, seed or plant debris in the form of mycelium, pycnidia, and various teleomorphic stages (Figure 1.3) (Kaiser, 1997). *A. rabiei* may remain viable for up to 2 years in infected chickpea debris but remains viable for <1 year when buried in at least 3-4 cm of soil (Gossen and Miller, 2004). *A. rabiei* may also survive by infection of chickpea seed coats, which allows later infection in chickpea fields.

The spread of *A. rabiei* is achieved by spores produced during primary infection of crop debris or seed (Nene, 1982). Seed transmission is most important as it ensures random distribution of the pathogen in a field to provide many primary infection sources. In fact,

movement of infected chickpea seed is responsible for introducing *A. rabiei* into Canada, Iran, Australia and the USA (Kaiser, 1997). Secondary spread of the disease is achieved through conidia and ascospores, which are dispersed by wetting, rain splash and wind. Ascochyta blight infection and disease development occurs at temperatures of 5-30ºC with an optimum of 20ºC, whilst 17 h of moisture is essential to produce severe infection (Pande *et al.*, 2005).

Tripathi *et al.* (1987) reported that only *Cicer* species were hosts for *A. rabiei*, but artificial inoculation of *A. rabiei* on lentil, field pea, vetch, common bean and cowpea has been achieved (Pande *et al.*, 2005). Disease symptoms were also observed on three common bean (*Phaseolus vulgaris* L.) cultivars after *A. rabiei* inoculation (Khan *et al.*, 1999).

1.2.7.1 Disease management

The management and control of ascochyta blight may be attempted through three different strategies; physical and cultural practices, chemical control, and genotype resistance. As each independent method is not entirely effective, all three measures must be integrated to produce a maximised disease management strategy (Gan *et al.*, 2006). Physical and cultural practices involve field sanitation, the use of clean seed, and sowing properties. The use of disease free seed is essential to prevent the introduction of the pathogen to new chickpea-growing areas (Gan *et al.*, 2006). Fields used for seed production should be positioned in arid areas where minimal rainfall occurs during flowering and fruiting periods, or at harvest. Additionally, furrow irrigation should be used rather than overhead sprinkling to minimise disease development (Chaube and Mishra, 1992). The exposure of chickpea seeds to sunlight and heat may reduce the seedborne inoculum of infected seed (Tripathi *et al.*, 1987).

Sowing time is also problematic for farmers, as the conditions that favour chickpea growth also favour disease development (Tekeoglu *et al.*, 2000). With effective disease control

farmers could sow in early winter without risking an epidemic, where yield may be increased by 50% compared to sowing in spring (Reddy and Singh, 1993). Further, the highest yields are obtained by sowing at a density of 5 cm (spaces within rows) x 20 cm (row spacings) (Eser *et al.*, 1991), where wider rows correlate with less disease (Gan *et al.*, 2006). The rotation of chickpea with non-host crops may also reduce the inoculation potential of *A. rabiei*. A 1-2 year period of non-host crops is recommended for warm moist areas, and a 3-4 year period for cooler climates where chickpea stubble breaks down slowly (Gan *et al.*, 2006).

The use of chemicals to control ascochyta blight usually refers to the application of fungicides. Fungicide application is usually required under favourable environmental conditions for disease, where the use of partially resistant cultivars does not provide adequate control for the disease (Gan *et al.*, 2006). Although several fungicides have proved effective for disease control, the need for repeated protective application under epiphytotic conditions often makes them uneconomical (Gan *et al.*, 2006). In Australia, chickpea varieties susceptible to *A. rabiei* have been successfully grown by strategic application of protective fungicides such as chlorothalonil and mancozeb several times in the growing season (Bretag *et al.*, 2000). Seedborne *A. rabiei* infection can also be effectively controlled using carbendazim and thiram (1:1), captan, iprodione, and propiconazole (Singh and Singh, 1990). Research into the use of fungicides to control *A. rabiei* has revealed that chemical control should be used only as a complement to genotype resistance (Shtienberg *et al.*, 2000).

1.3 Host resistance

Ascochyta blight resistance of chickpea is determined by a diverse range of anatomical, physiological, biochemical and genetic factors. Metabolic activities involved in host resistance include the induction of a hypersensitive response (HR), cell wall reinforcement, induction of phytoalexins, and proteins that inhibit pathogen growth or virulence (refer to

section 1.4.1). Hohl *et al.* (1990) studied infection in both susceptible and resistant chickpea genotypes and found no difference in the rate of formation of appressoria.

1.3.1 Anatomical and physiological characters

Anatomical characters involved in chickpea resistance to ascochyta blight include thickness of leaf cuticles, epithelium, and palisade cells to provide a mechanical barrier to *A. rabiei* penetration. Resistant cultivars possess increased numbers of xylem elements and xylem parenchyma cells, as well as a thicker stem epidermis and hypodermis (Angelini *et al.*, 1993). Susceptible chickpeas had a thinner outer cell wall and smaller area of cell lumen in the second outer cell layer (Venora and Porta-Puglia, 1993). Lignin, an integral part of plant cell walls, has also been implicated in *A. rabiei* defence, where histological studies found that *A. rabiei* was unable to penetrate highly lignified chickpea tissue, which was more abundant in resistant genotypes (Ilarslan and Dolar, 2002).

Chickpea resistance to ascochyta blight also correlates positively with respiration rate and total carbohydrate content. In a resistant genotype, it was observed that respiration rate and total carbohydrate content increased by the second dpi due to an HR, whilst the susceptible genotype only displayed a similar increase by the fifth dpi (Dolar and Gurcan, 1995).

1.3.2 Metabolic characters

In susceptible chickpea genotypes, *A. rabiei* invades and colonizes leaves, causing leaf spots and pycnidia 6-8 dpi (Hohl *et al.*, 1990). However, in resistant cultivars, leaves exhibit areas with autofluorescence within 24-48 hpi, which is typical of an HR (Hohl *et al.*, 1990). Incompatible plant-pathogen interactions often result in a rapid HR after an oxidative burst, which has also been reported in chickpea (Otte and Barz, 1996). The occurrence of an HR

was further confirmed by determining cell death by fluorescein staining, where resistant cells died rapidly at 12 hpi without any cell death in susceptible cells (Hohl *et al.*, 1990).

Further investigation of resistant genotypes revealed the synthesis of enzymes involved in primary and secondary metabolism, as well as enzymes involved in phytoalexin biosynthesis. Phytoalexins are metabolic compounds that play an important role in plant defence mechanisms (refer to section 1.4.1) and, in chickpea, resistant genotypes rapidly produce pterocarpan phytoalexins (medicarpin and maackiain) in higher quantities than susceptible genotypes (Dolar and Gurcan, 1993). Vogelsang *et al.* (1994) also reported rapid accumulation of medicarpin in cell walls of resistant chickpeas with no accumulation in susceptible cells.

In addition to the HR and phytoalexin biosynthesis, Hohl *et al.* (1990) also postulated the synthesis and secretion of antifungal compounds as part of the chickpea defence response. In fact, resistant genotypes have been shown to accumulate large quantities of phenolic isoflavone compounds compared with susceptible genotypes (Khirbat and Jalali, 1997). Further, induction of fungal cell wall-degrading hydrolytic enzymes and enzymes of the phenylpropanoid pathway have a role in conferring *A. rabiei* resistance. Cho *et al.* (2006) reported increased accumulation of flavanone 3-hydroxylase (F3H), a key enzyme of the phenylpropanoid pathway for antifungal flavonoid production, in an *A. rabiei* resistant chickpea. Activity of phenylalanine ammonia lyase (PAL) increased significantly in resistant compared to susceptible genotypes (Vogelsang *et al.*, 1994), whilst enzymes involved in the oxidative burst leading to an HR (peroxidase, copper amine oxidase, polyphenyloxidase, and catalase), have also been shown to have higher activity in resistant chickpea genotypes (Angelini *et al.*, 1993; Laurenzi *et al.*, 2001; Rea *et al.*, 2002; Sarwar *et al.*, 2003). Several pathogenesis-related proteins are also reported to be involved in chickpea resistance to

ascochyta blight, including the fungal cell wall degrading enzymes β-1,3-glucanase (Hanselle and Barz, 2001) and chitinase (Nehra *et al.*, 1994; Cho and Muehlbauer, 2004).

However, expression of the defence responses described above does not correlate with pathotype-specific resistance, indicating that other constitutive or unknown defence mechanisms may be involved in providing resistance to aggressive pathotypes (Jayakumar *et al.*, 2006). Therefore, opportunities exist for further study to identify potential unknown defence mechanisms in chickpea, as well as confirm previous observations.

1.3.3 Sources of resistance

The use of resistant cultivars is the most efficient and effective means of controlling ascochyta blight (Pande *et al.*, 2005). There exist numerous reports evaluating chickpea germplasm for resistance to *A. rabiei* in different chickpea-growing regions of the world (Reddy and Singh, 1984; 1993; Wadud and Riaz, 1988; Singh and Reddy, 1990; 1992; 1993; Gaur and Singh, 1996; Toker *et al.*, 1999). The majority of studies have evaluated genotypes in field trials under both natural infection and artificial inoculation. Disease severity is usually assessed using a 1 to 9 scale where 1 = immunity and 9 = plant death. At the International Center for Agricultural Research in the Dry Areas (ICARDA), >25000 lines have been screened for *A. rabiei* resistance, and 14 durable resistance sources have been identified (Pande *et al.*, 2005). In Australia, Nasir *et al.* (2000) identified 18 additional breeding lines with resistance to Australian *A. rabiei* pathotypes at the seedling stage.

The major problem of resistant cultivars is that resistance breaks down against new virulent races of *A. rabiei* that arise from mutation and genetic recombination. In fact, complete resistance to *A. rabiei* has not been found in chickpea, with the resistance present in superior cultivars only partial or incomplete (Jayakumar *et al.*, 2006). Additionally, there exists a high

degree of variation in resistance among cultivars, and resistance declines as the plant matures (Jayakumar *et al.*, 2006). Wild *Cicer* species have also been considered as sources for resistance, where high levels of resistance have been identified in genotypes of *C. bijugum*, *C. echinospermum*, *C. judicium* and *C. pinnatifidum* (Singh *et al.*, 1981; Collard *et al.*, 2001). Although interspecific crosses between wild species and *C. arietinum* have only been successful for *C. reticulatum* and *C. echinospermum* (Singh and Ocampo, 1993; Collard *et al.*, 2003), there is potential for transferring resistance genes from wild *Cicer* species into *C. arietinum*.

1.3.4 Genetics of resistance

Detailed information regarding the number, nature, and diversity of genes controlling resistance to ascochyta blight is essential for successful breeding programs. However, reports of the genetic basis of ascochyta blight resistance vary according to the chickpea genotype studied (Table 1.1). Initial studies suggested that resistance in desi chickpeas was controlled by a single dominant gene (Hafiz and Ashraf, 1953; Vir *et al.*, 1975). Singh and Reddy (1983) also reported a single dominant gene as the genetic control for resistance in four kabuli chickpea lines (ILC 72, ILC 183, ILC 200 and ICC 4935), but found that a single recessive gene conferred resistance in ILC 191. Subsequent research by Singh and Reddy (1989) identified that a single dominant gene was responsible for resistance to race 3 of *A. rabiei* in the four parent lines ILC 72, ILC 202, ILC 2956 and ILC 3279. However, when these parents were evaluated against six races of *A. rabiei*, each line showed different resistance patterns, which indicated the presence of other resistance genes.

Allelic studies have identified the presence of three independently segregating dominant genes for resistance in P 1215-1, EC 26446 and PG 82-1, and a recessive gene in BRG 8 (Tewari and Pandey, 1986). Other studies by Dey and Singh (1993) showed that two

complementary dominant genes conferred resistance in the genotypes GLG 84038 and GL 84099, whilst resistance in ICC 1468 was controlled by one dominant and one recessive gene. Further analysis indicated that inter-allelic interactions, additive gene effects, and dominance all influenced resistance in the three genotypes (Dey and Singh, 1993).

Quantitative inheritance of ascochyta blight resistance has also been proposed (Santra *et al.*, 2000). Recent studies on Recombinant Inbred Lines (RILs) suggest that several Quantitative Trait Loci (QTL) are involved in the control of resistance. Three sets of RILs were developed from two intraspecific crosses and one interspecific cross and, after evaluation of resistance, revealed that three recessive complementary major genes with some modifiers conferred resistance (Tekeoglu *et al.*, 2000). Further, absence of one or two of the major genes conferred susceptibility, whilst the presence of the modifiers determined the degree of resistance (Tekeoglu *et al.*, 2000). Conversely, Flandez-Glavez *et al.* (2003) studied an intraspecific population to find six QTL for ascochyta blight resistance in three genomic regions, where the major QTL showed additive gene action and dominance inter-locus interaction. Collard *et al.* (2003) also identified two QTL for seedling resistance and four QTL for stem resistance in an interspecific population. Cho *et al.* (2004) studied pathotype-specific genetic resistance mechanisms to identify one QTL conferring resistance to pathotype II of *A. rabiei*, and two QTL for resistance to pathotype I, including one QTL that was required for resistance to both pathotypes. Intraspecific RILs were also used to study pathotype-specific resistance, identifying two independent recessive major QTL with complementary gene action for resistance to pathotype II, as well as a single major QTL for resistance to pathotype I (Udupa and Baum, 2003). Another study using intraspecific RILs found two major QTL for *A. rabiei* resistance (Iruela *et al.*, 2006). Finally, interspecific RILs used in a very recent study found a previously unidentified major QTL for *A. rabiei* resistance (Cobos *et al.*, 2006).

These different estimates of the genetic basis of ascochyta blight resistance may result from the different fungal isolates and host genotypes used. Comparison of results is further complicated by the use of different classifications for resistance and susceptibility. The use of RIL populations has been identified as a strategy to enable resistance studies to be performed with replications in time and location (Tekeoglu *et al.*, 2000). Overall, previous studies indicate the existence of a range of different resistance sources, and opportunities exist for the characterisation of potential resistance genes that may be pyramided by breeding programs to enhance the level of resistance and increase durability.

1.3.5 Breeding for resistance

Ascochyta blight resistance breeding commenced in India in the early 1930s with the first release of a resistant cultivar (Luthra *et al.*, 1941). A further three resistant cultivars were released in the Soviet Union (Gushkin, 1946), but no ascochyta blight resistant cultivars were released in the Mediterranean region until 1984 (Singh and Reddy, 1991). Methods for breeding in self-pollinated crops such as chickpea include introduction and selection, hybridisation followed by pedigree, bulk/population and backcross methods, and mutation (Singh and Reddy, 1991). Slow progress in the development of resistance breeding has been due to the lack of a simple resistance screening technique, lack of resistant germplasm, and the evolution of new races of *A. rabiei* (Singh and Reddy, 1991).

Table 1.1 Varying reports of the genetic mechanisms controlling chickpea resistance to ascochyta blight.

Genetic mechanism/s	Genotype/s studied	Reference/s
Single dominant gene	ILC 72, ILC 183, ILC 200 and ILC 4935	(Singh and Reddy, 1983)
	ILC 72, ILC 202, ILC 2956 and ILC 3279	(Singh and Reddy, 1989)
	P 1215-1, EC 26446 and PG 82-1	(Tewari and Pandey, 1986)
Single recessive gene	ILC 191	(Singh and Reddy, 1983)
	BRG 8	(Tewari and Pandey, 1986)
Two dominant complementary genes	GLG 84038 and GL 84099	(Dey and Singh, 1993)
One dominant and one recessive gene	ICC 1468	(Dey and Singh, 1993)
Three recessive complementary genes with modifiers	RILs from PI 359075 x FLIP 84-92C, Blanco Lechoso x Dwelley and FLIP 84-92C x *C. reticulatum* (PI 489777)	(Tekeoglu *et al.*, 2000)
Three QTL	RILs from FLIP 84-92C x ILC 72	(Santra *et al.*, 2000)
Six QTL	RILs from Lasseter x ICC 12004	(Flandez-Galvez *et al.*, 2003)
Two/four QTL	F_2 from Lasseter x *C. echinospermum* (PI527930)	(Collard *et al.*, 2003)
One QTL for pathotype I Two recessive complementary QTL for pathotype II	RILs from ILC 1272 x ILC 3279	(Udupa and Baum, 2003)
Two QTL for pathotype I One QTL for pathotype II	RILs from PI 359075 x FLIP84-92C	(Cho *et al.*, 2004)
Two QTL	RILs from ILC 3279 x WR 315	(Iruela *et al.*, 2006)
One novel QTL	RILs from ILC 72 x *C. reticulatum* (Cr5-10)	(Cobos *et al.*, 2006)

Breeding for resistance to ascochyta blight has been a major focus in chickpea breeding programs in many countries, such as India, Syria, Canada, USA, Australia, Turkey and Pakistan. The most widely used sources of resistance have been supplied by the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT, India) and ICARDA (Syria). In Australia, the first variety released with a moderate level of resistance to ascochyta blight was 'Howzat' in 2001, but breeders have since selected a number of desi and kabuli lines with higher levels of resistance from ICRISAT and ICARDA breeding lines, as well as existing Australian varieties (K. Hobson, pers. comm.). Some of these lines are in the final stages of testing and will be released to help revive the local chickpea industry.

Marker-assisted breeding is a popular strategy for ascochyta blight resistance breeding in chickpea. Molecular markers, derived from DNA mutations, rearrangements, or errors in replication of tandemly-repeated DNA, are particularly useful considering their abundance and immunity to environmental factors or developmental stage of the plant (Winter and Kahl, 1995). Molecular markers linked to QTL contributing to ascochyta blight resistance have been discovered and may be used in marker-assisted breeding (Pande *et al.*, 2005). The markers will be important for enabling the pyramiding of resistance genes from diverse sources to reduce the time required to generate resistant cultivars.

The review will now focus on current knowledge regarding general plant-pathogen interactions, although specific examples from chickpea or related legumes have been included where available.

1.4 Plant-pathogen relationships

All pathogens have a restricted host range, where resistance (incompatibility) or susceptibility (compatibility) depends on two inherited factors; substrate requirements of the pathogen, and

the host-plant response. Two broad groups of pathogens have been identified based on their infection characteristics; necrotrophs, which kill plant cells and then parasitise them, and biotrophs, which inhabit the intercellular spaces and obtain nutrients from living plant cells (Brown and Ogle, 1997). Plants recognise the presence of a pathogen through the interaction of receptor proteins, encoded by resistance (*R*) genes, and pathogen-secreted elicitors. Elicitors may be non-specific, such as cell wall fragments, peptides, and glycoproteins, which trigger non-cultivar-specific defence responses (Tyler, 2002; Hahlbrock *et al.*, 2003; Montesano *et al.*, 2003; Nomura *et al.*, 2005). Other elicitors (or effectors) are gene-specific, and are conditioned by pathogen avirulence (*Avr*) genes (Dangl and Jones, 2001; Nomura *et al.*, 2005). Non-specific elicitors act by inducing signal transduction of membrane-bound host proteins, which indirectly helps to minimise disease, but it is the specific *Avr*-encoded effectors that are known to trigger the *R* gene-mediated plant defences (Dixon *et al.*, 1994). For example, an effector encoded by the *AvrB* locus of *Pseudomonas syringae* pv. *glycinea* was found to induce resistance in soybean cultivars possessing the *Rpg1-b* resistance gene product (Ong and Innes, 2006).

After pathogen contact, plants respond by series of highly coordinated molecular, cellular, and tissue based defences. Susceptible plants activate these defences too late, too little, or in the wrong place (Yang *et al.*, 1997). Pathogens respond by escaping or suppressing the plant response, or by rendering the plant response impotent (*e.g.* suppressing the HR; Jamir *et al.*, 2004). The plant-pathogen association fits the 'gene-for-gene' model of Harold Flor, which states that for host resistance to occur, complementary pairs of both pathogen (*Avr*) and host genes (*R*) are needed (Flor, 1947). A loss or change in a plant *R* gene, or a pathogen *Avr* gene, will lead to a compatible interaction (disease). It is for this reason that the two possible outcomes of a plant-pathogen interaction are;

1. The plant possesses a receptor (*R* gene product) that interacts with a pathogen protein (*Avr* gene product), resulting in rapid protective action (resistance). In this situation the pathogen is termed avirulent for the given plant. Such an outcome is evident in maize, where the product of a NADPH-dependant toxin reductase, encoded by the *Hm1* resistance gene, rapidly detoxifies the *Avr*-encoded HC-toxin of *Cochliobolus carbonum*, a cyclic tetrapeptide produced by the fungus to permit infection (Johal and Briggs, 1992).

2. The plant is affected by the pathogen and protective mechanisms are activated very slowly with only moderate effectiveness (susceptibility). In this situation the proteins of the pathogen are virulent for the given plant. An example of this is outlined by the suppression of an HR in *A. thaliana* by the cysteine protease product of the *AvrRpt2* gene of *Pseudomonas syringae* (Chisholm *et al.*, 2005).

1.4.1 Plant defences

The overall resistance mechanism is very complex and involves many interactions, including passive and active defences. Passive defences are pre-formed plant properties, such as physical barriers against invasion (waxy cuticle), inhibiting chemical defences (pH, nutrient deprivation), and constitutive antimicrobial compounds (Heath, 2000a). Conversely, active defences are considered the most important mechanism in host resistance (Koh and Somerville, 2006), which are activated by *Avr*-encoded elicitors and may either be rapid or delayed (Table 1.2; Figure 1.6). Rapid defences serve to kill the pathogen or inhibit its propagation. In the case of a fungal pathogen, these defences are termed fungicidal, and include;

Changes in membrane function: The host plant cell membrane is involved in pathogen recognition, signal transduction, permeability changes and enzyme activation (Grant and

Mansfield, 1999). Upon pathogen perception, membrane permeability changes to allow an influx of Ca^{2+} ions, which are a key signal involved in enzyme activation and defence-related gene expression (refer to Reddy (2001) for a comprehensive review). Specifically, Ca^{2+} is required for the activity of signal transducing protein kinases, which lead to protein phosphorylation and activation of defence-related genes (Grant and Mansfield, 1999). Pathogen inhibiting K^+ ions are also leaked from the cell (Dixon *et al.*, 1994). Although not yet directly observed in chickpea, such a response was reported in another legume, soybean, where rapid elevation of cytosolic Ca^{2+} followed treatment with fungal elicitors (Mithofer *et al.*, 1999). Similar observations have also been reported in *A. thaliana*, where the addition of an elicitor from *Fusarium oxysporum* resulted in the influx of Ca^{2+} ions leading to signal transduction of defence-related genes (Davies *et al.*, 2006).

The oxidative burst: The host plant produces reactive oxygen species (ROS) that act to kill pathogens, promote hypersensitive cell death, and signal the expression of defence-related genes (refer to Apel and Hirt (2004) for a comprehensive review). Hydrogen peroxide (H_2O_2) and the superoxide anion (O_2^-) are generated at levels sufficient to exercise an antimicrobial effect, and initiate host cell membrane lipid peroxidation, leading to hypersensitive cell death (Lam *et al.*, 1999; Neill *et al.*, 2002). During an oxidative burst, ROS scavenging enzymes (antioxidants) are also suppressed to allow the enhanced accumulation of ROS to induce an HR (Apel and Hirt, 2004). In chickpea, an oxidative burst has been observed in *A. rabiei* resistant genotypes, leading to the accumulation of defence-related proteins (Otte and Barz, 1996). An example of the importance of an oxidative burst was observed in tobacco, where knockout mutants lacking enzymes involved for the production of ROS showed reduced resistance to *Phytophthora infestans* (Yoshioka *et al.*, 2003). Peroxidase isozymes may also be involved in the formation of secondary compounds that limit the extent of pathogen spread (Tuzun, 2001). H_2O_2 has also been shown to induce numerous defence-related genes,

including receptor protein kinases and transcription factors (Apel and Hirt, 2004). Large-scale gene expression studies in *A. thaliana* have also identified >100 genes to be differentially regulated by H_2O_2 treatment (Desikan *et al.*, 2001).

Cell wall reinforcement: Cytoplasm accumulates at the pathogen penetration site, fortifying the cell wall against invasion and protecting against toxin diffusion. A number of different fortifications are produced, including papilla deposition between the host cell wall and plasma membrane, lignified callose deposits around invading hyphal tips, and hydroxyproline-rich (Hyp-rich) glycoproteins providing secondary cell wall thickening (Dixon and Lamb, 1990; Dixon *et al.*, 1994). Hyp-rich proteins include extensins, proline-rich, and glycine-rich proteins, which are all thought to play a role in restricting pathogen penetration of cell walls. In chickpea, Hyp-rich proteins have been found to accumulate after a rapid elicitor-induced oxidative burst (Otte and Barz, 2000). The role of lignin for cell wall strengthening was also observed in chickpea, where *A. rabiei* was unable to penetrate lignified tissue of resistant genotypes (refer to section 1.3).

The Hypersensitive Response (HR): A common indication of a resistance reaction is the presence of necrotic flecks at the sites of attempted pathogen colonisation, representing rapid plant cell death as a means of restricting pathogen growth. The HR (or 'programmed cell death') is induced at sites of pathogen invasion, where it is thought to directly kill invading pathogens and/or interfere with their acquisition of nutrients (refer to Heath (2000b) for a comprehensive review). The response involves cellular decompartmentalisation, browning, and cell death (about 12-24 h after attempted penetration). For necrotrophic pathogens that do not require host cells to be alive, the HR is also important for inducing the expression defence-related genes (Heath, 2000b). The HR is typically induced by Ca^{2+} influx, production of ROS from an oxidative burst, and production of salicylic acid (SA) (Heath,

2000b). The HR has also been linked to Systemic Acquired Resistance (SAR), described below (Delaney *et al.*, 1994; Ryals *et al.*, 1996). However, little is understood of the biochemistry of the HR (Beers and McDowell, 2001), but research has likened it to the apoptosis mechanism observed in vertebrates (Lam *et al.*, 1999). An example of the HR was observed in chickpea plants resistant to *A. rabiei*, where a rapid browning response and development of hypersensitive leaf spots occurred 24 h after infection, a phenomenon not observed in susceptible chickpea (Vogelsang *et al.*, 1994).

Phytoalexins: Phytoalexins are toxic natural products synthesised via the phenylpropanoid pathway in response to pathogen invasion or elicitor treatment (refer to Dixon *et al.* (2002) for a comprehensive review). Their effectiveness depends on the speed, location, and magnitude of the response. Although hundreds of phytoalexins have been identified, the most characterised group are the pterocarpans, isoflavans, and isoflavanones of legumes (Dixon *et al.*, 2002). Most known phytoalexins are small organic compounds with a non-selective toxicity, and accumulate to a maximum 18-24 h after pathogen infection (Brown and Ogle, 1997). The role of phytoalexins in the plant defence response has been studied in several plant-pathogen interactions, where correlations between the degree of host resistance and the level phytoalexin accumulation have been found (Soylu, 2006). An example of pathogen-induced accumulation of phytoalexins in chickpea was described in section 1.3.

Delayed active defences are important in restricting pathogen spread and containing host damage. With respect to fungal pathogens, these defences are termed fungistatic, and include;

Pathogen containment: Infected areas are sealed off by cork cells to restrict pathogen colonisation. This action, known as suberisation, also prohibits secondary infection by opportunistic pathogens (Brown and Ogle, 1997).

Pathogenesis Related (PR) proteins: PR proteins are stored in vacuoles and act in dissolving fungal cell walls and eliciting an HR, but are also involved in stress responses such as wounding. Considering that PR protein gene expression if often highly associated with enhanced resistance, the expression of PR protein genes has been identified as an important molecular indicator for defence responses (refer to Selitrennikoff (2001) for a comprehensive review). Classically, PR proteins have been divided into five groups, PR1-PR5, based on serological and amino acid sequence analyses, but another six groups have recently been included as PR proteins (Selitrennikoff, 2001). Each of the five classic groups contains an acidic (found in the extracellular space) and basic (found in the vacuole) subclass (Selitrennikoff, 2001). Most groups possess antifungal activity against specific pathogens, whilst others possess enzymatic or inhibitory activity such as chitinases (PR3, PR4, PR8, and PR11) and glucanases (PR2) that act to dissolve fungal cell walls, as well as peroxidases (PR9), ribonuclease-like (PR10) and proteinase inhibitors (PR6) (Datta and Muthukrishnan, 1999). PR proteins usually accumulate to a maximum within days of pathogen infection, and several examples have been found in chickpea after *A. rabiei* infection, including β-1,3-glucanase and chitinase (refer to section 1.3). A recent example of the activity of PR proteins can be observed in rice, where a PR-4 was isolated and shown to possess *in vitro* antifungal activity against *Rhizoctonia solani* (Zhu *et al.*, 2006).

Systemic Acquired Resistance (SAR): SAR is a signal released at the infection site that travels via the phloem to all parts of the plant, protecting the plant from subsequent infections. SAR, which has the ability to confer quantitative protection against a broad spectrum of microorganisms, is known to reduce disease severity but is not considered as immunity. A review by Durrant and Dong (2004) summarises the current knowledge on SAR, indicating that the signal molecule salicylic acid (SA) is required for SAR, and is associated with the accumulation of PR proteins. In response to SA, a positive regulator protein (*NPR1*) interacts

with transcription factors to induce defence-related gene expression and SAR (Durrant and Dong, 2004). However, another study has shown that a lipid-based molecule may be the signal for SAR in some plants (Maldonado *et al.*, 2002). Nevertheless, much research is focused on the chemical or biological induction of SAR, through the application of SA. Its use has been successful in legumes, for example, where SA was exogenously applied to pea leaves and found to induce a systemic resistance to *Erysiphe pisi*, reducing the infection of untreated leaves by 20-30% (Frey and Carver, 1998). Biological SAR induction using *Trichoderma harzianum* has also been successful, for example, the protection of soybean against *Sclerotinia sclerotiorum* was increased by 40% in plants that were treated with *Trichoderma harzianum* compared to untreated plants (Menendez and Godeas, 1998).

1.4.2 Resistant cultivars and resistance genes

As described in section 1.3.3, the use of resistant cultivars is the most efficient and effective means of controlling ascochyta blight of chickpea. Plant resistance is controlled by Mendelian inherited *R* genes, but new pathotypes may overcome *R* genes over time, resulting in a cycle between host resistance and susceptibility. The loss of *R* gene effectiveness is essentially caused by their selection for virulent pathotypes, thus *R* genes must be able to rapidly evolve to regain effectiveness. This requirement is predominately due to the high selection pressure caused by the monoculture of cropping, leading to the frequent appearance of new virulent pathotypes (Crute and Pink, 1996). An example of such an occurrence is the *Xa4* gene in rice, whose effectiveness in conferring resistance to *Xanthomonas oryzae* pv. *oryzae* became ineffective with the development of a new pathotype (Li *et al.*, 1999).

Table 1.2 Active defences against pathogen invasion and the timing of their action.

Time after infection	Active defence response	Reference/s
Minutes	Change in membrane function	(Otte and Barz, 1996; Davies *et al.*, 2006)
	Oxidative burst	(Mithofer *et al.*, 1999; Yoshioka *et al.*, 2003)
Hours	Cell wall reinforcements	(Otte and Barz, 2000; Ilarslan and Dolar, 2002)
	Hypersensitive response	(Vogelsang *et al.*, 1994)
	Phytoalexin accumulation	(Dolar and Gurcan, 1993; Soylu, 2006)
Days	Pathogen containment	(Brown and Ogle, 1997)
	Pathogenesis related proteins	(Hanselle and Barz, 2001; Zhu *et al.*, 2006)
	Systemic acquired resistance	(Frey and Carver, 1998; Menendez and Godeas, 1998)

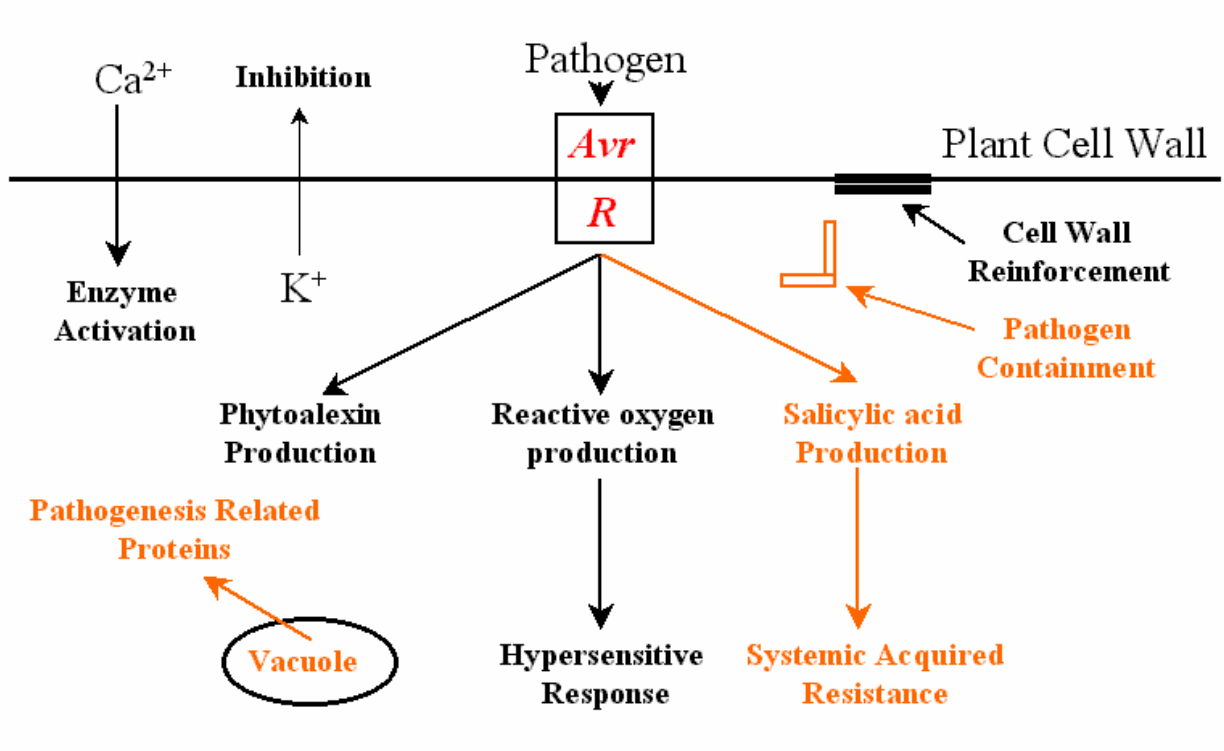

Figure 1.6 Summary of the rapid (black) and delayed (orange) active defences of the gene-for-gene response of a plant cell after pathogen recognition.

Loss of resistance also depends on whether resistance is vertical or horizontal. Vertical resistance is pathotype-specific, may be inherited as a single gene, and confers complete resistance to the plant. In roses, for example, several species are completely resistant to some, but not all pathotypes of *Diplocarpon rosae*, indicating that resistance is vertical and controlled by a single gene (Yokoya *et al.*, 2000). Horizontal/quantitative resistance is non-pathotype specific (does not require matching *R-Avr* genes), inherited as a polygenic trait, and confers a partial resistance to the plant. Horizontal resistance is less likely to be overcome by new pathotypes, as it does not impose as great a selection pressure as vertical resistance. Therefore, plant breeding programs often aim to 'pyramid' several minor and major *R* genes into single cultivars, thus achieving more durable resistance (Strange, 2006). With reference to the defeat of the *Xa4* gene in rice discussed above, a gene pyramiding technique was subsequently used to restore resistance, where durable resistance was achieved by the incorporation, into a single cultivar, of several moderately effective *R* genes as well as residual defeated genes such as *Xa4* (Li *et al.*, 1999).

1.4.2.1 Resistance gene classes

The study and isolation of over 40 *R* genes has revealed five main classes of *R* gene protein products that activate a similar range of defence mechanisms (Table 1.3). Reviews by Martin *et al.* (2003) and Hammond-Kosack and Jones (1997) provide in-depth accounts of the current state of knowledge in this area. Briefly, class 1 includes just one member, *Pto* from tomato, which possesses an intracellular serine/threonine-specific protein kinase capable of autophosphorylation (Loh and Martin, 1995), but no obvious capacity for recognition (Martin *et al.*, 1993). *R* genes of all other classes contain leucine-rich repeat (LRR) motifs, which are multiple repeats of ~24 amino acids believed to specify pathogen recognition. LRR domains of yeast, *Drosophila melanogaster,* and humans have all been shown to mediate protein-protein interactions, giving rise to the hypothesis that LRRs may serve as the binding site for

Avr gene products (Kobe and Deisenhofer, 1994). In fact, a single amino acid difference in the LRR domain of rice distinguished susceptible and resistant alleles to *Magnaporthe grisea* (Bryan *et al.*, 2000), providing strong evidence for the specific interaction of LRR domains with *Avr* gene products.

Class 2 *R* genes also contain a nucleotide binding site (NBS) and leucine zipper (LZ) region. NBS domains are known to possess ATP- or GTP-binding activity (Saraste *et al.*, 1990), indicating that nucleotide triphosphate binding is essential for their function, yet their role in resistance remains unclear (Martin *et al.*, 2003). The LZ region, occurring between the N-terminus and the LRR/NBS, is a heptad-repeat sequence that promotes the formation of coiled-coil structures that facilitate protein-protein interactions (Alber, 1992). In eukaryotes, LZ regions catalyse homo- and hetero-dimerisation of transcription factors, but little is understood of their role in plant *R* gene products (Martin *et al.*, 2003). The first discovered *R* genes of this class were the *A. thaliana RPS2* and *RPM1* genes that defend against *Pseudomonas syringae* carrying the *AvrRpt2* avirulence gene (Grant *et al.*, 1995; Bent *et al.*, 1996).

Class 3 *R* proteins contain a NBS/LRR, lack a LZ domain, but possess a large N-terminal domain similar to the cytoplasmic signalling domain of the *Drosophila* Toll protein and mammalian interleukin-1 receptors (IL-1R) (Whitham *et al.*, 1994). The *N* gene of tobacco (*Nicotiana tabacum*) is an *R* gene of this type, conferring resistance to tobacco mosaic virus. The *N* gene product shares a NBS and LRRs similar to those in *RPS2* and *RPM1*, but the amino terminal exhibits homology to Toll and the IL-1R, suggesting a role in signalling rather than ligand binding (Whitham *et al.*, 1994).

Unlike the first three classes, the final two classes of *R* genes encode extracellular LRR proteins that do not possess a NBS. Class 4 is made up of the *Cladosporium fulvum* defence genes (*Cf-9, Cf-2, Cf-4* and *Cf-5*) of tomato (*Lycopersicon esculentum*) (Martin *et al.*, 2003). These genes contain up to 24 extracellular LRR domains, possessing a C-terminus that contains a probable transmembrane domain and a short cytoplasmic tail (Dixon *et al.*, 1996). Class 5 possesses an additional kinase domain and, currently, the only member of this class is the *Xa21* leaf blight (*Xanthomonas oryzae* pv. *oryzae*) *R* gene of rice, which encodes a 1,025 amino acid protein that contains 23 cytoplasmic LRRs, a single transmembrane domain, and an intracellular serine/threonine kinase domain (Song *et al.*, 1995).

There also exist other *R* proteins outside these classes, such as the intracellular toxin reductase from maize (*Hm1*), which detoxifies the HC-toxin from *Cochliobolus carbonum* (Johal and Briggs, 1992; Meeley *et al.*, 1992). The *RPW8.1* and *RPW8.2* genes from *A. thaliana* also fall outside of classification, and confer broad-spectrum resistance against two pathogens, *Erysiphe cruciferarum* and *Erysiphe cichoracearum* (Xiao *et al.*, 2001).

Of the >40 cloned and characterised *R* genes, all but three are dominant genes (Buschges *et al.*, 1997; Deslandes *et al.*, 2002; Martin *et al.*, 2003; Iyer and McCouch, 2004). The products of most dominant *R* genes encode receptor-like proteins that interact directly with pathogen effectors, whilst the few recessive *R* genes encode proteins with different structures. The three recessive *R* genes identified so far include the barley *mlo* gene, *RRS1-R* from *A. thaliana* and *xa5* from rice, which all encode differing protein products. The dominant *Mlo* allele in barley encodes a transmembrane protein that acts as a negative regulator of the defence response to *Erysiphe graminis* f. sp. Hordei, whilst the recessive *mlo* allele is a loss-of-function mutant (Buschges *et al.*, 1997). The *RRS1-R* gene in *A. thaliana* encodes a novel nucleotide binding-leucine rich repeat-WRKY protein that confers resistance to *Ralstonia*

solanacearum (Deslandes *et al.*, 2002). Finally, the rice *xa5* gene encodes a gamma subunit of a transcription factor that confers resistance to *Xanthomonas oryzae* pv. *oryzae* races 1, 2, 3 and 5 (Iyer and McCouch, 2004).

The high degree of sequence homology amongst plant *R* genes has led to the development of a tool that can be used to 'fish' for similar *R* genes in other plant species. By targeting the conserved DNA sequences within plant *R* genes, opportunities exist for PCR amplification and isolation of similar genes in other plants (Leister *et al.*, 1996; Trognitz and Trognitz, 2005; Irigoyen *et al.*, 2006; Mammadov *et al.*, 2006). Importantly, the technique has been applied successfully in chickpea (Huettel *et al.*, 2002). The extracted product is known as a Resistance Gene Analogue (RGA), which may be sequenced and characterised. Subsequently, RGAs can be applied in further studies such as complementation, molecular mapping, 'knockout' mutants, positional cloning, or functional genomics to determine potential resistance activity.

1.4.3 Chemical elicitors of plant defence

Following *R* gene-mediated pathogen recognition, studies of signalling events responsible for active defence responses in plants have led to the identification of salicylic acid (SA), jasmonates (JA) and ethylene (E) as key regulators of these pathways (Schenk *et al.*, 2000; Salzman *et al.*, 2005; Jalali *et al.*, 2006). In fact, SA and JA are responsible for two major plant disease resistance mechanisms, SA-mediated systemic acquired resistance (SAR, described in section 1.4.1) (Durner *et al.*, 1997) and JA-mediated induced systemic resistance (ISR) (Pieterse *et al.*, 1998).

Table 1.3 Characteristics of the *R* gene classes, including the number (*n*) of members (Martin *et al.*, 2003). Class NA refers to unclassified *R* proteins.

Class (*n*)	**Example/s**	**Structural feature/s**	**Resistance function/s**	**Reference/s**
1 (*1*)	*Pto* (tomato)	Intracellular serine/threonine protein kinase	Autophosphorylation and signal transduction	(Martin *et al.*, 1993) (Loh and Martin, 1995)
2 (*22*)	*RPS2* and *RPM1* (*A. thaliana*)	Intracellular protein with LRRs, NBS, and LZ	Pathogen recognition, signal transduction, ATP- and GTP-binding activity; kinase activation, and promotion of coiled-coils for protein-protein interactions	(Grant *et al.*, 1995) (Bent *et al.*, 1996) (Saraste *et al.*, 1990) (Alber, 1992)
3 (*8*)	*N* (tobacco) *L6* (flax) *RPP5* (*A. thaliana*)	Intracellular protein with LRRs, NBS, and an amino terminal homologous to the *Drosophila* Toll protein	Pathogen recognition, signal transduction, ATP- and GTP-binding activity; kinase activation, and ligand binding to stimulate production of ROS and SAR	(Whitham *et al.*, 1994) (Lawrence *et al.*, 1995) (Parker *et al.*, 1997)
4 (*4*)	*Cf* (tomato)	Extracellular LRR protein with a transmembrane domain and short cytoplasmic carboxy terminus	Pathogen recognition	(Jones *et al.*, 1994) (Dixon *et al.*, 1996)
5 (*1*)	*Xa21* (rice)	Extracellular LRR protein with a transmembrane domain and a cytoplasmic kinase domain	Pathogen recognition and signal transduction	(Song *et al.*, 1995)
NA (*10*)	*Hm1* (maize)	Intracellular NADPH-dependent reductase	Toxin inactivation	(Johal and Briggs, 1992) (Meeley *et al.*, 1992)

SA, a precursor of aspirin widely distributed in the plant kingdom, is known to be a regulator of both systemic (SAR) and local resistance to pathogens (Ryals *et al.*, 1996; Jalali *et al.*, 2006). Evidence exists for the presence of upstream signal molecules that transmit an *R-Avr* recognition signal that leads to SA accumulation and expression of local resistance (Jalali *et al.*, 2006). The level of SA rises rapidly around necrotic lesions in plants and, although often required for SAR, SA is not translocated over long distances in plants and may interact with another systemic signal to induce the accumulation of SA in healthy plant tissue (Jalali *et al.*, 2006). In *A. thaliana*, the SA response is regulated by genes both upstream and downstream of SA synthesis (Jirage *et al.*, 1999; Shah *et al.*, 1999), and is found to be effective against biotrophic fungi and bacteria (Thomma *et al.*, 2001). SA has been found to induce genes associated with plant defence, such as those involved in phytoalexin biosynthesis, the oxidative burst and specific PR proteins (Schenk *et al.*, 2000; Salzman *et al.*, 2005).

Although SA is considered an important signalling molecule, JA and E signalling pathways are also involved in plant defence (Jalali *et al.*, 2006). JA has been implicated in defence responses to insects (Kessler *et al.*, 2004) and necrotrophic pathogens (Thomma *et al.*, 2001), and is synthesized via lipid signalling of the octadecanoid pathway (Creelman and Mullet, 1997). In *A. thaliana*, both resistance to insects and oomycete pathogens depend on defence signalling pathways involving JA (McConn *et al.*, 1997; Vijayan *et al.*, 1998). The exogenous application of JA induces a range of plant resistance genes (Schenk *et al.*, 2000; Salzman *et al.*, 2005) that can result in enhanced resistance to insects (Thomma *et al.*, 2000) and microbial pathogens (Baldwin, 1998). Specifically, JA activates defence-signalling proteins such as protein kinases (Salzman *et al.*, 2005), proteins involved in the oxidative burst (Schenk *et al.*, 2000), as well as antimicrobial proteins such as defensins (Manners *et al.*, 1998), PR proteins (Bower *et al.*, 2005; Salzman *et al.*, 2005), protease inhibitors (Farmer

and Ryan, 1992; Salzman *et al.*, 2005) and phytoalexins (Schenk *et al.*, 2000; Salzman *et al.*, 2005).

E is a gaseous plant hormone that is involved in defence against both biotic and abiotic stresses, and also affects a range of metabolic processes including germination, flower/leaf senescence, ripening, leaf abscission, and root nodulation (Johnson and Ecker, 1998; Bleecker and Kende, 2000; Wang *et al.*, 2002). E biosynthesis occurs by the conversion of methionine to *S*-adenosyl-L-methionine, production of 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase, and formation of E by ACC oxidase (Yang and Hoffman, 1984). The production of ACC by ACC synthase is considered the rate-limiting step (Yang SF and Hoffman, 1984). Perception of ethylene in plants occurs via a family of receptors according to a negative system, where the receptors become inactivated in the presence of ethylene to unblock downstream suppression (Hua and Meyerowitz, 1998). Positive signal transduction then occurs through transcription factors such as ethylene responsive element binding proteins (EREBPs), which leads to gene regulation (Hua and Meyerowitz, 1998). E has been shown to induce numerous plant defence and PR protein genes, as well as stimulate JA-related genes (Schenk *et al.*, 2000; Van Zhong and Burns, 2003; Salzman *et al.*, 2005).

The induction of SA/JA/E signalling pathways may also be pathogen-dependent, where the SA pathway is mainly induced by biotrophic pathogens and the JA and E pathways by necrotrophic pathogens (Thaler and Bostock, 2004). Crosstalk between defence pathways controlled by JA, SA and E has also been proposed, by means such as; (1) sharing components of pathways, (2) simultaneous modulation of different pathways, (3) negative modulation of one pathway by another, (4) synergistic action of signal molecules, and/or (5) enhancement of one pathway when others are not induced (Jalali *et al.*, 2006). As a result of studies in *A. thaliana*, SA is considered to block JA synthesis (Heck *et al.*, 2003), but there is also evidence that JA inhibits SA regulation of certain PR protein genes (Niki *et al.*, 1998).

Further, microarray analyses have indicated that SA and JA co-regulate large numbers of genes in *A. thaliana* (Schenk *et al.*, 2000) and sorghum (Salzman *et al.*, 2005). SA/JA/E signalling studies have also been performed in chickpea, and are described in section 5.1.

Considering the activity of SA/JA/E as inducers of plant defence mechanisms, treatment with these compounds essentially by-passes pathogen recognition to induce defence-related gene expression. In fact, microarray studies have shown that the majority of differentially expressed genes after treatment with SA/JA/E are involved in signal recognition and transduction (Schenk *et al.*, 2000; Salzman *et al.*, 2005). Subsequently, opportunities exists to employ functional genomics and quantitative methods for global and simultaneous analysis of large sets of genes, such as microarrays, to enhance the identification of regulatory pathways involved in defence-related gene expression.

1.5 Functional genomics

Considering that the resistance of many chickpea cultivars has broken down against new races of *A. rabiei*, an enhanced understanding of the chickpea defence response at the genomic level may improve the development of cultivars with durable resistance. At the genomic level, plant defence responses are complex and diverse, and every gene involved, from recognition to signalling to direct involvement, forms part of a coordinated response network.

The range of defence-related processes (refer to section 1.3) and varying reports on the genetic control of chickpea resistance to ascochyta blight (refer to section 1.3.4), indicate the presence of a complex network of signal transduction and transcriptional activation following pathogen perception to result in active defence responses. Currently, the genes and pathways of gene activation controlling effective resistance remain unknown, providing opportunity for further studies. Some approaches, including differential screening of cDNA libraries

(Ichinose *et al.*, 2000) and the placement of RGAs onto existing linkage maps (Rajesh *et al.*, 2002), have identified some genes that may be involved in *A. rabiei* defence. Chickpea Bacterial Artificial Chromosome (BAC) libraries have also been constructed to facilitate the physical mapping and positional cloning of identified resistance genes (Rajesh *et al.*, 2004; Lichtenzveig *et al.*, 2005). Quantitative methods for analysis of gene expression profiles, through functional genomics and microarray analysis, have the capacity to improve the overall understanding of the coordinated defence response at a molecular level (Michelmore, 2000). Although not performed in chickpea, microarray analysis has been successful in studying the defence responses of plants such as tomato (Gibly *et al.*, 2004), rice (Fujiwara *et al.*, 2004), maize (Baldwin, 1998), cassava (Lopez *et al.*, 2005), soybean (Moy *et al.*, 2004) and *A. thaliana* (Huitema *et al.*, 2003), to name a few.

The field of genomics involves investigations into the function of large numbers of genes in a simultaneous fashion. Structural genomics includes genetic mapping, physical mapping and sequencing, whilst functional genomics is concerned with the role of individual genes or groups of genes in the development of organisms (Draghici, 2003). Understanding of the functional roles of genes is very limited compared to the knowledge of sequence information, for example, there are 25,498 predicted genes in the *A. thaliana* genome but only 69% have been functionally classified according to sequence similarity in other organisms, and only 9% have been characterised experimentally (The Arabidopsis Genome Initiative, 2000). The challenge is to analyse and interpret the large-scale gene sequence data being produced to discover and understand functional roles of genes. The central dogma for the flow of genetic information is from DNA to RNA to proteins, where transcription is the process of using the information coded in a gene to create an mRNA sequence. This is termed as ‘expression’ of a gene, and is a key regulatory mechanism used by organisms to sustain and execute cellular function (Aharoni and Vorst, 2001). Although the encoded protein product dictates the final

expression of a gene, measurement of mRNA abundance has proven to be a valuable molecular tool (Aharoni and Vorst, 2001). Subsequently, microarrays are extremely useful for rapidly and simultaneously analysing the expression profile of whole genomes under the influence of a particular factor, such as disease pressure.

1.5.1 Microarray technology

Microarray technology is a hybridisation-based method that combines miniaturisation and the use of fluorescent dyes for labelling. Earlier hybridisation-based methods included DNA (Southern) and RNA (Northern) gel blot analysis where, for example, a unique labelled nucleic acid (probe) in solution was hybridised to a total RNA sample (target) that was attached to a membrane support. The outcome of this experiment only provides gene expression information for one probe, but array-based methods use a reversed strategy, where complex mixtures of target are hybridised to large numbers of probes on a solid support, gaining information on the abundance of many mRNA transcripts in parallel (Aharoni and Vorst, 2001).

Two prominent technologies are available for microarrays, one being a photolithographic method for high-density synthesis of up to a few hundred thousand oligonucleotides (Fodor *et al.*, 1991), which also allows for the sensitive detection of DNA mismatches in DNA variation analyses. However, a drawback is the requirement for prior sequence knowledge as well as complicated construction methodologies (Lipshutz *et al.*, 1999). Conversely, the alternate method of mechanically depositing (printing) pre-synthesised nucleic acid probes onto a solid surface (Duggan *et al.*, 1999) is more flexible for the fabrication of microarrays. Probes usually represent PCR-amplified products of either genomic DNA or inserts from cDNA libraries (*e.g.* Expressed Sequence Tags), but oligonucleotides can also be used. After

amplification probes are usually checked for integrity by gel electrophoresis, followed by purification and concentration before printing.

Array probes generally do not represent whole-genomes due to their expense and/or the absence of available cDNA clones. Subsequently, 'boutique' arrays usually contain probes that represent a subset of genes that may have been selected from a specific tissue, developmental stage, or from a cDNA library enriched for genes involved in the process under study, such as a disease response. However, the disadvantage of 'boutique' arrays is the potential limit of information regarding the process under study, especially when applied to genotypes other than those used as probe sources. Array probes may either be functionally characterised (Expressed Sequence Tags; refer to section 1.5.5) or remain anonymous until identified as important candidate genes. Considering that genomic DNA sequence probes may represent non-coding sequences, their use in gene expression experiments may be unsuccessful. Further, available cDNA probes from species closely related to that under study might also be used, where a high degree of sequence homology may allow for cross-species hybridisation (Zhu *et al.*, 2001). This approach may utilise available probes from model genomes, but precaution must be taken considering sequence conservation between related species is not consistent on a gene-for-gene basis (Clarke and Zhu, 2006). An alternative approach is to pre-select differentially expressed genes by suppression subtractive hybridisation (SSH) (refer to section 2.1).

1.5.2 Microarray construction

In mechanical deposition printing, robots are used to dip either solid or split pins into the DNA solution for loading, followed by direct contact with the solid array surface to dispense sub-nanolitre droplets at a pitch of 100-250 µm (Aharoni and Vorst, 2001). Figures 1.7 – 1.10 show examples of a printing robot, pin tool, split pin, and printing mechanism used for

mechanical deposition. cDNA microarrays are usually fabricated on glass slides that are coated with poly-lysine or amino silanes, which act to improve the adhesion of probes, restrict droplet spread and reduce background noise (Wu *et al.*, 2001). Following printing, the probes are immobilised by UV crosslinking or baking. Non-contact mechanical printing methods also exist, such as those based on ink-jet technology (Agilent®, Palo Alto, CA) where four cartridges are loaded with different nucleotides (A, T, C and G) and move across the array to project deposit nucleotides where they are required (Okamoto *et al.*, 2000). Another approach is the electrochemical synthesis method (CombiMatrix®, Bothel, WA), in which solutions containing specific bases are washed over the array surface and electrodes are activated in the necessary positions to allow the sequences to be constructed base by base (Liu *et al.*, 2006).

During photolithographic array fabrication, probes are photochemically synthesised on the chip without the need for cloning, spotting or PCR. The elimination of these steps is advantageous as it greatly reduces the noise observed in the cDNA system, thus improving data reliability. Affymetrix® (Santa Clara, CA) pioneered this technology and incorporated the probe match/mismatch strategy, where for each perfect matching (PM) reference probe there is a mismatch probe (MM) with a single nucleotide change (Draghici, 2003). This PM/MM system has allowed for studies to detect allelic variation in sequences that lead to phenotypic differences both within and between species, the most common being point mutations (commonly referred to a single-nucleotide polymorphisms) (Aharoni and Vorst, 2001). However, Affymetrix® have not yet produced a chip suitable for studying chickpea DNA.

Figure 1.7 A BioRobotics® MicroGrid II Compact array printing robot (Genomic Solutions, Ann Arbor, MI).

Figure 1.8 A BioRobotics® MicroGrid II Compact loaded pin tool for mechanical deposition of DNA (Genomic Solutions, Ann Arbor, MI).

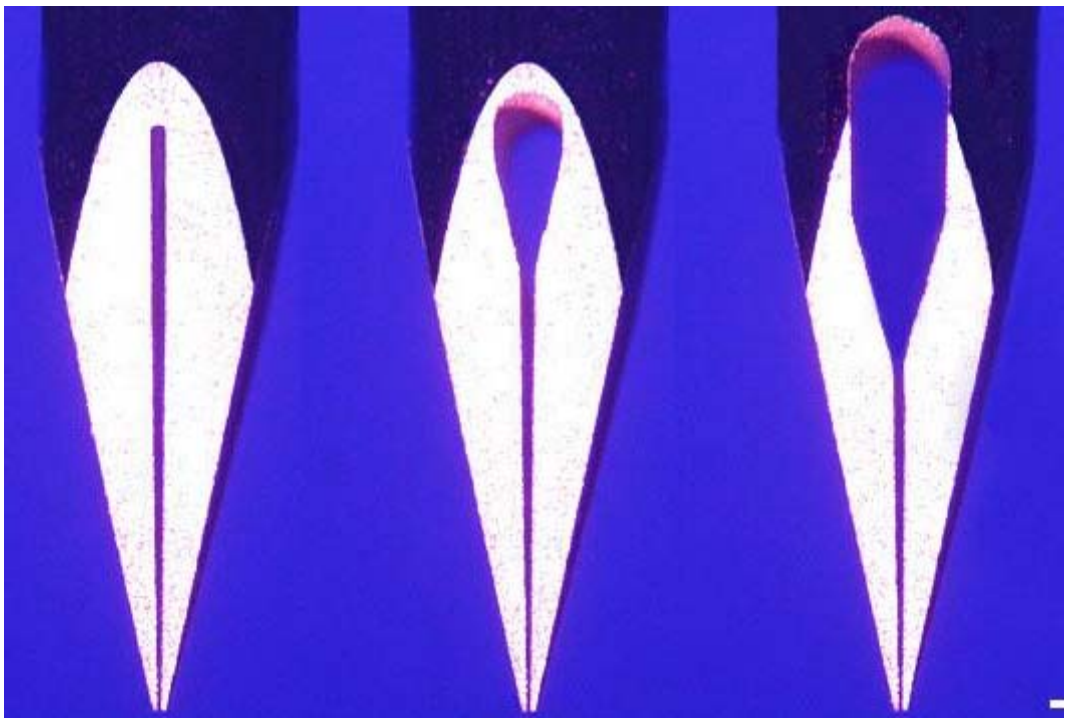

Figure 1.9 Close-up representation of three split pins with various sized reservoirs. Solution is drawn into the pin reservoir via capillary action (figure courtesy of TeleChem International Inc.).

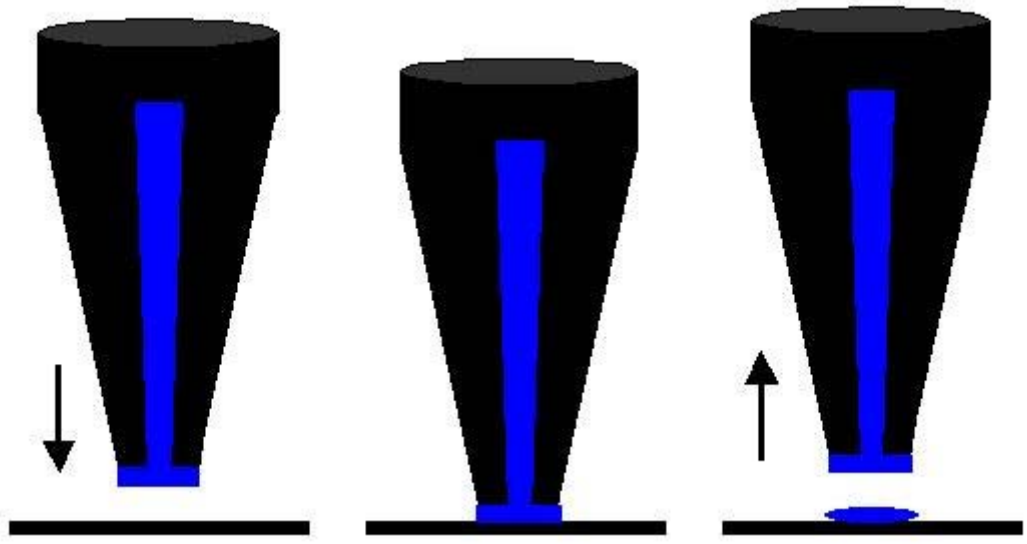

Figure 1.10 Summary of the mechanism for mechanical printing using split pins, showing a loaded pin depositing solution onto a solid substrate. Pins have flat tips to allow a layer of sample to form at the end of the pin, and printing to proceed by gentle surface contact (figure courtesy of TeleChem International Inc.).

1.5.3 Microarray assay and data analysis

The assay used for both types of array methodologies is based on the specific hybridisation of labelled target to the immobilised probe on the array, enabling the quantification of many individual mRNA transcripts in a single hybridisation (Figure 1.11). Further, the use of multiple fluorescent dyes with different emission and excitation characteristics enables the simultaneous analysis of two targets labelled with different dyes, a common practice for cDNA microarrays (Figure 1.11). In a typical experiment, two RNA samples (either total RNA or mRNA) are extracted from different biological sources (reference and test, for instance), which must be representative of the system under study. Fluorescent dye is incorporated either directly during first-strand cDNA synthesis, or indirectly by using amine-modified nucleotides for first-strand synthesis followed by chemical attachment of NHS-ester dyes in a later step (Aharoni and Vorst, 2001). The dyes commonly used are Cyanine-3 and Cyanine-5 due to their high incorporation efficiencies and distinct emission wavelengths. Relatively high amounts of RNA per sample are required for good results (10-50 µg total RNA or 0.5-2.5 µg mRNA), but target amplification methods have been developed to study samples derived from only a few cells (Hertzberg *et al.*, 2001).

Following labelling, the two independent samples (reference and test) are mixed and hybridised to the array under a coverslip, and the slide is incubated in a moisture-sealed chamber (Aharoni and Vorst, 2001). Photolithographic arrays use a different labelling and hybridisation procedure based on the incorporation of biotinylated ribonucleotides, in which only one sample is hybridised per array. Microarrays are susceptible to both technical and biological variations, therefore replication is required to minimise this variation. Technical replications are commonly used, where the same biological sample is assayed multiple times, as well as biological replication, where independent biological samples are assayed multiple

times (Clarke and Zhu, 2006). Biological replication is most important for downstream statistical analysis and to allow conclusions to be generally applied to whole populations.

After hybridisation of a cDNA array, the amount of sample hybridised to each probe is detected by fluorescence scanning. The strength of fluorescence emission at the two wavelengths represents the amount of bound target from each sample (reference and test), and is converted into a digital output. Image analysis software is then employed for quantification, which involves grid layover, calculation of pixel intensities and subtraction of background signal (Aharoni and Vorst, 2001). Normalisation then corrects for non-biological variations, including channel bias, unequal labelling efficiency, and unequal amounts of starting RNA. Several methods of normalisation are used depending on the assumptions regarding the samples under study (refer to sections 3.1, 4.1 and 4.2.4), including global hybridisation signal, 'housekeeping' genes and linear regression (Draghici, 2003). Normalisation is essential for enabling the comparison of results between arrays, which is important, for example, if comparing the response of different genotypes to disease (Clarke and Zhu, 2006). Finally, expression ratios (reference versus test) are produced, which are less prone to experimental variation than absolute expression values.

Interpretation of expression ratios to infer meaningful conclusions is achieved by firstly applying a fold change threshold for differential expression. Fold change thresholds are commonly identified by assessing the variability of the hybridisation system, performed by carrying out a hybridisation where identical RNA samples are used as reference and test. This 'self-self' hybridisation allows a measurement of the extent of expression ratio variation from expected equal ratios (Salzman *et al.*, 2005). The inclusion of adequate replication then allows the use of statistical tests to support fold change analysis, such as *t* tests, ANOVA, and multiple testing corrections (refer to section 4.2.5). Potentially important genes are then

selected, from which patterns of gene expression can be explored using clustering algorithms, such as hierarchical clustering, *k*-means clustering, principal component analysis (PCA) and self-organising maps (SOM) (Draghici, 2003). Detailed discussions of the clustering algorithms can be found in Clarke and Zhu (2006). Briefly, hierarchical clustering assembles a dataset by direct comparison and grouping, and has advantages for use on relatively small datasets. *K*-means and SOM are partition clustering methods that reduce complexity based on information from related gene groups. *K*-means requires a user-defined number of partitions (*k*) that should be calculated to allow for accurate groupings. PCA is not a true clustering method, but a decomposition technique that reduces the data into its major components, where the first component accounts for the most variation, the second component for the second most variation, and so on. Whilst these clustering methods are all distinct, it is often observed that they result in the identification of similar trends in datasets - the hierarchical branching may resemble the *k*-means groups, which may resemble the SOM nodes and PCA components (Clarke and Zhu, 2006). After clustering, groups of co-regulated genes are identified that, although possess distinct functions, may share the same regulatory mechanisms, such as common promoter elements that interact with the same transcription factors (Clarke and Zhu, 2006). In contrast to data generation, microarray data analysis to identify candidate important genes can be a lengthy process, involving the removal of non-interesting genes, reducing the dimensionality of the data, identifying gene expression patterns, and understanding the biological significance of the findings (Clarke and Zhu, 2006). To enable the independent assessment of data quality and maximise data usage, Minimum Information about a Microarray Experiment (MIAME) standards have been developed and are often required for publication of data (Brazma *et al.*, 2001).

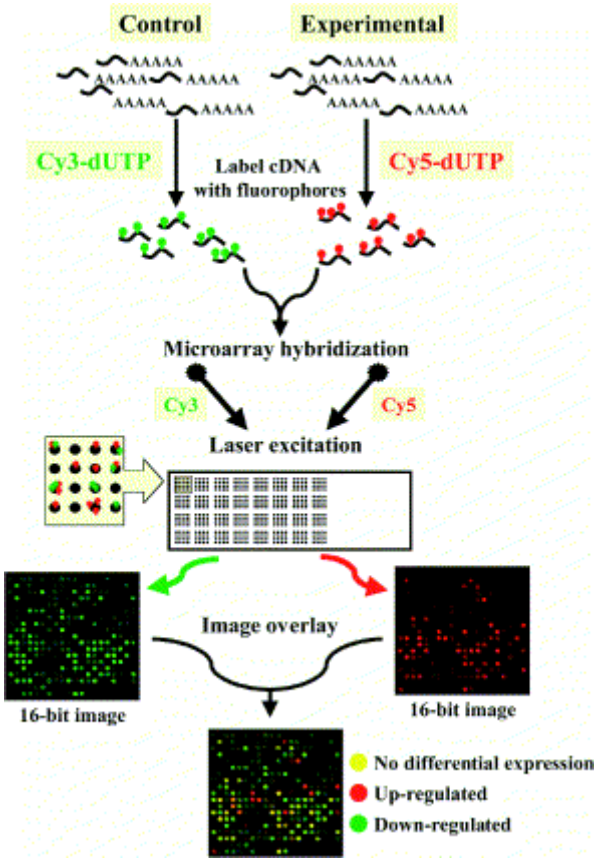

Figure 1.11 Summary of a typical DNA microarray hybridisation assay comparing reference (control) and experimental mRNA samples, followed by data acquisition for downstream statistical analysis (Wu *et al.*, 2001).

1.5.4 Confirmation of microarray data (Real-time PCR)

In order to provide further evidence for gene expression analysis, results from microarray experiments are often confirmed by Quantitative RT-PCR (or 'Real-time' RT-PCR). Currently, the reliability of microarray experiments may sometimes be questioned, considering the potential for cross-hybridisation between members of gene families on cDNA microarrays (Gachon *et al.*, 2004). However, the use of microarrays is justified by their capability to analyse thousands of genes simultaneously, whilst real-time PCR is limited to far fewer genes. Therefore, real-time PCR is often used to confirm the microarray observations of a selection of genes to indicate the validity of the microarray results as a whole (Dowd *et al.*, 2004; Fujiwara *et al.*, 2004; Lopez *et al.*, 2005; Salzman *et al.*, 2005). In addition to

microarray confirmatory studies, real-time PCR is also used to further analyse the expression kinetics of certain 'interesting' genes within different genotypes and tissue types (Goda *et al.*, 2002; Goto and Naito, 2002; Schenk *et al.*, 2003).

The real-time PCR methodology is based on the measurement of amplified product at each cycle of the PCR reaction, by recording the light emitted by a fluorochrome incorporated into the newly synthesised products. Thus, the exponential phase of the amplification can be followed in 'real-time', allowing the precise measurement of the amount of starting material. Real-time PCR also has high detection sensitivity due to the amplification step, which makes it useful for analysing the expression of genes from small quantities of RNA (Gachon *et al.*, 2004). Currently, intercalating agents and fluorogenic probes are used as fluorochrome molecules to detect amplification. SYBRgreen® is the intercalating agent of choice (Wittwer *et al.*, 1997), as it binds to all double-stranded DNA with high affinity and has a relatively low cost. However, the disadvantage of SYBRgreen® is that it also binds to any potential non-specific amplicons, thus causing potential error in signal measurement. Fluorogenic probes solve this problem as they specifically bind to the target sequence, however, they require the design of labelled oligonucleotide probes specific for each target, rendering them uneconomical unless used for high-throughput studies (Gachon *et al.*, 2004). Therefore, the SYBRgreen® method is commonly used for microarray confirmation in plant studies (Dowd *et al.*, 2004; Fujiwara *et al.*, 2004; Lopez *et al.*, 2005; Salzman *et al.*, 2005). Whilst numerous studies have observed significantly similar gene expression results between microarray and real-time PCR, others have found real-time PCR data with higher induction ratios compared to microarrays, although a strong correlation still existed (Wang *et al.*, 2003; Dowd *et al.*, 2004; Lopez *et al.*, 2005).

1.5.5 EST analysis

Functional genomics and microarray analysis provide opportunities for illuminating the chickpea resistance mechanism to ascochyta blight, possibly providing information concerning the resistance pathway/s employed by the plant, as well as the function of genes involved. However, before such analyses can be performed, an extensive library of chickpea gene sequence data must be available for the construction of microarray probes. Subsequently, a common first step in functional genomics is Expressed Sequence Tag (EST) analysis, which involves large-scale single-pass sequencing of randomly selected clones from cDNA libraries constructed from mRNA isolated at a particular developmental stage. Functional identification of sequenced clones is being made easier by the availability of rapidly growing sequence databases, such as GenBank™, that allow for the detection of regions showing sequence similarity in functionally related gene products, thus leading to the assignment of putative functions for many anonymous cDNA clones.

Despite the disadvantage of ESTs not representing full-length gene sequences, EST analysis has become a popular method for gene discovery and mapping in many organisms. For plants such as rice, maize and *A. thaliana*, comprehensive sets of EST sequences are available and have been used for the generation of molecular markers (Cato *et al.*, 2001; Yu *et al.*, 2004), identification of gene families (Epple *et al.*, 1997), single nucleotide polymorphism (SNP) development (Cho *et al.*, 1999), and the study of gene expression with microarrays (Schenk *et al.*, 2000; Lan *et al.*, 2004). ESTs may be particularly useful for the generation of molecular markers since, (1) an EST marker genetically associated with a trait is likely to represent a gene that directly affects that trait, and (2) EST markers are derived from highly conserved coding DNA sequences, which is likely to render them highly transportable across pedigrees compared to other markers derived from non-expressed sequences (*e.g.* Simple Sequence Repeat markers) (Cato *et al.*, 2001). Subsequently, the use of gene sequences derived from

ESTs holds much promise for identifying the actual genes controlling a desired trait. Further, the use of EST-derived markers for the development of higher density (saturated) linkage maps will provide researchers with a greater arsenal of tools for QTL mapping and effective use of marker assisted selection (MAS) (Collard *et al.*, 2005; Dita *et al.*, 2006).

Only few reports have described the use of functional genomics (or transcriptomics) to gain insights into legume-pathogen interactions (Ameline-Torregrosa *et al.*, 2006) and, to date, the study of the chickpea defence response to ascochyta blight through EST generation and microarray analysis has not been performed. The generation of sequence information alone, whilst valuable as a starting point, does not provide information regarding gene function, signalling networks and biochemical pathways associated with a stress (Dita *et al.*, 2006). Subsequently, the identification of differentially expressed genes, using microarrays, may provide an opportunity to identify chickpea genes effective against ascochyta blight.

1.6 Rationale for study

Ascochyta blight is a major disease of chickpea that limits worldwide production. Attempts to develop cultivars with a high level of durable resistance have been unsuccessful, despite the existence of highly resistant genotypes. Important reasons behind this obstacle are the conflicting reports concerning the genetics of resistance, as well as the limited understanding concerning the genes and pathways of gene activation involved in an effective defence response. Several reviews have identified that "EST generation is a key step to understand the genetic organization and assess the functions of genes in legumes" (Ameline-Torregrosa *et al.*, 2006), and that "the future will see more impact of transcriptomics in chickpea breeding including the application of microarrays" (Millan *et al.*, 2006). However, chickpea functional genomics in still very much in its infancy, and no reports exist describing large-scale EST generation and the use of microarrays to better understand the genes involved in ascochyta

blight resistance. Considering the gaps in knowledge regarding the mechanism of chickpea resistance to ascochyta blight and the opportunities for study identified in this review, the aims of this study were to;

1. Further sequence, assemble and functionally characterise ESTs from a cDNA library previously synthesised from stem/leaf tissue of an ascochyta blight resistant chickpea genotype. This study may uncover chickpea-specific defence-related ESTs to assist in further understanding of the defence mechanism, and for use in gene expression studies.

2. Construct a small-scale microarray to analyse and compare the expression of defence-related genes in a resistant and susceptible chickpea over a time-course after *A. rabiei* inoculation. This study may enable the identification of defence-related genes with potential involvement in effective resistance.

3. Construct a large-scale microarray representing all chickpea ESTs (unigenes), defence related ESTs from a related legume (*Lathyrus sativus*; grasspea), and numerous Resistance Gene Analogues (RGAs) from another related legume (*Lens culinaris*; lentil). Study gene expression in four chickpeas (resistant and susceptible), including a wild relative, over a time-course after *A. rabiei* inoculation. This study may enable the identification of genes and gene activation pathways with potential involvement in effective resistance.

4. Utilise the large-scale microarray to study gene expression after the exogenous application of SA, JA, and E in three chickpeas (resistant and susceptible). This study may enable the identification of important genes and pathways involved in defence and further characterise the mechanism of *A. rabiei* resistance.

5. Interpret the results of this study, in light of previous knowledge, to synthesise a hypothesis/model for the molecular control of chickpea resistance to *A. rabiei* that may be tested in future studies.

Chapter 2

Sequencing, functional characterisation, and clustering of ESTs from a *C. arietinum* cDNA library enriched for defence-related transcripts.

2.1 Introduction

As described in Chapter 1, the level of resistance in cultivated chickpeas is not sufficient to withstand disease pressure under conditions favourable to ascochyta blight, but the world collection of chickpea germplasm contains resistant genotypes. Studies of one such genotype, ICC3996, have revealed a strong capacity for *A. rabiei* resistance (Nasir *et al.*, 2000; Collard *et al.*, 2001), indicating that ICC3996 may be a valuable source of defence-related genes for use in the development of chickpea cultivars that are resistant to ascochyta blight.

EST generation and functional genomics was also described in Chapter 1 as a potential method for elucidating the mechanism of ascochyta blight resistance in chickpea (refer to section 1.5.5). To date, the study of chickpea defence to ascochyta blight through EST analysis and microarray expression experiments has not been performed. Whilst the National Center for Biotechnology Information EST database (GenBank dbEST) contains 36,181,620 ESTs (April 28, 2006; http://www.ncbi.nlm.nih.gov/dbEST/dbEST_summary.html), of which rice (1,183,548), wheat (853,316), maize (734,267), and *A. thaliana* (622,966) are the largest collections for plant species, chickpea is represented by just 724 ESTs. Such a low number of available chickpea ESTs exposes the need for a larger collection of sequence information before highly effective functional genomics strategies can be employed in chickpea research.

Therefore, the objective of this study was to uncover and characterise ESTs from the resistant chickpea genotype, ICC3996, which may be involved in the defence response against *A. rabiei*. Firstly, an existing cDNA library of ICC3996, enriched for the presence of defence-related transcripts, was built on to generate an additional 928 ESTs (total collection of 1021). The ESTs were functionally annotated based on homology to existing sequences in public databases and clustered into unigenes. Although such a library may lack the depth of a completely sequenced genome, it may still provide informative gene sequence data for studying the *A. rabiei* defence response at a much lower cost. A similar library, known as a Suppression Subtractive Hybridisation (SSH) cDNA library, has been synthesised in one other highly resistant chickpea (ILC3279), and consists of just 35 sequences considered to have general defensive functions (Ichinose *et al.*, 2000). An SSH library exploits the differences between a control cDNA sample, such as an uninoculated sample, and a test sample, such as an inoculated or highly resistant chickpea (Diatchenko *et al.*, 1996), to isolate sequences that are differentially expressed between the test and control samples. However, considering that an overall defence response can involve hundreds of genes, from recognition to signalling to direct involvement, an SSH library of just 35 sequences may not encompass the entire *A. rabiei* resistance mechanism (Caldo *et al.*, 2004; Dowd *et al.*, 2004; Fujiwara *et al.*, 2004; Gibly *et al.*, 2004; Jammes *et al.*, 2005; Lopez *et al.*, 2005; Ralph *et al.*, 2006).

The availability of EST sequence data is also particularly useful for the generation of Simple Sequence Repeat (SSR), or microsatellite, molecular markers. SSRs are stretches of DNA consisting of exact simple tandemly repeated short DNA motifs of 1–6 bp in length (Tautz and Renz, 1984). SSRs are considered to be very important DNA markers for genetic mapping because they are highly polymorphic, highly abundant, inherited in a co-dominant fashion, dispersed evenly throughout a genome, and are easily detected by PCR using two unique primers that flank and define the SSR locus (Collard *et al.*, 2005). The rapidly

increasing amount of EST sequence information becoming publicly available represents a rich source for SSR discovery. In fact, EST collections have been exploited to generate SSRs for use in genetic mapping from numerous crops including apple (Newcomb *et al.*, 2006), soybean (Tian *et al.*, 2004), barley (Thiel *et al.*, 2003), rice (Panaud *et al.*, 1996) and maize (Senior *et al.*, 1996). Subsequently, the ESTs generated in this study were also scanned for SSR discovery.

2.2 Materials and methods

2.2.1 Source of *C. arietinum* enriched cDNA library

A cDNA library of *C. arietinum* (ICC3996), and 93 ESTs, were previously constructed and generated by Tristan Coram (Honours project, RMIT University, 2001). Briefly, ICC3996 seeds were cultivated in a glasshouse (20 ± 4ºC) for 14 days (six- to eight-leaf stage) before inoculation with *A. rabiei*. The inoculation procedure was firstly optimised to obtain an infection that was representative of field conditions. 500 mg of stem/leaf tissue was extracted from inoculated ICC3996 plants at 24 h and 48 h post-inoculation. The inoculation of ICC3996 before tissue collection was performed to enrich for the presence of defence-related transcripts. Total RNA was extracted from the tissue samples before using the SMART™ PCR cDNA Synthesis Kit (Clontech, Mountain View, CA) to generate double-stranded cDNA. The resulting cDNA was ligated into pGEM®-T Easy Vector (Promega, Madison, WI) and transformed into *E. coli* JM109 cells (Promega, Madison, WI) according to manufacturer's instructions.

2.2.2 Evaluating the inserts of additional clones

Ninety-three clones were previously sequenced (Tristan Coram, 2001), but extensive additional sequencing of clones was performed in this study. Before sequencing, the presence and size of cDNA inserts within >1000 randomly selected clones were assessed. When each

transformed colony was picked for subculturing into LB/ampicillin broth (Appendix 1), cells were first transferred into sterile 0.2 mL PCR tubes containing 10 µL Milli-Q water by immersing the toothpick tip into the water before transferring to the LB/ampicillin broth. The cells were used as a template for PCR amplification using T7 forward and SP6 reverse primers. These primers are complementary to the T7 and SP6 promoter regions that flank the multiple cloning site of the pGEM®-T Easy Vector (Promega, Madison, WI) (Figure 2.1).

The 25 µL PCR reaction mixture contained; 2.5 µL 10X PCR buffer (Invitrogen Life Technologies, Carlsbad, CA), 2.4 mM $MgCl_2$, 0.24 mM dNTP (Promega, Madison, WI), 0.8 µM each of the T7 and SP6 primers (Geneworks, Adelaide, Australia), 1 unit of *Taq* DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA) and 10 µL Milli-Q water/colony mix. The PCR amplifications were performed in a Perkin Elmer 2400 thermal cycler (Perkin Elmer, Wellesley, MA) under the following conditions; 1 cycle of initial denaturation at 94°C for 12 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min.

To each PCR product, 5 µL of loading dye was added and 10 µL of the mixture was pipetted into wells of a 1% agarose gel (1 g agarose, 100 mL 1X TBE) and run in 1X TBE buffer (Appendix 2) at 100 V. Gels were post-stained in a solution of 300 mL 1X TBE containing 40 µL of 10 mg/mL ethidium bromide for 20 min, followed by de-staining in Milli-Q water for 20 min. Gels were viewed under a UV-light transilluminator and images captured using the Gel-Doc™ system (BIO-RAD, Hercules, CA). Amplification products represented the insert plus ~200 bp corresponding to the distance from the primer sites to the insertion site (Figure 2.1). Therefore, products >400 bp were sequenced, considering mRNA sequences >200 bp are more likely to represent full-length genes (Glick and Pasternak, 1998) (Figure 2.2).

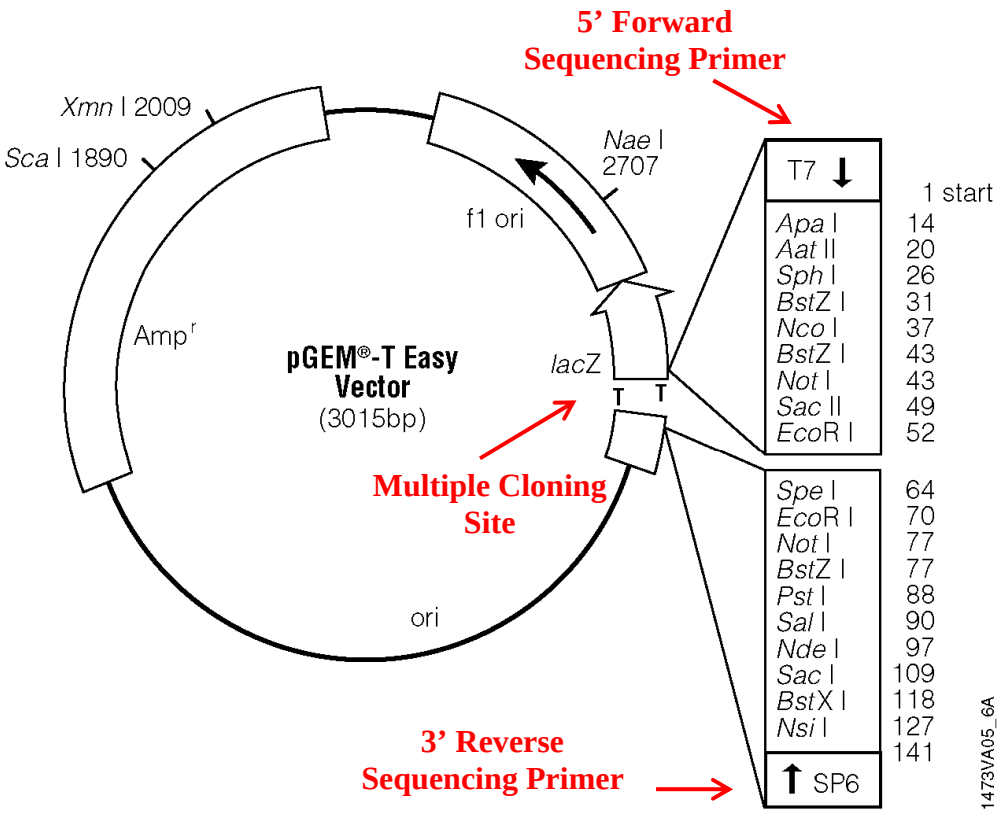

Figure 2.1 pGEM®-T Easy Vector (Promega, Madison, WI) showing the multiple cloning site flanked by restriction sites and T7/SP6 forward/reverse primer sites (www.promega.com).

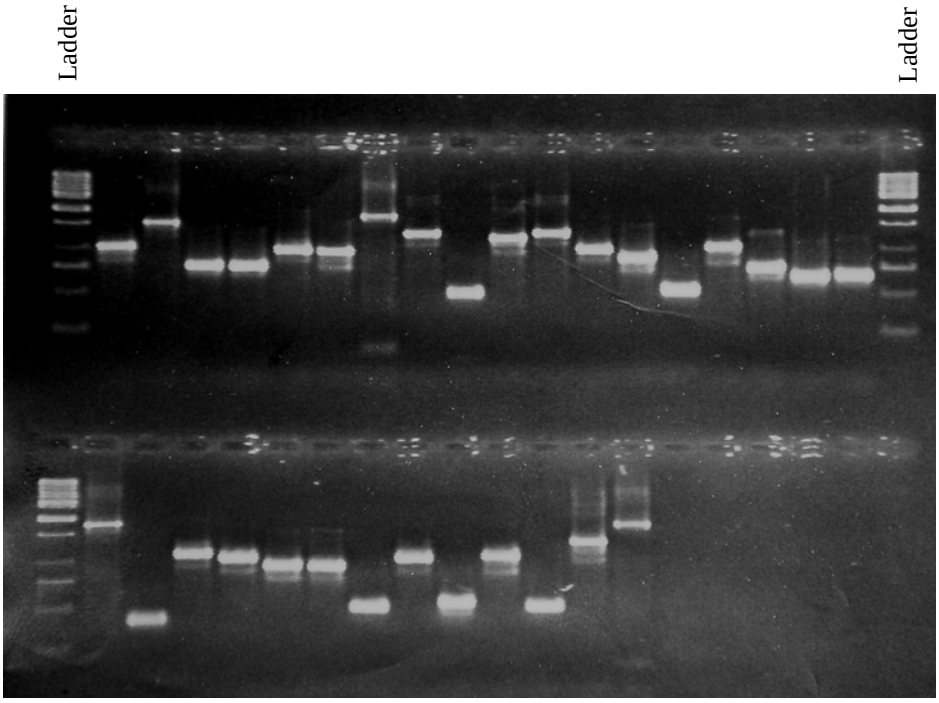

Figure 2.2 Example of cDNA inserts from pGEM®-T Easy Vector (Promega, Madison, WI) plasmids amplified using T7 forward and SP6 reverse primers.

2.2.3 Sequencing of additional clones

Plasmids that contained inserts >200 bp were isolated from LB/Ampicillin broths of *E. coli* JM109 cells originating from single colonies using the QIAprep Miniprep Kit (Qiagen, Valencia, CA), following the manufacturer's instructions. Subsequently, purified plasmid DNA was subjected to single-pass sequencing from the 5' end of the plasmid according to a modified ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit Protocol (Applied Biosystems, Foster City, CA). The conditions for each 15 µL PCR sequencing reaction were; 6 µL Terminator Ready Reaction Mix (DNA Sequencing Facility, Monash University, Victoria, Australia), 300 ng plasmid DNA, 1 µL 50 ng/µL T7 primer (Geneworks, Adelaide, Australia) and 5 µL Milli-Q water. The recommended cycling conditions were 25 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 min.

The PCR sequencing reactions were followed by DNA precipitation according to the Ethanol/Sodium Acetate method (Applied Biosystems, Foster City, CA). The resulting DNA pellets were sequenced with an ABI Prism 377 DNA Sequencer (School of Biomolecular and Biomedical Sciences, Griffith University, Queensland, Australia). Sequences identified as defence-related were subjected to further sequencing, using an additional 5' (T7) read as well as two 3' (SP6) reads.

2.2.4 Sequence analysis

Low quality sequence reads were manually removed, and vector sequences were removed using CodonCode Matcher™ (BioManager™ 2.0, Australian National Genomic Information Service, University of Sydney, NSW). CodonCode Matcher™ is a general-purpose utility for comparing a set of nucleotide sequence reads with vector sequence/s to produce vector-masked versions of the sequences. Each independent EST was then characterised using BLASTN and BLASTX to determine sequence homology with existing entries in the

GenBank® Main, GenBank® ESTs (dbEST), SwissProt® and SpTrEMBL® databases. Database hits were ranked by Expectation (e) value, and were regarded as significantly similar to the input sequence if P < 1.0e-10. The e value describes the number of database hits that are 'expected' by chance (noise), for example, an e value of 1 indicates that one match with a similar score can be expected simply by chance, but and e value of 0 indicates that no matches would occur by chance (Attwood and Parry-Smith, 1999).

Each EST was assigned a putative cellular function based on the significant database hit with the lowest e value, and the functional categories used were based on the Munich Information Center for Protein Sequences (MIPS) classification system applied to the *A. thaliana* genome (Mewes *et al.*, 2002). ESTs that matched to hypothetical proteins were classified as 'unclear' whilst ESTs with no significant match were classified as 'unknown'. The putative defence-related ESTs were deposited into GenBank (dbEST) with accession numbers CV793585-CV793610.

2.2.5 EST clustering

To identify the number of non-redundant ESTs, all sequenced and classified ESTs were clustered and assembled into unigenes (contigs and singlets) using CodonCode Assembler™ (BioManager™ 2.0, Australian National Genomic Information Service, University of Sydney, NSW). ESTs producing an alignment of >50 overlapping bases and >95% identity with another EST were assembled. After clustering, unigenes were functionally characterised and classified according to the method outlined for the independent ESTs. For each independent EST and unigene, all passport information including source, sequence read, functional category and putative identification, were catalogued in a custom designed 'PulseDB' database (Microsoft Access, Redmond, WA) for straightforward recall and searching of library data (Figure 2.3).

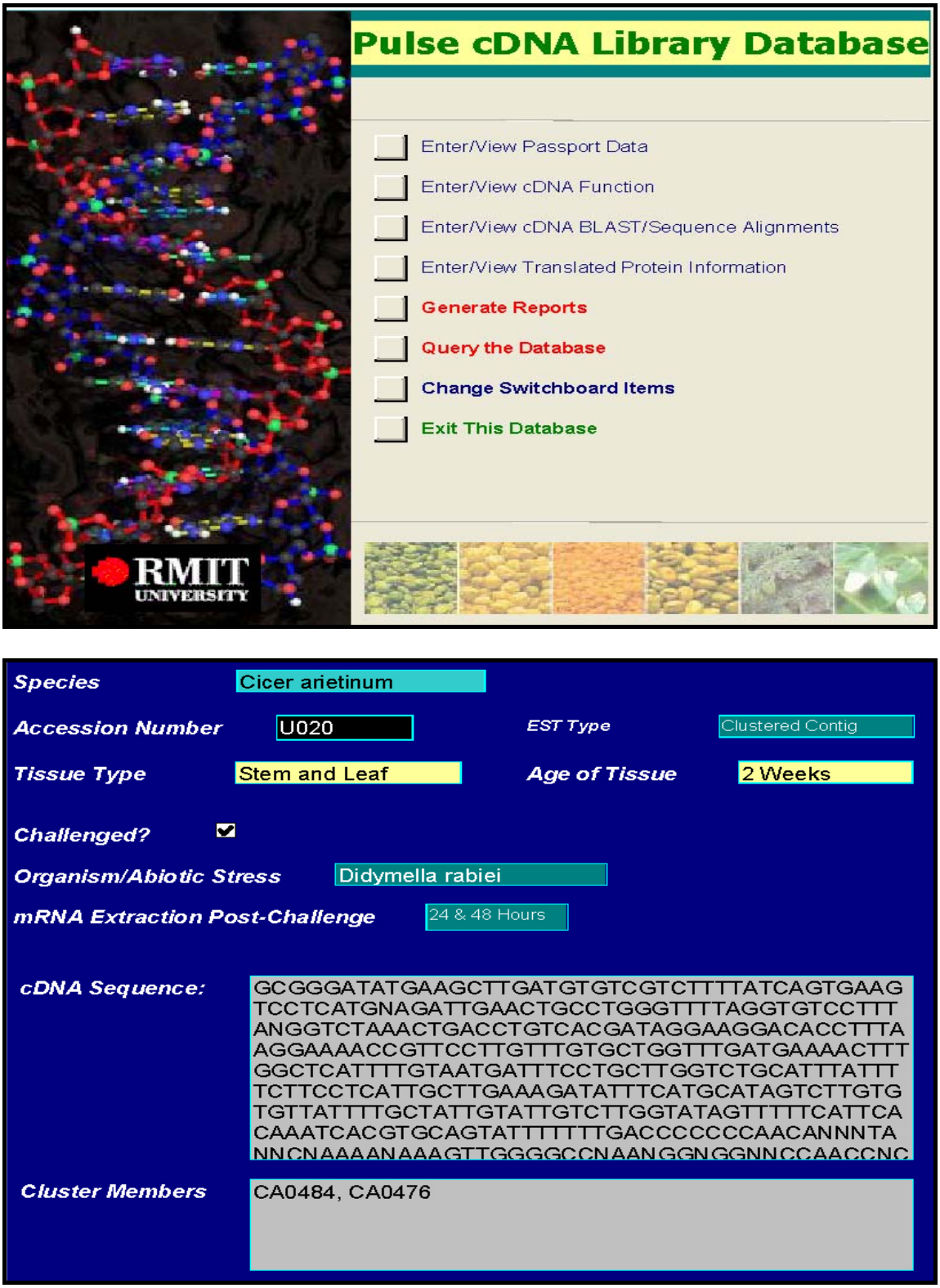

Figure 2.3 Examples of the 'PulseDB' database interface showing the main switchboard (top) and a passport data window (bottom).

2.2.6 Identification of SSRs

The Exact Tandem Repeats Analyzer (E-TRA) program (Karaca *et al.*, 2005) was used to locate SSRs in the unigene sequence data. The program allowed searching of unigene sequences to identify exact SSR motifs of 1–5 bp long. Valid SSRs were defined as being mononucleotide repeats >15 bp, dinucleotide repeats >14 bp, trinucleotide repeats >15 bp, tetranucleotide repeats >16 bp, and pentanucleotide repeats >20 bp, as similarly defined in other studies (Cardle *et al.*, 2000; Tian *et al.*, 2004).

2.3 Results

2.3.1 Sequencing of additional clones

Of the >1000 randomly selected additional clones, PCR amplification revealed insert sizes ranging from 100-2500 bp, but only those with an insert >200 bp were sequenced based on the assumption that most functional proteins (exons) are of this length (Glick and Pasternak, 1998). The clones were sequenced from the 5' end to generate an additional 1105 cDNA transcripts ranging from 200-2000 bp, but this number was reduced to 928 after removing poor-quality sequence reads. The overall sequence success rate was 84%.

2.3.2 Functional classification of independent ESTs

The 93 existing ESTs (Tristan Coram, RMIT University, 2001) were merged with the additional 928 generated in this study, and all 1021 independent ESTs were then subjected to functional classification and clustering (Figure 2.4). Sequence searches revealed that 450 (44%) possessed no significant database hit that would allow functional classification, and therefore may represent novel gene sequences or 5' untranslated regions. This category may be further divided into ESTs that significantly matched putative or hypothetical proteins (9% Unclear), and ESTs that did not match any nucleotide or protein sequence (35% Unknown). The remaining 571 independent ESTs (56%) showed significant homology to existing

sequences from public databases. Of these, the largest category was 'transcription' (16%), followed by 'energy' (13%), 'cellular metabolism' (9%), 'protein synthesis/fate' (6%), 'cell rescue/death/ageing' (4%), 'cellular communication/signal transduction' (3%), 'transport facilitation' (2%), and 'cell cycle & DNA processing' (1%). The putative defence-related sequences, targeted by this study, accounted for 26 (3%) of the 1,021 independent ESTs. The four independent sequence reads generated for each of these transcripts were aligned to produce single combined and accurate sequence, coding for potential antimicrobial, receptor, and defence-activating proteins.

2.3.3 EST clustering and functional classification of unigenes

Clustering and assembling of the 1021 independent ESTs produced 516 unigenes, with GenBank (dbEST) accession numbers CV793587-CV793591, CV793593-CV793595, CV793597-CV793603, CV793605- CV793610, and DY475047-DY475553. The unigenes ranged from 200-1800 bp, with an average length of 755 bp. The majority of the unigenes were from singletons (78%), whilst 17% were generated from 2-3 homologous ESTs, 4% were generated from 4-10 homologous ESTs, and only 1% of the unigenes were generated from more than 10 homologous sequences. Analysis and characterisation of the 516 unigenes (Figure 2.5) revealed that 50% possessed no significant functional database hit; of which 12% were classified as 'unclear' and the remaining 38% were 'unknown'. For convenience, all unigenes and ESTs in this and subsequent Chapters will be referred to according to their putative assigned function, although this is yet to be determined in chickpea.

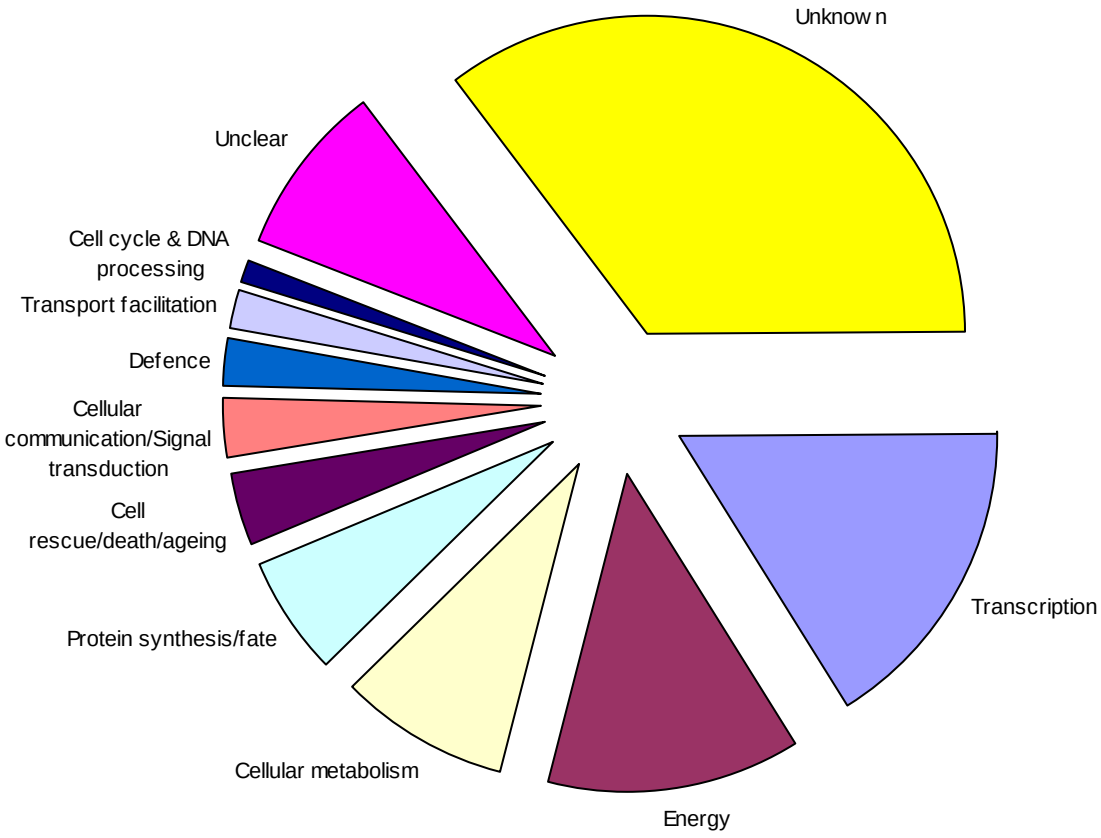

Figure 2.4 Functional distribution of the 1,021 independent *Cicer arietinum* (ICC3996) ESTs.

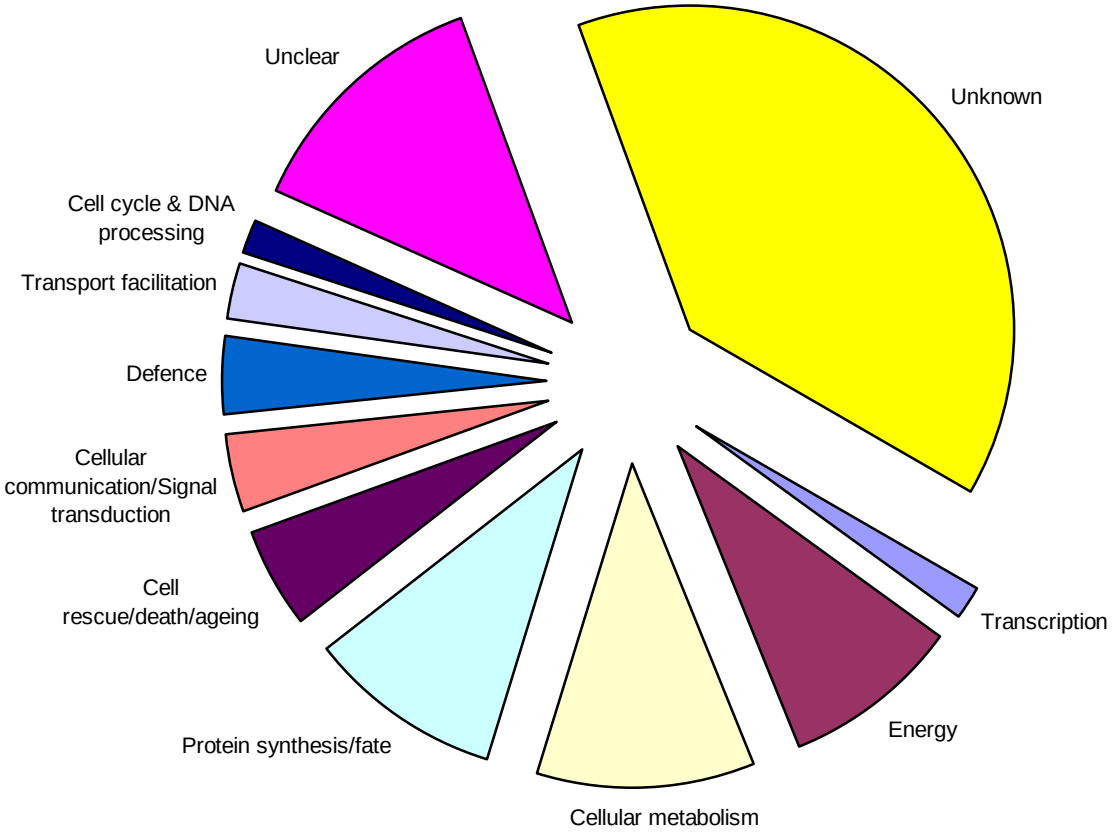

Figure 2.5 Functional distribution of the 516 *Cicer arietinum* (ICC3996) unigenes.

Of the 50% that were functionally annotated, the largest category was ‘cellular metabolism’ (11%), made up of various putative enzymes and metabolic proteins. Cytochrome-like proteins were the most common, especially cytochrome P450s, and several metabolic pathways were represented, including fatty acid metabolism, nitrogen fixation, amino acid biosynthesis, sterol biosynthesis and fruit development. The next largest category was ‘protein synthesis/fate’ (10%), of which the majority encoded putative nuclear, mitochondrial, and chloroplast ribosomal proteins. The ‘energy’ (9%) category included unigenes of the photosynthesis/ATP-synthesis/electron-transport pathways, such as the chlorophyll a/b binding protein, ATP synthase, Rubisco, and ferredoxin.

Proteins implicated in stress responses formed the majority of the ‘cell rescue/death/ageing’ (5%) category, the most common examples resembling auxin-repressed proteins, heat-shock proteins, and wound-induced proteins. Another 4% represented ‘cellular communication/signal transduction’, including protein kinases, and other putative membrane-bound signalling proteins. Few proteins were involved in ‘transport facilitation’ (3%), some examples being aquaporin, sugar transport proteins, and ion-channel proteins. The ‘transcription’ (2%) category included messenger RNAs and transcription factors, whilst ‘cell cycle & DNA processing’ (2%) included putative DNA methylation proteins. Clustering of the 26 putative defence-related ESTs resulted in 20 unigenes (Table 2.1), representing 4% of the unigene set.

A comparison can be made between the functional distributions of the independent ESTs and clustered unigenes to show those categories represented by highly expressed genes, as these categories will have a lesser value in the clustered distribution compared to the independent EST distribution (Figure 2.6). This was clearly apparent for ‘protein synthesis/fate’, ‘energy’, and ‘transcription’ where the numbers of independent ESTs are more than double the number

of unigenes. The members of these categories, which include various ribosomal/messenger RNA molecules as well as transcripts involved in photosynthesis and respiration, are sampled more frequently in random sequencing, supporting their high level of expression and potential involvement in general housekeeping processes. Additionally, other general housekeeping categories such as 'cellular metabolism' and 'protein synthesis/fate', were also sampled considerably more in the independent EST set compared to the unigenes. Interestingly, the 'unknown' category also fits this scenario, suggesting that many of its members may belong to housekeeping categories, or become highly expressed after *A. rabiei* challenge.

Detailed examination of the eight unigenes that contain more than 10 independent ESTs (Table 2.2) shows that the most highly expressed EST is a chloroplast mRNA of the 'transcription' category. Other highly expressed ESTs include ribosomal RNAs ('protein synthesis/fate'), putative enzymes of the 'cellular metabolism' category, and a chlorophyll protein and Rubisco enzyme ('energy').

Table 2.1 *Cicer arietinum* (ICC3996) defence-related unigenes after BLASTN and BLASTX sequence homology searches.

Unigene (GenBank accession)	Database match	Matching database accession	e value	Copy number
CV793587	*A. thaliana* extensin-like disease resistance protein	O82202[c]	6e-28	1
CV793588	*G. max* gamma-thionen defensin/protease inhibitor	Q39807[c]	2e-11	1
CV793589	*N. tabacum Avr9/Cf-9* rapidly elicited protein 65	Q9FQZ0[c]	2e-12	2
CV793590	*B. oleracea* pathogen-induced translation initiation factor *nps45*	SUI1_BRAOL[b]	4e-34	1
CV793591	*V. unguiculata* S1-3 pathogen-induced protein	Q9MB24[c]	4e-20	1
CV793593	*A. thaliana* putative disease resistance protein	DR29_ARATH[b]	6e-13	1
CV793594	*C. arietinum* transcription factor EREBP-1	Q8GTE5[c]	2e-95	3
CV793595	*M. sativa* caffeoyl-CoA-methyltransferase	CAMT_MEDSA[b]	5e-98	2
CV793597	*P. sativum* pathogenesis-related protein 4A	Q9M7D9[c]	5e-61	1
CV793598	*C. arietinum* β-1-3-glucanase	Q9XFW9[c]	2e-26	1
CV793599	*O. sativa* protein with leucine zipper	Q8RZJ0[c]	3e-48	1
CV793600	*S. tuberosum* pathogen-induced transcription factor	Q9LL86[c]	2e-11	1
CV793601	*E. esula* leucine-zipper containing protein	Q945B7[c]	2e-13	1
CV793602	*M. domestica* cinnamyl alcohol dehydrogenase (CAD1)	O65152[c]	2e-34	1
CV793603	*C. arietinum* nematode resistance protein *Hs1pro-1*	Q94BW7[c]	7e-16	1
CV793605	*O. sativa* multi-resistance ABC transporter protein	Q943U4[c]	7e-12	1
CV793607	*A. thaliana* putative flavonol glucosyl transferase	HQGT_ARATH[b]	4e-25	1
CV793608	*S. tuberosum* SNAKIN2 antimicrobial peptide precursor	Q93X17[c]	5e-23	2
CV793609	*A. thaliana* elicitor-induced receptor protein	Q9FH56[c]	1e-11	1
CV793610	*M. sativa* pathogenesis-related protein class 10	PR1_MEDSA[b]	1e-30	2

[a] Matching to GenBank database accession.
[b] Matching to SwissProt database accession.
[c] Matching to SpTrEMBL database accession.

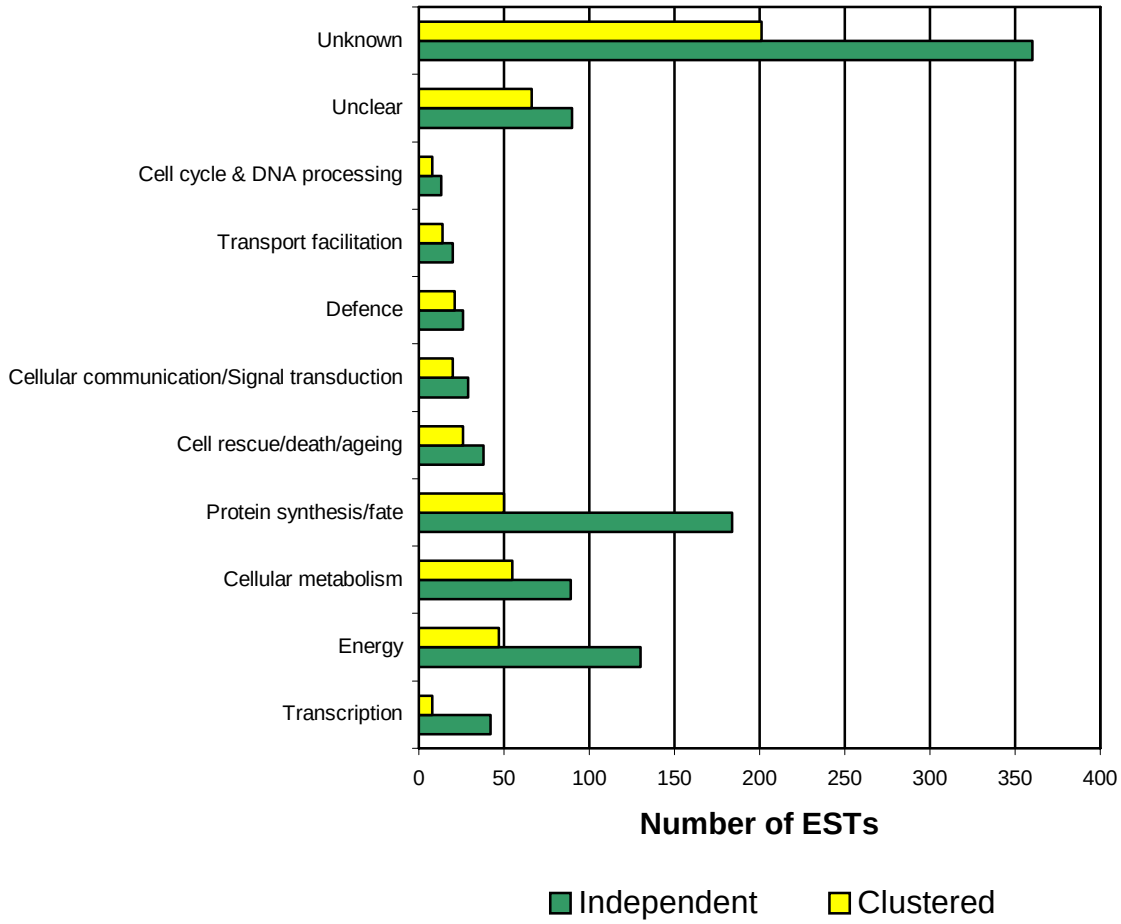

Figure 2.6 Comparison of functional distributions amongst independent ESTs and clustered unigenes.

Table 2.2 Description of the unigene clusters containing more than ten independent ESTs.

Number of ESTs in cluster	Cluster identification from BLASTN or BLASTX	e value	Functional category
173	*C. arietinum* chloroplast 4.5S/5S/16S/23S messenger RNA	4e-11	Transcription
34	*C. arietinum* 26S ribosomal RNA	1e-101	Protein synthesis/fate
31	*C. arietinum* putative deoxycytidylate deaminase	2e-48	Cellular metabolism
24	*L. esculentum* chlorophyll a/b binding protein	2e-83	Energy
17	*O. sativa* ribosomal RNA intron-encoded homing endonuclease	4e-26	Protein synthesis/fate
16	*P. sativum* UDP-glucose 4-epimerase (EC 5.1.3.2)	1e-26	Cellular metabolism
15	*C. arietinum* ribulose 1,5-bisphosphate carboxylase small subunit precursor (EC 4.1.1.39)	1e-95	Energy
15	*A. thaliana* mitochondrial 26S ribosomal RNA	4e-12	Protein synthesis/fate

2.3.4 Comparison with other plant species

The availability of the annotated *A. thaliana* genome, as well as large EST sets for other leguminous plant species, enabled an estimation of the level of gene conservation and similarity between *C. arietinum* and other related plant species. The *C. arietinum* (ICC3996) unigenes were compared with the TIGR Gene Indices of *A. thaliana*, *Medicago truncatula*, *Lotus japonicus* and *Glycine max* (http://www.tigr.org/tdb/tgi) using TBLASTX. The similarities of each species to the *C. arietinum* (ICC3996) unigenes are shown in Figure 2.7. An important observation was the high level of weak/no similarity detected for the model legumes *M. truncatula* (47.5%) and *L. japonicus* (58.1%), indicating that *C. arietinum* may possess many genes with little homology to genes within these model legumes. Integration of the search results revealed that 33.9% of the unigenes were conserved in all five species, whilst 4.5% were conserved only in legume species, including defence-related, cell signalling/communication and cellular metabolism unigenes, as well as hypothetical proteins.

Interestingly, 57.6% of the *C. arietinum* (ICC3996) unigenes were not significantly similar to the *A. thaliana* genome, and may represent genes for morphological features or metabolic processes specific to leguminous species. Although *A. thaliana* represents a model for flowering plants, it may not possess all the desired characteristics of other plant species, and subsequently may be unsuitable for use in the study of those characteristics. Alternatively, the sequencing of untranslated regions, or the presence of non-annotated *A. thaliana* genes could cause absences in sequence similarities. The unigenes conserved between *C. arietinum* (ICC3996) and *A. thaliana* were classified into their functional categories, and the levels of similarities for each category are shown in Figure 2.8. The most highly conserved categories included ‘transcription’, ‘protein synthesis/fate’, ‘energy’ and ‘cellular metabolism’, whilst the least conserved categories included ‘defence’, ‘cell rescue/death/ageing’ and ‘cellular communication/signal transduction’.

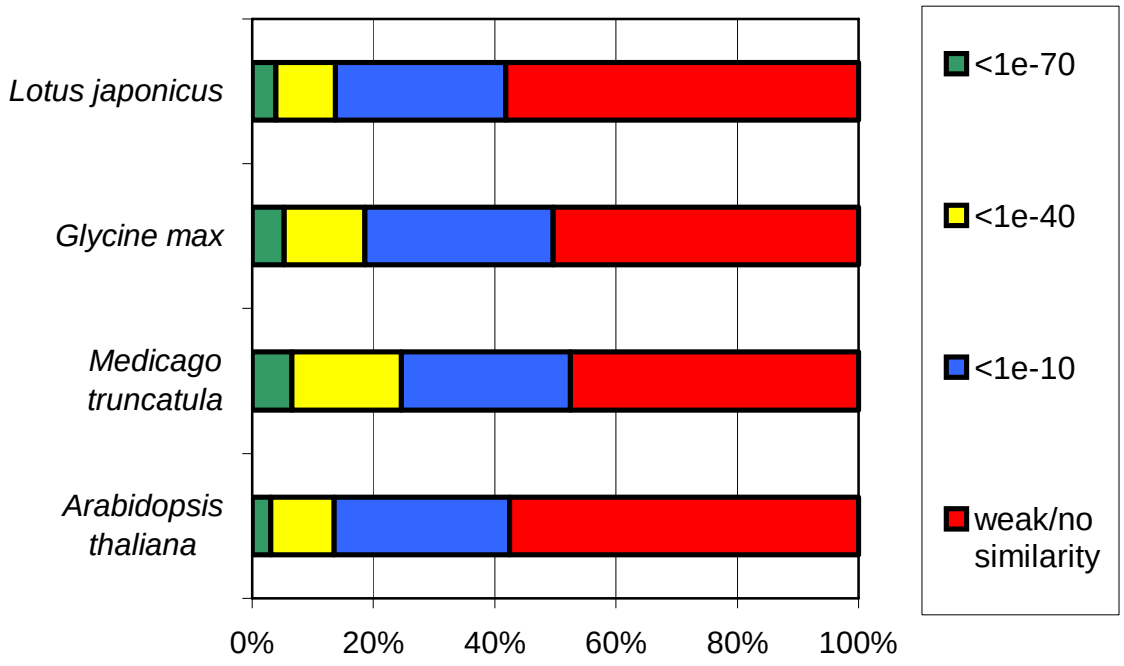

Figure 2.7 Distribution of conservation between *C. arietinum* (ICC3996) unigenes and the Gene Indices of *A. thaliana*, *M. truncatula*, *L. japonicus* and *G. max* according to similarity levels determined by TBLASTX e values.

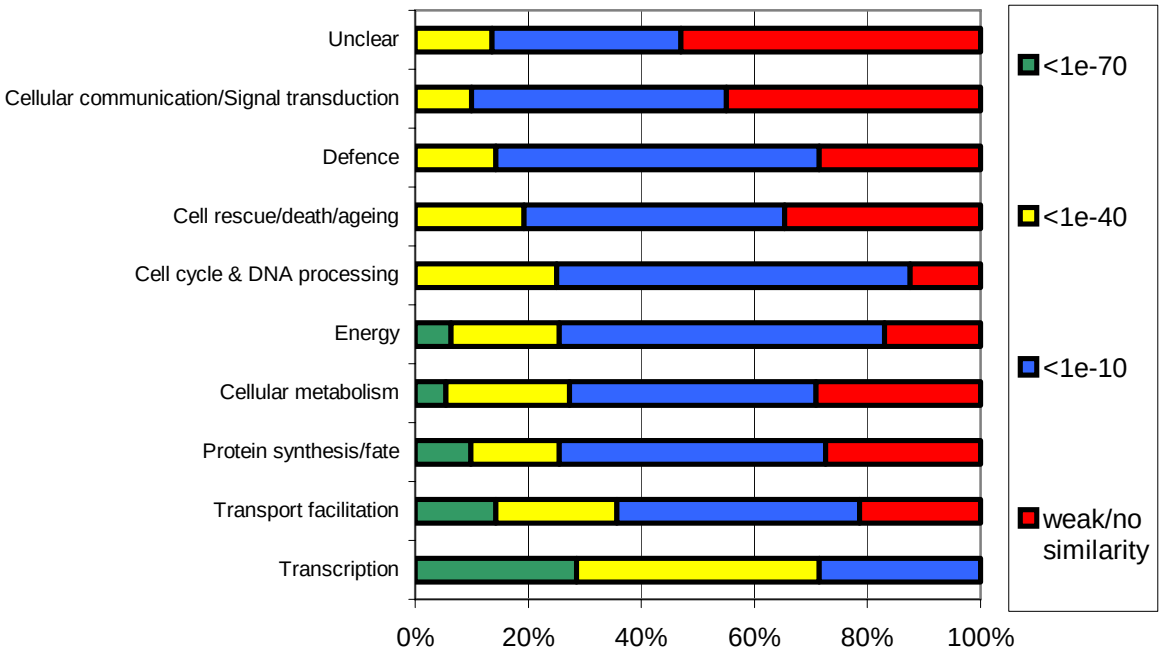

Figure 2.8 Levels of similarity for the unigenes conserved between *C. arietinum* (ICC3996) and *A. thaliana* according to functional categories. Similarity levels were determined by TBLASTX e values.

Figure 2.9 shows a comparison between the functional distributions of the clustered *C. arietinum* (ICC3996) unigenes, clustered unigenes from another legume known as white clover (*Trifolium repens*) (Sawbridge *et al.*, 2003), and the categorised genes from the *A. thaliana* genome (The Arabidopsis Genome Initiative, 2000) (note that some functional categories from *C. arietinum* and *T. repens* were merged in line with the *A. thaliana* data). The most obvious result from this comparison is the abundance of 'unknown' *C. arietinum* (ICC3996) and *T. repens* transcripts compared to *A. thaliana*. Importantly, the 'defence & cell rescue/death/ageing' category was also sampled more highly in the present study compared to *T. repens* and *A. thaliana*.

2.3.5 Identification of SSRs

The search for SSRs within the 516 chickpea unigenes revealed 14 SSRs, of which 10 were found within 10 separate singletons, and four within four individual contigs (Table 2.3). This corresponded to an overall SSR identification frequency of 2.7%, with one SSR found in every 40.1 singletons (2.5%), and one in every 28.8 contigs (3.5%). Other studies on plant species have reported SSR frequencies among ESTs of 11% in rice, 7% in *Medicago truncatula*, 5% in maize, 6% in soybean, 4% in tomato, 3% in cotton, 3% in poplar, 3% in sugarcane, and 2% in grape (Cardle *et al.*, 2000; Scott *et al.*, 2000; Cordeiro *et al.*, 2001; Tian *et al.*, 2004). Only exact SSRs were considered in the present study, and the frequency of occurrence according to motif length was 0% for mononucleotide SSRs, 29% dinucleotide, 43% trinucleotide, 14% tetranucleotide, and 14% pentanucleotide.

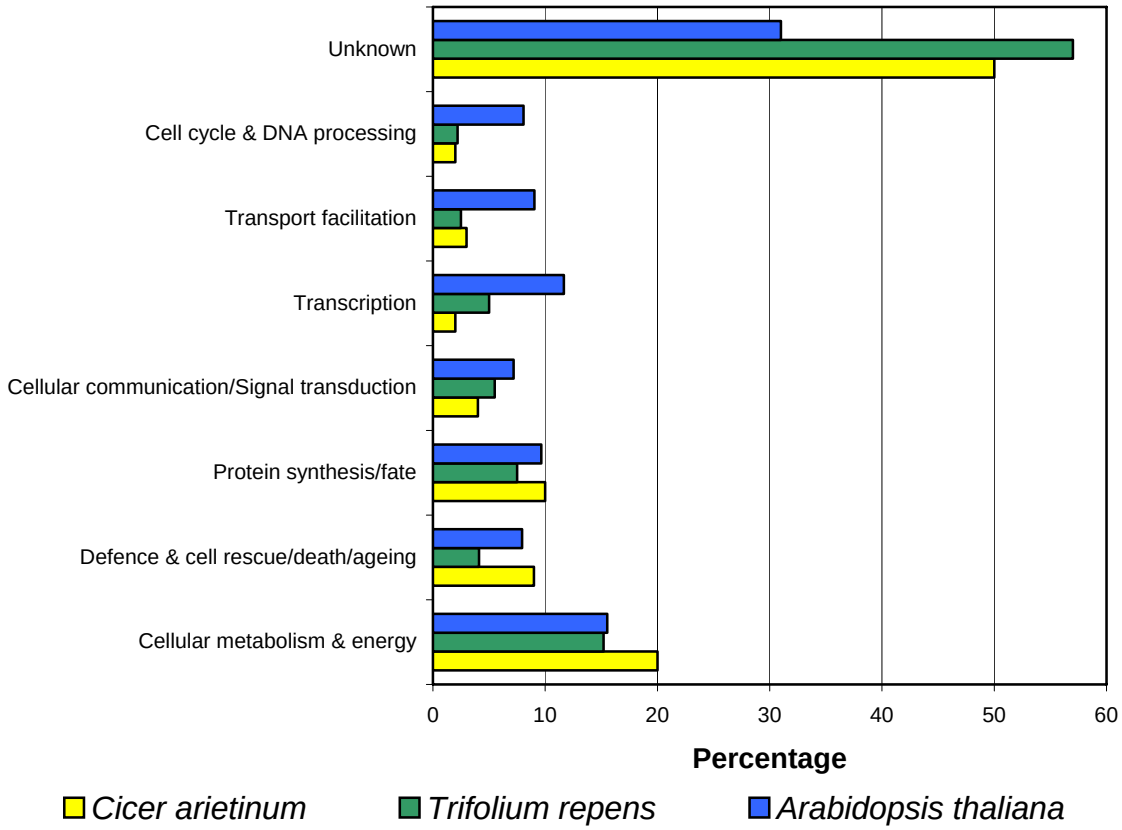

Figure 2.9 Comparison of functional distributions of clustered *C. arietinum* (ICC3996) unigenes after *A. rabiei* challenge, clustered *T. repens* unigenes from a mixed library of biotic/abiotic stress treatments excluding *A. rabiei* inoculation, and classified genes of the *A. thaliana* genome.

Table 2.3 Summary of the occurrence of SSRs within the 516 chickpea unigenes, showing the putative function of the unigene from which each SSR was identified.

SSR motif	Source (GenBank accession)	Number of repeats	Putative function of unigene
AG	Singleton (DY475284)	9	Unclear
TC	Singleton (DY475328)	7	Ubiquitin conjugating protein
TC	Singleton (DY475448)	8	Unclear
CAC	Singleton (DY475409)	5	Unclear
GAA	Singleton (DY475546)	10	Unclear
TAT	Singleton (DY475477)	5	Asparagine synthetase
TAT	Singleton (DY475174)	8	Aquaporin membrane protein
TGT	Singleton (CV793608)	5	SNAKIN2 antimicrobial peptide precursor
CCCT	Singleton (DY475273)	4	Unclear
ATTAC	Singleton (DY475510)	4	30S ribosomal protein S13
TA	Contig (DY475079)	7	Unknown
TAT	Contig (DY475133)	7	Unknown
CTAT	Contig (DY475128)	4	Photosystem I reaction centre subunit IV
GAAA	Contig (DY475124)	4	Aquaporin membrane protein

2.4 Discussion

A collection of 1021 independent ESTs was clustered and assembled to generate 516 unigenes. Clustering allowed the detection of highly expressed transcripts, and the comparison between functional distributions of the independent ESTs and clustered unigenes provided evidence for this (Figure 2.6). It was expected that functional categories mainly involved with general housekeeping activities would show the highest level of expression, and this was apparent by the 'transcription', 'energy', 'cellular metabolism' and 'protein synthesis/fate' category comparisons. The specific ESTs with the highest expression (Table 2.2) were also as expected. These ESTs all belonged to general housekeeping categories and possess well-characterised functions in common plant activities. An unexpected observation

was the independent ESTs to clustered unigenes comparison of the 'unknown' category in Figure 2.6, where the majority of ESTs were expected to possess a non-housekeeping role to justify their anonymity amongst databases. A possible explanation for this may be that the *A. rabiei* inoculation of the plant caused a substantial increase in the expression of numerous unknown defence-related transcripts, resulting in the skewing of the data toward high levels of expression in the 'unknown' category.

The large amount of nucleotide sequence data in public databases enabled the comparison of the *C. arietinum* (ICC3996) unigenes to the entire genome content of *A. thaliana*, as well as the current EST collections of *M. truncatula*, *L. japonicus* and *G. max* (Figure 2.7). The highest level of similarity was observed in *M. truncatula*, followed by *G. max*, whilst *L. japonicus* and *A. thaliana* possessed the least similarity. It was expected that the three leguminous species would show the highest levels of similarity, as was observed for *M. truncatula* and *G. max*. The lower similarity level observed for *L. japonicus* may be attributed to a smaller EST collection, and it is important to recognise that the similarity levels observed do not reflect phylogenetic relationships, but rather the coverage of EST sequencing for each species. Further, the high levels of weak/no similarity observed for the two model legumes, *M. truncatula* and *L. japonicus* may indicate a significant divergence in *C. arietinum* gene content. In fact, the levels of similarity observed for the model legumes were only marginally superior than observed for *A. thaliana*, indicating a possible insufficiency of homology for their use in the study of economically important legumes. As described in Chapter 1 (section 1.5.1), sequence conservation between related species is not consistent on a gene-for-gene basis; therefore the use of these models for the study of chickpea may be limited.

The expanding collections of ESTs for the model legumes may eventually provide adequate homology to the chickpea transcriptome, but the results of this study suggest a significant proportion of chickpea genes will remain non-homologous to those collections, and demonstrates the need for chickpea-specific ESTs to study *A. rabiei* resistance. The comparisons also revealed several gene candidates that were absent in *A. thaliana* but present in all legume species, including defence-related and cellular communication/signal transduction unigenes that may be functionally specific for the protection of leguminous plants only. Further, several cellular metabolism unigenes were identified only in the legumes, indicating a possible role in a legume-specific metabolic pathway such as nodulation. Numerous other legume-specific hypothetical proteins may also represent genes involved in these pathways.

The level of similarity between the functionally annotated *C. arietinum* (ICC3996) unigenes and the *A. thaliana* genome (Figure 2.8) may reflect the speed of gene evolution, based on the assumption that slow evolving genes show a high level of conservation, and fast evolving genes show a low conservation level (The Arabidopsis Genome Initiative, 2000). The most highly conserved categories contained unigenes encoding structural, ribosomal, photosynthetic, translational and metabolic proteins, whilst the least conserved categories contained unigenes encoding defence and stress-related proteins, as well as signalling proteins such as protein kinases. These observations are similar to those witnessed in soybean (Tian *et al.*, 2004) and *L. japonicus* (Asamizu *et al.*, 2004), and lend support to the theory that genes related to basic processes have not significantly evolved, whereas regulatory genes have (The Arabidopsis Genome Initiative, 2000).

Most pertinent to the aims of this study were the potential defence-related ESTs. To enrich for these sequences, post-inoculation tissue samples of a resistant chickpea (ICC3996) were

used as starting material to generate cDNA. Stem and leaf tissue samples were taken at two time points (24 h and 48 h) based on known active defence timing (refer to section 1.3), and pooled in an attempt to capture a broad range of gene sequences that may be involved in different branches of potential defence-related pathways. Although, after EST clustering, only 4% of the unigenes were defence-related, the availability of the functional distributions of clustered *T. repens* unigenes from a mixed library of various biotic/abiotic stress treatments (Sawbridge *et al.*, 2003) and categorised genes of the *A. thaliana* genome (The Arabidopsis Genome Initiative, 2000) provided a guide to the success of the enrichment. The distribution of the *T. repens* unigenes is important considering that it also represents a legume species that was challenged with a range of stresses, and Figure 2.9 shows a higher level of defence-related unigenes sampled by this study in comparison to *T. repens*. The comparison to *A. thaliana* also shows a slightly higher level of defence-related sampling for *C. arietinum* (ICC3996), which was not the case for several other categories. Therefore, it appears that the enrichment achieved some level of success, which is important as it may enable the sequencing of fewer clones per isolation of a defence-related transcript. The comparison to the *A. thaliana* genome also indicates that the present study successfully sampled all classes of genes, but transcripts of all categories apart from 'unknown', 'cellular metabolism & energy', 'protein synthesis/fate', and 'defence & cell rescue/death/ageing' were under-represented. The high over-representation of the 'unknown' category may reflect the relatively large amount of information available for *A. thaliana* sequences, or may also support a successful enrichment, as potential unknown defence-related transcripts may have been sampled in favour of transcripts in other categories. However, the *T. repens* distribution also shows an over-representation of the 'unknown' category, indicating that legumes may possess many novel transcripts in comparison to *A. thaliana* that are yet to be functionally annotated.

The putative defence-related ESTs identified in this study (Table 2.1) represented a variety of plant defence mechanisms and pathways, and can be further grouped into specific categories based on their inferred mode of action. The Extensin-like protein (CV793587), Caffeoyl-CoA-methyltransferase (CCoAOMT) (CV793595), and Cinnamyl alcohol dehydrogenase (CAD1) (CV793602) are all putatively involved in the synthesis of lignin or cell walls. Lignin, a complex three-dimensional polymer that forms a principal component of some plant cell walls, has been implicated in chickpea resistance to *A. rabiei* (refer to section 1.3), is synthesised via the phenylpropanoid pathway, and is often deposited around invading hyphal tips in response to fungal infection (Humphreys and Chapple, 2002). CCoAOMT and CAD1 are both enzymes that belong to the phenylpropanoid pathway, which is also responsible for the biosynthesis of antimicrobial phytoalexins. Although the lignin pathway contains several other enzymes, the isolation of CCoAOMT is important considering that it has previously been identified as part of an elicitor-induced plant defence response (Pakusch *et al.*, 1991). The isolation of CAD1 is also significant in characterising a potential chickpea lignin deposition response, as it acts as a multifunctional enzyme within the phenylpropanoid pathway. Extensin proteins are cell wall proteins rich in hydroxyproline (refer to section 1.4.1), and often contain leucine rich repeats (LRRs) that are common to several *R* gene classes and believed to be involved in pathogen recognition (refer to section 1.4.2.1; Stratford *et al.*, 2001). The chickpea extensin isolated in this study did match to an extensin with LRRs (Table 2.1), but the full-length gene sequence would have to be isolated before confirming the presence of LRRs. Additionally, another chickpea extensin, similar to the one isolated in this study, was found to proliferate rapidly after an oxidative burst, thus implicating the protein as part of a defence response (Otte and Barz, 2000).

Pathogenesis-related protein 4A (CV793597), *β*-1,3-glucanase (CV793598), and pathogenesis-related protein class 10 (CV793610) may all be grouped as pathogenesis-related

(PR) proteins (refer to section 1.4.1). There exist numerous classes of PR proteins, and Pathogenesis-related protein 4A (CV793597) represents class four, which are known to possess chitinase activity against fungal cell walls. The *β*-1,3-glucanase (CV793598) isolated in this study also acts to dissolve fungal cell walls and is a member of class two. Pathogenesis protein class 10 (CV793610) belongs to class 10 PR proteins that are acidic, intracellular, and usually members of a multigene family (Elbez *et al.*, 2002). Additionally, they also contain highly conserved phosphate-binding motifs and may possess an RNAse activity considering their structural similarity to ribonucleases (Van Loon and Van Strien, 1999).

The *β*-1,3-glucanase (CV793598) has been previously isolated from chickpea, where it was found to accumulate strongly after inoculation with *A. rabiei* (Hanselle and Barz, 2001), as well as in response to the fusarium wilt fungal disease (Singh *et al.*, 2003). Although cDNAs of PR proteins 4A and class 10 have previously been isolated from *Pisum sativum* and *Medicago sativa* respectively (Table 2.1), they have not been isolated from chickpea and may be important for *A. rabiei* defence. One other defence-related EST may be classed as a PR protein - the gamma-thionen defensin/protease inhibitor (CV793588). Proteins of this type may be involved in pathogen defence by preventing the hydrolysis of plant cell proteins by fungal toxins (Koiwa *et al.*, 1997; Pelegrini and Franco, 2005). Protease inhibitors have been widely characterised as defence-related proteins, and CV793588 is the first cDNA for a putative protein to be isolated from chickpea.

Five of the defence-related unigenes may be grouped into a putative signalling or defence-activating category; Translation initiation factor *nps45* (CV793590), Transcription factor EREBP-1 (CV793594), *Avr9/Cf-9* rapidly elicited protein 65 (CV793589), Pathogen-induced transcription factor (CV793600), and Elicitor-induced receptor protein (CV793609). The most characterised protein from this group is the Transcription factor EREBP-1 (Ethylene

Responsive Element Binding Protein). EREBP transcription factors are a subfamily of the AP2 transcription factor family that encode proteins involved in the regulation of disease resistance pathways. They share a conserved 58-59 amino-acid domain that binds *cis*-elements of the GCC box of pathogenesis-related gene promoters (Singh *et al.*, 2002), and their expression is regulated by plant hormones (salicylic acid, jasmonate, and ethylene) as well as pathogen challenge (Gutterson and Reuber, 2004). Although an EREBP sequence has been isolated from chickpea, its activity in chickpea is yet to be studied. However, in tobacco, an EREBP was induced by salicylic acid treatment and acted to induce expression of several PR proteins (Park *et al.*, 2001). Further, EREBP transcription factors were induced by pathogen infection and acted to stimulate defensive responses in rice, tomato and poplar (Thara *et al.*, 1999; Kim *et al.*, 2000; Ralph *et al.*, 2006). Subsequently, the chickpea EREBP-1 transcription factor isolated in this study may play a role in the activation of defence against *A. rabiei*.

Although the remaining four ESTs of this subgroup are potentially defence-related, they have not previously been isolated from chickpea, nor have they been characterised well in other plants. CV793590 identifies a translation initiation factor induced by the *Brassica oleracea*–*Xanthomonas campestris* pv. *campestris* interaction (Abdullah *et al.*, 1998). Translation initiation factors have been shown to contribute significantly to the level of expression of a gene (Kawaguchi and Bailey-Serres, 2002), hence it is possible that CV793590 may play a role in regulating levels of defence-related protein products in chickpea. CV793600 shows similarity to a transcription factor that is up-regulated in potato tubers after fungal infection (Godoy *et al.*, 2000). Considering that transcription factors are integral to the induction of stress responses in plants (Stein *et al.*, 2005), CV793600 possibly encodes an important protein involved in the regulation of defence-related gene expression. CV793609 identifies an *A. thaliana* protein that may be induced by elicitor treatment (Sato *et al.*, 2000), and

CV793589 identifies the *Avr9/Cf-9* rapidly elicited protein 65 isolated from tobacco. This protein was first identified from the *Lycopersicon esculentum–Cladosporium fulvum* interaction as being induced upon interaction of the *Cf-9* protein and *Avr9* avirulence gene product according to the gene-for-gene hypothesis (Durrant *et al.*, 1999). The action and effectiveness of this protein remain uncharacterised, but the existence of a tobacco and chickpea homolog shows that the protein is not specific to tomato.

The largest sub-group of the defence-related ESTs were those of putative defensive functions that had not been fully characterised, including; S1-3 pathogen-induced protein (CV793591), Putative disease resistance protein (CV793593), Protein with leucine zipper (CV793599), Leucine-zipper containing protein (CV793601), Multi-resistance ABC transporter protein (CV793605), and SNAKIN2 antimicrobial peptide precursor (CV793608). CV793591 matches to an uncharacterised pathogen-induced protein from cowpea (*Vigna unguiculata*), CV793593 matches to a putative disease resistance protein from *A. thaliana*, and CV793608 shows significant similarity to a precursor of an antimicrobial compound from potato (*Solanum tuberosum*) with uncharacterised activity (Table 2.1). CV793605 resembles a putative multi-resistance ABC transporter protein from rice (*Oryza sativa*), known to potentially control transport of antimicrobial secondary metabolites across cell membranes in plants under biotic stress (Crouzet *et al.*, 2006). The leucine–zipper proteins, CV793599 and CV793601 (Table 2.1), have different sequences but both possess the leucine-zipper domains that are characteristic of class 2 disease resistance proteins (refer to section 1.4.2.1). Further, leucine-zipper proteins are identified as basic region/leucine-zipper motif (bZIP) transcription factors that are involved in several plant processes including pathogen defence (Jakoby *et al.*, 2002). None of the six proteins of this subgroup have been formerly isolated in chickpea.

One of the defence-related ESTs was implicated in phytoalexin production; the putative flavonol glucosyl transferase (CV793607). Phytoalexins are toxic antibiotics that accumulate in cell membranes, and their production occurs via the flavonoid branch of the phenylpropanoid pathway (refer to section 1.4.1). CV793607 is a UDP flavonoid glucosyl transferase (UFGT) type enzyme that plays a central role in the production of flavonol glycosides and anthocyanins, which are both important in pigmentation and have been implicated in phytoalexin production (Winkel-Shirley, 2002). CV793607 shows significant sequence similarity to the UDP flavonol glucosyl transferase isolated by Horvath and Chua (1996) that was induced by the application of salicylic acid. Considering that salicylic acid is considered an elicitor of plant defences (refer to section 1.4.3), the UDP flavonol glucosyl transferase isolated in this study may represent an important enzyme involved in the formation of a chickpea phytoalexin, which are known to be involved in *A. rabiei* defence (refer to section 1.3.2). The final defence-related EST that cannot be grouped with any others is the Nematode resistance protein (CV793603). This protein has previously been isolated from chickpea (Table 2.1) and, considering that it has only been implicated in nematode resistance, is unlikely to be involved in defence against *A. rabiei*.

Scanning of the 516 unigenes enabled the identification of 14 SSRs, at an overall frequency of 2.7%. This frequency, although low, was in the range of 2–11% observed for SSR identification from ESTs in other plant studies (section 2.3.5). The most common SSR motif was a trinucleotide motif, which was also the case in other plant species including barley, rice, maize, sugarcane and *A. thaliana* (Chin, 1996; Cardle *et al.*, 2000; Temnykh *et al.*, 2000; Cordeiro *et al.*, 2001; Thiel *et al.*, 2003). The dominance of the trinucleotide motif in SSRs derived from ESTs may be caused by the need to suppress non-trinucleotide SSRs in coding regions of genes (corresponding to ESTs), due to the risk of frameshift mutations that may

occur by insertion of additional repeats of mono-, di-, tetra-, or penta-nucleotide motifs (Metzgar *et al.*, 2000).

Importantly, EST SSRs are associated with a transcribed gene, and thus can be used in genetic mapping to identify loci directly associated with a gene. However, not all EST SSRs can be successfully converted to useable markers, but reported frequencies of marker conversion are in the range of 60-65% (Cordeiro *et al.*, 2001; Thiel *et al.*, 2003). This study identified a potentially important SSR associated with the SNAKIN2 antimicrobial peptide precursor, which represents a defence-related protein with potential involvement in *A. rabiei* defence that will be studied further. If this protein is implicated in *A. rabiei* defence, the presence of an associated SSR may enable the development of a DNA marker that can be used in genetic mapping to identify the gene locus and determine alleles that are important for *A. rabiei* resistance. Subsequently, the DNA marker could be used in marker-assisted selection (MAS) breeding programs aimed at targeting potential *A. rabiei* resistance alleles. However, the focus of the present study was on gene expression experiments, thus the potential for converting identified SSRs into DNA markers was not pursued.

2.5 Conclusion

In summary, a collection of chickpea ESTs was generated from which potential *A. rabiei* defence-related unigenes were uncovered. The enrichment of the cDNA library increased the isolation of a wide range of defence-related unigenes from the resistant chickpea genotype, indicating that many biochemical pathways may be involved in the defence response. The defence-related unigenes included putative transcripts involved in pathogen recognition, defence signalling and the phenylpropanoid pathway, as well as several putative PR proteins and antimicrobial transcripts. It is important to note that motif analysis of functional protein products of all transcripts could not be performed without the isolation of full-length gene

sequences. The unigenes of this study represent single-pass partial sequence 'tags' and therefore may not entirely represent a full-length gene sequence.

Similarity comparisons of the chickpea unigenes revealed that a high proportion of the chickpea transcriptome may be insufficiently homologous to model legumes, limiting the use of their EST collections for the study of chickpea. In addition to the putative defence-related unigenes, perhaps the most important group of unigenes was those of 'unknown' and 'unclear' identity. The unigenes making up these categories may include novel defence-related genes, and similarly, the 'cellular communication/signal transduction' and 'cell rescue/death/ageing' categories may contain unigenes that are essential to the coordination of defence responses. The next step in this study will involve the use of cDNA microarrays to study expression patterns of the defence-related unigenes. By studying up- or down-regulation in resistant and susceptible chickpea genotypes over a range of post-inoculation time points, it may be possible to identify genes involved in *A. rabiei* defence.

In addition to microarray analysis and outside the scope of this study, the defence-related unigenes may also be applied to genetic mapping experiments where, if polymorphic between parents, they may act as markers to identify QTL associated with *A. rabiei* defence. In fact, in a step toward DNA marker development, 14 SSRs were identified from the chickpea unigenes. The unigenes may also be used in SNP discovery, which involves the amplification and analysis of genomic DNA sequences homologous to each unigene from various chickpea genotypes, restriction fragment length polymorphism (RFLP) mapping, and cleaved amplified polymorphic sequence (CAPS) mapping. These alternative applications are all directed toward producing molecular markers linked to *A. rabiei* defence, and the defence-related unigenes generated in this study may provide a valuable resource for such purposes.

Chapter 3

Optimisation of the *A. rabiei* inoculation procedure and microarray expression analysis of putative defence-related unigenes from *C. arietinum*.

3.1 Introduction

As reported in Chapter 2, the chickpea genotype ICC3996 possesses a strong capacity for *A. rabiei* resistance. Extensive research has been performed on the development of reliable disease assessments for ascochyta blight of chickpea (Singh *et al.*, 1981; Reddy and Singh, 1984; Tekeoglu *et al.*, 2000) and Reddy and Singh (1984) developed a 9-scale disease index that enabled researchers to standardise their inoculation procedures to provide consistent and comparable results. The scale ranges from 1.0 (no disease) to 9.0 (plants dead), and has been used to evaluate *A. rabiei* resistance in numerous chickpea genotypes (Collard *et al.*, 2001). The scale has been applied by the Department of Primary Industry (DPI), Horsham, for local chickpea evaluation trials (K. Hobson, pers. comm.), where scores over 5.0 were regarded as moderately to highly susceptible, and scores under 5.0 as moderately to highly resistant. Such trials, performed under field conditions, found that most cultivated chickpea genotypes score a 6.0-9.0, but some uncultivated breeding genotypes scored as low as 2.0 (K. Hobson, pers. comm.). The findings of Collard *et al.* (2001), a study that assessed *A. rabiei* infection in 114 *Cicer* genotypes, support the disease ratings found at DPI.

The availability of the set of chickpea unigenes from Chapter 2 enables the development of an efficient and accurate method of gene expression profiling, including the identification of genes whose expression is changed in response to disease pressure, which in turn suggests functional involvement. The expression pattern of several genes may also be used as an

indicator of the state of a cell or tissue, such as resistance or susceptibility to a disease. However, the development of a reliable inoculation procedure and disease assessment protocol is integral to any plant pathogenicity study (Brown and Ogle, 1997). Additionally, the ability to reproduce natural infection conditions under controlled (glasshouse) conditions is equally important when studying disease resistance. In order for the genetic samples (RNA) of the present study to be considered accurate and representative, the chickpea plants must be exposed to *A. rabiei* infection consistent with known field conditions. Subsequently, the first objective of this study was to develop and optimise the inoculation procedure for a range of chickpea genotypes with varying *A. rabiei* resistance levels, so that disease assessment resembled that achieved in known field conditions at DPI (Horsham).

DNA microarrays are powerful tools for comprehensive characterisation of different plant processes, such as pathogen defence, at the transcriptional level (Clarke and Zhu, 2006). The second part of this study involved the use of chickpea unigenes functionally classified defence-related (refer to Chapter 2) in microarray experiments. Only defence-related unigenes were selected to enable optimisation and assessment of microarray techniques before committing to the expense of large-scale microarray construction. The aim was to generate expression profiles over a time-course, after inoculation with *A. rabiei* spores, in the ascochyta blight resistant ICC3996 chickpea genotype and a susceptible chickpea cultivar known as Lasseter. As reported in Chapter 1 (section 1.5.3), data normalisation is essential and several methods exist depending on the samples studied. In this study the defence-related genes were expected to be differentially expressed, thus a normalisation protocol using a set of normalisation controls (or 'housekeeping genes') is common (Draghici, 2003). The subsequent detection of unigenes showing differential expression patterns in ICC3996 compared to Lasseter over the time-course led to the identification of genes with potential involvement in conferring *A. rabiei* resistance to ICC3996.

3.2 Optimisation of *A. rabiei* inoculation procedure

3.2.1 Materials and methods

3.2.1.1 Plant material

Seeds of four chickpea genotypes (Table 3.1) were obtained from the Australian Temperate Field Crops Collection (ATFCC, Horsham, Victoria, Australia). Lasseter is a large-seeded commonly cultivated desi-type, whilst ICC3996 is a small-seeded desi-type used as an uncultivated breeding line (K. Hobson, pers. comm.). Several disease evaluations have reported ICC3996 as resistant to ascochyta blight, whilst Lasseter is highly susceptible (Table 3.1). The two remaining genotypes were included in this study only for *A. rabiei* inoculation procedure optimisation, but are employed for microarray analysis in Chapter 4. Of these, FLIP94-508C is a small-seeded desi-type genotype that DPI commercially released in 2005 as an ascochyta blight resistant cultivar, under the name Genesis 508™. ILWC245 represents a wild relative of *C. arietinum*, which possesses resistance to ascochyta blight and may be crossed to *C. arietinum* to introgress potential resistance genes (Collard *et al.*, 2001).

Table 3.1 Summary of the four chickpea genotypes used for the *A. rabiei* inoculation procedure optimisation, showing available *A. rabiei* disease score data.

Chickpea genotype	Seed type/size	Comment	Disease score/s*
C. arietinum ICC3996	Desi/Small	Highly resistant breeding line	A: 3.9 B: 3.0 C: 4.6
C. arietinum Lasseter	Desi/Large	Highly susceptible cultivar	A: 9.0 B: 8.3 C: 7.0
C. arietinum FLIP94-508C	Desi/Small	Moderately resistant 2005 commercial release developed by DPI (Horsham)	A: 4.9 B: NA C: NA
C. echinospermum ILWC245	NA	Moderately resistant wild relative	A: NA B: NA C: 4.8

* A = Field score from DPI (2005) with no fungicide applied (K. Hobson, pers. comm.)
B = Score from Nasir *et al.*, 2000
C = Score from Collard *et al.*, 2001

3.2.1.2 Fungal isolates

Seven isolates of *A. rabiei* were collected from field samples of seven different chickpea genotypes at DPI, Horsham, Victoria, in 2003. To ensure the isolation of lowly and highly virulent *A. rabiei* isolates, the cultivars selected for spore isolation included those known to be susceptible to *A. rabiei*, as well as several that were known to be resistant. A single-spore culture of each isolate was prepared by initial culture on V8 agar (Appendix 1), followed by immersing the fungal culture in sterile distilled water and releasing spores by disrupting pycnidia with a glass spreader. The resulting spore suspension was diluted to a concentration of 1.0 x 10^4 spores mL^{-1}, and volumes of 200 µL were dispensed onto 2% water agar plates and spread evenly with a sterile glass spreader. Plates were then incubated at 20°C with a 12 h light/dark cycle under Philips 'TL' near UV (blacklight) to induce spore germination. After 24 h, single germinating spores were excised from the water agar and transferred to V8 agar and incubated at 20°C. Healthy stem and leaf tissue taken from Lasseter (*A. rabiei* susceptible cultivar) were surface sterilised by a 1 min soak in 70% ethanol, 5 min in 2% NaOCl (bleach) containing 1 mL 1% Tween 20, followed by three 2 min washes in sterile distilled water, before being placed on each fungal plate in order to maintain the pathogenicity of each isolate. Plates were incubated under the same conditions described above.

Mixed spore suspensions, containing equal numbers of spores from each of the seven isolates were prepared from 14-day-old fungal cultures by adding 10 mL of sterile distilled water to each plate and disrupting the pycnidia with a glass spreader. The seven resulting suspensions were filtered through four layers of muslin cloth and collected in a beaker. Using a haemocytometer, three separate samples were prepared from each of the seven isolate suspensions in sterile distilled water, exhibiting variations of spore concentration; 0.5 x 10^5 spores mL^{-1}, 1.0 x 10^5 spores mL^{-1}, and 2.0 x 10^5 spores mL^{-1}. The seven suspensions for each concentration were then equally mixed, resulting in three separate inoculums. A control

inoculum of sterile distilled water was also prepared. Mixed spore suspensions were used in an attempt to provoke a broad defence response from inoculated plants, rather than a potential isolate-specific response. Note that, due to previous experiments performed by Tristan Coram (Honours project, RMIT University, 2001) demonstrating that the addition of Tween 20 (detergent) to inoculums resulted in severe infection even in resistant plants, a surfactant was not added to the inoculums of this study.

3.2.1.3 Experimental design

Thirty-two seeds of each chickpea genotype (Table 3.1) were surface sterilised by soaking in 70% ethanol for 3 min, 2% NaOCl (bleach) for 10 min, and three subsequent washes in sterile distilled water for 2 min each. The seeds were then placed on sterile wetted filter paper in sterile petri-dishes, and left to germinate in the dark for 48 h at room temperature. Germinated seeds were then sown in 15 cm diameter pots filled with sterilised potting mix (110°C for 45 min). Sowing was performed randomly and all plants were grown in a glasshouse (20 ± 4°C) for 14 days (six- to eight-leaf stage).

The *A. rabiei* inoculation optimisation experiment was constructed in a completely randomised design, with four treatments (three spore suspensions and one control), two replicates, and four plants per replicate. Plants were inoculated using a plastic pump sprayer until run-off, and different suspension concentrations were used to determine a treatment that reproduced field conditions. Control plants were sprayed with sterile distilled water. Following inoculation, the initiation of infection was assisted by placing all plants in a black tub covered with black plastic to provide dark, humid (>90%) conditions for 48 h. In a randomised set up, each pot was then returned to a glasshouse.

3.2.1.4 Disease assessment

The severity of infection was assessed at 14 days post-inoculation (dpi) using the 9-class disease index scale from Reddy and Singh (1984); 1 = immune, no symptoms of disease; 2 = highly resistant, infection on only 1-10% of leaves; 3 = resistant, infection on only on 11-20% of leaves; 4 = moderately resistant, infection on 21-30% of leaves and stem(s); 5 = tolerant, infection on 31-40% of leaves and stems and/or stem girdling; 6 = moderately susceptible, infection on 41-50% of leaves and stems and/or stem girdling and breakage; 7 = susceptible, infection on 51-75% of leaves and stems including stem girdling and breakage; 8 = highly susceptible, infection of 76-98% of leaves and stems, including stem girdling and breakage; 9 = plant death. Analysis of variance (ANOVA) was performed on the disease scores obtained using Minitab v. 11.2 (Minitab Inc., State College PA). Fischer's least significant difference test ($LSD_{0.05}$) was used to identify significant differences in mean disease score values between genotypes.

3.2.2 Results

3.2.2.1 Disease assessment

For all treatments, disease symptoms were first observed four dpi as yellow flecks on the leaves of all chickpea genotypes. As the disease progressed, the lesions on the leaves enlarged and stem lesions appeared, some showing sporulation of *A. rabiei* (Figure 3.1). In the susceptible genotype Lasseter, the lesions on the infected stems often progressed to a point where the whole stem dried out, girdled and collapsed by 14 dpi (Figure 3.2). Subsequently, all Lasseter plants were dead by 14 dpi in treatments 2 and 3, and severely infected in treatment 1. Lesions on all other genotypes were smaller and superficial, but varied in size and frequency depending on treatment and genotype (Figures 3.3 – 3.4). ICC3996 showed the least infection in all treatments, whilst FLIP94-508C and ILWC245 showed similar levels of infection in all treatments. Importantly, all control plants were uninfected.

Figure 3.1 Sporulating *A. rabiei* stem lesions on a Lasseter plant at 14 dpi.

Figure 3.2 A Lasseter plant displaying stem girdling at 14 dpi as a result of *A. rabiei* infection.

Figure 3.3 ICC3996 plants showing small, superficial flecks on leaves and stems at 14 dpi after *A. rabiei* infection.

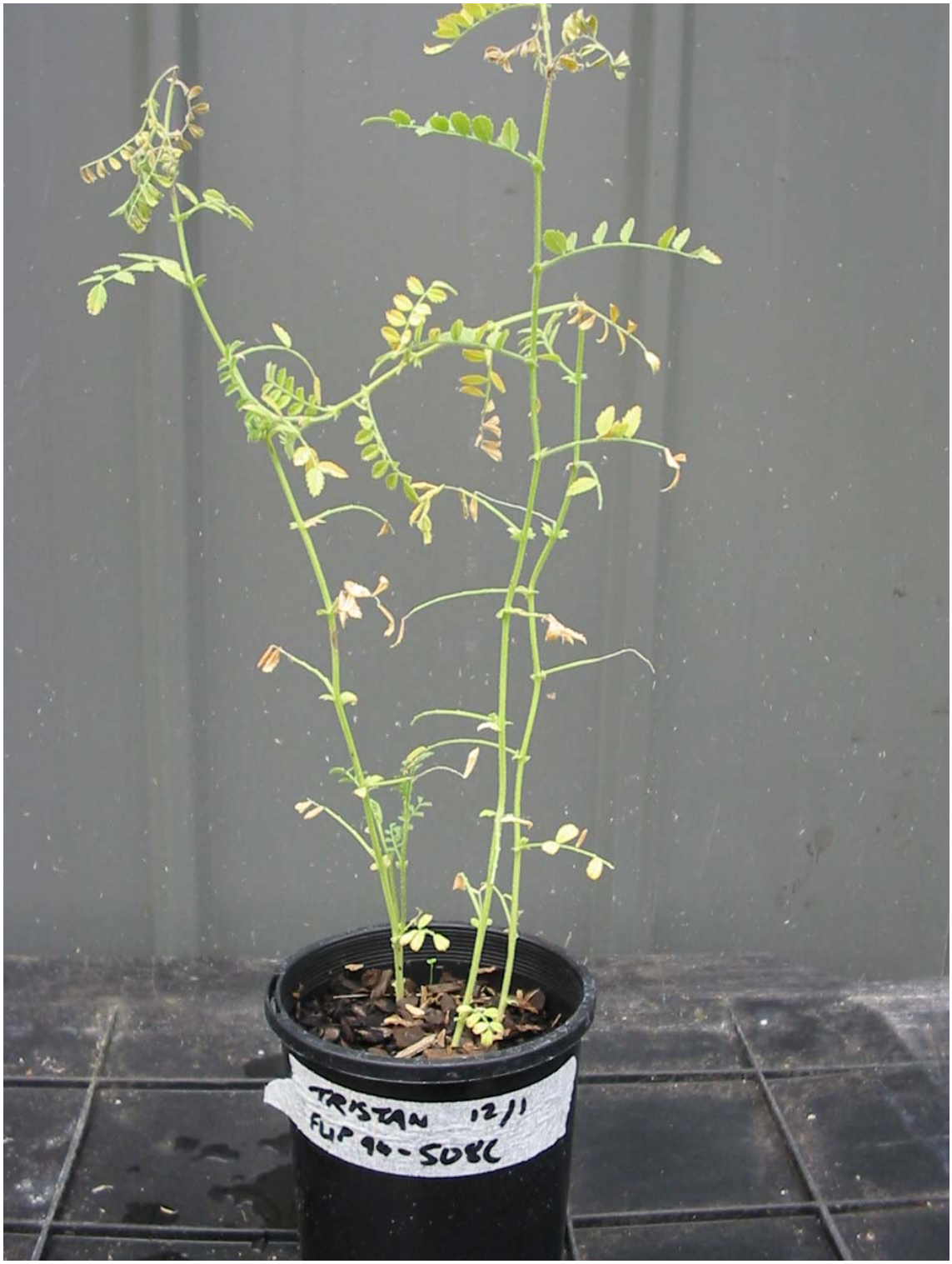

Figure 3.4 FLIP94-508C plants showing small, superficial flecks on leaves and stems at 14 dpi after *A. rabiei* infection.

A Kolmorgov-Smirnov Normality test (Minitab v. 11.2, State College PA) performed on the residuals from the model showed that the data fitted a normal distribution. Additionally, Bartlett's test showed equal variances of disease scores between the genotypes and treatments (P<0.05). Subsequently, the ANOVA (Table 3.2) showed that significant variance was attributable to the chickpea genotype used, the treatment, and the genotype x treatment interaction. Subsequently, comparisons could be made between the mean disease scores of each chickpea genotype for each treatment (Figure 3.5). Such comparisons revealed that Lasseter exhibited significantly higher (P<0.05) levels of disease compared to all other genotypes over the three experimental treatments. Of the three remaining genotypes, ICC3996 showed a significantly lower mean disease score than FLIP94-508C in all treatments, but only for treatments 2 and 3 when compared to ILWC245. Mean disease scores for FLIP94-508C and ILWC245 were only significantly different for treatment 2, where FLIP94-508C showed a higher mean disease score.

In order to identify the treatment that most accurately reproduced disease scores obtained in field conditions, or by previous disease evaluation studies, a comparison between the mean disease scores recorded for each treatment and the expected disease score was made (Table 3.3). Expected disease scores were regarded as those obtained in field conditions (DPI, Horsham) for ICC3996, FLIP94-508C and Lasseter, but for ILWC245, the expected disease score was regarded as that previously evaluated by Collard *et al.* (2001) (Table 3.1). Comparisons revealed that, across all genotypes, treatment 2 (1.0×10^5 spores mL^{-1}) produced the most comparable disease scores to those expected.

Table 3.2 ANOVA table for disease scores from the four chickpea genotypes, with four treatments (including control) and eight replicates per treatment.

Source of variation	Degrees of freedom	Sum of squares	Mean square	F	P
Treatment	3	505.281	168.427	410.58	0.000
Genotype	3	274.281	91.427	222.87	0.000
Replicate	7	0.219	0.031	0.08	0.999
Treatment x genotype	9	98.156	10.906	26.59	0.000
Genotype x replicate	21	4.719	0.225	0.55	0.937
Treatment x replicate	21	4.219	0.201	0.49	0.965
Error	63	25.844	0.410		
Total	127	912.719			

$LSD_{0.05} = 0.53$

Bartlett's test: test Statistic = 4.38, *P*-value = 0.885

Table 3.3 Comparison between the recorded mean disease scores for each genotype and treatment and the expected disease score for each genotype. 'Total' represents the sum of differences for each treatment irrespective of +/-.

Genotype	Expected score	Difference from expected score		
		Treatment 1	*Treatment 2*	*Treatment 3*
ICC3996	3.9	-0.9	-0.1	+0.4
FLIP94-508C	4.9	-1.3	+0.1	+0.6
ILWC245	4.8	-1.4	-0.4	+1.0
Lasseter	9.0	-0.9	0.0	0.0
Total		**4.5**	**0.6**	**2.0**

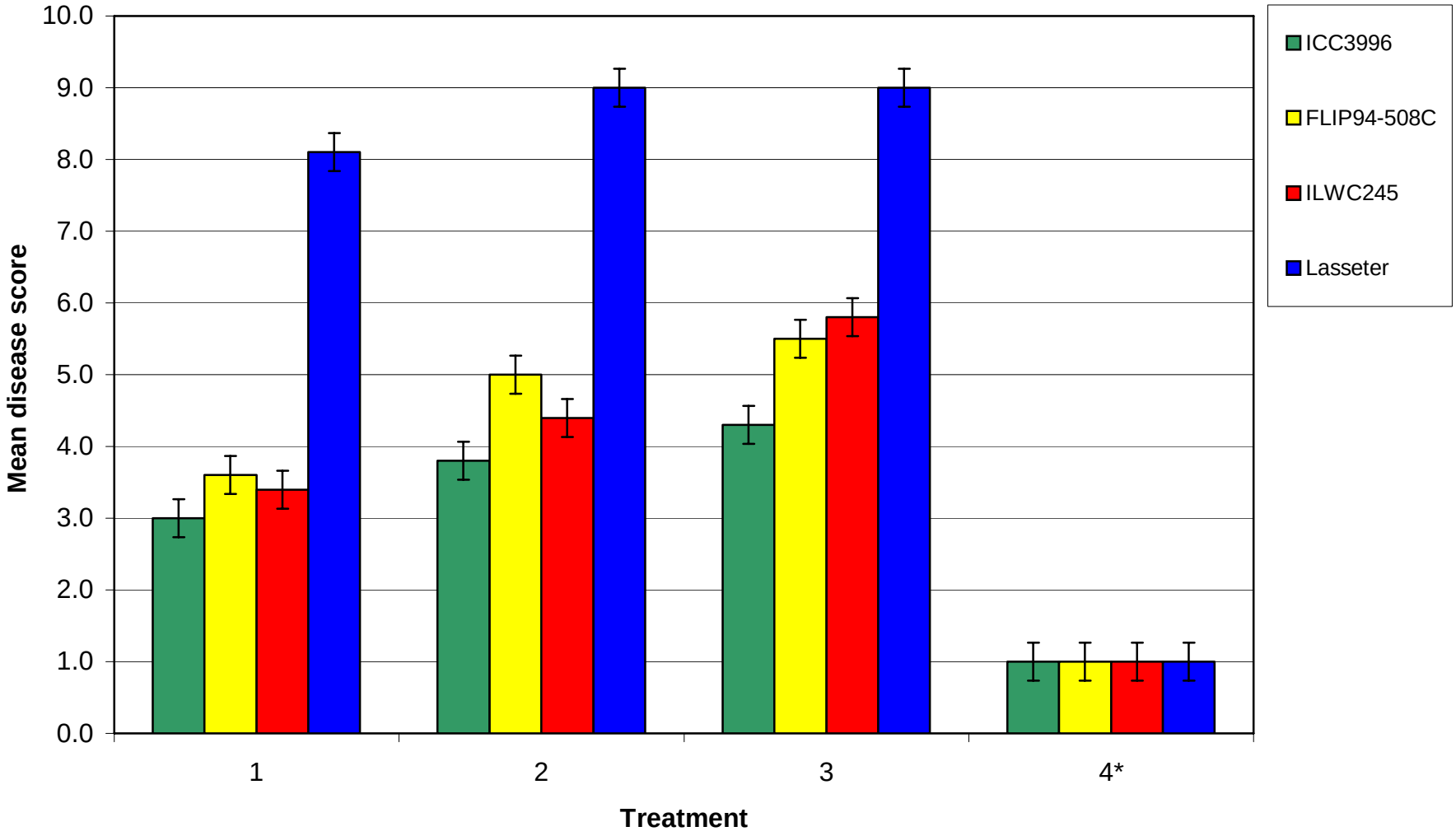

Figure 3.5 Mean disease scores for each chickpea genotype and treatment ($LSD_{0.05}$ bars are shown). Overlapping LSD bars indicate that means are not significantly different at $P = 0.05$.

3.2.3 Discussion

The chickpea cultivar, Lasseter, showed significantly higher susceptibility to *A. rabiei* compared to the three other genotypes, consistent with findings from Nasir *et al.* (2000), Collard *et al.* (2001), and the field score data from DPI (Horsham, 2005). ICC3996 showed the lowest mean disease scores in all treatments, further confirming the identification of this genotype as a source of *A. rabiei* resistance (Collard *et al.*, 2001; Nasir *et al.*, 2000; K. Hobson, pers. comm.). Although the two remaining genotypes showed higher mean disease scores than ICC3996, they were still classed as moderately resistant to *A. rabiei*. The mean disease scores of these two genotypes were only significantly different for treatment 2, where the wild relative (ILWC245) was more resistant to *A. rabiei*.

The present study used a similar inoculation technique and disease scoring method as described by Collard *et al.* (2001), but differences in disease severity were observed. Collard *et al.* used a single-isolate inoculum of 2.0×10^5 spores mL^{-1} and reported mean disease scores for ICC3996, Lasseter, and ILWC245 of 4.6, 7.0 and 4.8, respectively. This inoculum was equivalent to treatment 3 of the present study, which reported mean diseases scores of 4.3, 9.0 and 5.8 for the same genotypes. Therefore, this study observed higher disease scores for Lasseter and ILWC245, but a marginally lower score for ICC3996. The differences in disease severity may be due to the use of a mixed inoculum of seven *A. rabiei* isolates in this study, compared to a single isolate used by Collard *et al.* (2001). Subsequently, the higher scores observed for Lasseter and ILWC245 may have been caused by a higher susceptibility to some of the isolates used in this study. However, the similar disease score obtained for ICC3996 may indicate that it possesses resistance to a broad range of isolates. Further pathogenicity studies on the *A. rabiei* isolates used in this study is required before confirming differences in virulence.

The rationale for using multiple *A. rabiei* isolates in this study was that, in the field, plants could be infected by >1 genotype of the pathogen simultaneously. Subsequently, a mixed inoculum is more likely to represent field conditions. Further, the use of seven isolates may provoke a broader defence response that includes the expression of numerous defence-related genes. Such a broad defence response was important for subsequent gene expression studies, so that the identification of defence-related genes was maximised.

3.2.4 Conclusion

In summary, the aim of this study was to identify an inoculation procedure that accurately reproduced known field conditions, and the results indicated that treatment 2 (1.0×10^5 spores mL^{-1}) was optimal. Treatment 2 produced mean disease scores within 0.1–0.4 of those expected (Table 3.3), and was used in subsequent microarray gene expression studies.

3.3 Microarray expression analysis of putative defence-related unigenes

3.3.1 Materials and methods

3.3.1.1 Chickpea cultivation, inoculation and RNA extraction

Chickpea genotype ICC3996 was employed for microarray analysis as an *A. rabiei* resistant genotype, whilst Lasseter was used as a susceptible genotype. Thirty plants each of ICC3996 and Lasseter were cultivated and inoculated in a glasshouse (20 ± 4ºC) with three plants per pot and ten replicate pots, of which five replicates served as an uninoculated controls. Cultivation and inoculation was performed as described in sections 3.2.1.2 and 3.2.1.3, but only the optimal inoculum (1.0×10^5 spores mL^{-1}) concentration was used. Uninoculated controls were sprayed with sterile distilled water. After inoculation, approximately 500 mg of stem/leaf tissue was extracted from three plants per time-point of each genotype at 12, 24, 48, and 96 h post-inoculation (hpi), and immediately frozen in liquid nitrogen. The time-course was chosen based on the known timing of active defence responses in chickpea (refer to

section 1.3), whilst stem and leaf tissue were combined based on reports that chickpea stems and leaflets express the same defence mechanisms (Ilarslan and Dolar, 2002). Additionally, stem and leaf tissue samples were also taken from three uninoculated plants per time-point of ICC3996 and Lasseter. To confirm that *A. rabiei* infection had been effective, plants were checked for expected disease symptoms at 14 dpi based on the results of the inoculation optimisation experiment.

Inoculation and tissue collection was repeated twice, corresponding to two biological replicates. Within each biological replicate, total RNA was extracted from pooled stem/leaf tissue samples for ICC3996 and Lasseter (including control samples) using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA), where pools corresponded to the four post-inoculation time-points. The integrity and size distribution of total RNA samples was assessed by denaturing-agarose gel electrophoresis. A 2 µL aliquot of total RNA, mixed with 8 µL of RNase-free water (Qiagen, Valencia, CA) and 3 µL 5X RNA loading buffer (Appendix 2), was pipetted into wells of a 1.2% formaldehyde agarose (FA) gel (Appendix 2) and run in 1X FA gel running buffer (Appendix 2) at 100 V. Gels were post-stained by soaking in a solution of 300 mL 1X TBE containing 40 µL of 10 mg/mL ethidium bromide for 20 min, followed by de-staining in Milli-Q water for 20 min. Stained gels were viewed under a UV-light transilluminator and the images captured using the Gel-Doc™ system (BIO-RAD, Hercules, CA). Resulting bands were assessed for integrity and conformance to a control gel photo of plant ribosomal species supplied in the RNeasy Plant Mini Kit (Qiagen, Valencia, CA). Figure 3.6 shows an example of high quality total RNA isolated from chickpea tissue. Subsequently, the concentrations of all high quality total RNA samples were assessed by reading the absorbance of 5 µL aliquots diluted to 500 µL with RNase-free water. An absorbance of 1 unit at 260 nm corresponded to 40 µg of RNA. Additionally, the

spectrophotometer OD_{260}/OD_{280} ratios indicated the purity of the samples, where ratios of 1.9-2.1 were considered acceptable (RNeasy Mini Handbook, Qiagen, Valencia, CA, 2001).

Since the quality of RNA starting material is a major factor determining the rate of success in microarray experiments (Aharoni and Vorst, 2001), total RNA quality was also confirmed by ion-pair reversed-phase High Performance Liquid Chromatography (HPLC) (Azarani *et al.*, 2000). RNA chromatography is a fast and highly accurate method for RNA analysis, where degraded samples are detected by the absence of sharp elution peaks (Figure 3.7) (Azarani *et al.*, 2000). HPLC was performed at the Australian Genome Research Facility (AGRF, Melbourne, Australia). Briefly, 1 μg of total RNA was diluted with sterile water to 100 ng per μL and injected into the denaturing HPLC operating at 75ºC. The stationary phase consisted of a nonporous alkylated poly matrix to bind the total RNA, and the mobile phase, consisting of buffer A (0.1M triethylammonium acetate pH 7.0) and B (0.1M triethylammonium acetate pH 7.0 with 25% acetonitrile), flowed through the matrix. The ratio of buffer A:B decreased over time so that the acetonitrile in buffer B slowly negated the binding of the total RNA with matrix. Samples were eluted according to the size of the RNA fragments in the sample, and were detected at 260 nm. The integrity of the RNA is then assessed by examining the slope preceding the elution peak, where a sharp incline to the peak indicates high quality RNA, but a slow incline to the peak indicates degraded RNA.

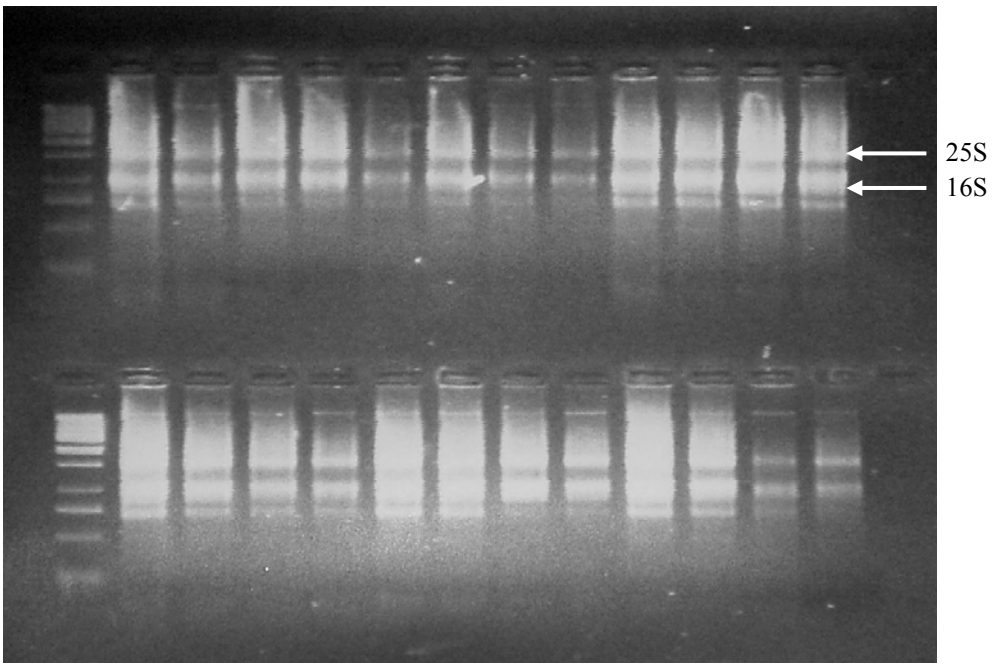

Figure 3.6 Example of high quality total RNA samples extracted from chickpea tissue, run on a 1.2% formaldehyde agarose gel and stained with ethidium bromide. Lanes on the left of each row represent 1 kb DNA ladder, whilst the indicated bands represent rRNA bands.

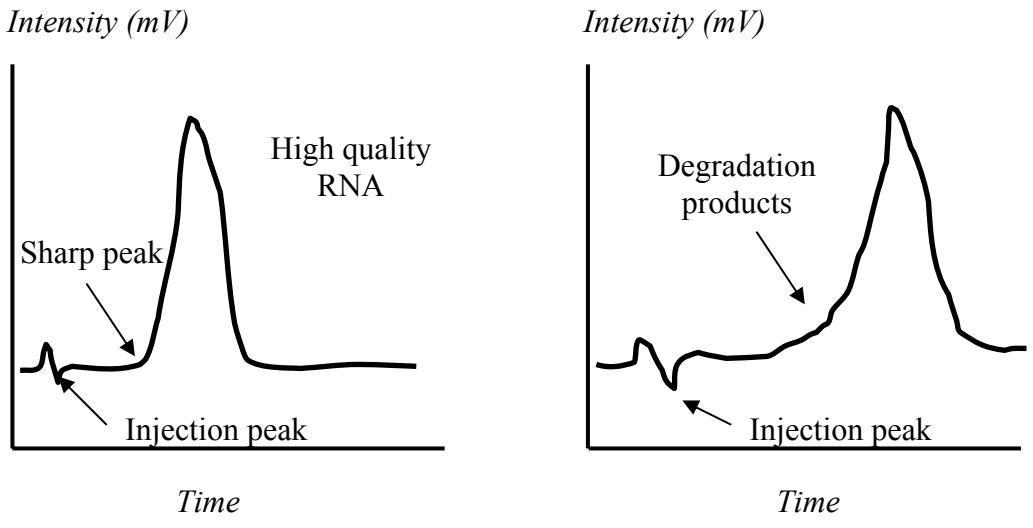

Figure 3.7 Graphical representation of the elution peaks obtained during ion-pair reversed-phase High Performance Liquid Chromatography (HPLC). The sharp peak on the left represents a high quality RNA sample, whilst the peak on the right represents a degraded RNA sample.

3.3.1.2 Preparation of probes for microarray construction

From the previously synthesised and characterised EST collection (refer to Chapter 2), the 20 unigenes classified as defence-related (refer to Table 2.1) were used as probes in the construction of microarrays according to minimum information about a microarray experiment guidelines (MIAME) (Brazma *et al.*, 2001). If applicable, a single EST was used to represent each contig and, in total, the cDNA of 25 ESTs was used after the selection of five housekeeping ESTs as internal normalisation control probes (Table 3.4).

The cDNA inserts of the 25 ESTs were amplified from their respective purified pGEM®-T Easy (Promega, Madison, WI) vector plasmids (prepared in Chapter 2) to >2000 ng by four replicate 110 µL PCR reactions. The PCR primer (Clontech, Mountain View, CA) was used for amplification instead of the T7/SP6 primers of the pGEM®-T Easy plasmid (Promega, Madison, WI) used to assess inserts in section 2.2.2. The Clontech PCR primer was able to bind to the adaptors initially ligated at both ends of all cDNAs in the construction of the cDNA library (Tristan Coram, Honours project, RMIT University, 2001). Subsequently, this primer specifically amplified the cDNA inserts without any flanking plasmid DNA sequences. Each 110 µL PCR reaction contained; 11.0 µL 10X PCR buffer (Invitrogen Life Technologies, Carlsbad, CA), 3.3 µL 50 mM $MgCl_2$, 2.2 µL 10 mM dNTP (Promega, Madison, WI), 5.5 µL 10 µM PCR primer (Clontech, Mountain View, CA), 0.75 units of *Taq* DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA), 2.2 µL purified plasmid, and 85.65 µL sterile Milli-Q water. The PCR amplifications were performed in a Perkin Elmer 2400 thermal cycler (Perkin Elmer, Wellesley, MA) under the following conditions; 1 cycle of initial denaturation at 94°C for 120 s, followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 30 s and extension at 72°C for 90 s, followed by a final extension at 72°C for 10 min.

Table 3.4 Identity of the 25 ESTs used for microarray construction, where CON01 to CON05 are normalisation controls and DEF01 to DEF20 represent the 20 defence-related unigenes.

EST	Putative identity	Category	GenBank accession
CON01	5.8S/18S/26S ribosomal RNA	Control	N/A
CON02	RUBISCO small subunit	Control	N/A
CON03	Chloroplast 4.5S/5S/16S/23S mRNA	Control	N/A
CON04	Chlorophyll a/b binding protein	Control	N/A
CON05	ATP Synthase C chain	Control	N/A
DEF01	Extensin-like disease resistance protein	Defence	CV793587
DEF02	Gamma-thionen defensin/protease inhibitor	Defence	CV793588
DEF03	*Avr9/Cf-9* rapidly elicited protein 65	Defence	CV793589
DEF04	Pathogen-induced translation initiation factor *nps45*	Defence	CV793590
DEF05	S1-3 pathogen-induced protein	Defence	CV793591
DEF06	Putative disease resistance protein	Defence	CV793593
DEF07	Transcription factor EREBP-1	Defence	CV793594
DEF08	Caffeoyl-CoA-methyltransferase	Defence	CV793595
DEF09	Pathogenesis-related protein 4A	Defence	CV793597
DEF10	β-1-3-glucanase	Defence	CV793598
DEF11	Protein with leucine zipper	Defence	CV793599
DEF12	Pathogen-induced transcription factor	Defence	CV793600
DEF13	Leucine-zipper containing protein	Defence	CV793601
DEF14	Cinnamyl alcohol dehydrogenase (CAD1)	Defence	CV793602
DEF15	Nematode resistance protein *Hs1pro-1*	Defence	CV793603
DEF16	Multi-resistance ABC transporter protein	Defence	CV793605
DEF17	Putative flavonol glucosyl transferase	Defence	CV793607
DEF18	SNAKIN2 antimicrobial peptide precursor	Defence	CV793608
DEF19	Elicitor-induced receptor protein	Defence	CV793609
DEF20	Pathogenesis-related protein class 10	Defence	CV793610

Replicate PCR reactions were then combined and purified with Montage™ multiscreen-$PCR_{\mu 96}$ plates (Millipore, Billerica, MA) and a vacuum manifold (Qiagen, Valencia, CA). PCR products were applied to the membrane-filter wells of the plate under a vacuum of –20 inches Hg (-500 mbar) for 10 min. After the liquid had drained from the wells, 50 uL of sterile Milli-Q water was added to each well to wash the filter (water was used for washing as other buffers may affect microarray slide chemistry). Washing was repeated after the liquid from the first wash had drained. The vacuum seal was then broken and the bottom of the plate was blotted on paper towel. To elute the bound PCR products, 50 uL of sterile Milli-Q water was applied to each well (not under vacuum) and the plate was shaken at moderate speed for 10 min on a platform shaker.

Subsequently, 2 uL aliquots of the eluted samples were assessed by gel electrophoresis for the presence of specific products (single bands), and to determine approximate PCR product concentration with the GeneRuler 1kb ladder (Fermentas, Ontario, Canada). Eight µL of sterile Milli-Q water and 3 µL of loading dye was added to each PCR product and pipetted into wells of a 1% agarose gel (1 g agarose, 100 mL 1X TBE) and run in 1X TBE buffer (Appendix 2) at 100 V. Gels were post-stained and images captured as described in section 3.3.1.1. Subsequently, any samples that did not show single bands, or were of unsatisfactory concentration, were re-amplified.

Negative controls to be included on the microarray included a blank dimethylsulphoxide (DMSO) buffer spot, digested pGEM®-T Easy plasmid, and the PCR primer used for amplification of cDNA inserts. For the PCR primer, 2000 ng (2.8 uL of the 100 mM stock) was used and, because of its already pure form, did not require any purification before printing. For the pGEM®-T Easy plasmid controls, 2000 ng of uncut plasmid was separately digested with the restriction enzymes *AluI* and *HaeIII* (Fermentas, Ontario, Canada), in order

to cut the plasmid into fragments of size suitable for printing. *AluI* and *HaeIII* are both 4 bp cutters, and the digests consisted of 2000 ng plasmid made up to 50 uL with sterile Milli-Q water, 5 uL of reaction buffer, and 3 uL of restriction enzyme. Digestions were allowed to proceed at 37°C for 3 h. Aliquots of digested product were checked by gel electrophoresis for successful digestion, according to the method described earlier in this section. Finally, successfully digested products were purified using the Qiaquick PCR purification kit (Qiagen, Valencia, CA) according to manufacturer's guidelines.

All satisfactorily amplified cDNA inserts (probes) and negative controls were transferred to a V-bottom polypropylene 384-well plate (Corning Incorporated Life Sciences, Acton, MA) in microarray configuration. The samples were then allowed to dry down overnight in a laminar flow cabinet, before being resuspended in 8 µL 50% (v/v) DMSO:water at 250 ng/µL. Re-suspension was performed by adding 5 uL of autoclave- and filter-sterilised water (to eliminate any dust that can interfere with microarray printing) to each sample, mixing by pipetting, sealing the plate, and leaving overnight at 4ºC on a platform shaker (250 rpm). 100% DMSO was then added to a final concentration of 50%, the plate was re-sealed and wrapped in moist paper towel to prevent evaporation, and stored at -20ºC.

3.3.1.3 Printing of microarrays

Microarray grids were printed onto GAPS II amino-silanized slides (Corning Incorporated Life Sciences, Acton, MA) using a Virtek Chipwiter (Virtek Vision International Inc.) with one pin. Printing was performed at the AGRF (Melbourne, Australia). For each sub-grid, microarray probes and negative controls were deposited in duplicate with a volume of approximately 6 nL and diameter of 200 µm. Three sub-grids were printed per slide (Figure 3.8). After printing, slides were treated according to the guidelines for the GAPS II coated slides (Corning Incorporated Life Sciences, Acton, MA), which involved steaming of the

array surface by holding the array side down over a beaker of boiling sterile water for 5 s (until condensation was observed across the slide) and snap-drying for 5 s at 100ºC on a heating block (printed side up). This action re-hydrated the probes to ensure the even distribution of DNA within the spots. The spotted DNA was then immobilised by UV cross-linking at 70 mJ and baking at 80ºC for 4 h. Finally, printed slides were stored in a dust-free desiccated environment for no longer than one month until use.

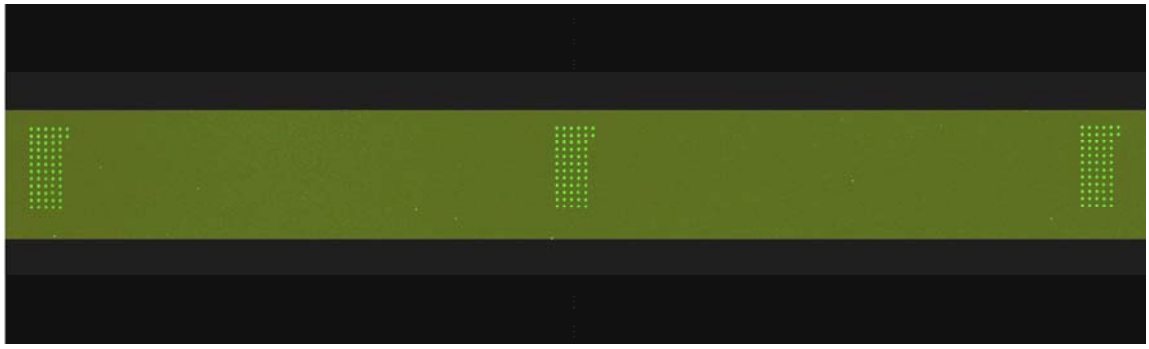

Figure 3.8 A non-hybridised microarray slide showing the three sub-grids printed per slide.

3.3.1.4 Microarray target preparation and hybridisation

Total RNA of 50 µg from each post-inoculation tissue sample and corresponding uninoculated control (refer to section 3.3.1.1) was reverse transcribed using Superscript II reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA) and oligo(dT) 23mer (Invitrogen Life Technologies, Carlsbad, CA). Amino-allyl dUTP (Sigma-Aldrich, St Louis, MO) was incorporated during the reverse transcription process. Briefly, the RNA and 5 µg of oligo(dT) primer were denatured at 70ºC for 10 min and cooled on ice before adding first strand buffer to a final concentration of 1X, aa-dUTP/dNTPs mix (final concentrations of 0.5 mM dATP, 0.5 mM dGTP, 0.5 mM dCTP, 0.2 mM dTTP, 0.3 mM aa-dUTP), DTT to a final concentration of 10 mM, and 150 units Superscript II in a total reaction volume of 30 µL.

Reverse transcription was carried out at 42ºC for 2 h before hydrolysis of RNA template with NaOH for 15 min at 65ºC and neutralisation with HEPES (pH 7.0). The cDNA was purified and post-labelled using the Qiaquick PCR purification kit (Qiagen, Valencia, CA) and Cy3/Cy5 mono-NHS esters (Amersham Pharmacia, Buckinghamshire, UK) resuspended in 0.1 M Na_2CO_3 (pH 9.0). cDNA samples were applied to QIAquick columns and washed/dried according to manufacturer's instructions, before adding the appropriate resuspended CyDye to the column membrane and incubating for 1 h at room temperature in the dark. Following incubation, labelled samples were eluted, appropriate Cy3 and Cy5 targets were combined (to represent a post-inoculated sample and uninoculated control from the same genotype and time-point), and purification was repeated.

Slides were pre-hybridised by blocking in 5X SSC, 0.1% SDS, 25% Formamide, 1% BSA for 45 min at 42ºC, rinsed in distilled water and dried with an air gun. Purified combined targets were resuspended in 2X hybridisation buffer (5X SSC, 0.2% SDS, 50% formamide, 25 µg Cot1 DNA (Invitrogen Life Technologies, Carlsbad, CA), 0.4 mg polyA (Sigma-Aldrich, St Louis, MO), 0.5 mg salmon sperm DNA (Sigma-Aldrich, St Louis, MO), made up to 60 µL with sterile water) and applied to the slide (covering three sub-grids) after denaturation at 100ºC for 2 min. The entire slide was covered by a 60 x 25 mm Lifter slip (Grale Scientific, Australia) and incubated in a 42ºC water bath for 16-20 h in a waterproof and humidified hybridisation chamber (Corning Incorporated Life Sciences, Acton, MA) in the dark. Each hybridisation was performed with six technical replications (three sub-grids) and two biological replicates were performed for each hybridisation, incorporating dye-swapping (i.e. reciprocal labelling of Cy3 and Cy5) to eliminate any dye bias.

3.3.1.5 Scanning and data analysis

After hybridisation, slides were washed for 5 min in each of 1X SSC/0.2% SDS and 0.1X SSC/0.2% SDS, and twice for 2 min in 0.1X SSC. Washed slides were rinsed in distilled water and immediately dried with an air gun. Slides were scanned at 532 nm (Cy3 green laser) and 660 nm (Cy5 red laser) using an Affymetrix® 428™ (Santa Clara, CA) array scanner (Figure 3.9), and captured with the Affymetrix® Jaguar™ software (v. 2.0, Santa Clara, CA). Within the Jaguar™ software slides were firstly preview scanned (50 µm resolution) to locate the sub-grids on the slide surface. Each sub-grid was then scanned separately at 10 µm resolution with a line average of three (three repeat scans per image line) for both the Cy3 and Cy5 channels. The gain setting (controlling the signal strength) was firstly set at automatic for both channels, then at 66 db for both channels after optimisation. Jaguar™ generated a separate image for each channel of each scan, which were subsequently saved as TIFF files.

For image analysis, the saved images from Jaguar™ were opened using Imagene™ v. 5.5 (BioDiscovery, Marina Del Rey, CA). For each sub-grid, both the Cy3 and Cy5 images were opened together and overlaid to produce a composite image. Spot diameter (pixels) within the image was determined with the 'ruler' tool before generating a grid defined by the number of columns, rows, and spot diameter (14.0 pixels). The grid was then positioned over the sub-grid by the 'automatically place grid' tool (local flexibility set to 5.0 pixels) to ensure optimal spot recognition. Some grid spots were then manually adjusted by visual inspection of their alignment with array spots. The corresponding gene ID file, generated with Microsoft Excel (Microsoft, Redmond, WA), was then loaded to assign a particular identification to each spot within the array. Spots were individually quantified using the fixed circle method; sample values were measured as the mean of pixels within the spot circle and the local background in a five-pixel diameter ring that began five pixels outside the spot circle.

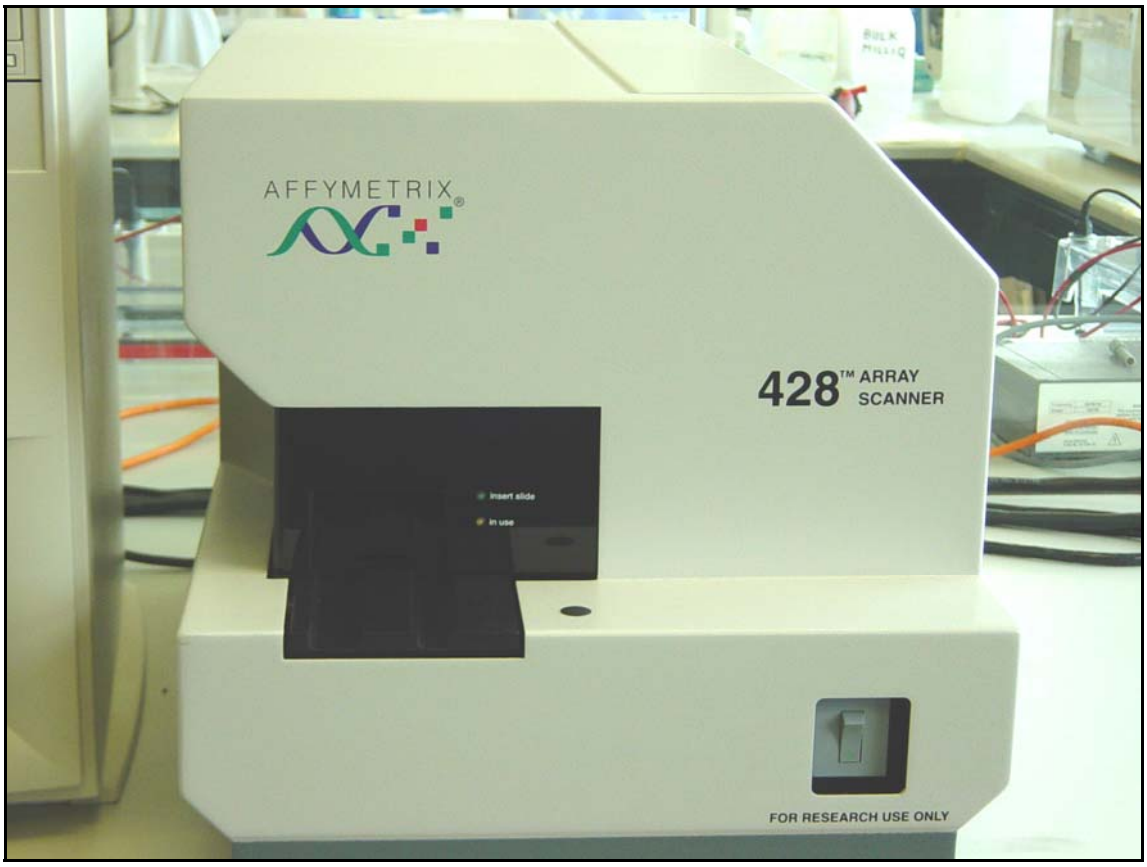

Figure 3.9 The Affymetrix® 428™ (Santa Clara, CA) array scanner at RMIT University used for microarray scanning.

During quantification, auto segmentation was performed, which partitioned the image into regions of specified meaning, namely spot signal versus background. This view showed the user what pixels were valued as signal, background and ignored in the quantification process. Once segmentation was complete, suspicious spots were identified and flagged by various types of automated and manual flagging. Under the 'quality flags' tool, options selected for automatic flagging included;

1. Empty spots: Low-expressed or missing spots were flagged based on the sensitivity threshold $R<2$, where R = (signal mean-background mean)/background standard deviation. The R threshold was adjusted until all negative controls were flagged as 'empty'.

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

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

Automatic multichannel flagging was set to flag a spot in both channels if it was 'poor' in one channel, 'empty' in both channels, or 'negative' in both channels. Spots with mean signal intensity less than two times the local background were manually flagged. Each spot was then given a code under the flagging column of the quantification data according to Table 3.5. The quantification data was then displayed in a spreadsheet, containing numerous measurement values for each spot, including mean signal intensity values and flagging codes. This data was then saved as a common tab delimited text file before post-image analysis.

Table 3.5 Description of the possible codes given to each quantified spot based on manual or automatic flagging.

Code	Flag	Type
0	No flag	NA
1	No reason	Manual
2	Empty spot	Automatic
3	Poor spot	Automatic
4	Negative spot	Automatic
5	Empty spot	Manual
6	Poor spot	Manual
7	Negative spot	Manual

Quantification data was imported into Genesight™ 3 (BioDiscovery, Marina Del Rey, CA) for post-image analysis. The dataset builder was used to load image files into experiment groups so that replicate data could be combined. For each genotype and time-point, the dataset was organised into ratio data for Cy3 and Cy5. The data preparation tool was then used to perform a specific ordered series of transformations;

1. Local background correction: Background intensity of each spot was subtracted from the signal intensity. This was the most accurate form of background correction as it allowed for variations of background intensity over the slide area.

2. Omit flagged spots: Flagged spots from Imagene™ v. 5.5 (BioDiscovery, Marina Del Rey, CA) were filtered out of the dataset, ensuring only high quality spots remained.

3. Ratio: A ratio between treatment and control mean signal intensities was created.

4. Normalisation: The normalisation controls were selected for normalisation of channel bias as these genes were assumed to be evenly expressed in both control and treated RNA samples. Normalisation was performed by dividing the ratio values of all genes by the mean ratio of the normalisation controls.

5. Combine replicates: Data for replicate spots was combined by taking the average of the replicated spots to produce a single value with a coefficient of variance (cv). Substituting a set of values with a single value caused a loss of information, but to alleviate this a cv was also calculated.

After performing data transformations, the data for each array feature was reported as the expression ratio of treatment/control, where a gene up-regulated by a factor of two in a treated sample had a value of 2.0 and a gene down-regulated by a factor of 2 had a value of 0.5. Most studies use this 2-fold increase or decrease as a cut-off for up and down regulation (Maguire *et al.*, 2002; Scheideler *et al.*, 2002; Lopez *et al.*, 2005). However, to determine the significant cut-offs for up- or down-regulation in this study, a separate hybridisation was performed using identical total RNA for both Cy3 and Cy5 labelling (refer to section 1.5.3). This self-self hybridisation allowed the inherent noise and sensitivity of the microarray system to be determined, and was performed by three separate self-self hybridisations. Subsequently, ESTs showing up- or down-regulation at one or more time-points in ICC3996 or Lasseter were subjected to time-course analysis. Additionally, the expression ratios of all ESTs in all time-points were used for hierarchical cluster analysis, performed with SPSS® v. 13.0 (SPSS Inc., Chicago, IL) using average distance linkage between groups and Euclidean metrics. Hierarchical clustering was selected based on the relatively small and simple nature of the dataset (refer to section 1.5.3).

3.3.2 Results

3.3.2.1 Microarray construction and hybridisation

The transcript level for each cDNA was calculated firstly as the mean intensity of the duplicated spots, then the mean intensity of the technical replications, and finally the mean intensity of the two biological replicates. Figure 3.10 shows an example of a scan for a hybridised slide used for signal quantification, whilst Figure 3.11 shows an example of a ratio scatter plot obtained after data transformation for determining up- and down-regulation. The expression ratio data for all 20 defence-related ESTs at each time-point for both ICC3996 and Lasseter can be found in Appendix 3.

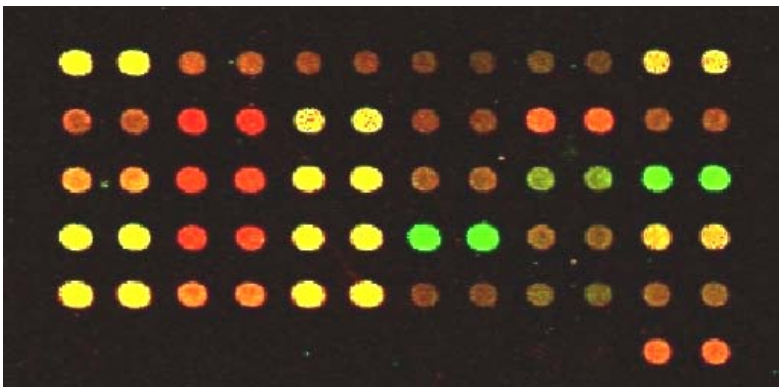

Figure 3.10 Example of a scan for a hybridised sub-grid opened and overlaid in Imagene™ v. 5.5 (BioDiscovery, Marina Del Rey, CA), where green spots indicate an abundance of Cy3-labelled target, red spots indicate an abundance of Cy5-labelled target, and yellow spots indicate equal abundance of Cy3 and Cy5-labelled targets.

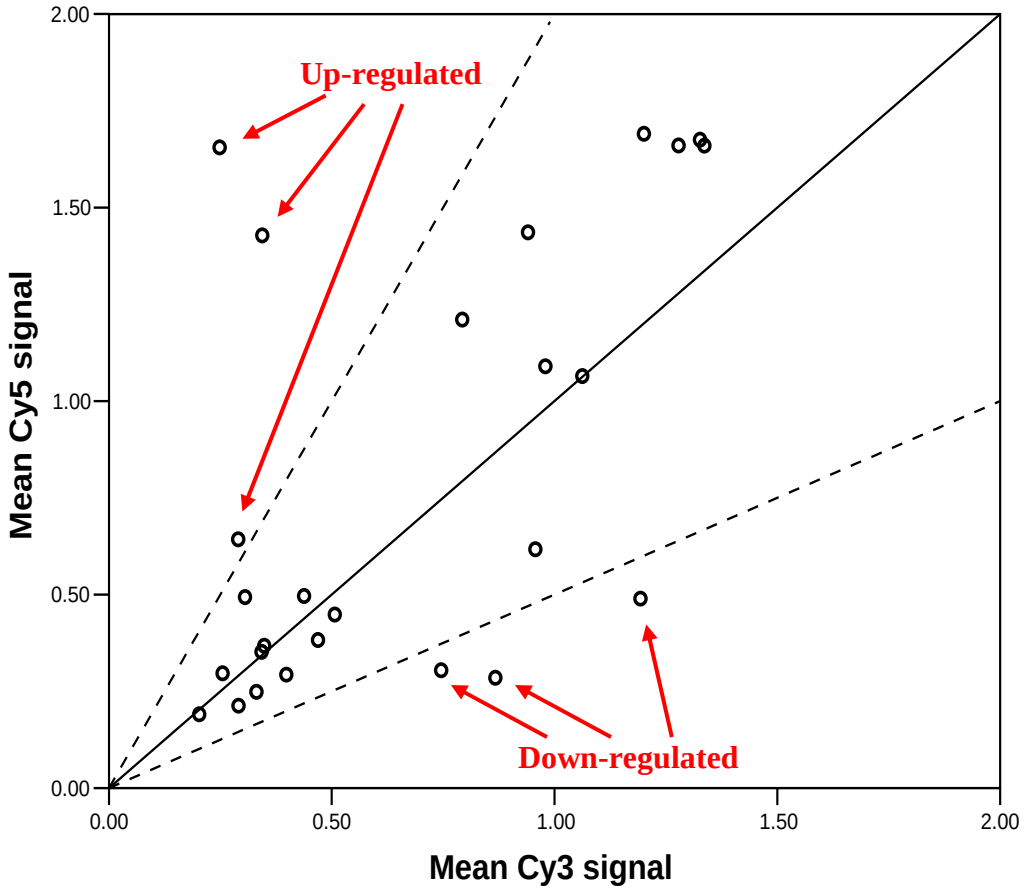

Figure 3.11 Example of a scatter plot of mean signal intensity (Cy3 v. Cy5) from a Lasseter 24 hpi hybridisation. Broken lines show the two-fold difference range from equal ratio (solid line), and up- and down-regulated spots are indicated.

The result of the separate replicated hybridisation to determine the significant cut-offs for up- or down-regulation yielded a scatter plot with all spots lying within a two-fold difference range (Figure 3.12), therefore, cDNAs were regarded as differentially expressed where they showed a greater than two-fold increase or decrease compared to control samples. These cut-offs translate into up-regulated cDNAs having a ratio ≥2.0, and down-regulated cDNAs ≤0.5. Additionally, the distribution of the ratio data for the five normalisation controls in every hybridisation for both ICC3996 and Lasseter also resulted in a scatter plot where all spots lay within a two-fold difference range (Figure 3.13).

3.3.2.2 Time-course analysis

Of the 20 specific defence-related ESTs included in the microarray, 10 exhibited differential expression in at least one time-point of either ICC3996 or Lasseter compared to uninoculated control samples. The remaining 10 ESTs failed to show differential expression over the sampled time-points. Figure 3.14, representing the number of up- or down-regulated ESTs in both ICC3996 and Lasseter at each time-point, shows that differential expression peaked 24 hpi, and the majority of ESTs had returned to normal regulation by 96 hpi. Additionally, Figure 3.14 shows that the main response to *A. rabiei* inoculation amongst the differentially expressed ESTs was up-regulation (19 instances) in favour of down-regulation (9 instances).

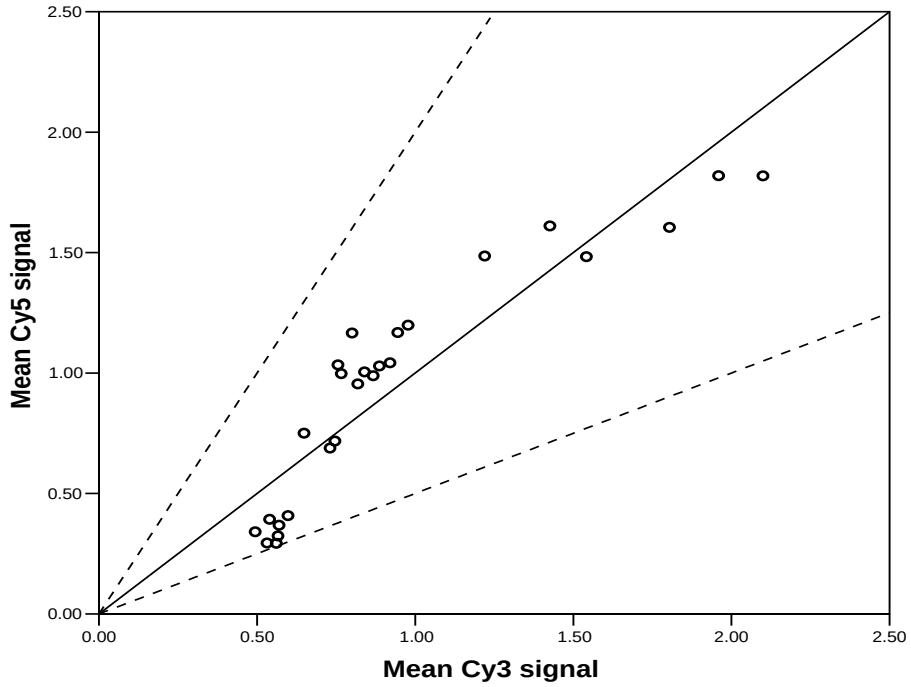

Figure 3.12 Scatter plot of mean signal intensity (Cy3 v. Cy5) from the self-self hybridisations. Broken lines show the two-fold difference range from equal ratio (solid line).

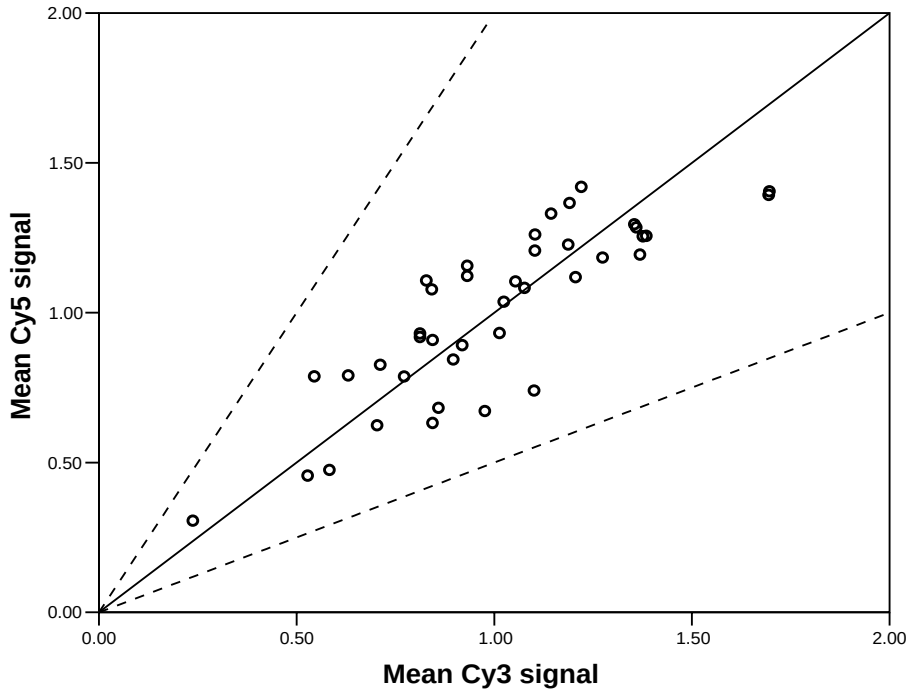

Figure 3.13 Scatter plot of mean signal intensity (Cy3 v. Cy5) for the five normalisation controls over every hybridisation for ICC3996 and Lasseter. Broken lines show the two-fold difference range from equal ratio (solid line).

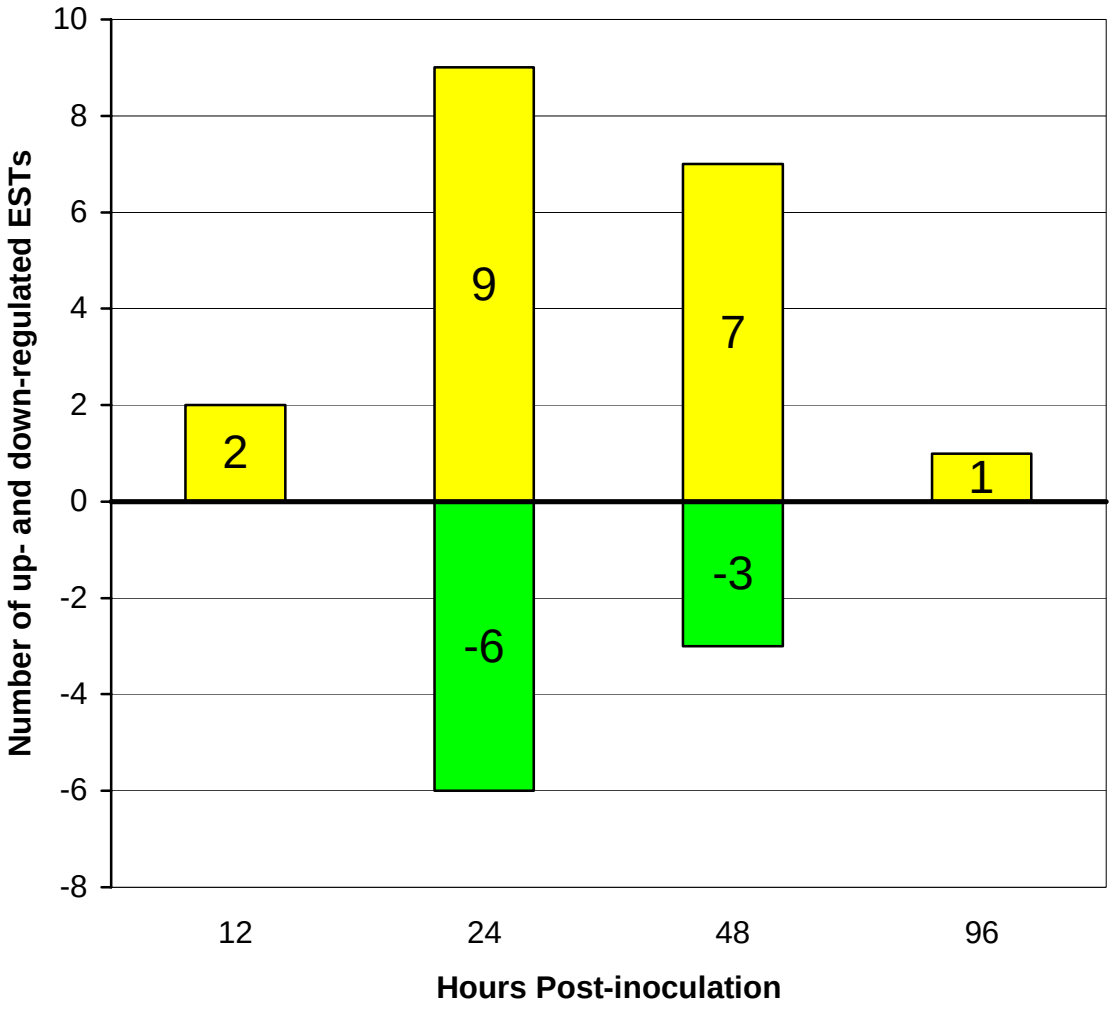

Figure 3.14 Distribution of the number of differentially expressed ESTs in both ICC3996 and Lasseter over the time-course, where up-regulated ESTs are shaded in yellow and down-regulated in green.

The 10 differentially expressed cDNAs were subjected to time-course analysis to independently compare expression levels in ICC3996 and Lasseter (Figures 3.15 – 3.24). Of the 10 ESTs, seven were up-regulated (DEF09, DEF10, DEF11, DEF16, DEF18, DEF19, and DEF20), whilst three were down-regulated (DEF08, DEF13, and DEF17). Importantly, the three down-regulated ESTs showed the same pattern of reduced expression in both ICC3996 and Lasseter. However, only two of the seven up-regulated ESTs showed similar differential expression in both ICC3996 and Lasseter (DEF10 and DEF20). DEF09 displayed up-regulation in Lasseter and no change in ICC3996, DEF16 showed a significantly greater up-regulation in Lasseter than the up-regulation observed in ICC3996, whilst DEF11, DEF18, and DEF19 showed up-regulation in ICC3996 and no significant change in Lasseter. Figures 3.15 – 3.24 also show that every differentially expressed EST achieved up- or down-regulation by 24 hpi at the latest, and all but DEF16 returned to normal regulation by 96 hpi.

3.3.2.3 Hierarchical clustering

To statistically analyse the gene expression dataset and divide it into groups of similar observations, agglomerative hierarchical clustering was performed. To calculate dissimilarities between observations, average linkage between groups and Euclidean metrics were used, and the higher order hierarchical branching identified five different clusters (Figure 3.25). Cluster I contains the 10 ESTs whose expression did not significantly change over the time-course. Cluster II includes three ESTs (DEF11, DEF18, and DEF19) temporarily up-regulated in ICC3996 only, before returning to baseline expression at 96 hpi. Clusters III and IV were represented by ESTs that showed down-regulation (DEF08, DEF13, and DEF17), and up-regulation (DEF10 and DEF20) respectively in both ICC3996 and Lasseter, before both clusters returned to normal regulation at 96 hpi. Finally, cluster V contained two ESTs significantly up-regulated in Lasseter for all or most of the time-course, and either brief up-regulation (DEF16) or unchanged regulation (DEF09) in ICC3996.

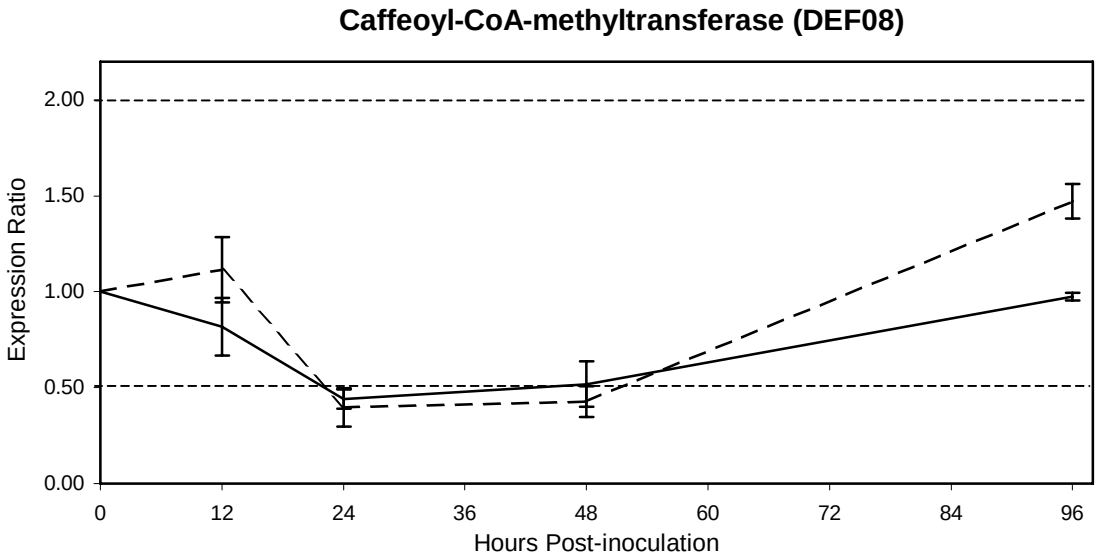

Figure 3.15 Time-course plot of the Caffeoyl-CoA-methyltransferase EST showing mean expression ratios (treatment/control) in ICC3996 (solid line) and Lasseter (broken line). Standard error bars are included for each measurement. Dashed lines at 2.00 and 0.50 expression ratio represent up- and down-regulation respectively in relation to control samples.

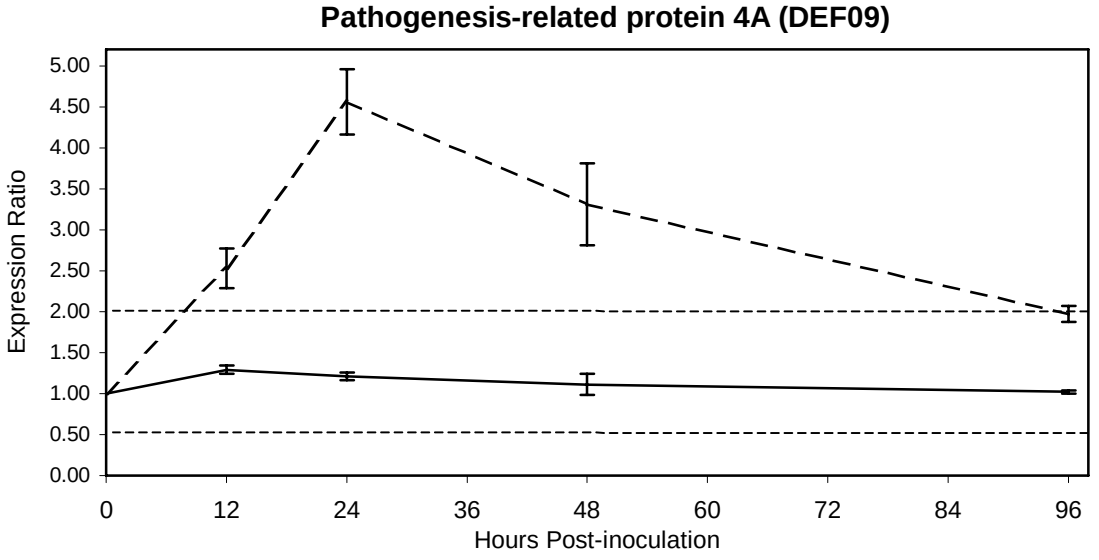

Figure 3.16 Time-course plot of the Pathogenesis-related protein 4A EST showing mean expression ratios (treatment/control) in ICC3996 (solid line) and Lasseter (broken line). Standard error bars are included for each measurement. Dashed lines at 2.00 and 0.50 expression ratio represent up- and down-regulation respectively in relation to control samples.

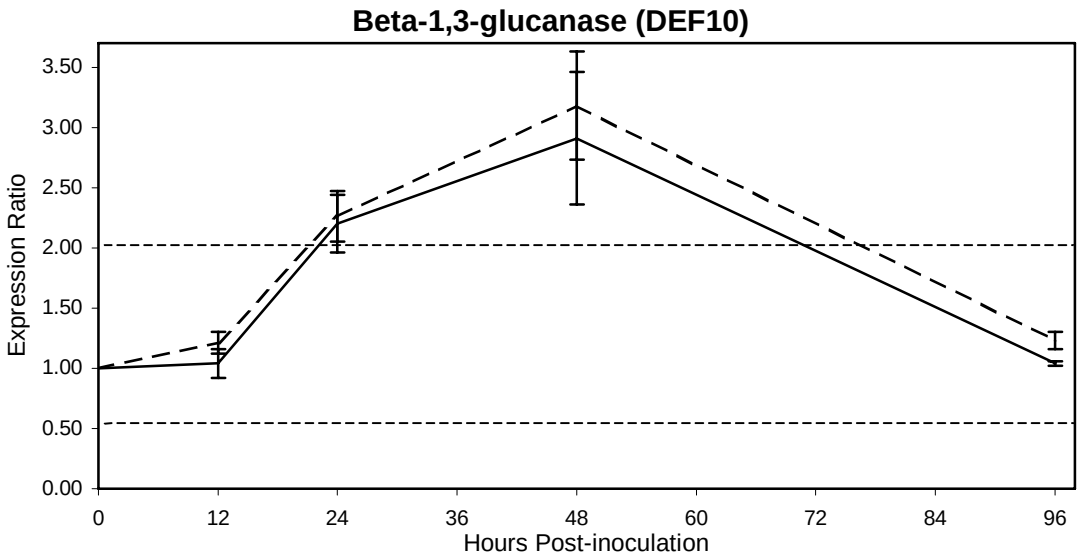

Figure 3.17 Time-course plot of the β-1,3-glucanase EST showing mean expression ratios (treatment/control) in ICC3996 (solid line) and Lasseter (broken line). Standard error bars are included for each measurement. Dashed lines at 2.00 and 0.50 expression ratio represent up- and down-regulation respectively in relation to control samples.

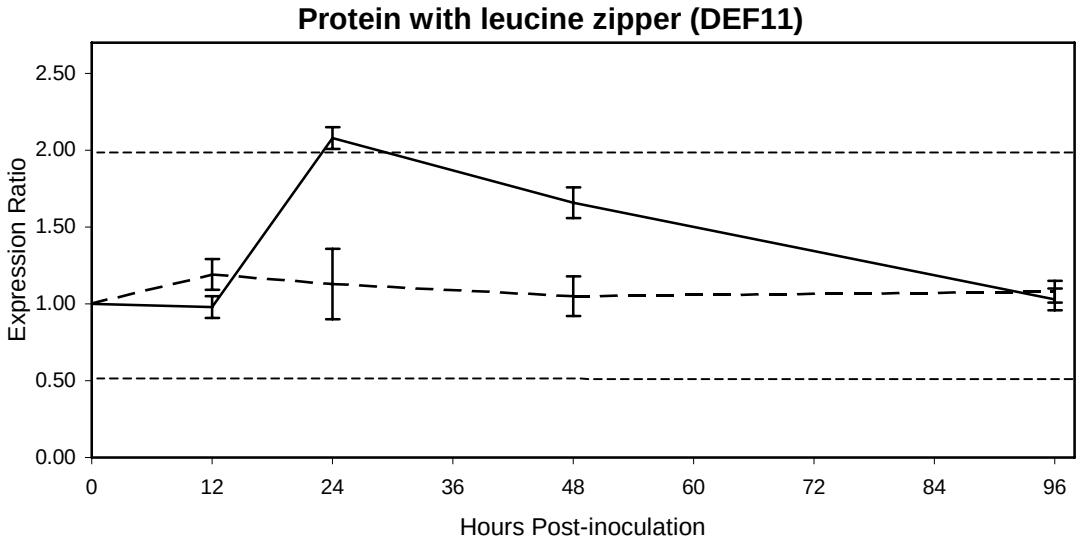

Figure 3.18 Time-course plot of the Protein with leucine-zipper EST showing mean expression ratios (treatment/control) in ICC3996 (solid line) and Lasseter (broken line). Standard error bars are included for each measurement. Dashed lines at 2.00 and 0.50 expression ratio represent up- and down-regulation respectively in relation to control samples.

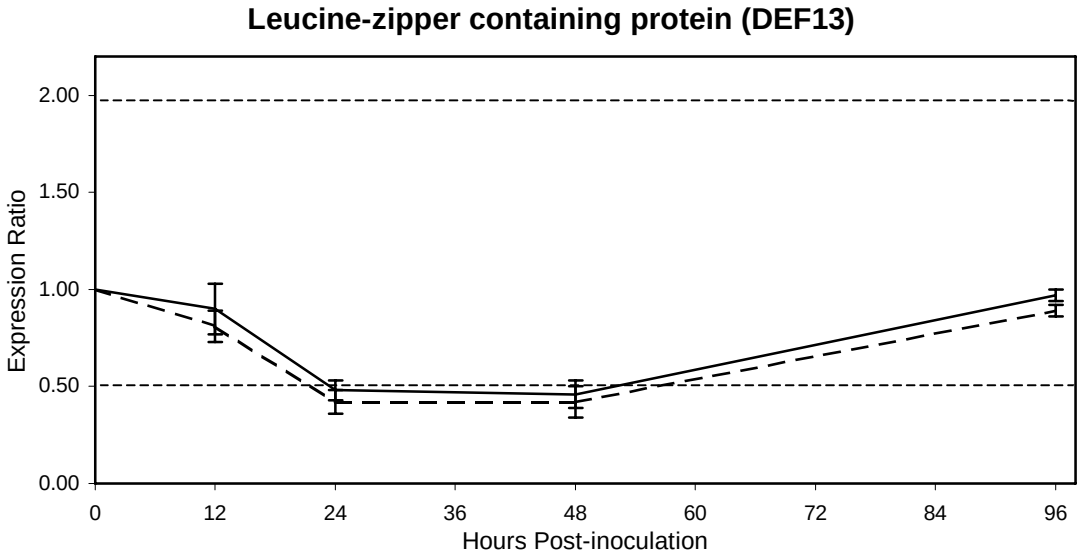

Figure 3.19 Time-course plot of the Leucine-zipper containing protein EST showing mean expression ratios (treatment/control) in ICC3996 (solid line) and Lasseter (broken line). Standard error bars are included for each measurement. Dashed lines at 2.00 and 0.50 expression ratio represent up- and down-regulation respectively in relation to control samples.

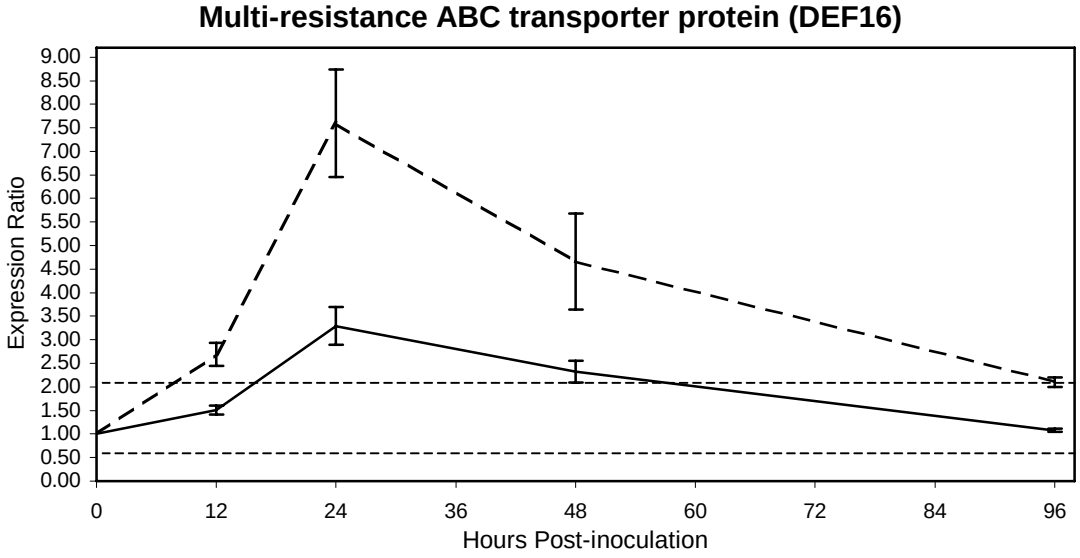

Figure 3.20 Time-course plot of the Multi-resistance ABC transporter protein EST showing mean expression ratios (treatment/control) in ICC3996 (solid line) and Lasseter (broken line). Standard error bars are included for each measurement. Dashed lines at 2.00 and 0.50 expression ratio represent up- and down-regulation respectively in relation to control samples.

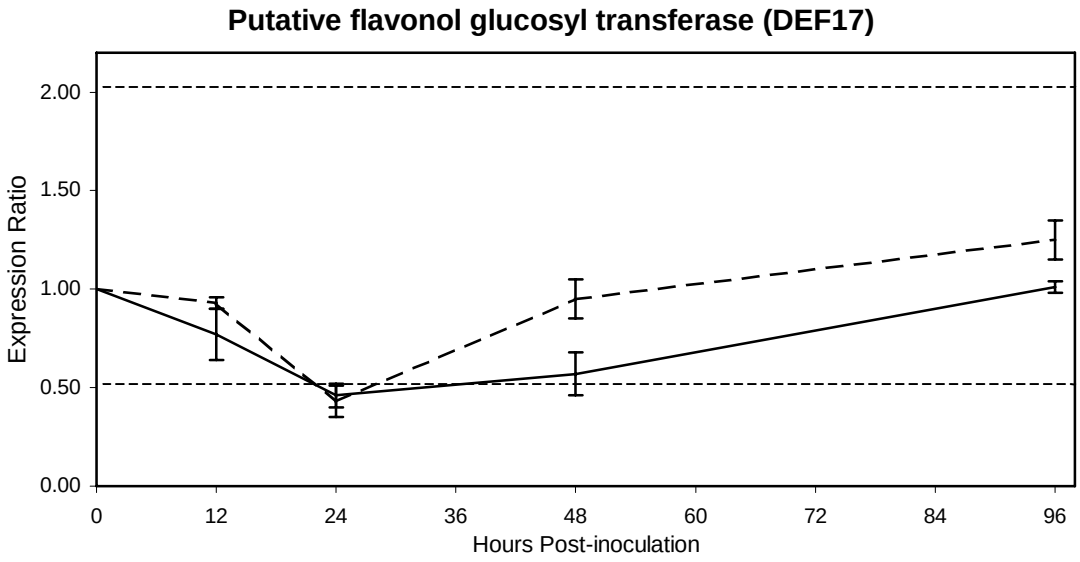

Figure 3.21 Time-course plot of the Putative flavonol glucosyl transferase EST showing mean expression ratios (treatment/control) in ICC3996 (solid line) and Lasseter (broken line). Standard error bars are included for each measurement. Dashed lines at 2.00 and 0.50 expression ratio represent up- and down-regulation respectively in relation to control samples.

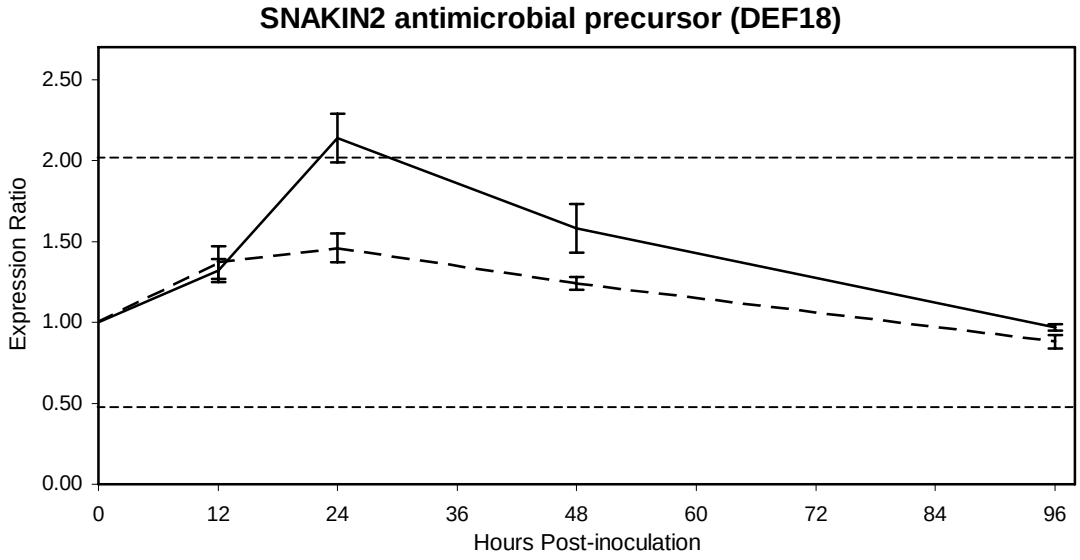

Figure 3.22 Time-course plot of the SNAKIN2 antimicrobial peptide EST showing mean expression ratios (treatment/control) in ICC3996 (solid line) and Lasseter (broken line). Standard error bars are included for each measurement. Dashed lines at 2.00 and 0.50 expression ratio represent up- and down-regulation respectively in relation to control samples.

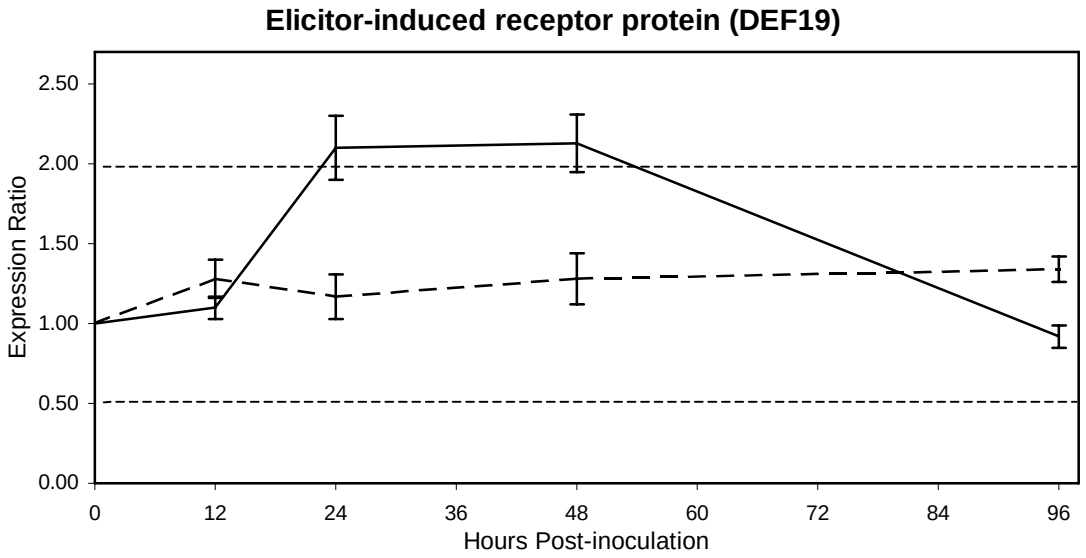

Figure 3.23 Time-course plot of the Elicitor-induced receptor protein EST showing mean expression ratios (treatment/control) in ICC3996 (solid line) and Lasseter (broken line). Standard error bars are included for each measurement. Dashed lines at 2.00 and 0.50 expression ratio represent up- and down-regulation respectively in relation to control samples.

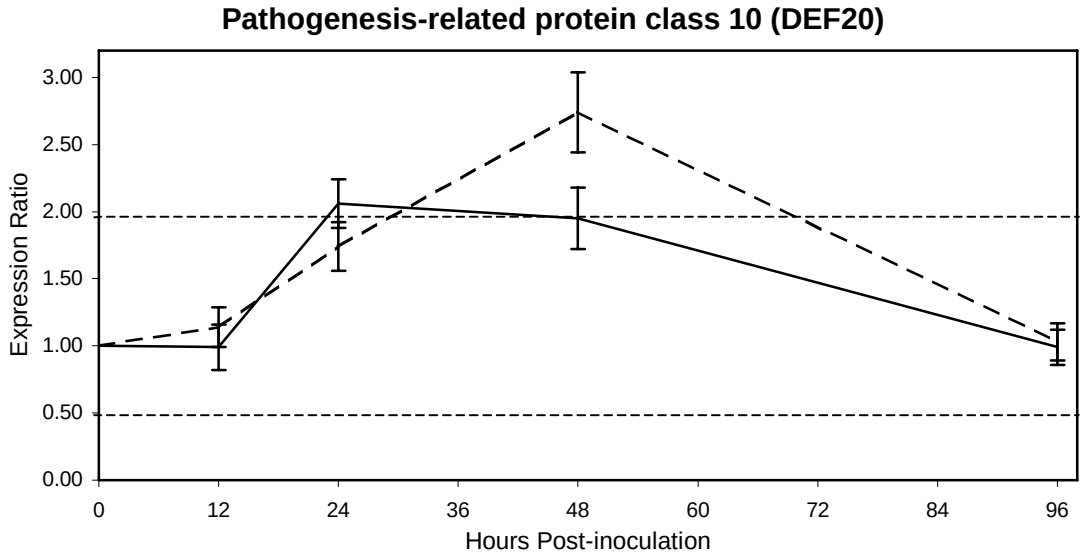

Figure 3.24 Time-course plot of the Pathogenesis-related protein class 10 EST showing mean expression ratios (treatment/control) in ICC3996 (solid line) and Lasseter (broken line). Standard error bars are included for each measurement. Dashed lines at 2.00 and 0.50 expression ratio represent up- and down-regulation respectively in relation to control samples.

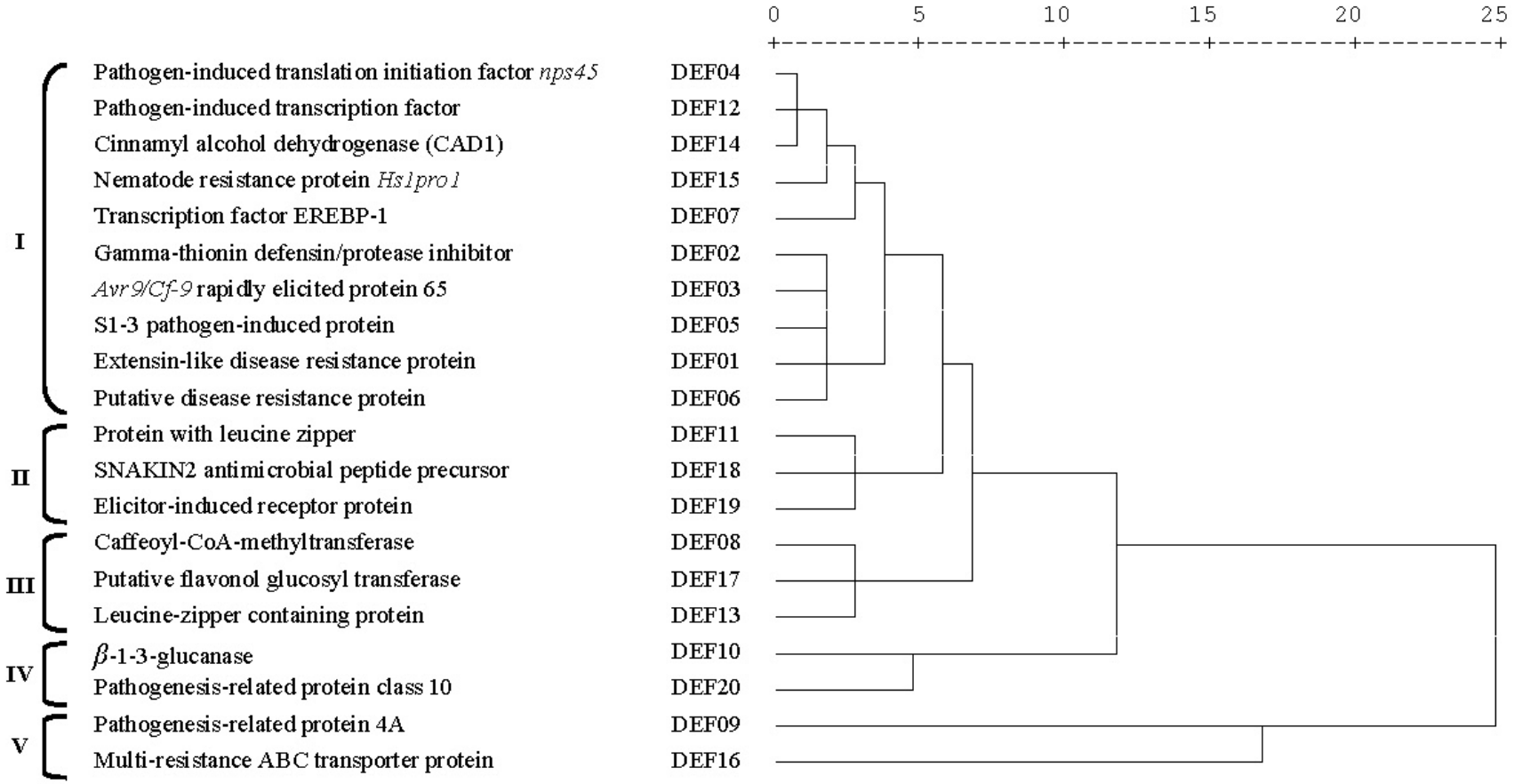

Figure 3.25 Dissimilarity dendrogram for the expression dataset of the defence-related ESTs in ICC3996 and Lasseter, showing hierarchical clustering into five groups of similar observations. The steps of the dendrogram show the combined clusters and the values of the distance coefficients at each step, where the values have been rescaled to numbers between 0 and 25, preserving the ratio of the distances between steps.

3.3.3 Discussion

The aim of this study was to examine the changes that occur in the transcript level of 20 previously identified *A. rabiei* defence-related unigenes (Chapter 2). The ascochyta blight resistant chickpea, ICC3996, and susceptible cultivar, Lasseter, were inoculated with *A. rabiei* spores before extracting total RNA over a time-course. Microarray technology was used to assess the expression of the 20 defence-related ESTs in each RNA sample when compared to uninoculated control RNA samples. The use of a time-course also enabled the putative detection of gene induction over the sampled period.

The five normalisation controls included in the present study (5.8S/18S/26S rRNA, Rubisco, chloroplast 4.5S/5S/16S/23S mRNA, chlorophyll a/b, and ATP synthase) are all involved in general biochemical pathways and housekeeping activities, thus their expression levels after *A. rabiei* inoculation were not expected to alter. Importantly, the expression ratios of these ESTs over the times-series did not exceed a two-fold change in either direction (Figure 3.13), which provided validation of the sampling and hybridisation methods used. This observation also supports the up- and down-regulation cut-off values defined by the self-self hybridisation (Figure 3.12), giving increased significance to values that did show a greater than two-fold expression change. The blank buffer spots incorporated as negative controls were all automatically flagged by the scanning analysis software (Imagene™ v. 5.5, BioDiscovery, Marina Del Rey, CA), a result that confirmed the desired level of hybridisation stringency.

Considering that the ESTs employed in this study were functionally annotated as defence-related, it was assumed that the main observed response to *A. rabiei* challenge would be significant differential expression. However, 10 of the ESTs failed to display any sign of either increased or decreased expression, which may be explained by the limitation of the time-course used or by the absence of a specific role for those ESTs in the *A. rabiei* infection

response. Additionally, it is important to note that significantly different levels of basal expression between ICC3996 and Lasseter could not be determined by this study, as hybridisations were not performed between control samples of ICC3996 and Lasseter. Thus, some of the 10 non-differentially expressed ESTs may possibly possess high constitutive expression that allows them to be effective in the *A. rabiei* response.

Four of the non-differentially expressed ESTs; *Avr9/Cf-9* rapidly elicited protein 65 (DEF03), Pathogen-induced translation initiation factor *nps45* (DEF04), Transcription factor EREBP-1 (DEF07), and Pathogen-induced transcription factor (DEF12) are putatively involved in signal transduction/defence-activating pathways. Considering that signal transduction follows pathogen recognition as a very early stage in a plant-pathogen interaction, a possible explanation for the lack of observed expression changes for these ESTs may be that the time-course was unable to capture these changes. The earliest sampling in this study was 12 hpi, but in *A. thaliana* it has been shown that the earliest detectable changes in gene expression for an incompatible pathogen reaction are as early as 6 hpi (Tao *et al.*, 2003). Therefore, the use of earlier sampling points may have revealed differential expression for these ESTs.

The Extensin-like disease resistance protein (DEF01) and Cinnamyl alcohol dehydrogenase (CAD1) (DEF14) were two other non-differentially expressed ESTs that are involved in cell-wall resistance. Extensins are Hyp-rich cell-wall proteins implicated in pathogen recognition, whilst CAD1 is an enzyme of the phenylpropanoid pathway responsible for lignin biosynthesis (refer to section 2.4). Again, considering extensins are involved in pathogen recognition, earlier sampling may have captured differential expression. However, the unchanging CAD1 expression may be explained by another upstream enzyme of the lignin biosynthesis pathway, Caffeoyl-CoA-methyltransferase (DEF08). DEF08 was found to be down-regulated in both ICC3996 and Lasseter (Figure 3.15), indicating that lignin

biosynthesis was not being altered, and hence there existed no requirement for CAD1 up-regulation. However, a previous study implicated the involvement of lignin for *A. rabiei* resistance in chickpea (refer to section 1.3), where the higher level of lignin in resistant genotypes was thought to contribute to effective defence. However, the previous study found cytological evidence of lignin at seven dpi, indicating that the up-regulation of lignin biosynthesis may not occur during the time-course of the present study (12-96 hpi). However, the previous study also identified differing cell wall compositions between susceptible and resistant genotypes, which may be pre-formed (constitutive) cellular properties. Further, a separate study has shown that resistant chickpeas possess a thicker stem epidermis and hypodermis than susceptible ones, which is also constitutive (refer to section 1.3). Therefore, increases lignin levels may be a constitutive property of resistant genotypes, although lignin biosynthesis may also be up-regulated at a later time-point than included in this study.

The Nematode resistance protein *HsPro-1* (DEF15) and Gamma-thionen/defensin protease inhibitor (DEF02) also possessed unchanged expression over the time-course. This observation was not unexpected, considering that DEF15 has previously only been implicated in chickpea resistance to nematodes, and protease inhibitors are generally only involved in insect defence (Koiwa *et al.*, 1997). DEF15 and DEF02 were included in this study only on the possibility that they may represent broad-spectrum disease resistance proteins. The final two ESTs with unchanging expression were the S1-3 pathogen-induced protein (DEF05) and Putative disease resistance protein (DEF06), indicating that these putative proteins may not be involved in chickpea defence against *A. rabiei*.

The previous classification of the 20 ESTs as defence-related (Chapter 2) was supported by the observation that 10 (50%) showed up- or down-regulation in ICC3996 or Lasseter for at least one time-point. Furthermore, the distribution of the number of differentially expressed

ESTs (Figure 3.14) showed that there existed a tendency toward up-regulation rather than down-regulation. This demonstrates that the *A. rabiei* inoculation provoked a significant response that could be witnessed over a wide-range of ESTs involved in various defensive pathways.

Hierarchical clustering statistically confirmed that 10 of the 20 ESTs were not differentially expressed (Cluster I, Figure 3.25), whilst five were co-regulated with either increased (Cluster IV) or decreased (Cluster III) expression in both the *A. rabiei* resistant (ICC3996) and susceptible (Lasseter) chickpea. Such co-regulation may imply that ESTs of these clusters are not effective in *A. rabiei* defence, as they cannot be used to explain the phenotypic difference between ICC3996 and Lasseter. The co-regulated ESTs displaying up-regulation were PR proteins β-1,3-glucanase (DEF10) and Pathogenesis-related protein 10 (DEF20). Although β-1,3-glucanase has previously been found to accumulate in chickpea after *A. rabiei* infection (Hanselle and Barz, 2001), that study did not compare resistant and susceptible varieties, and the present results suggest that DEF10 and DEF20 possess limited effectiveness in *A. rabiei* resistance.

The down-regulated ESTs in ICC3996 and Lasseter were Caffeoyl-CoA-methyltransferase (DEF08), Putative flavonol glucosyl transferase (DEF17), and Leucine-zipper containing protein (DEF13). DEF17 is involved in the production of flavonol glycosides and anthocyanins, which have been implicated in phytoalexin production (Winkel-Shirley, 2002). Although phytoalexins have been implicated in *A. rabiei* defence (refer to section 1.3), the down-regulation of DEF17 provides no evidence for phytoalexin accumulation in this study. However, the presence of other phytoalexin-related genes not included in this study may have been induced. Both DEF08 and DEF17 are part of the phenylpropanoid pathway that, together with the earlier described CAD1 (DEF14), does not appear to be up-regulated in

response to *A. rabiei* infection in this study. The leucine-zipper protein (DEF13) is a bZIP transcription factor, and considering these proteins can have various regulatory roles ranging from pathogen defence to flower development (Jakoby *et al.*, 2002), DEF13 may not be involved in defence. In fact, DEF13 may have been down-regulated to allow for more efficient energy utilisation in the defence pathways, as is often observed for non-defensive proteins (Katagiri, 2004).

Cluster V (Figure 3.25) is of interest as it contains two ESTs that showed significantly higher expression in the susceptible Lasseter compared to ICC3996; Pathogenesis-related protein 4A (DEF09) and Multi-resistance ABC transporter protein (DEF16). DEF09 is a class four (chitinase) PR protein and DEF16 is a putative uncharacterised transporter protein with potential involvement in transport of antimicrobial proteins (refer to Chapter 2). The increased expression of these proteins in Lasseter implies a lack of effectiveness in *A. rabiei* resistance, considering that Lasseter is highly susceptible to ascochyta blight. Overall, of the four ESTs up-regulated in Lasseter, three were PR proteins (DEF09, DEF10, DEF20) and one was a putative antimicrobial protein transporter (DEF16).

The most important group of ESTs are members of Cluster II, as these ESTs were up-regulated in the resistant ICC3996 and showed no change in Lasseter. Subsequently, these ESTs may possess an effective role in *A. rabiei* resistance. The first member of this cluster is the Protein with leucine-zipper (DEF11), another bZIP transcription factor. These proteins, made up of a basic region that binds DNA and a leucine-zipper dimerization motif, have been studied in *A. thaliana* where they regulate a variety of plant processes (Jakoby *et al.*, 2002). There exists a group of the bZIP transcription factors that participate in pathogen defence, specifically by regulating the production of salicylic acid (SA) to induce the expression of PR proteins (Jakoby *et al.*, 2002). Studies of one *A. thaliana* protein, *NPR1*, which is essential

for regulating PR protein gene expression, have shown that *NPR1* interacts strongly with a bZIP transcription factor (Zhang *et al.*, 1999; Despres *et al.*, 2003). Thus, the up-regulation of DEF11 in ICC3996 may indicate that it is involved in activating effective defence mechanisms against *A. rabiei*, such as PR proteins.

The second member of Cluster II is a SNAKIN2 antimicrobial peptide precursor (DEF18) whose activity has been studied extensively in potato (Berrocal-Lobo *et al.*, 2002). SNAKIN2 peptides are basic globular antimicrobial peptides rich in *Cys* residues that form stabilising disulphide bridges, but the exact mechanism of their action remains unknown. However, it is known that SNAKIN2 is induced by fungal infection of potato tubers, and that a SNAKIN2 protein has been associated with potato leaves infected with *Phytophthora infestans* (Berrocal-Lobo *et al.*, 2002). The evidence to date suggests that SNAKIN2 is involved in pathogen defence, so the up-regulation of DEF18 in ICC3996 compared to Lasseter may indicate that DEF18 has some effect in *A. rabiei* resistance.

The final member of Cluster II is the Elicitor-induced receptor protein (DEF19). This protein was first isolated from *A. thaliana*, where it was putatively identified according to sequence structure alone (Sato *et al.*, 2000). DEF19 represents the only subsequent isolation of a protein matching the *A. thaliana* protein, thus information on the biochemical activity of DEF19 is scarce. However, the identification of DEF19 as an elicitor-induced receptor protein indicates that it may be membrane-bound, involved in signal transduction, and up-regulated by pathogen-secreted elicitor molecules. The up-regulation of DEF19 in ICC3996 does support a potential role for DEF19 in effective *A. rabiei* resistance, but further study on this protein is required to identify the mode of its action and confirm any potential involvement in the chickpea resistance mechanism to ascochyta blight.

A major observation for the differentially expressed ESTs was that they all achieved either up- or down-regulation by 12 or 24 hpi (Figure 3.14). This reflects a rapid growth and proliferation of *A. rabiei* within the host tissues, resulting in a significant host response at these time-points. Other post-inoculation gene expression studies have also reported this rapid change in gene expression, such as the soybean and *Phytophthora sojae* interaction, where expression changes peaked at 24 hpi (Moy *et al.*, 2004). In the present study, the expression changes also peaked at either 24 or 48 hpi, and the majority of the differentially expressed ESTs returned to baseline expression by 96 hpi. This may be a consequence of using probes sourced from 24-48 hpi tissue, or may indicate that, for the ESTs included in this study, the potential defence mechanism against *A. rabiei* is occurs within 48 hpi.

3.3.4 Conclusion

In summary, this study represented the first use of cDNA microarrays to study the chickpea resistance response to ascochyta blight. Expression profiles were generated for 20 defence-related unigenes, leading to the identification of potentially effective, and ineffective, unigenes in conferring *A. rabiei* resistance. The results indicate that significant differences exist between the response of the *A. rabiei* resistant (ICC3996) and susceptible (Lasseter) chickpea. In particular, ICC3996 expressed three defence-related ESTs not observed in Lasseter, which may form part of an effective ascochyta blight resistance response, and will be studied further. This study successfully enabled the optimisation of microarray hybridisation techniques to validate the use of larger-scale experiments. Considering that the plant response to pathogen challenge is associated with massive changes in gene expression (Katagiri, 2004), the next step will involve the generation of a large-scale cDNA microarray incorporating all chickpea unigenes from all functional categories (refer to Chapter 2). The sampling of a wide range of ESTs may aid in the identification of pathways of defence activation to enhance understanding of the overall mechanism of resistance.

Chapter 4

Large-scale microarray expression profiling of chickpea unigenes differentially regulated during a resistance response to *A. rabiei*.

4.1 Introduction

Plant resistance or susceptibility to disease is dictated by the genetic backgrounds of both pathogen and host. Pathogen recognition occurs via *R-Avr* gene interactions, where plant *R* proteins possess highly conserved motifs (refer to section 1.4.2.1) and pathogen *Avr* effectors lack structural similarity (Bonas and Lahaye, 2002). Interestingly, many *Avr* gene products are actually required for effective virulence on susceptible hosts that lack a corresponding *R* gene (Kjemtrup *et al.*, 2000). Of the common active defence responses described in Chapter 1 (section 1.4.1), transcription factors play an integral role in the signalling and control of these pathways (Singh *et al.*, 2002; Li *et al.*, 2006), which are also mediated by plant hormones, elevation of cytosolic calcium, and activation of protein kinases (Grant and Mansfield, 1999; Rivas and Thomas, 2005). The speed and coordination of pathogen perception, signal transduction and transcriptional activation is vital to successful plant resistance. At the genomic level, plant defence responses are complex and diverse, and every gene involved in the defence response, from recognition to signalling to direct involvement, forms part of a coordinated response network.

Several active defence responses in chickpea have been defined (refer to section 1.3) and, as reported in Chapter 1, *A. rabiei* resistant genotypes exist, including wild relatives, which may be bred with cultivated varieties to incorporate potential resistance genes (Collard *et al.*, 2001). Although chickpea breeding programs in major growing areas are focused on

producing *A. rabiei* tolerant varieties, the genes and pathways of gene activation controlling effective resistance remain unknown. Understanding the chickpea defence response at the transcript level may assist in developing resistant cultivars, but reports of the genetic basis for resistance vary according to the chickpea genotype and *A. rabiei* pathotype studied (refer to section 1.3.4). Therefore, quantitative methods for analysis of expression profiles may have the capacity to improve the overall understanding of the coordinated defence response at a molecular level (refer to section 1.5).

Chapter 2 reported the characterisation of a set of chickpea unigenes, and Chapter 3 reported a successful small-scale cDNA microarray study of the chickpea response to *A. rabiei* infection for 20 defence-related unigenes. This study involved the construction of a cDNA microarray representing the non-redundant set of chickpea unigenes, as well as putative defence-related ESTs from grasspea (*Lathyrus sativus* L.), and 41 Resistance Gene Analogue (RGA) sequences from lentil (*Lens culinaris* Med.). Although chickpea ESTs were shown to be substantially divergent from model legumes in Chapter 2, successful hybridisation of chickpea targets to the grasspea and lentil probes was expected considering their higher degree of relatedness within the Papilionoideae subfamily of the Fabaceae. Gene expression changes were investigated in four chickpea genotypes (corresponding to the four genotypes used for inoculation procedure optimisation in Chapter 3) over a time-course after inoculation with *A. rabiei* spores. Compared to Chapter 3, a different method of data normalisation was used in this study because of the inclusion of ESTs representing potential non defence-related functional categories that are unlikely to be differentially expressed. Therefore, a global normalisation was applied, which assumes that the majority of genes are not differentially expressed and that the entire population accurately represents the channel bias (Draghici, 2003). This study provided novel insights into the molecular mechanisms controlling chickpea defence.

4.2 Materials and methods

4.2.1 Plant material, inoculation and RNA extraction

Seeds of *C. arietinum* genotypes ICC3996 (IC), Lasseter (LA), and FLIP94-508C (FL), as well as *C. echinospermum* ILWC245 (IL) (refer to Table 3.1) were germinated and cultivated according to section 3.2.1. The experiment was designed so that seeds of each genotype were sown in 15 cm diameter pots (three seeds per pot) in sterile soil, with 24 replicate pots, of which 12 served as uninoculated controls. All plants were grown in a glasshouse (20 ± 4ºC) for 14 days (six- to eight-leaf stage) before inoculation with *A. rabiei*. Inoculation with the seven isolates of *A. rabiei* was also performed as described in section 3.2.1, using the optimal inoculum concentration of 1.0 x 10^5 spores mL^{-1}. Uninoculated controls were sprayed with sterile distilled water.

Entire stem and leaf tissue was extracted from six plants per time-point at each of 6, 12, 24, 48, and 72 hpi and immediately frozen in liquid nitrogen. Additionally, stem and leaf tissue was taken from six uninoculated plants per time-point of each genotype. Sampling times were selected based on the results of the small-scale microarray study (refer to Chapter 3), as well as the histopathology information on active defence response timing (refer to section 1.3). To confirm that *A. rabiei* infection had been effective, plants were checked for expected disease symptoms at 14 dpi. The entire inoculation experiment and tissue collection was repeated three times on newly cultivated plants, corresponding to three biological replicates. Within each biological replicate, total RNA was extracted from pooled stem/leaf tissue samples for each genotype (including control samples) as described in section 3.3.1, where pools corresponded to the four post-inoculation time-points. The quantity and quality of each RNA sample was also determined as in section 3.3.1. As reported in Chapter 3, LA was susceptible to *A. rabiei* with a score of 9.0, whilst IC was resistant with a score of 3.8, FL was moderately resistant with a score of 5.0, and IL was moderately resistant with a score of 4.4.

4.2.2 Microarray construction

A total of 715 ESTs, 41 RGA DNA sequences, and 12 controls were used in the construction of 768-feature microarrays according to minimum information about a microarray experiment guidelines (MIAME) (Brazma *et al.*, 2001). Of the 715 ESTs, 516 represented the unigenes characterised in Chapter 2. Where applicable, a single clone was selected to represent each contig. A further 43 chickpea (IC) cDNAs whose sequencing reactions had previously failed were also included in this microarray, as they may still represent important defence-related genes that could be re-sequenced if required. The remaining 156 ESTs represented potential defence-related ESTs from a grasspea (*Lathyrus sativus*) cDNA library constructed from the *Mycosphaerella pinodes* resistant genotype ATC80878 (Skiba *et al.*, 2005), provided by Dr B. Skiba (RMIT University, Victoria, Australia). Finally, the 41 RGA sequences were amplified from genomic DNA of lentil (*Lens culinaris*) genotypes ILL6002 (*A. lentis* susceptible) and ILL7537 (*A. lentis* resistant), provided by Mr B. Mustafa (University of Melbourne, Victoria, Australia). The 12 controls included negative, printing, and blank buffer controls. A complete description of the 768 microarray features can be found in Appendix 4.

Considering that the *L. sativus* cDNA clones were present in the same vector as the chickpea clones, the cDNA inserts (probes) of all ESTs were amplified to >2000 ng and purified according to section 3.3.1.2. The 41 RGA sequences were amplified to >2000 ng from lentil DNA using specific primers designed to target potential plant resistance gene motifs (B. Mustafa, pers. comm.). The RGA probes were then purified and prepared for microarray printing as for the EST probes, which involved visualisation on agarose gels to confirm the presence of single bands, drying down all samples in 384-well microarray plates, and re-suspension in 10 µL 50% (v/v) dimethylsulphoxide:water at 250 ng/µL (refer to section 3.3.1.2).

Unlike the microarray constructed in Chapter 3, the probes in this study were arrayed onto amino-silanized slides (Corning Incorporated Life Sciences, Acton, MA) using a BioRobotics® MicroGrid II Compact (Genomic Solutions, Ann Arbor, MI) with four *Micro*spot™ 2500 pins (Genomic Solutions, Ann Arbor, MI) at RMIT University (Victoria, Australia). Blank buffer spots, digested vector and PCR primer were prepared and incorporated as negative controls according to section 3.3.1.2. Additionally, a Cy5-10T labelled oligonucleotide (250 ng/μL) was incorporated as a printing control, which would always fluoresce under the Cy5 red laser (660 nm). For each sub-grid, probes and controls were deposited once with a volume of approximately 6 nL and diameter of 200 μm. Six sub-grids were printed per slide. After printing, probes were re-hydrated, immobilised onto the slide surface, and stored as described in section 3.3.1.3.

4.2.3 Microarray target preparation and hybridisation

Total RNA (50 μg) from each post-inoculation tissue sample and corresponding uninoculated control was reverse transcribed and fluorescent-labelled to generate microarray targets according to the method described in section 3.2.1.4. Slides were pre-hybridised, targets applied to the microarray, and hybridisation carried out as in section 3.2.1.4, but targets in this study were applied to cover six sub-grids. Each hybridisation was performed with six technical replicates (corresponding to the six sub-grids on each microarray slide) and three biological replicates, incorporating dye-swapping (i.e. reciprocal labelling of Cy3 and Cy5) to eliminate any dye bias (Figure 4.1). Overall, 360 images were analysed from 60 slides, resulting in 18 data points for each time-point of each genotype.

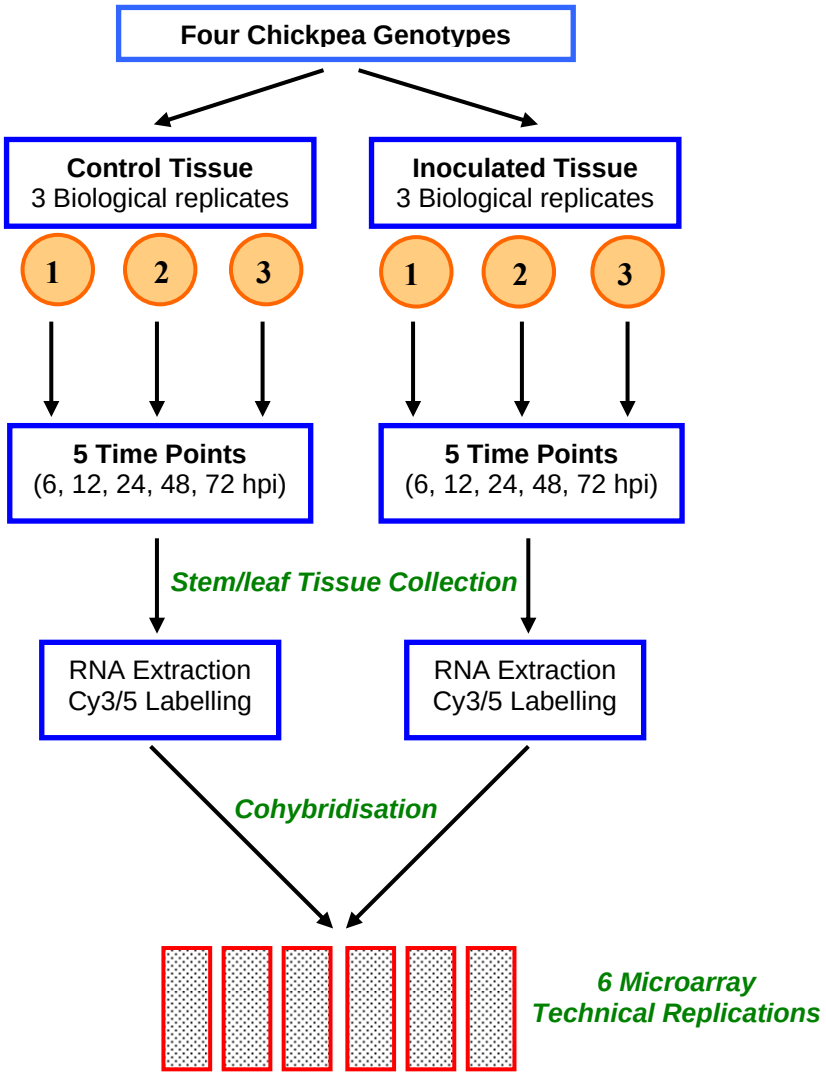

Figure 4.1 Experimental design and replication. Experiments were performed in a reference design where the samples for the uninoculated controls at each time-point acted as references against samples taken at each time-point for the treated samples.

4.2.4 Scanning and data analysis

Slides were scanned, captured, and image analysis performed as in section 3.3.1.5. However, due to different printing settings and spot diameters, sample values were measured as the mean of pixels within the spot circle and the local background in a three-pixel diameter ring that began three pixels outside the spot circle during fixed circle quantification. Additionally, the automatic flagging setting for 'empty spots' was altered to an *R* threshold of $R<4$, in order to flag all negative controls of this study as 'empty'. All other automatic and manual flagging remained as per the method in section 3.3.1.5. Data transformation of the quantified spot data using GeneSight™ 3 (BioDiscovery, Marina Del Rey, CA) was performed as described in section 3.3.1.5, except that a shifted log (base 2) of the ratio dataset was performed, meaning that the 2-fold differential expression levels were 1.0 for up-regulation and –1.0 for down-regulation, with 0 as the point of equal (unchanged) regulation. The log transformation was performed in this study as it made the distribution symmetrical and almost normal, and was very convenient for expressing fold changes (Draghici, 2003).

In the present study, a global normalisation was applied, considering the presence of many ESTs representing potential non defence-related functional categories that are unlikely to be differentially expressed (refer to section 4.1). As described in Chapter 1 (section 1.5.3), normalisation was essential to make arbitrary comparisons between experiments, and can be achieved by various methods. A method of normalisation considered for this study were 'divide by mean', where values for genes in one channel are divided by the mean value of all genes of that channel. However, this approach was not used as it adjusts overall intensity problems but does not address dye non-linearity (Draghici, 2003). Additionally, linear regression normalisation was not used, as it is only applicable if differential expression is not expected and also does not correct for any dye non-linearity (Draghici, 2003).

Subsequently, important considerations for normalisation are the different biochemical properties of the fluorescent dyes that affect the data collected. A raw scatter plot in a two-channel array will usually show a tendency toward one dye caused either by unequal amounts of starting RNA or different labelling efficiencies for Cy3 and Cy5. The data on the plot is usually consistently off the diagonal, and the 'cloud' of data points resemble a banana or comma shape (Draghici, 2003). Experiments that incorporate dye-swaps and a Lowess normalisation can account for this bias (Draghici, 2003).

Lowess normalisation (LOcally WEighted polynomial regreSSion) was used in this study, which divides the data into a number of overlapping intervals and fits a polynomial function. In Lowess, the degrees of the polynomials are restricted to 1 to avoid over-fitting and excessive twisting and turning, and will divide the data domain into such narrow intervals so that the polynomial approximation is accurate (Draghici, 2003). Thus, the smoothness of the curve is directly proportional to the number of points considered for each local polynomial. Considering that Lowess can 'straighten out' the data on a scatter plot, it is currently the normalisation method of choice (Draghici, 2003), used in microarray studies on plants such as soybean (Moy *et al.*, 2004), sorghum (Buchanan *et al.*, 2005; Salzman *et al.*, 2005), and *Medicago truncatula* (Lohar *et al.*, 2006). However, Lowess does not produce a regression function that can be applied to all data sets, and is also susceptible to outliers, which is a reason why flagged spots must be removed before performing this normalisation (Draghici, 2003).

After performing data transformations, the data for each array feature was reported as the $\log_2$ expression ratio of treatment/control, where a gene up-regulated by a factor of two in a treated sample had a value of 1.0 and a gene down-regulated by a factor of 2 had a value of -1.0. As described in Chapter 3, most studies use a fold change (FC) cut-off of 2 for up- and down-

regulation, but as in Chapter 3 (section 3.3.2), the significant FC cut-offs for up- or down-regulation in this study were determined by three separate self-self hybridisations. It is important to recognise that the results of the Chapter 3 self-self hybridisation could not be applied here because of the use of a different microarray in this study. The resulting self-self ratio dataset was used to determine the 99% confidence interval for mean expression ratio of each array feature. This was performed by calculating sample standard errors and using the Z distribution to identify the confidence interval for each array sample (Appendix 5). The confidence distribution was then examined to identify the FC cut-off thresholds for differential expression.

4.2.5 Identification of differentially expressed ESTs

In general, microarray experiments generate large multivariate datasets, from which important differentially expressed (DE) genes must be identified (Yang *et al.*, 2005). Subsequently, the identification of DE genes can be divided into two parts; ranking and selection. Ranking involves the specification of a statistic or measure, which captures evidence for DE on a per gene basis, whilst selection requires specification of a procedure (*e.g.* stipulation of a critical value) for arbitrating what constitutes 'significant' DE (Yang *et al.*, 2005).

The ranking method employed in this study was based on an FC cut-off for expression determined by self-self hybridisations. Specifically, the expression datasets were used to determine the 95% confidence interval for mean expression ratio of each array feature (see formula in Appendix 5), and those ESTs whose confidence interval extended beyond the determined FC cut-off were identified as DE. However, ranking by FC alone implicitly assigns equal variance to every gene (Yang *et al.*, 2005). Subsequently, *t* statistics were also used, taking into account differing gene-specific variation across arrays (Yang *et al.*, 2005),

and are commonly used for assessing DE in plant microarray studies (Dudoit *et al.*, 2002; Fujiwara *et al.*, 2004; Gibly *et al.*, 2004; Buchanan *et al.*, 2005; Salzman *et al.*, 2005).

The main drawback of using *t* statistics for ranking lies in the unstable variance estimates that arise when sample sizes are small, which can be common in microarray experiments due to high costs and/or limited RNA material. Penalised statistics methods (*e.g.* Statistical Analysis of Microarrays (Tusher *et al.*, 2001)) have been developed to provide a more reliable variance estimate, either by variance stabilising functions or error fudge factors and Bayesian methods (Yang *et al.*, 2005). Linear models such as analysis of variance (ANOVA) have also been used for ranking, for example, fixed effect ANOVA including terms for dye, array, treatment, and gene main effects, as well as interactions (Kerr *et al.*, 2000). However, due to the increased technical and biological replication in this study, sample sizes were relatively large (n = 18, where n represents the number of data-points for each array feature). Therefore, *t* statistics could be reliably used without experiencing large effects of outliers.

The specific ranking method applied in this study (Appendix 5) involved, firstly, the generation of a report for each genotype x time-point dataset containing ratio data, channel-specific (control and treatment) means, and corresponding coefficient of variance (cv) values. The report was imported into Microsoft Excel (Microsoft, Redmond, WA) and equality of variance tests between channel means was performed for each array feature using the F distribution. In all cases, equal variances were observed, enabling the pooling of sample variances. Students *t* statistics were then calculated for each feature, and *P* values were obtained from the *t* distribution for use in the selection of DE ESTs.

Informal selection approaches include graphical representations of ranking statistics via Q-Q plots or volcano plots, whilst formal approaches involve testing hypotheses of equal

expression through multiple testing corrections (Yang *et al.*, 2005). Problems arise when performing hundreds of significance tests for ranking, for example, using a $P<0.05$ in this study (768 features) would imply the acceptance of 38 (5%) false positives, which is not acceptable. To account for this problem, two different approaches have emerged; one based on Westfall and Young (1993) to control type I error rates, and one that develops and extends the notion of False Discovery Rate (FDR) by Benjamani and Hochberg (1995). The corrections that seek to control type I error rates are single step methods that assume the variables are independent, which does not hold for arrays involving interacting genes (Draghici, 2003). However, the FDR is able to cope with gene interaction and is computationally efficient. Subsequently, FDR multiple testing corrections were applied in this study after ranking by FC cut-off and *t* statistics.

For each experimental dataset corresponding to a specific genotype x time-point, FDR was applied to adjust the *P* values obtained from *t* tests (Appendix 5). Firstly, the ESTs were listed in ascending order by their *t* test *P* value. Secondly, the *P* value of each EST was compared to a threshold that depended on the position of the EST in the list. The thresholds were (1/R x α) for the first gene, then (2/R x α) for the second, and so on, where R represented the number of ESTs in the list and α was the desired significance level (0.05). To pass the threshold the original *P* value from the *t* test must have been less than the individual threshold for each gene (*e.g.* $p_1 < (1/R) \times \alpha$, $p_2 < (2/R) \times \alpha$ and so on). Overall, DE ESTs were then identified as those with a 95% confidence interval for mean fold change (FC) that extended beyond the cut-off determined by the self-self hybridisation, and also passed the Students *t* test ($P<0.05$) and FDR correction.

Lists of DE ESTs for each genotype at each time-point were then compiled into a single non-redundant EST list. This list was then used to create a dataset of mean expression ratios for

the ESTs in each time-point of each genotype. To identify co-regulated ESTs, Figure of Merit (FOM) and *k*-means clustering (Euclidean metrics) were performed separately for each genotype with the MeV software (Saeed *et al.*, 2003) available from The Institute for Genomic Research (TIGR) (www.tigr.org/software/microarrays.html). Unlike Chapter 3, *k*-means clustering was performed in this study considering the greater complexity of the dataset, and FOM allowed the calculation of optimal *k* values (refer to section 1.5.3).

4.2.6 Quantitative RT-PCR

Seven EST targets were selected for confirmation by qRT-PCR. The primers for quantitative detection had GC contents of 50-60%, Tm>50ºC, no more than two G or C nucleotides in the last five 3' bases, no stretch of G or C nucleotides longer than three bases, and targeted amplicons of 75-150 bp. For each genotype and time-point, 5 µg total RNA from one of the biological replicates used for microarray hybridisations was converted to cDNA template using oligodT 23-mer (Invitrogen Life Technologies, Carlsbad, CA) and Superscript II reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA). Briefly, 5 µg of total RNA was added to 1 µL oligodT 23-mer (500 mg/ml) and 1 µL dNTP mix (10 mM each dATP, dTTP, dCTP, dGTP), and made up to 12 µL with sterile water. This mixture was heated at 65°C for 5 min and chilled on ice for 5 min. To the tube was then added 4 µL 5X first-strand buffer and 2 µL 0.1M DTT, followed by incubation at 42°C for 2 min, addition of 1 µL (200 units) Superscript II reverse transcriptase and incubation at 42°C for 2 h. Reactions were stopped by heating tubes at 70°C for 15 min.

Resulting cDNA was purified using the Qiaquick PCR purification kit (Qiagen, Valencia, CA) according to manufacturer's guidelines, and diluted to 250 µL in sterile water. Triplicate qRT-PCR reactions were performed for each clone of interest using Sybr Green Mastermix (Stratagene, La Jolla, CA) with primers (0.4 µM each) and 5 µL of cDNA. Control reactions

containing untranscribed RNA confirmed that no interfering genomic DNA products were present. PCR was performed on the BIO-RAD MyiQ™ instrument (BIO-RAD, Hercules, CA) with the following cycling program: 95ºC for 10 min followed by 40 cycles of 45 s at 95ºC, 1 m at 55ºC, and 1 m at 72ºC. Amplification products were subjected to melting curve analysis by applying decreasing temperature from 95ºC to 45ºC (0.5ºC/10 s) and continuous fluorescence recording. Additionally, 2 uL aliquots of amplification products were checked for single products by gel electrophoresis according to the method described in section 3.3.1.2. Relative fold change in accumulation of target under a given treatment was standardised against cDNA derived from water-sprayed control tissue. Expression levels obtained were normalised using actin (reference), which showed similar expression levels at all time-points after infection as revealed by microarray analysis.

The comparative C_T method ($\Delta\Delta C_T$ method) was used to calculate fold changes, which eliminates the need for standard curves, but can only be used if PCR amplification efficiencies are relatively equal between target and reference (actin) (Applied Biosystems, 2004). To determine equal amplification efficiency between each primer pair (target) and the reference, validation experiments were performed. Validations involved the use of a dilution series (1.0, 0.5, 0.1, 0.0) of a single cDNA template, and triplicate qPCR reactions were performed on each dilution for each target and reference. The C_T (cycle threshold) values of the target and reference for each dilution were used to calculate ΔC_T ($C_{T\ target}$–$C_{T\ reference}$). The ΔC_T values of all dilutions of each target *vs*. reference were plotted against log dilution to create a semi-log regression line. The slope of the lines were calculated in Microsoft Excel (Redmond, WA), and absolute values <0.1 indicated successful validation (Applied Biosystems, 2004).

Validated targets were then used to detect relative fold changes between treated and control cDNA samples (0.1 template dilutions). Mean C_T and C_T standard deviations were calculated

from the triplicate qPCRs for each sample. ΔC_T values were then calculated as above, and the standard deviation was calculated from the target and reference C_T standard deviation values using the formula; $s = sqrt(s_1^2+s_2^2)$. The $\Delta\Delta C_T$ values (relative fold change) could then be calculated by; $\Delta\Delta C_T = \Delta C_T$ treated sample-ΔC_T control sample (Applied Biosystems, 2004).

4.3 Results

4.3.1 Experimental design

A standardised system of plant growth, fungal inoculation and replication was developed in order to minimise experimental variability and ensure accurate measurements of changes in mRNA abundance (Figure 4.1). The inherent noise and sensitivity of the microarray system was determined by three separate self-self hybridisations. The combined result of these hybridisations yielded a 99% confidence distribution where 97.6% of the signals fell within 1.5 FC and 100% were within 1.8 FC (Figure 4.2). Based on this result, a 1.8 FC cut-off was selected for differential expression in addition to the significance (*t*) test and FDR multiple testing correction. These cut-offs translated into up-regulated cDNAs having a $\log_2$ ratio $\geq$0.85, and down-regulated cDNAs $\leq$-0.85.

4.3.2 Microarray construction and analysis

The microarray consisted of 715 cDNA clones (559 from chickpea and 156 from grasspea) and 41 lentil RGA DNA sequences, and gel analysis of PCR-amplified products showed that all contained single inserts (Figure 4.3). Transcript level for each cDNA was calculated firstly as the average intensity of the six technical replicates, then the average intensity of the three biological replicates. Figure 4.4 shows an example of a scan viewed using Imagene™ v. 5.5 (BioDiscovery, Marina Del Rey, CA). All MIAME guidelines were observed and the datasets were deposited into the Gene Expression Omnibus, National Center for Biotechnology Information (series no. GSE4660).

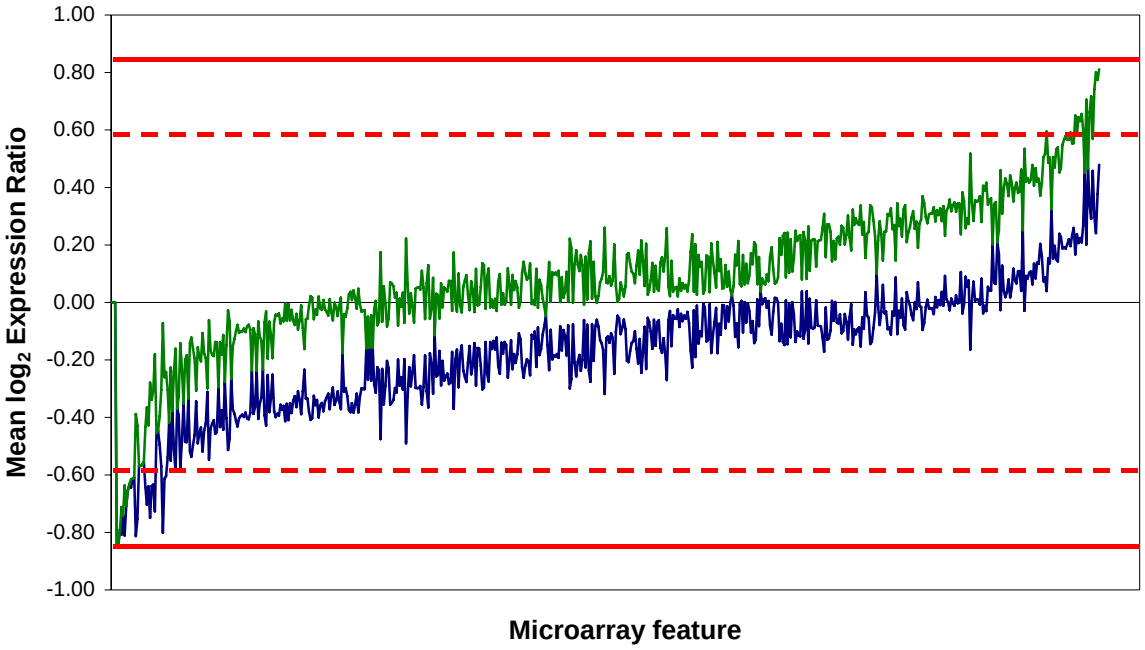

Figure 4.2 Combined distribution of the 99% confidence intervals of mean $\log_2$ ratios for all array features of the self-self hybridisations. Green line represents the upper 99% confidence limit and blue line represents the lower limit. Broken red horizontal line indicates the point representing a 1.5 FC, and the unbroken red horizontal line indicates the 1.8 FC line.

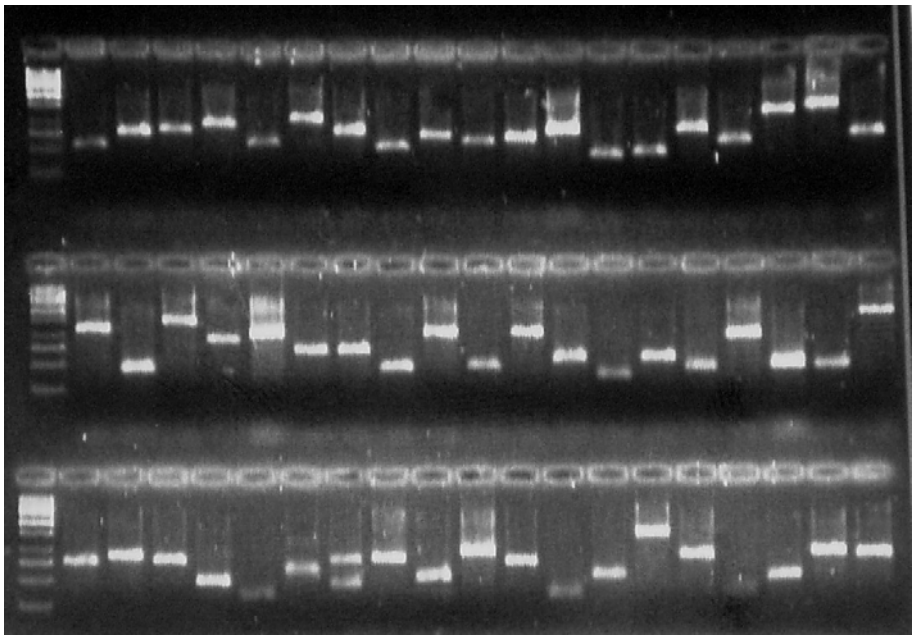

Figure 4.3 Gel photo showing an example of PCR-amplified cDNA inserts (left hand lanes contain ladder). Each insert must have a single product (band) to be included for printing.

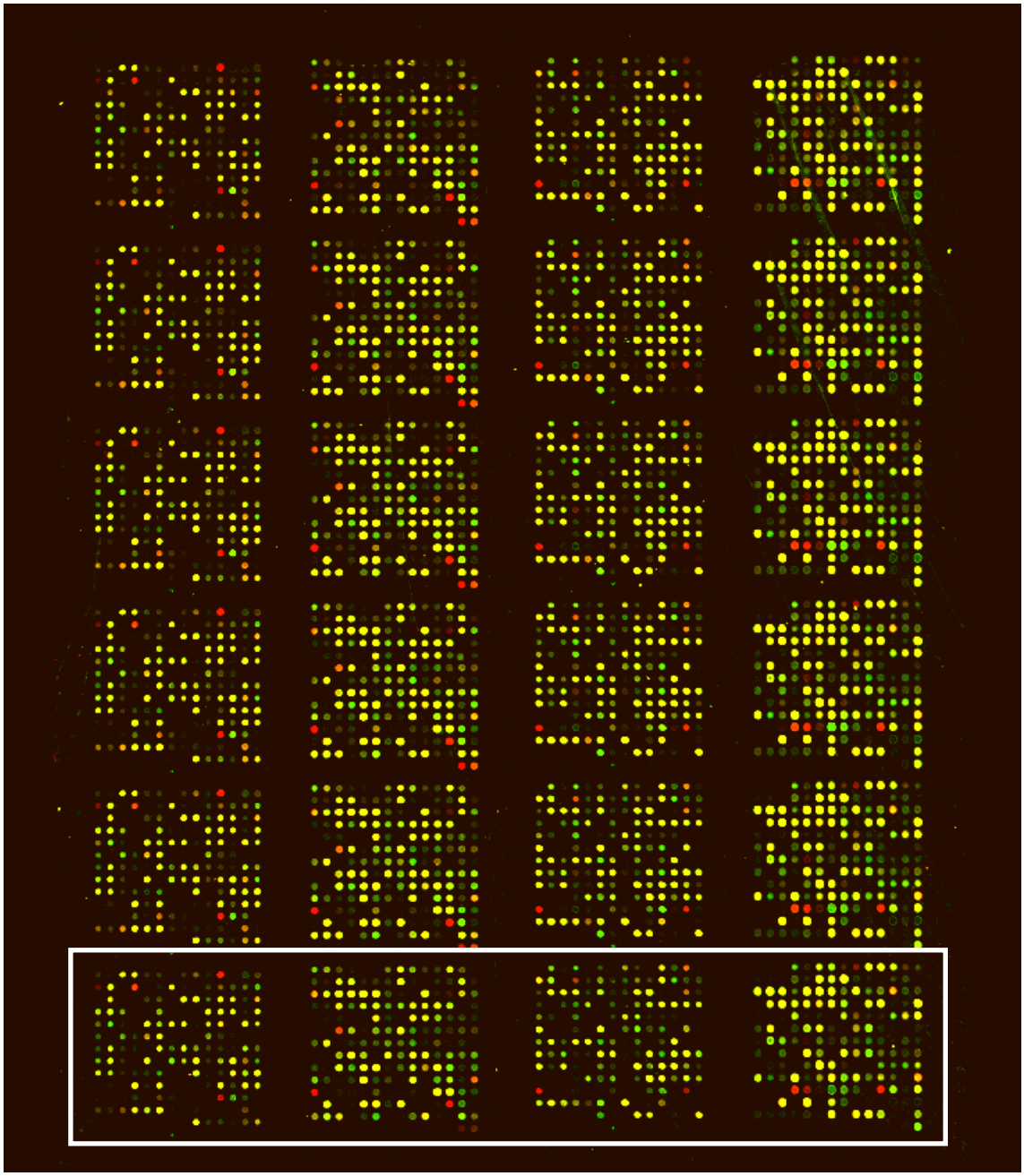

Figure 4.4 Example of a scan viewed with Imagene™ v. 5.5 (BioDiscovery, Marina Del Rey, CA), showing six microarray sub-grids. The white rectangle borders one sub-grid that contains all 768 features.

Table 4.1 reports the number of microarray features (probes) that were undetected in each chickpea genotype over all time-points, as well as the source of the probes. Only small percentages of *C. arietinum* probes were undetected in each genotype, the highest being for IL (4.3%), which is the wild relative chickpea genotype. Only 0.5% of the *C. arietinum* probes were undetected in IC. The levels of undetected features for the *L. sativus* probes were higher, again with IL as the highest (12.8%) but LA as the lowest (7.7%). All lentil RGA sequence probes were undetected in all genotypes (see discussion). Importantly, labelled cDNA targets did not hybridise to any negative controls on the microarray including blank buffer, digested vector and PCR primer sequences.

An FC cut-off and Students *t* test ($P<0.05$) ranking with FDR multiple testing correction selection was used to identify DE ESTs. The 1.8 FC cut-off was determined by three separate replicated self-self hybridisations (with six technical replications each), whilst the *t* test with FDR allowed only the selection of statistically significant DE ESTs. Although a commonly used FC cut-off is 2.0 (Maguire *et al.*, 2002; Scheideler *et al.*, 2002; Lopez *et al.*, 2005), several recent studies have shown reliable use of a lower FC cut-off of 1.5 - 2.0 (Gibly *et al.*, 2004; Salzman *et al.*, 2005; Lohar *et al.*, 2006), particularly if several replicates are included. A total of 192 ESTs were found to be DE in at least one time-point from at least one chickpea genotype. Several ESTs were found to be DE at more than one time-point for each genotype whereas others were DE at specific times during the response to *A. rabiei* infection. Tables 4.2 – 4.5 describe the characteristics of the DE ESTs for each genotype and time-point.

Table 4.1 Undetected microarray probes from each source, where undetected corresponds to mean fluorescence intensity less than two times the mean local background intensity in all time-points and all replications.

	Microarray probe source		
Genotype	***C. arietinum***	***L. sativus***	***L. culinaris***
IC (*C. arietinum*)	3 (0.5%)	18 (11.5%)	41 (100%)
LA (*C. arietinum*)	6 (1.1%)	12 (7.7%)	41 (100%)
FL (*C. arietinum*)	6 (1.1%)	18 (11.5%)	41 (100%)
IL (*C. echinospermum*)	24 (4.3%)	20 (12.8%)	41 (100%)

Table 4.2 List of differentially expressed ESTs in *C. arietinum* IC after inoculation with *A. rabiei.* Fold change represents the $\log_2$ of mean expression ratio of treated *vs* control samples, and 95% +/- is the interval above and below the mean corresponding to the 95% confidence interval. For 'clone source', CA indicates *C. arietinum* (chickpea) and LS is *L. sativus* (grasspea).

Time-point	**Regulation**	**Fold change**	**95% +/-**	**Clone source**	**Putative function**	**GenBank accession**
06 hpi	Up	1.34	0.08	CA	Chlorophyll a/b binding protein	DY475534
	Down	-1.11	0.10	LS	Ripening-related protein	DY396347
		-0.85	0.11	CA	Hypothetical transmembrane protein	DY475478
		-0.83	0.19	CA	Unknown	DY475401
		-0.79	0.08	LS	Nitrate transporter NRT1-1	DY396335
		-0.71	0.15	LS	Ripening-related protein	DY396377
12 hpi	Up	0.70	0.25	CA	Chlorophyll a/b binding protein	DY475534
		0.69	0.34	LS	Disease resistance response protein DRRG49-C	DY396265
		0.58	0.29	CA	18S nuclear rRNA	DY475150
		0.43	0.44	LS	NADH dehydrogenase	DY396279
	Down	-1.23	0.27	CA	Phosphate-induced protein	DY475076
		-0.86	0.05	CA	RNA/ssDNA binding protein	DY475357
24 hpi	Up	1.31	0.26	LS	Pathogenesis-related protein 4A	DY396384
		1.09	0.27	LS	Pathogenesis-related protein 4A	DY396388
		1.08	0.32	LS	Pathogenesis-related protein	DY396301
		0.99	0.34	LS	Disease resistance response protein DRRG49-C	DY396265
		0.97	0.25	CA	Pathogenesis-related protein 4A	CV793597
		0.86	0.26	CA	SNAKIN2 antimicrobial peptide	CV793608

					precursor	
		0.82	0.30	CA	Protein with leucine-zipper	CV793599
	Down	-1.56	0.14	CA	S-adenosylmethionine decarboxylase	DY475170
		-1.20	0.06	CA	Asparagine synthetase	DY475108
		-1.14	0.10	CA	Apocytochrome F	DY475181
		-0.94	0.44	CA	Hypothetical transmembrane protein	DY475478
		-0.90	0.30	CA	S-adenosylmethionine synthetase	DY475190
		-0.77	0.35	CA	Serine/threonine protein kinase	DY475384
		-0.77	0.37	CA	Superoxide dismutase copper chaperone precursor	DY475397
		-0.75	0.35	CA	Zinc finger protein	DY475091
48 hpi	Up	1.43	0.19	LS	Hypothetical proline-rich protein	DY396288
		1.25	0.38	LS	Pathogenesis-related protein 4A	DY396388
		1.17	0.39	LS	Pathogenesis-related protein 4A	DY396384
		1.02	0.20	CA	Polymorphic antigen membrane protein	DY475248
		1.00	0.19	CA	Unknown	DY475532
		0.89	0.19	CA	Unknown	DY475365
		0.87	0.19	CA	β-1,3-glucanase	CV793598
		0.86	0.28	LS	Pathogenesis-related protein 4A	DY396372
		0.73	0.18	LS	Pathogenesis-related protein	DY396305
	Down	-1.23	0.18	CA	RNA/ssDNA binding protein	DY475357
		-1.08	0.18	CA	Photosystem II reaction centre I protein	DY475116
		-1.04	0.13	CA	Asparagine synthetase	DY475108
		-0.88	0.16	CA	Hypothetical transmembrane protein	DY475478
		-0.86	0.13	CA	Phosphate-induced protein	DY475172
		-0.80	0.37	CA	Protein with leucine-zipper	CV793599
		-0.78	0.10	CA	WD repeat protein	DY475550
		-0.61	0.26	CA	Thiazole biosynthetic enzyme	DY475242
		-0.58	0.38	CA	Thylakoid protein	DY475305
		-0.54	0.38	CA	S1-3 pathogen-induced protein	CV793591
		-0.53	0.43	CA	ATP synthase	DY475245
72 hpi	Up	0.89	0.17	LS	NADH Dehydrogenase	DY396279
		0.86	0.06	CA	18S rRNA	DY475557
		0.79	0.15	CA	Unknown	DY475533
		0.75	0.26	CA	Unknown	DY475157
		0.72	0.22	LS	Ca-binding carrier protein	DY396262
	Down	-1.43	0.05	CA	Proline oxidase	DY475225
		-1.37	0.21	CA	Asparagine synthetase	DY475108
		-1.22	0.21	CA	Protein with leucine-zipper	CV793599
		-1.20	0.11	CA	Glutathione S-transferase	DY475250
		-1.13	0.16	CA	Nematode resistance protein *Hs1pro-1*	CV793603
		-1.05	0.08	CA	S-adenosylmethionine synthetase	DY475190
		-1.03	0.17	CA	Zinc finger protein	DY475091
		-1.02	0.32	CA	Hypothetical transmembrane protein	DY475478

	-1.00	0.12	CA	Homocysteine methyltransferase	DY475276
	-0.82	0.14	CA	4-alpha-glucanotransferase	DY475302
	-0.79	0.38	CA	*β*-1,3-glucanase	CV793598
	-0.75	0.25	CA	Wound-induced protein	DY475220
	-0.73	0.14	CA	Fructose-1,6-bisphosphatase	DY475543
	-0.69	0.16	CA	Cytosolic fructose 1,6-bisphosphatase	DY475548
	-0.66	0.39	CA	Carbonic anhydrase-like protein	DY475403
	-0.63	0.23	CA	Serine/threonine protein kinase	DY475384

Table 4.3 List of differentially expressed ESTs in *C. arietinum* LA after inoculation with *A. rabiei*. Fold change represents the $\log_2$ of mean expression ratio of treated *vs* control samples, and 95% +/- is the interval above and below the mean corresponding to the 95% confidence interval. For 'clone source', CA indicates *C. arietinum* (chickpea) and LS is *L. sativus* (grasspea).

Time-point	**Regulation**	**Fold change**	**95% +/-**	**Clone source**	**Putative function**	**GenBank accession**
06 hpi	Down	-1.14	0.03	LS	EREBP-4	DY396400
		-0.77	0.21	CA	Chlorophyll a/b binding protein	DY475534
		-0.62	0.29	CA	Superoxide dismutase copper chaperone precursor	DY475397
12 hpi	Down	-0.95	0.41	LS	Ripening-related protein	DY396344
24 hpi	Up	1.13	0.10	CA	Unclear	DY475322
		0.93	0.11	LS	Pathogenesis-related protein 4A	DY396281
		0.91	0.14	CA	Pathogenesis-related protein 4A	CV793597
		0.64	0.33	CA	Unknown	DY475483
	Down	-0.96	0.04	CA	Unknown	DY475536
		-0.95	0.08	CA	Formyltetrahydrofolate deformylase	DY475493
		-0.90	0.39	CA	Unclear	DY475522
		-0.88	0.08	CA	NADH dehydrogenase	DY475316
		-0.86	0.19	CA	26S rRNA	DY475540
		-0.86	0.25	CA	Unknown	DY475339
		-0.85	0.22	CA	RNA/ssDNA binding protein	DY475357
		-0.84	0.12	CA	Similar to endopeptidase	DY475396
		-0.80	0.30	CA	Photosystem I reaction centre subunit IX	DY475047
		-0.77	0.10	CA	S-adenosylmethionine synthetase	DY475190
		-0.77	0.14	CA	Zinc finger protein	DY475091
		-0.67	0.22	CA	S-adenosylmethionine decarboxylase	DY475170
		-0.59	0.30	CA	ATP Synthase C chain	DY475464
48 hpi	Up	1.80	0.15	CA	Pathogenesis-related protein 4A	CV793597
		1.48	0.13	LS	Pathogenesis-related protein 4A	DY396372
		1.39	0.20	LS	Pathogenesis-related protein 4A	DY396388
		1.23	0.17	CA	Unknown	DY475365

		1.22	0.05	LS	Pathogenesis-related protein 4A	DY396384
		1.21	0.01	LS	Pathogenesis-related protein 4A	DY396281
		0.92	0.33	CA	Sorting nexin protein	DY475523
		0.80	0.12	CA	Asparagine synthetase	DY475475
		0.52	0.39	LS	Hypothetical proline-rich protein	DY396288
	Down	-1.16	0.15	CA	Unknown	DY475535
		-1.12	0.16	CA	Asparagine synthetase	DY475108
		-0.94	0.12	CA	Unknown	DY475125
		-0.79	0.07	CA	Chlorophyll a/b binding protein	DY475554
		-0.78	0.20	CA	Hypothetical transmembrane protein	DY475478
		-0.74	0.27	CA	Proline oxidase	DY475225
		-0.61	0.30	CA	RNA/ssDNA binding protein	DY475357
72 hpi	Up	1.20	0.33	CA	Pathogenesis-related protein 4A	CV793597
		0.99	0.61	LS	Pathogenesis-related protein 4A	DY396384
		0.75	0.50	LS	Pathogenesis-related protein 4A	DY396372
		0.65	0.27	LS	Pathogenesis-related protein 4A	DY396281
		0.63	0.58	LS	Pathogenesis-related protein 4A	DY396388
	Down	-1.12	0.20	CA	26S rRNA	DY475211
		-1.02	0.23	CA	Unknown	DY475125
		-0.81	0.12	CA	26S rRNA	DY475153
		-0.41	0.54	CA	18S rRNA	DY475542

Table 4.4 List of differentially expressed ESTs in *C. arietinum* FL after inoculation with *A. rabiei*. Fold change represents the $\log_2$ of mean expression ratio of treated *vs* control samples, and 95% +/- is the interval above and below the mean corresponding to the 95% confidence interval. For 'clone source', CA indicates *C. arietinum* (chickpea) and LS is *L. sativus* (grasspea).

Time-point	Regulation	Fold change	95% +/-	Clone source	Putative function	GenBank accession
06 hpi	Up	0.83	0.09	CA	Chloroplast 4.5S, 5S, 16S and 23S mRNA	DY475544
	Down	-0.87	0.25	CA	Superoxide dismutase copper chaperone precursor	DY475397
12 hpi	Down	-0.77	0.28	CA	18S rRNA	DY475542
24 hpi	Up	1.91	0.06	LS	Hypothetical proline-rich protein	DY396288
		1.71	0.12	LS	Pathogenesis-related protein 4A	DY396388
		1.22	0.23	LS	Pathogenesis-related protein 4A	DY396372
		1.11	0.36	LS	Pathogenesis-related protein 4A	DY396384
		1.09	0.40	CA	Pathogenesis-related protein 4A	CV793597
		1.03	0.27	LS	Pathogenesis-related protein 4A	DY396281
		0.85	0.22	LS	Pathogenesis-related protein	DY396301

		0.84	0.25	LS	Environmental stress-inducible protein	DY396298
		0.83	0.11	CA	Unclear	DY475217
	Down	-1.25	0.05	CA	Unclear	DY475186
		-1.10	0.09	CA	Unknown	DY475535
		-1.02	0.04	CA	Unclear	DY475322
		-0.97	0.03	CA	Proline oxidase	DY475225
		-0.97	0.14	CA	26S rRNA	DY475540
		-0.91	0.13	CA	Nematode resistance protein *Hs1pro-1*	CV793603
		-0.78	0.30	CA	Extensin-like protein	CV793587
		-0.78	0.15	CA	Hypothetical transmembrane protein	DY475478
		-0.77	0.10	CA	Dehydrin cold-induced protein	DY475092
		-0.75	0.16	CA	Unknown	DY475481
		-0.74	0.14	LS	Small GTP-binding protein	DY396367
		-0.69	0.23	CA	Phosphate-induced protein	DY475172
48 hpi	Up	0.90	0.11	CA	Unclear	DY475186
		0.83	0.33	CA	Unknown	DY475462
		0.76	0.44	CA	Unknown	DY475365
		0.72	0.39	CA	26S rRNA	DY475153
		0.59	0.29	CA	Unclear	DY475323
	Down	-1.48	0.34	CA	Carbonic anhydrase	DY475213
		-1.12	0.26	CA	ATP synthase	DY475245
		-1.11	0.15	CA	Flavonol glucosyl transferase	CV793607
		-1.05	0.46	CA	Hypothetical transmembrane protein	DY475478
		-0.97	0.28	CA	Serine/threonine protein kinase	DY475384
		-0.96	0.12	CA	S-adenosylmethionine synthetase	DY475190
		-0.88	0.20	CA	Zinc finger protein	DY475091
		-0.87	0.32	CA	Protein with leucine-zipper	CV793599
		-0.86	0.07	CA	Unknown	DY475535
		-0.83	0.08	CA	Mitochondrial 26S rRNA	DY475087
		-0.82	0.29	CA	GPI-anchored membrane protein	DY475246
		-0.82	0.08	CA	Unknown	DY475094
		-0.79	0.36	CA	Phosphate-induced protein	DY475172
		-0.70	0.24	CA	S-adenosylmethionine decarboxylase	DY475170
72 hpi	Up	0.92	0.07	CA	18S rRNA	DY475557
		0.87	0.19	LS	Small GTP-binding protein	DY396367
	Down	-1.00	0.13	CA	Phosphate-induced protein	DY475172
		-0.97	0.10	CA	Unknown	DY475535
		-0.86	0.06	CA	Unknown	DY475538

Table 4.5 List of differentially expressed ESTs in *C. arietinum* IL after inoculation with *A. rabiei*. Fold change represents the $\log_2$ of mean expression ratio of treated *vs* control samples and 95% +/- is the interval above and below the mean corresponding to the 95% confidence interval. For 'clone source', CA indicates *C. arietinum* (chickpea) and LS is *L. sativus* (grasspea).

Time-point	Regulation	Fold change	95% +/-	Clone source	Putative function	GenBank accession
06 hpi	Down	-1.26	0.43	LS	Ripening-related protein	DY396377
		-0.94	0.04	CA	Unknown	DY475539
		-0.91	0.26	CA	26S rRNA	DY475540
		-0.85	0.09	CA	Unknown	DY475532
		-0.81	0.08	CA	26S rRNA	DY475153
		-0.71	0.25	CA	Chloroplast genome DNA	DY475541
		-0.68	0.24	CA	*Avr9/Cf9* rapidly elicited protein 65	CV793589
		-0.52	0.45	LS	Ripening-related protein	DY396344
12 hpi	Up	1.30	0.49	CA	Polymorphic antigen membrane protein	DY475248
		0.78	0.18	CA	Unknown	DY475084
		0.68	0.42	LS	Pathogenesis-related protein 4A	DY396384
24 hpi	Up	1.73	0.22	LS	Pathogenesis-related protein 4A	DY396372
		1.56	0.15	CA	Pathogenesis-related protein 4A	CV793597
		1.55	0.13	LS	Pathogenesis-related protein 4A	DY396384
		1.51	0.09	LS	Pathogenesis-related protein 4A	DY396388
		0.69	0.17	LS	Disease resistance response protein DRRG49-C	DY396265
	Down	-0.88	0.22	CA	Unclear	DY475095
		-0.85	0.33	CA	Fructose-1,6-bisphosphatase	DY475543
		-0.73	0.15	CA	Unknown	DY475115
		-0.60	0.26	CA	Photosystem II reaction centre I protein	DY475116
		-0.60	0.29	CA	Unclear	DY475515
48 hpi	Down	-0.92	0.12	CA	NADH dehydrogenase	DY475316
		-0.88	0.14	CA	Proline oxidase	DY475225
		-0.74	0.13	CA	Zinc finger protein	DY475091
		-0.70	0.24	CA	GPI-anchored membrane protein	DY475246
		-0.69	0.27	CA	Polymorphic antigen membrane protein	DY475248
		-0.65	0.24	CA	Nematode resistance protein *Hs1pro-1*	CV793603
72 hpi	Up	1.19	0.06	CA	Pathogenesis-related protein 4A	CV793597
		0.96	0.04	LS	Pathogenesis-related protein	DY396301
	Down	-0.92	0.06	CA	Homocysteine methyltransferase	DY475276

Figure 4.5 shows the kinetic trend of differential expression for each chickpea genotype. For LA, no up-regulation was observed until 24 hpi, but up-regulation was achieved as early as 6 hpi for IC and FL and 12 hpi for IL. Up-regulation peaked at 48 hpi in IC and LA, compared to 24 hpi in FL and IL. Down-regulation occurred as early as 6 hpi in all genotypes, peaking at 24 hpi in LA and FL, 72 hpi in IC, and 6 hpi in IL. Globally, the proportion of DE ESTs were relatively low at 6 and 12 hpi (10% and 6% of total DE ESTs, respectively), before a considerable increase at 24 and 48 hpi (33% and 31% respectively), and then a fall to 20% at 72 hpi.

Any of the 43 previously non-sequenced chickpea cDNAs that showed differential expression were accurately re-sequenced and functionally identified according to the methods described in Chapter 2. Of the 192 DE ESTs, 20 were 'Unknown' (no significant homology to sequences in public databases) and nine were 'Unclear' (significant homology to hypothetical proteins only). Putative genes previously implicated in resistance responses were found to be DE, including up-regulation in all genotypes of PR proteins (CV793597, DY396301, DY396384, DY396388, DY396372, DY396305 and DY396281), up-regulation of the *Pisum sativum-Fusarium solani* disease resistance response protein DRRG49-C (DY396265) in IC and IL, up-regulation of SNAKIN2 antimicrobial peptide precursor (CV793608) in IC only, and up-regulation of hypothetical proline-rich protein (DY396288) in IC, FL and LA. Up- and down-regulation was observed for protein with leucine-zipper (CV793599) in IC and FL, and β-1,3-glucanase (CV793598) in IC. Down-regulation was witnessed for Ethylene Responsive Element Binding Protein 4 (EREBP-4) (DY396400) in LA, *Avr9/Cf9* rapidly elicited protein 65 (CV793589) in IL, and nematode resistance protein *Hs1pro-1* (CV793603) in all genotypes but LA. Other down-regulated putative defence-related genes encoded an extensin-like protein (CV793587) and flavonol glucosyl transferase (CV793607) in FL, as well as S1-3 pathogen-induced protein (CV793591) in IC.

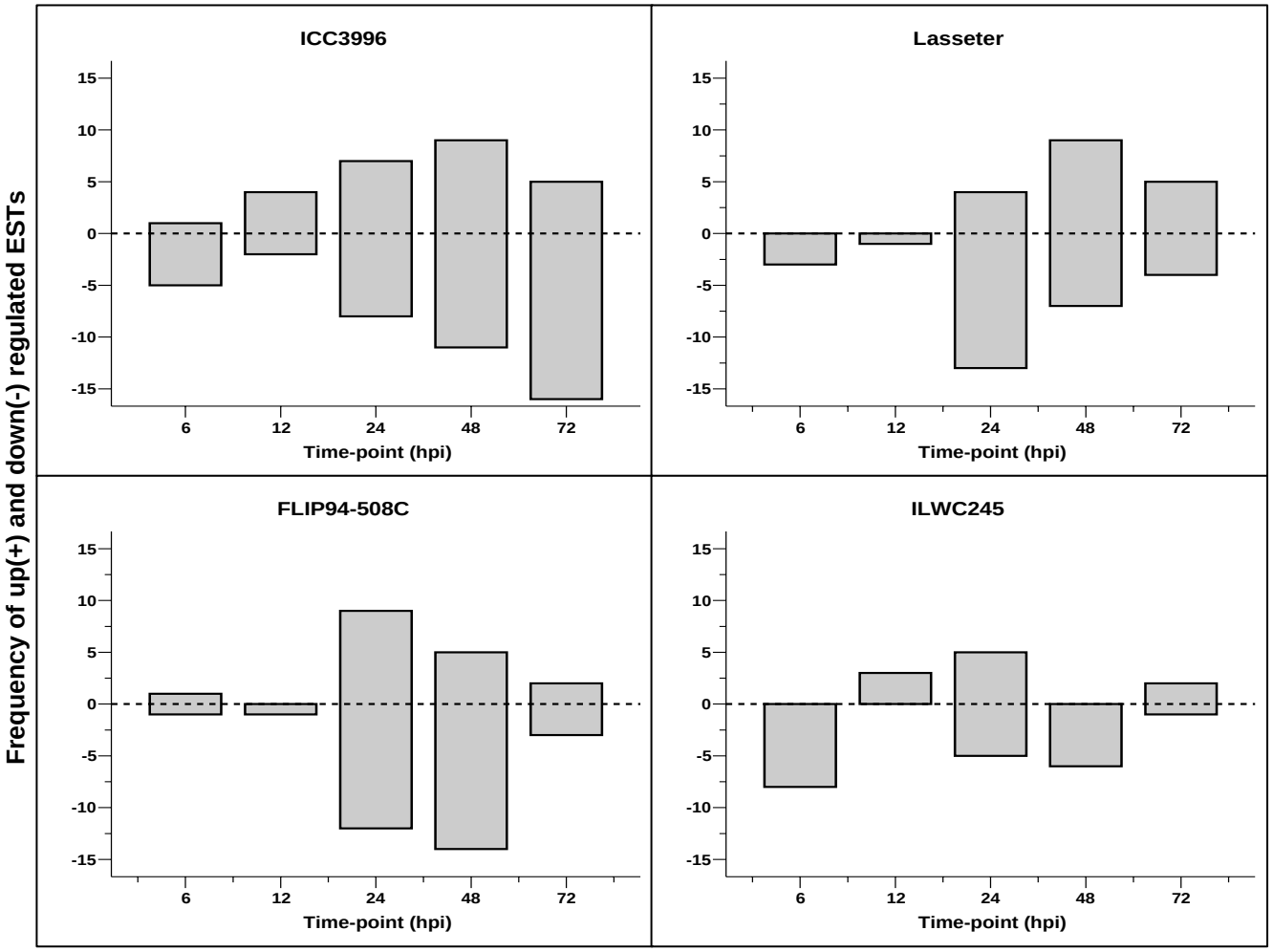

Figure 4.5 Kinetic trends of differential expression for each chickpea genotype over the time-course after inoculation with *A. rabiei*.

Eleven ESTs involved with cell rescue/death/ageing were down-regulated in IC, compared with four in LA, seven in FL, and four in IL. Examples were antioxidant proteins glutathione S-transferase (DY475250) and superoxide dismutase copper chaperone precursor (DY475397), as well as other proteins including ripening-related proteins (DY396347, DY396377 and DY396344), proline oxidase (DY475225), and phosphate-induced protein (DY475076). The only cell rescue/death/ageing EST to be up-regulated was the environmental stress-inducible protein (DY396298) in FL. Cellular communication/signal transduction and transport-associated ESTs were again mainly down-regulated at different times in different genotypes, including nitrate transporter NRT1-1 (DY396335), zinc-finger protein (DY475091), and a serine/threonine protein kinase (DY475384). Examples of up-regulated proteins in this category were polymorphic antigen membrane protein (DY475248) in IC and IL, and sorting nexin protein (DY475523) in LA. ESTs involved in energy production and protein synthesis/fate were both up- and down-regulated in IC and FL, but down-regulated only in LA and IL (examples including DY475534, DY396279, DY475150, DY475116 and DY475245). Ten cellular metabolism ESTs were down-regulated in IC without any instances of up-regulation, including DY475108, DY475170 and DY475181. However, only three cellular metabolism ESTs were down-regulated in LA (DY475170, DY475396 and DY475108), two in FL (DY475170 and DY475213), and one in IL (DY475543). Little differential regulation was detected for the cell cycle/DNA processing and transcription-associated ESTs. Only two and three occurrences of down-regulation were observed in IC and LA respectively (DY475357 and DY475493), no differential regulation in IL, and just one instance of up-regulation only in FL (DY475544).

4.3.3 Comparison to results of Chapter 3

A comparison of the expression data for the time-points (24 and 48 hpi) and chickpea genotypes (IC and LA) that overlapped between this study and the previous small-scale study

of defence-related cDNAs from Chapter 3 was made (Table 4.6). Comparisons were made for the ESTs previously identified as differentially expressed in Chapter 3, revealing that the majority of the up- and down-regulation trends from the present study were in line with the differential regulation reported in Chapter 3, although they were not considered as significantly DE in this study. Possible reasons for this observation are discussed in section 4.4.

4.3.4 Quantitative RT-PCR

To confirm the reliability of results from the microarray expression analyses, seven ESTs that showed varying levels of regulation were selected for qRT-PCR. The comparative C_T method ($\Delta\Delta$ C_T method) of quantitation was used, and validation of all seven targets was successful. Figure 4.6 shows an example of a validation curve achieved for one target (see Appendix 6 for all validation curves). C_T values were automatically generated by the MyiQ™ instrument (BIO-RAD, Hercules, CA), and Figure 4.7 shows an example of amplification curves and C_T value determination.

The reference target chosen for normalisation of quantified data was actin, whose expression was constant under pathogen inoculation. Amplified qRT-PCR products were examined for specificity of product by both melting curve analysis and gel electrophoresis. Melting curve analysis was performed directly after amplification as part of the qPCR protocol, where the presence of a single dissociation peak indicated the specific amplification of a single product (Figure 4.8). Amplification products were also checked for single products using gel electrophoresis. Subsequently, data for any sample not showing specific amplification was discarded from analysis and qPCR was repeated.

Table 4.6 Comparison between the expression observations for ESTs classed as DE in Chapter 3 and the expression observations of the present study. DE in Chapter 3 was determined by a mean FC >2 (arrows indicate up- or down-regulation and 'nd' indicates non-DE). Chapter 4 values correspond to $\log_2$ mean FC values and * indicates statistically significant DE. NA indicates that data was eliminated due to quality control.

GenBank accession	**Putative function**	**Comparison of observations**				
		Time hpi	***IC***		***LA***	
			Chap 3	**Chap 4**	**Chap 3**	**Chap 4**
CV793595	Caffeoyl-CoA-	**24**	↓	-0.30	↓	-0.28
(DEF08)	methyltransferase	**48**	nd	NA	↓	-0.30
CV793597	Pathogenesis-related protein	**24**	nd	0.95*	↑	0.91*
(DEF09)	4A	**48**	nd	0.81	↑	1.80*
CV793598	*β*-1-3-glucanase	**24**	↑	0.39	↑	NA
(DEF10)		**48**	↑	0.87*	↑	NA
CV793599	Protein with leucine zipper	**24**	↑	0.82*	nd	-0.10
(DEF11)		**48**	nd	-0.81*	nd	-0.67
CV793601	Leucine-zipper containing	**24**	↓	-0.07	↓	0.17
(DEF13)	protein	**48**	↓	-0.35	↓	-0.07
CV793605	Multi-resistance transporter	**24**	↑	0.24	↑	0.28
(DEF16)	protein	**48**	↑	0.23	↑	0.47
CV793607	Putative flavonol glucosyl	**24**	↓	-0.17	↓	-0.17
(DEF17)	transferase	**48**	nd	NA	nd	-0.48
CV793608	SNAKIN2 antimicrobial	**24**	↑	0.86*	nd	0.22
(DEF18)	peptide precursor	**48**	nd	0.13	nd	-0.07
CV793609	Elicitor-induced receptor	**24**	↑	0.20	nd	-0.03
(DEF19)	protein	**48**	↑	0.17	nd	-0.14
CV793610	Pathogenesis-related protein	**24**	↑	0.16	↑	0.28
(DEF20)	class 10	**48**	↑	0.09	↑	0.57

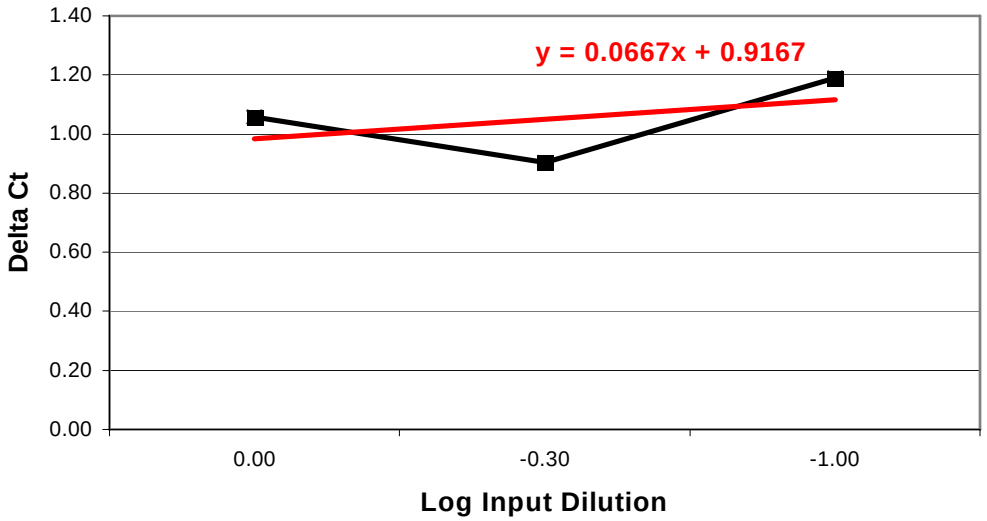

Figure 4.6 Example of a validation standard curve generated over a dilution series for target CV793597 (Pathogenesis-related protein 4A). The equation of the red linear trendline for the data shows that the absolute value of the slope is <0.1.

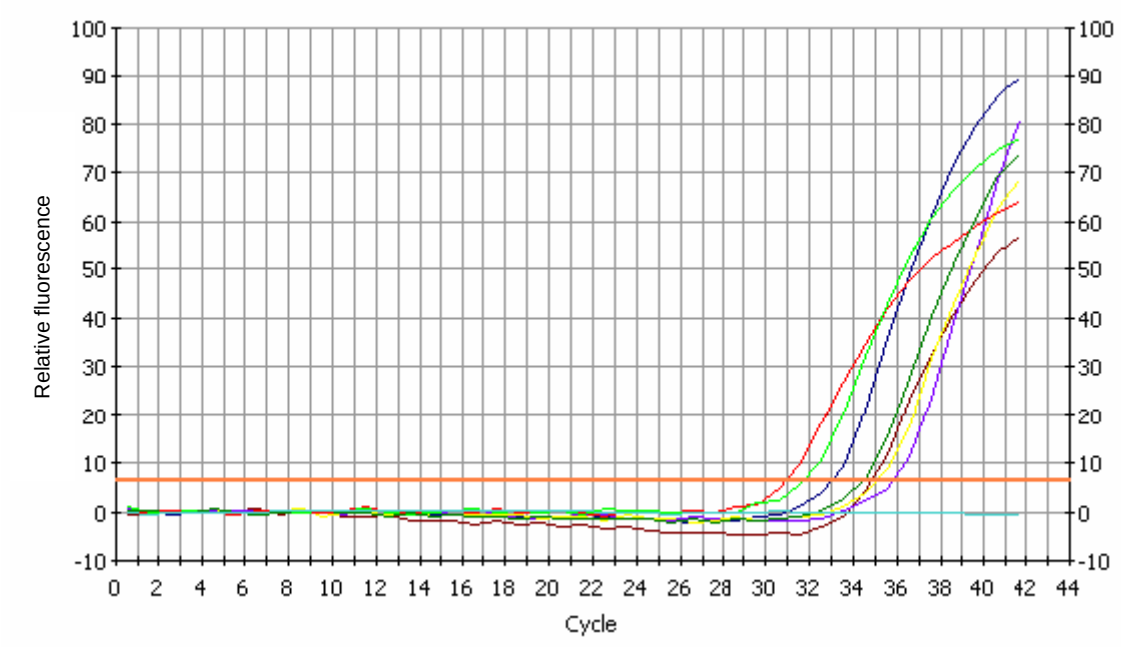

Figure 4.7 Example of amplification curves for seven samples (coloured lines) generated by the MyiQ™ instrument (BIO-RAD, Hercules, CA). The solid red line represents the threshold used to calculate C_T values (C_T was the cycle number where the amplification curve crossed the threshold line).

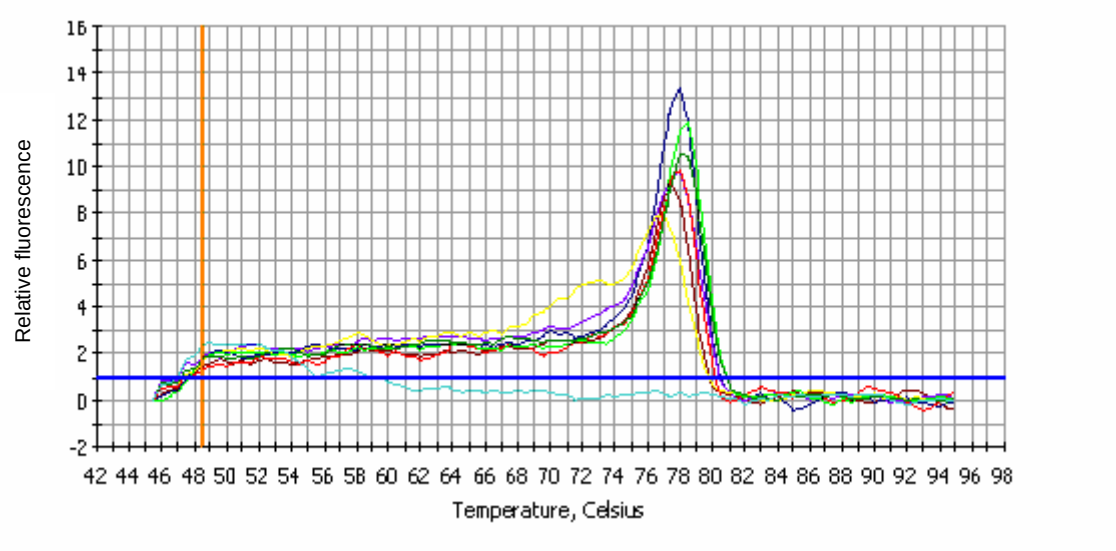

Figure 4.8 Example of melting curves for seven samples (coloured lines) generated by the MyiQ™ instrument (BIO-RAD, Hercules, CA). The presence of sharp single fluorescence peaks in this example indicated the presence of specific amplified products.

Table 4.7 summarises the qRT-PCR results and provides a comparison of the $\log_2$ mean FC ratio (relative to controls) from the microarray observations and the qRT-PCR results. Comparisons were made at 24, 48, and 72 hpi for each chickpea genotype. Differential expression observed in microarray analyses were confirmed by qRT-PCR in all cases but for *β*-1,3-glucanase (CV793598) at 72 hpi in IC. The overall patterns of up/down regulation were conserved for all seven EST targets in each time-point of each genotype, although the ratios observed for qRT-PCR were generally more exaggerated than those obtained for microarray analyses. A total of 63 comparisons between microarray and qRT-PCR fold-inductions were made (excluding all absent data), where 54 (86%) showed conserved direction of regulation. Of the nine contradictory comparisons, eight were due to ratios close to zero, and the only major contradiction was for the SNAKIN2 antimicrobial peptide precursor in FL at 24 hpi, which showed a ratio of 0.92 from qRT-PCR and –0.15 from microarray.

Table 4.7 Expression ratios of selected ESTs assessed by microarray and qRT-PCR. Array values indicate mean $\log_2$ fold change (FC) ratio relative to untreated controls and qRT-PCR values indicate $\log_2$ ratios of $2^{(\Delta C_t control/\Delta C_t treatment)}$. na indicates absence of valid data, whilst array values in bold and underline indicate DE ESTs after statistical analysis.

GenBank accession	**Putative function**	***IC***						***LA***					
		24 hpi		**48 hpi**		**72 hpi**		**24 hpi**		**48 hpi**		**72 hpi**	
		Array	**qPCR**	**Array**	**qPCR**	**Array**	**qPCR**	**Array**	**qPCR**	**Array**	**qPCR**	**Array**	**qPCR**
DY475157	Unknown	na	na	na	0.77	**0.75**	2.27	na	na	na	-1.11	-0.03	0.06
DY475186	Unclear	0.27	0.51	0.68	1.04	-0.77	-0.58	0.25	0.23	0.41	1.16	-1.04	-0.94
DY475248	Polymorphic antigen membrane protein	na	1.37	**1.02**	2.32	-0.58	-0.13	0.31	0.56	na	0.58	0.70	1.08
DY475259	Unclear	0.02	-0.04	na	-0.16	-0.01	0.12	0.22	0.25	na	0.25	0.27	0.18
CV793597	Pathogenesis-related protein 4A	**0.95**	2.37	0.81	2.27	-0.17	-0.13	**0.91**	2.35	**1.80**	3.45	**1.20**	2.98
DY396305	β-1,3-glucanase	0.39	1.13	**0.87**	2.47	**-0.79**	-0.57	-0.79	-2.15	na	na	na	na
CV793608	SNAKIN2 antimicrobial peptide precursor	0.86	2.26	0.13	0.40	-0.16	0.03	0.22	0.34	-0.06	-0.17	0.04	-0.04

		FL						***IL***					
		24 hpi		**48 hpi**		**72 hpi**		**24 hpi**		**48 hpi**		**72 hpi**	
		Array	**qPCR**	**Array**	**qPCR**	**Array**	**qPCR**	**Array**	**qPCR**	**Array**	**qPCR**	**Array**	**qPCR**
DY475157	Unknown	0.31	0.17	na	0.60	0.06	-0.01	-0.16	-0.54	na	-0.16	na	na
DY475186	Unclear	**-1.25**	-1.55	**0.90**	1.81	0.04	0.08	1.22	2.31	0.16	0.12	-0.67	-1.06
DY475248	Polymorphic antigen membrane protein	na	-0.27	-0.67	-2.66	na	na	0.19	0.41	**-0.69**	-1.82	na	na
DY475259	Unclear	na	-0.18	na	-0.21	-0.16	-0.31	0.09	-0.10	na	0.05	0.04	-0.01
CV793597	Pathogenesis-related protein 4A	**1.09**	3.00	-0.20	-0.14	0.16	0.45	**1.56**	3.64	-0.49	-0.30	**1.15**	1.72
DY396305	β-1,3-glucanase	0.63	1.74	-0.61	-1.06	na	na	-0.37	-0.98	-0.67	-2.44	na	na
CV793608	SNAKIN2 antimicrobial peptide precursor	-0.15	0.92	0.13	0.10	0.06	0.04	0.13	0.24	0.09	0.43	0.11	0.44

4.3.5 Cluster analysis of microarray data

Overall, the microarray analysis of chickpea and grasspea cDNAs led to the identification of large groups of *A. rabiei* modulated ESTs in each chickpea genotype surveyed. To identify common expression kinetics among the DE ESTs for each genotype, a non-redundant list of all DE ESTs from at least one time-point in any of the four chickpea genotypes was generated. For each of the 97 non-redundant DE ESTs, mean $\log_2$ expression ratios of *A. rabiei*-treated versus uninoculated control expression values from each time-point of each genotype were built into a single dataset. Separate *k*-means clustering analyses were then applied to the dataset for each genotype.

Firstly, Figure of Merit (FOM) was applied to determine the optimal number of clusters for the *k*-means algorithm for each genotype. FOM is a measure of the fit of expression patterns for the clusters produced by a particular algorithm, and estimates the predictive power of the clustering algorithm for a maximum of *n* clusters (Saeed *et al.*, 2003). FOM is computed by removing each sample in turn from the data set, clustering based on the remaining data and calculating the fit of the withheld sample to the clustering pattern obtained for the other samples (Saeed *et al.*, 2003). Adjusted FOM values were given for each number of *n* clusters up to a maximum of $n = 20$, where lower FOM values indicate higher predictive power. The FOM outputs for each genotype showed steep increases in predictive power up to a certain *n* clusters before levelling out. To determine the optimal *n* clusters for each genotype in this study, the step decreases in adjusted FOM values were assessed for each increase in *n* clusters, and the optimal *n* was selected as the point before FOM value step decrease became <0.05 (Table 4.8). This resulted in cluster numbers of seven for IC, eight for LA, six for FL and seven for IL. *K*-means clustering with Euclidean metrics was then applied to each genotype using the optimal *n* clusters (Figures 4.9 – 4.12; Appendix 7 for full cluster data).

Table 4.8 Mean FOM values (10 iterations) obtained for *n* clusters for each chickpea genotype. The point before step decrease in mean FOM became <0.05 was regarded the optimal *n*, marked with an *.

IC			***LA***		
Clusters (*n*)	**Mean FOM value**	**Step decrease**	**Clusters (*n*)**	**Mean FOM value**	**Step decrease**
1	2.42	na	1	1.97	0
2	2.00	-0.42	2	1.54	-0.43
3	1.82	-0.18	3	1.39	-0.15
4	1.68	-0.14	4	1.28	-0.11
5	1.58	-0.10	5	1.21	-0.07
6	1.50	-0.08	6	1.14	-0.07
7*	1.42	-0.08	7	1.09	-0.05
8	1.38	-0.04	**8***	1.04	-0.05
9	1.33	-0.05	9	1.02	-0.02
10	1.31	-0.02	10	0.98	-0.04

FL			***IL***		
Clusters (*n*)	**Mean FOM value**	**Step decrease**	**Clusters (*n*)**	**Mean FOM value**	**Step decrease**
1	2.14	0	1	1.86	0
2	1.78	-0.36	2	1.57	-0.29
3	1.52	-0.26	3	1.38	-0.19
4	1.40	-0.12	4	1.29	-0.09
5	1.31	-0.09	5	1.22	-0.07
6*	1.23	-0.08	6	1.15	-0.07
7	1.19	-0.04	**7***	1.09	-0.06
8	1.16	-0.03	8	1.06	-0.03
9	1.12	-0.04	9	1.02	-0.04
10	1.09	-0.03	10	0.98	-0.04

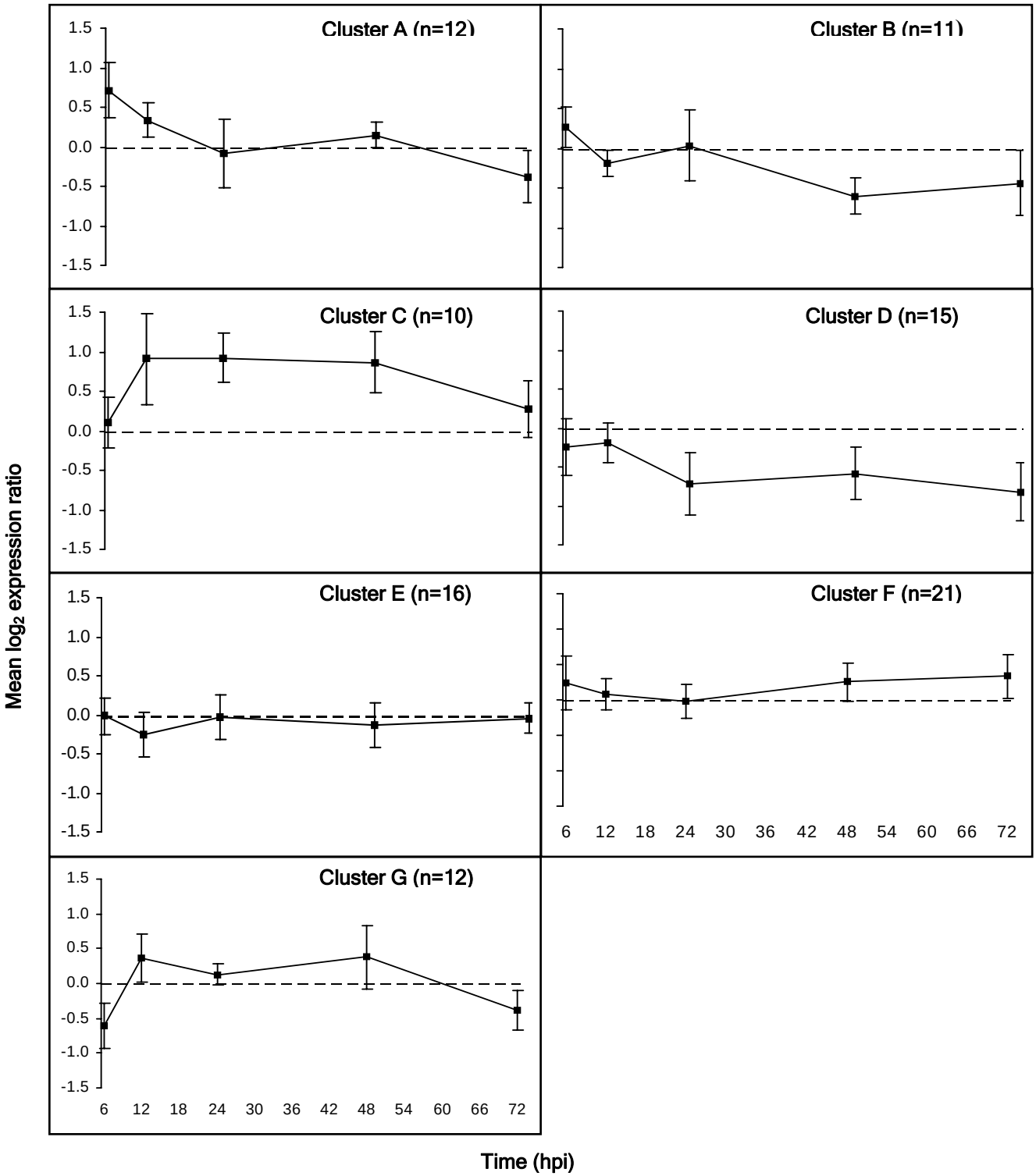

Figure 4.9 Cluster analysis of expression profiles for the 97 DE ESTs in IC after inoculation with *A. rabiei*. ESTs were grouped into clusters by applying the *k*-means algorithm to the dataset of mean $\log_2$ expression ratios measured at different post-inoculation time-points relative to control samples. For each cluster, graphs represent the mean expression of cluster members and standard error at each time-point measured. The number of ESTs in each cluster (n) is indicated.

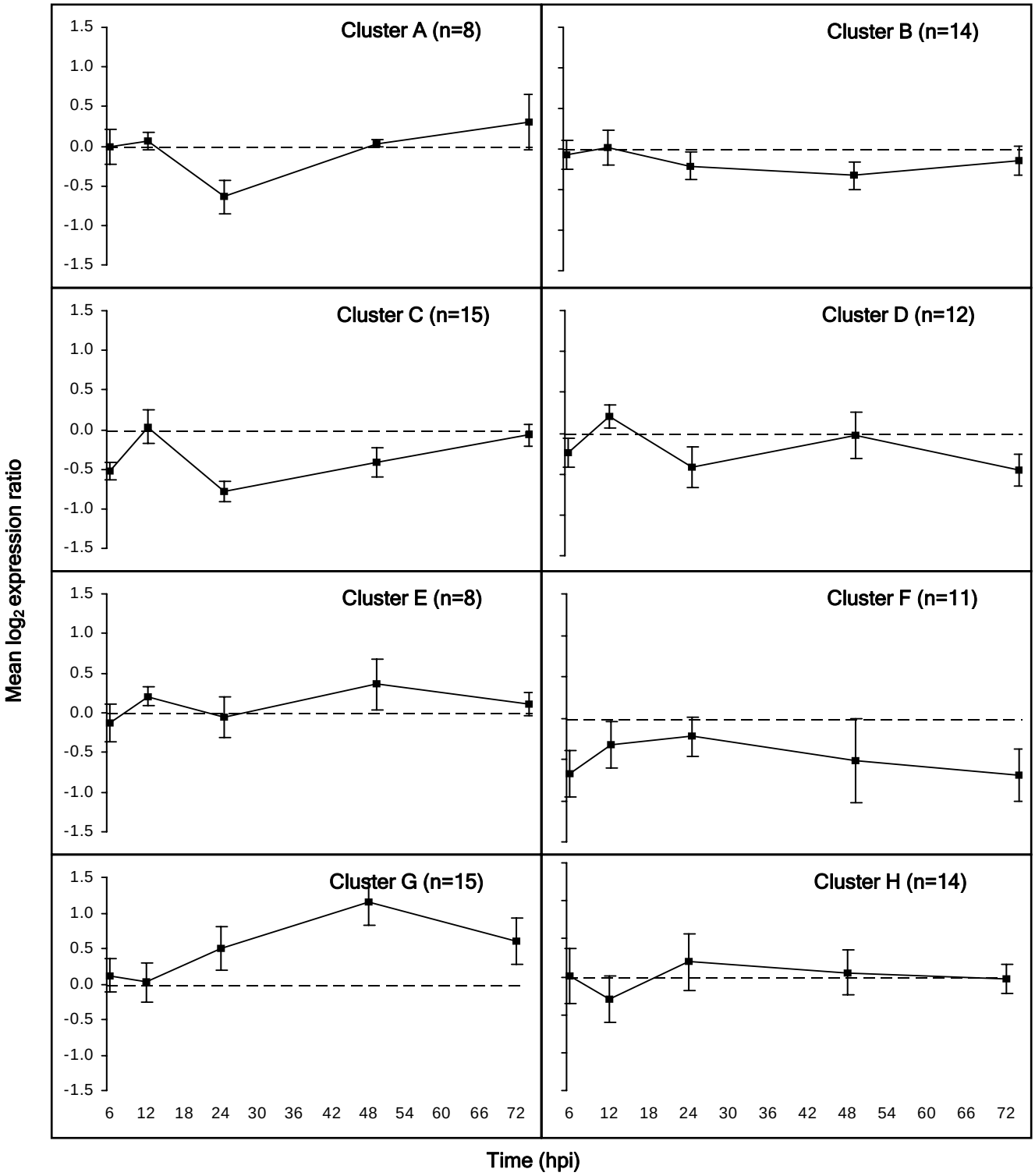

Figure 4.10 Cluster analysis of expression profiles for the 97 DE ESTs in LA after inoculation with *A. rabiei*. ESTs were grouped into clusters by applying the *k*-means algorithm to the dataset of mean $\log_2$ expression ratios measured at different post-inoculation time-points relative to control samples. For each cluster, graphs represent the mean expression of cluster members and standard error at each time-point measured. The number of ESTs in each cluster (n) is indicated.

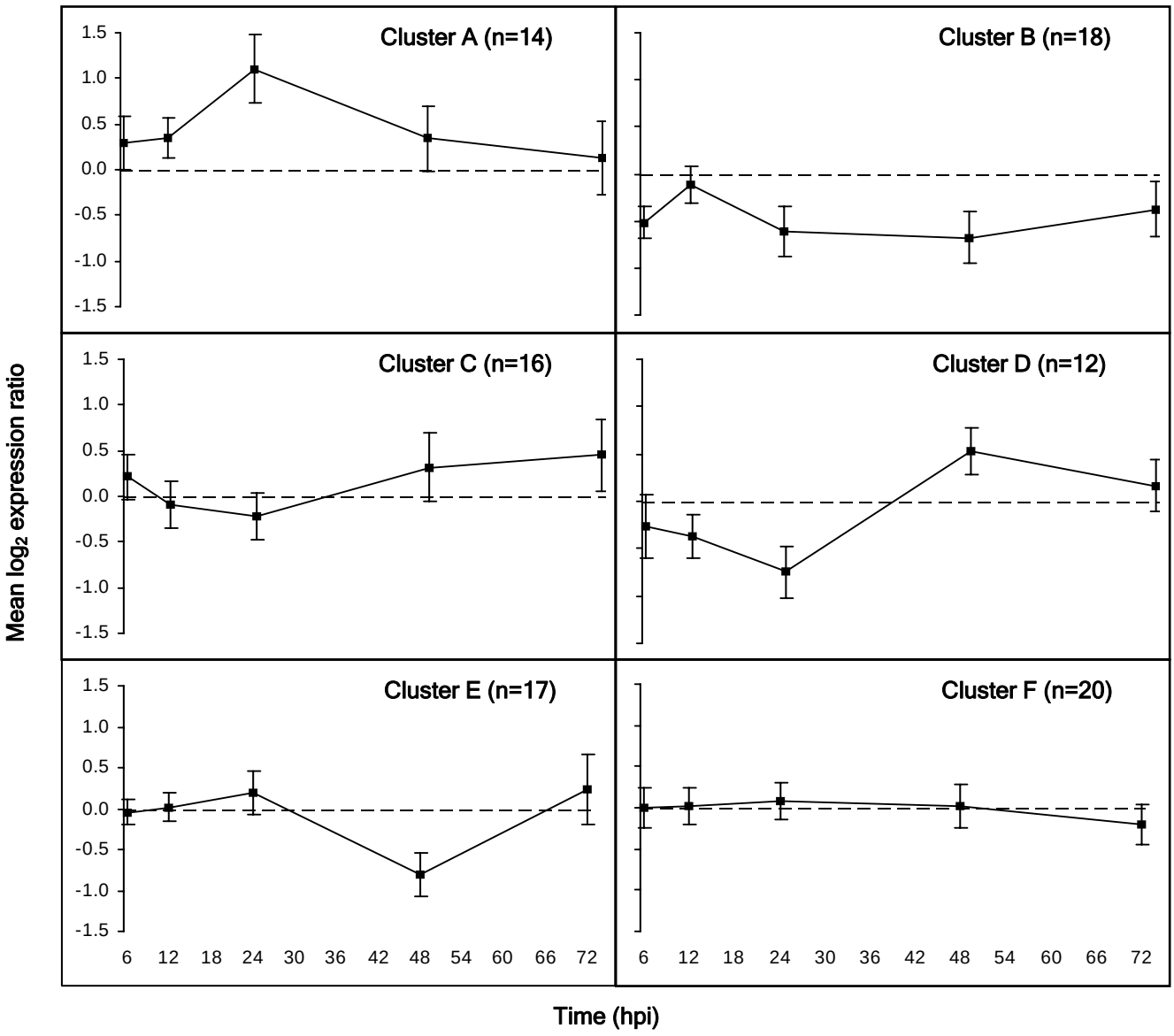

Figure 4.11 Cluster analysis of expression profiles for the 97 DE ESTs in FL after inoculation with *A. rabiei*. ESTs were grouped into clusters by applying the *k*-means algorithm to the dataset of mean $\log_2$ expression ratios measured at different post-inoculation time-points relative to control samples. For each cluster, graphs represent the mean expression of cluster members and standard error at each time-point measured. The number of ESTs in each cluster (n) is indicated.

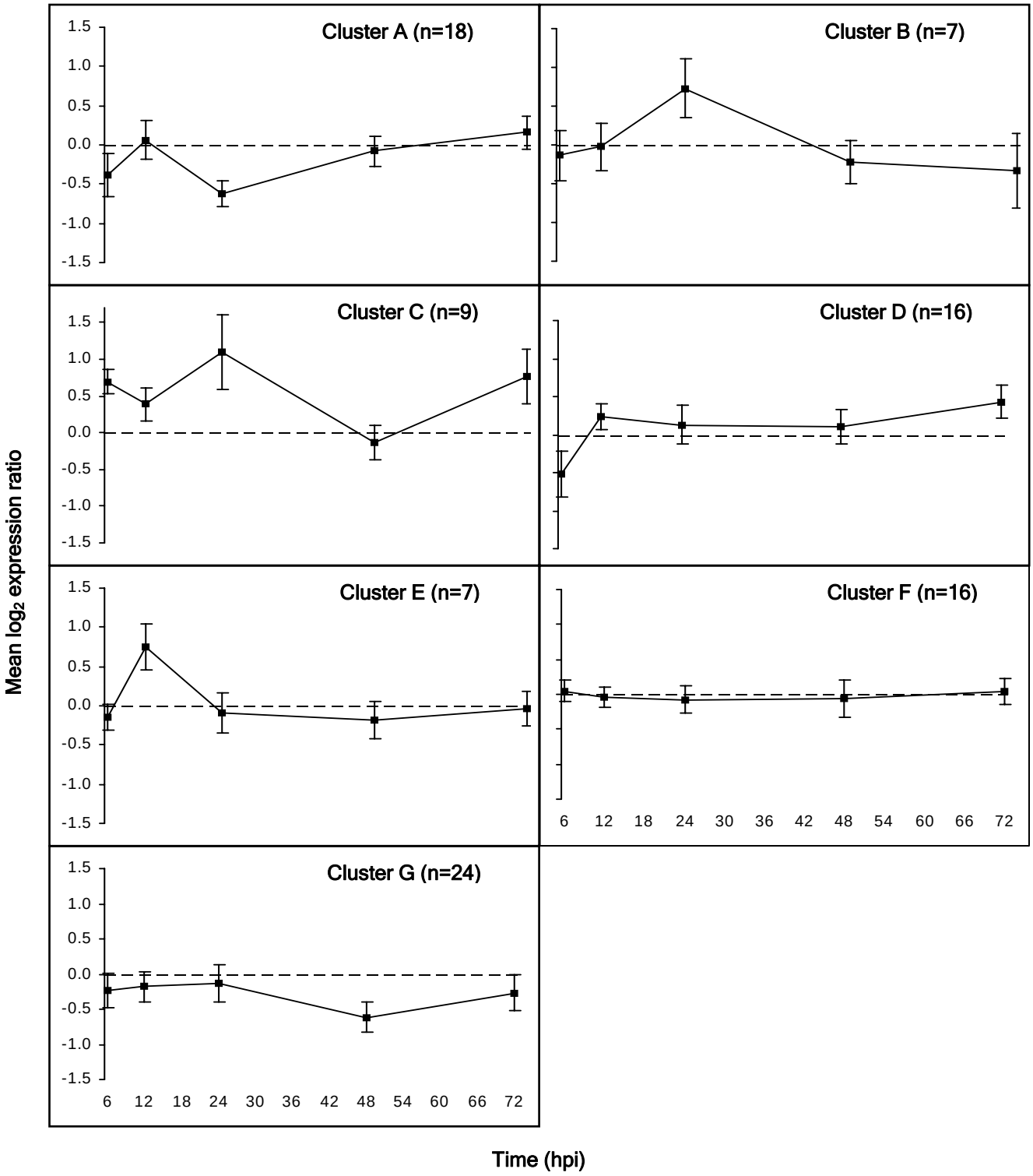

Figure 4.12 Cluster analysis of expression profiles for the 97 DE ESTs in IL after inoculation with *A. rabiei*. ESTs were grouped into clusters by applying the *k*-means algorithm to the dataset of mean $\log_2$ expression ratios measured at different post-inoculation time-points relative to control samples. For each cluster, graphs represent the mean expression of cluster members and standard error at each time-point measured. The number of ESTs in each cluster (n) is indicated.

4.3.5.1 IC clusters

Cluster C was formed by transcripts that were up-regulated for the majority of the time-course, and included several PR proteins, disease resistance response protein DRRG49-C, and a hypothetical proline-rich protein. Cluster D contained down-regulated transcripts (24-72 hpi) such as a nematode resistance protein, several metabolic enzymes, abiotic stress-related proteins (including wound-induced protein, phosphate-induced protein, and proline oxidase), and cellular communication proteins (including a transmembrane protein and protein kinase). Cluster A contained transcripts that were induced early (6-12 hpi) after *A. rabiei* inoculation, including chlorophyll a/b binding protein (energy), several unknown proteins, and several ribosomal RNAs involved in protein synthesis. Cluster F showed slight up-regulation at 48-72 hpi, and included numerous unknown transcripts, abiotic stress-inducible proteins, ribosomal RNAs and a GTP-binding protein (cellular communication). Cluster B contained putative defence-related proteins (*Avr9/Cf9* rapidly-elicited protein, S1-3 homolog, and flavonol glucosyl transferase) that showed late (48-72 hpi) down-regulation, as well as other metabolic and cellular respiration-related proteins. Cluster G transcripts showed early down-regulation (6 hpi) followed by slight up-regulation (12-48 hpi) and late down-regulation (72 hpi), and included the PR protein β-1,3-glucanase, transcription factor EREBP-4, several unknown proteins, and ripening-related proteins. Finally, cluster E showed relatively little change in expression, and included an abiotic stress-inducible protein, cellular communication/transport proteins (including sorting nexin protein and extensin-like protein), as well as numerous metabolic proteins.

4.3.5.2 LA clusters

Cluster G contained transcripts that were up-regulated from 24-72 hpi and mainly included putative defence-related, unknown, and cellular communication/transport proteins. Down-regulated transcripts were found in cluster F (6-72 hpi), examples being transcription factor

EREBP-4, metabolic proteins, unknown proteins, abiotic stress-inducible proteins, and a ribosomal RNA protein (protein synthesis). Other clusters containing down-regulated transcripts were clusters A (24 hpi), B (24-48 hpi), C (6, 24-48 hpi), D (24, 72 hpi), and H (12 hpi), which contained putative defence-related ESTs (SNAKIN2 antimicrobial peptide precursor, flavonol glucosyl transferase, S1-3 protein homolog, glutathione S-transferase, *β*-1,3-glucanase, *Avr9/Cf9* rapidly elicited protein, leucine-zipper protein, and nematode resistance protein), cellular communication/transport proteins (protein kinase, extensin-like protein, nitrate transporter, and zinc finger protein), abiotic stress-inducible proteins, metabolic/cellular respiration proteins, ribosomal RNAs and several unknown proteins. Finally, cluster E contained transcripts that were slightly up-regulated (48 hpi), such as a GTP-binding protein and GPI-anchored membrane protein (cellular communication), as well as unknown and metabolic proteins.

4.3.5.3 FL clusters

Transcripts up-regulated early after *A. rabiei* inoculation (6-48 hpi) were found in cluster A, and included PR proteins, an environmental stress-inducible protein, a proline-rich protein, unknown proteins, and a chloroplast mRNA. Late up-regulated transcripts (48-72 hpi) belonged to cluster C, examples being a GTP-binding protein (cellular communication), several unknown proteins, as well as cellular respiration and metabolic proteins (including ATP synthase and ribosomal RNAs). Members of cluster D were down-regulated early (6-24 hpi) before showing up-regulation at 48 hpi, and included putative ripening related proteins, an 18S and 26S rRNA (protein synthesis), an RNA/ssDNA binding protein (cell cycle and DNA processing), and unknown proteins. Cluster B (6, 24-72 hpi) and E (48 hpi) were formed of down-regulated transcripts including putative defence-related proteins (flavonol glucosyl transferase, S1-3 protein homolog, glutathione S-transferase, *β*-1,3-glucanase, leucine-zipper protein, and nematode resistance protein), cellular communication/transport

proteins (extensin-like protein, transmembrane protein, GPI-anchored membrane protein, polymorphic antigen membrane protein, and nitrate transporter protein), abiotic stress-inducible proteins (including phosphate-induced protein), several metabolic and cellular respiration proteins (including chlorophyll a/b binding protein, NADH dehydrogenase, ATP synthase, and a thylakoid protein), as well as unknown proteins. Finally, cluster F was made up of transcripts with relatively unaltered expression over the time-course, and mainly consisted of metabolic proteins, but also included putative defence-related proteins (SNAKIN2 antimicrobial peptide precursor and disease resistance response protein DRRG49-C), cellular communication/transport proteins (EREBP-4, sorting nexin protein), and abiotic stress-inducible proteins.

4.3.5.4 IL clusters

Transcripts up-regulated for the majority of the post-inoculation time-course belonged to cluster C, mainly consisting of putative defence-related proteins (PR proteins and disease resistance response protein DRRG49-C), but also containing a NADH dehydrogenase. Cluster E was formed of transcripts up-regulated early (12 hpi) after *A. rabiei* inoculation, such as a polymorphic antigen membrane protein (cellular communication), an 18S ribosomal RNA, metabolic enzymes, and unknown proteins. Transcripts slightly up-regulated at 24 hpi belonged to cluster B, and included two putative defence-related proteins (flavonol glucosyl transferase and S1-3 protein homolog), a histone H2A protein (cell cycle and DNA processing), as well as metabolic and protein synthesis proteins. Transcripts of cluster D were down-regulated at 6 hpi followed by up-regulation at 72 hpi, including the *Avr9/Cf9* rapidly elicited protein (defence), proline-rich protein (defence), cellular communication proteins (EREBP-4 and GTP-binding protein), metabolic proteins, protein synthesis-associated rRNAs, abiotic stress-inducible proteins, and unknown proteins. Down-regulated transcripts were observed in clusters A (6, 24 hpi) and G (48 hpi), which consisted of putative defence-

related proteins (glutathione S-transferase, *β*-1,3-glucanase, leucine-zipper protein, and nematode resistance protein), cellular communication/transport proteins (extensin-like protein, transmembrane protein, GPI-anchored membrane protein, sorting nexin protein, zinc finger protein, and nitrate transporter protein), abiotic stress-inducible proteins (including phosphate-induced protein), several metabolic and cellular respiration proteins (including proline oxidase, chlorophyll a/b binding protein, ATP synthase, and photosystem II reaction center proteins), as well as unknown proteins. The transcripts of the final cluster F were all unchanged in their expression, and included the SNAKIN2 antimicrobial peptide precursor (defence), cellular communication proteins (including a protein kinase), abiotic stress-induced proteins, metabolic enzymes, and unknown proteins.

4.3.6 ESTs differentially expressed between chickpea genotypes

The availability of mean expression profiles and 95% confidence intervals of DE ESTs for each genotype enabled a comparison between genotypes to identify potentially important *A. rabiei*-inducible genes in the resistant genotypes compared to the susceptible LA genotype. Although direct hybridisations between targets of different genotypes at different time-points were not performed, a comparison between expression profiles still provided a guide to transcripts whose expression was significantly altered, in reference to controls, in one genotype compared to another.

A total of seven putative PR proteins were identified as DE in this study (CV793597, DY396301, DY396384, DY396388, DY396372, DY396305 and DY396281), representing six cDNAs from grasspea and one chickpea unigene. The expression patterns of all seven PR proteins were similar within each genotype and, by calculating the average of the mean $\log_2$ expression ratios of all seven at each time-point, it was possible to identify common expression kinetics for the PR proteins in each genotype (Figure 4.13). PR protein expression

peaked at 12 hpi in IC, 24 hpi in FL and IL, and at 48 hpi in LA. LA also displayed a significantly lower level of PR protein induction to all other genotypes at 12-24 hpi.

A comparison of the expression profiles for SNAKIN2 antimicrobial peptide precursor (CV793608) (Figure 4.14) showed that the *A. rabiei*-resistant IC significantly induced the transcription of this gene at 24 hpi compared to relatively unaltered expression in the other genotypes. A hypothetical proline-rich protein (PRP) (DY396288) (Figure 4.15) showed significant induction in FL (24 and 72 hpi) and IC (48 hpi) compared to other genotypes at these time-points. Other significant comparisons of putative 'defence' category ESTs included exclusive up-regulation of a leucine-zipper containing protein (LZP) (CV793599) in IC at 24 hpi, β-1,3-glucanase (CV793598) in IC at 48 hpi, and disease resistance response protein DRRG49-C (DY396265) in IL (24 hpi) and IC (12 and 24 hpi).

Important comparisons from other functional categories included distinct up-regulation of a polymorphic antigen membrane protein (PAMP) (DY475248) at 12 hpi in IL and 48 hpi in IC, up-regulation of an environmental stress-inducible protein (ESP) (DY396298) in FL at 24 hpi, a putative Ca-binding carrier protein (DY396262) in IC (72 hpi), and down-regulation of the antioxidant proteins glutathione S-transferase (GST) (DY475250) in IC (72 hpi) and superoxide dismutase copper chaperone precursor (SDCC) (DY475397) in FL (6 hpi), IC (24 hpi) and LA (6 hpi). A serine/threonine protein kinase (DY475384) was also down-regulated in FL (48 hpi) and IC (24 and 72 hpi), whilst a chlorophyll a/b binding protein (DY475534) was found to be significantly up-regulated in IC (6-12 hpi) but down-regulated in LA (6 hpi). Several unknown/unclear ESTs were also up-regulated in each genotype, and *k*-means clustering provided a guide their possible function (Table 4.9).

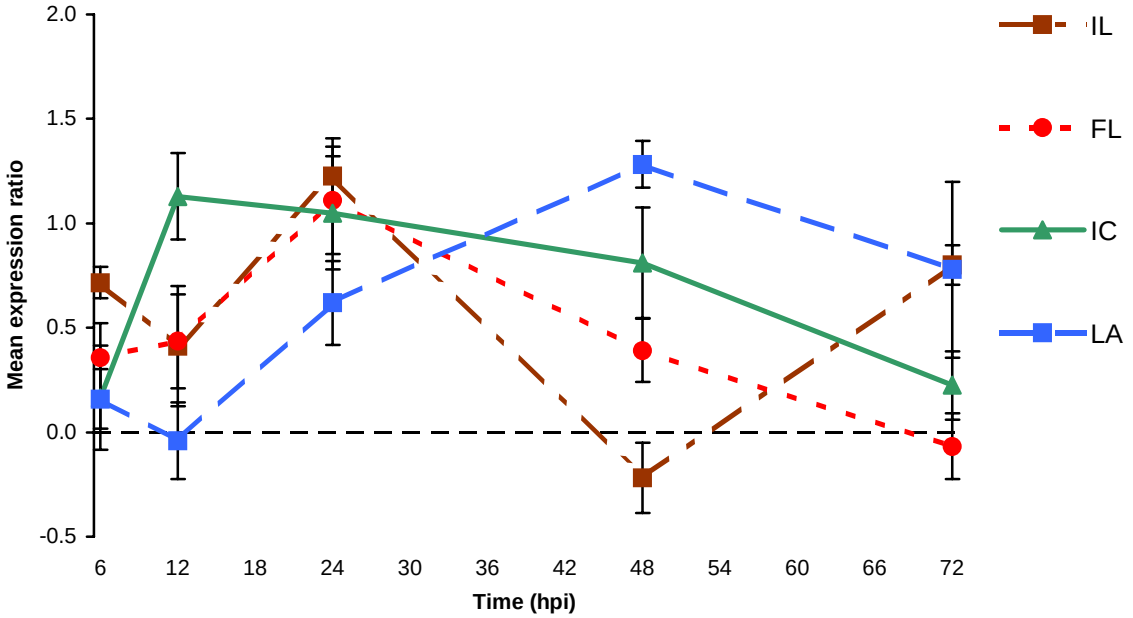

Figure 4.13 Mean (log_2) expression profiles observed for the seven DE pathogenesis-related proteins in each chickpea genotype after inoculation with *A. rabiei*, calculated as the mean expression ratio of all seven features in each genotype at each time-point. Error bars represent the 95% confidence interval for the mean.

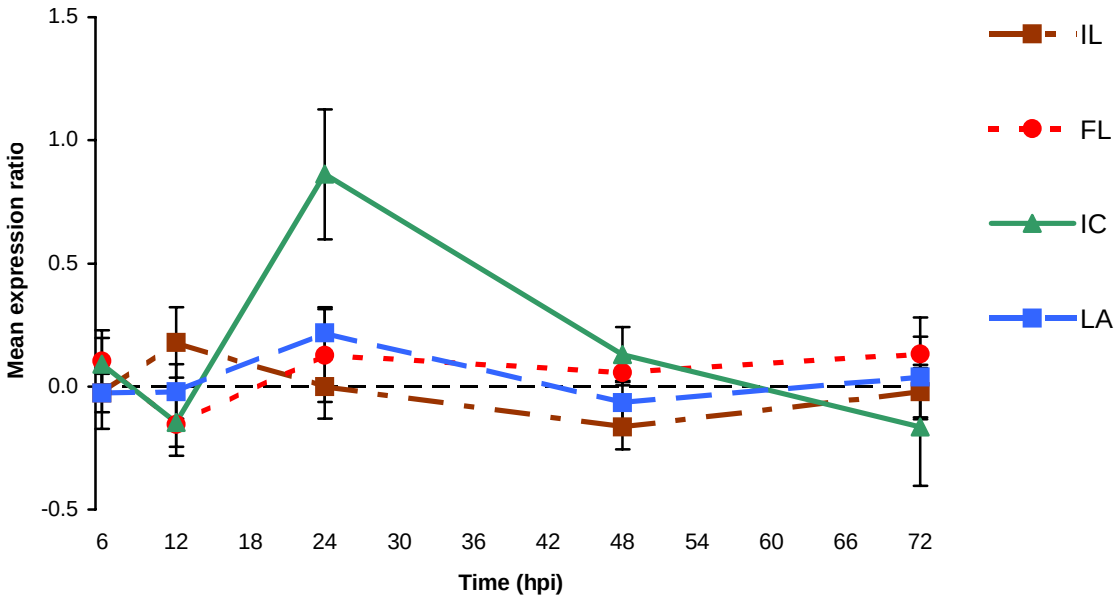

Figure 4.14 Mean (log_2) expression profiles observed for SNAKIN2 antimicrobial peptide precursor in each chickpea genotype after inoculation with *A. rabiei*. Error bars represent the 95% confidence interval for the mean.

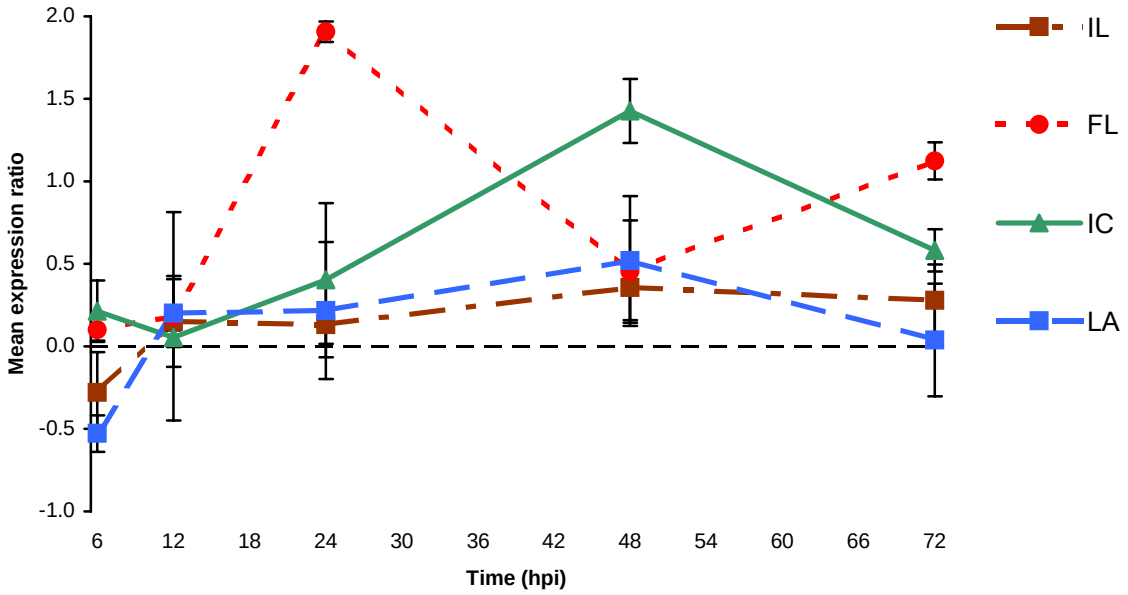

Figure 4.15 Mean (log_2) expression profiles observed for hypothetical proline-rich protein in each chickpea genotype after inoculation with *A. rabiei*. Error bars represent the 95% confidence interval for the mean.

Table 4.9 Summary of the unknown/unclear ESTs up-regulated by *A. rabiei* inoculation in each genotype, and observations from *k*-means clustering.

GenBank accession	Genotype (time-point)	Clustered with
DY475532	IC (48 hpi)	Defence-related/stress-inducible ESTs (cluster G)
DY475157	IC (72 hpi)	Metabolic/stress-inducible ESTs (cluster F)
DY475533	IC (72 hpi)	Metabolic/stress-inducible ESTs (cluster F)
DY475322	LA (24 hpi)	Defence-related ESTs (cluster H)
DY475483	LA (24 hpi)	Defence-related ESTs (cluster G)
DY475217	FL (24 hpi)	Defence-related ESTs (cluster A)
DY475186	FL (48 hpi)	Stress-inducible ESTs (cluster D)
DY475462	FL (48 hpi)	Metabolic ESTs (cluster C)
DY475323	FL (48 hpi)	Metabolic ESTs (cluster C)
DY475084	IL (12 hpi)	Defence-related/metabolic ESTs (cluster E)
DY475365	IC (24 hpi)	Metabolic/stress-inducible ESTs (cluster F)
	LA (24 hpi)	Defence-related ESTs (cluster G)
	FL (24 hpi)	Defence-related ESTs (cluster A)

4.4 Discussion

Large-scale transcriptional responses to pathogens in chickpea have not previously been documented. As reported in Chapter 1 (section 1.5), the generation of limited collections of ESTs for use in the development of 'boutique' microarrays has become a widely used strategy in large-scale transcriptional profiling, although they may only provide partial information regarding the system under study. The 768-feature microarrays constructed in this study were exploited to profile the defence responses of three chickpea and one wild relative genotype to *A. rabiei* inoculation over a time-course. However, considering the diversity of functional categories represented by the ESTs, the chickpea and grasspea EST resources could be applied widely, for example, to study gene expression associated with grain quality, and in response to other economically important diseases such as fusarium wilt (*Fusarium oxysporum* f.sp. *ciceri*).

An experimental system was implemented that minimised environmental effects and was representative of ascochyta blight in the field. A high level of data quality and reproducibility was achieved through the use of biological and technical replication, the inclusion of negative controls, and strict selection criteria for DE genes. The observations from the microarray data were validated by qRT-PCR for seven genes showing varying levels of regulation, indicating the strong reliability of the microarray data. Although the expression ratio data obtained by qRT-PCR was higher than that for corresponding microarray ratios in many cases, regulation trends were still strongly correlated. Such a phenomenon has also been reported in other studies (Dowd *et al.*, 2004; Lopez *et al.*, 2005).

It was expected that the chickpea targets in this study would hybridise to the grasspea and lentil probes, and the levels of undetected features for each probe source (Table 4.1) showed a high level of hybridisation for *C. arietinum*-*C. arietinum* target and probe. Theoretically, IC

targets should have hybridised to all chickpea probes considering that they were derived from this genotype. However, three undetected probes were observed, all of which possessed 'unknown' function. It is possible that these three cDNAs were unintentionally derived from *A. rabiei* mRNA during construction of the cDNA library, but even if that were true, such a low level of potentially contaminating *A. rabiei* cDNAs suggests that the overall library is of high quality. Alternatively, the undetected features may have been due to the stringent criteria for identifying positive spots or a printing failure for those cDNAs. A slightly lower level of hybridisation to the *C. arietinum* probes was observed for the wild-relative chickpea, which may be attributed to IL being a different species to IC, LA and FL. The hybridisation to grasspea probes, although lower than to chickpea probes, was very high and demonstrated the ability to cross-hybridise between closely related species (refer to section 1.5.1). However, hybridisation to all lentil probes failed, possibly due to insufficient homology with the chickpea targets. Alternatively, amplification of the lentil RGA probes from genomic DNA may have resulted in the inclusion of non-coding regions and, retrospectively, these probes should have been amplified from RNA.

In this study 97 of the 715 (13.6%) chickpea and grasspea derived cDNAs evaluated by microarray were differentially expressed in at least one genotype. Individually, 9.5% were DE in IC, 6.4% in LA, 6.7% in FL and 4.2% in IL. Considering that the probes were not sourced from SSH libraries (refer to section 2.1) and did not exclusively represent defence-related ESTs, these levels indicate a considerable number of genes differentially expressed in response to pathogen attack, especially for IC. Comparison of expression regulation for the time-points (24 and 48 hpi) and genotypes (IC and LA) that overlap between this study and a the previous small-scale study of defence-related cDNAs (refer to Chapter 3) revealed that the regulation trends from this study were in line with the observations of the previous study. However, less of the defence-related cDNAs were regarded as DE in this study, which may be

attributed to the higher number of technical/biological replications of this study that acted to reduce the false positive rate, or the use of a stringent global Lowess normalisation protocol to generate more conservative FC ratios (Clarke and Zhu, 2006).

The kinetics of differential expression (Figure 4.5) after *A. rabiei* inoculation showed a larger proportion of down-regulated ESTs for each genotype, which may be ascribed to the transcriptional sacrifice of numerous 'housekeeping' and general metabolic ESTs included on the array in favour of the potential defence-related ESTs that were mainly up-regulated in each genotype (Mysore *et al.*, 2003; Dowd *et al.*, 2004; Gibly *et al.*, 2004; Lopez *et al.*, 2005). The majority of differential expression was observed from 24-72 hpi, which may be attributed to the use of chickpea and grasspea probes derived from cDNA libraries that were generated from 24-48 hpi (chickpea) and 48-72 hpi (grasspea) tissue (Skiba *et al.*, 2005). Alternatively, the kinetic trends may represent the timing of pathogen recognition and subsequent transcriptional changes associated with the *A. rabiei* defence response in each genotype. It has been shown that *A. rabiei* spores germinate by 12 hpi (Pandey *et al.*, 1987) and penetrate by 24 hpi (Kohler *et al.*, 1995), followed by autofluorescence and synthesis of antifungal compounds at 24-48 hpi (Hohl *et al.*, 1990). The transcriptional changes observed in this study do fit this time-line, where early (6-12 hpi) changes may reflect initial responses following recognition of pathogen contact, and the major responses at 24-48 hpi correlate with observed timings of pathogen penetration and signalling cascades that result in an oxidative burst, induction of an HR, and synthesis of antifungal proteins.

Other studies of post-inoculation gene expression also report rapid changes in gene expression over the first 48 hours (Dowd *et al.*, 2004; Gibly *et al.*, 2004; Moy *et al.*, 2004). The greater proportion of differential expression observed in IC at 6-12 hpi may indicate rapid pathogen recognition in comparison to LA and FL. Additionally, the irregular kinetic pattern observed

for the wild relative (IL) may be the result of alternative defence mechanisms, possibly including strong passive and/or basal defences. It is important to recognise that different levels of basal expression between genotypes could not be determined by this study, as hybridisations were not performed between control samples. Resistant chickpea genotypes have been shown to possess increased basal levels of certain defence-related compounds, such as copper amine oxidase, in comparison to susceptible types (Laurenzi *et al.*, 2001; Rea *et al.*, 2002). Alternatively, the irregular kinetic pattern of IL may demonstrate a weakness of the 'boutique' array for studying a species different to that used as the probe source (refer to section 1.5.1). Because the chickpea probes were constructed from IC cDNA, the array could only reveal expression patterns for genes in common between IC and IL. Therefore, IL may possess unique defence mechanisms that cannot be detected by this array.

In this study, IC was the most resistant genotype to *A. rabiei*, FL and IL were moderately resistant, and LA was classified as susceptible. Therefore it is reasonable to infer that the observed transcriptional responses of IC, IL and FL may involve potentially effective genes for *A. rabiei* resistance, whilst genes of the LA response may be ineffective. Considering this, comparisons of defence-related gene expression between genotypes revealed that the resistant genotypes significantly induce the transcription of several putative PR proteins at an earlier time-point than LA (Figure 4.13). *K*-means clustering revealed the rapid activation of these antifungal compounds in resistant genotypes, which may contribute significantly to their ability to defend against infection. It has been shown in other species that plant susceptibility may result from a delay in defence responses (Yang *et al.*, 1997).

The specific microarray expression profile of the PR protein β-1,3-glucanase also revealed exclusive up-regulation in the *A. rabiei*-resistant genotype IC, whilst qRT-PCR confirmed this expression and also indicated up-regulation in FL. β-1,3-glucanase has previously been

implicated in chickpea resistance to *A. rabiei* (Hanselle and Barz, 2001; Cho and Muehlbauer, 2004), and may possess a role in effective resistance. However, Chapter 3 also reported this EST to be up-regulated in the *A. rabiei* susceptible LA. Although *β*-1,3-glucanase was slightly up-regulated in LA in the present study it was not classified as DE for possible reasons discussed earlier. Considering the increased robustness of this experimental system and the observation that *β*-1,3-glucanase was not up-regulated in LA by qRT-PCR, the result of Chapter 3 may have been a false positive.

The SNAKIN2 antimicrobial peptide precursor included in this study was previously shown to be up-regulated in response to *A. rabiei* infection (refer to Chapter 3). The microarray (Figure 4.14) and qRT-PCR (Table 4.7) results of the present study confirmed that this transcript is significantly up-regulated by the *A. rabiei*-resistant genotype IC. Considering that IC is the most resistant genotype used, this protein may be integral to the resistance mechanism. As reported in Chapter 3, SNAKIN2 proteins are antimicrobial peptides induced by pathogen infection. SNAKIN2 also synergistically accumulates with SNAKIN1, a protein with which it shares only 38% sequence similarity (Berrocal-Lobo *et al.*, 2002). Therefore, the up-regulation of SNAKIN2 in IC may also be associated with an up-regulation of a yet to be isolated SNAKIN1-like protein. qRT-PCR also found SNAKIN2 to be up-regulated at 24 hpi in FL (Table 4.7). Discrepancies between results from microarray and qRT-PCR are widely recognised and attributed to cross hybridisation of gene family members on microarrays, differences in hybridisation on array surfaces rather than in qRT-PCR solutions, and better quantitation of low abundance transcripts by RT-PCR (Salzman *et al.*, 2005). Subsequently, the observed difference between microarray and qPCR results for SNAKIN2 could be due to a low copy number in FL, which caused its up-regulation to be undetected by microarray. Overall, qPCR data is considered more reliable than microarray data (Gachon *et*

al., 2004), thus further investigation of SNAKIN2 regulation in FL would be required to confirm any possible up-regulation.

This study also identified a hypothetical proline–rich protein (PRP) as being significantly up-regulated in IC and FL (Figure 4.15). PRPs are structural proteins of the primary cell wall, which are involved in the strengthening of cell walls to restrict pathogen penetration (Otte and Barz, 2000). In chickpea, the H_2O_2 from an elicitor-induced oxidative burst has been shown to directly control the insolubilisation of a PRP in cell walls (Otte and Barz, 1996). Considering that *A. rabiei* penetration has been shown to occur at 24 hpi (Kohler *et al.*, 1995), significant up-regulation of the PRP by 24 hpi in FL may act to effectively restrict penetration. Further, the up-regulation of this protein by 48 hpi in IC may not restrict initial penetration but could have an effect on restricting ongoing penetration of neighbouring cells. Assuming that the expression of this PRP is most likely mediated by the H_2O_2 from an oxidative burst, the production of reactive oxygen species (ROS) should occur before the observed up-regulation of the PRP.

ROS cause damage to both plant and pathogen cells, and the protection of uninfected plant cells is achieved through antioxidant proteins (Apel and Hirt, 2004). Evidence for an oxidative burst in this study is demonstrated by the expression profiles of superoxide dismutase copper chaperone precursor (SDCC) and glutathione S-transferase (GST). These proteins represent antioxidant compounds, whose down-regulation has been shown to be crucial for controlling the increase of cellular H_2O_2 levels leading to an HR (Fath *et al.*, 2001; Neill *et al.*, 2002; Apel and Hirt, 2004). SDCC was significantly down-regulated at 6 hpi in FL and LA, and at 48 hpi in IC. GST was down-regulated only in IC at 72 hpi. These results may suggest an early accumulation of H_2O_2 in FL and the *A. rabiei*-susceptible LA, which corresponds to the previously observed timing (24-48 hpi) of a subsequent HR in chickpea

(Hohl *et al.*, 1990). However, the oxidative burst in LA does not lead to up-regulation of a cell wall-strengthening PRP, possibly contributing to susceptibility. The later timing of SDCC and GST down-regulation in IC may be explained by the observation that, in resistant plants, ROS accumulation is biphasic, where a rapid burst is followed by a more prolonged burst (Baker and Orlandi, 1995). Therefore, the observed timing of SDCC and GST down-regulation in IC may correlate with a second burst of ROS production. The initial burst of ROS production in IC may have occurred before the first sampling time of this study, possibly indicating a very rapid pathogen recognition and signal transduction mechanism in IC. Considering that ROS also act as messengers to induce defence-related gene expression (refer to section 1.4.1), the rapid up-regulation of PR proteins in IC (12 hpi, Figure 4.13) may also provide support for a very rapid initial oxidative burst. The lack of evidence for a second burst in LA and FL may contribute to the susceptibility of LA, and may also explain the lower level of resistance in FL compared to IC. Alternatively, a second burst in these genotypes may occur after the time-course of this study.

Other proteins whose regulation may be predictive of *A. rabiei* resistance included a disease resistance response protein DRRG49-C, leucine-zipper protein (LZP), environmental stress-inducible protein (ESP), and polymorphic antigen membrane protein (PAMP). DRRG49-C was initially isolated from *Fusarium solani* infected peas (*Pisum sativum*), where it was expressed in response to pathogen challenge (Chiang and Hadwiger, 1990). Pea southern blot data suggested it is a member of a gene family and Chiang and Hadwiger (1990) postulated that it is involved in transcriptional activation. In this study, DRRG49-C was significantly up-regulated in IL at 24 hpi and in IC at 12-24 hpi. The absence of up-regulation in LA suggests a possible involvement of this protein in *A. rabiei* resistance, although further work is needed to characterise its activity. The LZP was significantly up-regulated in IC at 24 hpi in a previous study (refer to Chapter 3), which was confirmed here, but this study also

observed down-regulation at 48-72 hpi in IC and at 48 hpi in FL. The LZP may represent a bZIP transcription factor, which are proteins composed of a basic DNA-binding region and a leucine-zipper dimerisation motif. *A. thaliana* bZIP transcription factors regulate a variety of processes including the production of salicylic acid (SA) and induction of the expression of PR proteins (Fan and Dong, 2002; Jakoby *et al.*, 2002; Despres *et al.*, 2003). Further, a bZIP transcription factor was recently reported to be involved in pepper (*Capsicum annum* L.) resistance to *Xanthomonas campestris* pv. *vesicatoria* (Lee *et al.*, 2006). Although the LZP characterised here requires further work to be positively characterised as a bZIP transcription factor, its up-regulation in IC at 24 hpi may induce the production of PR proteins.

The ESP was also found to be exclusively up-regulated in FL at 24 hpi, but little is known of the function of this protein other than its expression after environmental stress. However, its up-regulation in response to *A. rabiei* infection may indicate a possible role in the defence response of FL. Finally, the PAMP was significantly up-regulated in IL at 12 hpi (then down-regulated at 48 hpi) and IC (48 hpi), and resembles a surface protein that has previously been isolated from the malaria parasite and shown to contain a leucine-zipper motif (McColl and Anders, 1997). The differential expression of PAMP was confirmed by qRT-PCR where up-regulation in LA at 72 hpi was also observed. Although further study needs to be performed to characterise this putative PAMP protein in chickpea, it may represent a membrane-targeted protein involved in defence-related transcriptional activation.

The putative Ca-binding carrier protein (DY396262) found to be up-regulated in the *A. rabiei*-resistant IC (72 hpi) may also be involved in the defence mechanism. As described in Chapter 1 (section 1.4.1), the cellular influx of Ca^{2+} ions is a key signal involved in enzyme activation and defence-related gene expression. Further, the increase of cytoplasmic Ca^{2+} is sensed by various Ca-binding proteins that act to 'decode' the Ca^{2+} signal and restore Ca^{2+}

levels to a normal state (Reddy, 2001). Four classes of Ca-binding proteins have been identified in plants; (1) calmodulin (CaM), (2) CaM-like, (3) Ca-regulated protein kinases, and (4) Non CaM-like (Reddy, 2001). Several CaM and CaM-like genes have been reported to be up-regulated by pathogen infection in tobacco (Yamakawa *et al.*, 2001), and oxidative stress in *A. thaliana* (Desikan *et al.*, 2001), where they were shown to affect ROS and phytoalexin production, as well as defence-related gene activation. Subsequently, DY396262 may represent a Ca-binding protein with potential involvement in defence-related gene activation in chickpea. However, the relatively late (72 hpi) up-regulation of this transcript in IC indicates that it may regulate a delayed defence response, or only be involved in restoring cellular Ca^{2+} levels to a normal state after defence activation has occurred.

Interestingly, a serine/threonine protein kinase (DY475384) was found to be significantly down-regulated in FL (48 hpi) and IC (24 and 72 hpi). Protein kinases in plant cells act by accepting input information from receptors that sense environmental conditions and other factors, and converting it into outputs such as metabolic changes and gene expression (Hardie, 1999). Some plant protein kinases have been shown to be important for the regulation of defence-related gene expression, such as the *Pto* resistance gene from tomato (Martin, 1999). The down-regulation of the protein kinase in *A. rabiei*-resistant genotypes in this study suggests its involvement in other cellular signalling rather than pathogen defence.

Two energy-production proteins, chlorophyll a/b binding protein (CAB) and NADH dehydrogenase, were also found to correlate with *A. rabiei* resistance. CAB was up-regulated in IC from 6-12 hpi and down-regulated in the *A. rabiei*-susceptible LA at 6 hpi, whilst NADH dehydrogenase was up-regulated in IC only at 12 and 72 hpi. The up-regulation of these proteins in the resistant genotype may provide support for the observation by Dolar and Gurcan (1995) that respiration rate in resistant cultivars increased by 48 hpi due to an HR

(refer to section 1.3). Therefore, the 6-12 hpi up-regulation of CAB and early up-regulation of NADH dehydrogenase at 12 hpi in IC may indicate a rapid HR, further supporting the potential presence of a rapid oxidative burst discussed earlier. Susceptible cultivars were shown to increase respiration by 120 hpi, which was outside the time-course of this study and could not be confirmed. The absence of up-regulation of these proteins in FL or IL may contribute to their lower (moderate) level of resistance compared to IC.

The several unknown/unclear ESTs that were significantly up-regulated in the *A. rabiei*-resistant genotypes compared to LA (confirmed by qRT-PCR for DY475157) may also possess functional involvement in an effective resistance response, especially considering that the currently known chickpea defence mechanisms do not explain pathotype-specific resistance (refer to section 1.3). As reported in Chapter 1 (section 1.5.3), groups of co-regulated ESTs identified by clustering may share the same regulatory mechanisms, such as common promoter elements that interact with the same transcription factors. Considering this, *k*-means clustering revealed that several unknown/unclear ESTs may be important for successful resistance as they were exclusively co-regulated with up-regulated defence-related transcripts in *A. rabiei*-resistant genotypes (such as DY475217, DY475084 and DY475532) compared to LA. Further, the exclusive co-regulation of DY475483 and DY475532 with up-regulated defence-related ESTs in LA indicated that these ESTs might be ineffective for *A. rabiei* resistance. Considering that resistant genotypes have been shown to produce phenolic compounds (refer to section 1.3) and reduce the level of the *A. rabiei*-toxin solanapyrone C (refer to section 1.2.6), some of the important unclear/unknown proteins may encode phenolic or detoxifying compounds. Further work on these proteins, such as isolation of full-length gene sequences and protein expression, must be performed in order to characterise their potential function in the defence response.

Two of the grasspea ESTs included on the microarray represented a putative chitinase and catalase, but were not found to be DE contrary to the reports of up-regulation of these proteins in the chickpea response to *A. rabiei* (refer to section 1.3). Similarly, a grasspea chalcone reductase of the phenylpropanoid pathway, reported to be involved in phytoalexin production in chickpea (refer to section 1.3), was not DE in this study. In fact, as in Chapter 3, this study found no evidence for phytoalexin accumulation over the time-course used. These results may indicate possible differences in the cDNA sequences of the chickpea chitinase, catalase and chalcone reductase compared to grasspea that would be detrimental to hybridisation, or may suggest that the chickpea transcripts were up-regulated at time-points not included in this study. Resistant chickpeas have also been reported to show increased levels of other enzymes involved in promoting ROS accumulation (refer to section 1.3), but the set of ESTs in this study did not include these enzymes so this observation could not be confirmed. Similarly, another enzyme of the phenylpropanoid pathway, phenylalanine ammonia-lyase (PAL), which has been implicated in phytoalexin biosynthesis in chickpea (refer to section 1.3), was unable to be detected.

As in Chapter 3, this study also found a lack of evidence for the up-regulation of lignification in the *A. rabiei* defence response as demonstrated by the unchanged regulation of central enzymes of this pathway (Caffeoyl-CoA-methyltransferase and Cinnamyl-alcohol-dehydrogenase). However, as reported in Chapter 3, pre-formed (constitutive) lignin levels may be important for *A. rabiei* defence, and up-regulation of lignin biosynthesis may occur at a later time-point than included in this study. Protein ubiquitination and degradation is emerging as a regulator of plant defence responses. Polyubiquinated proteins are often degraded, but recent studies have extended the function of ubiquitin from simply providing a degradation signal to activation of defence responses (Devoto *et al.*, 2003). Other microarray studies have found genes of the ubiquitination pathway to be induced after pathogen infection

(Mysore *et al.*, 2003; Lopez *et al.*, 2005), but this study found no significant change in expression of several ubiquitination-related genes included on the microarray. However, considering the ubiquitination pathway involves numerous genes, the potential for ubiquitin-regulated defence-related gene expression remains a possibility.

The wild chickpea relative, IL, induced less DE defence-related ESTs compared to the other genotypes with *A. rabiei* resistance (IC and FL). As discussed earlier, this observation may be attributed to IL being a different species to the chickpea genotype used as the microarray probe source or, alternatively, the differing physiology of the species that may provide stronger passive defences. In fact, thicker stem epidermis and hypodermis has been shown to correlate with chickpea resistance to *A. rabiei* (Angelini *et al.*, 1993). The observed resistance of IL did not appear to be explained by the expression pattern of ESTs included in this study and may be conferred by the presence of unique defence-related genes not included in the microarray, or by high levels of basal gene expression that could not be assessed in this study.

K-means clustering confirmed that the majority of genes showing decreased or unaltered regulation belonged to classes of general metabolic or 'housekeeping' genes. Observations such as this have been reported in other microarray studies (Dowd *et al.*, 2004; Gibly *et al.*, 2004; Lopez *et al.*, 2005), and suggest that during *A. rabiei* infection chickpea reduces its growth processes and redirects resources to the defence response, an effect that must be closely monitored in crop plants considering the potential reduction in fitness of the plant (Heil and Baldwin, 2002).

It is difficult to relate the results of this study to previous reports on the genetic basis of *A. rabiei* resistance, especially considering the conflict on the mode and number of genes

controlling resistance (refer to section 1.3.4). Reports of dominant gene/QTL control range from one to three genes, and recessive gene/QTL control also from one to three (refer to section 1.3.4), suggesting that there exists a range of different resistance sources. Considering that this study examined the expression of genes, it did not have to power to discriminate between dominant or recessive gene control, nor between alleles. However, the study may provide a guide to the number of expressed genes involved in *A. rabiei* resistance. Numerous potential *A. rabiei*-resistance predictive genes were identified from different genotypes in this study, including PR proteins, SNAKIN2, PRP, DRRG49-C, LZP, ESP, PAMP, Ca-binding protein, and several unknown/unclear ESTs, which indicated that overall resistance may be controlled by more than one *R* gene. Alternatively, susceptible genotypes may express fewer doses of defence-related genes compared to resistant genotypes, may possess alleles that cause ineffectiveness of protein products, or resistant genotypes may simply possess a single *R* gene that is able to regulate a wide range of defence responses.

To date, more than 40 plant *R* genes have been cloned and characterised, of which all but three are dominant (refer to section 1.4.2.1). The products of most dominant *R* genes encode receptor-like proteins that interact directly with pathogen effectors, whilst the few recessive *R* genes encode proteins with different structures, including a loss-of-function mutant that allows negative regulation of a defence response to become unblocked (refer to section 1.4.2.1). Considering this, an alternate explanation for the observed differential regulation of defence-related genes in this study may be through the action of a mutant gene that causes non-expression of 'defence-suppressing' genes or small interfering RNAs (siRNAs), which are known to regulate gene expression in plants via post-transcriptional gene silencing (Dugas and Bartel, 2004). For example, the up-regulation of SNAKIN2 or PRP may be allowed by the absence of siRNA transcripts encoded by alleles in susceptible chickpeas. Therefore, because siRNA-mediated gene silencing is post-transcriptional, susceptible genotypes may

show a similar up-regulation of defence-related transcripts as resistant types, although they are not all translated. Considering that siRNA transcripts themselves are also never translated, it may be possible that some of the unknown/unclear cDNAs are actually siRNAs.

Overall, the results of this study that show differences in the timing and level of up-regulation of numerous defence-related transcripts that may account for *A. rabiei* resistance provide more support for the action of multiple expressed *R* genes rather than a single, or non-expressed, gene.

4.5 Conclusion

The microarray analyses performed with the limited number of available cDNA clones from chickpea and grasspea show that genes involved in the defence response of chickpea to *A. rabiei* are similar to those governed by *R*-gene mediated resistance, including the production of ROS (oxidative burst) and the HR, down-regulation of 'housekeeping' gene expression, and expression of PR proteins. The comparison between the compatible *A. rabiei*-LA and incompatible *A. rabiei*-IC/FL/IL interactions led to the identification of certain gene expression 'signatures' that are exclusively present in the incompatible interactions, including rapid expression of PR proteins, as well as up-regulation of β-1,3-glucanase, SNAKIN2, PRP, DRRG49-C, LZP, ESP, PAMP, Ca-binding protein, and several unknown/unclear proteins. The microarray-based differential expression of some ESTs were confirmed by qRT-PCR. The results also confirm histopathology studies of the chickpea defence response and, although the microarray was unlikely to contain all *A. rabiei* defence-related genes, provide novel insights to the molecular control of these events. Numerous putative genes were identified whose involvement in the chickpea-*A. rabiei* interaction had not previously been described.

Considering that studies of signalling events responsible for active defence responses in plants have identified salicylic acid (SA), jasmonate (JA) and ethylene (E) as key regulators of these pathways (refer to section 1.4.3), the next step in this study will attempt to identify the regulatory pathways leading to the induction of the potentially important defence-related ESTs through the study of gene regulation after exogenous application of SA, JA and E. Other steps forward, which are outside the scope of this study, would involve the functional characterisation and validation of important genes by methods such as genetic transformation and the mapping of polymorphisms associated with resistance in populations segregating for resistance. Overall, the information generated in this study enhances the understanding of this plant-pathogen relationship and may aid breeding programs directed toward producing resistant cultivars.

Chapter 5

Large-scale microarray expression profiling of chickpea unigenes differentially regulated by defence-signalling compounds to reveal pathways of defence-related gene regulation.

5.1 Introduction

As described is Chapter 1 (section 1.4.3), studies of signalling events following *R-Avr* pathogen recognition that induce local and systemic active defence responses in plants have led to the identification of salicylic acid (SA), jasmonates (JA) and ethylene (E) as key regulators of these pathways (Schenk *et al.*, 2000; Salzman *et al.*, 2005; Jalali *et al.*, 2006).

The previous microarray studies of Chapters 3 and 4 identified putative genes potentially involved in effective *A. rabiei* resistance in chickpea. These included the rapid synthesis of PR proteins, presence of an oxidative burst, and the synthesis of putative cell-wall strengthening proteins, antimicrobial proteins, and numerous proteins of unknown identity. However, to further identify and characterise the regulatory pathways of putative genes involved in *A. rabiei* defence, this study used microarray technology to quantify expression profiles of the chickpea response to treatments with the defence signalling compounds SA, methyl jasmonate (MeJA), and the immediate ethylene precursor aminocyclopropane carboxylic acid (ACC).

Microarray-based studies of responses to defence signalling compounds have been successful in other plants for characterising defence-related gene activation (Schenk *et al.*, 2000; Salzman *et al.*, 2005). In chickpea, studies do exist on the gene expression analysis of certain

potential defence-related genes after *A. rabiei* inoculation, SA treatment, and JA treatment (Cho and Muehlbauer, 2004; Cho *et al.*, 2006). In these studies, gene expression levels were determined by RNA blots and RT-PCR and revealed that, whilst differential expression was observed for all treatments amongst the genes studied, resistance to *A. rabiei* did not correlate with SA- and JA-mediated regulation of the defence-related genes. However, the majority of putative *A. rabiei* defence-related genes identified in Chapters 3 and 4 were not analysed in previous studies, therefore, the mechanisms of their regulation in chickpea remain unknown.

The large-scale microarray constructed in Chapter 4 was used again in this study. However, the lentil RGA probes were not included due to their hybridisation failure in Chapter 4. Specifically, this study investigated the gene expression changes in the three chickpea genotypes studied in Chapter 4 over a time-course after treatment with SA, MeJA and ACC. The wild relative, ILWC245, was not included considering that the results of Chapter 4 indicated that the microarray expression patterns appeared to explain little of the observed resistance of this genotype. The study represents the first large-scale microarray study of chickpea gene expression in response to defence signalling compounds, and provides novel insights into the molecular mechanisms regulating chickpea defence.

5.2 Materials and methods

5.2.1 Hydroponic plant culture and treatments

Chickpea genotypes ICC3996 (IC), Lasseter (LA), and FLIP94-508C (FL) were used in this study. Seeds of each genotype were surface-sterilised according to section 3.2.1.3, germinated for 2 d on wetted sterile filter paper, and then transferred to rock wool plugs (fibrous rock strands bonded together to form plugs) embedded in 7 L aerated hydroponic containers (24 seedlings per container) containing 0.5X Hoagland solution (Appendix 1). All plants were grown in a glasshouse (20 ± 4ºC) for 10 days (six- to eight-leaf stage) before

treatments with SA (Sigma-Aldrich, St Louis, MO), MeJA (Sigma-Aldrich, St Louis, MO), and the E precursor ACC (Sigma-Aldrich, St Louis, MO). Nutrient solutions were refreshed 3 d prior to treatments, which were performed by adding stock solutions of SA, MeJA or ACC to the nutrient solutions of 10 d old chickpea seedlings to final concentrations of 1 mM SA, 100 µM MeJA and 0.5 mM ACC. Treatment concentrations were selected based on similar studies in sorghum (Salzman *et al.*, 2005) and *A. thaliana* (Schenk *et al.*, 2000). ACC was used in place of gaseous E to avoid unwanted effects of having to seal plants in containers. Control plants were also grown in hydroponic containers under the same conditions except for the addition of treatment compounds. Control, MeJA and ACC treated plants were kept separate to avoid effects of volatile E or MeJA on other plants.

5.2.2 Experimental design and replication

Each 24-plant hydroponic container was set up with eight replicate plants of each genotype. For each of three biological replications (separately conducted experiments), four containers were included, of which three containers were used for the treatments and one for untreated controls. Four plants of each genotype were harvested from each container at 3 and 27 hours post treatment (hpt), and then pooled for RNA extraction. Sampling times were selected based on the rapid gene expression changes observed in similar studies (Schenk *et al.*, 2000; Van Zhong and Burns, 2003; Bower *et al.*, 2005; Salzman *et al.*, 2005), as well as histopathology information on the chickpea defence response timing against *A. rabiei* (refer to section 1.3). A total of 18 conditions were evaluated in this study, comprising three chickpea genotypes X three treatments X two harvest time-points X one tissue type (Figure 5.1). All condition hybridisations were performed with six technical replicates (corresponding to six microarray sub-grids) and three biological replicates, incorporating dye-swapping (i.e. reciprocal labelling of Cy3 and Cy5) to eliminate any dye bias. Overall, 324 images were analysed from 54 slides, resulting in 18 data points for each condition.

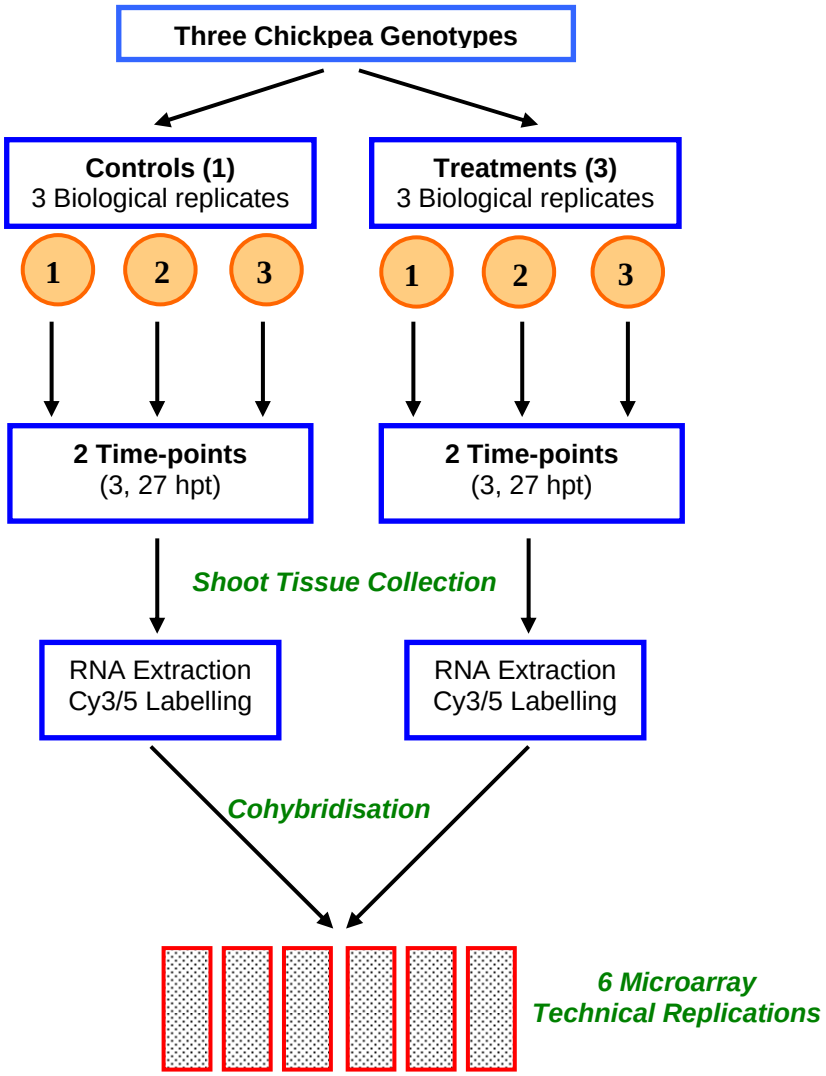

Figure 5.1 Abbreviated summary of experimental design and replication used for plant culture, treatment, tissue collection and microarray replication. Experiments were performed in a reference design where the samples for the untreated controls at each time-point acted as references against samples taken at each time-point for the three treated samples (SA, MeJA and ACC).

5.2.3 Microarray construction, target preparation and hybridisation

Microarrays were constructed according to the method described in Chapter 4 (section 4.2.2), except that the lentil RGA probes were eliminated due to previous hybridisation failure. For target preparation, total RNA was extracted from separately pooled shoot samples for each condition (each genotype at each time-point for each treatment) using the RNeasy Plant Mini Kit (Qiagen, Valencia, CA). The quantity and quality of the total RNA samples were assessed by OD_{260}/OD_{280} ratios and gel electrophoresis respectively, as per the methods of section 3.3.1. Subsequently, fluorescent-labelled targets were prepared and hybridised to the microarray slides according to section 4.2.3.

5.2.4 Scanning and data analysis

Slides were scanned, images captured, and data transformations performed as described in the methods of section 4.2.4. According to Chapter 4 (section 4.2.4), the significant FC cut-offs for up- or down-regulation in this study were determined by a separate self-self hybridisation performed using identical total RNA for both Cy3 and Cy5 labelling. The results of the Chapter 4 self-self hybridisation were not applied in this study because of the differing experimental systems. The self-self ratio dataset was used to determine the 99% confidence interval for mean expression ratio of each array feature, which was examined to identify FC cut-off thresholds for differential expression.

5.2.5 Identification of differentially expressed ESTs

To identify differentially expressed (DE) ESTs, the ranking and selection methods of section 4.2.5 were applied. Briefly, expression ratio results were filtered to eliminate ESTs whose 95% confidence interval for mean FC did not extend to the threshold determined by the self-self hybridisation, followed by Students *t* test with False Discovery Rate (FDR) multiple testing correction to retain only ESTs in which expression changes versus untreated control

were significant at $P<0.05$. The lists of DE ESTs for each condition were then compiled into a single non-redundant list, which was used to create a dataset of mean ratios for the DE ESTs in all conditions. Additionally, ESTs that were undetected in five or more out of the six genotype X time-point conditions of each treatment were excluded from analysis. The use of two time-points only in this study enabled the patterns of expression in each genotype to be identified without the need for data clustering performed in Chapters 3 and 4.

5.2.6 Quantitative RT-PCR

Eleven EST targets were selected for confirmation by qRT-PCR, including three that were assessed in Chapter 4 (section 4.2.6). The primers for quantitative detection and cDNA template were generated according to the methods of section 4.2.6. Triplicate qRT-PCR reactions were also performed for each clone of interest as described in section 4.2.6. Control reactions containing untranscribed RNA confirmed that no interfering genomic DNA products were present. Amplification products were subjected to melting curve analysis, as well as confirming single products by gel electrophoresis (refer to section 4.2.6). Relative fold change in accumulation of target under a given treatment was standardised against cDNA derived from untreated control tissue, and calculated by the comparative C_T method ($\Delta\Delta C_T$ method; refer to section 4.2.6). Gene expression levels obtained were normalised using the actin gene, which showed similar expression levels in all conditions as revealed by microarray analysis.

5.3 Results

5.3.1 Experimental design

A standardised system of hydroponic plant culture (Figure 5.2), signalling compound treatment and replication was developed in order to minimise experimental variability and ensure accurate measurements of changes in mRNA abundance (Figure 5.1). The inherent

noise and sensitivity of the microarray system was determined by three separate self-self hybridisations. The combined result of these hybridisations yielded a 99% confidence distribution where 97.3% of the signals fell within 1.5 FC and 99.4% were within 2.0 FC (Figure 5.3). Based on this result, a 2.0 FC cut-off was selected for differential expression in addition to the significance (*t*) test and FDR multiple testing correction. These cut-offs translated into up-regulated ESTs having a $\log_2$ ratio ≥1, and down-regulated cDNAs ≤-1.

5.3.2 Microarray construction and analysis

To study gene expression patterns of the chickpea response to exogenous treatments with defence signalling compounds, microarrays were prepared using chickpea and grasspea clones previously used for microarray construction in Chapter 4. This study profiled the changes in gene expression occurring in three genotypes, one with resistance (IC) to *A. rabiei*, one with moderate resistance (FL), and one susceptible genotype (LA). The transcript level for each cDNA was calculated firstly as the average intensity of the six technical replicates, then the average intensity of the three biological replicates. All MIAME guidelines were observed and, at the time of writing, the expression datasets were not yet deposited into the Gene Expression Omnibus, National Center for Biotechnology Information.

Table 5.1 summarises the level of undetected probes for each genotype according to source, where only small percentages of *C. arietinum* probes were undetected in each genotype, the highest being for FL (5.2%). Only 3.0% of the *C. arietinum* probes were undetected in IC. The levels of undetected features for the *L. sativus* probes were higher, again with FL as the highest (12.2%) and IC as the lowest (6.4%). Importantly, labelled cDNA targets did not hybridise to any negative controls on the microarray including blank buffer, digested vector and PCR primer sequences.

Figure 5.2 Hydroponic culture containers used for each biological replication. Each 24-plant container was set up with eight replicate plants of each chickpea genotype. Three containers were used for treatments and one for untreated controls.

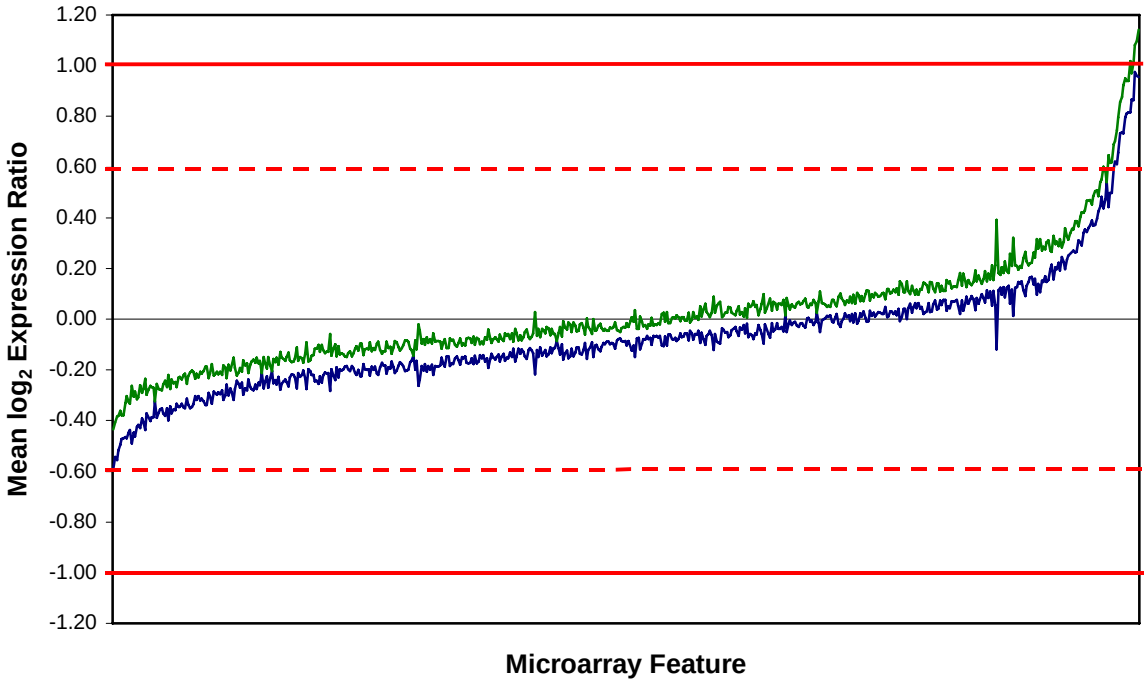

Figure 5.3 Combined distribution of the 99% confidence intervals of mean $\log_2$ ratios for all array features of the self-self hybridisations. Green line represents the upper 99% confidence limit and blue line represents the lower limit. Broken red horizontal line indicates the point representing a 1.5 FC, and the unbroken red horizontal line indicates the 2.0 FC line.

Table 5.1 Undetected microarray probes from each source, where undetected corresponds to mean fluorescence intensity less than two times mean local background intensity in all time-points and replications.

	Microarray probe source	
Genotype	***C. arietinum***	***L. sativus***
IC (*C. arietinum*)	17 (3.0%)	10 (6.4%)
LA (*C. arietinum*)	23 (4.1%)	15 (9.4%)
FL (*C. arietinum*)	29 (5.2%)	19 (12.2%)

A FC cut-off and Students *t* test ($P<0.05$) ranking with FDR multiple testing correction selection was used to identify DE ESTs. The 2.0 FC cut-off was determined by separate replicated self-self hybridisations that estimated the sensitivity of the microarray system, whilst the *t* test with FDR allowed only the selection of statistically significant DE ESTs. Of the 715 experimental microarray elements, 425 (59.4%) were found to be DE in at least one condition, although several ESTs were found to be DE in more than one condition. Figure 5.4 summarises the relationship of observed DE ESTs within each genotype and Appendix 8 reports the characteristics of the DE ESTs. Down-regulation was most prominent for all treatments, where ACC and SA altered the expression levels of substantially more ESTs than MeJA. The kinetic trend of differential expression in each genotype (Table 5.2) revealed that, for IC, a greater proportion of DE ESTs were observed at 27 hpt, and down-regulation was most prominent for all conditions except ACC 27 hpt. LA showed a larger amount of DE ESTs at 3 hpt and greater down-regulation in all conditions. FL was similar to IC with more DE ESTs observed at 27 hpt, but was similar to LA with down-regulated ESTs outnumbering up-regulated ESTs in all conditions.

In all genotypes, the largest group of uniquely up-regulated ESTs was for ACC treatment, whilst the largest group of uniquely down-regulated ESTs was for SA treatment in IC and LA, and ACC treatment in FL (Figure 5.4). Of the co-regulated ESTs, smaller proportions were up-regulated in IC (10% of all up-regulated ESTs) and LA (7%) compared to down-regulated (24% and 19% respectively). However, co-regulated ESTs in FL were equally distributed (12% up and down). Of the co-up-regulated ESTs for IC, the majority were induced by ACC-MeJA, but by MeJA-SA for LA and FL. Co-down-regulated ESTs were most common for ACC-SA in all genotypes (Figure 5.4). Further, no ESTs were commonly up-regulated by all three treatments in FL, and only one in IC and LA. Five ESTs were commonly down-regulated by all treatments in IC, but only one in each of LA and FL.

Table 5.2 Kinetic trends of significant DE ESTs for each experimental condition.

Condition	**Number of observations**						
	ICC3996		**Lasseter**		**FLIP94-508C**		
	03 hpt	**27 hpt**	**03 hpt**	**27 hpt**	**03 hpt**	**27 hpt**	**Total**
SA Up-regulated	2	21	24	16	19	7	**89**
SA Down-regulated	28	86	77	38	49	45	**323**
MeJA Up-regulated	4	8	7	2	5	4	**30**
MeJA Down-regulated	23	34	13	3	5	5	**83**
ACC Up-regulated	11	76	35	10	13	18	**163**
ACC Down-regulated	33	35	82	21	38	126	**335**
Total	**101**	**260**	**238**	**90**	**129**	**205**	

Specifically, several putative defence-related ESTs were co-induced in IC, including a disease resistance response protein DRRG49-C (ACC-SA), multi-resistance ABC transporter protein (ACC-SA), SNAKIN2 antimicrobial peptide precursor (ACC-MeJA), and two PR proteins (ACC-MeJA and ACC-MeJA-SA, respectively). Fewer defence-related transcripts were co-induced in FL and LA, including a PR protein (ACC-SA) and glutathione S-transferase (ACC-MeJA-SA) in LA, and a PR protein (ACC-SA) and multi-resistance ABC transporter protein (ACC-SA) in FL. Most co-repressed ESTs in each genotype were either unknown or associated with general 'housekeeping' processes. However, one important observation was the co-down-regulation of the antioxidant superoxide dismutase in the *A. rabiei* resistant IC (MeJA-SA) and moderately resistant FL (ACC-SA), which was described in Chapter 4 to potentially allow the accumulation of reactive oxygen species (ROS) involved in the oxidative burst (Neill *et al.*, 2002).

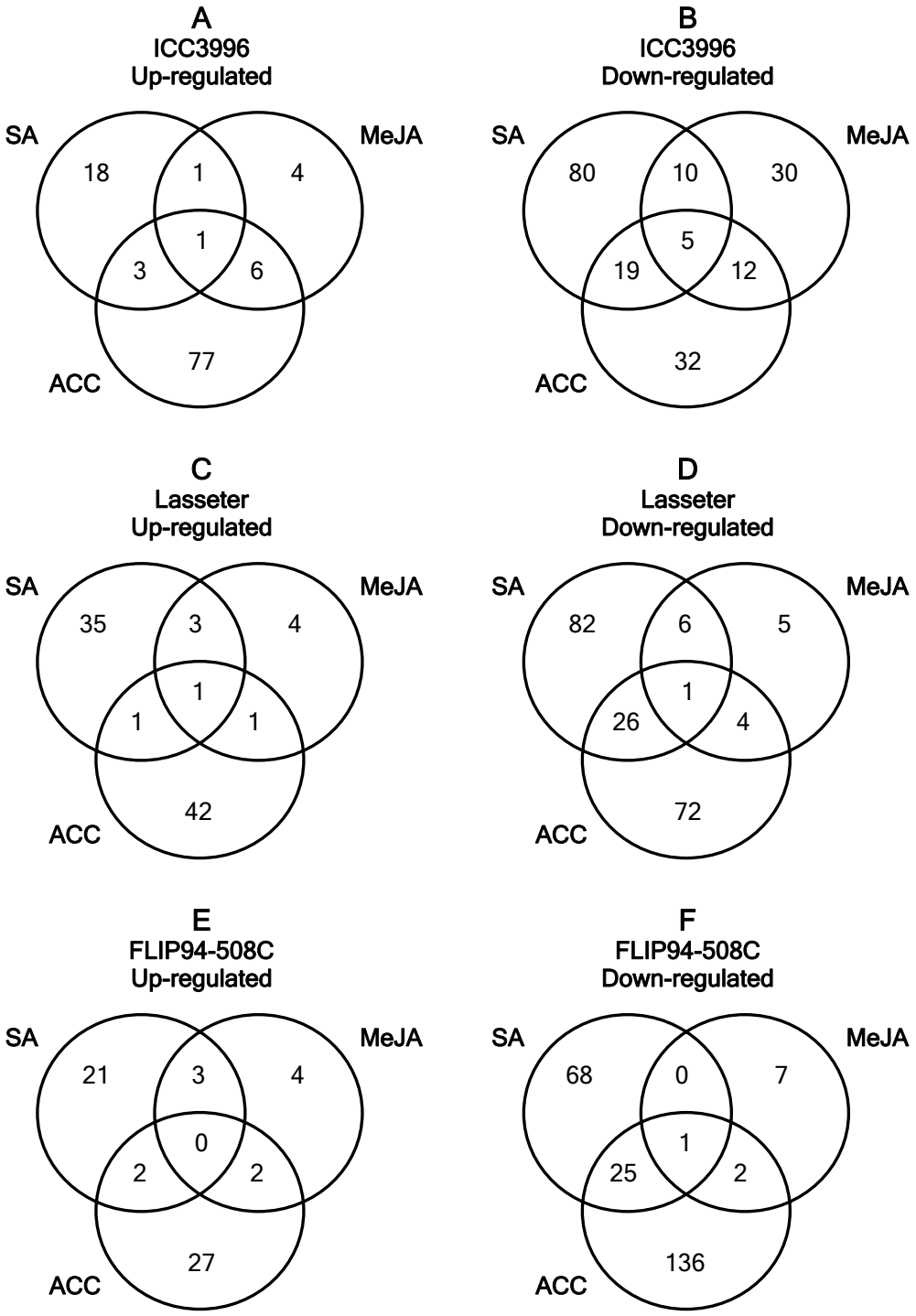

Figure 5.4 Regulation of the DE ESTs for each genotype after application with ACC, MeJA and SA. DE ESTs were determined by a 2.0 FC cut-off, significance test (P<0.05) and FDR multiple testing correction. Up-regulated (A, C and E) and down-regulated (B, D and F) ESTs are shown according to treatment, where time-points have been combined.

To supplement the data presented in Figure 5.4, Figure 5.5 reports the relationship of DE ESTs within each treatment to show the level of co-regulation between genotypes. Again, Figure 5.5 shows that ACC and SA regulated the expression of substantially more transcripts than MeJA, and that down-regulation was most prominent for all treatments. The key observation from Figure 5.5 is the relatively low proportions of co-regulation between genotypes for all treatments. For example, in the ACC up-regulation category, IC only shared the regulation of 17 out of 75 ESTs with LA, and only 16 with FL. These results indicated that the application of the defence signalling compounds induced transcriptional responses in the three genotypes that were not completely conserved, suggesting that each genotype possesses different pathways of defence-related gene activation. Of the co-regulated ESTs between genotypes, a smaller proportion were up-regulated by ACC treatment (15% of all up-regulated ESTs) compared to down-regulated (29%). For SA treatment, relatively equal proportions were up- and down-regulated (34% and 33% respectively). However, very few ESTs were co-regulated between genotypes by MeJA (4% up-regulated and 3% down-regulated). Several putative defence-related ESTs were both co-regulated between some genotypes and uniquely regulated in different genotypes, and are described in section 5.3.3.

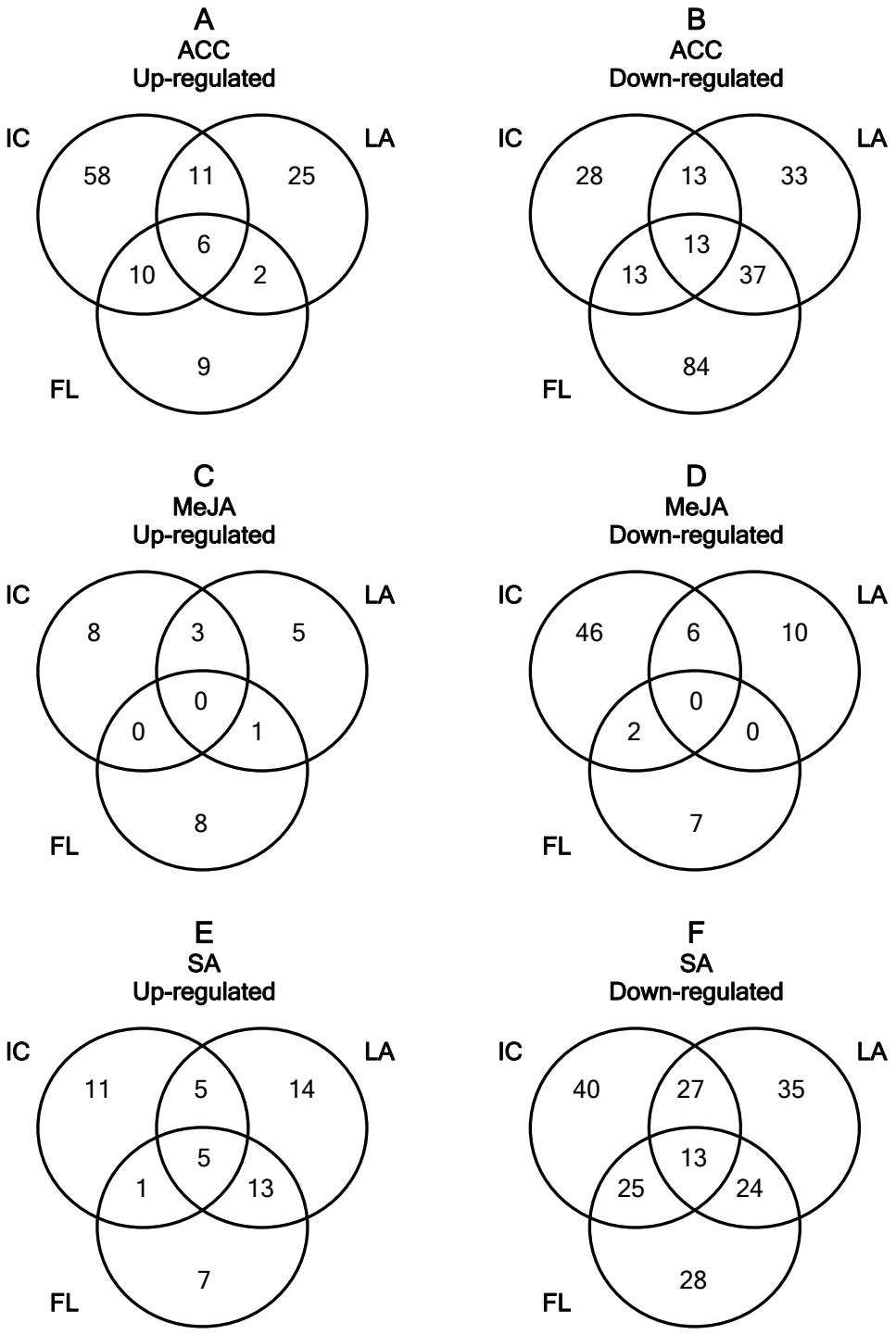

Figure 5.5 Regulation of the 425 DE ESTs for each treatment (ACC, MeJA and SA). DE ESTs were determined by a 2.0 FC cut-off, significance test (P<0.05) and FDR multiple testing correction. Up-regulated (A, C and E) and down-regulated (B, D and F) ESTs are shown according to genotype, where time-points have been combined.

All chickpea and grasspea ESTs present on the microarray were previously assigned putative cellular functions based on sequence similarities with genes in public databases, and classified into functional categories (refer to Chapter 2). Further, any of the previously non-sequenced chickpea cDNAs included on the microarray that showed differential expression were accurately re-sequenced and functionally identified according to the method described in Chapter 2. Figure 5.6 shows the proportion of DE ESTs for each experimental condition according to their functional categories, where percentages represent the number of DE ESTs of each category in relation to the total number of ESTs from each category present on the array. 'Unknown' represents no significant homology to sequences in public databases, and 'unclear' represents significant homology to hypothetical proteins only. For the ACC treatment, most categories were evenly represented, supporting the role of ethylene in diverse cellular processes. Most categories showed a prominence for down-regulation, especially for the FL and LA genotypes. Within the 'defence' category, only IC showed a greater amount of up- than down-regulation. MeJA treatment provoked very weak responses from all categories, including no DE ESTs from the 'transcription' category. Within the 'defence' category, more up- than down-regulation was again only observed for the *A. rabiei* resistant IC genotype. Finally, the SA treatment resulted in substantial regulation of ESTs of all categories, but down-regulation was most prominent in all categories except 'cell rescue/death/ageing' and 'defence'. Within the 'defence' category, greater proportions of up-regulation were observed for IC and FL.

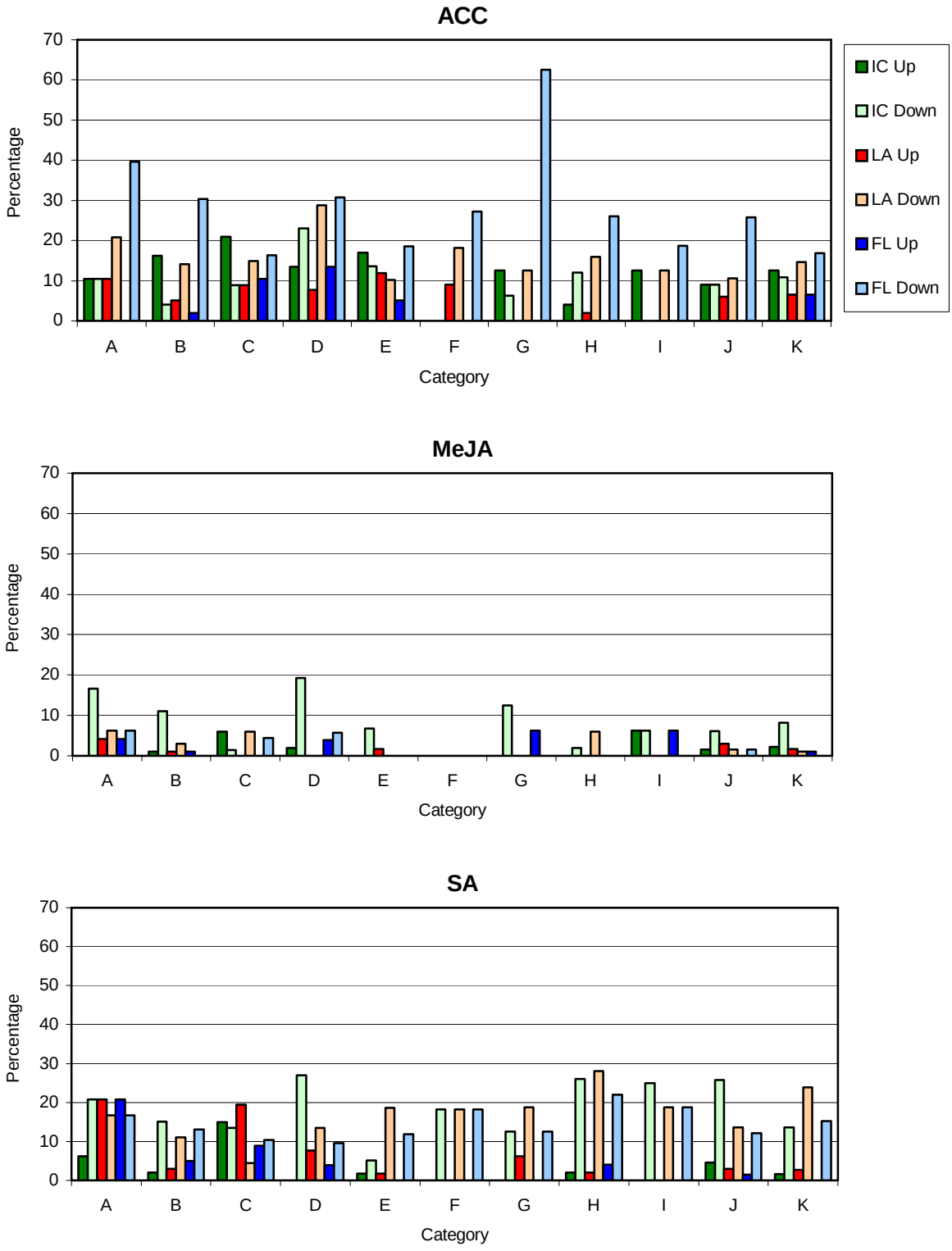

Figure 5.6 Proportion of up- and down-regulated ESTs for each genotype (IC, LA and FL) according to treatment (ACC, MeJA and SA) and functional category (A-Cell rescue/death/ageing, B-Cellular metabolism, C-Defence, D-Energy, E-Protein synthesis/fate, F-Transcription, G-Transport facilitation, H-Cellular communication/Signal transduction, I-Cell cycle & DNA processing, J-Unclear, K-Unknown).

5.3.3 Functional groups regulated by defence signalling compounds

As revealed in Figure 5.6, the defence signalling compounds regulated ESTs from various categories of biochemical function within the three chickpea genotypes studied. Additionally, that data in Figure 5.5 indicated that many of these ESTs were not co-regulated between genotypes. The focus of this study was on the identification of ESTs potentially involved in pathogen defence, which were divided into several sub-classes described below.

5.3.3.1 Regulation of cellular redox state

Several ESTs involved in the accumulation of ROS and regulation of oxidative state were DE after treatment with the three defence signalling compounds. Approximately equal incidences of up- and down-regulation were observed for these ESTs, but MeJA regulated few of these ESTs. A glycolate oxidase (DY396348), known to produce hydrogen peroxide (H_2O_2) involved in the oxidative burst and pathogen defence (Neill *et al.*, 2002), was up-regulated by ACC treatment in both the *A. rabiei* resistant (IC) and susceptible (LA) genotypes, although significant up-regulation was reached by 3 hpt in LA and 27 hpt in IC. Additionally, two cysteine proteases (DY475458 and DY475066), implicated as mediators of pathogen-induced cell death in plants (Solomon *et al.*, 1999; Huckelhoven *et al.*, 2001; Sheokand and Brewin, 2003), were exclusively up-regulated by ACC in IC (27 hpt).

Antioxidant ESTs involved in the regulation of cellular ROS levels were also regulated exclusively by ACC, including a catalase (DY396413) and cationic peroxidase (DY475306) up-regulated only in IC at 27 hpt. A glutathione peroxidase (DY396331) was down-regulated at 27 hpt in IC and FL after ACC treatment, but up-regulated at 3 hpt in FL and LA by SA. Of two glutathione S-transferases (DY396404 and DY475250), DY396404 was up-regulated in IC (3 hpt) and down-regulated in LA (3 hpt) in response to ACC, and up-regulated in FL (27 hpt) and LA (27 hpt) by SA. DY475250, confirmed by qRT-PCR, was up-regulated in all

genotypes by SA, and in LA (3 hpt) only after MeJA treatment. The antioxidants superoxide dismutase (DY475155, confirmed by qRT-PCR) and superoxide dismutase copper chaperone precursor (DY475397) were down-regulated at 27 hpt by ACC in IC and FL (DY475155) and up-regulated at 3 hpt in LA (DY475397). DY475397 was also down-regulated in IC (27 hpt) by MeJA and SA, as well as down-regulated in FL (3 hpt) by SA. Finally, two ESTs encoding water-channel aquaporin-like proteins, DY475124 and DY475512, were up-regulated by ACC (IC 27 hpt) and SA (LA 3 hpt) respectively. These proteins have been implicated in trans-membrane transport of ROS (Henzler and Steudle, 2000).

5.3.3.2 Defence signalling/activation

ESTs with putative involvement in the activation of defence mechanisms were regulated by the three treatments. However, most ESTs of this sub-class were down-regulated, and the majority were regulated by ACC and SA. Two ethylene responsive element binding proteins (EREBPs), which represent transcription factors known to be stimulated by E (Van Zhong and Burns, 2003) and regulate disease resistance pathways (Gutterson and Reuber, 2004), were exclusively up-regulated by ACC 3 hpt in IC and LA (DY396395 and DY396400), of which DY396400 was confirmed by qRT-PCR. Additionally, DY396395 was down-regulated at both 3 and 27 hpt in FL.

Other defence activating ESTs up-regulated by ACC included a translation initiation factor SUI1 homolog (EB085043) in the *A. rabiei* resistant IC (27 hpt), and a putative translational activator (EB085015) in the susceptible LA genotype (3 hpt). Two transcription factors were down-regulated by both ACC and SA; DY396263 was down-regulated in FL (ACC 27 hpt), LA (SA 3 hpt) and IC (SA 27 hpt), whilst DY396309 was down-regulated in LA (ACC 3 hpt and SA 27 hpt). Two leucine-zipper containing proteins (CV793601 and CV793599) may also represent putative (bZIP) transcription factors that are known to be involved in pathogen

defence (Fan and Dong, 2002; Jakoby *et al.*, 2002; Despres *et al.*, 2003; Lee *et al.*, 2006). Of these, CV793601 was found to be up-regulated by ACC in IC (27 hpt) and FL (3-27 hpt), whilst CV793599 was down-regulated by SA treatment at 27 hpt in both IC and FL.

Numerous protein kinase-like proteins, which have been shown to be involved in various cellular processes including defence signalling (Hardie, 1999; Romeis, 2001), were also DE. Six such proteins were regulated by ACC, of which only one (DY475103) showed up-regulation (IC 27 hpt). Similarly, only one of the nine kinase-like proteins regulated by SA treatment showed up-regulation, again in IC at 27 hpt (DY475384). Only two protein kinases were regulated by MeJA, of which both were down-regulated. Putative GTP-binding proteins, known to have possible involvement in plant defence signalling (Sano and Ohashi, 1995; Bovie *et al.*, 2004; Zhao *et al.*, 2005), were also highly down-regulated. Four instances were observed in ACC treated samples and one in SA treated samples. However, one GTP-binding protein (DY396367) was up-regulated by SA in FL (3 hpt).

An EST representing a WD-repeat protein (DY475550), known to regulate a range of cellular processes (Neer *et al.*, 1994), was down-regulated by SA in the *A. rabiei* susceptible LA (3 hpt). This study also found an EST homologous to the *Cf9* resistance gene cluster of tomato (DY396352), which represents leucine-rich repeat (LRR) pathogen resistance genes (Jones *et al.*, 1994), to be down-regulated by MeJA treatment in LA (3 hpt) and by SA treatment in IC (27 hpt) and LA (27 hpt). Finally, two putative calmodulin-like proteins were specifically up-regulated in IC (27 hpt) by SA (DY396411 and DY396364), and may be involved in Ca^{2+} signalling for plant defence (Reddy, 2001; Kim *et al.*, 2002).

5.3.3.3 Secondary metabolic pathways involved in defence

The main metabolic pathway represented by DE ESTs of this sub-class was the phenylpropanoid pathway, involved in the biosynthesis of defence-related compounds including phytoalexins and lignin (refer to section 1.4.1). Two ESTs representing proteins with homology to a lignin producing enzyme of this pathway, caffeoyl-coA-methyltransferase (CV793595 and DY396415), were down-regulated by ACC (FL 27 hpt) and SA treatment (IC 27 hpt and FL 27 hpt). However, cinnamyl-alcohol-dehydrogenase (CV793602), involved in a later step of lignin biosynthesis, was up-regulated in the *A. rabiei* resistant IC genotype (27 hpt) after ACC treatment, also confirmed by qRT-PCR. Additionally, a putative flavonol glucosyl transferase (CV793607), potentially important in the formation of isoflavonoid phytoalexins, was solely up-regulated by ACC in the *A. rabiei* resistant IC (27 hpt). However, CV793607 was not induced by SA in this study although a similar protein was reported to be induced by SA in chickpea (Horvath and Chua, 1996). Finally, a cytochrome P450 monoxygenase (DY475136), known to be important in several steps of the phenylpropanoid pathway (Feldmann, 2001), was up-regulated by ACC treatment in all genotypes (IC 27 hpt, LA 3-27 hpt and FL 27 hpt) and confirmed by qRT-PCR. In fact, a cytochrome P450 was shown to play a role in the defence response of pepper (*Capsicum annum* L.) to *Xanthomonas axonopodis* pv. *glycines* (Kim *et al.*, 2006).

5.3.3.4 Defence-related structural ESTs

Several ESTs with putative involvement in cell wall strengthening or transport were both induced or repressed by the three treatments. As with most other sub-classes, these ESTs were mainly down-regulated in all treatments, with few observations for MeJA. Exclusively regulated by ACC were two proline-rich proteins (DY396288 and DY475348), a glycine-rich cell wall protein (DY396342) and a histidine-rich glycoprotein precursor protein (DY475271). However, only the histidine-rich glycoprotein precursor was up-regulated (LA

3 hpt). A multi-resistance ABC transporter protein (CV793605), known to potentially control transport of antimicrobial secondary metabolites across cell membranes in plants under biotic stress (Crouzet *et al.*, 2006), was up-regulated by ACC treatment in IC (3 hpt) and FL (27 hpt), and by SA treatment in IC (27 hpt) and FL (27 hpt). Also regulated by both ACC and SA were two putative membrane-related proteins (DY475478 and DY475119). DY475478 was down-regulated by ACC (FL 3-27 hpt) and SA (IC 3-27 hpt and LA 3 hpt), whilst DY475119 was also down-regulated by ACC (FL 27 hpt) and SA (LA 3-27 hpt). Solely up-regulated by ACC in the *A. rabiei* resistant IC genotype at 27 hpt was a hypothetical multispanning membrane protein (DY475410), as well as a nuclear transport factor (DY475059).

Other ESTs of this sub-class were a putative membrane protein (DY396429) that was down-regulated by both MeJA (LA 3 hpt) and SA (LA 3-27 hpt and IC 27 hpt), and a GPI-anchored membrane protein (DY475246) that was down-regulated by SA treatment in LA (3 hpt) and (FL 3 hpt). SA treatment also resulted in the down-regulation of a hypothetical membrane-spanning ring-finger protein (DY475508) in LA (3 hpt). The final EST of this sub-class was a DnaJ-like chaperone protein (DY475488), involved in intercellular transport of macromolecules and previously found to accumulate in tomato (*Lycopersicon esculentum*) after inoculation by tomato spotted wilt virus (von Bargen *et al.*, 2001). In this study, this transcript was up-regulated after MeJA treatment in the moderately *A. rabiei* resistant FL genotype (27 hpt), confirmed by qRT-PCR.

5.3.3.5 Specific antimicrobial ESTs

ESTs with direct involvement in pathogen defence constituted the largest sub-class of defence-related DE ESTs. SA treatment resulted in up-regulation of most of these ESTs, whilst ACC and MeJA treatments showed relatively equal incidences of up- and down-

regulation. Again, few ESTs were regulated by MeJA when compared to ACC and SA. The most common type of ESTs in this sub-class represented putative pathogenesis-related (PR) proteins. Six PR proteins were regulated by ACC treatment, five by MeJA, and nine by SA. PR proteins up-regulated by ACC included DY396305 in all genotypes (27 hpt), CV793597 (confirmed by qRT-PCR) in IC and FL (27 hpt), DY396281 in IC and FL (27 hpt), and DY396388 in only the *A. rabiei* resistant IC (27 hpt). CV793597 was also up-regulated by MeJA (IC 3 hpt) and SA (IC 3 hpt and LA 3-27 hpt). Similarly, DY396281 was additionally up-regulated by MeJA (IC 3 hpt) and SA (LA 27 hpt and FL 27 hpt), whilst DY396305 and DY396388 were both up-regulated by SA at 27 hpt in LA. PR proteins specifically up-regulated by SA treatment were DY396301 (IC 27 hpt, LA 3-27 hpt and FL 3 hpt), DY396343 (LA 3 hpt), DY396372 (IC 27 hpt, LA 27 hpt and FL 3 hpt), CV793610 (LA 27 hpt), and DY396384 (LA 27 hpt). Of these, DY396301 was down-regulated by both ACC and MeJA treatment, DY396343 was down-regulated solely by ACC, and DY396372 and CV793610 by MeJA.

Five ESTs with homology to disease resistance response proteins from pea (*Pisum sativum*) were differentially regulated. Particularly, disease resistance response protein DRRG49-C (DY396265, confirmed by qRT-PCR) was up-regulated by ACC in the *A. rabiei* resistant IC at 27 hpt, as well as by SA in IC (27 hpt), LA (3-27 hpt) and FL (3 hpt). Another EST with homology to the same protein (EB085032) was up-regulated by ACC in LA (3 hpt), down-regulated by ACC in FL (3 hpt), and up-regulated by SA in FL (3 hpt). Of the three other disease resistance response proteins, one was down-regulated by ACC and MeJA treatments (DY396296), and two were down-regulated by ACC only (DY396276 and DY396277). CV793591, an EST with homology to the S1-3 defence-related protein from cowpea (*Vignia unguiculata*), was up-regulated by ACC in IC (3 hpt), but also down-regulated in this genotype at 27 hpt by SA. A unigene included in this study (CV793608) was homologous to

a SNAKIN2 antimicrobial peptide previously isolated from potato (*Solanum tuberosum*). This unigene (confirmed by qRT-PCR) was up-regulated by MeJA in the *A. rabiei* resistant IC (3-27 hpt), by ACC in all genotypes, and down-regulated by SA in FL (3 hpt). Other ESTs of this sub-class coded for wound-induced proteins (DY475220 and DY475254), but only DY475254 was up-regulated (FL 27 hpt). Additionally, an EST with homology to subtilisin inhibitors (DY396374) was found to be down-regulated by MeJA and SA treatment only in the *A. rabiei* susceptible LA genotype.

This study also found three ESTs to encode defence-related proteins that were exclusively up-regulated in the *A. rabiei* resistant IC genotype at 27 hpt after SA treatment. These included PR proteins beta-glucan binding protein (DY396299) and putative chitinase (DY396275), as well as a putative disease resistance response protein previously isolated from *A. thaliana* (CV793593). Additionally, a gamma thionen type defensin/protease inhibitor (CV793588) was also up-regulated in IC (27 hpt) after ACC treatment, with potential involvement in preventing the hydrolysis of plant cell proteins by fungal toxins (Koiwa *et al.*, 1997; Pelegrini and Franco, 2005).

5.3.3.6 ESTs not previously associated with defence

ESTs coding for numerous ubiquitin and polyubiquitin-like proteins were up-regulated by ACC treatment. Such proteins have been classically associated with protein degradation, but are now implicated in plant signalling pathways mediating responses to light, hormones, and pathogens (Devoto *et al.*, 2003). Specifically, three polyubiquitins and three ubiquitins were solely up-regulated in the *A. rabiei* resistant IC genotype (DY396302, DY396378, DY396428, DY396326, DY396368 and DY396424), of which DY396302 was confirmed by qRT-PCR. Additionally, ACC also up-regulated a putative ubiquinol-cytochrome C reductase complex protein (DY396401) and a putative acyl-activating enzyme (EB085018) in IC.

Of MeJA up-regulated ESTs not previously involved in plant defence, a histone H2A protein and two ripening-related proteins were up-regulated in FL (DY396268, DY396344 and DY396347). ESTs outside major sub-classes induced by SA included transcripts for a Beta-galactosidase (DY475141 and EB085056) in FL and LA, three ripening-related proteins (DY396344, DY396347 and DY396377) in FL and LA, and a dehydrin cold-induced protein (DY475092) in FL and LA. Numerous ESTs with unknown or unclear functions were also up-regulated by the three treatments. Of all genotypes and time-points, 54 unknown/unclear ESTs were induced by ACC, 14 by MeJA, and 24 by SA. These included several transcripts exclusively up-regulated in the *A. rabiei* resistant IC and moderately resistant FL genotypes.

5.3.4 Comparison to ESTs previously implicated in *A. rabiei* defence

The study in Chapter 4 utilised the same microarray (excluding the lentil probes) and genotypes (except for ILWC245) as this study to identify DE ESTs after inoculation with *A. rabiei* spores over a time-course. Numerous ESTs with potential involvement in the *A. rabiei* defence response of each genotype (IC, LA and FL) were identified. Subsequently, with the results from the present study, a comparison was made in attempt to identify the signalling compounds (ACC, MeJA and/or SA) that may be responsible for the regulation of those ESTs (Table 5.3). Up-regulation of several PR proteins was considered important in *A. rabiei* defence (refer to Chapter 4), and these putative proteins were also up-regulated in this study. However, the treatment responsible for up-regulation varied with each PR protein. Instances of up-regulation were observed with all treatments for IC, but with ACC and MeJA for FL, and only SA for LA (Table 5.3). Some PR proteins also showed combinations of up- and down-regulation within and between treatments. Disease resistance response protein DRRG49-C (DY396265) and SNAKIN2 antimicrobial peptide precursor (CV793608), previously induced by *A. rabiei* inoculation in the resistant IC genotype (refer to Chapter 4), were up-regulated by ACC/SA, and ACC/MeJA respectively (Table 5.3).

Table 5.3 ESTs previously identified as potentially involved in *A. rabiei* defence (Chapter 4) and their regulation by defence signalling compounds (ACC, MeJA and SA) in the present study. Comparisons were made between previously identified up- and down-regulated ESTs ('*A. rabiei* condition') for each genotype (IC, LA and FL) and their observed regulation in this study, where 'na' indicates no differential regulation observed.

***A. rabiei* condition**	**Putative function**	**GenBank accession**	**Regulated by**	**Regulation type**
IC Up	Pathogenesis-related protein 4A	DY396388	ACC	Up
			SA	Down
	Pathogenesis-related protein 4A	CV793597	ACC	Up
			MeJA	Up
			SA	Up
	Pathogenesis-related protein 4A	DY396372	SA	Up
	Pathogenesis-related protein 4A	DY396384	na	
	Pathogenesis-related protein	DY396301	SA	Up
	Pathogenesis-related protein	DY396305	ACC	Up
	Disease resistance response protein	DY396265	ACC	Up
	DRRG49-C		SA	Up
	SNAKIN2 antimicrobial peptide precursor	CV793608	ACC	Up
			MeJA	Up
	Hypothetical proline-rich protein	DY396288	ACC	Down
	Protein with leucine-zipper	CV793599	SA	Down
	Polymorphic antigen membrane protein	DY475248	na	
	β-1,3-glucanase	CV793598	na	
	Ca-binding carrier protein	DY396262	ACC	Down
			SA	Down
	Unknown	DY475533	SA	Down
	Unknown	DY475532	na	
	Unknown	DY475365	na	
	Unknown	DY475157	na	
IC Down	Superoxide dismutase copper chaperone precursor	DY475397	MeJA	Down
			SA	Down
	Glutathione S-transferase	DY475250	SA	Up
LA Up	Pathogenesis-related protein 4A	DY396281	MeJA	Down
			SA	Up
	Pathogenesis-related protein 4A	CV793597	SA	Up
	Pathogenesis-related protein 4A	DY396372	SA	Up
	Pathogenesis-related protein 4A	DY396388	SA	Up
	Pathogenesis-related protein 4A	DY396384	SA	Up
	Hypothetical proline-rich protein	DY396288	na	
	Unknown	DY475483	SA	Down
	Unknown	DY475365	ACC	Up

	Unclear	DY475322	na	
LA Down	Superoxide dismutase copper chaperone precursor	DY475397	ACC	Up
FL Up	Pathogenesis-related protein 4A	DY396372	MeJA	Down
			SA	Up
	Pathogenesis-related protein 4A	CV793597	ACC	Up
	Pathogenesis-related protein 4A	DY396281	ACC	Down/Up
			SA	Down/Up
	Pathogenesis-related protein 4A	DY396388	na	
	Pathogenesis-related protein 4A	DY396384	na	
	Pathogenesis-related protein	DY396301	MeJA	Down
			SA	Up
	Hypothetical proline-rich protein	DY396288	ACC	Down
	Environmental stress-inducible protein	DY396298	ACC	Down
			SA	Down
	Unknown	DY475462	ACC	Down
			SA	Down
	Unknown	DY475365	na	
	Unclear	DY475217	SA	Down
	Unclear	DY475186	ACC	Down
	Unclear	DY475323	na	
FL Down	Superoxide dismutase copper chaperone precursor	DY475397	ACC	Down
			SA	Down

Other important observations for previously up-regulated transcripts included a hypothetical proline-rich protein (DY396288) down-regulated by ACC in IC and FL, a polymorphic antigen membrane protein (DY475248) unregulated in IC, an environmental stress inducible protein (DY396298) down-regulated in FL by ACC and SA, a leucine-zipper protein (CV793599; also identified in Chapter 3) down-regulated by SA in IC, and a Ca-binding carrier protein (DY396262) down-regulated by ACC and SA in IC. Another important up-regulated transcript was the elicitor-induced receptor protein (CV793609) identified in IC in Chapter 3. However, this transcript was not regulated by any of the treatments in this study. Important transcripts down-regulated by *A. rabiei* inoculation in Chapter 4 included a superoxide dismutase copper chaperone precursor (DY475397) that was also down-regulated by MeJA and SA in IC, ACC and SA in FL, but up-regulated by ACC in LA. Additionally, a down-regulated glutathione S-transferase (DY475250) was up-regulated in ICC by SA. Of 11 unknown and unclear ESTs induced by *A. rabiei* inoculation in all genotypes, only one (DY475365) was also up-regulated in this study (ACC LA).

5.3.5 Quantitative RT-PCR

To confirm the reliability of results from the microarray expression data, 11 ESTs with varying levels of up/down-regulation among the three treatments were selected for qRT-PCR. As in Chapter 4 (section 4.3.4), the comparative C_t method ($\Delta\Delta$ C_t method) of quantitation was used, and validation of the eight primer pairs unique to this study (three pairs were already validated in Chapter 4) was successful (Appendix 6). C_T values were determined as in Chapter 4 (section 4.3.4). The reference gene chosen for normalisation of quantified data was actin, whose expression was constant under all treatments. Amplified qRT-PCR products were examined for specificity of product by both melting curve analysis and gel electrophoresis (refer to Chapter 4), and data for any sample not showing specific amplification was discarded from analysis and qPCR was repeated. Table 5.4 summarises the

qRT-PCR results and provides a comparison of the $\log_2$ mean FC ratio (relative to controls) from the microarray observations and the qRT-PCR results. Comparisons were made at 27 hpt for each treatment X genotype condition. The expression of all statistically significant DE ESTs from microarray analysis was confirmed by qRT-PCR, but ratios observed for qRT-PCR were generally more exaggerated than those from microarray analyses. Of a total of 90 comparisons between microarray and qRT-PCR expression ratios (excluding all absent data), 82 (91%) showed conserved direction of regulation, confirming the reliability of the microarray data. The majority of the eight contradictory comparisons resulted from comparisons between ratios close to zero.

Table 5.4 Expression ratios of selected ESTs assessed by microarray and qRT-PCR. Array values indicate mean log_2 FC ratio relative to untreated controls and qRT-PCR values indicate log_2 ratios of $2^{(\Delta C_t control/\Delta C_t treatment)}$. na indicates absence of valid data, whilst array values in bold and underline indicate DE ESTs after statistical analysis.

GenBank accession	Putative function	Treatment					
		ACC					
		IC 27 hpt		**LA 27 hpt**		**FL 27 hpt**	
		Array	**qPCR**	**Array**	**qPCR**	**Array**	**qPCR**
DY396265	Disease resistance protein DRRG49-C	0.87	1.15	1.97	2.50	0.03	0.09
DY396302	Polyubiquitin	**1.63**	2.40	0.16	0.06	0.44	0.23
DY396400	EREBP-4	-0.72	-1.02	na	-4.00	-0.69	-0.93
DY475136	Cytochrome P450	**2.47**	4.21	**2.12**	3.52	**1.53**	3.99
DY475155	Superoxide dismutase	**-1.99**	-2.80	-0.64	-1.61	**-1.29**	-2.02
DY475250	Glutathione S-transferase	-0.59	-0.07	**0.80**	1.06	0.05	0.24
DY475259	Unclear	-0.26	0.39	-1.06	-0.02	0.60	0.52
CV793597	Pathogenesis-related protein 4A	**2.33**	3.37	1.53	1.74	**0.91**	2.99
CV793602	Cinnamyl-alcohol-dehydrogenase	**1.67**	2.70	0.39	0.67	1.31	2.37
CV793608	SNAKIN2 antimicrobial peptide	**3.93**	5.15	2.18	1.89	**2.84**	4.45
DY475488	DnaJ-like protein	-0.06	0.24	0.78	0.22	-0.66	0.18
		MeJA					
		IC 27 hpt		**LA 27 hpt**		**FL 27 hpt**	
		Array	**qPCR**	**Array**	**qPCR**	**Array**	**qPCR**
DY396265	Disease resistance protein DRRG49-C	-0.37	-0.73	-0.03	0.04	-0.76	-0.95
DY396302	Polyubiquitin	0.63	1.23	0.04	-0.07	0.04	0.23
DY396400	EREBP-4	na	na	na	na	na	na
DY475136	Cytochrome P450	0.37	0.55	0.21	0.35	0.03	0.04
DY475155	Superoxide dismutase	-0.07	-0.25	-0.08	-0.04	-0.07	-0.26
DY475250	Glutathione S-transferase	0.10	0.02	0.23	0.10	-0.10	0.00
DY475259	Unclear	na	0.18	-0.23	-0.03	0.20	0.11
CV793597	Pathogenesis-related protein 4A	-0.96	-0.79	0.18	-0.19	-0.55	-0.34
CV793602	Cinnamyl-alcohol-dehydrogenase	0.46	0.66	0.17	0.26	0.04	-0.02
CV793608	SNAKIN2 antimicrobial peptide	**1.22**	2.89	0.41	0.74	0.47	0.84
DY475488	DnaJ-like protein	na	-1.07	0.10	0.02	**1.48**	1.08
		SA					
		IC 27 hpt		**LA 27 hpt**		**FL 27 hpt**	
		Array	**qPCR**	**Array**	**qPCR**	**Array**	**qPCR**
DY396265	Disease resistance protein DRRG49-C	**1.60**	2.83	**2.90**	3.49	0.99	3.32
DY396302	Polyubiquitin	0.12	0.20	0.42	0.53	0.82	1.41
DY396400	EREBP-4	na	na	na	na	na	na
DY475136	Cytochrome P450	-0.03	-0.29	-1.04	-1.18	0.86	0.73
DY475155	Superoxide dismutase	-0.28	-0.74	-0.13	-0.59	-1.03	-0.53
DY475250	Glutathione S-transferase	**2.09**	3.65	**3.98**	4.26	**3.31**	4.53
DY475259	Unclear	**-3.28**	-5.02	-2.29	-4.28	-0.27	-4.53
CV793597	Pathogenesis-related protein 4A	0.75	2.39	**3.34**	2.43	1.56	1.91
CV793602	Cinnamyl-alcohol-dehydrogenase	0.72	2.39	1.05	2.03	1.74	2.68
CV793608	SNAKIN2 antimicrobial peptide	0.81	1.71	1.67	1.51	0.44	0.98
DY475488	DnaJ-like protein	-0.14	-0.31	-1.72	-0.72	-1.48	-0.44

5.4 Discussion

The availability of a set of chickpea unigenes (refer to Chapter 2) and grasspea ESTs (provided by Dr B. Skiba, RMIT University, Victoria, Australia) enabled the construction of a 727-feature microarrays that, in the present study, were exploited to profile the response of three chickpea genotypes to treatments with the defence signalling compounds ACC, MeJA and SA. An experimental system was employed that minimised environmental effects and reduced any gene expression changes not resulting from the treatments. Root tissue was not used considering the microarray probes were sourced from stem/leaf tissue (refer to Chapter 2), and an aim of this study was to associate the results with foliar disease responses. Data quality and reproducibility was strengthened through the use of replication, negative controls, and strict selection criteria for DE ESTs. Confirmation of the microarray observations by qRT-PCR was also performed for 11 ESTs showing varying levels of regulation. The comparison between methods revealed common expression kinetics for all significant microarray regulation, indicating the strong reliability of the microarray data. As reported in Chapter 4, the expression ratio data obtained by qRT-PCR was higher than that for corresponding microarray ratios in many cases.

The microarray cDNA probes originated from libraries constructed of *A. rabiei*-infected chickpea (IC 24/48 hpi) and *M. pinodes*-infected grasspea (ATC80878 48/72 hpi) stem/leaf tissue. The lentil RGA probes were not included considering that cross-hybridisation of the chickpea targets to lentil probes was not successful in Chapter 4. However, cross-hybridisation to grasspea probes was successful in Chapter 4. Subsequently, the proportion of undetected features for each probe source (Table 5.1) indicated that cross-hybridisation to the grasspea probes was again successful (6.4-12.2% undetected).

In this study 425 of the 715 (59.4%) experimental microarray features were DE in at least one condition, although some features were DE in more than one treatment and/or time-point. Such a high level of differential regulation has also been reported in other studies involving these defence signalling compounds (Schenk *et al.*, 2000; Salzman *et al.*, 2005). According to treatment, 69.7% were DE by ACC, 15.8% by MeJA and 57.6% by SA. The substantially lower amount of differential regulation achieved by MeJA may be caused by the use of a lower concentration of this treatment compound compared to ACC and SA. Alternatively, the result may indicate that fewer cellular functions are regulated by jasmonates in chickpea, or that ESTs representing genes of JA-related pathways were underrepresented on the array. In fact, a previous small-scale study of chickpea responses to SA and MeJA treatment found a substantially higher amount of differential regulation for SA (Cho and Muehlbauer, 2004).

The prominence of down-regulation for most conditions (Table 5.2; Figure 5.4) also suggested that the treatments may force the plant to sacrifice the transcription of some 'housekeeping' genes, and that these genes are abundantly represented on the array (also reported in Chapter 4). According to genotype, the *A. rabiei* resistant IC and moderately resistant FL showed more differential expression at 27 hpt, whilst differential expression was more prominent at 3 hpt in the susceptible LA. This may suggest that the response of LA is not sustained as long as that in IC and FL, which may contribute to its susceptibility. Additionally, the only experimental condition showing more up-regulation than down was IC (27 hpt) after ACC treatment. Considering that IC is resistant to *A. rabiei*, this result may indicate that this genotype is able to induce a greater range of potential defence-related genes in response to this defence signalling compound.

The co-regulation of ESTs between treatments for each genotype (Figure 5.4) revealed that large proportions of ESTs were independently regulated by ACC, MeJA or SA. Of the co-

regulated ESTs, the ACC-SA category contained the most co-regulated ESTs for all genotypes but, within genotypes, IC showed a more substantial spread between co-regulation categories than LA and FL. These results supported reports of cross-talk and overlap between signalling pathways observed in other studies (Schenk *et al.*, 2000; Salzman *et al.*, 2005; Jalali *et al.*, 2006). This was particularly evident for ACC-SA, and the finding that some ESTs were co-regulated by MeJA-SA contradicts the hypothesis that SA is antagonistic to JA (Pena-Cortez *et al.*, 1998), possibly indicating that antagonism is restricted to certain genes only. Furthermore, the higher level overlap of ESTs regulated by the three defence signalling compounds in IC may contribute to its ability to mount a broader and more successful defence response. In fact, more co-induced transcripts were defence-related in IC compared to FL and LA. The lower level of cross-talk observed for FL may also provide further evidence for the presence of a specific defence response mediated by unknown signalling mechanisms.

The proportion of regulated ESTs for each treatment X genotype according to functional categories (Figure 5.6) revealed that, even though the global regulation trend was down-regulation, members of the 'defence' category were up-regulated at a higher frequency than down-regulated for all treatments in the *A. rabiei* resistant IC, and for SA treatment in the moderately resistant FL genotype. Subsequently, evidence exists that these treatments provoked defence-like responses in chickpea. SA and ACC treatments induced substantial proportions of 'defence' and 'cell rescue/death/ageing' transcripts amongst all genotypes, which supports the reported importance of SA in localised and systemic defence responses (Jalali *et al.*, 2006). Specifically, SA mainly regulated ESTs involved in the oxidative burst, PR proteins and putative antimicrobial proteins, which has also been reported in other studies (Schenk *et al.*, 2000; Salzman *et al.*, 2005). ACC treatment induced defence-related transcripts involved in the induction of PR proteins, and putative genes controlling the oxidative burst and phenylpropanoid pathway. However, some PR proteins were down-

regulated after ACC treatment, possibly due to the hypothesis that E may confer resistance to certain pathogens and not others (Wang *et al.*, 2002). As expected, ACC induced EREBP transcription factors (one confirmed by qRT-PCR), also observed by Van Zhong and Burns (2003). ACC also induced numerous transcripts from all other functional categories, which may be due to the diverse role of E in growth and development. In fact, other studies have reported significant effects of E on genes involved in primary metabolism (Van Zhong and Burns, 2003)

For the MeJA treatment, very little up-regulation was observed across all categories, and only the IC genotype showed a higher induction of 'defence' transcripts. Subsequently, the only induced ESTs related to defence were PR proteins and some ESTs representing genes involved in the oxidative burst. Induction of genes such as these by JA has been previously reported (Bower *et al.*, 2005; Salzman *et al.*, 2005), although this study did not observe regulation of other genes reported to be involved in defence signalling or phytoalexin biosynthesis. Overall, numerous ESTs encoding putative PR proteins, antimicrobial proteins, and oxidative burst-related proteins were induced by more than one treatment, indicating some common links between the three signalling pathways.

The co-regulation of ESTs between genotypes for each treatment (Figure 5.5) revealed that the regulation of most ESTs was not conserved between genotypes and indicated the presence of differing gene induction networks in each genotype. When comparing the responses of each genotype, it was apparent that the genotypes with resistance to *A. rabiei* were able to induce a much broader range of defence-related transcripts in response to the various signalling compounds. This was particularly evident for the highly resistant IC, which exclusively induced many defence-related ESTs putatively involved in the oxidative burst, defence signalling, and phenylpropanoid pathways, as well as specific PR/antimicrobial and

structural/transport proteins. The moderately resistant FL genotype also induced a greater range of defence-related transcripts compared to the susceptible LA genotype. The defence-signalling compounds all represent secondary defence-signalling molecules, thus their application essentially by-passed the pathogen recognition required for the induction of defence responses to *A. rabiei*. Considering this, the signalling compounds should elicit maximised defence responses from each genotype without the interference of any pathogen-associated molecules. Therefore, the observed differing responses suggest that each genotype possesses a different pathway of defence-related gene expression that is independent of pathogen recognition. However, it is important to recognise that the induction of these defence-related ESTs in response to ACC/MeJA/SA treatments does not imply their involvement in the *A. rabiei* defence response.

To identify putative genes involved in *A. rabiei* defence for these genotypes, Chapter 4 reported an expression profiling study in response to *A. rabiei* inoculation. Subsequently, linking the results of the present study enabled the possibility of inferring the signalling pathway/s responsible for the regulation of those genes putatively involved in *A. rabiei* defence (Table 5.3). For IC, some potentially important induced transcripts were regulated by all treatments, suggesting that signalling pathways mediated by ACC/MeJA/SA are all involved in the IC response to *A. rabiei*. This observation contradicts a previous report that the SA pathway is mainly induced by biotrophic pathogens (refer to section 1.4.3). However, several of the important IC transcripts were not regulated by any treatment, indicating that other *A. rabiei*-specific signalling events may be required for their induction. In the moderately resistant FL genotype, many of the *A. rabiei* induced transcripts were down-regulated by the treatments of this study. Subsequently, FL may also possess defence-signalling mechanisms that are a specifically induced after *A. rabiei* recognition. Of the *A. rabiei* induced ESTs in LA, most resembled PR proteins that we found to be induced by SA

treatment only. This result suggests that an SA-mediated defence response exists in the susceptible LA genotype, an observation also reported by Cho and Muehlbauer (2004), who found that SA regulation of defence-related genes was independent of *A. rabiei* resistance.

The specific transcripts that were previously reported as potentially predictive of *A. rabiei* resistance included the PR proteins, *β*-1,3-glucanase, SNAKIN2 antimicrobial peptide, hypothetical proline-rich protein (PRP), disease resistance response protein DRRG49-C, leucine-zipper containing protein (LZP), environmental stress-inducible protein (ESP), polymorphic antigen membrane protein (PAMP), Ca-binding protein, and several unknown/unclear proteins (refer to Chapter 4). Rapid expression of PR proteins in resistant genotypes (IC and FL) was considered important for *A. rabiei* resistance, and were induced by one or more signalling compound in IC, by SA only in LA, but both induced and repressed by one or more treatments in FL. Therefore, the rapid expression of these proteins in resistant genotypes may be due to rapid *A. rabiei*-specific signalling following recognition, or the presence of ACC-mediated signalling in addition to SA.

Two important *A. rabiei*-induced ESTs in the resistant IC were SNAKIN2 and DRRG49-C, of which SNAKIN2 was co-induced by both ACC- and MeJA-mediated signalling, and DRRG49-C co-induced by ACC- and SA-mediated signalling (both confirmed by qRT-PCR). The PRP and LZP, also induced by *A. rabiei* in IC and FL, were repressed by ACC and SA treatment respectively, suggesting that their induction may be via a pathogen-specific signal. Similarly, the ESP induced by *A. rabiei* in FL, was repressed by both ACC and SA treatment. Both the *β*-1,3-glucanase and PAMP, up-regulated by *A. rabiei* in IC, were not regulated by any treatment of this study, again suggesting the involvement of other signalling pathways.

Interestingly, the Ca-binding protein, up-regulated by *A. rabiei* in IC, was down-regulated by both ACC and SA treatment in this study. This result suggested that elevation of cytosolic Ca^{2+} was not induced by the signalling compounds used, which may be attributed to the elevation of Ca^{2+} being a defence-activating signal in itself (Reddy, 2001), possibly requiring pathogen perception to be triggered. However, two calmodulin-like proteins (DY396411 and DY396364) were up-regulated in IC after SA treatment in this study, which also represent Ca-binding proteins and contradict the absence of elevated Ca^{2+} after SA treatment. Subsequently, the Ca-binding protein found to be down-regulated in this study may only become induced after specific pathogen perception, whilst the calmodulin-like proteins can be induced by other signalling mechanisms. Further studies would be required to confirm such an observation.

Of the antioxidant proteins that were down-regulated by *A. rabiei* inoculation to allow the accumulation of ROS (refer to Chapter 4), the superoxide dismutase copper chaperone precursor was down-regulated by MeJA/SA in IC and by ACC/SA in FL, but the glutathione S-transferase was up-regulated by SA treatment in IC. However, the *A. rabiei*-mediated down-regulation of glutathione S-transferase in IC may be controlled by a different signalling pathway or may be a pathogen-specific response. Finally, of the several unknown/unclear transcripts induced by *A. rabiei* inoculation in the resistant genotypes, none were induced by treatments of this study.

Overall, the highly resistant IC genotype appears to possess a broad range defence-related genes regulated by treatments of this study and, of those putatively involved in *A. rabiei* defence, some are regulated by one or more treatments, whilst others may be regulated by other pathogen-specific mechanisms. FL possesses less defence-related transcripts, and those regulated by *A. rabiei* infection appear to be induced by a signalling pathway undetected by

this study. Finally, the susceptible LA possesses the fewest defence-related transcripts and, of those important in the *A. rabiei* response, SA-mediated signalling is prominent. However, both FL and LA may possess unique defence-related transcripts that were unable to be detected by the 'boutique' array used in this study (refer to section 4.4).

The observations of this study indicate that, although E, JA and SA are partially involved in the signalling of chickpea defence responses to *A. rabiei*, they are not responsible for mediating the entire response that may lead to resistance. Additionally, small-scale transcriptional studies of defence-related genes in chickpea after SA and MeJA treatment, also reported that resistance to *A. rabiei* did not correlate with SA- and JA-mediated regulation of the defence-related genes (Cho and Muehlbauer, 2004; Cho *et al.*, 2006). Subsequently, elucidation of the entire mechanism responsible for *A. rabiei* resistance is difficult, and may involve pathways of pathogen recognition and signal transduction that are mediated by pathogen-specific transcription factors or protein kinases. The necrotrophic nature of *A. rabiei* may contribute to the involvement of unknown mechanisms, considering that resistance mechanisms to necrotrophs have not been conclusively identified (Mayer *et al.*, 2001; Govrin and Levine, 2002). Further, accumulation of antifungal phytoalexins in chickpea has been shown to occur via transformation of a constitutively accumulated pool of isoflavonoids rather than being induced after pathogen infection (Mackenbrock and Barz, 1991). Subsequently, some chickpea defence responses to pathogens may occur constitutively or without the need for known signalling pathways.

5.5 Conclusion

The microarray analyses performed with the available cDNA clones from chickpea and grasspea show that E, JA and SA signalling mediate the expression of numerous putative defence-related genes, as well as genes of other cellular processes. The *A. rabiei* resistant

genotypes, IC and FL, showed a more substantial range of defence-related gene induction by all treatments, indicating that they may possess stronger abilities to resist infection than LA. Further, the involvement of E, JA, and SA signalling was identified for the regulation of some important *A. rabiei* responsive genes, as well as cross-talk between these pathways, especially for IC. This study also found evidence to suggest the involvement of *A. rabiei*-specific signalling mechanisms for the induction of several genes that were previously implicated in *A. rabiei* resistance. The microarray-based differential expression of some ESTs was confirmed by qRT-PCR.

Overall, this study characterised the regulatory mechanisms of many chickpea genes that may be important in defence against various pathogens, as well as other cellular functions. Although the size of the microarray was limited, the results provided novel insights to the molecular control of chickpea cellular processes, which may assist the understanding of chickpea defence mechanisms and allow enhanced development of disease resistant cultivars. The next, and final, step in this study will involve the drawing together of all results to synthesise a hypothetical model of chickpea resistance to *A. rabiei*. Such a model may be used as the basis for further studies on candidate resistance genes, which will be discussed in detail.

Chapter 6

General discussion and synthesis of resistance model.

In Chapter 1 of this thesis I reviewed the current state of knowledge regarding ascochyta blight of chickpea. A key finding from the review was that attempts to develop cultivars with a high level of durable resistance have been unsuccessful, despite the existence of highly resistant genotypes. Important reasons behind this obstacle were identified as the conflicting reports concerning the genetics of resistance, as well as the limited understanding concerning the genes, and pathways of gene activation, involved in an effective defence response. Subsequently, I highlighted significant opportunities for further characterisation of the chickpea defence response using a functional genomics approach.

Before functional genomics techniques could be applied to chickpea, a resource of annotated cDNA clones was required. In Chapter 2 I satisfied this requirement through the sequencing and characterisation of >1000 cDNA clones (ESTs) to result in a library of 516 unigenes. The library included 20 specific defence-related unigenes that may be important for ascochyta blight resistance. Additionally, 14 SSRs were identified that could be used for the development of molecular markers, including one SSR within the SNAKIN2 defence-related unigene. A key finding from this study was that a high proportion of the chickpea transcriptome may be insufficiently homologous to model legumes, which would limit the use of their EST collections for the study of chickpea. This finding validated the generation of chickpea-specific ESTs and highlighted the caveat described in Chapter 1 (section 1.5.1) that sequence conservation between related species is not consistent on a gene-for-gene basis.

In Chapter 3 I described an optimisation of both the chickpea inoculation procedure and microarray methodologies. The use of genetic material representative of the system under study was crucial for gaining meaningful results from microarray studies. Therefore, it was important to optimise the inoculation of chickpea genotypes to resemble disease progression observed in the field. Upon identifying an optimal inoculation procedure, the 20 defence-related unigenes were used to construct small-scale microarrays with the aim of both validating the microarray techniques and providing gene expression profiles for the unigenes in a resistant and susceptible chickpea genotype. This study successfully identified differential expression patterns between genotypes and found three unigenes with potential involvement in ascochyta blight defence (protein with leucine-zipper, SNAKIN2 antimicrobial peptide precursor, and elicitor-induced receptor protein).

Considering that an overall defence response can involve hundreds of genes, from recognition to signalling to direct involvement (refer to section 2.1), the validation of the microarray methods in Chapter 3 enabled the construction of large-scale microarrays comprising all chickpea unigenes. Further, the availability of potential defence-related ESTs and RGAs from related legumes (grasspea and lentil, respectively) provided an opportunity to explore the potential for cross-species hybridisation to these probes on the microarray (refer to section 1.5.1). Although chickpea ESTs were shown to be substantially divergent from model legumes in Chapter 2, successful hybridisation to grasspea and lentil probes was expected considering their higher degree of relatedness (refer to section 3.1). Expression profiles for three chickpeas and one wild relative were generated. Hybridisation to grasspea probes was successful but all lentil probes failed, most likely due to the presence of non-coding regions in these RGAs (refer to section 4.4). A total of 97 differentially expressed ESTs were identified, and the inclusion of greater replication, more stringent statistical tests and qRT-PCR led to the

results of this study being considered more reliable than Chapter 3, although the regulation trends of Chapter 3 did correlate with the results.

The results indicated that genes involved in the active defence response are similar to those governed by *R*-gene mediated resistance, including the production of ROS (oxidative burst) and the HR, down-regulation of 'housekeeping' gene expression, and expression of PR proteins. The comparison between compatible and incompatible interactions identified certain gene expression 'signatures' that may be predictive of resistance, including rapid expression of PR proteins, as well as up-regulation of β-1,3-glucanase, SNAKIN2, PRP, DRRG49-C, LZP, ESP, PAMP, Ca-binding protein, and several unknown/unclear proteins. The results confirmed histopathology studies of the chickpea defence response and, although the microarray was unlikely to contain all *A. rabiei* defence-related genes, provided novel insights to the molecular control of these events. However, the disadvantages of using a 'boutique' array (refer to section 1.5.1) for studying a species different to that used as the probe source were highlighted in this study. Because the chickpea probes were constructed from ICC3996 cDNA, the array could only reveal expression patterns for genes in common between other genotypes and ICC3996. Therefore, the expression patterns of the ESTs for the wild relative appeared to explain little of the observed resistance of this genotype. In retrospect, the array may have been more informative if the probes were sourced from mixed cDNA libraries of all genotypes under study.

To further characterise the regulation of the potential defence-related genes identified in Chapters 3 and 4, the study of Chapter 5 involved treatment of the three chickpea genotypes (excluding the wild relative) with known defence signalling compounds. Expression profiles were generated using the microarray of Chapter 4 (excluding failed lentil probes), resulting in differential expression of 425 ESTs. Comparison between genotypes revealed the presence of

a wider range of inducible defence responses in the resistant chickpea (ICC3996) compared to the moderately resistant and susceptible genotypes. The susceptible genotype appeared to possess the weakest arsenal of inducible defences. Using the results of this study to identify possible regulation of the important ESTs for *A. rabiei* defence indicated the presence of other pathogen-specific signalling mechanisms in addition to E, JA and SA. Treatments with the defence signalling compounds essentially by-passed pathogen recognition and should have induced maximised defence-responses in all genotypes. Therefore, the lower arsenal of defence-related gene expression observed in the susceptible genotype may be a result of 'breaks' in the pathways of defence-related gene activation. The observations that resistant and susceptible genotypes possessed differing responses to the signalling compounds, and that the susceptible genotype was able to mount some defence to *A. rabiei* (Chapter 4) indicate that the 'breaks' may not related to pathogen recognition, but to signal transduction. The susceptible genotype appears to lack the ability to regulate several signalling-related genes, which lead to more rapid and diverse defence responses in resistant genotypes. Possible locations for signal-transduction 'breaks' are highlighted in the model (described below).

To draw together and summarise the findings of this thesis study, I constructed a model to represent a hypothetical mechanism for chickpea resistance to *A. rabiei* (Figure 6.1). The model was synthesised based on evidence gathered in this study, as well as previously identified defence mechanisms in chickpea. The model represents a hypothesis that may form the basis of further studies to either confirm or reject aspects within it, and is in no way intended to represent a definitive model of the defence response. A detailed description of the model follows the figure.

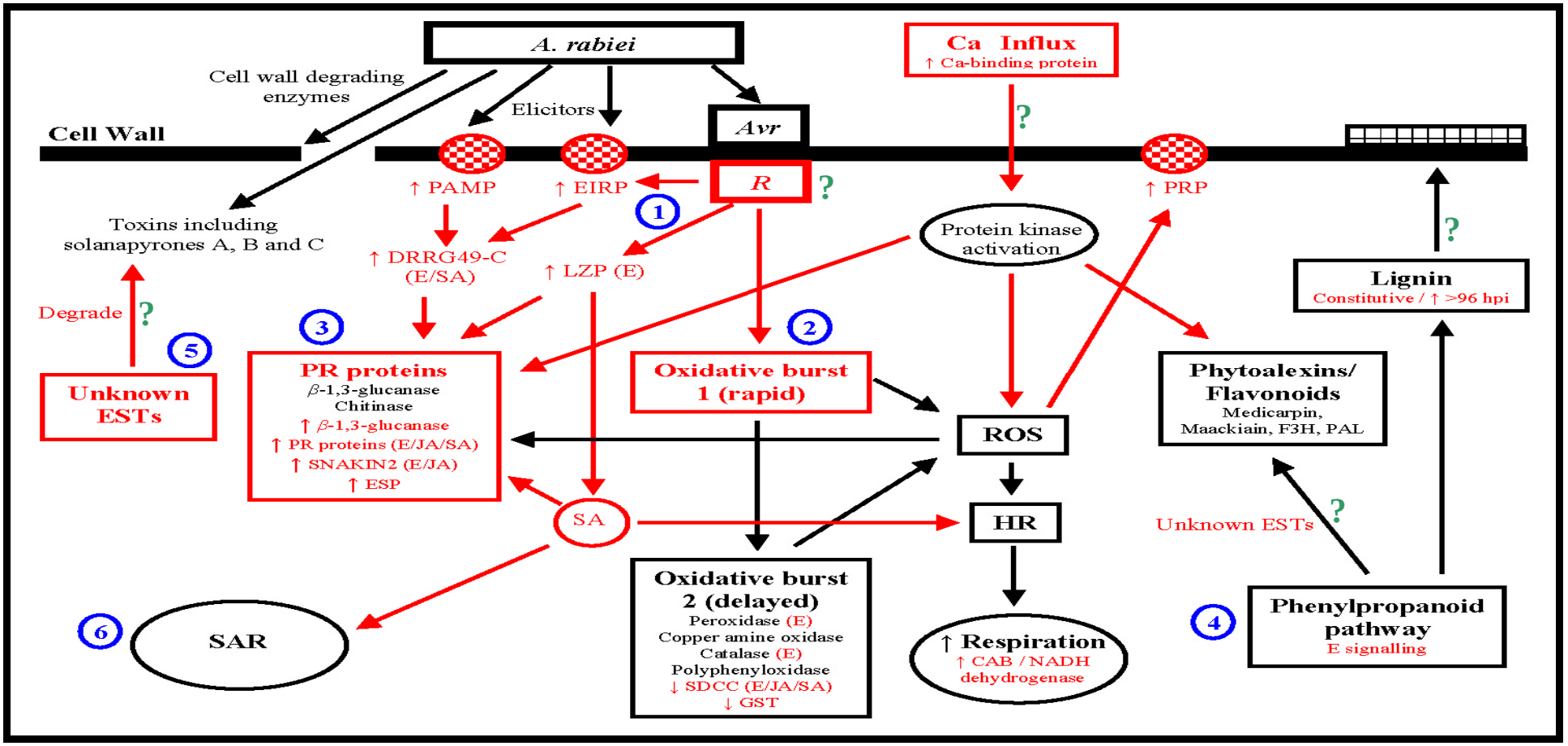

Figure 6.1 Hypothetical model of an effective chickpea defence response to *A. rabiei*, where black sections represent previous knowledge and red sections represent information derived from this study. Blue numbers indicate portions of the model that are explained below and green question marks indicate areas of weak evidence. Arrows before gene names indicate up- or down-regulation and possible signalling compounds are indicated in brackets after each gene name where available. Expanded gene abbreviations are also shown on the legend below.

Figure 6.1 Legend

Abbreviation	Name
EIRP	Elicitor-induced receptor protein
PAMP	Polymorphic antigen membrane protein
LZP	Leucine-zipper protein
DRRG49-C	Disease resistance response protein DRRG49-C
SNAKIN2	SNAKIN2 antimicrobial peptide precursor
ESP	Environmental stress-inducible protein
SDCC	Superoxide dismutase copper chaperone precursor
GST	Glutathione S-transferase
CAB	Chlorophyll a/b binding protein
PRP	Proline-rich protein
ROS	Reactive oxygen species
HR	Hypersensitive response
SAR	Systemic acquired resistance
SA	Salicylic acid
E	Ethylene
JA	Jasmonate

1. Perception: *A. rabiei* is known to secrete elicitors and cell wall degrading enzymes (refer to section 1.2.6), which may represent the *Avr* gene product. The chickpea *R* gene product stimulates the production of EIRP, via a transcription factor or protein kinase, to allow for secondary perception of pathogen-secreted elicitors and amplified signalling cascades in resistant genotypes. The up-regulation of PAMP may also indicate a role in secondary perception and signal transduction. EIRP and PAMP are not induced by E/JA/SA, indicating *A. rabiei*-specific regulation. Further, EIRP and PAMP are not up-regulated in susceptible genotypes, indicating a possible absence of secondary perception and signalling amplification. A change in membrane permeability following perception allows the cellular influx of Ca^{2+}, which stimulates signal transduction via protein kinases to induce active defences including PR proteins, phytoalexins and ROS generating enzymes. After successful activation, Ca^{2+} levels are returned to normal state by the Ca-binding protein, which is also regulated by an *A. rabiei*-specific mechanism.

2. Oxidative burst and HR: Rapid signal transduction following *R* gene perception results in a rapid biphasic oxidative burst in resistant genotypes (refer to section 4.4). The first phase

of the oxidative burst allows the accumulation of ROS via down-regulation of antioxidants including SDCC (controlled by E/JA/SA) and GST (*A. rabiei*-specific), which in turn promotes the induction of PR proteins and the HR. The HR may also be induced by the production of SA (described below). ROS also act to up-regulate a cell wall strengthening PRP in resistant genotypes via *A. rabiei*-specific signalling that acts to restrict pathogen penetration. The result of an effective HR is correlated with the rapid increase in cellular respiration, observed by the *A. rabiei*-specific up-regulation of CAB and NADH dehydrogenase. The second phase oxidative burst acts to sustain the HR. Susceptible genotypes commence an oxidative burst at a later time-point, indicating a delay in signal transduction. Additionally, a PRP is not induced in susceptible genotypes, possibly due a limited accumulation of ROS.

3. PR proteins: Transduction of the perception signal also results in the rapid synthesis of antimicrobial PR proteins. The LZP may deliver this signal through bZIP transcription factor activity, stimulated by E signalling from the recognition event. Further, the production of SA via the LZP (discussed below) may also result in the up-regulation of some PR proteins that were shown to respond to SA treatment (Chapter 5). Signal transduction following PAMP and EIRP stimulation may also contribute in maintaining high levels of PR proteins through the transcriptional activator DRRG49-C, which is also regulated by E/SA in resistant genotypes. Numerous PR proteins are rapidly up-regulated in resistant genotypes and act to kill the invading pathogen, by means such as degrading fungal cell walls. The PR proteins are regulated by both E/JA/SA and *A. rabiei*-specific signalling, where susceptible genotypes delay the up-regulation of some PR proteins, and do not up-regulate others at all (*e.g.* SNAKIN2 and ESP). Susceptible genotypes also do not up-regulate LZP, therefore may not possess the required transcription factors for rapid induction of a wide-range of antimicrobial proteins.

4. Phenylpropanoid pathway: E signalling stimulates the activity of this pathway, leading to the synthesis of phytoalexins and flavonoids (no direct evidence found in this study). Pre-formed lignin synthesised by this pathway provides a physical barrier to pathogen penetration, and resistant genotypes possess a higher quantity than susceptible genotypes. Lignin production may also be up-regulated >96 hpi to further restrict pathogen penetration.

5. Unknown ESTs: *A. rabiei* secretes toxins into chickpea cells, including solanapyrones A, B and C (refer to section 1.2.6). Resistant genotypes are able to detoxify solanapyrone C, possibly through the activity of an unknown protein that is exclusively up-regulated in resistant genotypes. Important unknown proteins up-regulated in response to *A. rabiei* were not regulated by E/JA/SA, indicating that they may also be involved in rapid *A. rabiei*-specific signal transduction in resistant genotypes (not shown in figure). These transcripts may be particularly important since the known chickpea defence mechanisms do not explain pathotype-specific resistance (refer to section 1.3).

6. SAR: Through bZIP transcription factor activity (refer to section 4.4), the LZP may regulate the production of SA as a signal to promote SAR (refer to section 1.4.1). The production of SA may also induce the HR (refer to section 1.4.1).

As reported in Chapter 4, relating the results of gene expression studies to differing reports on the genetic control of resistance is difficult. The defence response postulated in Figure 6.1 is based on classic resistance mechanisms including the oxidative burst, HR, PR proteins and phenylpropanoid pathway. These responses are usually controlled by dominant receptor-like *R* genes, with few examples of recessive control (refer to section 1.4.2.1). Therefore, *A. rabiei* recognition may occur through a dominant *R* gene product such as the extensin isolated in Chapter 2. The extensin possesses LRR motifs for pathogen recognition and, considering

that it was not differentially expressed, supports the ability for pathogen perception in both resistant and susceptible genotypes. However, the presence signal transduction 'breaks' in susceptible genotypes together with reports of numerous incomplete resistance sources, suggest that resistance may be controlled by >1 gene under either dominant or recessive control. For example, the putative LZP transcription factor may lack an effective promoter in susceptible genotypes, or may encode a dysfunctional protein due to sequence mutation. The effective LZP allele could be recessive, where susceptible genotypes possess a dominant allele to mask the recessive allele, but resistant genotypes are homozygous recessive. Alternatively, the effective allele could be dominant, where susceptible genotypes are homozygous recessive.

To move forward from the results of this study I propose that consideration should firstly be given to the identification of copy number and allelic forms of the candidate resistance genes. A simple study to assess the copy number of the candidate genes in resistant and susceptible genotypes could involve genomic Southern blots. The presence of increased copy numbers in resistant genotypes may indicate a gene dosage effect, where up-regulation of an important resistance gene is achieved through the presence of multiple copies of the gene in resistant genotypes. A possible method for identifying alleles could involve the isolation of full-length genes, including upstream regulatory regions, from recently developed chickpea BAC libraries. Full-length sequences could then be used to design primers to amplify and sequence the gene from a range of different genotypes, allowing the possible identification of polymorphisms (*e.g.* SNPs) that represent alleles. The identification of alleles is important, as susceptible genotypes may possess mutated alleles of important genes that cause a loss of function. Additionally, the study of the gene regulatory regions may reveal the presence of ineffective promoters, and may also allow the identification of transcription factors known to interact with specific motifs within the regulatory regions.

Alleles associated with resistance could be identified using SNP genotyping methods, where the presence of certain alleles may correlate with phenotypic resistance. SNP polymorphisms could be assayed in populations segregating for resistance to *A. rabiei* to infer potential effectiveness of alleles for resistance, as well as dominant/recessive genetic control. Other molecular markers, such as the SSR from SNAKIN2, may be exploited to screen existing mapping populations to determine if the gene co-locates with existing QTL for *A. rabiei* resistance. Considering that gene expression level does not necessarily correlate to protein expression due to post-transcriptional modifications, functional analysis of protein products of full-length candidate genes may also be valuable. Additionally, reverse genetics approaches, such as gene knockouts or viral-induced gene silencing, could be exploited to validate the potential function of the candidate genes for conferring resistance.

If the proposed studies described above revealed a lack of difference between alleles of candidate genes and an absence of any gene dosage effect, then a gene silencing mechanism may be present in susceptible genotypes that is under the control of active repressors, DNA methylation, or siRNA. For example, susceptible genotypes may possess a dominant allele that encodes a suppressor of certain transcription factors involved in defence-related gene activation (similar to the barley *Mlo* gene, refer to section 1.4.2.1). Conversely, resistant genotypes may express the recessive allele, a loss of function mutant, which allows defence-related expression to proceed. A dominant allele in susceptible genotypes may also be responsible for targeted methylation of defence-related genes, effectively hindering their transcription. Finally, the dominant allele may encode siRNA transcripts that destroy defence-related transcripts before translation can occur in susceptible genotypes (refer to section 4.4). The possible presence of these mechanisms in cultivars such as Lasseter may have been brought about by their constant selection for yield and growth characters. The

selection for these traits may have indirectly selected for mutants that block defence pathways to allow for greater allocation of metabolic resources to yield and growth.

In summary, the results of this thesis have enhanced the body of knowledge regarding resistance of chickpea to *A. rabiei*. The limited understanding of the genes, and pathways of gene activation, involved in resistance has been improved through the identification of candidate resistance genes, and synthesis of a model describing the molecular control of a potential defence response. Previous knowledge of chickpea defence responses has been confirmed, and novel genes and mechanisms of defence have been identified. The hypothetical model identifies signal transduction as a key to resistance, which may be controlled by multiple dominant or recessive genes, and provides a basis for further studies to characterise and test the importance of the candidate genes. Subsequently, validation of candidate resistance genes may enable the pyramiding of resistance genes and breeding of cultivars with durable resistance to ascochyta blight.

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Appendix 1. Media recipes

LB/Ampicillin Broth (1L)

10 g Tryptone
5 g Yeast Extract
5 g NaCl

Make up to 1 L with water.
Adjust to pH 7.0 using 1 M NaOH, autoclave at 121°C for 15 minutes.
Cool and add Ampicillin to final concentration of 100 μg/mL.

V8 Juice Agar (1L)

200 mL of V8 juice
800 mL of tap water
17 g of Bacteriological agar

Adjust to pH 6.0 using 1 M NaOH, autoclave at 121°C for 15 minutes.
Makes up to 60 plates.

Hoagland Solution (0.5X; 1 L)

Add the following nutrients;

Macronutrients

- 3.0 mL 1.0 M KNO_3
- 2.0 mL 1.0 M $Ca(NO_3)_2 \cdot 4H_2O$
- 1.0 mL 1.0 M $NH_4H_2PO_4$
- 0.5 mL 1.0 M $MgSO_4 \cdot 7H_2O$

Micronutrients

- 165 μL 25 mM KCl
- 165 μL 12.5 mM H_3BO_3
- 165 μL 1.0 mM $MnSO_4 \cdot H_2O$
- 165 μL 1.0 mM $ZnSO_4 \cdot 7H_2O$
- 165 μL 0.25 mM $CuSO_4 \cdot 5H_2O$
- 165 μL 0.25 mM H_2MoO_4
- 0.5 mL 64 mM NaFeDTPA

Add Milli-Q water to 1 L.
Adjust to pH 6.0 using 1 M NaOH.

Appendix 2. Composition of gel electrophoresis buffers

10X FA gel buffer

200 mM 3-[N-Morpholino]propanesulfonic acid (MOPS) (free acid)
50 mM sodium acetate
10 mM EDTA

Adjust to pH 7.0 using 1 M NaOH.

1X FA gel running buffer (1 L)

100 mL 10X FA gel buffer
20 mL 37% (=12.3 M) formaldehyde
880 mL RNase-free water

5X RNA loading buffer (10 mL)

16 µL saturated bromophenol blue
80 µL 500 mM EDTA, pH 8.0
720 µL 37% (= 12.3% M) formaldehyde
2 mL 100% glycerol
3084 µL formamide
4 mL 10X FA gel buffer

Add RNase-free water to 10 mL.

1.2% FA gel

1.2 g agarose
10 mL 10X FA gel buffer

Add RNase-free water to 100 mL.
Microwave to melt agarose, cool to 65°C in waterbath.
Add 1.8 mL of 37% (12.3 M) formaldehyde and 1 µL of ethidium bromide (10 mg/mL). Mix well and pour into gel mould.

5X TBE buffer (1 L)

54 g Tris base
27.5 g boric acid
20 mL 0.5 M EDTA

Add Milli-Q water to 1 L.

Appendix 3. Expression ratio data for the 20 defence-related unigenes

EST	Mean expression ratio*							
	ICC3996				Lasseter			
	12 hpi	24 hpi	48 hpi	96 hpi	12 hpi	24 hpi	48 hpi	96 hpi
DEF01	0.69	1.11	1.20	0.81	0.60	0.96	0.95	0.98
DEF02	0.73	1.11	1.14	1.08	0.63	0.79	0.58	1.11
DEF03	0.94	1.45	1.29	1.14	1.06	0.98	1.09	1.22
DEF04	1.24	1.17	1.09	0.99	1.22	1.34	1.20	0.90
DEF05	0.75	0.96	1.15	1.14	0.80	0.83	1.00	1.20
DEF06	0.97	1.27	1.25	0.96	0.99	0.84	1.07	1.02
DEF07	1.17	1.69	1.33	0.97	1.20	1.77	1.13	1.06
DEF08	0.82	0.44	0.52	0.98	1.12	0.40	0.43	1.47
DEF09	1.29	1.21	1.11	1.02	2.53	4.56	3.31	1.95
DEF10	1.04	2.20	2.91	1.04	1.21	2.26	3.18	1.23
DEF11	0.98	2.08	1.66	1.03	1.19	1.13	1.05	1.08
DEF12	1.24	1.13	1.07	0.97	1.17	1.32	1.18	0.89
DEF13	0.90	0.48	0.46	0.97	0.81	0.42	0.42	0.89
DEF14	1.18	1.21	0.95	0.99	1.22	1.20	1.10	0.91
DEF15	1.19	1.06	1.01	0.94	1.50	1.04	1.23	1.10
DEF16	1.51	3.29	2.32	1.08	2.69	7.60	4.66	2.10
DEF17	0.77	0.46	0.57	1.01	0.93	0.43	0.95	1.25
DEF18	1.32	2.14	1.58	0.97	1.37	1.46	1.24	0.88
DEF19	1.10	2.10	2.13	0.92	1.28	1.17	1.28	1.34
DEF20	1.00	2.06	1.95	0.99	1.14	1.74	2.74	1.03

* Ratio values represent the mean ratio calculated from, firstly, the mean intensity of the duplicated spots, then the mean intensity of the technical replications, and finally the mean intensity of the two biological replicates. Underlined ratios indicate significant up- or down-regulation.

Appendix 4. Characteristics of the 768 microarray features

For 'source', CA indicates *Cicer arietinum* (chickpea), LS indicates *Lathyrus sativus* (grasspea), and LC indicates *Lens culinaris* (lentil).

Meta Row	Meta Column	Row	Column	GenBank Accession	Gene Name	Source	Biosequence Type	Reporter Usage	Control Type
1	1	1	1	DY396334	Aquaporin-like transmembrane channel protein	LS	cDNA clone	Experimental	NA
1	1	1	2	DY396423	Gibberellin-regulated protein 3 precursor	LS	cDNA clone	Experimental	NA
1	1	1	3	NA	NBS-LRR putative RGA Aj516088	LC (ILL7537)	Genomic PCR product	Experimental	NA
1	1	1	4	NA	Lipoxygenase	LC (ILL7537)	Genomic PCR product	Experimental	NA
1	1	1	5	NA	NBS-LRR putative RGA LR1	LC (ILL7537)	Genomic PCR product	Experimental	NA
1	1	1	6	NA	NBS-LRR putative RGA Aj516061	LC (ILL6002)	Genomic PCR product	Experimental	NA
1	1	1	7	NA	NBS-LRR putative RGA Aj516063	LC (ILL6002)	Genomic PCR product	Experimental	NA
1	1	1	8	NA	NBS-LRR putative RGA Aj516065	LC (ILL6002)	Genomic PCR product	Experimental	NA
1	1	1	9	NA	NBS-LRR putative RGA Aj516070	LC (ILL6002)	Genomic PCR product	Experimental	NA
1	1	1	10	NA	NBS-LRR putative RGA Aj516073	LC (ILL6002)	Genomic PCR product	Experimental	NA
1	1	2	1	DY396360	Poly(A)-binding protein	LS	cDNA clone	Experimental	NA
1	1	2	2	DY396394	Transcription initiation factor TFIID 85 KDA subunit	LS	cDNA clone	Experimental	NA
1	1	2	3	DY396387	Similarity to RNA-binding protein	LS	cDNA clone	Experimental	NA
1	1	2	4	DY396378	Polyubiquitin	LS	cDNA clone	Experimental	NA
1	1	2	5	DY396376	Polyubiquitin	LS	cDNA clone	Experimental	NA
1	1	2	6	DY396371	Polyubiquitin	LS	cDNA clone	Experimental	NA
1	1	2	7	DY396414	Splicing factor RSZ33	LS	cDNA clone	Experimental	NA
1	1	2	8	DY396410	Polyubiquitin	LS	cDNA clone	Experimental	NA
1	1	2	9	DY396293	Thioredoxin	LS	cDNA clone	Experimental	NA
1	1	2	10	DY396290	Splicing factor-like protein	LS	cDNA clone	Experimental	NA
1	1	2	11	DY396282	18.2 KDA class I heat shock protein	LS	cDNA clone	Experimental	NA
1	1	2	12	DY396279	NADH dehydrogenase	LS	cDNA clone	Experimental	NA
1	1	2	13	DY396386	Amine oxidase	LS	cDNA clone	Experimental	NA
1	1	2	14	DY396338	Senescence-associated protein DIN1	LS	cDNA clone	Experimental	NA
1	1	3	1	DY396374	Subtilisin inhibitors I and II (ASI-I and ASI-II)	LS	cDNA clone	Experimental	NA
1	1	3	2	DY396379	Putative auxin-repressed protein	LS	cDNA clone	Experimental	NA
1	1	3	3	DY396382	Protein kinase-like protein	LS	cDNA clone	Experimental	NA
1	1	3	4	DY396405	PR1A precursor	LS	cDNA clone	Experimental	NA

1	1	3	5	DY396389	Polygalacturonase inhibitor protein	LS	cDNA clone	Experimental	NA
1	1	3	6	DY396392	Multi resistance protein (F20D22.11 protein)	LS	cDNA clone	Experimental	NA
1	1	3	7	DY396288	Hypothetical proline-rich protein	LS	cDNA clone	Experimental	NA
1	1	3	8	DY396302	Polyubiquitin	LS	cDNA clone	Experimental	NA
1	1	3	9	DY396432	Transcription initiation factor IIF, beta subunit	LS	cDNA clone	Experimental	NA
1	1	3	10	DY396286	Ubiquitin	LS	cDNA clone	Experimental	NA
1	1	3	11	DY396274	Ubiquitin-specific protease 6	LS	cDNA clone	Experimental	NA
1	1	3	12	DY396322	Metallothionein-like protein 1	LS	cDNA clone	Experimental	NA
1	1	3	13	DY396320	salt-inducible protein-like	LS	cDNA clone	Experimental	NA
1	1	3	14	DY396318	Transcription initiation factor IIF beta subunit	LS	cDNA clone	Experimental	NA
1	1	4	1	NA	Unknown	CA	cDNA clone	Experimental	NA
1	1	4	2	EB085055	26S rRNA	CA	cDNA clone	Experimental	NA
1	1	4	3	NA	Unknown	CA	cDNA clone	Experimental	NA
1	1	4	4	EB085058	Unclear	CA	cDNA clone	Experimental	NA
1	1	4	5	DY396283	Protein kinase C inhibitor-like protein	LS	cDNA clone	Experimental	NA
1	1	4	6	DY396289	Putative auxin-repressed protein	LS	cDNA clone	Experimental	NA
1	1	4	7	DY396292	Putative auxin-repressed protein	LS	cDNA clone	Experimental	NA
1	1	4	8	DY396296	Disease resistance response protein 39 precursor	LS	cDNA clone	Experimental	NA
1	1	4	9	DY396299	Beta-glucan binding protein	LS	cDNA clone	Experimental	NA
1	1	4	10	DY396301	Pathogenesis-related protein	LS	cDNA clone	Experimental	NA
1	1	4	11	DY396305	Pathogenesis-related protein	LS	cDNA clone	Experimental	NA
1	1	4	12	DY396311	Disease resistance response protein 230 precursor	LS	cDNA clone	Experimental	NA
1	1	4	13	DY396365	Serine acetyl transferase	LS	cDNA clone	Experimental	NA
1	1	4	14	DY396369	Putative WD-repeat protein	LS	cDNA clone	Experimental	NA
1	1	5	1	EB085019	Chloroplast DNA	CA	cDNA clone	Experimental	NA
1	1	5	2	EB085021	Unknown	CA	cDNA clone	Experimental	NA
1	1	5	3	EB085038	Chloroplast DNA	CA	cDNA clone	Experimental	NA
1	1	5	4	EB085039	Unknown	CA	cDNA clone	Experimental	NA
1	1	5	5	DY475538	Unknown	CA	cDNA clone	Experimental	NA
1	1	5	6	NA	Unknown	CA	cDNA clone	Experimental	NA
1	1	5	7	NA	Unknown	CA	cDNA clone	Experimental	NA
1	1	5	8	EB085043	Translation initiation factor SUI1	CA	cDNA clone	Experimental	NA
1	1	5	9	NA	Unknown	CA	cDNA clone	Experimental	NA
1	1	5	10	EB085045	Unclear	CA	cDNA clone	Experimental	NA
1	1	5	11	EB085051	Unknown	CA	cDNA clone	Experimental	NA
1	1	5	12	EB085066	4.5S, 5S, 16S and 23S rRNA	CA	cDNA clone	Experimental	NA

1	1	5	13	EB085053	Unknown	CA	cDNA clone	Experimental	NA
1	1	5	14	NA	Unknown	CA	cDNA clone	Experimental	NA
1	1	6	1	NA	Unknown	CA	cDNA clone	Experimental	NA
1	1	6	2	NA	Unknown	CA	cDNA clone	Experimental	NA
1	1	6	3	NA	Unknown	CA	cDNA clone	Experimental	NA
1	1	6	4	NA	Unknown	CA	cDNA clone	Experimental	NA
1	1	6	5	EB085060	Unknown	CA	cDNA clone	Experimental	NA
1	1	6	6	NA	Unknown	CA	cDNA clone	Experimental	NA
1	1	6	7	EB085027	5.8S, 18S and 25S rRNA	CA	cDNA clone	Experimental	NA
1	1	6	8	NA	Unknown	CA	cDNA clone	Experimental	NA
1	1	6	9	NA	Unknown	CA	cDNA clone	Experimental	NA
1	1	6	10	EB085065	18S rRNA	CA	cDNA clone	Experimental	NA
1	1	6	11	DY475554	Chlorophyll a/b binding protein	CA	cDNA clone	Experimental	NA
1	1	6	12	NA	Unknown	CA	cDNA clone	Experimental	NA
1	1	6	13	DY475536	Unknown	CA	cDNA clone	Experimental	NA
1	1	6	14	DY475532	Unknown	CA	cDNA clone	Experimental	NA
1	1	7	1	DY475350	Unknown	CA	cDNA clone	Experimental	NA
1	1	7	2	DY475353	Unknown	CA	cDNA clone	Experimental	NA
1	1	7	3	DY475360	Unknown	CA	cDNA clone	Experimental	NA
1	1	7	4	DY475363	Unknown	CA	cDNA clone	Experimental	NA
1	1	7	5	DY475365	Unknown	CA	cDNA clone	Experimental	NA
1	1	7	6	DY475369	Unknown	CA	cDNA clone	Experimental	NA
1	1	7	7	DY475436	Unknown	CA	cDNA clone	Experimental	NA
1	1	7	8	DY475439	Unknown	CA	cDNA clone	Experimental	NA
1	1	7	9	DY475446	Unknown	CA	cDNA clone	Experimental	NA
1	1	7	10	DY475459	Unknown	CA	cDNA clone	Experimental	NA
1	1	7	11	DY475462	Unknown	CA	cDNA clone	Experimental	NA
1	1	7	12	DY475472	Unknown	CA	cDNA clone	Experimental	NA
1	1	7	13	DY475481	Unknown	CA	cDNA clone	Experimental	NA
1	1	7	14	DY475483	Unknown	CA	cDNA clone	Experimental	NA
1	1	8	1	DY475171	Unknown	CA	cDNA clone	Experimental	NA
1	1	8	2	DY475178	Unknown	CA	cDNA clone	Experimental	NA
1	1	8	3	DY475187	Unknown	CA	cDNA clone	Experimental	NA
1	1	8	4	DY475191	Unknown	CA	cDNA clone	Experimental	NA
1	1	8	5	DY475260	Unknown	CA	cDNA clone	Experimental	NA
1	1	8	6	DY475268	Unknown	CA	cDNA clone	Experimental	NA

1	1	8	7	DY475275	Unknown	CA	cDNA clone	Experimental	NA
1	1	8	8	DY475279	Unknown	CA	cDNA clone	Experimental	NA
1	1	8	9	DY475281	Unknown	CA	cDNA clone	Experimental	NA
1	1	8	10	DY475288	Unknown	CA	cDNA clone	Experimental	NA
1	1	8	11	DY475291	Unknown	CA	cDNA clone	Experimental	NA
1	1	8	12	DY475295	Unknown	CA	cDNA clone	Experimental	NA
1	1	8	13	DY475342	Unknown	CA	cDNA clone	Experimental	NA
1	1	8	14	DY475347	Unknown	CA	cDNA clone	Experimental	NA
1	1	9	1	DY475323	Unclear	CA	cDNA clone	Experimental	NA
1	1	9	2	DY475333	Unclear	CA	cDNA clone	Experimental	NA
1	1	9	3	DY475552	Unclear	CA	cDNA clone	Experimental	NA
1	1	9	4	DY475522	Unclear	CA	cDNA clone	Experimental	NA
1	1	9	5	DY475528	Unclear	CA	cDNA clone	Experimental	NA
1	1	9	6	DY475054	Unknown	CA	cDNA clone	Experimental	NA
1	1	9	7	DY475056	Unknown	CA	cDNA clone	Experimental	NA
1	1	9	8	DY475062	Unknown	CA	cDNA clone	Experimental	NA
1	1	9	9	DY475067	Unknown	CA	cDNA clone	Experimental	NA
1	1	9	10	DY475079	Unknown	CA	cDNA clone	Experimental	NA
1	1	9	11	DY475157	Unknown	CA	cDNA clone	Experimental	NA
1	1	9	12	DY475159	Unknown	CA	cDNA clone	Experimental	NA
1	1	9	13	DY475165	Unknown	CA	cDNA clone	Experimental	NA
1	1	9	14	DY475167	Unknown	CA	cDNA clone	Experimental	NA
1	1	10	1	DY475209	Lipid transfer protein	CA	cDNA clone	Experimental	NA
1	1	10	2	DY475290	GTP-binding protein	CA	cDNA clone	Experimental	NA
1	1	10	3	DY475447	Protein transport protein	CA	cDNA clone	Experimental	NA
1	1	10	4	DY475488	DNAJ-like protein	CA	cDNA clone	Experimental	NA
1	1	10	5	DY475523	Sorting nexin protein	CA	cDNA clone	Experimental	NA
1	1	10	6	DY475065	Unclear	CA	cDNA clone	Experimental	NA
1	1	10	7	DY475086	Unclear	CA	cDNA clone	Experimental	NA
1	1	10	8	DY475097	Unclear	CA	cDNA clone	Experimental	NA
1	1	10	9	DY475259	Unclear	CA	cDNA clone	Experimental	NA
1	1	10	10	DY475264	Unclear	CA	cDNA clone	Experimental	NA
1	1	10	11	DY475272	Unclear	CA	cDNA clone	Experimental	NA
1	1	10	12	DY475274	Unclear	CA	cDNA clone	Experimental	NA
1	1	10	13	DY475292	Unclear	CA	cDNA clone	Experimental	NA
1	1	10	14	DY475319	Unclear	CA	cDNA clone	Experimental	NA

1	1	11	1	DY475489	Chlorophyll a/b binding protein	CA	cDNA clone	Experimental	NA
1	1	11	2	DY475518	Chloroplast DNA between the RUBISCO large subunit and ATPase (beta) genes	CA	cDNA clone	Experimental	NA
1	1	11	3	DY475063	Chloroplast 30S ribosomal protein S12	CA	cDNA clone	Experimental	NA
1	1	11	4	DY475104	Ribosomal protein L41	CA	cDNA clone	Experimental	NA
1	1	11	5	DY475117	40S ribosomal protein S15	CA	cDNA clone	Experimental	NA
1	1	11	6	DY475122	Amino acid transferase	CA	cDNA clone	Experimental	NA
1	1	11	7	DY475420	26S ribosomal protein	CA	cDNA clone	Experimental	NA
1	1	11	8	DY475425	60S ribosomal protein L23	CA	cDNA clone	Experimental	NA
1	1	11	9	DY475442	Translation initiation factor	CA	cDNA clone	Experimental	NA
1	1	11	10	DY475499	S28 ribosomal protein	CA	cDNA clone	Experimental	NA
1	1	11	11	DY475506	Chloroplast 50S ribosomal protein	CA	cDNA clone	Experimental	NA
1	1	11	12	DY475510	30S ribosomal protein S13	CA	cDNA clone	Experimental	NA
1	1	11	13	DY475524	40S ribosomal protein S27	CA	cDNA clone	Experimental	NA
1	1	11	14	DY475101	Chloroplast 16S rRNA	CA	cDNA clone	Experimental	NA
1	1	12	1	DY475500	Zinc-binding dehydrogenase	CA	cDNA clone	Experimental	NA
1	1	12	2	DY475530	Thiamine biosynthesis protein	CA	cDNA clone	Experimental	NA
1	1	12	3	CV793610	Class 10 pathogenesis related protein	CA	cDNA clone	Experimental	NA
1	1	12	4	CV793594	Transcription factor of the AP2/EREBP1 DNA binding domain	CA	cDNA clone	Experimental	NA
1	1	12	5	DY475047	Photosystem I reaction centre subunit IX	CA	cDNA clone	Experimental	NA
1	1	12	6	DY475058	Chloroplast CP12 mRNA	CA	cDNA clone	Experimental	NA
1	1	12	7	DY475069	Thioredoxin	CA	cDNA clone	Experimental	NA
1	1	12	8	DY475083	Ferredoxin-NADP reductase (EC 1.18.1.2)	CA	cDNA clone	Experimental	NA
1	1	12	9	DY475128	Photosystem I reaction centre subunit IV	CA	cDNA clone	Experimental	NA
1	1	12	10	DY475132	Photosystem I reaction centre subunit XI	CA	cDNA clone	Experimental	NA
1	1	12	11	DY475142	Photosystem II D2 protein	CA	cDNA clone	Experimental	NA
1	1	12	12	DY475148	Photosystem II protein	CA	cDNA clone	Experimental	NA
1	1	12	13	DY475454	Chlorophyll a/b binding protein	CA	cDNA clone	Experimental	NA
1	1	12	14	DY475480	Photosystem II core complex protein psbY	CA	cDNA clone	Experimental	NA
1	1	13	1	DY475379	Thymidylate kinase	CA	cDNA clone	Experimental	NA
1	1	13	2	DY475550	WD repeat protein	CA	cDNA clone	Experimental	NA
1	1	13	3	DY475155	Superoxide dismutase (EC 1.15.1.1)	CA	cDNA clone	Experimental	NA
1	1	13	4	DY475179	Acetyl transferase	CA	cDNA clone	Experimental	NA
1	1	13	5	DY475181	Apocytochrome F	CA	cDNA clone	Experimental	NA
1	1	13	6	DY475199	Squalene epoxidase enzyme (EC 1.14.99.7)	CA	cDNA clone	Experimental	NA

1	1	13	7	DY475212	Glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12)	CA	cDNA clone	Experimental	NA
1	1	13	8	DY475240	Ribose 5-phosphate isomerase (EC 5.3.1.6)	CA	cDNA clone	Experimental	NA
1	1	13	9	DY475234	Glycine cleavage system H protein	CA	cDNA clone	Experimental	NA
1	1	13	10	DY475547	Fructose-1,6-bisphosphate aldolase (EC 4.1.2.13)	CA	cDNA clone	Experimental	NA
1	1	13	11	DY475443	Succinate dehydrogenase subunit 3	CA	cDNA clone	Experimental	NA
1	1	13	12	DY475457	Lipoamide dehydrogenase (EC 1.8.1.4)	CA	cDNA clone	Experimental	NA
1	1	13	13	DY475475	Asparagine synthetase (EC 6.3.5.4)	CA	cDNA clone	Experimental	NA
1	1	13	14	DY475551	Homogentisate 1,2 dioxygenase (EC 1.13.11.5)	CA	cDNA clone	Experimental	NA
1	1	14	1	DY475112	Nucleotide-sugar epimerase	CA	cDNA clone	Experimental	NA
1	1	14	2	DY475244	Nucleotide-sugar dehydratase	CA	cDNA clone	Experimental	NA
1	1	14	3	DY475300	Actin	CA	cDNA clone	Experimental	NA
1	1	14	4	DY475372	Adenosylhomocysteinase	CA	cDNA clone	Experimental	NA
1	1	14	5	DY475049	Metallothionein protein (MT-2)	CA	cDNA clone	Experimental	NA
1	1	14	6	DY475076	Phosphate-induced protein	CA	cDNA clone	Experimental	NA
1	1	14	7	DY475092	Dehydrin cold-induced protein	CA	cDNA clone	Experimental	NA
1	1	14	8	DY475137	Auxin repressed protein	CA	cDNA clone	Experimental	NA
1	1	14	9	DY475509	PPF1 - post floral protein	CA	cDNA clone	Experimental	NA
1	1	14	10	DY475077	Protein kinase	CA	cDNA clone	Experimental	NA
1	1	14	11	DY475103	Protein kinase	CA	cDNA clone	Experimental	NA
1	1	14	12	DY475198	SNAP25 protein	CA	cDNA clone	Experimental	NA
1	1	14	13	DY475248	Polymorphic antigen membrane protein	CA	cDNA clone	Experimental	NA
1	1	14	14	DY475320	Serine/threonine protein kinase	CA	cDNA clone	Experimental	NA
1	2	1	1	NA	NBS-LRR putative RGA Aj516078	LC (ILL7537)	Genomic PCR product	Experimental	NA
1	2	1	2	NA	NBS-LRR putative RGA Aj516084	LC (ILL7537)	Genomic PCR product	Experimental	NA
1	2	1	3	NA	NBS-LRR putative RGA Aj516078	LC (ILL6002)	Genomic PCR product	Experimental	NA
1	2	1	4	NA	NBS-LRR putative RGA Aj516083	LC (ILL6002)	Genomic PCR product	Experimental	NA
1	2	1	5	NA	NBS-LRR putative RGA Aj516088	LC (ILL6002)	Genomic PCR product	Experimental	NA
1	2	1	6	NA	Copper amine oxidase	LC (ILL6002)	Genomic PCR product	Experimental	NA
1	2	1	7	NA	Isoflavone synthase	LC (ILL6002)	Genomic PCR product	Experimental	NA
1	2	1	8	NA	Printing Control	NA	Oligo	Control	Printing
1	2	1	9	NA	Blank	NA	Blank	Control	Negative
1	2	1	10	NA	Blank	NA	Blank	Control	Negative
1	2	2	1	DY396406	Metallothionein-like protein 1	LS	cDNA clone	Experimental	NA
1	2	2	2	DY396402	Alpha-amylase	LS	cDNA clone	Experimental	NA
1	2	2	3	DY396399	Cornifin alpha (small proline-rich protein 1)	LS	cDNA clone	Experimental	NA

1	2	2	4	DY396270	Putative deoxycytidylate deaminase	LS	cDNA clone	Experimental	NA
1	2	2	5	DY396267	Enolase	LS	cDNA clone	Experimental	NA
1	2	2	6	DY396428	Polyubiquitin	LS	cDNA clone	Experimental	NA
1	2	2	7	DY396420	Similarity to heat shock related protein	LS	cDNA clone	Experimental	NA
1	2	2	8	DY396317	Putative glutaredoxin	LS	cDNA clone	Experimental	NA
1	2	2	9	DY396419	Putative tonoplast intrinsic protein	LS	cDNA clone	Experimental	NA
1	2	2	10	NA	NBS-LRR putative RGA Aj516061	LC (ILL7537)	Genomic PCR product	Experimental	NA
1	2	2	11	NA	NBS-LRR putative RGA Aj516064	LC (ILL7537)	Genomic PCR product	Experimental	NA
1	2	2	12	NA	NBS-LRR putative RGA Aj516067	LC (ILL7537)	Genomic PCR product	Experimental	NA
1	2	2	13	NA	NBS-LRR putative RGA Aj516071	LC (ILL7537)	Genomic PCR product	Experimental	NA
1	2	2	14	NA	NBS-LRR putative RGA Aj516073	LC (ILL7537)	Genomic PCR product	Experimental	NA
1	2	3	1	DY396411	Calmodulin-binding protein/ER66	LS	cDNA clone	Experimental	NA
1	2	3	2	DY396416	Disease resistance response protein 230 precursor	LS	cDNA clone	Experimental	NA
1	2	3	3	DY396422	Protein kinase-like protein	LS	cDNA clone	Experimental	NA
1	2	3	4	DY396426	Subtilisin inhibitors I and II (ASI-I and ASI-II)	LS	cDNA clone	Experimental	NA
1	2	3	5	DY396427	Lectin-like protein	LS	cDNA clone	Experimental	NA
1	2	3	6	DY396430	Chalcone reductase	LS	cDNA clone	Experimental	NA
1	2	3	7	DY396310	Polyubiquitin	LS	cDNA clone	Experimental	NA
1	2	3	8	DY396306	Epoxide hydrolase	LS	cDNA clone	Experimental	NA
1	2	3	9	DY396342	Glycine-rich cell wall protein GRP 1.8	LS	cDNA clone	Experimental	NA
1	2	3	10	DY396340	Cytochrome B5	LS	cDNA clone	Experimental	NA
1	2	3	11	DY396337	Alpha-amylase precursor	LS	cDNA clone	Experimental	NA
1	2	3	12	DY396326	Ubiquitin	LS	cDNA clone	Experimental	NA
1	2	3	13	DY396368	Ubiquitin	LS	cDNA clone	Experimental	NA
1	2	3	14	DY396363	Magnesium chelatase subunit	LS	cDNA clone	Experimental	NA
1	2	4	1	DY396262	Probable Ca-binding mitochondrial carrier AT2G35800	LS	cDNA clone	Experimental	NA
1	2	4	2	DY396265	Disease resistance response protein DRRG49-C	LS	cDNA clone	Experimental	NA
1	2	4	3	DY396275	Putative chitinase	LS	cDNA clone	Experimental	NA
1	2	4	4	DY396277	Disease resistance response protein 39 precursor	LS	cDNA clone	Experimental	NA
1	2	4	5	DY396314	Immunophilin	LS	cDNA clone	Experimental	NA
1	2	4	6	DY396331	Glutathione peroxidase	LS	cDNA clone	Experimental	NA
1	2	4	7	DY396335	Nitrate transporter NRT1-1	LS	cDNA clone	Experimental	NA
1	2	4	8	DY396436	Putative nuclear transport factor 2	LS	cDNA clone	Experimental	NA
1	2	4	9	DY396345	Protein kinase-like protein (Serine/Threonine kinase PBS1)	LS	cDNA clone	Experimental	NA

1	2	4	10	DY396351	Putative protein kinase	LS	cDNA clone	Experimental	NA
1	2	4	11	DY396358	Laccase-like protein	LS	cDNA clone	Experimental	NA
1	2	4	12	DY396362	Protein kinase-like protein	LS	cDNA clone	Experimental	NA
1	2	4	13	DY396395	EREBP-4	LS	cDNA clone	Experimental	NA
1	2	4	14	DY396384	Pathogenesis-related protein 4A	LS	cDNA clone	Experimental	NA
1	2	5	1	NA	Unknown	CA	cDNA clone	Experimental	NA
1	2	5	2	EB085037	Unknown	CA	cDNA clone	Experimental	NA
1	2	5	3	EB085046	Unclear	CA	cDNA clone	Experimental	NA
1	2	5	4	NA	Unknown	CA	cDNA clone	Experimental	NA
1	2	5	5	NA	Unknown	CA	cDNA clone	Experimental	NA
1	2	5	6	NA	Unknown	CA	cDNA clone	Experimental	NA
1	2	5	7	DY475539	Unknown	CA	cDNA clone	Experimental	NA
1	2	5	8	NA	Unknown	CA	cDNA clone	Experimental	NA
1	2	5	9	EB085050	Chloroplast DNA	CA	cDNA clone	Experimental	NA
1	2	5	10	NA	Unknown	CA	cDNA clone	Experimental	NA
1	2	5	11	NA	Normalisation control 2	CA	cDNA clone	Control	Normalisation
1	2	5	12	NA	Digested pGEM-T Easy Vector II (Promega) Plasmid *AluI*	NA	Digested Plasmid	Control	Negative
1	2	5	13	NA	SMART (Clontech) PCR primer	NA	Oligo	Control	Negative
1	2	5	14	DY396260	Subtilisin Inhibitors I and II (ASI-I and ASI-I)	LS	cDNA clone	Experimental	NA
1	2	6	1	EB085028	Unknown	CA	cDNA clone	Experimental	NA
1	2	6	2	EB085029	Unknown	CA	cDNA clone	Experimental	NA
1	2	6	3	EB085030	Unknown	CA	cDNA clone	Experimental	NA
1	2	6	4	NA	Unknown	CA	cDNA clone	Experimental	NA
1	2	6	5	NA	Unknown	CA	cDNA clone	Experimental	NA
1	2	6	6	NA	Unknown	CA	cDNA clone	Experimental	NA
1	2	6	7	NA	Unknown	CA	cDNA clone	Experimental	NA
1	2	6	8	EB085032	Disease resistance response protein DRRG49-C	CA	cDNA clone	Experimental	NA
1	2	6	9	EB085023	60S rRNA	CA	cDNA clone	Experimental	NA
1	2	6	10	DY475533	Unknown	CA	cDNA clone	Experimental	NA
1	2	6	11	EB085061	Unknown	CA	cDNA clone	Experimental	NA
1	2	6	12	NA	Unknown	CA	cDNA clone	Experimental	NA
1	2	6	13	EB085026	Unknown	CA	cDNA clone	Experimental	NA
1	2	6	14	NA	Unknown	CA	cDNA clone	Experimental	NA
1	2	7	1	DY475391	Unknown	CA	cDNA clone	Experimental	NA
1	2	7	2	DY475399	Unknown	CA	cDNA clone	Experimental	NA

1	2	7	3	DY475407	Unknown	CA	cDNA clone	Experimental	NA
1	2	7	4	DY475414	Unknown	CA	cDNA clone	Experimental	NA
1	2	7	5	DY475426	Unknown	CA	cDNA clone	Experimental	NA
1	2	7	6	DY475431	Unknown	CA	cDNA clone	Experimental	NA
1	2	7	7	DY475485	Unknown	CA	cDNA clone	Experimental	NA
1	2	7	8	DY475491	Unknown	CA	cDNA clone	Experimental	NA
1	2	7	9	DY475553	Unknown	CA	cDNA clone	Experimental	NA
1	2	7	10	DY475519	Unknown	CA	cDNA clone	Experimental	NA
1	2	7	11	DY475521	Unknown	CA	cDNA clone	Experimental	NA
1	2	7	12	NA	Unknown	CA	cDNA clone	Experimental	NA
1	2	7	13	EB085014	Unknown	CA	cDNA clone	Experimental	NA
1	2	7	14	NA	Unknown	CA	cDNA clone	Experimental	NA
1	2	8	1	DY475230	Unknown	CA	cDNA clone	Experimental	NA
1	2	8	2	DY475236	Unknown	CA	cDNA clone	Experimental	NA
1	2	8	3	DY475243	Unknown	CA	cDNA clone	Experimental	NA
1	2	8	4	DY475255	Unknown	CA	cDNA clone	Experimental	NA
1	2	8	5	DY475298	Unknown	CA	cDNA clone	Experimental	NA
1	2	8	6	DY475303	Unknown	CA	cDNA clone	Experimental	NA
1	2	8	7	DY475311	Unknown	CA	cDNA clone	Experimental	NA
1	2	8	8	DY475315	Unknown	CA	cDNA clone	Experimental	NA
1	2	8	9	DY475327	Unknown	CA	cDNA clone	Experimental	NA
1	2	8	10	DY475331	Unknown	CA	cDNA clone	Experimental	NA
1	2	8	11	DY475337	Unknown	CA	cDNA clone	Experimental	NA
1	2	8	12	DY475339	Unknown	CA	cDNA clone	Experimental	NA
1	2	8	13	DY475373	Unknown	CA	cDNA clone	Experimental	NA
1	2	8	14	DY475382	Unknown	CA	cDNA clone	Experimental	NA
1	2	9	1	DY475444	Unclear	CA	cDNA clone	Experimental	NA
1	2	9	2	DY475473	Unclear	CA	cDNA clone	Experimental	NA
1	2	9	3	DY475081	Unknown	CA	cDNA clone	Experimental	NA
1	2	9	4	DY475085	Unknown	CA	cDNA clone	Experimental	NA
1	2	9	5	DY475094	Unknown	CA	cDNA clone	Experimental	NA
1	2	9	6	DY475100	Unknown	CA	cDNA clone	Experimental	NA
1	2	9	7	DY475106	Unknown	CA	cDNA clone	Experimental	NA
1	2	9	8	DY475125	Unknown	CA	cDNA clone	Experimental	NA
1	2	9	9	DY475133	Unknown	CA	cDNA clone	Experimental	NA
1	2	9	10	DY475051	Unknown	CA	cDNA clone	Experimental	NA

1	2	9	11	DY475203	Unknown	CA	cDNA clone	Experimental	NA
1	2	9	12	DY475208	Unknown	CA	cDNA clone	Experimental	NA
1	2	9	13	DY475215	Unknown	CA	cDNA clone	Experimental	NA
1	2	9	14	DY475219	Unknown	CA	cDNA clone	Experimental	NA
1	2	10	1	DY475114	Unclear	CA	cDNA clone	Experimental	NA
1	2	10	2	DY475126	Unclear	CA	cDNA clone	Experimental	NA
1	2	10	3	DY475175	Unclear	CA	cDNA clone	Experimental	NA
1	2	10	4	DY475205	Unclear	CA	cDNA clone	Experimental	NA
1	2	10	5	DY475217	Unclear	CA	cDNA clone	Experimental	NA
1	2	10	6	DY475222	Unclear	CA	cDNA clone	Experimental	NA
1	2	10	7	DY475226	Unclear	CA	cDNA clone	Experimental	NA
1	2	10	8	DY475235	Unclear	CA	cDNA clone	Experimental	NA
1	2	10	9	DY475367	Unclear	CA	cDNA clone	Experimental	NA
1	2	10	10	DY475380	Unclear	CA	cDNA clone	Experimental	NA
1	2	10	11	DY475388	Unclear	CA	cDNA clone	Experimental	NA
1	2	10	12	DY475549	Unclear	CA	cDNA clone	Experimental	NA
1	2	10	13	DY475409	Unclear	CA	cDNA clone	Experimental	NA
1	2	10	14	DY475418	Unclear	CA	cDNA clone	Experimental	NA
1	2	11	1	DY475312	60S ribosomal protein L14	CA	cDNA clone	Experimental	NA
1	2	11	2	DY475324	60S ribosomal protein L19	CA	cDNA clone	Experimental	NA
1	2	11	3	DY475344	Chloroplast 50S ribosomal protein L14	CA	cDNA clone	Experimental	NA
1	2	11	4	DY475354	40S ribosomal protein S27A	CA	cDNA clone	Experimental	NA
1	2	11	5	DY475371	60S ribosomal protein L38	CA	cDNA clone	Experimental	NA
1	2	11	6	DY475395	60S ribosomal protein L11	CA	cDNA clone	Experimental	NA
1	2	11	7	DY475109	Mitochondrial 26S rRNA	CA	cDNA clone	Experimental	NA
1	2	11	8	DY475146	Chloroplast 16S rRNA	CA	cDNA clone	Experimental	NA
1	2	11	9	DY475153	26S ribosomal RNA	CA	cDNA clone	Experimental	NA
1	2	11	10	DY475196	RNA polymerase beta subunit	CA	cDNA clone	Experimental	NA
1	2	11	11	DY475297	RNA binding protein	CA	cDNA clone	Experimental	NA
1	2	11	12	DY475419	DNA directed RNA polymerase	CA	cDNA clone	Experimental	NA
1	2	11	13	DY475074	Protein transport protein	CA	cDNA clone	Experimental	NA
1	2	11	14	DY475169	Potassium channel regulatory factor	CA	cDNA clone	Experimental	NA
1	2	12	1	CV793606	SNAKIN2 antimicrobial peptide precursor	CA	cDNA clone	Experimental	NA
1	2	12	2	CV793608	SNAKIN2 antimicrobial peptide precursor	CA	cDNA clone	Experimental	NA
1	2	12	3	CV793603	Nematode resistance protein *Hs1pro-1* homolog	CA	cDNA clone	Experimental	NA
1	2	12	4	CV793587	Extensin-like protein	CA	cDNA clone	Experimental	NA

1	2	12	5	DY475163	Chlorophyll a/b binding protein	CA	cDNA clone	Experimental	NA
1	2	12	6	DY475202	Chlorophyll a/b binding protein	CA	cDNA clone	Experimental	NA
1	2	12	7	DY475245	ATP synthase (EC 3.6.1.34)	CA	cDNA clone	Experimental	NA
1	2	12	8	DY475287	NADH-plastoquinone oxidoreductase subunit I (EC 1.6.5.3)	CA	cDNA clone	Experimental	NA
1	2	12	9	DY475304	Similar to ferredoxin-thioredoxin reductase	CA	cDNA clone	Experimental	NA
1	2	12	10	DY475316	NADH dehydrogenase	CA	cDNA clone	Experimental	NA
1	2	12	11	DY475402	Chloroplast DNA	CA	cDNA clone	Experimental	NA
1	2	12	12	DY475430	Chlorophyll a/b binding protein	CA	cDNA clone	Experimental	NA
1	2	12	13	DY475131	50S ribosomal protein L12	CA	cDNA clone	Experimental	NA
1	2	12	14	DY475238	Chloroplast 30S ribosomal protein S7	CA	cDNA clone	Experimental	NA
1	2	13	1	DY475136	Cytochrome P450	CA	cDNA clone	Experimental	NA
1	2	13	2	DY475149	UDP-glucose 4-epimerase (EC 5.1.3.2)	CA	cDNA clone	Experimental	NA
1	2	13	3	DY475286	Similar to alpha galactosidase	CA	cDNA clone	Experimental	NA
1	2	13	4	DY475306	Cationic peroxidase (EC 1.11.1.7)	CA	cDNA clone	Experimental	NA
1	2	13	5	DY475309	Xylose isomerase (EC 5.3.1.5)	CA	cDNA clone	Experimental	NA
1	2	13	6	DY475374	Cytochrome P450	CA	cDNA clone	Experimental	NA
1	2	13	7	DY475387	Peptidase-like protein	CA	cDNA clone	Experimental	NA
1	2	13	8	DY475396	Similar to endopeptidase	CA	cDNA clone	Experimental	NA
1	2	13	9	DY475403	Carbonic anhydrase like protein (EC 4.2.1.1)	CA	cDNA clone	Experimental	NA
1	2	13	10	DY475415	Beta glucosidase (EC 3.2.1.21)	CA	cDNA clone	Experimental	NA
1	2	13	11	CV793593	Homology to putative disease resistance protein from *A .thaliana*	CA	cDNA clone	Experimental	NA
1	2	13	12	CV793598	beta-1,3-glucanase	CA	cDNA clone	Experimental	NA
1	2	13	13	CV793600	Transcriptional activator	CA	cDNA clone	Experimental	NA
1	2	13	14	CV793602	Cinnamyl-alcohol-dehydrogenase	CA	cDNA clone	Experimental	NA
1	2	14	1	DY475172	Phosphate-induced protein	CA	cDNA clone	Experimental	NA
1	2	14	2	DY475192	Dehydration-induced protein	CA	cDNA clone	Experimental	NA
1	2	14	3	DY475220	Wound-induced protein	CA	cDNA clone	Experimental	NA
1	2	14	4	DY475237	Translation initiation factor	CA	cDNA clone	Experimental	NA
1	2	14	5	DY475254	Wound-induced protein	CA	cDNA clone	Experimental	NA
1	2	14	6	DY475278	Heat shock protein	CA	cDNA clone	Experimental	NA
1	2	14	7	DY475335	Heat shock protein	CA	cDNA clone	Experimental	NA
1	2	14	8	DY475453	Heat shock protein	CA	cDNA clone	Experimental	NA
1	2	14	9	DY475463	Similarity to protein-tyrosine-kinase receptor (EC 2.7.1.112)	CA	cDNA clone	Experimental	NA
1	2	14	10	DY475478	Hypothetical transmembrane protein	CA	cDNA clone	Experimental	NA

1	2	14	11	DY475525	Actin regulating protein	CA	cDNA clone	Experimental	NA
1	2	14	12	DY475068	L-allo-threonine aldolase (EC 4.1.2.5)	CA	cDNA clone	Experimental	NA
1	2	14	13	DY475105	Sucrose synthase (EC 2.4.1.14)	CA	cDNA clone	Experimental	NA
1	2	14	14	DY475108	Asparagine synthetase (EC 6.3.5.4)	CA	cDNA clone	Experimental	NA
1	3	1	1	DY396330	Thioredoxin H-type 1	LS	cDNA clone	Experimental	NA
1	3	1	2	DY396404	Glutathione S-transferase GST 8	LS	cDNA clone	Experimental	NA
1	3	1	3	NA	Copper amine oxidase	LC (ILL7537)	Genomic PCR product	Experimental	NA
1	3	1	4	NA	Isoflavone synthase	LC (ILL7537)	Genomic PCR product	Experimental	NA
1	3	1	5	NA	NBS-LRR putative RGA Aj516060	LC (ILL6002)	Genomic PCR product	Experimental	NA
1	3	1	6	NA	NBS-LRR putative RGA Aj516062	LC (ILL6002)	Genomic PCR product	Experimental	NA
1	3	1	7	NA	NBS-LRR putative RGA Aj516064	LC (ILL6002)	Genomic PCR product	Experimental	NA
1	3	1	8	NA	NBS-LRR putative RGA Aj516067	LC (ILL6002)	Genomic PCR product	Experimental	NA
1	3	1	9	NA	NBS-LRR putative RGA Aj516072	LC (ILL6002)	Genomic PCR product	Experimental	NA
1	3	1	10	NA	NBS-LRR putative RGA Aj516076	LC (ILL6002)	Genomic PCR product	Experimental	NA
1	3	2	1	DY396354	Polyubiquitin	LS	cDNA clone	Experimental	NA
1	3	2	2	DY396413	Catalase	LS	cDNA clone	Experimental	NA
1	3	2	3	DY396383	Putative extracellular dermal glycoprotein	LS	cDNA clone	Experimental	NA
1	3	2	4	DY396377	Ripening-related protein	LS	cDNA clone	Experimental	NA
1	3	2	5	DY396373	Metallothionein-like protein 1	LS	cDNA clone	Experimental	NA
1	3	2	6	DY396370	Ubiquitin-conjugating enzyme E2	LS	cDNA clone	Experimental	NA
1	3	2	7	DY396412	Poly(A)-binding protein	LS	cDNA clone	Experimental	NA
1	3	2	8	DY396408	Ubiquitin-specific protease 16	LS	cDNA clone	Experimental	NA
1	3	2	9	DY396284	Histone deacetylase 2 isoform B	LS	cDNA clone	Experimental	NA
1	3	2	10	DY396287	Kinesin-like protein	LS	cDNA clone	Experimental	NA
1	3	2	11	DY396280	Serine carboxypeptidase isolag	LS	cDNA clone	Experimental	NA
1	3	2	12	DY396396	Cysteine proteinase 15A precursor	LS	cDNA clone	Experimental	NA
1	3	2	13	DY396348	Glycolate oxidase	LS	cDNA clone	Experimental	NA
1	3	2	14	DY396435	L-ascorbate peroxidase cytosolic	LS	cDNA clone	Experimental	NA
1	3	3	1	DY396375	Putative protein kinase	LS	cDNA clone	Experimental	NA
1	3	3	2	DY396381	Small GTP-binding protein	LS	cDNA clone	Experimental	NA
1	3	3	3	DY396388	Pathogenesis-related protein 4A	LS	cDNA clone	Experimental	NA
1	3	3	4	DY396385	TMV resistance protein-like	LS	cDNA clone	Experimental	NA
1	3	3	5	DY396390	Disease resistance response protein 230 (DRR230-a)	LS	cDNA clone	Experimental	NA
1	3	3	6	DY396393	6-Phosphogluconate dehydrogenase	LS	cDNA clone	Experimental	NA
1	3	3	7	DY396303	Ubiquitin-like protein	LS	cDNA clone	Experimental	NA
1	3	3	8	DY396298	Environmental stress inducible protein	LS	cDNA clone	Experimental	NA

1	3	3	9	DY396295	Metallothionein-like protein	LS	cDNA clone	Experimental	NA
1	3	3	10	DY396278	Ubiquitin	LS	cDNA clone	Experimental	NA
1	3	3	11	DY396263	Transcription factor NTLIM1	LS	cDNA clone	Experimental	NA
1	3	3	12	DY396321	Dehydration stress-induced protein	LS	cDNA clone	Experimental	NA
1	3	3	13	DY396319	Polyubiquitin	LS	cDNA clone	Experimental	NA
1	3	3	14	DY396315	Auxin-responsive protein IAA9	LS	cDNA clone	Experimental	NA
1	3	4	1	DY475542	18S rRNA	CA	cDNA clone	Experimental	NA
1	3	4	2	EB085056	Beta-galactosidase (EC 3.2.1.23)	CA	cDNA clone	Experimental	NA
1	3	4	3	EB085057	Unknown	CA	cDNA clone	Experimental	NA
1	3	4	4	NA	Normalisation control 1	CA	cDNA clone	Control	Normalisation
1	3	4	5	DY396285	Protein kinase C inhibitor-like protein	LS	cDNA clone	Experimental	NA
1	3	4	6	DY396291	Putative ARF1 GTPase activating protein	LS	cDNA clone	Experimental	NA
1	3	4	7	DY396294	Putative steroid binding protein	LS	cDNA clone	Experimental	NA
1	3	4	8	DY396297	Isovaleryl-coa dehydrogenase	LS	cDNA clone	Experimental	NA
1	3	4	9	DY396300	ATHP3 (histidine-containing phosphotransfer protein like)	LS	cDNA clone	Experimental	NA
1	3	4	10	DY396304	Putative steroid binding protein	LS	cDNA clone	Experimental	NA
1	3	4	11	DY396307	Serine/threonine protein kinase	LS	cDNA clone	Experimental	NA
1	3	4	12	DY396313	Guanine nucleotide regulatory protein	LS	cDNA clone	Experimental	NA
1	3	4	13	DY396367	Small GTP-binding protein	LS	cDNA clone	Experimental	NA
1	3	4	14	DY396372	Pathogenesis-related protein 4A	LS	cDNA clone	Experimental	NA
1	3	5	1	EB085020	Unknown	CA	cDNA clone	Experimental	NA
1	3	5	2	EB085022	Unknown	CA	cDNA clone	Experimental	NA
1	3	5	3	NA	Unknown	CA	cDNA clone	Experimental	NA
1	3	5	4	EB085040	Unknown	CA	cDNA clone	Experimental	NA
1	3	5	5	NA	Unknown	CA	cDNA clone	Experimental	NA
1	3	5	6	EB085041	Unknown	CA	cDNA clone	Experimental	NA
1	3	5	7	EB085042	Phosphate-induced protein	CA	cDNA clone	Experimental	NA
1	3	5	8	EB085044	Unknown	CA	cDNA clone	Experimental	NA
1	3	5	9	DY475558	Unknown	CA	cDNA clone	Experimental	NA
1	3	5	10	NA	Unknown	CA	cDNA clone	Experimental	NA
1	3	5	11	EB085052	Unknown	CA	cDNA clone	Experimental	NA
1	3	5	12	EB085064	Unknown	CA	cDNA clone	Experimental	NA
1	3	5	13	EB085054	Chloroplast DNA	CA	cDNA clone	Experimental	NA
1	3	5	14	DY475541	Chloroplast DNA	CA	cDNA clone	Experimental	NA
1	3	6	1	DY475531	Unclear	CA	cDNA clone	Experimental	NA

1	3	6	2	EB085015	Translational activator	CA	cDNA clone	Experimental	NA
1	3	6	3	EB085016	Unknown	CA	cDNA clone	Experimental	NA
1	3	6	4	EB085017	Unclear	CA	cDNA clone	Experimental	NA
1	3	6	5	NA	Unknown	CA	cDNA clone	Experimental	NA
1	3	6	6	NA	Unknown	CA	cDNA clone	Experimental	NA
1	3	6	7	DY475556	NADH-plastoquinone oxidoreductase chain 1	CA	cDNA clone	Experimental	NA
1	3	6	8	EB085063	Unknown	CA	cDNA clone	Experimental	NA
1	3	6	9	NA	Unknown	CA	cDNA clone	Experimental	NA
1	3	6	10	EB085018	Acyl-activating enzyme	CA	cDNA clone	Experimental	NA
1	3	6	11	EB085034	Unknown	CA	cDNA clone	Experimental	NA
1	3	6	12	EB085035	Unknown	CA	cDNA clone	Experimental	NA
1	3	6	13	DY475537	Chloroplast Val-tRNA	CA	cDNA clone	Experimental	NA
1	3	6	14	DY475555	Chlorophyll a/b binding protein	CA	cDNA clone	Experimental	NA
1	3	7	1	DY475351	Unknown	CA	cDNA clone	Experimental	NA
1	3	7	2	DY475356	Unknown	CA	cDNA clone	Experimental	NA
1	3	7	3	DY475362	Unknown	CA	cDNA clone	Experimental	NA
1	3	7	4	DY475364	Unknown	CA	cDNA clone	Experimental	NA
1	3	7	5	DY475366	Unknown	CA	cDNA clone	Experimental	NA
1	3	7	6	DY475370	Unknown	CA	cDNA clone	Experimental	NA
1	3	7	7	DY475437	Unknown	CA	cDNA clone	Experimental	NA
1	3	7	8	DY475445	Unknown	CA	cDNA clone	Experimental	NA
1	3	7	9	DY475451	Unknown	CA	cDNA clone	Experimental	NA
1	3	7	10	DY475461	Unknown	CA	cDNA clone	Experimental	NA
1	3	7	11	DY475469	Unknown	CA	cDNA clone	Experimental	NA
1	3	7	12	DY475476	Unknown	CA	cDNA clone	Experimental	NA
1	3	7	13	DY475482	Unknown	CA	cDNA clone	Experimental	NA
1	3	7	14	DY475484	Unknown	CA	cDNA clone	Experimental	NA
1	3	8	1	DY475177	Unknown	CA	cDNA clone	Experimental	NA
1	3	8	2	DY475185	Unknown	CA	cDNA clone	Experimental	NA
1	3	8	3	DY475189	Unknown	CA	cDNA clone	Experimental	NA
1	3	8	4	DY475193	Unknown	CA	cDNA clone	Experimental	NA
1	3	8	5	DY475263	Unknown	CA	cDNA clone	Experimental	NA
1	3	8	6	DY475270	Unknown	CA	cDNA clone	Experimental	NA
1	3	8	7	DY475277	Unknown	CA	cDNA clone	Experimental	NA
1	3	8	8	DY475280	Unknown	CA	cDNA clone	Experimental	NA
1	3	8	9	DY475283	Unknown	CA	cDNA clone	Experimental	NA

1	3	8	10	DY475289	Unknown	CA	cDNA clone	Experimental	NA
1	3	8	11	DY475293	Unknown	CA	cDNA clone	Experimental	NA
1	3	8	12	DY475296	Unknown	CA	cDNA clone	Experimental	NA
1	3	8	13	DY475343	Unknown	CA	cDNA clone	Experimental	NA
1	3	8	14	DY475349	Unknown	CA	cDNA clone	Experimental	NA
1	3	9	1	DY475329	Unclear	CA	cDNA clone	Experimental	NA
1	3	9	2	DY475355	Unclear	CA	cDNA clone	Experimental	NA
1	3	9	3	DY475515	Unclear	CA	cDNA clone	Experimental	NA
1	3	9	4	DY475526	Unclear	CA	cDNA clone	Experimental	NA
1	3	9	5	DY475048	Unknown	CA	cDNA clone	Experimental	NA
1	3	9	6	DY475055	Unknown	CA	cDNA clone	Experimental	NA
1	3	9	7	DY475061	Unknown	CA	cDNA clone	Experimental	NA
1	3	9	8	DY475064	Unknown	CA	cDNA clone	Experimental	NA
1	3	9	9	DY475075	Unknown	CA	cDNA clone	Experimental	NA
1	3	9	10	DY475080	Unknown	CA	cDNA clone	Experimental	NA
1	3	9	11	DY475158	Unknown	CA	cDNA clone	Experimental	NA
1	3	9	12	DY475160	Unknown	CA	cDNA clone	Experimental	NA
1	3	9	13	DY475166	Unknown	CA	cDNA clone	Experimental	NA
1	3	9	14	DY475168	Unknown	CA	cDNA clone	Experimental	NA
1	3	10	1	DY475239	Membrane sugar-transport protein	CA	cDNA clone	Experimental	NA
1	3	10	2	DY475424	Beta adaptin like protein	CA	cDNA clone	Experimental	NA
1	3	10	3	DY475468	Cyclic ion channel protein	CA	cDNA clone	Experimental	NA
1	3	10	4	DY475512	Aquaporin 2 protein	CA	cDNA clone	Experimental	NA
1	3	10	5	DY475053	Unclear	CA	cDNA clone	Experimental	NA
1	3	10	6	DY475071	Unclear	CA	cDNA clone	Experimental	NA
1	3	10	7	DY475095	Unclear	CA	cDNA clone	Experimental	NA
1	3	10	8	DY475099	Unclear	CA	cDNA clone	Experimental	NA
1	3	10	9	DY475262	Unclear	CA	cDNA clone	Experimental	NA
1	3	10	10	DY475265	Unclear	CA	cDNA clone	Experimental	NA
1	3	10	11	DY475273	Unclear	CA	cDNA clone	Experimental	NA
1	3	10	12	DY475284	Unclear	CA	cDNA clone	Experimental	NA
1	3	10	13	DY475313	Unclear	CA	cDNA clone	Experimental	NA
1	3	10	14	DY475322	Unclear	CA	cDNA clone	Experimental	NA
1	3	11	1	DY475501	Chloroplast DNA for P700 chlorophyll a-apoproteins	CA	cDNA clone	Experimental	NA
1	3	11	2	DY475050	Chloroplast 30S ribosomal protein S3	CA	cDNA clone	Experimental	NA

1	3	11	3	DY475073	40S ribosomal protein S3	CA	cDNA clone	Experimental	NA
1	3	11	4	DY475110	60S ribosomal protein L17	CA	cDNA clone	Experimental	NA
1	3	11	5	DY475120	40S ribosomal protein S18	CA	cDNA clone	Experimental	NA
1	3	11	6	DY475123	60S ribosomal protein L10	CA	cDNA clone	Experimental	NA
1	3	11	7	DY475421	Acidic 60s ribosomal protein	CA	cDNA clone	Experimental	NA
1	3	11	8	DY475429	50S ribosomal protein L7Ae	CA	cDNA clone	Experimental	NA
1	3	11	9	DY475479	Serine:glyoxylate aminotransferase (EC 2.6.1.45)/alanine:glyoxylate aminotransferase (EC 2.6.1.44)	CA	cDNA clone	Experimental	NA
1	3	11	10	DY475504	S29 ribosomal protein	CA	cDNA clone	Experimental	NA
1	3	11	11	DY475507	Anthranilate phosphoribosyltransferase-like protein (EC 2.4.2.18)	CA	cDNA clone	Experimental	NA
1	3	11	12	DY475511	Histidine-containing phosphotransferprotein	CA	cDNA clone	Experimental	NA
1	3	11	13	DY475087	Mitochondrial 26S rRNA	CA	cDNA clone	Experimental	NA
1	3	11	14	DY475544	Chloroplast 4.5S, 5S, 16S and 23S mRNA	CA	cDNA clone	Experimental	NA
1	3	12	1	DY475516	Cytochrome C oxidase subunit	CA	cDNA clone	Experimental	NA
1	3	12	2	CV793595	Caffeoyl-CoA-Methyltransferase	CA	cDNA clone	Experimental	NA
1	3	12	3	CV793589	Homology to an Avr9/Cf9 rapidly elicited protein from *N. tabacum*	CA	cDNA clone	Experimental	NA
1	3	12	4	CV793591	S1-3 protein homolog induced by CMV infection in cowpea	CA	cDNA clone	Experimental	NA
1	3	12	5	DY475052	Oxygen splitting enhancer protein of photosystem II	CA	cDNA clone	Experimental	NA
1	3	12	6	DY475060	Oxygen splitting enhancer protein of photosystem II	CA	cDNA clone	Experimental	NA
1	3	12	7	DY475082	ATP synthase (EC 3.6.1.34)	CA	cDNA clone	Experimental	NA
1	3	12	8	DY475116	Photosystem II reaction centre I protein	CA	cDNA clone	Experimental	NA
1	3	12	9	DY475129	mRNA for light inducible protein precursor of photosystem II	CA	cDNA clone	Experimental	NA
1	3	12	10	DY475139	NADH dehydrogenase subunit/NADH-Plastoquinone oxidoreductase subunit	CA	cDNA clone	Experimental	NA
1	3	12	11	DY475144	Chloroplast psbB operon	CA	cDNA clone	Experimental	NA
1	3	12	12	DY475151	Chlorophyll a/b binding protein	CA	cDNA clone	Experimental	NA
1	3	12	13	DY475464	ATP Synthase C chain (lipid binding protein) (EC 3.6.1.34)	CA	cDNA clone	Experimental	NA
1	3	12	14	DY475487	Ferredoxin	CA	cDNA clone	Experimental	NA
1	3	13	1	DY475384	Serine/threonine protein kinase	CA	cDNA clone	Experimental	NA
1	3	13	2	DY475410	Multispanning membrane protein	CA	cDNA clone	Experimental	NA
1	3	13	3	DY475170	S-adenosylmethionine decarboxylase (EC 4.1.1.50)	CA	cDNA clone	Experimental	NA

1	3	13	4	DY475180	Cytochrome F	CA	cDNA clone	Experimental	NA
1	3	13	5	DY475184	Carboxytransferase	CA	cDNA clone	Experimental	NA
1	3	13	6	DY475200	Nodulin 21 protein	CA	cDNA clone	Experimental	NA
1	3	13	7	DY475213	Carbonic anhydrase (EC 4.2.1.1)	CA	cDNA clone	Experimental	NA
1	3	13	8	DY475221	UDP-glucose 4-epimerase (EC 5.1.3.2)	CA	cDNA clone	Experimental	NA
1	3	13	9	DY475242	Thiazole biosynthetic enzyme	CA	cDNA clone	Experimental	NA
1	3	13	10	DY475282	Trehalose-phosphatase (EC 3.1.3.12)	CA	cDNA clone	Experimental	NA
1	3	13	11	DY475449	Cytochrome P450	CA	cDNA clone	Experimental	NA
1	3	13	12	DY475458	Cysteine proteinase	CA	cDNA clone	Experimental	NA
1	3	13	13	DY475477	Asparagine synthetase (glutamine hydrolysing) (EC 6.3.5.4)	CA	cDNA clone	Experimental	NA
1	3	13	14	DY475498	Glucosyltransferase	CA	cDNA clone	Experimental	NA
1	3	14	1	DY475227	Myosin heavy-chain protein	CA	cDNA clone	Experimental	NA
1	3	14	2	DY475266	DNA binding protein	CA	cDNA clone	Experimental	NA
1	3	14	3	DY475357	RNA/ssDNA binding protein	CA	cDNA clone	Experimental	NA
1	3	14	4	DY475493	Formyltetrahydrofolate deformylase-like (EC 3.5.1.10)	CA	cDNA clone	Experimental	NA
1	3	14	5	DY475070	Dehydration-induced protein	CA	cDNA clone	Experimental	NA
1	3	14	6	DY475078	Auxin-repressed protein	CA	cDNA clone	Experimental	NA
1	3	14	7	DY475111	Wound-induced protein	CA	cDNA clone	Experimental	NA
1	3	14	8	DY475138	Aluminium-induced protein	CA	cDNA clone	Experimental	NA
1	3	14	9	DY475517	Farnesylated/isoprenylated protein	CA	cDNA clone	Experimental	NA
1	3	14	10	DY475091	Zinc finger protein	CA	cDNA clone	Experimental	NA
1	3	14	11	DY475119	Membrane-related protein CP5	CA	cDNA clone	Experimental	NA
1	3	14	12	DY475246	GPI-anchored membrane protein	CA	cDNA clone	Experimental	NA
1	3	14	13	DY475271	Histidine-rich glycoprotein precursor	CA	cDNA clone	Experimental	NA
1	3	14	14	DY475348	Proline-rich structural protein	CA	cDNA clone	Experimental	NA
1	4	1	1	NA	NBS-LRR putative RGA Aj516082	LC (ILL7537)	Genomic PCR product	Experimental	NA
1	4	1	2	NA	NBS-LRR putative RGA Aj516087	LC (ILL7537)	Genomic PCR product	Experimental	NA
1	4	1	3	NA	NBS-LRR putative RGA Aj516082	LC (ILL6002)	Genomic PCR product	Experimental	NA
1	4	1	4	NA	NBS-LRR putative RGA Aj516087	LC (ILL6002)	Genomic PCR product	Experimental	NA
1	4	1	5	NA	NBS-LRR putative RGA Aj516090	LC (ILL6002)	Genomic PCR product	Experimental	NA
1	4	1	6	NA	Lipoxygenase	LC (ILL6002)	Genomic PCR product	Experimental	NA
1	4	1	7	NA	NBS-LRR putative RGA LR1	LC (ILL6002)	Genomic PCR product	Experimental	NA
1	4	1	8	NA	Printing Control	NA	Oligo	Control	Printing
1	4	1	9	NA	Blank	NA	Blank	Control	Negative

1	4	1	10	NA	Blank	NA	Blank	Control	Negative
1	4	2	1	DY396403	Ubiquitin-carboxyl extension	LS	cDNA clone	Experimental	NA
1	4	2	2	DY396401	Ubiquinol-cytochrome C reductase complex 6.7 KDA protein	LS	cDNA clone	Experimental	NA
1	4	2	3	DY396397	Heat shock protein DNAJ homolog	LS	cDNA clone	Experimental	NA
1	4	2	4	DY396268	Histone H2A	LS	cDNA clone	Experimental	NA
1	4	2	5	DY396266	Nucleic acid binding protein-like	LS	cDNA clone	Experimental	NA
1	4	2	6	DY396424	Ubiquitin	LS	cDNA clone	Experimental	NA
1	4	2	7	DY396417	Glycogen synthase kinase-3 homolog MSK-3	LS	cDNA clone	Experimental	NA
1	4	2	8	DY396308	Xyloglucan endotransglycosylase LEXET2	LS	cDNA clone	Experimental	NA
1	4	2	9	NA	NBS-LRR putative RGA Aj516060	LC (ILL7537)	Genomic PCR product	Experimental	NA
1	4	2	10	NA	NBS-LRR putative RGA Aj516063	LC (ILL7537)	Genomic PCR product	Experimental	NA
1	4	2	11	NA	NBS-LRR putative RGA Aj516065	LC (ILL7537)	Genomic PCR product	Experimental	NA
1	4	2	12	NA	NBS-LRR putative RGA Aj516070	LC (ILL7537)	Genomic PCR product	Experimental	NA
1	4	2	13	NA	NBS-LRR putative RGA Aj516072	LC (ILL7537)	Genomic PCR product	Experimental	NA
1	4	2	14	NA	NBS-LRR putative RGA Aj516076	LC (ILL7537)	Genomic PCR product	Experimental	NA
1	4	3	1	DY396415	Caffeoyl-coa O-methyltransferase 4	LS	cDNA clone	Experimental	NA
1	4	3	2	DY396418	Protein transport protein SEC61 gamma subunit	LS	cDNA clone	Experimental	NA
1	4	3	3	DY396425	Disease resistance response protein 230 precursor	LS	cDNA clone	Experimental	NA
1	4	3	4	DY396347	Ripening-related protein	LS	cDNA clone	Experimental	NA
1	4	3	5	DY396429	Putative membrane related protein	LS	cDNA clone	Experimental	NA
1	4	3	6	DY396273	Putative senescence-associated protein	LS	cDNA clone	Experimental	NA
1	4	3	7	DY396309	Transcription initiation protein SPT4 homolog 1	LS	cDNA clone	Experimental	NA
1	4	3	8	DY396344	Ripening-related protein	LS	cDNA clone	Experimental	NA
1	4	3	9	DY396341	Polyubiquitin	LS	cDNA clone	Experimental	NA
1	4	3	10	DY396339	Magnesium chelatase subunit	LS	cDNA clone	Experimental	NA
1	4	3	11	DY396328	Polyubiquitin	LS	cDNA clone	Experimental	NA
1	4	3	12	DY396324	Dehydrin-cognate	LS	cDNA clone	Experimental	NA
1	4	3	13	DY396366	Putative Ubiquitin protein	LS	cDNA clone	Experimental	NA
1	4	3	14	DY396361	Heat shock factor binding protein	LS	cDNA clone	Experimental	NA
1	4	4	1	DY396264	Protein kinase precursor-like	LS	cDNA clone	Experimental	NA
1	4	4	2	DY396269	Putative auxin-repressed protein	LS	cDNA clone	Experimental	NA
1	4	4	3	DY396276	Disease resistance response protein 39 precursor	LS	cDNA clone	Experimental	NA
1	4	4	4	DY396281	Pathogenesis-related protein 4A	LS	cDNA clone	Experimental	NA
1	4	4	5	DY396325	Cutinase negative acting protein	LS	cDNA clone	Experimental	NA
1	4	4	6	DY396332	Lipid transfer protein	LS	cDNA clone	Experimental	NA

1	4	4	7	DY396336	RAC-GTP binding protein-like	LS	cDNA clone	Experimental	NA
1	4	4	8	DY396343	Pathogenesis-related protein	LS	cDNA clone	Experimental	NA
1	4	4	9	DY396350	Non-specific lipid-transfer protein precursor	LS	cDNA clone	Experimental	NA
1	4	4	10	DY396352	CF-9 resistance gene cluster	LS	cDNA clone	Experimental	NA
1	4	4	11	DY396359	Putative auxin-repressed protein	LS	cDNA clone	Experimental	NA
1	4	4	12	DY396364	ER66 protein/calmodulin binding protein	LS	cDNA clone	Experimental	NA
1	4	4	13	DY396400	EREBP-4	LS	cDNA clone	Experimental	NA
1	4	4	14	DY396407	Defence-related peptide 1	LS	cDNA clone	Experimental	NA
1	4	5	1	EB085036	Chloroplast 30S rRNA	CA	cDNA clone	Experimental	NA
1	4	5	2	NA	Unknown	CA	cDNA clone	Experimental	NA
1	4	5	3	EB085047	18S rRNA	CA	cDNA clone	Experimental	NA
1	4	5	4	NA	Unknown	CA	cDNA clone	Experimental	NA
1	4	5	5	EB085048	Unclear	CA	cDNA clone	Experimental	NA
1	4	5	6	EB085049	Unknown	CA	cDNA clone	Experimental	NA
1	4	5	7	NA	Unknown	CA	cDNA clone	Experimental	NA
1	4	5	8	DY475540	26S rRNA	CA	cDNA clone	Experimental	NA
1	4	5	9	NA	Unknown	CA	cDNA clone	Experimental	NA
1	4	5	10	NA	Unknown	CA	cDNA clone	Experimental	NA
1	4	5	11	NA	Normalisation control 3	CA	cDNA clone	Control	Normalisation
1	4	5	12	NA	Digested pGEM-T Easy Vector II (Promega) Plasmid *HaeIII*	NA	Digested Plasmid	Control	Negative
1	4	5	13	DY396259	GTP-binding protein SAR1A	LS	cDNA clone	Experimental	NA
1	4	5	14	DY396261	Receptor-like protein kinase	LS	cDNA clone	Experimental	NA
1	4	6	1	NA	Unknown	CA	cDNA clone	Experimental	NA
1	4	6	2	NA	Unknown	CA	cDNA clone	Experimental	NA
1	4	6	3	NA	Unknown	CA	cDNA clone	Experimental	NA
1	4	6	4	EB085031	Cytochrome P450	CA	cDNA clone	Experimental	NA
1	4	6	5	DY475557	18S rRNA, partial	CA	cDNA clone	Experimental	NA
1	4	6	6	NA	Unknown	CA	cDNA clone	Experimental	NA
1	4	6	7	DY475535	Unknown	CA	cDNA clone	Experimental	NA
1	4	6	8	EB085033	5.8S, 18S and 25S rRNA	CA	cDNA clone	Experimental	NA
1	4	6	9	EB085024	Unknown	CA	cDNA clone	Experimental	NA
1	4	6	10	DY475534	Chlorophyll a/b binding protein	CA	cDNA clone	Experimental	NA
1	4	6	11	EB085025	Unknown	CA	cDNA clone	Experimental	NA
1	4	6	12	EB085062	Unknown	CA	cDNA clone	Experimental	NA
1	4	6	13	NA	Unknown	CA	cDNA clone	Experimental	NA

1	4	6	14	NA	Unknown	CA	cDNA clone	Experimental	NA
1	4	7	1	DY475392	Unknown	CA	cDNA clone	Experimental	NA
1	4	7	2	DY475401	Unknown	CA	cDNA clone	Experimental	NA
1	4	7	3	DY475412	Unknown	CA	cDNA clone	Experimental	NA
1	4	7	4	DY475416	Unknown	CA	cDNA clone	Experimental	NA
1	4	7	5	DY475428	Unknown	CA	cDNA clone	Experimental	NA
1	4	7	6	DY475432	Unknown	CA	cDNA clone	Experimental	NA
1	4	7	7	DY475490	Unknown	CA	cDNA clone	Experimental	NA
1	4	7	8	DY475503	Unknown	CA	cDNA clone	Experimental	NA
1	4	7	9	DY475513	Unknown	CA	cDNA clone	Experimental	NA
1	4	7	10	DY475520	Unknown	CA	cDNA clone	Experimental	NA
1	4	7	11	DY475529	Unknown	CA	cDNA clone	Experimental	NA
1	4	7	12	EB085013	26S rRNA	CA	cDNA clone	Experimental	NA
1	4	7	13	NA	Unknown	CA	cDNA clone	Experimental	NA
1	4	7	14	NA	Unknown	CA	cDNA clone	Experimental	NA
1	4	8	1	DY475232	Unknown	CA	cDNA clone	Experimental	NA
1	4	8	2	DY475241	Unknown	CA	cDNA clone	Experimental	NA
1	4	8	3	DY475253	Unknown	CA	cDNA clone	Experimental	NA
1	4	8	4	DY475256	Unknown	CA	cDNA clone	Experimental	NA
1	4	8	5	DY475299	Unknown	CA	cDNA clone	Experimental	NA
1	4	8	6	DY475310	Unknown	CA	cDNA clone	Experimental	NA
1	4	8	7	DY475314	Unknown	CA	cDNA clone	Experimental	NA
1	4	8	8	DY475326	Unknown	CA	cDNA clone	Experimental	NA
1	4	8	9	DY475330	Unknown	CA	cDNA clone	Experimental	NA
1	4	8	10	DY475336	Unknown	CA	cDNA clone	Experimental	NA
1	4	8	11	DY475338	Unknown	CA	cDNA clone	Experimental	NA
1	4	8	12	DY475340	Unknown	CA	cDNA clone	Experimental	NA
1	4	8	13	DY475377	Unknown	CA	cDNA clone	Experimental	NA
1	4	8	14	DY475390	Unknown	CA	cDNA clone	Experimental	NA
1	4	9	1	DY475448	Unclear	CA	cDNA clone	Experimental	NA
1	4	9	2	DY475495	Unclear	CA	cDNA clone	Experimental	NA
1	4	9	3	DY475084	Unknown	CA	cDNA clone	Experimental	NA
1	4	9	4	DY475089	Unknown	CA	cDNA clone	Experimental	NA
1	4	9	5	DY475098	Unknown	CA	cDNA clone	Experimental	NA
1	4	9	6	DY475102	Unknown	CA	cDNA clone	Experimental	NA
1	4	9	7	DY475115	Unknown	CA	cDNA clone	Experimental	NA

1	4	9	8	DY475130	Unknown	CA	cDNA clone	Experimental	NA
1	4	9	9	DY475143	Unknown	CA	cDNA clone	Experimental	NA
1	4	9	10	DY475156	Unknown	CA	cDNA clone	Experimental	NA
1	4	9	11	DY475206	Unknown	CA	cDNA clone	Experimental	NA
1	4	9	12	DY475210	Unknown	CA	cDNA clone	Experimental	NA
1	4	9	13	DY475216	Unknown	CA	cDNA clone	Experimental	NA
1	4	9	14	DY475223	Unknown	CA	cDNA clone	Experimental	NA
1	4	10	1	DY475118	Unclear	CA	cDNA clone	Experimental	NA
1	4	10	2	DY475173	Unclear	CA	cDNA clone	Experimental	NA
1	4	10	3	DY475186	Unclear	CA	cDNA clone	Experimental	NA
1	4	10	4	DY475214	Unclear	CA	cDNA clone	Experimental	NA
1	4	10	5	DY475218	Unclear	CA	cDNA clone	Experimental	NA
1	4	10	6	DY475546	Unclear	CA	cDNA clone	Experimental	NA
1	4	10	7	DY475233	Unclear	CA	cDNA clone	Experimental	NA
1	4	10	8	DY475257	Unclear	CA	cDNA clone	Experimental	NA
1	4	10	9	DY475376	Unclear	CA	cDNA clone	Experimental	NA
1	4	10	10	DY475386	Unclear	CA	cDNA clone	Experimental	NA
1	4	10	11	DY475389	Unclear	CA	cDNA clone	Experimental	NA
1	4	10	12	DY475400	Unclear	CA	cDNA clone	Experimental	NA
1	4	10	13	DY475411	Unclear	CA	cDNA clone	Experimental	NA
1	4	10	14	DY475438	Unclear	CA	cDNA clone	Experimental	NA
1	4	11	1	DY475317	40S ribosomal protein S8	CA	cDNA clone	Experimental	NA
1	4	11	2	DY475334	Chloroplast 30S ribosomal protein S7	CA	cDNA clone	Experimental	NA
1	4	11	3	DY475346	Elongation factor (translation initiation factor)	CA	cDNA clone	Experimental	NA
1	4	11	4	DY475359	50S ribosomal protein L27	CA	cDNA clone	Experimental	NA
1	4	11	5	DY475394	60S ribosomal protein L39	CA	cDNA clone	Experimental	NA
1	4	11	6	DY475406	FKBP-type peptidyl-prolyl cis-trans isomerase (EC 5.2.1.8)	CA	cDNA clone	Experimental	NA
1	4	11	7	DY475545	Chloroplast 4.5S, 5S, 16S and 23S mRNA	CA	cDNA clone	Experimental	NA
1	4	11	8	DY475150	18S nuclear rRNA	CA	cDNA clone	Experimental	NA
1	4	11	9	DY475154	Chloroplast 4.5S/5S/16S/23S mRNA	CA	cDNA clone	Experimental	NA
1	4	11	10	DY475211	26S rRNA	CA	cDNA clone	Experimental	NA
1	4	11	11	DY475375	Sucrose responsive transcription factor	CA	cDNA clone	Experimental	NA
1	4	11	12	DY475059	Nuclear transport factor	CA	cDNA clone	Experimental	NA
1	4	11	13	DY475124	Aquaporin	CA	cDNA clone	Experimental	NA
1	4	11	14	DY475174	Aquaporin membrane protein	CA	cDNA clone	Experimental	NA

1	4	12	1	CV793607	Flavonol glucosyl transferase	CA	cDNA clone	Experimental	NA
1	4	12	2	CV793609	Similar to elicitor-inducible receptor-like protein	CA	cDNA clone	Experimental	NA
1	4	12	3	CV793590	Protein translation factor homolog (translation initiation factor nps45)	CA	cDNA clone	Experimental	NA
1	4	12	4	CV793588	Gamma-thionen type defensin/protease inhibitor	CA	cDNA clone	Experimental	NA
1	4	12	5	DY475176	Chloroplast genome DNA	CA	cDNA clone	Experimental	NA
1	4	12	6	DY475224	Plastocyanin	CA	cDNA clone	Experimental	NA
1	4	12	7	DY475285	Photosystem I reaction centre subunit VI-2	CA	cDNA clone	Experimental	NA
1	4	12	8	DY475294	NADH-ubiquinone oxidoreductase (EC 1.6.5.3)	CA	cDNA clone	Experimental	NA
1	4	12	9	DY475305	Thylakoid protein	CA	cDNA clone	Experimental	NA
1	4	12	10	DY475345	Photosystem I assembly protein ycf3	CA	cDNA clone	Experimental	NA
1	4	12	11	DY475423	ATP synthase (EC 3.6.1.34)	CA	cDNA clone	Experimental	NA
1	4	12	12	DY475434	Proton pump interactor protein	CA	cDNA clone	Experimental	NA
1	4	12	13	DY475201	60S ribosomal protein L34	CA	cDNA clone	Experimental	NA
1	4	12	14	DY475258	40S ribosomal protein S11	CA	cDNA clone	Experimental	NA
1	4	13	1	DY475141	Beta-galactosidase (EC 3.2.1.23)	CA	cDNA clone	Experimental	NA
1	4	13	2	DY475152	Cytidine deaminase enzyme	CA	cDNA clone	Experimental	NA
1	4	13	3	DY475302	4-alpha-glucanotransferase (EC 2.4.1.25)	CA	cDNA clone	Experimental	NA
1	4	13	4	DY475308	Glutamate dehydrogenase (EC 1.4.1.3)	CA	cDNA clone	Experimental	NA
1	4	13	5	DY475321	Mitochondrial glyoxylase	CA	cDNA clone	Experimental	NA
1	4	13	6	DY475548	Cytosolic fructose 1,6-bisphosphatase (EC 3.1.3.11)	CA	cDNA clone	Experimental	NA
1	4	13	7	DY475393	Cytochrome C biogenesis protein ccsA	CA	cDNA clone	Experimental	NA
1	4	13	8	DY475398	Glutamine synthetase (glutamate ammonia ligase) (EC 6.3.1.2)	CA	cDNA clone	Experimental	NA
1	4	13	9	DY475408	Xylosidase	CA	cDNA clone	Experimental	NA
1	4	13	10	DY475417	Probable 3-hydroxyisobutyrate dehydrogenase (HIBADH) mitochondrial precursor (EC 1.1.1.31)	CA	cDNA clone	Experimental	NA
1	4	13	11	CV793597	Pathogenesis-related protein 4A	CA	cDNA clone	Experimental	NA
1	4	13	12	CV793599	Protein containing leucine-zipper motif	CA	cDNA clone	Experimental	NA
1	4	13	13	CV793601	Leucine-zipper containing protein	CA	cDNA clone	Experimental	NA
1	4	13	14	CV793605	Multi-resistance protein ABC transporter	CA	cDNA clone	Experimental	NA
1	4	14	1	DY475190	S-adenosylmethionine synthetase enzyme (EC 2.5.1.6)	CA	cDNA clone	Experimental	NA
1	4	14	2	DY475207	Endoxyloglucan transferase involved in water-stress	CA	cDNA clone	Experimental	NA
1	4	14	3	DY475225	Proline oxidase	CA	cDNA clone	Experimental	NA
1	4	14	4	DY475250	Glutathione S-transferase (EC 2.5.1.18)	CA	cDNA clone	Experimental	NA
1	4	14	5	DY475276	Homocysteine methyltransferase	CA	cDNA clone	Experimental	NA

1	4	14	6	DY475328	Ubiquitin conjugating protein	CA	cDNA clone	Experimental	NA
1	4	14	7	DY475397	Superoxide dismutase copper chaperone precursor	CA	cDNA clone	Experimental	NA
1	4	14	8	DY475474	Heat shock protein	CA	cDNA clone	Experimental	NA
1	4	14	9	DY475470	Protein kinase mRNA	CA	cDNA clone	Experimental	NA
1	4	14	10	DY475508	Hypothetical protein with a membrane spanning ring-H2 finger domain	CA	cDNA clone	Experimental	NA
1	4	14	11	DY475066	Cysteine proteinase	CA	cDNA clone	Experimental	NA
1	4	14	12	DY475096	Glyceraldehyde 3-phosphate dehydrogenase (EC 1.2.1.12)	CA	cDNA clone	Experimental	NA
1	4	14	13	DY475543	Fructose-1,6-bisphosphatase (EC 3.1.3.11)	CA	cDNA clone	Experimental	NA
1	4	14	14	DY475113	Cytochrome C oxidase subunit	CA	cDNA clone	Experimental	NA

Appendix 5. Formulas

Calculating confidence intervals for mean expression ratios (Microsoft Excel. Redmond, WA)

1. Calculate the ratio standard error (SE) for each array feature using the ratio coefficient of variance (cv) value obtained from GeneSight™ 3;

SE = cv x mean

2. Calculate interval using the Z distribution and $n = 18$ (n is the number of data-points for each array feature; 3 biological replications of 6 technical replications each = 18);
For 99% confidence intervals;

Interval = mean +/- 2.58 x SE / sqrt(n)

For 95% confidence intervals;

Interval = mean +/- 1.96 x SE / sqrt(n)

Ranking method for identification of DE ESTs (Microsoft Excel. Redmond, WA)

1. Apply FC cut-off determined by self-self hybridisations

2. Import dataset into Microsoft Excel and determine equal/unequal variances for each array feature by comparing sample variances (control and treatment) using the F distribution;

Calculate the F statistic $F = s^2_1 / s^2_2$ using

$$F = \frac{(cv_{control} \text{ x sample mean}_{control})^2}{(cv_{test} \text{ x sample mean}_{test})^2}$$

Calculate the degrees of freedom for each variable ($n_1 - 1$, $n_2 - 1$). Considering that for each array feature there were 6 technical replicates and 3 biological replicates, $n = 18$ for both control and treatment.

$df_{control} = 18 - 1 = 17$
$df_{test} = 18 - 1 = 17$

Calculate F statistic probability using the F distribution tables. This was a two-tailed test so calculated F at P=0.025 for each tail to give a total P=0.05. Using these parameters the F statistic must be between 0.32 and 2.72 to assume equal variance between control and treatment means at P=0.05.

$F_{0.975\ (17,17)} = 2.72$
$F_{0.025\ (17,17)} = 1 / F_{0.975\ (17,17)} = 1 / 2.72 = 0.35$

Calculate the F statistic for each array feature using the 'FDIST' function.

Use the 'IF' function to determine if the F statistic probabilities are within the 0.35 – 2.72 interval. If the result is 'TRUE' then variance is equal.

Assuming equal sample variances, pool the sample variances according to

$$s^2_p = \frac{(n_1 - 1)*s^2_1 + (n_2 - 1)*s^2_2}{n_1 + n_2 - 2}$$

Considering that both control (n_1) and treatment (n_2) are 18, use the 'AVERAGE' function to pool variances.

$$\text{AVERAGE } (cv_{control} \text{ x sample mean}_{control})^2 + (cv_{test} \text{ x sample mean}_{test})^2$$

3. Calculate the *t* statistic for each sample using a two-sample *t* test assuming equal variances;

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

Convert each *t* statistic value into a positive number by squaring and the taking the square root.

Calculate the *P* value for each *t* statistic using the 'TDIST' function where x = sample *t* statistic, df = 18 + 18 –2 = 34, and tails = 2.

Selection method for identification of DE ESTs (Microsoft Excel. Redmond, WA)

1. For each dataset, sort the ESTs in ascending order according to *P* value.

2. Apply a FDR multiple testing correction;

Number the ranked ESTs from 1 to *R*.

Use arbitrary *P* value cut-off for DE of $P<0.05$.

Compare the *P* value of each EST to a threshold that depends on the position of the gene in the list. The thresholds are (1/R x α) for the first gene, then (2/R x α) for the second and so on, where R is the number of genes in the list and α is the desired significance level (0.05).

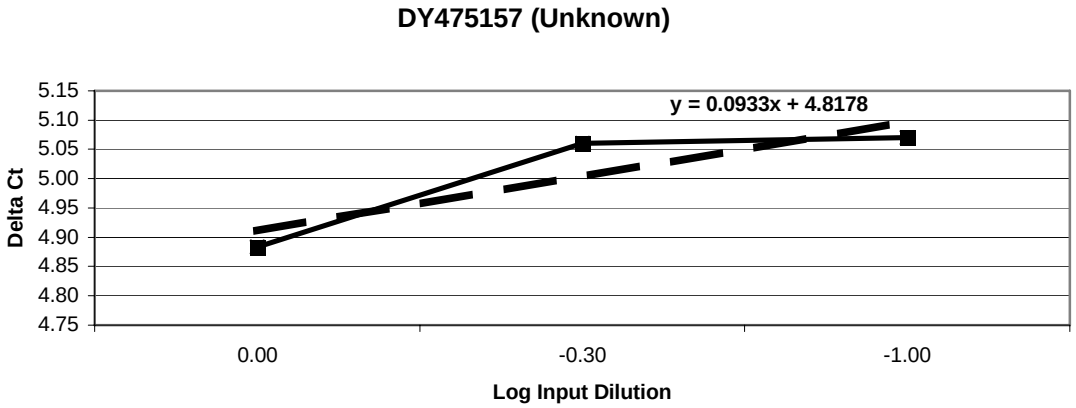
To pass the threshold and be accepted as DE, the observed *P* value must be less than the individual threshold for each EST.

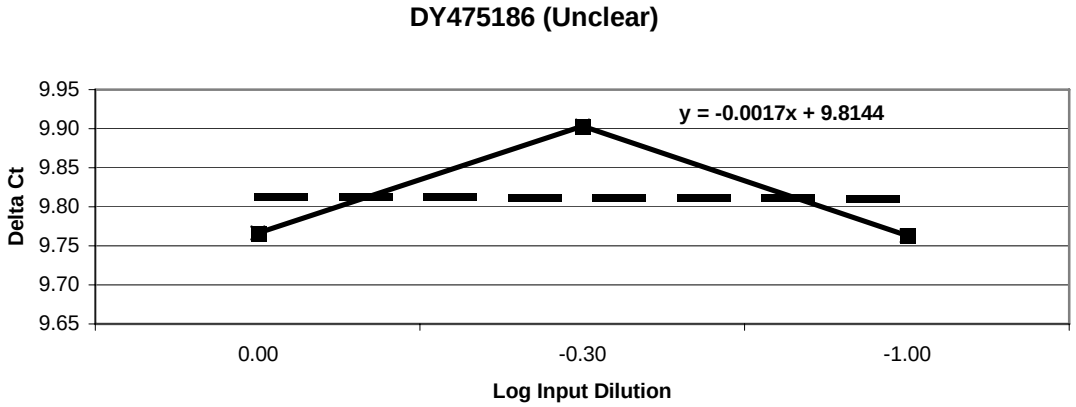
e.g. $p_1 < (1/R) \times \alpha$, $p_2 < (2/R) \times \alpha$)

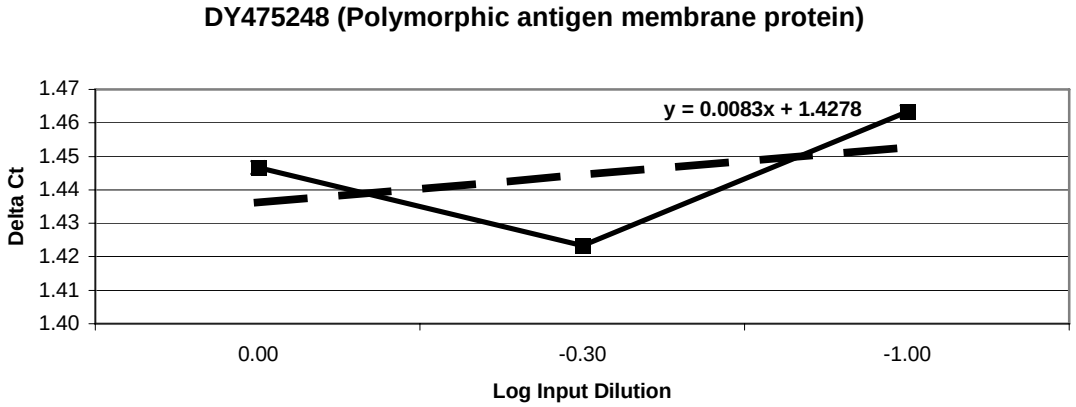
Appendix 6. Validation standard curves for quantitative RT-PCR

Linear trendlines (broken lines) and equations for the data are shown.

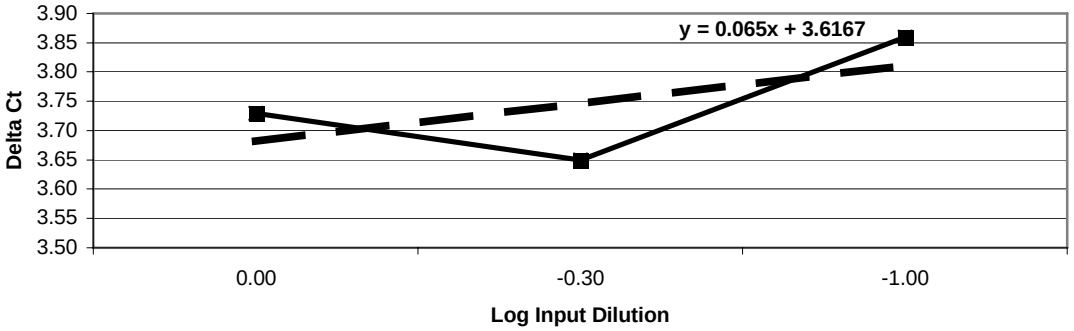
Chapter 4



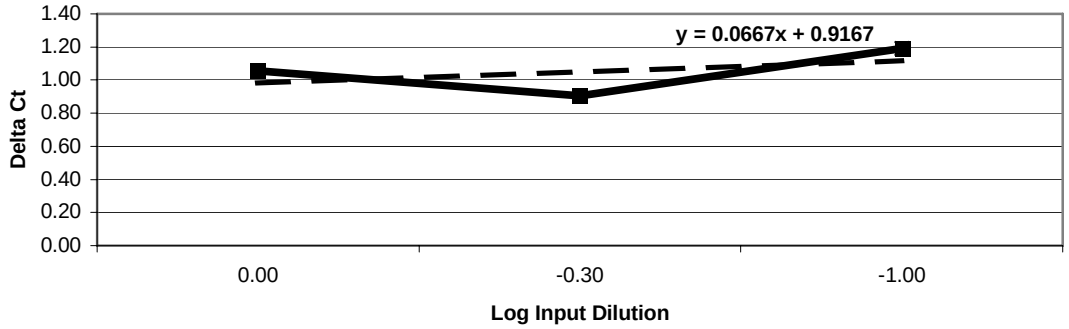




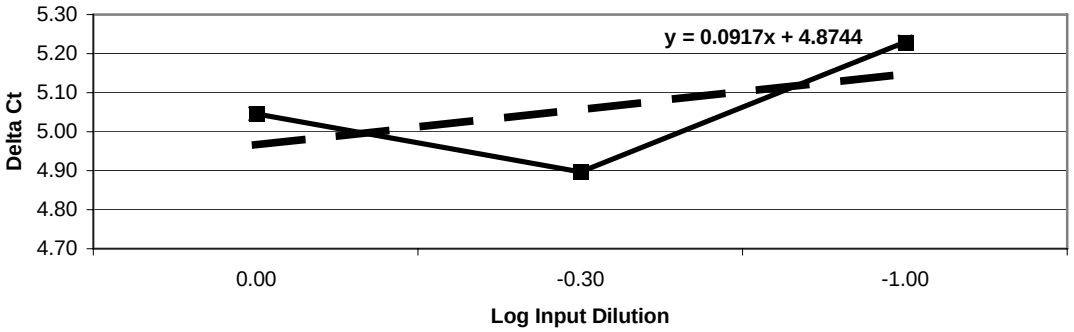
DY475259 (Unclear)



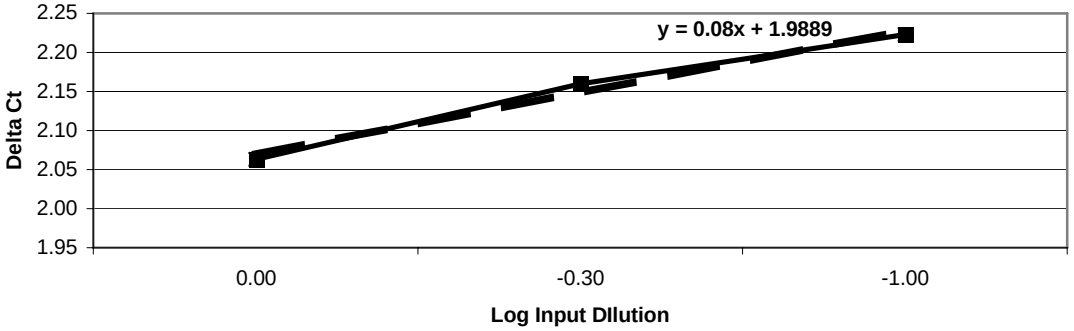
CV793597 (Pathogenesis-related protein 4A)



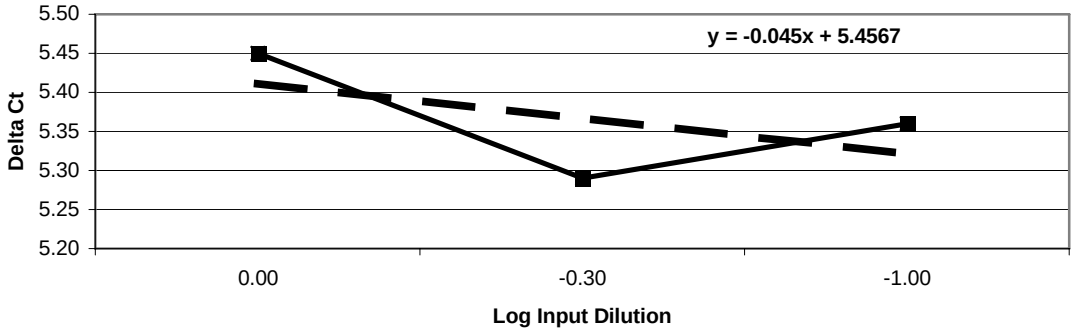
DY396305 (*β*-1,3-glucanase)



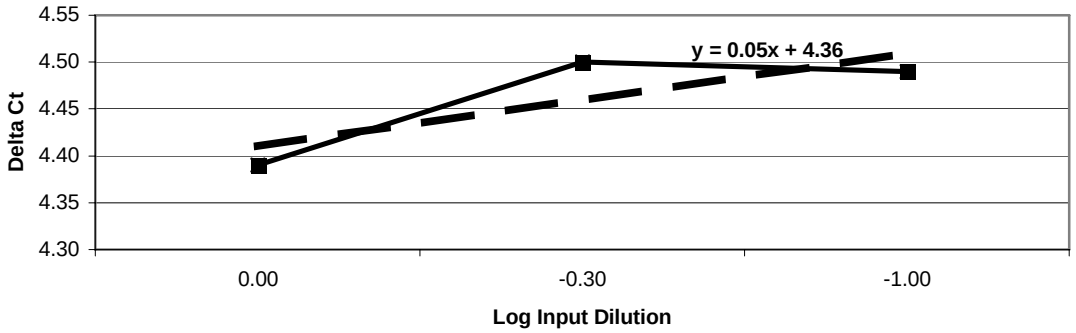
CV793608 (SNAKIN2 antimicrobial peptide precursor)



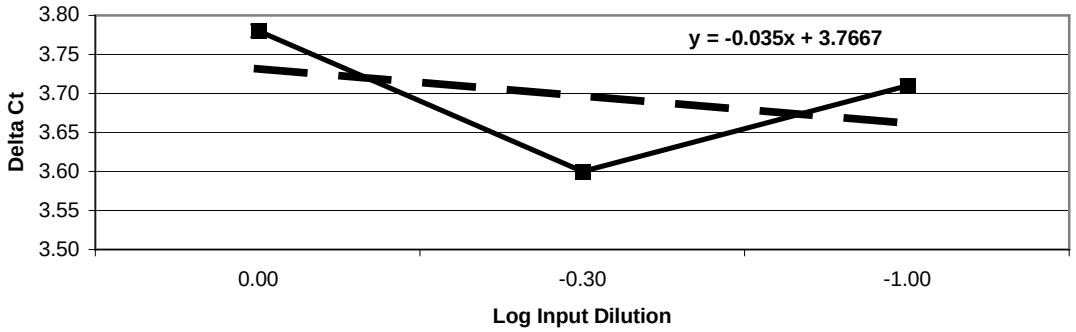
DY396265 (Disease resistance response protein DRRG49-C)



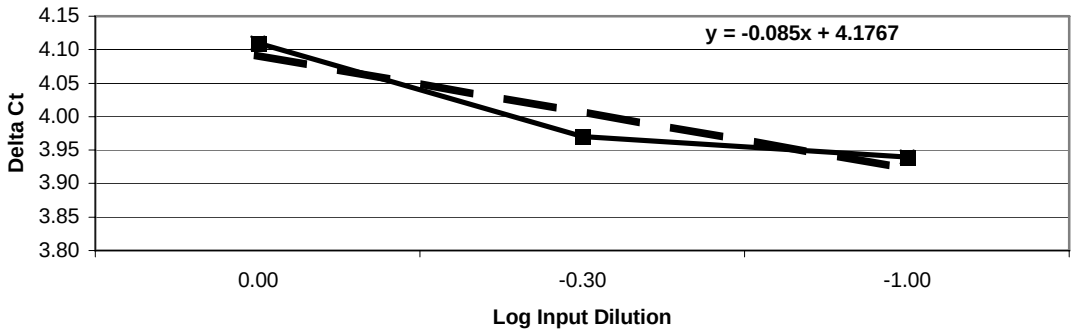
DY396302 (Polyubiquitin)



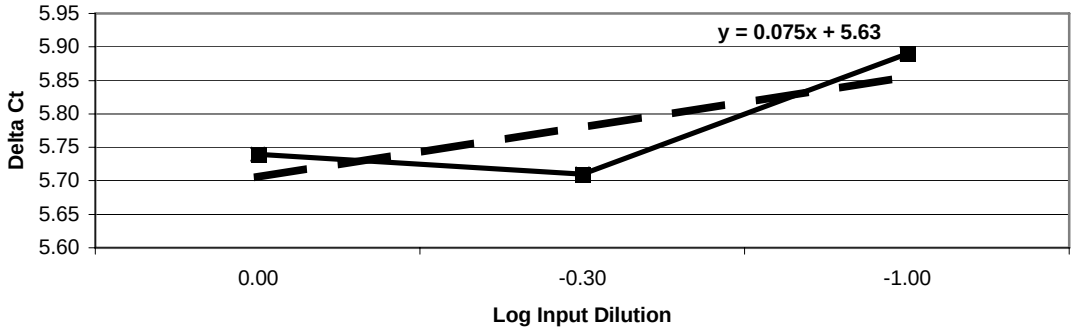
DY396400 (EREBP-4)



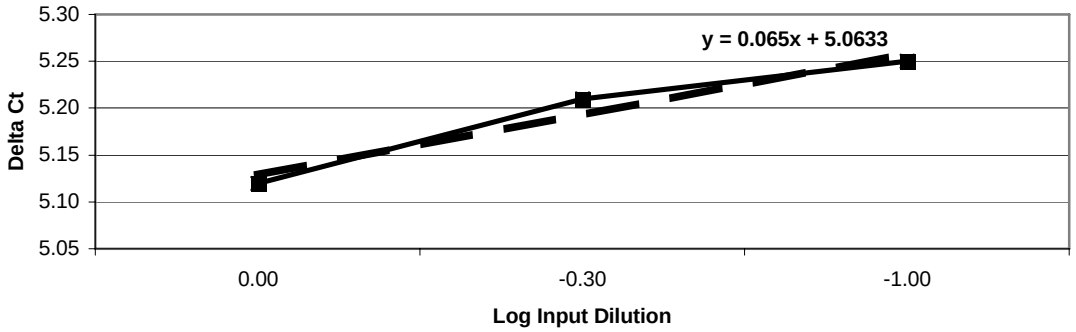
DY475136 (Cytochrome P450)



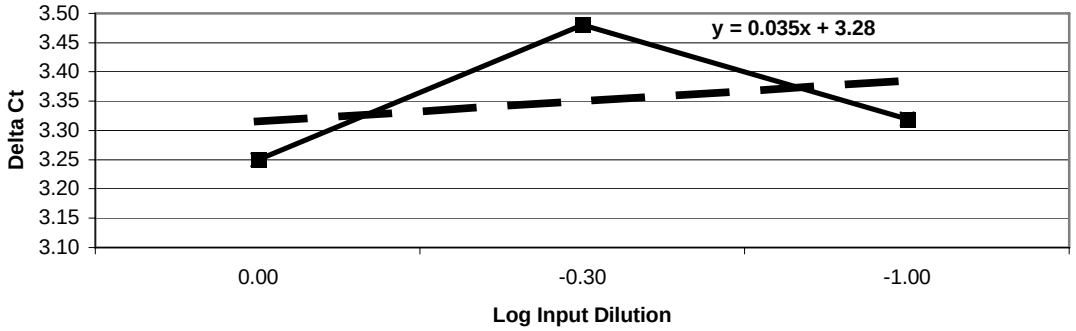
DY475155 (Superoxide dismutase)



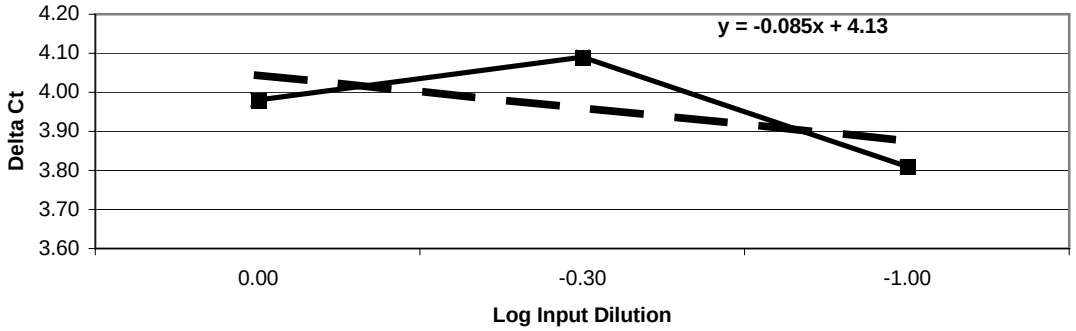
DY475250 (Glutathione S-transferase)



CV793602 (Cinnamyl-alcohol-dehygrogenase)



DY475488 (DnaJ-like protein)



Appendix 7. Cluster members for each chickpea genotype

EST ID refers to the GenBank accessions, whilst values for each cluster member at each time-point represent the mean FC expression ratio, where Na indicates absence of valid data.

ICC3996

Cluster A					
EST ID	**06 hpi**	**12 hpi**	**24 hpi**	**48 hpi**	**72 hpi**
DY475181	1.24	0.13	-1.17	Na	-0.20
DY475153	0.75	0.23	0.28	0.16	-0.09
DY475481	0.32	0.49	0.06	0.30	-0.12
DY475125	0.62	0.45	Na	0.40	-0.55
DY475211	0.45	0.18	-0.12	0.17	-0.63
DY475250	0.45	0.31	-0.10	-0.03	-1.20
DY475542	1.06	0.61	0.26	0.25	-0.26
DY475150	0.70	0.58	-0.05	0.21	-0.23
DY475548	0.81	0.06	-0.27	-0.12	-0.69
DY475534	1.34	0.70	0.33	0.15	-0.17
DY475535	0.48	0.19	Na	-0.04	-0.16
DY475115	0.30	0.14	-0.02	0.20	-0.23

Cluster B					
EST ID	**06 hpi**	**12 hpi**	**24 hpi**	**48 hpi**	**72 hpi**
DY475543	0.59	0.00	-0.29	-0.29	-0.73
DY475302	0.17	-0.05	-0.06	-0.46	-0.82
DY475550	-0.01	-0.40	0.14	-0.78	-0.01
DY475403	Na	-0.35	0.05	-0.52	-0.66
DY475245	Na	-0.22	-0.16	-0.53	-0.10
CV793589	Na	-0.04	0.91	-0.46	Na
DY475305	0.48	-0.44	-0.51	-0.58	0.06
CV793591	Na	-0.22	-0.16	-0.54	-0.36
DY475116	0.08	-0.11	-0.17	-1.08	-0.22
CV793599	Na	0.03	0.82	-0.80	-1.22
CV793607	Na	-0.31	-0.17	Na	-0.37

Cluster C					
EST ID	**06 hpi**	**12 hpi**	**24 hpi**	**48 hpi**	**72 hpi**
DY396265	-0.14	0.69	0.98	0.39	-0.28
DY396372	Na	1.75	1.21	0.86	0.27
DY396279	Na	0.43	0.46	1.18	0.89
DY396281	Na	0.82	1.09	0.33	0.08
DY396288	0.21	0.06	0.40	1.43	0.58
DY396301	-0.23	0.72	1.08	0.51	0.51
DY396384	Na	1.52	1.30	1.17	0.41
DY396388	Na	1.64	1.08	1.25	0.47
CV793597	0.14	1.08	0.95	0.81	-0.17
DY396305	0.58	0.38	0.65	0.73	-0.01

Cluster D					
EST ID	**06 hpi**	**12 hpi**	**24 hpi**	**48 hpi**	**72 hpi**
DY475108	0.29	-0.36	-1.23	-1.04	-1.37
DY475172	-0.66	-0.45	-0.30	-0.86	-0.54
DY475478	-0.85	-0.32	-0.96	-0.88	-1.02
DY475220	-0.15	-0.05	-0.23	-0.13	-0.75
DY475091	-0.13	0.08	-0.78	-0.19	-1.03
DY475170	0.11	-0.25	-1.60	-0.50	-0.54

DY475190	Na	-0.23	-0.93	-0.19	-1.05
DY475225	0.19	-0.18	-0.47	-0.69	-1.43
DY475276	Na	0.01	-0.16	-0.46	-1.00
CV793603	-0.40	0.18	-0.46	-0.55	-1.13
DY475357	-0.50	-0.86	-0.46	-1.23	0.03
DY475384	Na	0.05	-0.79	-0.21	-0.63
DY475397	-0.48	-0.18	-0.79	-0.68	-0.62
DY475242	Na	-0.02	-0.79	-0.61	-0.63
DY475493	-0.11	-0.30	Na	Na	-0.61

Cluster E

EST ID	06 hpi	12 hpi	24 hpi	48 hpi	72 hpi
DY475246	0.00	-0.30	-0.34	-0.18	-0.45
CV793608	0.09	-0.15	0.86	0.13	-0.16
DY475396	0.09	-0.36	0.07	Na	-0.26
DY475464	-0.02	-0.05	-0.15	0.19	-0.01
DY475076	0.00	-1.23	0.00	-0.13	-0.25
DY475213	-0.13	-0.17	-0.13	-0.32	0.01
DY475316	-0.12	-0.07	Na	Na	-0.19
DY475322	-0.23	-0.12	-0.25	-0.08	Na
DY475541	0.03	-0.09	0.11	0.01	0.01
DY475087	-0.30	-0.13	0.09	0.17	-0.09
CV793587	Na	-0.28	Na	Na	-0.02
DY475538	0.23	-0.33	-0.11	-0.18	0.09
DY396268	Na	-0.16	-0.19	Na	0.05
DY475523	0.22	-0.25	-0.12	Na	0.15
DY475515	-0.53	-0.07	-0.19	-0.77	0.07
DY475094	0.43	-0.33	-0.03	-0.31	0.35

Cluster F

EST ID	06 hpi	12 hpi	24 hpi	48 hpi	72 hpi
DY475095	-0.09	0.23	-0.08	-0.07	0.24
DY475522	0.33	-0.24	0.02	Na	0.19
DY475539	0.23	0.14	-0.02	0.10	0.16
DY396367	0.21	0.42	-0.03	-0.04	0.42
DY475339	0.47	-0.14	-0.27	0.25	0.07
DY475047	0.28	0.41	-0.06	0.26	0.09
DY475544	1.25	0.06	-0.39	Na	0.67
DY475462	0.65	0.21	-0.30	0.06	0.31
DY475533	Na	0.16	-0.01	-0.02	0.79
DY475557	Na	-0.10	Na	Na	0.86
DY475540	0.08	0.35	-0.26	0.14	-0.02
DY396262	-0.29	-0.11	-0.11	0.26	0.72
DY475157	-0.14	-0.17	Na	Na	0.75
DY475365	0.54	-0.40	0.01	0.89	0.43
DY396298	Na	0.20	0.39	Na	0.40
DY475323	Na	0.12	0.41	Na	0.53
DY475475	0.33	0.30	0.32	0.41	-0.01
DY475536	Na	0.06	0.14	Na	Na
DY475217	0.04	-0.15	0.23	0.37	0.06
DY475483	-0.05	0.13	-0.22	0.62	0.12
DY475092	-0.01	0.18	-0.19	0.38	-0.16

Cluster G

EST ID	06 hpi	12 hpi	24 hpi	48 hpi	72 hpi
CV793598	Na	0.10	0.39	0.87	-0.79
DY475401	-0.83	0.45	0.18	0.07	-0.18
DY475532	-0.23	0.03	-0.04	1.00	-0.13

DY475186	-0.06	0.04	0.27	0.68	-0.77
DY475248	Na	-0.14	Na	1.02	-0.58
DY475084	-0.36	0.57	0.18	0.04	-0.21
DY396347	-1.11	0.95	0.12	0.11	-0.43
DY396377	-0.71	0.80	0.20	0.08	-0.49
DY475554	-0.53	0.19	0.07	0.57	0.09
DY396344	-0.87	0.62	0.06	-0.02	-0.39
DY396400	-0.56	0.56	-0.16	-0.26	Na
DY396335	-0.79	0.12	Na	Na	Na

Lasseter

Cluster A					
EST ID	**06 hpi**	**12 hpi**	**24 hpi**	**48 hpi**	**72 hpi**
DY475557	Na	0.25	Na	Na	0.37
DY475384	-0.20	-0.01	-0.54	0.09	0.56
DY475084	0.15	-0.05	-0.54	0.02	-0.05
DY475091	-0.12	0.19	-0.77	Na	0.73
DY475250	-0.25	0.01	-0.32	0.05	0.77
DY475522	0.40	0.09	-0.90	Na	0.01
DY475464	-0.04	0.04	-0.59	-0.03	-0.04
DY475396	-0.04	-0.04	-0.84	Na	0.08

Cluster B					
EST ID	**06 hpi**	**12 hpi**	**24 hpi**	**48 hpi**	**72 hpi**
DY475543	-0.26	0.22	0.03	-0.30	-0.36
DY475548	-0.08	0.29	-0.22	-0.25	-0.32
DY475076	Na	0.12	0.07	-0.20	-0.25
DY475157	-0.13	0.00	Na	Na	-0.03
DY475245	-0.07	0.00	-0.33	Na	-0.06
DY475276	-0.34	0.25	-0.09	-0.11	-0.19
DY475087	0.30	-0.53	-0.35	-0.48	-0.07
DY475220	0.20	-0.06	-0.16	-0.12	0.02
DY475305	-0.19	0.03	-0.46	-0.32	-0.11
DY475550	-0.05	0.08	-0.06	-0.26	0.18
DY396262	0.00	-0.20	-0.26	Na	-0.01
DY475213	-0.10	-0.11	-0.44	-0.48	0.00
CV793599	-0.16	0.23	-0.10	-0.67	-0.40
DY475542	Na	-0.04	-0.34	-0.39	-0.41

Cluster C					
EST ID	**06 hpi**	**12 hpi**	**24 hpi**	**48 hpi**	**72 hpi**
DY475540	-0.45	0.08	-0.86	-0.29	-0.23
DY475357	-0.56	-0.07	-0.85	-0.61	0.12
DY475242	-0.49	0.39	-0.68	-0.42	0.11
CV793598	Na	-0.18	-0.79	Na	Na
DY475534	-0.77	0.32	-0.79	-0.41	-0.14
DY475536	-0.40	-0.30	-0.96	Na	0.07
DY475170	Na	-0.03	-0.67	-0.67	0.05
DY475190	-0.42	-0.03	-0.77	Na	0.12
CV793603	-0.50	-0.11	-0.58	Na	Na
DY475316	-0.43	0.34	-0.88	Na	-0.14
DY475397	-0.62	-0.10	-0.53	-0.22	-0.21
DY475493	-0.49	-0.10	-0.95	Na	-0.13
DY396335	-0.57	0.28	Na	Na	-0.18
DY475150	Na	-0.07	-0.76	-0.50	-0.17
DY475481	Na	0.07	-0.80	-0.15	-0.21

Cluster D					
EST ID	**06 hpi**	**12 hpi**	**24 hpi**	**48 hpi**	**72 hpi**
DY475462	-0.56	0.42	-0.32	-0.06	-0.57
DY396268	Na	0.10	-0.30	Na	-0.37
DY475116	Na	0.41	-0.18	Na	-0.50
DY475181	Na	0.13	-0.37	Na	-0.51
DY475339	-0.31	0.19	-0.86	0.45	-0.24
DY475541	-0.08	0.03	-0.51	0.09	-0.17
DY475047	-0.07	0.29	-0.80	0.36	-0.23
DY475153	-0.38	0.40	-0.08	-0.22	-0.81
DY475302	-0.13	0.01	-0.34	-0.07	-0.49
DY475095	-0.06	0.13	-0.66	-0.38	-0.62
CV793589	Na	0.22	-0.27	-0.10	Na
DY475539	-0.30	0.18	-0.33	-0.30	-0.45

Cluster E					
EST ID	**06 hpi**	**12 hpi**	**24 hpi**	**48 hpi**	**72 hpi**
DY475246	-0.13	0.08	-0.13	0.00	0.15
DY475403	-0.22	0.31	-0.43	0.73	0.16
DY475544	Na	0.21	0.26	Na	-0.16
DY475323	0.13	0.03	-0.06	Na	0.24
DY475533	-0.06	0.23	Na	Na	0.13
DY475538	0.06	0.20	-0.17	0.05	0.32
DY396288	-0.53	0.20	0.22	0.52	0.04
DY396367	Na	0.42	Na	0.52	0.04

Cluster F					
EST ID	**06 hpi**	**12 hpi**	**24 hpi**	**48 hpi**	**72 hpi**
DY396377	Na	-0.70	-0.03	0.03	-0.72
DY396400	-1.14	-0.66	-0.01	-0.11	Na
DY475125	Na	-0.04	-0.24	-0.94	-1.02
DY475478	Na	0.00	-0.41	-0.78	-0.42
DY475535	-0.77	-0.27	-0.29	-1.16	-0.43
DY475108	-0.55	-0.20	-0.25	-1.12	Na
DY475172	-0.53	-0.17	-0.48	-0.25	-0.53
DY475186	-0.77	-0.28	0.25	0.41	-1.04
DY475211	Na	-0.15	-0.16	-0.22	-1.12
DY475225	-0.30	-0.20	-0.62	-0.74	-0.70
DY475554	Na	-0.84	-0.26	-0.79	-0.24

Cluster G					
EST ID	**06 hpi**	**12 hpi**	**24 hpi**	**48 hpi**	**72 hpi**
DY396281	Na	-0.18	0.93	1.21	0.65
DY396301	-0.17	0.37	0.90	0.89	0.44
DY396384	Na	-0.22	Na	1.22	0.99
DY396388	Na	-0.40	0.11	1.39	0.63
CV793597	0.16	0.19	0.91	1.80	1.20
DY475483	0.01	0.14	0.64	Na	0.07
DY396265	-0.21	0.12	0.52	0.73	0.82
DY396372	Na	-0.28	0.23	1.48	0.75
DY475248	Na	-0.44	0.31	Na	0.70
DY475365	0.36	-0.07	0.00	1.23	0.40
DY475523	0.22	0.21	0.27	0.92	0.04
DY396298	Na	0.40	0.67	Na	0.68
DY396305	0.49	0.23	0.63	0.96	0.78
DY475475	0.11	0.27	0.37	0.80	0.36
DY475532	Na	0.09	0.50	Na	Na

Cluster H					
EST ID	**06 hpi**	**12 hpi**	**24 hpi**	**48 hpi**	**72 hpi**
CV793607	Na	-0.33	-0.17	Na	0.05
DY475322	-0.68	-0.03	1.13	-0.21	Na
CV793587	-0.11	-0.32	Na	Na	0.02
DY475515	-0.35	0.00	0.43	Na	-0.09
DY396279	0.50	-0.27	0.45	Na	0.09
DY396344	Na	-0.95	-0.04	0.27	-0.36
DY396347	Na	-0.44	-0.02	0.12	-0.19
DY475217	0.12	-0.16	0.40	Na	0.05
DY475092	0.20	-0.87	-0.33	0.18	-0.32
DY475115	0.51	0.01	0.31	-0.40	-0.18
CV793608	-0.03	-0.02	0.22	-0.06	0.04
DY475401	-0.13	-0.38	0.11	0.56	-0.07
DY475094	0.12	-0.05	0.13	-0.04	0.26
CV793591	Na	-0.28	-0.09	Na	0.28

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Cluster A					
EST ID	**06 hpi**	**12 hpi**	**24 hpi**	**48 hpi**	**72 hpi**
DY396281	0.44	0.60	1.03	0.14	-0.46
DY396301	0.00	0.10	0.85	0.62	-0.04
DY396305	0.61	0.06	0.75	0.27	0.26
DY396372	Na	0.34	1.22	0.62	-0.09
DY396384	Na	0.72	1.11	0.53	-0.14
DY396388	Na	0.53	1.71	0.75	-0.17
DY475217	0.01	0.06	0.83	Na	0.01
CV793597	0.39	0.70	1.09	-0.20	0.16
DY475365	0.30	0.24	0.83	0.76	0.09
DY475522	0.26	0.30	Na	Na	0.20
DY396298	-0.07	0.36	0.84	0.11	0.60
DY475544	0.83	0.47	Na	-0.24	Na
CV793589	Na	0.26	Na	Na	Na
DY396288	0.10	0.18	1.91	0.45	1.12

Cluster B					
EST ID	**06 hpi**	**12 hpi**	**24 hpi**	**48 hpi**	**72 hpi**
DY475172	-0.58	-0.29	-0.69	-0.79	-1.00
DY475250	-0.50	-0.01	-0.06	-0.45	-0.73
DY475397	-0.87	-0.01	-0.63	-0.70	-0.14
DY475478	-0.33	-0.41	-0.78	-1.05	-0.22
DY475108	-0.60	-0.32	-0.61	-0.56	Na
DY475190	-0.54	0.12	-0.72	-0.96	-0.16
DY475225	-0.64	-0.44	-0.97	-0.84	-0.52
DY475245	Na	0.09	-0.33	-1.12	-0.40
DY475276	-0.65	-0.06	-0.40	-0.44	-0.20
CV793599	-0.34	-0.24	Na	-0.87	-0.24
DY475543	-0.63	-0.21	-0.42	-0.30	-0.15
DY475242	Na	-0.18	-0.38	-0.75	-0.07
DY475246	-0.43	-0.08	-0.33	-0.82	-0.16
DY475305	-0.58	0.28	-0.53	-0.26	-0.38
DY475535	-0.23	0.12	-1.10	-0.86	-0.97
CV793603	Na	-0.24	-0.91	-0.45	Na
CV793587	Na	-0.06	-0.78	Na	Na
DY475302	-0.35	-0.16	-0.66	-0.29	-0.26

Cluster C					
EST ID	**06 hpi**	**12 hpi**	**24 hpi**	**48 hpi**	**72 hpi**
DY396367	Na	0.06	-0.74	0.59	0.87
DY475047	0.14	-0.15	0.01	0.35	0.08
DY475153	0.56	-0.52	-0.11	0.72	0.84
DY475532	0.33	-0.13	Na	Na	0.35
DY475533	0.02	0.14	0.00	0.42	0.52
DY475557	Na	0.21	-0.20	Na	0.92
DY475125	Na	-0.25	Na	-0.41	1.31
DY475211	0.06	-0.20	0.09	0.03	0.62
DY475542	0.65	-0.77	-0.19	0.32	-0.07
DY475396	0.12	-0.04	Na	Na	0.37
DY475462	0.32	-0.02	-0.34	0.83	0.44
DY475464	0.16	0.07	-0.06	0.07	0.24
DY396377	-0.25	-0.12	Na	0.10	0.52
DY475323	Na	0.19	-0.18	0.59	0.03
DY475401	0.23	-0.01	-0.21	0.68	-0.01
DY475339	0.19	-0.02	-0.66	-0.14	0.15

Cluster D					
EST ID	**06 hpi**	**12 hpi**	**24 hpi**	**48 hpi**	**72 hpi**
DY475084	-0.32	-0.58	-0.29	0.11	0.16
DY475092	-0.12	-0.62	-0.77	0.58	0.27
DY396347	-1.11	-0.65	-0.49	0.34	0.56
DY475186	-0.51	-0.30	-1.25	0.90	0.04
DY475150	-0.35	-0.31	-0.41	0.45	-0.11
DY475481	0.02	-0.39	-0.75	0.78	0.01
DY475539	0.10	-0.07	-0.75	0.53	0.36
DY475540	-0.09	-0.37	-0.97	0.80	-0.38
DY396344	-0.12	-0.37	-0.82	0.54	0.48
DY475095	0.13	-0.27	-0.67	0.55	0.28
DY475322	-0.30	-0.62	-1.02	0.23	Na
DY475357	-0.45	0.08	-0.80	Na	0.13

Cluster E					
EST ID	**06 hpi**	**12 hpi**	**24 hpi**	**48 hpi**	**72 hpi**
DY475248	Na	0.03	Na	-0.67	Na
DY475384	-0.09	0.10	0.05	-0.97	-0.62
DY475094	-0.08	0.06	0.21	-0.82	0.11
CV793591	Na	0.17	Na	-0.45	0.46
DY475091	-0.16	-0.08	0.22	-0.88	-0.37
DY475116	Na	-0.21	Na	-0.48	0.50
DY475170	Na	-0.15	Na	-0.70	0.15
DY475213	0.02	0.01	-0.23	-1.48	0.01
CV793607	-0.10	0.45	-0.20	-1.11	-0.07
DY475554	-0.18	-0.18	0.18	-0.68	0.97
DY475541	-0.27	-0.24	-0.02	-0.79	0.82
DY396335	Na	-0.04	Na	Na	0.08
DY475087	0.12	0.02	0.16	-0.83	0.32
CV793598	0.04	-0.05	0.63	-0.61	Na
DY475157	Na	0.14	0.31	Na	0.06
DY396279	Na	0.22	0.45	Na	0.63
DY475115	0.29	-0.03	0.52	Na	0.41

Cluster F					
EST ID	**06 hpi**	**12 hpi**	**24 hpi**	**48 hpi**	**72 hpi**
DY475538	0.12	-0.23	-0.04	0.00	-0.86
DY475316	-0.18	0.07	Na	-0.27	-0.02

DY475220	0.12	-0.07	-0.04	-0.22	-0.34
DY475076	Na	0.07	-0.25	-0.28	-0.28
DY475548	-0.53	-0.38	-0.08	-0.15	-0.32
DY475181	Na	0.07	Na	-0.07	Na
DY475550	-0.13	0.24	-0.13	-0.30	-0.05
DY475403	-0.15	0.59	Na	-0.31	-0.03
DY475483	0.05	0.26	0.06	0.40	-0.23
DY475515	-0.32	0.29	-0.04	Na	-0.36
DY475534	0.32	-0.20	-0.19	0.37	-0.40
DY475536	Na	-0.05	0.20	-0.05	0.07
DY475493	-0.18	0.25	-0.05	Na	-0.27
DY475475	0.40	0.10	0.36	0.16	-0.38
DY396262	Na	-0.02	0.44	0.22	-0.21
DY396268	0.14	-0.08	0.02	Na	-0.04
DY396400	-0.05	-0.26	0.01	0.09	Na
DY475523	0.19	0.02	0.40	0.46	-0.11
CV793608	0.10	-0.15	0.13	0.06	0.13
DY396265	0.18	0.00	0.47	0.32	0.07

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Cluster A					
EST ID	**06 hpi**	**12 hpi**	**24 hpi**	**48 hpi**	**72 hpi**
DY475535	Na	Na	Na	Na	Na
DY475539	-0.94	0.25	-0.63	-0.23	0.39
DY475541	-0.71	-0.27	-0.43	-0.08	-0.17
DY475544	Na	Na	Na	Na	Na
DY475115	-0.23	0.19	-0.73	Na	Na
DY475534	-0.21	0.24	-0.54	0.18	0.41
DY475540	-0.91	0.39	-0.70	0.30	0.23
DY475095	-0.19	0.24	-0.88	0.08	0.39
DY475339	-0.38	-0.21	-0.77	-0.24	-0.25
DY475357	-0.24	-0.48	-0.52	-0.22	0.17
DY475047	-0.28	0.25	-0.35	-0.30	0.18
DY475543	-0.31	0.40	-0.85	-0.23	0.07
DY475150	-0.38	0.12	-0.47	0.10	0.36
DY475515	-0.31	-0.07	-0.60	-0.20	-0.17
DY475076	-0.01	-0.16	-0.57	-0.14	0.15
DY396335	-0.30	0.02	Na	Na	0.16
DY396262	Na	-0.02	Na	Na	0.15
DY475125	Na	0.08	Na	Na	0.31

Cluster B					
EST ID	**06 hpi**	**12 hpi**	**24 hpi**	**48 hpi**	**72 hpi**
CV793591	-0.48	-0.16	1.26	-0.42	-0.01
DY475403	-0.11	0.42	0.83	-0.45	-1.27
DY396268	Na	-0.30	0.42	Na	0.03
DY475186	-0.21	-0.49	1.22	0.16	-0.67
CV793607	-0.27	0.05	0.31	-0.38	-0.07
DY475087	Na	0.12	0.45	0.11	-0.05
DY475475	0.38	0.18	0.62	-0.37	-0.24

Cluster C					
EST ID	**06 hpi**	**12 hpi**	**24 hpi**	**48 hpi**	**72 hpi**
DY396265	0.48	0.40	0.69	-0.09	0.51
DY396305	1.00	0.39	0.49	-0.18	0.25
DY396279	0.75	0.20	0.57	0.11	0.90
DY396281	0.70	0.69	1.11	Na	Na

DY396301	0.57	0.35	0.66	-0.30	0.96
DY396372	0.47	-0.02	1.73	Na	Na
DY396384	0.83	0.68	1.55	0.10	Na
DY396388	0.64	0.46	1.51	Na	Na
CV793597	0.80	0.32	1.56	-0.49	1.19

Cluster D

EST ID	06 hpi	12 hpi	24 hpi	48 hpi	72 hpi
DY396288	-0.28	0.15	0.13	0.36	0.28
DY396400	-0.32	0.25	0.15	0.31	Na
DY475542	-0.08	0.30	-0.13	Na	0.53
DY475481	-0.64	0.42	-0.28	0.28	0.45
DY396298	-0.40	-0.15	0.28	0.26	0.47
DY475153	-0.81	0.31	0.08	0.19	0.27
DY475462	Na	0.29	0.03	Na	0.43
DY396344	-0.52	0.29	0.54	-0.16	0.78
DY396347	-0.28	0.51	0.36	-0.27	0.82
DY396367	-0.34	0.30	-0.02	Na	Na
DY396377	-1.26	0.32	0.50	-0.07	0.43
DY475181	-0.39	0.26	-0.03	Na	Na
DY475211	-0.65	0.00	-0.18	0.21	0.14
DY475092	-0.26	0.09	0.40	-0.10	0.15
CV793589	-0.68	Na	Na	Na	Na
DY475532	-0.85	Na	Na	Na	Na

Cluster E

EST ID	06 hpi	12 hpi	24 hpi	48 hpi	72 hpi
DY475248	Na	1.30	0.19	-0.69	Na
DY475302	0.10	0.38	0.08	-0.16	-0.09
DY475548	Na	0.82	-0.37	-0.11	Na
DY475533	Na	0.55	-0.14	-0.01	-0.15
DY475554	-0.22	0.61	-0.49	-0.04	0.07
DY475084	-0.19	0.78	0.08	-0.07	-0.28
DY475557	-0.26	0.77	0.04	-0.23	0.27

Cluster F

EST ID	06 hpi	12 hpi	24 hpi	48 hpi	72 hpi
DY475094	0.06	-0.03	0.06	0.00	-0.10
DY475550	-0.28	-0.21	0.02	-0.28	-0.01
DY475483	0.03	-0.02	-0.40	-0.13	-0.19
DY475220	0.03	-0.20	0.22	0.00	0.12
CV793608	-0.03	0.18	0.00	-0.17	-0.02
DY475396	0.16	0.01	0.06	-0.41	0.52
DY475157	Na	-0.01	-0.16	Na	Na
DY475170	Na	-0.14	Na	-0.11	-0.06
DY475213	0.11	-0.18	-0.09	-0.40	0.03
DY475365	0.33	-0.08	0.01	0.49	0.25
DY475384	0.18	-0.25	-0.23	-0.21	-0.15
DY475323	Na	0.11	-0.08	Na	Na
DY475536	Na	-0.08	Na	Na	Na
DY475538	0.06	0.11	-0.39	0.19	0.07
DY475322	Na	-0.01	-0.26	Na	Na
DY475401	-0.11	0.21	0.16	0.27	0.04

Cluster G

EST ID	06 hpi	12 hpi	24 hpi	48 hpi	72 hpi
DY475225	-0.79	0.16	-0.54	-0.88	-0.37
DY475242	-0.05	0.07	0.07	Na	-0.41

DY475091	0.07	-0.27	-0.28	-0.74	Na
DY475108	Na	-0.34	0.06	-0.55	Na
DY475116	Na	0.02	-0.60	-0.72	-0.22
DY475172	-0.41	-0.55	-0.45	-0.62	Na
DY475245	Na	-0.26	0.22	-0.83	-0.04
CV793599	Na	-0.03	-0.17	-0.53	Na
CV793603	Na	-0.26	Na	-0.65	Na
DY475316	-0.28	-0.33	-0.02	-0.92	-0.37
DY475478	-0.26	-0.05	0.27	-0.78	-0.39
DY475493	0.24	-0.27	0.12	Na	-0.57
DY475522	-0.32	-0.16	-0.23	Na	-0.06
DY475246	0.12	-0.11	0.03	-0.70	-0.03
DY475305	-0.30	-0.35	-0.40	Na	-0.07
CV793598	-0.02	-0.25	-0.37	-0.67	Na
CV793587	0.05	-0.58	Na	Na	Na
DY475464	-0.25	-0.08	-0.33	-0.32	-0.18
DY475190	-0.44	-0.43	0.09	Na	-0.63
DY475217	-0.44	-0.01	-0.13	Na	-0.06
DY475250	-0.25	0.18	0.10	-0.44	-0.23
DY475276	-0.37	0.08	-0.49	-0.15	-0.92
DY475397	-0.45	-0.39	0.12	-0.40	-0.04
DY475523	-0.38	-0.11	-0.14	Na	0.04

Appendix 8. Differentially expressed ESTs after ACC, SA and MeJA treatments

Lists of DE ESTs in all chickpea genotypes after treatment with ACC, SA and MeJA. Fold change represents the $\log_2$ of mean expression ratio of treated *vs* control samples, and 95% +/- is the interval above and below the mean corresponding to the 95% confidence interval. For 'clone source', CA indicates *Cicer arietinum* and LS indicates *Lathyrus sativus*.

ACC

Time-point	Condition	Fold change	95% +/-	Clone source	Putative function	GenBank accession
03 hpt	IC Up	1.93	0.16	CA	Multi-resistance ABC transporter protein	CV793605
		1.78	0.15	CA	Oxygen splitting enhancer of photosystem II	DY475060
		1.31	0.12	LS	EREBP-4	DY396395
		1.22	0.07	CA	Fructose-1,6-bisphosphatase	DY475543
		1.20	0.09	LS	EREBP-4	DY396400
		1.20	0.20	CA	S1-3 homolog	CV793591
		1.14	0.11	CA	Unknown	EB085028
		1.14	0.07	CA	Cytosolic fructose 1,6-bisphosphatase	DY475548
		1.13	0.19	CA	Unclear	DY475175
		0.95	0.24	LS	Glutathione S-transferase	DY396404
		0.87	0.19	LS	Histidine-containing phosphotransfer protein	DY396300
	LA Up	2.23	0.34	CA	Unknown	EB085028
		1.68	0.50	CA	Thylakoid protein	DY475305
		1.46	0.36	CA	Unclear	DY475175
		1.45	0.23	CA	Superoxide dismutase copper chaperone precursor	DY475397
		1.34	0.37	CA	Unknown	EB085044
		1.23	0.49	CA	Unknown	DY475365
		1.22	0.42	CA	PPF1 – post floral protein	DY475509
		1.20	0.16	CA	Unknown	DY475191
		1.18	0.42	LS	Glycolate oxidase	DY396348
		1.16	0.45	CA	Unclear	DY475355
		1.14	0.23	CA	Cytochrome P450	DY475136
		1.08	0.13	LS	EREBP-4	DY396400
		1.08	0.37	CA	Unknown	DY475160
		1.07	0.09	CA	Disease resistance response protein DRRG49-C	EB085032
		1.06	0.36	CA	26S rRNA	EB085013
		1.05	0.09	CA	Histidine-containing phosphotransfer protein	DY475511
		1.05	0.34	CA	50S ribosomal protein	DY475359
		1.04	0.39	CA	Unknown	EB085034
		1.02	0.36	CA	Ribose 5-phosphate isomerase	DY475240
		0.96	0.32	CA	Similar to ferredoxin-thioredoxin reductase	DY475304
		0.92	0.11	CA	Unknown	DY475064
		0.90	0.59	CA	Unknown	EB085014
		0.88	0.16	LS	Histidine-containing phosphotransfer protein	DY396300
		0.88	0.34	CA	Chloroplast 4.5S/5S/16S/23S mRNA	DY475154
		0.86	0.27	CA	Translational activator	EB085015
		0.86	0.25	CA	Unknown	DY475461
		0.83	0.41	CA	Succinate dehydrogenase subunit 3	DY475443
		0.83	0.32	CA	Histidine-rich glycoprotein precursor	DY475271
		0.82	0.19	LS	EREBP-4	DY396395
		0.82	0.27	CA	50S ribosomal protein	DY475131
		0.80	0.28	CA	Unknown	DY475075
		0.79	0.31	CA	SNAKIN2 antimicrobial peptide precursor	CV793608
		0.76	0.31	CA	Chloroplast genome DNA	DY475176
		0.72	0.30	LS	Magnesium chelatase subunit	DY396363

	0.71	0.36	CA	Chloroplast DNA	EB085054
FL Up	1.81	0.50	CA	Leucine-zipper containing protein	CV793601
	1.74	0.55	CA	Unknown	EB085057
	1.51	0.05	CA	Unknown	DY475293
	1.46	0.39	CA	Photosystem I reaction centre subunit VI-2	DY475285
	1.43	0.47	CA	Photosystem I reaction centre subunit XI	DY475132
	1.32	0.19	CA	Anthranilate phosphoribosyltransferase	DY475507
	1.21	0.27	CA	Unknown	EB085030
	1.19	0.06	CA	Asparagine synthetase	DY475477
	1.10	0.28	CA	Chloroplast CP12 mRNA	DY475058
	1.02	0.28	CA	Unknown	EB085051
	1.00	0.38	CA	Oxygen splitting enhancer of photosystem II	DY475052
	0.96	0.29	CA	Chlorophyll a/b binding protein	DY475202
	0.75	0.26	CA	Light inducible protein of photosystem II	DY475129
IC Down	-3.10	0.97	CA	Unknown	DY475236
	-2.55	0.47	CA	Unknown	DY475339
	-2.14	0.23	CA	26S rRNA	DY475540
	-2.09	0.25	CA	18S nuclear rRNA	DY475150
	-1.85	0.35	CA	26S rRNA	DY475211
	-1.74	0.55	CA	Chloroplast Val-tRNA	DY475537
	-1.61	0.80	CA	Phosphate-induced protein	DY475172
	-1.57	0.33	CA	Chlorophyll a/b binding protein	DY475534
	-1.56	0.21	LS	Cornifin alpha (small proline-rich protein)	DY396399
	-1.56	0.69	CA	Unknown	DY475481
	-1.51	0.16	CA	Unknown	DY475539
	-1.46	0.54	LS	Protein kinase-like protein	DY396362
	-1.28	0.25	CA	Unclear	DY475099
	-1.19	0.41	CA	Proton pump interacting protein	DY475434
	-1.14	0.28	CA	Unknown	DY475347
	-1.12	0.63	CA	Probable 3-hydroxyisobutyrate dehydrogenase mitochondrial precursor	DY475417
	-1.11	0.36	CA	18S rRNA	DY475542
	-1.09	0.05	CA	Unknown	DY475311
	-1.06	0.30	CA	Unknown	DY475431
	-1.05	0.21	CA	Phosphate-induced protein	EB085042
	-1.04	0.39	LS	Ca-binding carrier protein	DY396262
	-0.99	0.24	CA	5.8S, 18S and 25S rRNA	EB085033
	-0.96	0.13	LS	RAC-GTP binding protein-like	DY396336
	-0.94	0.11	LS	Serine/threonine protein kinase	DY396307
	-0.93	0.13	CA	Peptidase-like protein	DY475387
	-0.90	0.22	CA	Unknown	EB085061
	-0.90	0.28	CA	ATP Synthase C chain (lipid binding protein)	DY475464
	-0.81	0.19	CA	Unknown	DY475253
	-0.81	0.36	CA	Unknown	DY475513
	-0.80	0.27	CA	Unknown	DY475338
	-0.79	0.29	CA	Unclear	DY475217
	-0.75	0.45	CA	Unclear	DY475095
	-0.71	0.55	CA	Chlorophyll mRNA	EB085059
LA Down	-6.15	0.73	CA	Oxygen splitting enhancer of photosystem II	DY475060
	-3.36	0.79	CA	Unknown	DY475481
	-3.33	0.54	LS	Disease resistance response protein DRRG49-C	DY396265
	-3.18	0.63	CA	Unknown	DY475513
	-3.03	1.17	LS	Pathogenesis-related protein	DY396301
	-2.90	1.01	CA	Unclear	DY475444
	-2.66	0.67	LS	Transcription initiation protein SPT4	DY396309

-2.50	1.17	CA	Chloroplast Val-tRNA	DY475537
-2.40	0.57	LS	Pathogenesis-related protein	DY396343
-2.36	0.33	CA	Unknown	DY475084
-2.35	0.39	CA	Asparagine synthetase	DY475475
-2.33	0.53	CA	Chlorophyll mRNA	EB085059
-2.28	0.89	CA	Unknown	EB085063
-2.26	0.44	CA	S-adenosylmethionine decarboxylase	DY475170
-2.21	0.18	CA	18S rRNA	DY475542
-2.17	0.59	LS	Poly(A)-binding protein	DY396412
-2.14	1.03	CA	Unknown	DY475401
-2.11	1.12	CA	Unknown	DY475356
-2.10	0.54	LS	Xyloglucan endotransglycosylase	DY396308
-2.08	0.50	LS	GTP-binding protein SAR1A	DY396259
-2.05	0.74	CA	Unknown	DY475327
-1.96	0.86	LS	Putative auxin-repressed protein	DY396359
-1.91	0.53	CA	Unknown	DY475185
-1.91	0.44	CA	S1-3 homolog	CV793591
-1.91	0.59	LS	Disease resistance response protein 39 precursor	DY396296
-1.89	0.58	CA	Unknown	DY475533
-1.84	0.20	CA	Unknown	DY475236
-1.82	0.70	CA	Unknown	DY475223
-1.79	0.71	CA	Unknown	EB085022
-1.77	0.74	CA	Phosphate-induced protein	DY475076
-1.76	1.64	LS	Putative auxin-repressed protein	DY396289
-1.65	0.86	CA	SNAKIN2 antimicrobial peptide precursor	CV793606
-1.56	0.43	CA	Unknown	DY475364
-1.56	0.17	CA	Phosphate-induced protein	DY475172
-1.54	0.63	CA	Unclear	DY475313
-1.52	0.50	CA	Chloroplast 16S rRNA	DY475101
-1.52	0.46	LS	Cytochrome B5	DY396340
-1.49	0.45	CA	Xylosidase	DY475408
-1.44	0.52	CA	Proline-rich structural protein	DY475348
-1.38	0.50	CA	Unknown	DY475414
-1.36	0.49	CA	Unknown	DY475159
-1.35	0.39	LS	Glutathione S-transferase	DY396404
-1.34	0.53	CA	Unknown	DY475206
-1.33	0.24	CA	RNA polymerase beta subunit	DY475196
-1.32	0.33	CA	Fructose-1,6-bisphosphatase	DY475543
-1.30	0.08	CA	Chloroplast 16S rRNA	DY475146
-1.26	0.55	LS	Thioredoxin	DY396293
-1.25	0.56	CA	Unknown	DY475203
-1.24	0.75	CA	Unknown	DY475472
-1.23	0.32	CA	Unclear	DY475546
-1.23	0.43	CA	Thioredoxin	DY475069
-1.21	0.06	CA	18S nuclear rRNA	DY475150
-1.21	0.61	CA	Chloroplast DNA between RUBISCO large subunit and ATPase (beta) genes	DY475518
-1.19	0.36	LS	Disease resistance response protein 39 precursor	DY396277
-1.15	0.10	CA	Phosphate-induced protein	EB085042
-1.15	0.08	CA	26S rRNA	DY475211
-1.13	0.26	LS	Small GTP-binding protein	DY396367
-1.11	0.32	LS	Lectin-like protein	DY396427
-1.11	0.54	CA	Unknown	EB085052
-1.08	0.39	CA	Similar to endopeptidase	DY475396
-1.02	0.24	CA	Unclear	DY475473
-1.02	0.08	CA	Unknown	DY475462
-1.01	0.25	CA	Unknown	DY475391
-0.98	0.56	LS	Putative auxin-repressed protein	DY396269

	-0.97	0.18	LS	Guanine nucleotide regulatory protein	DY396313
	-0.96	0.50	CA	NADH-plastoquinone oxidoreductase	DY475287
	-0.96	0.34	LS	Putative auxin-repressed protein	DY396292
	-0.93	0.21	CA	Unclear	DY475284
	-0.92	0.30	CA	Carbonic anhydrase like protein	DY475403
	-0.91	0.21	LS	Thioredoxin H-type 1	DY396330
	-0.89	0.39	CA	Unknown	DY475340
	-0.89	0.15	CA	Unknown	DY475263
	-0.89	0.32	CA	NADH dehydrogenase	DY475316
	-0.88	0.45	CA	Beta glucosidase	DY475415
	-0.86	0.36	LS	RAC-GTP binding protein-like	DY396336
	-0.86	0.22	CA	Unknown	DY475347
	-0.82	0.39	LS	TMV resistance protein-like	DY396385
	-0.82	0.20	LS	Ubiquitin	DY396326
	-0.82	0.31	LS	L-ascorbate peroxidase cytosolic	DY396435
	-0.79	0.56	LS	Senescence-associated protein DIN1	DY396338
	-0.76	0.33	LS	Transport protein SEC61 gamma subunit	DY396418
	-0.75	0.27	CA	Chloroplast DNA	EB085019
FL Down	-4.40	1.13	CA	Unknown	DY475485
	-4.21	1.28	LS	Pathogenesis-related protein	DY396343
	-3.89	1.49	LS	18.2 KDA class I heat shock protein	DY396282
	-3.53	0.98	LS	EREBP-4	DY396395
	-3.52	0.74	CA	Unclear	DY475175
	-3.41	1.18	LS	Polyubiquitin	DY396410
	-3.13	0.82	LS	Environmental stress inducible protein	DY396298
	-3.11	0.97	CA	Unknown	DY475084
	-2.91	1.26	LS	Putative ubiquitin protein	DY396366
	-2.85	0.59	CA	Superoxide dismutase copper chaperone precursor	DY475397
	-2.81	0.31	CA	NADH-plastoquinone oxidoreductase	DY475287
	-2.38	0.35	CA	NADH dehydrogenase	DY475316
	-2.37	0.59	CA	Unknown	DY475203
	-2.18	1.08	CA	Unknown	DY475185
	-1.88	1.47	CA	Unknown	DY475536
	-1.59	0.07	CA	Hypothetical transmembrane protein	DY475478
	-1.57	0.35	CA	Disease resistance response protein DRRG49-C	EB085032
	-1.48	0.31	CA	Unclear	DY475313
	-1.40	0.13	CA	Unclear	DY475205
	-1.38	0.15	CA	S-adenosylmethionine decarboxylase	DY475170
	-1.36	0.02	LS	Pathogenesis-related protein 4A	DY396281
	-1.32	0.23	CA	Membrane sugar-transport protein	DY475239
	-1.24	0.04	CA	Thioredoxin H-type 1	DY396330
	-1.20	0.04	CA	Similar to endopeptidase	DY475396
	-1.18	0.36	CA	L-allo-threonine aldolase	DY475068
	-1.18	0.03	LS	Hypothetical proline-rich protein	DY396288
	-1.09	0.40	CA	Amino acid transferase	DY475122
	-1.08	0.63	LS	Poly(A)-binding protein	DY396412
	-1.05	0.04	CA	Thiazole biosynthetic enzyme	DY475242
	-0.98	0.54	CA	Unclear	DY475319
	-0.96	0.04	CA	FKBP-type peptidyl-prolyl cis-trans isomerase	DY475406
	-0.94	0.31	CA	Protein kinase mRNA	DY475470
	-0.93	0.16	CA	Aquaporin membrane protein	DY475174
	-0.86	0.17	CA	Unclear	DY475367
	-0.82	0.39	CA	Chloroplast 4.5S, 5S, 16S and 23S mRNA	DY475545
	-0.81	0.21	CA	Unknown	DY475048
	-0.76	0.43	LS	Putative auxin-repressed protein	DY396269
	-0.75	0.54	CA	Unknown	DY475431

27 hpt	IC Up	3.93	0.34	CA	SNAKIN2 antimicrobial peptide precursor	CV793608
		2.70	0.62	LS	Pathogenesis-related protein	DY396305
		2.56	0.62	CA	Unknown	DY475520
		2.47	0.51	CA	Cytochrome P450	DY475136
		2.33	0.53	CA	Pathogenesis-related protein 4A	CV793597
		2.00	0.47	CA	Unclear	DY475222
		1.98	0.50	CA	Unclear	DY475226
		1.96	0.36	CA	Nuclear transport factor	DY475059
		1.95	0.44	LS	Glycolate oxidase	DY396348
		1.86	0.27	CA	Leucine-zipper containing protein	CV793601
		1.85	0.46	CA	Unknown	EB085037
		1.84	0.47	CA	Multispanning membrane protein	DY475410
		1.82	0.48	CA	Histidine-containing phosphotransfer protein	DY475511
		1.81	0.54	CA	Unknown	DY475054
		1.75	0.43	CA	Unknown	EB085035
		1.72	0.55	CA	Unknown	EB085034
		1.72	0.47	CA	Auxin-repressed protein	DY475078
		1.70	0.62	LS	Ubiquitin	DY396326
		1.69	0.43	CA	Flavonol glucosyl transferase	CV793607
		1.67	0.40	CA	Cinnamyl-alcohol-dehydrogenase (CAD)	CV793602
		1.66	0.25	LS	Pathogenesis-related protein 4A	DY396281
		1.66	0.39	CA	Cysteine protease	DY475458
		1.66	0.40	CA	60S rRNA	EB085023
		1.64	0.50	CA	Gamma-thionen type defensin/protease inhibitor	CV793588
		1.63	0.60	LS	Polyubiquitin	DY396302
		1.61	0.47	LS	Catalase	DY396413
		1.60	0.36	CA	Protein kinase	DY475103
		1.59	0.36	CA	Unknown	DY475160
		1.53	0.62	LS	Polyubiquitin	DY396378
		1.52	0.45	CA	Aquaporin	DY475124
		1.50	0.14	CA	Unknown	DY475191
		1.50	0.57	LS	Ubiquitin	DY396368
		1.47	0.40	CA	PPF1 - post floral protein	DY475509
		1.45	0.15	CA	Unknown	EB085028
		1.45	0.47	CA	Unclear	DY475086
		1.42	0.30	CA	Cysteine protease	DY475066
		1.42	0.41	CA	Unknown	DY475098
		1.41	0.46	CA	Unknown	DY475349
		1.40	0.47	CA	Auxin repressed protein	DY475137
		1.39	0.33	CA	Unknown	DY475061
		1.38	0.01	CA	Anthranilate phosphoribosyltransferase	DY475507
		1.38	0.37	CA	50S ribosomal protein L12	DY475131
		1.37	0.38	LS	Poly(A)-binding protein	DY396360
		1.36	0.22	CA	Acyl-activating enzyme	EB085018
		1.34	0.48	LS	Polyubiquitin	DY396428
		1.32	0.34	CA	Unknown	DY475133
		1.31	0.54	CA	Unknown	DY475315
		1.28	0.49	CA	S29 ribosomal protein	DY475504
		1.28	0.35	CA	Adenosylhomocysteinase	DY475372
		1.28	0.43	LS	Ubiquinol-cytochrome C reductase complex	DY396401
		1.28	0.29	CA	Unknown	DY475437
		1.27	0.18	CA	Unknown	DY475432
		1.26	0.39	CA	Acidic 60s ribosomal protein	DY475421
		1.24	0.20	CA	Photosystem II core complex protein psbY	DY475480
		1.24	0.18	CA	Photosystem I reaction centre subunit VI-2	DY475285
		1.21	0.29	CA	60S ribosomal protein L19	DY475324
		1.21	0.40	CA	Unknown	EB085040
		1.19	0.31	CA	Unknown	DY475426

	1.19	0.16	CA	Unknown	DY475472
	1.17	0.14	CA	Photosystem I reaction centre subunit XI	DY475132
	1.17	0.45	LS	Ubiquitin	DY396424
	1.14	0.40	CA	Translation initiation factor SUI1	EB085043
	1.13	0.35	CA	40S ribosomal protein S15	DY475117
	1.12	0.28	CA	Chloroplast CP12 mRNA	DY475058
	1.08	0.30	CA	Unknown	DY475416
	1.03	0.33	CA	Ribose 5-phosphate isomerase	DY475240
	1.03	0.28	CA	Unclear	EB085017
	1.00	0.14	CA	Unknown	DY475538
	1.00	0.12	CA	Unknown	DY475291
	0.95	0.31	CA	Chloroplast DNA	EB085065
	0.92	0.15	CA	Photosystem I reaction centre subunit IV	DY475128
	0.91	0.32	CA	Cationic peroxidase	DY475306
	0.90	0.13	CA	Unknown	EB085057
	0.89	0.32	LS	Pathogenesis-related protein 4A	DY396388
	0.87	0.40	LS	Disease resistance response protein DRRG49-C	DY396265
	0.81	0.20	CA	Unclear	DY475205
LA Up	2.41	0.12	LS	Pathogenesis-related protein	DY396305
	2.12	0.34	CA	Cytochrome P450	DY475136
	2.02	0.20	CA	Unclear	DY475367
	1.98	0.18	CA	Unknown	EB085028
	1.44	0.43	CA	Unknown	DY475521
	1.30	0.07	CA	SNAKIN2 antimicrobial peptide precursor	CV793606
	1.21	0.14	CA	Unclear	DY475205
	1.11	0.11	CA	Amino acid transferase	DY475122
	0.83	0.28	LS	Metallothionein-like protein	DY396322
	0.80	0.26	CA	Glutathione S-transferase	DY475250
FL Up	4.07	0.06	CA	Multi-resistance ABC transporter protein	CV793605
	2.84	0.13	CA	SNAKIN2 antimicrobial peptide precursor	CV793608
	2.45	0.13	CA	Unknown	EB085028
	1.90	0.08	LS	Pathogenesis-related protein	DY396305
	1.60	0.14	CA	Histidine-containing phosphotransfer protein	DY475511
	1.57	0.10	CA	Unknown	DY475191
	1.53	0.18	CA	Cytochrome P450	DY475136
	1.53	0.20	CA	Unknown	DY475054
	1.43	0.15	CA	Unknown	DY475521
	1.17	0.06	LS	Pathogenesis-related protein 4A	DY396281
	1.06	0.03	CA	Unknown	DY475293
	0.99	0.10	CA	4.5S, 5S, 16S and 23S rRNA	EB085066
	0.95	0.09	CA	Unknown	DY475055
	0.94	0.32	CA	Leucine-zipper containing protein	CV793601
	0.91	0.10	CA	Pathogenesis-related protein 4A	CV793597
	0.81	0.26	CA	Unknown	EB085057
	0.79	0.25	CA	Chloroplast CP12 mRNA	DY475058
	0.77	0.24	CA	Unknown	DY475432
IC Down	-4.26	1.10	CA	26S ribosomal RNA	DY475153
	-3.04	1.05	LS	TMV resistance protein-like	DY396385
	-2.77	0.30	LS	Protein kinase-like protein	DY396382
	-2.50	1.32	CA	Unclear	DY475273
	-2.45	0.66	LS	Glutathione peroxidase	DY396331
	-2.42	0.77	CA	Chloroplast 16S rRNA	DY475146
	-2.39	0.51	CA	Unknown	DY475339
	-2.16	0.72	LS	Thioredoxin	DY396293
	-2.11	0.74	CA	ATP synthase	DY475423

	-2.07	0.79	CA	Unclear	DY475376
	-2.04	0.55	CA	NADH dehydrogenase	DY475316
	-1.99	0.58	CA	Superoxide dismutase	DY475155
	-1.72	0.56	LS	Hypothetical proline-rich protein	DY396288
	-1.68	0.38	CA	Unknown	DY475187
	-1.66	0.26	LS	Putative membrane related protein	DY396429
	-1.66	0.53	CA	NADH-plastoquinone oxidoreductase	DY475287
	-1.61	0.67	CA	Aquaporin 2 protein	DY475512
	-1.54	0.68	CA	Heat shock protein	DY475278
	-1.52	0.51	CA	Unknown	EB085026
	-1.52	0.54	LS	Putative senescence-associated protein	DY396273
	-1.46	0.27	CA	Unknown	DY475451
	-1.45	0.48	CA	Unknown	DY475159
	-1.37	0.47	CA	Chloroplast DNA	DY475541
	-1.36	0.37	CA	Chloroplast DNA between RUBISCO large subunit and ATPase (beta) genes	DY475518
	-1.34	0.65	CA	Unknown	DY475366
	-1.34	0.16	LS	Disease resistance response protein 39 precursor	DY396296
	-1.29	0.36	CA	Chloroplast DNA	EB085050
	-1.29	0.36	CA	Unknown	EB085021
	-1.28	0.22	LS	Disease resistance response protein 39 precursor	DY396276
	-1.28	0.35	CA	Proline-rich structural protein	DY475348
	-1.26	0.24	CA	Chloroplast 30S ribosomal protein S12	DY475063
	-1.23	0.24	CA	Cytochrome C biogenesis protein ccsA	DY475393
	-1.07	0.17	CA	Unknown	DY475177
	-1.06	0.29	CA	Unclear	DY475313
	-0.93	0.27	CA	Unknown	DY475167
LA Down	-4.87	1.80	CA	Chlorophyll a/b binding protein	DY475554
	-3.45	0.71	CA	Unknown	DY475462
	-3.08	1.41	CA	Chloroplast DNA	EB085050
	-2.99	0.65	CA	Unknown	DY475481
	-2.98	0.62	LS	Nitrate transporter NRT1-1	DY396335
	-2.78	0.61	CA	Serine/threonine protein kinase	DY475320
	-2.69	1.05	CA	Unclear	DY475186
	-2.47	0.44	LS	Putative glutaredoxin	DY396317
	-2.42	0.46	CA	Apocytochrome F	DY475181
	-2.38	0.47	LS	Putative senescence-associated protein	DY396273
	-2.35	0.27	CA	Thymidylate kinase	DY475379
	-2.14	0.28	CA	Chloroplast Val-tRNA	DY475537
	-1.95	1.17	LS	Lectin-like protein	DY396427
	-1.69	0.19	CA	Chloroplast 16S rRNA	DY475101
	-1.57	0.28	CA	Chloroplast DNA for P700 chlorophyll a-apoprotein	DY475501
	-1.54	0.18	LS	Similarity to RNA-binding protein	DY396387
	-1.25	0.19	CA	Unknown	DY475236
	-1.15	0.21	CA	Chlorophyll a/b binding protein	DY475534
	-1.11	0.23	CA	Unclear	DY475273
	-0.92	0.11	LS	Serine carboxypeptidase isolag	DY396280
	-0.89	0.14	LS	Disease resistance response protein 39 precursor	DY396296
FL Down	-4.91	0.88	CA	Asparagine synthetase	DY475108
	-3.63	1.27	LS	Putative glutaredoxin	DY396317
	-3.54	0.44	CA	Unknown	DY475279
	-3.53	0.89	CA	Unknown	DY475462
	-3.15	0.70	CA	Unclear	DY475444
	-2.86	1.21	LS	Nitrate transporter NRT1-1	DY396335
	-2.74	0.26	LS	Auxin-responsive protein IAA9	DY396315
	-2.69	1.12	CA	Protein transport protein	DY475074

-2.41	0.31	LS	Putative membrane related protein	DY396429
-2.41	0.55	LS	Ripening-related protein	DY396377
-2.36	0.75	LS	Alpha-amylase precursor	DY396337
-2.33	0.73	CA	Unclear	DY475367
-2.33	0.83	CA	Unclear	DY475400
-2.27	0.48	CA	Unknown	DY475353
-2.25	0.53	CA	Unknown	DY475485
-2.24	0.42	CA	Chloroplast mRNA	EB085059
-2.23	0.10	LS	Putative auxin-repressed protein	DY396379
-2.23	0.62	CA	Chloroplast 16S rRNA	DY475146
-2.20	0.45	LS	Senescence-associated protein DIN1	DY396338
-2.13	0.44	CA	18S rRNA	DY475542
-2.11	0.55	CA	Unknown	DY475236
-2.09	0.65	LS	Thioredoxin	DY396293
-2.02	0.39	LS	TMV resistance protein-like	DY396385
-2.01	0.53	CA	Hypothetical transmembrane protein	DY475478
-2.01	0.71	CA	Cytosolic fructose 1,6-bisphosphatase	DY475548
-2.01	0.39	CA	Nucleotide-sugar epimerise	DY475112
-2.00	0.43	LS	Aquaporin-like transmembrane protein	DY396334
-1.97	0.67	CA	Thiazole biosynthetic enzyme	DY475242
-1.97	0.49	LS	EREBP-4	DY396395
-1.96	0.58	LS	Putative auxin-repressed protein	DY396359
-1.96	0.59	CA	Unclear	DY475376
-1.95	0.44	CA	S1-3 homolog	CV793591
-1.94	0.29	CA	Photosystem II reaction centre I protein	DY475116
-1.93	0.72	CA	UDP-glucose 4-epimerase	DY475149
-1.93	0.52	CA	Glucosyltransferase	DY475498
-1.92	0.38	CA	Beta glucosidase	DY475415
-1.92	0.30	LS	Transcription factor NTLIM1	DY396263
-1.91	0.35	CA	Chloroplast 50S ribosomal protein L14	DY475344
-1.87	0.44	CA	Unknown	EB085029
-1.85	0.35	LS	Ripening-related protein	DY396344
-1.83	0.54	CA	Unclear	DY475186
-1.82	0.36	CA	Unknown	DY475206
-1.81	0.25	CA	Dehydrin cold-induced protein	DY475092
-1.80	0.28	CA	Endoxyloglucan transferase	DY475207
-1.79	0.38	LS	Ripening-related protein	DY396347
-1.78	0.34	CA	Photosystem I reaction centre subunit IX	DY475047
-1.76	0.34	LS	Dehydrin-cognate	DY396324
-1.76	0.27	LS	Putative auxin-repressed protein	DY396289
-1.72	0.36	CA	Unknown	EB085060
-1.68	0.22	LS	Lectin-like protein	DY396427
-1.67	0.47	LS	GTP-binding protein SAR1A	DY396259
-1.66	0.45	LS	Hypothetical proline-rich protein	DY396288
-1.59	0.04	LS	Small GTP-binding protein	DY396367
-1.57	0.24	CA	Photosystem I assembly protein ycf3	DY475345
-1.56	0.37	CA	Unclear	EB085045
-1.56	0.47	LS	Putative auxin-repressed protein	DY396269
-1.56	0.87	CA	Unclear	EB085048
-1.54	0.29	CA	NADH-plastoquinone oxidoreductase	DY475287
-1.53	0.14	CA	Fructose-1,6-bisphosphatase	DY475543
-1.45	0.28	LS	Glutathione peroxidase	DY396331
-1.45	0.29	CA	Chloroplast 30S rRNA	EB085036
-1.44	0.25	CA	Chloroplast DNA	EB085050
-1.43	0.43	CA	Aquaporin 2 protein	DY475512
-1.42	0.32	CA	Unknown	EB085021
-1.42	0.24	LS	Serine carboxypeptidase isolag	DY396280
-1.42	0.66	CA	Unclear	EB085046

-1.38	0.45	CA	Unknown	DY475336
-1.34	0.19	CA	Unknown	DY475360
-1.30	0.48	CA	RNA polymerase beta subunit	DY475196
-1.29	0.40	CA	Superoxide dismutase	DY475155
-1.29	0.06	CA	Chlorophyll a/b binding protein	DY475534
-1.29	0.35	CA	Beta-galactosidase	EB085056
-1.27	0.31	CA	Chloroplast DNA	EB085038
-1.25	0.52	CA	Unknown	DY475401
-1.25	0.32	LS	Non-specific lipid-transfer protein precursor	DY396350
-1.25	0.36	CA	Unclear	DY475095
-1.24	0.20	CA	NADH dehydrogenase	DY475316
-1.22	0.26	CA	Unclear	DY475175
-1.20	0.60	CA	Unknown	DY475255
-1.18	0.17	CA	Unknown	DY475084
-1.15	0.33	CA	Unknown	DY475476
-1.13	0.12	CA	Membrane-related protein CP5	DY475119
-1.13	0.22	CA	Apocytochrome F	DY475181
-1.09	0.15	CA	Aquaporin membrane protein	DY475174
-1.06	0.41	CA	S-adenosylmethionine decarboxylase	DY475170
-1.06	0.12	LS	Metallothionein-like protein	DY396406
-1.06	0.33	LS	Ca-binding carrier protein	DY396262
-1.06	0.19	CA	Unknown	DY475177
-1.05	0.30	CA	Unclear	DY475097
-1.04	0.34	CA	Cytochrome P450	EB085031
-1.04	0.57	CA	Unknown	DY475275
-1.04	0.22	CA	Unknown	DY475159
-1.02	0.05	CA	Chloroplast DNA for P700 chlorophyll a-apoprotein	DY475501
-1.01	0.20	LS	Polyubiquitin	DY396410
-1.01	0.50	CA	60S ribosomal protein L11	DY475395
-1.00	0.17	CA	Xylosidase	DY475408
-0.99	0.30	CA	GTP-binding protein	DY475290
-0.99	0.37	CA	Serine:glyoxylate aminotransferase/ alanine:glyoxylate aminotransferase	DY475479
-0.97	0.23	LS	Disease resistance response protein 39 precursor	DY396277
-0.97	0.10	LS	Poly(A)-binding protein	DY396412
-0.96	0.33	CA	4-alpha-glucanotransferase	DY475302
-0.96	0.23	LS	RAC-GTP binding protein-like	DY396336
-0.96	0.25	CA	Unknown	DY475185
-0.95	0.26	CA	Unknown	EB085039
-0.95	0.25	CA	40S ribosomal protein S11	DY475258
-0.94	0.16	CA	60S ribosomal protein L34	DY475201
-0.94	0.36	CA	Unknown	DY475340
-0.94	0.28	CA	Unknown	DY475461
-0.93	0.35	LS	Putative tonoplast intrinsic protein	DY396419
-0.93	0.15	LS	Polyubiquitin	DY396354
-0.91	0.15	CA	Chloroplast 30S ribosomal protein S12	DY475063
-0.91	0.16	CA	Chloroplast 16S rRNA	DY475101
-0.89	0.24	CA	Zinc-binding dehydrogenase	DY475500
-0.86	0.26	CA	Proline oxidase	DY475225
-0.86	0.19	CA	Carbonic anhydrase like protein	DY475403
-0.86	0.21	LS	Guanine nucleotide regulatory protein	DY396313
-0.85	0.27	CA	Unknown	DY475536
-0.84	0.22	CA	Chlorophyll a/b binding protein	DY475555
-0.82	0.18	LS	Protein kinase-like protein	DY396382
-0.81	0.37	CA	Unclear	DY475473
-0.80	0.20	LS	Cytochrome B5	DY396340
-0.76	0.45	LS	Glycine-rich cell wall protein GRP 1.8	DY396342
-0.76	0.27	CA	Unknown	EB085041

		-0.75	0.37	LS	Polyubiquitin	DY396328
		-0.75	0.34	CA	Caffeoyl-CoA-Methyltransferase	CV793595
		-0.74	0.39	CA	Cytochrome P450	DY475449

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Time-point	Condition	Fold change	95% +/-	Clone source	Putative function	GenBank accession
03 hpt	IC Up	1.12	0.38	CA	Pathogenesis-related protein 4A	CV793597
		0.77	0.25	CA	Glutathione S-transferase	DY475250
	LA Up	3.92	0.14	LS	Ripening-related protein	DY396347
		3.73	0.14	LS	Ripening-related protein	DY396344
		3.47	0.10	CA	Dehydrin cold-induced protein	DY475092
		2.88	0.21	LS	Lectin-like protein	DY396427
		2.87	0.22	LS	Ripening-related protein	DY396377
		2.48	0.12	CA	Unknown	DY475353
		1.95	0.11	CA	Photosystem II reaction centre I protein	DY475116
		1.76	0.56	LS	Thioredoxin H-type 1	DY396330
		1.63	0.09	LS	Pathogenesis-related protein	DY396343
		1.51	0.34	LS	Pathogenesis-related protein	DY396301
		1.46	1.18	LS	Disease resistance response protein DRRG49-C	DY396265
		1.44	0.12	CA	Chloroplast DNA	EB085050
		1.35	0.59	CA	Carbonic anhydrase like protein	DY475403
		1.28	0.14	CA	Aquaporin 2 protein	DY475512
		1.24	0.48	LS	Glutathione peroxidase	DY396331
		1.20	0.36	CA	Unknown	DY475084
		1.18	0.41	CA	Pathogenesis-related protein 4A	CV793597
		1.17	0.11	LS	Dehydrin-cognate	DY396324
		1.13	0.13	CA	Beta-galactosidase	DY475141
		1.07	0.04	CA	Unknown	DY475338
		1.01	0.02	CA	Unclear	DY475259
		0.94	0.09	LS	Putative auxin-repressed protein	DY396359
		0.77	0.27	LS	Xyloglucan endotransglycosylase LEXET2	DY396308
		0.43	0.62	CA	5.8S, 18S and 25S rRNA	EB085027
	FL Up	3.05	0.16	LS	Ripening-related protein	DY396344
		2.90	0.28	LS	Ripening-related protein	DY396347
		2.62	0.24	LS	Ripening-related protein	DY396377
		2.18	0.10	LS	Thioredoxin H-type 1	DY396330
		1.44	0.07	LS	Disease resistance response protein DRRG49-C	DY396265
		1.42	0.06	LS	Pathogenesis-related protein	DY396301
		1.40	0.32	CA	Dehydrin cold-induced protein	DY475092
		1.32	0.21	LS	Lectin-like protein	DY396427
		1.16	0.08	CA	Disease resistance response protein DRRG49-C	EB085032
		1.05	0.10	LS	Pathogenesis-related protein 4A	DY396372
		0.97	0.21	CA	Beta-galactosidase	EB085056
		0.95	0.14	LS	Dehydrin-cognate	DY396324
		0.83	0.24	LS	Alpha-amylase precursor	DY396337
		0.82	0.27	CA	Chlorophyll a/b binding protein	DY475555
		0.81	0.28	CA	Beta-galactosidase	DY475141
		0.80	0.30	CA	Carbonic anhydrase like protein	DY475403
		0.75	0.34	LS	Senescence-associated protein DIN1	DY396338
		0.71	0.48	LS	Small GTP-binding protein	DY396367
		0.50	0.67	LS	Glutathione peroxidase	DY396331
	IC Down	-5.03	0.88	LS	RAC-GTP binding protein-like	DY396336

	-4.48	0.79	CA	Unknown	DY475451
	-4.04	0.47	CA	Squalene epoxidase	DY475199
	-4.00	0.57	CA	Unknown	DY475513
	-3.45	0.57	CA	Unclear	DY475323
	-3.28	0.55	LS	Environmental stress inducible protein	DY396298
	-3.28	1.48	CA	Hypothetical transmembrane protein	DY475478
	-3.20	0.73	CA	Unclear	DY475205
	-2.79	0.58	CA	Unknown	DY475080
	-2.54	0.63	CA	Unclear	DY475313
	-2.07	0.32	CA	Unknown	DY475177
	-2.05	0.70	CA	NADH-ubiquinone oxidoreductase	DY475294
	-2.04	0.89	CA	Unknown	DY475289
	-1.90	0.40	CA	Unknown	DY475263
	-1.82	0.48	CA	Cytochrome C oxidase subunit	DY475113
	-1.57	0.19	CA	Unclear	DY475400
	-1.50	0.33	CA	Unclear	DY475319
	-1.45	0.35	CA	SNAKIN2 antimicrobial peptide precursor	CV793606
	-1.44	0.25	CA	Chloroplast DNA for P700 chlorophyll a-apoprotein	DY475501
	-1.41	0.30	CA	Asparagine synthetase	DY475475
	-1.23	0.83	CA	Photosystem I reaction centre subunit IX	DY475047
	-1.08	0.45	CA	Unclear	DY475546
	-0.98	0.12	LS	Protein kinase precursor-like	DY396264
	-0.98	0.15	CA	Unknown	DY475187
	-0.95	0.06	CA	ATP synthase	DY475082
	-0.89	0.17	CA	Unknown	DY475185
	-0.84	0.18	LS	Putative ARF1 GTPase activating protein	DY396291
	-0.81	0.54	LS	Pathogenesis-related protein 4A	DY396388
LA Down	-6.50	1.62	CA	Unknown	EB085049
	-5.46	0.60	CA	Unknown	DY475080
	-5.41	0.24	CA	Protein kinase	DY475077
	-4.87	1.75	LS	Beta-glucan binding protein	DY396299
	-4.28	1.65	CA	Hypothetical membrane spanning ring-H2 finger protein	DY475508
	-4.28	0.61	CA	Unclear	DY475205
	-4.23	0.99	LS	Subtilisin inhibitors I and II	DY396374
	-4.22	0.12	CA	Chloroplast 16S rRNA	DY475101
	-4.03	0.43	CA	18S nuclear rRNA	DY475150
	-3.71	0.64	CA	Hypothetical transmembrane protein	DY475478
	-3.59	1.01	CA	Unclear	DY475367
	-3.53	0.16	CA	Unknown	DY475451
	-3.47	0.51	LS	18.2 KDA class I heat shock protein	DY396282
	-3.47	0.12	CA	Unknown	DY475483
	-3.37	0.97	CA	Squalene epoxidase enzyme	DY475199
	-3.31	0.10	CA	Membrane sugar-transport protein	DY475239
	-3.21	0.57	CA	Unknown	DY475513
	-3.12	0.57	CA	Cytochrome C oxidase subunit	DY475113
	-2.95	0.49	LS	Putative membrane related protein	DY396429
	-2.94	0.95	LS	Transcription factor NTLIM1	DY396263
	-2.70	0.71	CA	Beta adaptin like protein	DY475424
	-2.67	1.18	CA	WD repeat protein	DY475550
	-2.63	0.95	CA	Unknown	DY475055
	-2.47	0.09	CA	Phosphate-induced protein	EB085042
	-2.43	0.26	CA	Unknown	EB085052
	-2.27	0.28	CA	Unknown	DY475206
	-2.23	0.54	CA	UDP-glucose 4-epimerase	DY475221
	-2.21	0.04	CA	Endoxyloglucan transferase	DY475207
	-2.19	1.24	CA	Unknown	DY475437

	-2.19	0.87	CA	Unknown	DY475289
	-2.17	0.39	CA	Amino acid transferase	DY475122
	-2.12	0.21	CA	Nodulin 21 protein	DY475200
	-2.08	0.29	CA	Unknown	EB085024
	-1.99	0.14	CA	Unknown	DY475177
	-1.95	0.31	CA	Unknown	EB085053
	-1.91	0.20	CA	Phosphate-induced protein	DY475172
	-1.90	0.45	CA	Similar to alpha galactosidase	DY475286
	-1.89	0.64	CA	Similar to endopeptidase	DY475396
	-1.89	0.74	CA	Unknown	DY475446
	-1.85	0.49	CA	Similarity to protein-tyrosine-kinase receptor	DY475463
	-1.77	0.88	CA	Unknown	EB085062
	-1.75	0.64	CA	Unknown	DY475260
	-1.69	0.25	CA	Unknown	EB085026
	-1.69	0.82	CA	Unknown	DY475171
	-1.68	0.46	CA	Cytochrome F	DY475180
	-1.67	0.08	CA	Unknown	EB085021
	-1.66	0.15	CA	Unclear	DY475313
	-1.65	0.78	CA	Mitochondrial 26S rRNA	DY475087
	-1.58	0.37	CA	Unknown	DY475263
	-1.57	0.12	CA	Membrane-related protein CP5	DY475119
	-1.49	0.30	LS	Putative senescence-associated protein	DY396273
	-1.47	0.31	CA	Unknown	DY475315
	-1.46	0.49	LS	NADH dehydrogenase	DY396279
	-1.44	0.64	LS	Auxin-responsive protein IAA9	DY396315
	-1.41	0.41	CA	Nucleotide-sugar dehydratase	DY475244
	-1.39	0.15	CA	Unknown	EB085016
	-1.37	0.33	CA	Probable 3-hydroxyisobutyrate dehydrogenase mitochondrial precursor	DY475417
	-1.29	0.45	CA	Dehydration-induced protein	DY475070
	-1.28	0.52	CA	Unclear	DY475526
	-1.26	0.76	CA	GPI-anchored membrane protein	DY475246
	-1.21	0.06	CA	NADH-plastoquinone oxidoreductase	DY475287
	-1.13	0.68	CA	Unknown	DY475062
	-1.09	0.13	CA	Unknown	EB085061
	-1.05	0.20	CA	Unknown	DY475364
	-1.04	1.29	CA	Unclear	DY475217
	-1.02	0.03	CA	Unknown	DY475048
	-0.97	0.50	LS	RAC-GTP binding protein-like	DY396336
	-0.96	1.26	CA	Unknown	EB085064
	-0.96	0.14	CA	Unknown	DY475051
	-0.95	0.09	CA	Unknown	DY475130
	-0.94	0.26	CA	DNA binding protein	DY475266
	-0.93	0.18	CA	Unknown	DY475336
	-0.91	0.74	CA	Cyclic ion channel protein	DY475468
	-0.82	0.19	CA	Unknown	DY475519
	-0.67	0.32	CA	Chlorophyll a/b binding protein	DY475534
	-0.67	0.94	CA	Mitochondrial glyoxylase	DY475321
	-0.46	0.66	CA	Unclear	DY475522
FL Down	-3.24	1.06	CA	Formyltetrahydrofolate deformylase	DY475493
	-3.13	1.48	LS	Protein kinase C inhibitor-like protein	DY396285
	-2.91	0.25	CA	Unknown	DY475521
	-2.84	0.12	LS	RAC-GTP binding protein-like	DY396336
	-2.80	0.20	CA	Unknown	DY475048
	-2.63	0.91	CA	Superoxide dismutase copper chaperone precursor	DY475397
	-2.09	0.74	CA	Cytochrome C oxidase subunit	DY475113
	-1.98	0.25	CA	Unknown	DY475536

		-1.93	0.31	LS	Environmental stress inducible protein	DY396298
		-1.91	0.55	CA	Protein kinase	DY475077
		-1.64	0.34	CA	ATP synthase	DY475245
		-1.60	0.58	CA	Chloroplast 30S ribosomal protein S12	DY475063
		-1.42	0.57	CA	DNA directed RNA polymerase	DY475419
		-1.41	0.36	CA	Fructose-1,6-bisphosphate aldolase	DY475547
		-1.39	0.01	LS	Laccase-like protein	DY396358
		-1.33	0.34	LS	18.2 KDA class I heat shock protein	DY396282
		-1.20	0.24	LS	Kinesin-like protein	DY396287
		-1.18	0.18	LS	Putative ARF1 GTPase activating protein	DY396291
		-1.18	0.14	LS	Nucleic acid binding protein-like	DY396266
		-1.17	0.40	CA	Unclear	DY475531
		-1.11	0.19	LS	Poly(A)-binding protein	DY396412
		-1.11	0.25	CA	NADH-plastoquinone oxidoreductase	DY475287
		-1.10	0.39	CA	Similarity to protein-tyrosine-kinase receptor (EC 2.7.1.112)	DY475463
		-1.07	0.30	CA	Unknown	DY475315
		-1.05	0.30	CA	Unclear	DY475217
		-1.04	0.47	CA	Unclear	DY475409
		-1.03	0.37	CA	Unknown	DY475206
		-1.03	0.62	LS	Pathogenesis-related protein 4A	DY396281
		-1.02	0.34	CA	Unknown	EB085025
		-1.01	0.33	CA	4.5S, 5S, 16S and 23S rRNA	EB085066
		-1.01	0.57	CA	Trehalose-phosphatase	DY475282
		-0.99	0.23	CA	Glutamate dehydrogenase	DY475308
		-0.98	0.33	CA	Unclear	DY475355
		-0.97	0.29	CA	Unknown	EB085024
		-0.96	0.41	CA	Apocytochrome F	DY475181
		-0.96	0.32	CA	Unknown	DY475055
		-0.95	0.28	CA	SNAKIN2 antimicrobial peptide precursor	CV793608
		-0.94	0.33	CA	Anthranilate phosphoribosyltransferase	DY475507
		-0.90	0.20	CA	Cyclic ion channel protein	DY475468
		-0.89	0.25	CA	Peptidase-like protein	DY475387
		-0.87	0.20	LS	Auxin-responsive protein IAA9	DY396315
		-0.86	0.24	CA	Unknown	DY475349
		-0.84	0.26	CA	Mitochondrial glyoxylase	DY475321
		-0.81	0.23	CA	GPI-anchored membrane protein	DY475246
		-0.79	0.22	CA	Unknown	DY475299
		-0.79	0.29	CA	Unknown	EB085062
		-0.78	0.27	CA	Unknown	DY475437
		-0.77	0.26	CA	Unknown	DY475390
		-0.70	0.33	CA	UDP-glucose 4-epimerase	DY475221
27 hpt	IC Up	3.84	0.08	LS	Putative chitinase	DY396275
		3.28	0.18	CA	Multi-resistance ABC transporter protein	CV793605
		3.20	0.08	CA	Unknown	DY475288
		2.91	0.10	CA	Phosphate-induced protein	DY475076
		2.77	0.09	LS	Beta-glucan binding protein	DY396299
		2.29	0.16	LS	Xyloglucan endotransglycosylase LEXET2	DY396308
		2.13	0.10	CA	Unclear	EB085058
		2.09	0.42	CA	Glutathione S-transferase	DY475250
		2.08	0.05	CA	Unknown	DY475451
		2.00	0.09	CA	Unknown	DY475521
		1.92	0.15	CA	Serine/threonine protein kinase	DY475384
		1.82	0.16	LS	Pathogenesis-related protein 4A	DY396372
		1.70	0.11	LS	Pathogenesis-related protein	DY396301
		1.63	0.35	CA	Asparagine synthetase	DY475477
		1.60	0.14	LS	Disease resistance response protein DRRG49-C	DY396265

	1.52	0.06	CA	Unclear	DY475329
	1.47	0.10	CA	5.8S, 18S and 25S rRNA	EB085027
	1.28	0.14	CA	Unclear	DY475205
	1.20	0.09	LS	ER66 protein/calmodulin-like protein	DY396364
	1.09	0.11	LS	Calmodulin-like protein/ER66	DY396411
	0.96	0.12	CA	Putative disease resistance protein	CV793593
LA Up	4.00	0.18	LS	Glutathione S-transferase	DY396404
	3.98	0.51	CA	Glutathione S-transferase	DY475250
	3.61	0.34	LS	Pathogenesis-related protein 4A	DY396281
	3.58	0.16	LS	Pathogenesis-related protein 4A	DY396372
	3.51	0.16	LS	Pathogenesis-related protein 4A	DY396384
	3.34	0.94	CA	Pathogenesis-related protein 4A	CV793597
	3.30	0.10	LS	Pathogenesis-related protein 4A	DY396388
	3.09	0.24	CA	Phosphate-induced protein	DY475076
	3.01	0.46	CA	Unclear	EB085058
	2.90	0.37	LS	Disease resistance response protein DRRG49-C	DY396265
	2.72	0.37	LS	Pathogenesis-related protein	DY396301
	2.72	0.23	CA	Unknown	DY475288
	2.57	0.60	LS	Pathogenesis-related protein	DY396305
	1.80	0.38	CA	Ferredoxin	DY475487
	1.76	0.85	CA	Class 10 pathogenesis related protein	CV793610
	1.66	0.62	CA	Unknown	EB085020
FL Up	3.31	0.78	CA	Glutathione S-transferase	DY475250
	3.31	0.24	LS	Glutathione S-transferase	DY396404
	3.29	0.11	CA	Multi-resistance ABC transporter protein	CV793605
	2.53	0.35	CA	Wound-induced protein	DY475254
	2.52	0.36	CA	Unclear	EB085058
	2.52	0.56	CA	Glutamate dehydrogenase	DY475308
	2.36	0.43	LS	Pathogenesis-related protein 4A	DY396281
IC Down	-4.55	0.12	LS	Protein kinase precursor-like	DY396264
	-4.14	0.41	CA	Probable 3-hydroxyisobutyrate dehydrogenase mitochondrial precursor	DY475417
	-4.00	0.70	LS	Putative glutaredoxin	DY396317
	-3.55	0.21	LS	Putative membrane related protein	DY396429
	-3.28	0.44	CA	Unclear	DY475259
	-3.20	0.10	LS	Protein kinase C inhibitor-like protein	DY396283
	-3.15	0.12	LS	Putative protein kinase	DY396375
	-3.14	0.48	CA	Formyltetrahydrofolate deformylase	DY475493
	-3.10	0.31	CA	Unclear	DY475313
	-3.02	0.25	LS	Cornifin alpha (small proline-rich protein)	DY396399
	-2.97	0.43	CA	Sorting nexin protein	DY475523
	-2.89	0.71	LS	TMV resistance protein-like	DY396385
	-2.84	0.46	CA	Unknown	DY475279
	-2.83	0.53	CA	Chlorophyll mRNA	EB085059
	-2.74	0.47	LS	Auxin-responsive protein IAA9	DY396315
	-2.67	0.36	LS	Putative protein kinase	DY396351
	-2.60	0.78	CA	L-allo-threonine aldolase	DY475068
	-2.55	0.34	CA	Squalene epoxidase	DY475199
	-2.54	0.89	CA	Unclear	DY475175
	-2.45	0.21	LS	Ca-binding carrier protein	DY396262
	-2.43	0.19	CA	Unclear	DY475409
	-2.43	0.37	CA	Similar to endopeptidase	DY475396
	-2.43	0.56	CA	NADH-plastoquinone oxidoreductase	DY475287
	-2.36	0.21	CA	Unknown	DY475431
	-2.23	0.59	CA	Chloroplast DNA for P700 chlorophyll a-apoprotein	DY475501

-2.18	0.42	LS	Amine oxidase	DY396386
-2.17	0.18	LS	Laccase-like protein	DY396358
-2.07	0.27	CA	Hypothetical transmembrane protein	DY475478
-2.01	0.36	CA	Unclear	DY475546
-1.94	0.23	LS	*Cf9* resistance gene cluster	DY396352
-1.89	1.12	CA	Serine/threonine protein kinase	DY475320
-1.83	0.56	CA	Unknown	DY475533
-1.78	0.47	LS	Splicing factor-like protein	DY396290
-1.69	0.75	CA	Chloroplast DNA between RUBISCO large subunit and ATPase (beta) genes	DY475518
-1.62	0.36	CA	Unclear	DY475323
-1.62	0.33	CA	Unclear	DY475444
-1.60	0.41	CA	ATP synthase	DY475082
-1.59	0.47	CA	Unknown	DY475253
-1.52	0.32	CA	18S rRNA	DY475542
-1.51	0.36	LS	Caffeoyl-CoA-Methyltransferase	DY396415
-1.51	0.25	CA	Fructose-1,6-bisphosphate aldolase	DY475547
-1.48	0.17	LS	Environmental stress inducible protein	DY396298
-1.44	0.18	CA	NADH dehydrogenase	DY475316
-1.36	0.90	CA	ATP synthase	DY475245
-1.35	0.20	CA	Unclear	DY475284
-1.35	0.53	CA	Protein containing leucine-zipper motif	CV793599
-1.35	0.24	LS	Thioredoxin	DY396293
-1.32	0.32	LS	Heat shock protein DnaJ homolog	DY396397
-1.31	0.12	CA	Beta-galactosidase	DY475141
-1.27	0.44	CA	Unclear	DY475217
-1.24	0.19	LS	Senescence-associated protein DIN1	DY396338
-1.23	0.38	CA	Membrane sugar-transport protein	DY475239
-1.17	0.42	CA	Unknown	DY475048
-1.16	0.45	CA	Xylosidase	DY475408
-1.15	0.06	LS	Histone H2A	DY396268
-1.13	0.41	CA	Phosphate-induced protein	EB085042
-1.11	0.45	CA	Cytochrome F	DY475180
-1.11	0.27	CA	Fructose-1,6-bisphosphatase	DY475543
-1.11	0.19	LS	Putative auxin-repressed protein	DY396379
-1.09	0.10	CA	Chlorophyll a/b binding protein	DY475534
-1.08	0.34	CA	DNA directed RNA polymerase	DY475419
-1.04	0.80	CA	Unknown	DY475513
-1.01	0.15	LS	Poly(A)-binding protein	DY396412
-1.00	0.39	CA	Unknown	DY475293
-0.99	0.34	CA	Unknown	DY475171
-0.99	0.19	CA	Unknown	DY475338
-0.98	0.14	CA	Unknown	EB085044
-0.98	0.13	LS	Transcription factor NTLIM1	DY396263
-0.94	0.16	CA	Unknown	DY475191
-0.94	0.48	CA	Unknown	DY475206
-0.94	0.27	LS	Ripening-related protein	DY396377
-0.93	0.18	CA	Wound-induced protein	DY475220
-0.92	0.23	CA	Unknown	EB085024
-0.92	0.16	CA	RNA binding protein	DY475297
-0.91	0.23	CA	Unknown	DY475260
-0.91	0.27	CA	Unknown	DY475055
-0.90	0.17	CA	S1-3 homolog	CV793591
-0.88	0.30	CA	Anthranilate phosphoribosyltransferase	DY475507
-0.88	0.14	CA	Chloroplast DNA	EB085054
-0.88	0.30	CA	Unclear	DY475099
-0.86	0.23	LS	Protein kinase C inhibitor-like protein	DY396285
-0.86	0.22	LS	Kinesin-like protein	DY396287

	-0.81	0.44	CA	Unknown	EB085052
	-0.81	0.27	CA	Unknown	DY475062
	-0.79	0.33	CA	Unclear	DY475526
	-0.75	0.34	CA	Superoxide dismutase copper chaperone precursor	DY475397
LA Down	-4.17	1.09	CA	Unknown	DY475539
	-4.09	1.60	CA	26S ribosomal RNA	DY475153
	-4.08	1.34	CA	Unknown	DY475051
	-3.91	0.68	CA	ATP synthase	DY475082
	-3.71	1.00	CA	L-allo-threonine aldolase	DY475068
	-3.65	1.02	CA	Unclear	DY475313
	-2.96	0.90	LS	RAC-GTP binding protein-like	DY396336
	-2.84	0.92	CA	18S nuclear rRNA	DY475150
	-2.40	0.36	LS	Protein kinase-like protein	DY396362
	-2.10	0.10	CA	Unknown	EB085021
	-1.96	1.01	CA	Unknown	DY475263
	-1.95	0.27	CA	Unknown	DY475159
	-1.93	0.31	CA	Unknown	DY475347
	-1.89	0.24	CA	26S rRNA	DY475211
	-1.82	0.32	CA	Unknown	EB085024
	-1.81	0.54	CA	Chloroplast 16S rRNA	DY475101
	-1.81	0.41	CA	Unknown	DY475055
	-1.70	1.45	LS	Putative membrane related protein	DY396429
	-1.69	0.21	CA	26S rRNA	DY475540
	-1.67	0.28	CA	18S rRNA	DY475542
	-1.65	0.36	CA	Unknown	EB085063
	-1.63	0.30	CA	26S rRNA	EB085055
	-1.54	0.29	CA	Chlorophyll a/b binding protein	DY475534
	-1.52	0.47	CA	Membrane-related protein CP5	DY475119
	-1.42	0.13	CA	Unknown	DY475451
	-1.41	0.19	CA	Unclear	DY475274
	-1.40	0.67	CA	Phosphate-induced protein	EB085042
	-1.39	0.06	CA	Unclear	DY475367
	-1.31	0.83	CA	Chlorophyll mRNA	EB085059
	-1.30	0.25	LS	Similarity to RNA-binding protein	DY396387
	-1.24	0.08	CA	Unknown	EB085049
	-1.23	0.52	CA	Chloroplast Val-tRNA	DY475537
	-1.15	0.23	CA	Cytochrome C biogenesis protein ccsA	DY475393
	-1.14	0.65	CA	Unknown	DY475533
	-1.12	0.40	LS	Protein kinase C inhibitor-like protein	DY396285
	-0.97	0.49	LS	Transcription initiation protein SPT4	DY396309
	-0.94	0.24	CA	Unknown	DY475236
	-0.75	0.41	LS	*Cf9* resistance gene cluster	DY396352
FL Down	-5.51	0.55	CA	Protein containing leucine-zipper motif	CV793599
	-3.55	1.05	CA	Unknown	DY475051
	-3.39	0.55	CA	Unknown	DY475125
	-3.36	0.41	CA	Chloroplast DNA for P700 chlorophyll a-apoprotein	DY475501
	-3.00	1.28	CA	Unknown	DY475253
	-2.91	0.47	CA	26S rRNA	DY475211
	-2.88	0.29	LS	Putative senescence-associated protein	DY396273
	-2.83	0.61	CA	Cytochrome C biogenesis protein ccsA	DY475393
	-2.80	0.90	LS	Dehydration stress-induced protein	DY396321
	-2.63	0.47	LS	Putative protein kinase	DY396351
	-2.58	0.62	LS	Putative protein kinase	DY396375
	-2.57	0.63	CA	Unknown	EB085049
	-2.48	0.66	CA	Unknown	EB085063
	-2.45	1.50	CA	Homogentisate 1,2 dioxygenase	DY475551

		-2.39	0.25	CA	Unknown	DY475431
		-2.15	1.25	LS	Alpha-amylase	DY396402
		-2.12	0.77	CA	Unknown	DY475293
		-2.08	0.88	CA	Unknown	EB085021
		-2.03	0.24	CA	Unclear	DY475522
		-2.02	0.17	CA	Chloroplast Val-tRNA	DY475537
		-1.93	0.34	CA	Unclear	DY475095
		-1.84	0.83	LS	Protein kinase C inhibitor-like protein	DY396283
		-1.71	0.43	LS	Serine carboxypeptidase isolag	DY396280
		-1.71	0.86	CA	Unclear	DY475546
		-1.54	0.32	CA	Unknown	DY475048
		-1.51	0.14	CA	Chloroplast 4.5S, 5S, 16S and 23S mRNA	DY475545
		-1.49	0.31	LS	Cornifin alpha (small proline-rich protein)	DY396399
		-1.46	0.11	CA	Unknown	DY475462
		-1.42	0.39	CA	Amino acid transferase	DY475122
		-1.42	0.03	CA	Chloroplast 16S rRNA	DY475101
		-1.36	0.30	CA	Unknown	DY475536
		-1.36	0.16	CA	26S rRNA	EB085013
		-1.31	0.59	CA	Unclear	DY475313
		-1.27	0.27	CA	Unknown	DY475236
		-1.21	0.40	CA	Unknown	DY475338
		-1.19	0.48	LS	Protein kinase C inhibitor-like protein	DY396285
		-1.18	0.12	CA	Unknown	DY475539
		-1.17	0.35	CA	Unknown	EB085064
		-1.14	0.55	CA	Sorting nexin protein	DY475523
		-1.12	0.23	LS	Caffeoyl-CoA-Methyltransferase	DY396415
		-0.91	0.60	CA	Phosphate-induced protein	DY475172
		-0.89	0.30	LS	Serine/threonine protein kinase	DY396307
		-0.89	0.36	CA	Chlorophyll mRNA	EB085059
		-0.80	0.50	CA	Phosphate-induced protein	EB085042
		-0.77	0.44	LS	ER66 protein/calmodulin binding protein	DY396364

MeJA

Time-point	Condition	Fold change	95% +/-	Clone source	Putative function	GenBank accession
03 hpt	IC Up	1.18	0.25	CA	SNAKIN2 antimicrobial peptide precursor	CV793608
		1.16	0.06	LS	Pathogenesis-related protein 4A	DY396281
		1.07	0.45	CA	Pathogenesis-related protein 4A	CV793597
		0.89	0.15	CA	Unclear	EB085058
	LA Up	1.88	0.15	CA	Glutathione S-transferase	DY475250
		1.30	0.31	CA	Unknown	DY475401
		1.17	0.23	CA	Unclear	EB085058
		0.98	0.17	CA	Phosphate-induced protein	DY475076
		0.98	0.11	CA	Unknown	DY475485
		0.84	0.23	CA	Unclear	DY475186
		0.80	0.64	CA	18S rRNA	DY475542
	FL Up	1.41	0.11	LS	Ripening-related protein	DY396344
		1.38	0.08	LS	Thioredoxin H-type 1	DY396330
		1.26	0.15	LS	Cytochrome B5	DY396340
		0.96	0.17	LS	Histone H2A	DY396268
		0.72	0.28	LS	Ripening-related protein	DY396347
	IC Down	-1.34	0.38	LS	Cytochrome B5	DY396340
		-1.21	0.18	LS	Putative auxin-repressed protein	DY396269

		-1.20	0.19	LS	Dehydrin-cognate	DY396324
		-1.19	0.33	LS	Disease resistance response protein 39 precursor	DY396296
		-1.17	0.04	LS	Putative senescence-associated protein	DY396273
		-1.13	0.24	CA	Chloroplast DNA	EB085050
		-1.12	0.15	LS	Putative auxin-repressed protein	DY396359
		-1.10	0.22	LS	Putative nuclear transport factor 2	DY396436
		-1.05	0.10	LS	Thioredoxin H-type 1	DY396330
		-1.05	0.06	CA	Xylosidase	DY475408
		-1.04	0.16	CA	Photosystem I assembly protein ycf3	DY475345
		-1.02	0.12	CA	Chloroplast 16S rRNA	DY475146
		-1.00	0.24	CA	Unknown	DY475339
		-1.00	0.17	CA	Photosystem I reaction centre subunit IX	DY475047
		-0.99	0.14	CA	Beta-galactosidase	EB085056
		-0.99	0.12	LS	NADH dehydrogenase	DY396279
		-0.98	0.13	LS	Polyubiquitin	DY396354
		-0.94	0.20	LS	Putative auxin-repressed protein	DY396292
		-0.94	0.14	LS	Putative auxin-repressed protein	DY396289
		-0.92	0.13	LS	Poly(A)-binding protein	DY396412
		-0.89	0.24	CA	Unclear	DY475175
		-0.82	0.18	CA	NADH-plastoquinone oxidoreductase	DY475556
		-0.70	0.34	LS	Alpha-amylase precursor	DY396337
	LA Down	-1.68	0.37	LS	Putative senescence-associated protein	DY396273
		-1.52	0.19	CA	Unknown	DY475084
		-1.43	0.60	CA	Xylosidase	DY475408
		-1.38	0.37	LS	Protein kinase-like protein	DY396362
		-1.37	0.38	LS	*Cf9* resistance gene cluster	DY396352
		-1.20	0.13	CA	Fructose-1,6-bisphosphatase	DY475543
		-1.18	0.39	LS	Laccase-like protein	DY396358
		-1.13	0.17	LS	Pathogenesis-related protein 4A	DY396281
		-0.97	0.33	CA	Probable 3-hydroxyisobutyrate dehydrogenase mitochondrial precursor	DY475417
		-0.95	0.17	CA	Unclear	DY475175
		-0.93	0.34	LS	Ca-binding carrier protein	DY396262
		-0.74	0.26	LS	Putative membrane related protein	DY396429
		-0.69	0.34	CA	Unknown	DY475177
	FL Down	-1.11	0.06	CA	Auxin repressed protein	DY475137
		-0.94	0.10	CA	Chloroplast DNA	EB085019
		-0.93	0.22	CA	NADH-plastoquinone oxidoreductase	DY475287
		-0.93	0.07	CA	Class 10 pathogenesis related protein	CV793610
		-0.86	0.23	CA	Unclear	DY475274
27 hpt	IC Up	2.27	0.29	CA	Oxygen splitting enhancer of photosystem II	DY475060
		1.96	0.56	CA	Unknown	EB085053
		1.53	0.48	CA	Unknown	DY475160
		1.22	0.70	CA	SNAKIN2 antimicrobial peptide precursor	CV793608
		1.00	0.37	CA	Adenosylhomocysteinase	DY475372
		0.94	0.06	CA	Unknown	DY475401
		0.92	0.20	CA	Carbonic anhydrase like protein	DY475403
		0.76	0.39	CA	Unknown	DY475472
	LA Up	0.93	0.17	CA	Carbonic anhydrase like protein	DY475403
		0.82	0.18	CA	Unknown	DY475191
	FL Up	1.54	0.07	CA	ATP synthase	DY475423
		1.48	0.03	CA	DnaJ-like protein	DY475488
		1.13	0.04	CA	Unknown	DY475293

	0.70	0.40	CA	Unknown	DY475191
IC Down	-4.58	0.82	LS	Nitrate transporter NRT1-1	DY396335
	-4.45	1.01	CA	Unknown	DY475364
	-4.41	1.20	CA	Probable 3-hydroxyisobutyrate dehydrogenase mitochondrial precursor	DY475417
	-4.16	0.54	CA	Unknown	EB085052
	-3.96	1.32	CA	Unclear	DY475273
	-3.85	0.83	CA	NADH-plastoquinone oxidoreductase	DY475287
	-3.58	1.50	LS	Putative senescence-associated protein	DY396273
	-3.56	0.32	CA	Unknown	DY475390
	-3.47	0.87	CA	Protein kinase	DY475077
	-3.00	0.46	CA	NADH dehydrogenase	DY475316
	-2.91	1.26	CA	Superoxide dismutase copper chaperone precursor	DY475397
	-2.89	0.39	CA	Peptidase-like protein	DY475387
	-2.68	0.98	CA	Apocytochrome F	DY475181
	-2.53	0.23	CA	Unknown	EB085021
	-2.32	0.68	CA	Unclear	DY475284
	-2.23	0.12	CA	26S rRNA	DY475211
	-2.15	0.36	CA	Unknown	DY475084
	-2.06	0.82	CA	Unknown	DY475283
	-1.91	0.37	CA	Unknown	DY475513
	-1.86	0.19	CA	Xylosidase	DY475408
	-1.79	0.18	CA	26S ribosomal RNA	DY475153
	-1.78	0.06	CA	Unknown	DY475539
	-1.70	0.15	CA	Unknown	DY475451
	-1.57	0.42	CA	Unknown	EB085062
	-1.56	0.16	CA	Unknown	EB085064
	-1.56	0.33	CA	Unknown	DY475336
	-1.52	0.74	CA	Chloroplast DNA	DY475541
	-1.47	0.41	CA	Cytochrome F	DY475180
	-1.47	0.75	CA	Unknown	DY475289
	-1.02	0.33	CA	Unknown	DY475178
	-0.95	0.64	CA	18S rRNA	EB085047
	-0.75	0.47	CA	Unclear	EB085048
	-0.64	0.72	CA	Chloroplast DNA	EB085050
	-0.54	0.79	CA	Cytochrome C oxidase subunit	DY475113
LA Down	-1.47	0.29	CA	Wound-induced protein	DY475220
	-1.17	0.35	LS	Putative auxin-repressed protein	DY396289
	-0.86	0.17	LS	Subtilisin inhibitors I and II	DY396374
FL Down	-1.10	0.12	CA	Chloroplast DNA	EB085019
	-1.00	0.03	LS	Putative auxin-repressed protein	DY396379
	-0.90	0.20	LS	Pathogenesis-related protein 4A	DY396372
	-0.87	0.21	LS	Putative auxin-repressed protein	DY396359
	-0.76	0.23	LS	Pathogenesis-related protein	DY396301