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FACULTY OF MEDICINE, HEALTH & LIFE SCIENCES

School of Biological Sciences

The Role of Membrane Proteins in the *Erisiphe cichoracearum/ Arabidopsis thaliana* interaction

By

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ABSTRACT

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THE ROLE OF MEMBRANE PROTEINS IN THE *ERISIPHE CICHORACEARUM / ARABIDOPSIS THALIANA* INTERACTION

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Powdery mildew fungi are biotrophic pathogens that infect a wide variety of economically important plants. When the fungus invades a plant it forms a complex interface between the host plant and the pathogen, known as the haustorial complex. The pathogen acts as an additional sink, competing with host sinks such as roots and flowers, resulting in considerable modification of photoassimilate production and partitioning between the host tissues. This study has investigated the transcriptional response of *Arabidopsis thaliana* to *E. cichoracearum* infection, using microarray analysis and quantitative real-time PCR. The focus has been on the role of membrane transporters during the infection process. It has been shown that the response to two different powdery mildews, *E. cichoracearum* and *E. orontii* is broadly similar. This study has confirmed that a number of transporter genes are up-regulated in response to powdery mildew infections, as well as several members of the *PDF1.2* family of defensins. Analysis by quantitative real-time PCR confirmed the microarray results for most of the genes investigated.

Homozygous insertional mutants were isolated in six membrane transporter genes (*ACA12*, *LHT*1, *GLR1.4*, *PTR3*, *ALA10* and *At2g18680*) and one invertase gene *AtcwINV1* showing regulation in response to *E. cichoracearum* infection. It was confirmed that no full-length cDNA for the respective genes was present in the mutants.

Mutants in *LHT1*, *AtcwINV1* and *GLR1.4* all displayed a difference in growth and development compared to wild-type plants, whereas for the other mutants, no marked differences were observed.

A new method for monitoring the development of *E. cichoracearum* on Arabidopsis was developed, based on measurements of individual powdery colonies. The growth of *E. cichoracearum* was investigated on all seven of the insertional mutants and was not found to be significantly different compared to wild-type plants.

The previously observed increased apoplastic concentrations of amino acids induced by *ptr3* mutants do not increase the growth of *E. cichoracearum* on Arabidopsis, and host systems which scavenge amino acids from the apoplasm probably do not contribute to powdery mildew resistance.

Interestingly, *ptr3* mutants did not show increased resistance to *E. cichoracearum*, as has previously been sown to other types of pathogens (necrotrophs).

In summary, this study demonstrates that several host-specific transporter genes, from a number of different membrane-transporter-gene families are induced as a result of powdery mildew infection. However, the progress of infection of *E. cichoracearum* is not significantly affected in Arabidopsis insertional mutants for these genes. This highlights the complexity and redundancy in the host response to *E. cichoracearum*.

Contents

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Abbreviations

AAP	Amino acid permease
AAT	Amino acid transporter
ABA	Abscisic acid
ABC	ATP-binding cassette
ABRE	ABA response element
ACA	Autoinhibited $Ca2^{+}$ ATPase
AFGC	Arabidopsis functional genome consortium
AGT	Appressorial germ tube
AHA	Arabidopsis H^{+}-ATPase
ALA	Aminophospholipid ATPase1
AMT	Arabidopsis membrane transporter
ANT	Neutral amino acid transporter
APC	Amino acid, polyamine and choline transporter
ARF	Auxin response factor
AROS	Array ready oligo set
At	Arabidopsis thaliana
ATF	Amino acid transporter family
ATPase	Adenosine triphosphate
ATPase	Adenosine triphosphatase
AUX	Auxin transporters
BLAST	Basic local alignment search tool
bp	Base pairs
CATMA	Complete Arabidopsis transcriptome microarray
cDNA	Complementary DNA
CNGC	Cyclic nucleotide gated channel
Ct	Cycle threshold
cwINV	Cell-wall invertase
DD	Differential display
dH2O	Distilled water
DiOC6	3,3'-dihexyloxacarbocyanin iodide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease

dNTP	Deoxynucleotidetriphosphate
DPI	Days post infection
DTT	Dithiothreitol
dUTP	Deoxyuridinetriphosphate
E	Efficiency
ECA	ER-type calcium ATPase
EDR	Enhanced disease resistance
EDS	Enhanced disease susceptibility
EDTA	Ethylene diamine tetra-acetic acid
EHM	Extrahaustorial membrane
EHMAT	Extrahaustorial matrix
EMBL	European molecular biology laboratory
ER	Endoplasmic reticulum
EST	Expressed sequence tag
ET	Ethylene
FLG	Flagellin
GA3	Gibberellic acid
GARE	Gibberellin acid responsive element
GEO	Gene expression omnibus
GFP	Green fluorescent protein
Glc	Glucose
GLR	Glutamate receptor
GO	Gene ontology
GST	Gene specific tag
GUS	β-glucuronidase
HC	Haustorial complex
HM	Haustorial membrane
HPI	Hours post infection
HR	Hypersensitive response
HRGP	Hydroxyproline-rich glycoprotein
HXT	Hexose transporter
iGluR	Inhibitory glutamate receptor
IPTG	Isopropyl-β-D-thiogalactoside
JA	Jasmonic acid

kb	Kilobase pairs
kDa	Kilo Daltons
LB	Luria Bertani
LHT	Lysine and histidine transporter
LIMS	Laboratory information management system
LOWESS	Locally weighted scatter plot smoothing
LPS	Lipopolysaccharide
MADS	MCM1, agamous, deficiens and SRF
MAMP	Microbe associated molecular pattern
MAP kinase	Mitogen activated protein kinase
MATDB	MIPS Arabidopsis thaliana database
MATE	Multidtrug and toxin efflux
MeJA	Methyl jasmonate
MES	2-(N-Morpholino) ethanesulfonic acid
MFS	Major facilitator superfamily
MIAME	Minimum information about microarray experiments
MJ	Methyl jasmonate
MOPS	3-(N-morpholino) propanesulphonic acid
MPK	Mitogen activated protein kinase 4
mRNA	Messenger RNA
MS	Murashige and Skoog
MST	Monosaccharide transporter
NaOH	Sodium hydroxide
NASC	Nottingham Arabidopsis stock centre
NCBI	National centre for biotechnology information
NDR	Non-race specific disease resistance
NO	Nitric oxide
NPR	Non-producer of PR1
NPTII	Neomycin phosphotransferase
OB	Oxidative burst
OD	Optical density
OMG	Oxy-methyl-D-glucose
OPT	Oligo-peptide transporter
PAD	Phtoalexin deficient

PCD	Programmed cell death
PCR	Polymerase chain reaction
PDF	Plant defencin
PGT	Primary germ tube
PhoA	Alkaline phosphatase
PM	Plasma membrane
PMA	Plasma membrane associated+C99
pmf	Proton motive force
PMR	Powdery mildew resistant
PR	Pathogenesis-related
ProTs	Proline transporters
PTR	Peptide transporter
rDNA	Ribosomal DNA
RLK	Receptor-like kinase
RLP	Receptor-like protein
RNA	Ribonucleic acid
RNAse	Ribonuclease
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
RT	Reverse transcriptase
S	Sulphur
SA	Salicylic acid
SAGE	Serial analysis of gene expression
SAIL	Syngenta Arabidopsis insertion library
SAR	Systemic acquired resistance
SDS	Sodium dodecyl sulphate
SEN	Senescence-associated gene
SIR	SAR independent resistance
SMD	Stanford microarray database
SNARE	Soluble N-ethylmaleimide-sensitive factor attachment protein receptors
SSC	Saline-sodium citrate
SSI	Suppressor of salicylate insensitivity of NPR1-5
STP:	Sugar transport protein

SUC	Sucrose transporter
TAE:	Tris/acetate/EDTA
TAIR	The Arabidopsis information resource
TE	Tris/EDTA
TF	Transcription factor
TMH	Transmembrane Helix
TPI	Triose phosphate isomerase
Tris	Tris(hydroxymethyl) aminomethane
UV	Ultra violet

Chapter 1

General Introduction

1.0 Background

Powdery mildews (Erysiphales) are an order of obligate biotrophic pathogenic plant fungi, current taxonomic classification - Eukaryota; Fungi; Ascomycota; Pezizomycotina; Leotiomycetes; Erysiphaceae (NCBI Taxonomy ID: 62708). However, the classification at both the family and genus level for species within the order has changed frequently over time, as new techniques such as electron microscopy and sequence analysis have provided more data (Matsuda & Takamatsu, 2003). Braun (2002) provides a recent and comprehensive discussion of the current and past taxonomy of the powdery mildews. *Erysiphe cichoracearum* has been reclassified as *Golovinomyces cichoracearum* by Braun *et al.* (2002), but in current literature and this work, it is still referred to as *E. cichoracearum.*

Powdery mildews have an enormous host range of nearly 10,000 angiosperm plants (Amano, 1986). Globally, powdery mildews are one of the most economically important plant pathogens (see Table 1.1 for a list of some of the economically important hosts), but precise information on the global losses caused by powdery mildews is scarce, due to the various logistical problems that exist, such as poor record keeping (Pinstrup-Andersen, 1999). However, the most current independent publication (Oerke *et al.*, 1994) suggests that of 8 economically important species (wheat, corn, rice, barley, soybean, cotton, potatoes and coffee), approximately 13% of attainable output was lost in the years 1988-1990 due to plant pathogens in general,. This loss was estimated at some $39 bn annually. Although varieties of crops resistant to powdery mildews have become available (Hsam & Zeller, 2002), information on the impact this has on overall economic losses is scarce (Pinstrup-Andersen, 1999) and resistance may be quickly overcome (Hsam & Zeller, 2002). Given that the losses of all plants that are due to powdery mildews probably surpasses any other single type of plant disease (Agrios, 1997), powdery mildews represent one of the largest individual parts of the economic loss due to all plant pathogens. These losses provide a strong economic imperative for both more effective and more cost-effective control measures, as well as a need to address concerns arising from the widespread application of toxic chemicals currently used to control powdery mildews. The understanding of disease mechanisms should increasingly contribute to the development of these measures.

The mycelium produced by powdery mildew grows only on the plant surface, never invading the plant tissue further than epidermal cells. However, the fungi do produce a specialised interface within the plant, called a haustorium. Formed in the epidermal cells of plants, it is thought that they are the site of the nutrient transfer which sustains the obligate biotroph (Manners & Gay, 1978; Spencer-Phillips & Gay, 1980) and reduces crop

Table 1.1 Powdery Mildews affect important crops. Examples of some economically important powdery mildew hosts, and their most common powdery mildew pathogen.

Host (common name)	Infecting Powdery Mildew (scientific name)
Wheat	*Blumeria graminis* f.sp. *tritici*
Barley	*B. graminis* f. sp. *hordei*
Pea	*Erysiphe pisi*
Cucumber	*Erysiphe cichoracearum*
	≡ *G. cichoracearum*
Endive	"
Lettuce	"
Melon	"
Potato	"
Pumpkin	"
Squash	"
Broccoli	*Erysiphe cruciferarum*
Brussels sprout	"
Cauliflower	"
Radish	"
Turnip	"
Rose	*Sphaerotheca pannosa* f. sp. *rosae*
Grape Vine	*Unicinula necator*
Tomato	*Erysiphe lycopersici*

yield. Typical examples of a powdery mildew infection are shown in Figure 1.1. As ascomycetes, powdery mildews have a varied life cycle in natural circumstances and this is exemplified by the rose powdery mildew, *Sphaerotheca pannosa* f. sp. *rosae* (Figure 1.2). The fungus produces a white mycelium on the surface of the plant tissues, sending haustoria into epidermal cells. Normally, reproduction is achieved by the production of 5 – 10 haploid conidia in chains, on short conidiophores (Agrios, 1997). Overwintering however, is achieved by overwintering mycelium which produces conidia in the spring, or by sexual reproduction of two hyphae producing a dikaryotic (N + N), as opposed to diploid (2N) cleistothecium. This matures by the spring to produce a small number of haploid ascospores per cleistothecium, which germinate and form the same white mycelium found at the start (Figure 1.2) (Agrios, 1997). Different species may preferentially use one form of overwintering over another, as the role of the cleistothecium in some species is controversial; some species fail to develop viable ascospores by the time a new season's host tissue becomes available for infection, a subject discussed by Jarvis *et al.* (2002). There is little published evidence on how *E. cichoracearum* (the powdery mildew in use in this study) overwinters, but it will depend on the climate in which the host is grown, as the reproduction method of powdery mildews is highly sensitive to the environmental conditions (Schnathorst, 1965).

As powdery mildew fungi are obligate pathogens, they cannot be cultured on artificial media; this makes the powdery mildew/ host interaction a complex and difficult experimental system on which to work. Because of the economic impact on cereals, studies in the past have focused on cereal systems such as barley (*Hordeum vulgare*/*B. graminis* f.sp. *hordei*) and wheat (*Triticum aestivum*/*B. graminis* f. sp. *tritici*), see Adam & Somerville (1996) for references. The body of molecular knowledge surrounding most aspects of these plants is not as extensive as that surrounding the model flowering plant Arabidopsis (Adam & Somerville, 1996). In the past these systems have also had less advanced tools for exploiting genetic, mutational and complementation analysis than those available for Arabidopsis (Adam & Somerville, 1996). Adam & Somerville (1996) have described the visible process of compatible infection (that is to say successful - see later) of *E. cichoracearum* on Arabidopsis in some detail, and detailed study of susceptible Arabidopsis accessions has also been conducted using the UCSC isolate of *E. cichoracearum* (Adam *et al.*, 1999). The impact of using the model plant Arabidopsis to study plant pathogen interactions has been the subject of previous reviews (Buell, 1998; Schulze-Lefert & Vogel, 2000), see Table 1.2.

This introduction will focus on the nature of successful (compatible) and unsuccessful (incompatible) powdery mildew infections and the effect this has on the host. In addition, the use of microarrays as a tool with which to study powdery mildew interactions will be discussed.

Figure 1.1 Powdery Mildew is typically a visible plant pathogen. Examples of powdery mildew infections. Top Left, Arabidopsis infected with the powdery mildew *E. cichoracearum*. Top right, Barley leaves infected with *B. graminis*. Bottom, left to right, healthy squash leaf, squash leaf 7 days after infection with *E. cichoracearum*, squash leaf

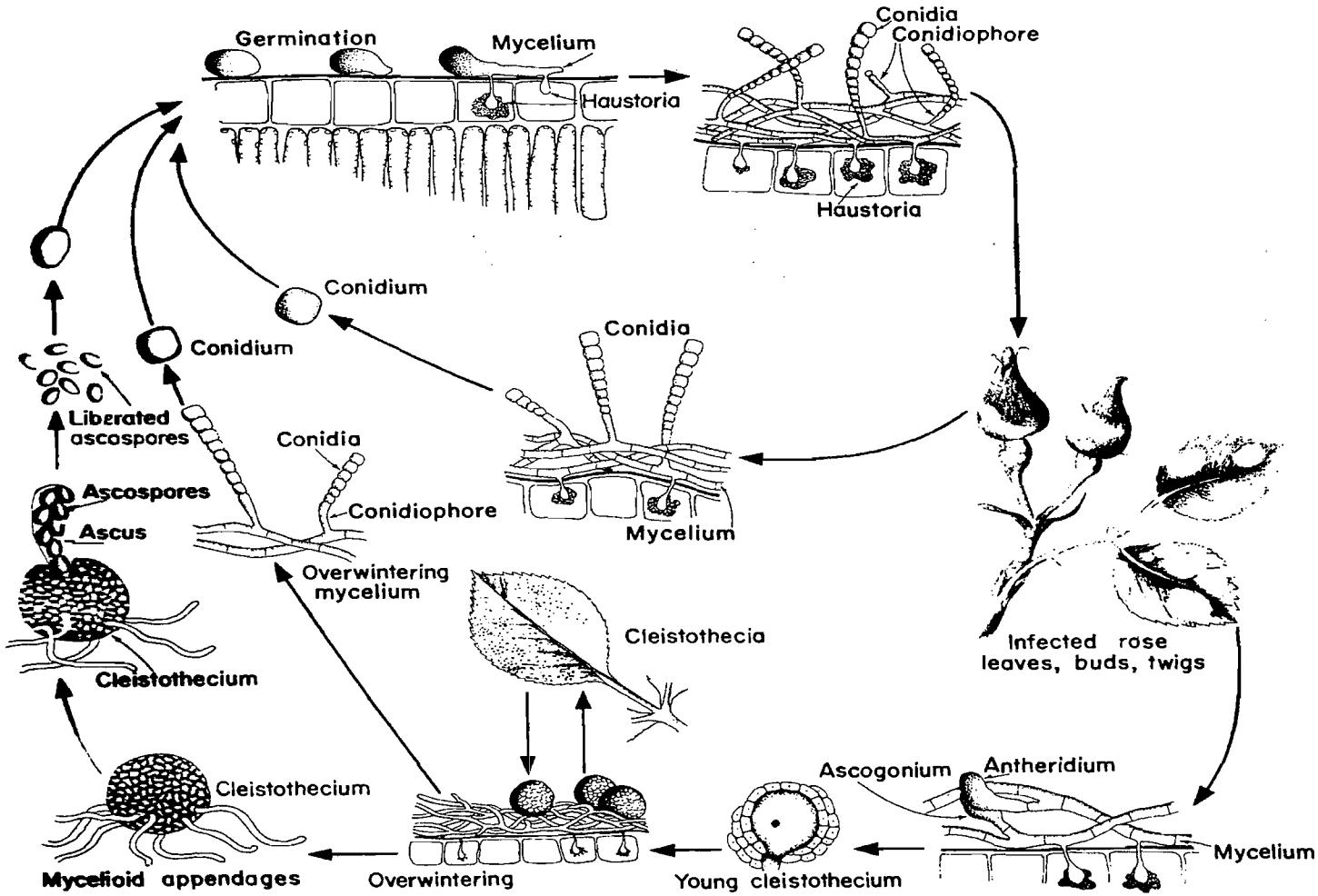

Figure 1.2 Disease cycle of powdery mildew. The disease cycle of powdery mildew is exemplified by the rose powdery mildew *Sphaerotheca pannosa* f. sp. *rosae* (reproduced from Agrios, 1997).

Table 1.2 Advantageous characteristics of Arabidopsis as a model plant with which to study plant pathogen interactions.

Advantageous characteristics of Arabidopsis
Similar defence mechanisms to other angiosperms (Buell, 1998)
Several pathogen-specific mapped loci (Buell, 1998)
Simple, Agrobacterium-mediated transformation (Clough & Bent, 1998)
A small, sequenced genome (Arabidopsis Genome Initiative, 2000)
Short life cycle
Characterised mutants (e.g. Cao *et al.*, 1994; Glazebrook *et al.*, 1996)
Commercially available knockout lines (www.nasc.ac.uk)
Entire genome microarray chips (www.affymetrix.com), (www.catma.org)

1.1 Plant disease and defence

Plant pathogen interactions can be referred to as compatible or incompatible (Heitefuss, 2001). Most plants are resistant to most potential pathogens (Heath, 2000) and plant pathogens can be split into three broad classes, biotrophs, necrotrophs and hemibiotrophs (Hammond-Kosack & Jones, 1997). Resistance of plants to pathogens can also be broadly categorised. Where plant resistance to a given pathogen is cultivar or accession specific, it is referred to as host resistance, race-specific resistance or vertical resistance (Bushnell, 2002; Mysore & Ryu, 2004). However, where resistance is shown by a whole species it is termed non-host resistance, race-non-specific or horizontal resistance (e.g. Bushnell, 2002; Mysore & Ryu, 2004). Non-host resistance is the form of resistance most plants show against most potential pathogens (Heath, 2000) and can have two forms: type I, which does not produce any visible symptoms or type II, which results in rapid cell death around attempted attack areas and is known as the hypersensitive response (HR) (Mysore & Ryu, 2004). For both type I and type II, the outcome is that no disease results (Hammond-Kosack & Parker, 2003). The defence against pathogens can consist of both pre-formed and active defences (see below).

1.1.1 Pre-formed defences

Pre-formed defences are the first barrier for a pathogen to overcome (Mysore & Ryu, 2004) and are based on the "constitutive structural or chemical attributes of a plant, irrespective of the presence of or attack by a pathogen" (Heitefuss, 2001), although sometimes a clear distinction between active and pre-formed components is not possible (Heitefuss, 2001). Examples of such attributes are the cell wall, the presence of inhibitory substances, limited availability of nutrients and the plant cytoskeleton providing a physical barrier against invading pathogens (Heath, 2000; Heitefuss, 2001; Strange, 2003; Mysore & Ryu, 2004). Plants also produce a large number of low molecular weight secondary metabolites which can have an antimicrobial activity, sometimes termed phytoanticipins, since they are present before pathogen attack (Taiz & Zeiger, 2002; Strange 2003). Such chemicals include but are not limited to: saponins, which disrupt cellular membranes that contain sterols; cyanogenic glycosides, which inhibit cellular respiration; pyrrolizidine alkaloids; polyacetylenes; benzoxazinones and glucosinolates (Osbourn, 1996; Wittstock & Gershenzon, 2002; Strange, 2003). Many of the above metabolites do not have a described mechanism of action and some are toxic to the plant itself and produced as inactive precursors (Wittstock & Gershenzon, 2002). Other pre-formed compounds, such as tannins antimicrobial proteins and peptides (such as thionins and defensins), have a higher molecular weight (Strange, 2003).

A good example of a pre-formed physical defence mechanism is the actin cytoskeleton, which has been shown to have a role in resistance; loss of actin cytoskeletal function by pharmaceutical inactivation compromises non-host resistance (Kobayashi &

Hakuno, 2003; Yun *et al.*, 2003). *Nicotiana plumbaginifolia* BY-2 cell lines allow the powdery mildew *E. cichoracearum* (normally compatible) to develop, but not the powdery mildew *E. pisi* (normally incompatible - tobacco is a non-host to *E. pisi*). However, after treatment with cytoskeleton polymerisation inhibitors, *E. pisi* could develop normally (Kobayashi & Hakuno, 2003). That cultured cells can change from being incompatible to compatible, while normal defence responses such as pathogenesis related (PR) gene expression after salicylic acid (SA) treatment (see below) could not be observed, alludes to the possible fundamental nature of cytoskeleton mediated incompatibility. Similar events have been shown in Arabidopsis against the incompatible wheat powdery mildew (*B. graminis* f. sp. *tritici*) (Yun *et al.*, 2003). The same cytoskeleton polymerisation inhibitors both singularly, and in a pronounced additive combination with the Enhanced Disease Susceptibility-1 mutant (*eds1-1*) (Glazebrook *et al.*, 1996) severely compromised non-host resistance to *B. graminis*, allowing the formation of secondary hyphae and viable conidia (Yun *et al.*, 2003). EDS1 is not itself part of a pre-formed defence mechanism, but is part of a signalling cascade activated by a subset of *R* (Resistance) genes, which would otherwise cause an accumulation of the disease response mediator SA (see below) (Hammond-Kosack & Parker, 2003). EDS1 itself has only a small positive effect on *B. graminis* development on Arabidopsis (Yun *et al.*, 2003).

1.1.2 Active defences

Active defences, by their nature, need to be stimulated in order to occur (as opposed to pre-formed defences, which do not). These responses are thought to be stimulated by two kinds of receptors, R genes and MAMP (Microbe-Associated Molecular Patterns) receptors (see later). While all known MAMP receptors are extracellular receptors which directly bind pathogen derived ligands, R genes may be intra- or extracellular and may sense intra- or extracellular molecules secreted by the pathogen, or can detect self or 'modified self' proteins intracellularly (Bent & Mackey, 2007). Many pathogens secrete effector proteins (produced by virulence genes) which can assist pathogenicity (Bent & Mackey, 2007), and it is either the effectors, or the effects of effector proteins on a host protein (such as a change in conformation) that are detected by R genes. If an effector is detected by the host, and if that detection reduces the virulence of the host, then the pathogen gene responsible for the production of the effector is then termed an avirulence gene (Bent & Mackey, 2007). Pathogens retain effector genes which reduce virulence because not all hosts will contain R genes against them (Bent & Mackey, 2007). The system of virulence, avirulence and resistance genes is known as the gene for gene hypothesis, originally proposed by Flor (Taiz & Zeiger, 2002).

Following recognition, defence responses are expressed through downstream signalling molecules which regulate expression of defence through cell wall strengthening using callose, lignins, suberins and hydroxyproline-rich glycoproteins (Zeyen *et al.*, 2002;

Strange, 2003), the expression of PR-genes, (van Loon & Van Strien, 1999), the production of phytoalexins (Strange, 2003), an oxidative burst (OB) (Mysore & Ryu, 2004), and expression of localised cell death HR (e.g. Takakura *et al.*, 2004). Defence responses can also lead to the expression of induced resistance (Jalali *et al.*, 2006). These receptors signal via overlapping routes, using SA, jasmonic acid (JA), ethylene (ET), nitric oxide (NO) and intermediates through mitogen activated protein (MAP) kinase cascades and other unknown routes to defence responses (Figure 1.3) (Thomma *et al.*, 2001; Hammond-Kosack & Parker, 2003). SA and JA/ET are involved in signalling defences against different sets of pathogens. SA mainly signals in defence against biotrophs and JA/ET normally signals defence against necrotrophs, although some overlaps exist (Rojo *et al.*, 2003). Local responses can also trigger systemic responses that prime the plant against subsequent attack (systemic acquired resistance, SAR) (Hammond-Kosack & Parker, 2003). The hypersensitive response, (described above as a symptom of active defence responses) is a form of programmed cell death (PCD) that can be associated with both successful and unsuccessful pathogen attacks (e.g. Greenberg & Yao, 2004). It is a rapid death of cells at the infection site, associated with pathogen limitation, defence gene activation and systemic signalling involved in SAR (Pennell & Lamb, 1997; Heath, 2000; Greenberg & Yao, 2004).

PR-genes mentioned above encode proteins of various functions, collectively referred to as PR-proteins, and defined as "proteins coded for by the host plant but induced specifically in pathological or related situations" (van Loon & Van Strien, 1999). They can accumulate both locally and systemically, the latter associated with the development of SAR. Van Loon and Van Strien (1999) give an excellent review on the families and functions of known PR-proteins.

The OB is the production of various reactive oxidants, normally extracellular (e.g. hydrogen peroxide, superoxide anions), in response to pathogen attack (Lamb & Dixon, 1997; Heath, 2000; Heitefuss, 2001; Hammond-Kosack & Parker, 2003; Greenberg & Yao, 2004). There are several functions to this burst, which has direct antimicrobial activity, can cross-link cell wall components (making it harder for pathogens to penetrate), can activate transcription-dependent defences and has a role in both HR and SAR (Lamb & Dixon, 1997).

SAR is a mechanism of induced defence, a process by which a normally compatible plant-pathogen interaction can be converted into an incompatible one (e.g. Ryals *et al.*, 1996; Durrant & Dong, 2004), and can be activated as either as a part of the HR or as a symptom of disease. Molecularly, SAR is characterized by the increased expression of a large number of PR-genes, in both local and systemic tissues. The *son1* mutant however, which expresses systemic resistance without PR-gene expression, referred to as SAR independent resistance (SIR), shows that PR-gene expression is not the only mechanism of systemic resistance (Kim & Delaney, 2002).

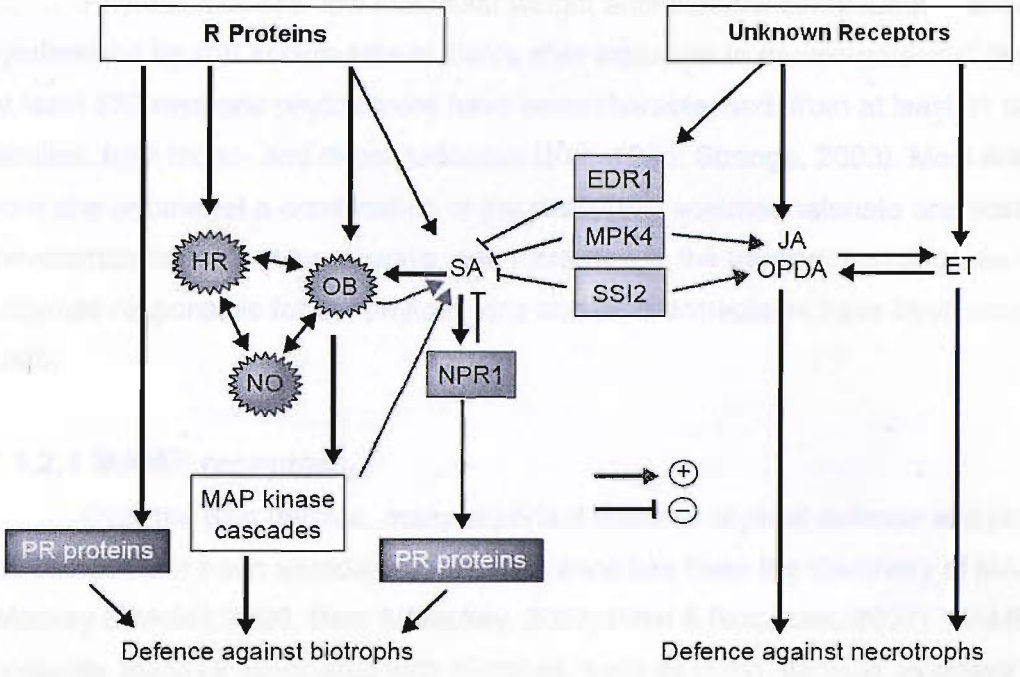

Figure 1.3 Simplified overview of the local signalling networks controlling activation of local defence responses. The network is considerably more complex than is represented in this overview and readers are directed to Hammond-Kosack & Parker (2003) for a fuller explanation. The points at which R proteins are shown to interact on the signalling network are points where individual R proteins are known to interact, and do not indicate that each R protein can act at all points shown. Nitric oxide (NO) itself is also a defence signal and can activate both the HR and Oxidative Burst (OB). Salicylic acid (SA) is thought to act through SA-binding proteins, which are not shown here. A different signal transduction network leads to the activation of parallel jasmonic acid (JA) and ethylene (ET) signalling cascades and can activate resistance to necrotrophic pathogens. EDR1, enhanced disease resistance; NDR1, non-race specific disease resistance; SSI2, suppressor of salicylate insensitivity of NPR1-5; MPK4, mitogen activated protein kinase 4, 12-oxophytodienoic acid (OPDA). Redrawn after Hammond-Kosack & Parker (2003).

Callose is often synthesised and deposited around a wound or site of pathogen attack (such as a penetration attempt by powdery mildews – see later) as localized wall appositions, known as papillae, see Strange, (2003). These papillae serve as both a physical barrier to fungal penetration and as sites for the accumulation of localised antimicrobial compounds (Zeyen *et al.*, 2002).

Phytoalexins are "low molecular weight antimicrobial compounds that are both synthesised by and accumulate in plants after exposure to microorganisms" (Kuc, 1995). At least 350 separate phytoalexins have been characterised, from at least 31 plant families, both mono- and di-cotyledonous (Kuc, 1995; Strange, 2003). Most are derived from one or other of a combination of the shikimate, acetate-malonate and acetate-mevalonate biosynthetic pathways, and increases in the transcription of genes encoding enzymes responsible for the phytoalexins and its intermediates have been observed (Kuc, 1995).

1.1.2.1 MAMP receptors

Over the past decade, many important features of plant defence and plant resistance have been elucidated. A key advance has been the discovery of MAMPs (Mackey & Mcfall, 2006; Bent & Mackey, 2007; Bittel & Robatzek, 2007). MAMPs are molecular features associated with microbes, such as chitin, flagellin, xylanase and lipopolysaccharide (LPS) (Zipfel *et al.*, 2004; Zipfel *et al.*, 2006; Bittel & Robatzek, 2007). The recognition of these features is a key component of basal resistance within plants (Jones & Dangl, 2006; Bent & Mackey, 2007). The recognised targets are normally key structural features of the microbe and are often both essential and invariant in structure, but not necessarily important for pathogenicity (Bittel & Robatzek, 2007). Because of this, genes for MAMP receptors are not regarded as R genes and genes for MAMPs are not regarded as avirulence genes (Bent & Mackey, 2007). MAMPs are recognised by MAMP receptors and to date all known MAMP receptors contain a single transmembrane domain with an extracellular ligand-binding domain (Bittel & Robatzek, 2007). Most also contain an intracellular serine/threonine kinase domain (receptor-like kinases; RLKs); other types are similarly structured but lack the cytoplasmic kinase domain (receptor-like proteins, RLPs) (Bittel & Robatzek, 2007). In Arabidopsis 610 RLKs and 56 RLPs have been identified (although not all of these are MAMP receptors) (Shiu & Bleecker, 2001; Fritz-Laylin *et al.*, 2005). Some RLK MAMP receptors have been shown to initiate a MAP kinase cascade in response to MAMP recognition (Asai *et al.*, 2002) and it is thought that this is the common means of signal transduction (Bent & Mackey, 2007). The exact signal transduction mechanism by which RLP MAMP receptors induce defence responses is at the moment unclear, but other kinase-less proteins require heteromeric co-receptors for function (Bittel & Robatzek, 2007).

Following MAMP detection and signal transduction, it is known that in the case of the flagellin receptor (*FLG2*) WRKY22 and WRKY29 transcription factors are up-regulated (Asai *et al.*, 2002). It seems likely that WRKY transcription factors (although exactly which ones is unclear) resulting from MAMP detection, can activate SA production (Eulgem & Somssich, 2007), callose deposition and the production of antimicrobial compounds (Bittel & Robatzek, 2007).

It is not clear how many would-be pathogens are not virulent solely because of MAMP recognition, but it is clear that virulent pathogens suppress MAMP mediated resistance through the secretion of effectors (Bent & Mackey, 2007). This suppression is presumed to be only partial, as the limited evidence currently available shows that the loss of the MAMP receptor for flagellin increases the virulence of the already virulent *Pseudomonas syringae* pv. tomato (Zipfel *et al.*, 2004)

1.1.3 Resistance and powdery mildews

The ability of powdery mildews to penetrate a plant and form infection structures is highly host dependent; the majority of plants show non-host resistance to powdery mildews. Indeed many Arabidopsis accessions are resistant to powdery mildew attack (Adam *et al.*, 1999). Work in this lab uses a comparison of compatible (*E. cichoracearum*/ Arabidopsis) against incompatible (*B. graminis*/ Arabidopsis) reactions, to try and isolate responses due to pathogen attack from responses due to successful interaction and the establishment of biotrophy.

Much research into powdery mildew has focused on defence and resistance in, or by the host. Some approaches have used molecular mapping to identify resistance loci. Wilson *et al.* (2001) identified three different loci, while Adam & Somerville (1996) found five loci correlating to powdery mildew resistance. Mutants with enhanced penetration resistance, where novel components of disease resistance such as synaptosome-associated proteins (SNAREs) have been identified (Collins *et al.*, 2003). Equally, enhanced susceptibility mutants have also been identified (Reuber *et al.*, 1998; Frye *et al.*, 2001), some with interesting signalling implications, such as MAP kinase mutants (Frye *et al.*, 2001). Some studies however have focused on functional assessment of genes rather than resistance genetics; Schultheiss *et al.* (2003) found that the PR-protein PR-1b was probably not antimicrobial and expression of the PR-protein PR-1b of barley could be induced by hydrogen peroxide, known to accumulate around penetration sites.

A series of interesting investigations of Arabidopsis resistance to powdery mildew have been conducted on the premise that the specificity of the relationship between powdery mildews (which as stated previously cannot be cultured on artificial media) and their hosts probably means that some host genes would be required for pathogen growth (Vogel & Somerville, 2000; Vogel *et al.*, 2002; Vogel *et al.*, 2004). It is possible that some of these genes might not be directly defence related, and represent genes not previously

thought to be involved in powdery mildew interactions. Mutations in these genes prevent the growth of *E. cichoracearum*. Three of the mutants with loci confined to single genes have had functions associated with the cell wall. PMR4 is a callose synthase and the resistance it provides is SA-dependent (Nishimura *et al.*, 2003). However, the other two genes PMR5 and PMR6 are of unknown function and are pectate lyase-like respectively. Interestingly neither is dependent on SA or JA/ET signalling and both have a similar effect of altering the cell wall of host plants to contain more pectin (Vogel *et al.*, 2002; Vogel *et al.*, 2004). The authors speculate that the mutants either cause the loss of susceptibility factor or activate a novel form of disease resistance (Vogel *et al.*, 2002; Vogel *et al.*, 2004).

1.2 Powdery mildew infection

1.2.1 Infection sequence

Several summaries have been published about the initial events in powdery mildew infections (Carver *et al.*, 1995; Tucker & Talbot, 2001; Green *et al.*, 2002). Within seconds of spore deposition (Carver *et al.*, 1999), enzymes - including cutinases - are released by conidia of *B. graminis* (Nicholson *et al.*, 1988; Pascholati *et al.*, 1992). Dixon and Lamb (1990) give a good review on the signals needed to start and continue infection events. The released enzymes have the ability to degrade host surfaces and their release coincides with a change in the conidial surface from hydrophobic to hydrophilic (Nicholson *et al.*, 1993). It has been reported that at this time (i.e. before germination), conidia can take up anionic, low molecular weight materials, which is thought to possibly aid host recognition (Nielsen *et al.*, 2000). The exudates deposited beneath the conidia also increase the hydrophilicity of the leaf surface (Kunoh *et al.*, 1988) which may help prepare the infection area for germling adhesion, penetration site recognition and penetration itself (Kunoh *et al.*, 1988; Nicholson *et al.*, 1993).

1.2.2 Germination and prehaustorial development

Most of the studies on germination and pre-haustorial developments have been conducted on *B. graminis* which, while having some unique features (Kunoh *et al.*, 1979), seems broadly in line with observations on other powdery mildews (Adam & Somerville, 1996). The contact of conidia with a solid or even organic surface is not a prerequisite for germination (Gay *et al.*, 1985; Carver & Ingerson, 1987; Nicholson *et al.*, 1993). In *B. graminis* between 0.5 and 1h after spore deposition, a primary germ tube (PGT) emerges (discussed in Nielsen *et al.*, 2000), which contacts the host leaf. However this appears to be unique within the Erysiphaceae (Kunoh *et al.*, 1979). The function of this PGT is unclear, but as reviewed in Carver & Bushnell (1983) and Carver (1995) the proposed (and not mutually exclusive) functions are: rapid fungal attachment to the host surface, the gaining of access to the host water supply and recognising the contact surface. The latter

is thought to consequentially induce intracellular signalling (Dixon & Lamb, 1990). This results in the elongation of the second formed germ tube, a prerequisite for appressorium formation. The other members of Erysipheae, (including *E. cichoracearum*) circumvent the need for a PGT. For other powdery mildews, either the proposed functions are unnecessary or the functions are performed in other ways before their appressoria develop.

In most cases, the secondary germ tube (the appressorial germ tube or AGT) emerges between 15 and 60 minutes after the PGT. Given that the PGT has recognised the host, the AGT elongates, secreting extracellular material of a largely unknown composition beneath it (Carver *et al.*, 1995). By 9 –10 hours post inoculation (HPI), the AGT has reached its mature length, and a hook shaped appressorial lobe is formed. After the appressorial lobe differentiates (9-10 HPI) the gross morphology remains constant until the germling either dies or penetrates the epidermis (Carver *et al.*, 1995). However a 2nd or 3rd appressorial lobe may form on the AGT and attempt penetration, if the first attempt is unsuccessful. *E. cichoracearum* is unusual within Erysipheae, as the first penetration rarely succeeds in a compatible host, and up to three attempts may be made before a haustorium is formed (Heath, 2002).

1.2.3 Formation of haustoria

Fungal development continues with attempted primary infection, by the emergence of a blunt ended penetration peg from beneath the appressorial lobe (reviewed in Bushnell, 1972). The peg appears to have no cell wall (McKeen & Rimmer, 1973) and emerges at about 12 – 15 HPI. It was shown by Pryce-Jones *et al.* (1999) that a combination of enzymatic and mechanical force is used to penetrate the host cell wall. In some fungal hemibiotrophic fungi, melanin impregnation allows the build-up of high osmotic pressure, which has been seen to result in a 16.8 (± 3.2) μN penetration force, concentrated at the tip of the penetration peg (Bechinger *et al.*, 1999). Complex pathways control the formation of haustoria (reviewed in Kahmann & Basse, 2001), which, when penetration is complete, allow the penetration peg to swell and initiate haustorial formation. The mature haustoria are thought to be the sites for nutrient transfer to the fungus (Bushnell, 1972; Mendgen *et al.*, 2000; Mendgen & Hahn, 2002) (see section 1.4).

1.3 Haustorial structure

Once the penetration of the epidermal cell wall is achieved, a lobed haustorium develops in the epidermal cell. The plant surrounds the haustorium in a membrane termed the extrahaustorial membrane, which appears to be an invagination of the cell membrane (this has been observed by numerous researchers, but see Gil and Gay (1977) and Manners and Gay (1977) for some early examples). The intervening space is known as the extrahaustorial matrix, a mixture of complex carbohydrates. This matrix is not able to

form part of the apoplast, as the extrahaustorial membrane is sealed directly to the haustorial membrane close to the point of entry, an area known as the neckband (Gil & Gay, 1977). The haustoria and appressorium are separated by a septum (Figure 1.4). The whole structure is known as the haustorial complex (HC) and has been successively isolated in some interactions like *Pisum sativum* (pea)/ *E. pisi* but not in others such as grape vine/ *Unicinula necator* (Gil & Gay, 1977). Excellent reviews on haustorial structure are given in Bushnell (1972), Harrison (1999) and Perfect & Green (2001).

1.3.1 Extrahaustorial membrane

The extrahaustorial membrane surrounds the normally heavily invaginated haustorial membrane, but differs from the plant membrane of which it is a continuation, at both the microscopic (Gil & Gay, 1977) and molecular level (e.g. Mackie *et al.*, 1991). It is thought that the convolutions in invaginated membranes act to increase the surface area and thus aid nutrient acquisition by the fungus (Manners & Gay, 1983). The extrahaustorial membrane can be up to 2.3x thicker than the plant plasma membrane (Gil & Gay, 1977), possibly due to the incorporation of extra carbohydrates (Gil & Gay, 1977) and it is resistant to some treatments such as detergents, which normally dissolve membranes (Gil & Gay, 1977). Additionally the extrahaustorial membrane does not contain the intra-membrane particles that are seen in many plant plasma membranes (Gil & Gay, 1977). The extrahaustorial membrane also contains protuberances that increase with haustorium maturity (Harder & Mendgen, 1982). In the case of lily leaves infected by *Puccinia hemerocallidis*, these extensions produce a tubular network that penetrates deep into the host cytoplast (Mims *et al.*, 2002).

Importantly, ATPase activity can be demonstrated on the plant plasma membrane of both infected and non-infected cells in several different interactions (Spencer-Phillips & Gay, 1981; Manners, 1989; Baka *et al.*, 1995), while the extrahaustorial membrane does not show any ATPase activity. This may be the result of exclusion of ATPase from the extrahaustorial membrane or the inhibition of ATPase activity (Baka *et al.*, 1995).

More evidence of molecular differentiation has come from the generation of monoclonal antibodies. Roberts *et al.* (1993) raised monoclonal antibodies against the whole haustorial complex and various antibodies were obtained that recognise specific components of the extrahaustorial matrix. For example, the UB11 antibody recognises a large glycoprotein (250 kDa) on the plant side of the extrahaustorial membrane; this binding is sensitive to periodate (ions which modify sugar rings), suggesting that parts of the carbohydrate are recognised by the antibody. This protein is present only in the early stages of infection. Other antibodies also showed time-dependent distribution on the extrahaustorial membrane, but time-independent distribution on the plant plasma membrane (e.g. the UB9 antibody). Other studies have shown differences in glycoprotein

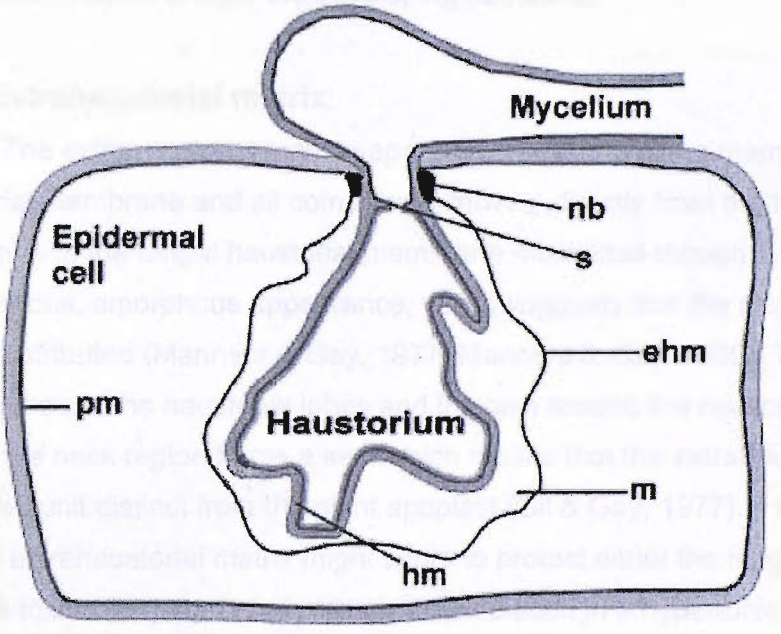

Figure 1.4 The Structure of Haustoria. ehm, extrahaustorial membrane; hm, haustorial membrane; pm, plasma membrane; m, interface matrix; nb, neckband; s, septum. Modified from Hall and Williams (2000).

incorporation between the extrahaustorial and plant plasma membrane (Green *et al.*, 1995).

These differences in protein content suggest that there may be specific targeting of proteins to the extrahaustorial membrane. The development of the extrahaustorial membrane is an expansion of the previous plasma membrane and its production requires a significant increase in membrane biosynthesis, discussed by Harrison (1999). It has been suggested that in likely conjunction with the Golgi apparatus, the endoplasmic reticulum (ER) supplies proteins and other membrane components to the extrahaustorial matrix (Manners & Gay, 1983). This idea is supported by confocal laser microscopy (Leckie *et al.*, 1995) showing the ER moving from an even distribution in uninfected cells to a dense network around the developing haustoria.

1.3.2 Extrahaustorial matrix

The extrahaustorial matrix separates the plant plasma membrane from the fungal haustorial membrane and all compounds moving directly from the host extrahaustorial membrane to the fungal haustorial membrane must pass though it. The matrix has a homogenous, amorphous appearance, which suggests that the major components are evenly distributed (Manners & Gay, 1977; Manners & Gay, 1980). The matrix is normally thickest around the haustorial lobes and thinnest around the neckbands. As mentioned before, the neck region forms a seal which means that the extrahaustorial matrix is a contained unit distinct from the plant apoplast (Gil & Gay, 1977). It has been suggested that the extrahaustorial matrix might serve to protect either the fungus or plant from possible toxins (Hajlaoui *et al.*, 1991). When placed in a hypertonic solution the extrahaustorial matrix of *E. pisi* swells without rupturing the haustoria, suggesting that the extrahaustorial matrix is fluid in nature (Gil & Gay, 1977). However, although small molecules such as uranyl ions are able to pass through the extrahaustorial matrix, horseradish peroxidase, a 40-kDa molecule cannot. This suggests the extrahaustorial matrix surrounding *E. pisi* is gel-like as opposed to a solution (Gay & Manners, 1987).

Some extrahaustorial matrices have been observed to contain threonine-hydroxyproline rich glycoproteins (THRGPs), which are thought to be produced by the plant (Hippe-Sanwald *et al.*, 1994). While both arabinogalactan and callose have been observed in the extrahaustorial matrix of *Uromyces vignae* (Stark-Urnau & Mendgen, 1995), neither of these has been observed in *E. pisi* extrahaustorial matrices, suggesting that the plant contributes to the extrahaustorial matrix in some, but not all biotrophic interactions.

1.3.3 Haustorial membrane

Monoclonal antibody studies have led the way in showing the molecular differentiation of the haustorial membrane. For example, three carbohydrate epitopes specific to haustorial cell walls have been identified in *Melampsora lini* (Murdoch & Hardham, 1998) as have two monoclonal antibodies which recognise epitopes in the cell wall and membranes of *E. pisi* (Mackie *et al.*, 1991).

Other specific properties include ATPase activity, which has been shown to exist by lead staining and electron microscopy. This can be seen in some infections (*E. pisi*), but not in others (*U. appendiculatus*) (Spencer-Phillips & Gay, 1980). ATPase activity was demonstrated in vesicles purified from isolated *U. viciae-fabae* haustoria (Struck *et al.*, 1996). These vesicles have shown four- and eight- fold more ATPase activity than vesicles purified from the germ tubes and uredospores, respectively. Some researchers have discussed the accuracy of the original lead-EM technique (Chauhan *et al.*, 1991; Baka *et al.*, 1995) after criticism that it is not specific for H^+-ATPase (Katz *et al.*, 1988). A single copy fungal plasma membrane ATPase, PMA1 has been cloned from *U. fabae* (Struck *et al.*, 1998) which was expressed at high levels in spores and germ tubes, but at low levels in haustoria. This possible discrepancy has led to the idea that post-transcriptional regulation may be involved (Schulze-Lefert & Panstruga, 2003). The relatively high level of ATPase activity is consistent with the idea that the haustorial membrane can actively transport nutrients.

Other evidence of molecular events at the haustorial membrane has come from a cDNA library prepared from haustorium RNA, which has identified several *in planta* expressed genes (Hahn & Mendgen, 1997) including a putative amino acid transporter, AAT2p (Hahn *et al.*, 1997).

1.4 Function of haustoria

Haustoria have long been proposed to be the site of nutrient transfer (Bushnell, 1972; Mendgen *et al.*, 2000; Mendgen & Hahn, 2002). They may also be involved in recognition of, or by, the host and may contribute to the establishment or maintenance of compatibility (Heath & Skalamera, 1997). For powdery mildews, it has been established that the haustorium is involved in nutrient transfer (Mount & Ellingboe, 1969; Spencer-Phillips & Gay, 1980). Indeed, both powdery mildew and downy mildews have also indicated dependence on haustoria (Andrews, 1975; Manners & Gay, 1978; Manners & Gay, 1980). In these experiments radioisotope entered the complexes even when hyphae were detached. Studies on the location of the isotope showed 70% of insoluble label had been incorporated into the haustorial body (Spencer-Phillips & Gay, 1980). Powdery mildew-infected wheat leaves showed that the change from a low to a high rate of transfer of ^{32}P and ^{35}S (supplied as salts to leaves) corresponds with the establishment and maturation of haustoria (Mount & Ellingboe, 1969). Other biotrophic fungi have also been

observed to take up amino acids (Burrell & Lewis, 1977), and to do this via haustoria (Mendgen, 1981). Other indirect evidence, such as the poor growth of pathogens without haustoria has been reviewed by Bushnell (1972).

More recent experiments have shown that a single epidermal cell (and thus a single haustorium) may support a whole colony of powdery mildew. Only single barley epidermal cells that had been biolistically transformed with a plasmid, which conferred susceptibility, supported a compatible interaction. Eventually this gave rise to a sporulating fungal colony (Shirasu *et al.*, 1999).

A matter of some debate has been the nature of the host carbon energy-source transferred to the fungus. Experiments by Manners and Gay (Manners & Gay, 1982) using $^{14}CO_2$ suggested sucrose as the major sugar transferred to the fungus, as it was the largest source of ^{14}C within the fungus. This did not however preclude other sugars, as they may have metabolised faster in the haustoria than in the host (Spencer-Phillips & Gay, 1980).

However, radiolabel from glucose was shown to be taken up more rapidly than fructose or sucrose into *E. pisi* mycelium (Aked & Hall, 1993b; Clark & Hall, 1998) and this has been supported by studies with potentiometric dyes. These indicate that glucose is taken up immediately by *B. graminis* haustoria in host barley cells, while sucrose and fructose require time to be metabolised (Mendgen & Nass, 1988). More recently, Sutton *et al.* (1999) proved that glucose rather than sucrose is transported from wheat to the wheat powdery mildew fungus, *B. graminis* f.sp. *tritici*. as sucrose was shown to be cleaved prior to uptake by the pathogen. Finally, Abood and Lösel (2003) showed the presence of glucose but only traces of sucrose in spores from cucumber leaves infected with the powdery mildew *Sphaerotheca fuliginea* using gas-liquid chromatography.

1.4.1 The nature of haustorial transport

In spite of the evidence indicating the role of haustoria in nutrient uptake, the exact mechanisms remain unknown. The sealed matrix suggests that solutes move through the extrahaustorial membrane (EHM) into the extrahaustorial matrix, do not mix with the apoplast and are transported actively into the fungus, although it is not clear how (Manners, 1989; Green *et al.*, 2002). Evidence suggests that the mode of transport for solute flow from the plant across the EHM is by an active or facilitated process, as discussed by Manners (1989). The lack of ATPase activity on the EHM (as discussed previously) suggests that this membrane is not likely to be actively transporting nutrients, while strong activity on the haustorial plasma membrane would be consistent with active uptake of nutrients across that membrane. However such activity may not be present in all interactions (Manners, 1989). Isolated haustorial complexes have been used to demonstrate passive uptake of sucrose and glucose, but this approach has severe limitations (Manners & Gay, 1983) as the damage to membranes, lack of mycelia as

metabolic sink and lack of host cell distorts the transport processes of haustoria. Other studies (Aked & Hall, 1993c) are consistent with passive carriers for glucose and sucrose shown by floating infected pea leaf disks on labelled solutions of glucose, 3-oxy-methyl-D-glucose, fructose and sucrose.

Active uptake by the fungus had appeared to be less credible by the finding that the haustorial membrane may also lack ATPase activity in a number of fungal biotrophic pathogens including *U. appendiculatus* (Spencer-Phillips & Gay, 1981), and *B. graminis* (Gay & Woods, 1987), as measured by lead precipitation. Spencer-Phillips & Gay (1981) proposed that the ATPase activity on the normal plasma membrane of infected cells could effect a potential difference at the EHM, which could be used to drive nutrient uptake. In this scenario, solutes would be off-loaded at the extrahaustorial matrix because of the host's diminished control of solute retention at this point, presumably through facilitated diffusion, due to the lack of ATPase activity (Manners, 1989; Green *et al.*, 2002). These solutes would then be available for uptake by the parasite. A study by Gay et al (1987) was thought to provide evidence for this. Using a pH sensitive fluorescent compound, the fungal haustoria and plant cytoplasm were thought to be coupled, as fusicoccin (a stimulator of plant plasma membrane ATPases though to be ineffective against fungal ATPases) altered the pH in the haustoria in synchrony with the host cell cytoplasm (Gay *et al.*, 1987). An increase in fluorescence after fusicoccin treatment was not observed in isolated haustoria alone (Gay *et al.*, 1987). However, exactly how the haustoria and host cell were ionically linked was never certain. It now seems that nutrient uptake to haustoria by specific H^+/solute symport as proposed by Patrick (1989) seems the most likely method of nutrient transport. This is due partly to the doubts about the techniques used to prove a lack of ATPase activity (Katz *et al.*, 1988) and the lack of convincing mechanism from past results (Gay *et al.*, 1987), but is also due to convincing current work, such as the findings of ATPase activity demonstrated in vesicles purified from isolated *U. viciae-fabae* haustoria (Struck *et al.*, 1996) and the amino acid permease identified in *U. fabae* (Hahn *et al.*, 1997). With the advances being made in molecular approaches, it may not be long before more is known concerning the mechanism of nutrient uptake by the haustorium.

1.4.2 Known fungal biotrophic transporters

The amino acid permease identified in *U. fabae* (Hahn *et al.*, 1997) was present on the periphery of the haustoria except the neckband. The sequence homology of this protein suggests that it is a symporter. This finding helps to support the proton symport model (Patrick, 1989); the symporter could use a proton gradient generated by the ATPase activity, which has been seen on the haustorial membrane in the *U. fabae* interaction. The cDNA library from *U. fabae* (Hahn *et al.*, 1997) has identified 31 different in planta-induced genes. Two of these (*AAT1p* and *AAT2p*) were found to encode proteins with high homology to certain fungal amino acid transporters. The role of AAT1p as an

amino acid transporter was confirmed by functional yeast complementation analysis, and its location confirmed by imunofluorescence, while electrophysiological studies in transfected *Xenopus* oocytes indicated that the uptake of amino acids involves a proton-symport mechanism (Struck *et al.*, 2002).

The only biotrophic fungal sugar transporters to be identified and fully characterized are *AmMst1*, a monosaccharide transporter of an ectomycorrhizal fungus, *Amanita muscaria* (Nehls *et al.*, 1998), and *UfHXT1* from the pathogenic fungus *U. fabae* (Voegele *et al.*, 2001). *AmMst1* was identified by a cDNA fragment, which was used to identify the full length cDNA by the use of primers designed to match highly conserved regions in fungal monosaccharide transporters. *UfHXT1* was identified in the haustorial cDNA library of *U. fabae* described earlier (Hahn *et al.*, 1997).

1.5 Physiological effects on the host of biotrophic interactions

1.5.1 Effects on host plant photosynthesis

Biotrophic infections of leaves have been shown to lead to a reduction in the rate of photosynthesis. Tang *et al.* (1996) showed that *Albugo candida* caused a decrease in the rate of photosynthesis in Arabidopsis leaves, at a time when soluble carbohydrates (especially hexose sugars) were actually accumulating. Scholes *et al.* (1994) showed that under conditions of saturating light and ambient CO_2, the rate of photosynthesis declined in mildewed leaves from 3 days post inoculation.

1.5.2 Assimilate partitioning

Biotrophic interactions are reported to have an effect on some key components of the system of assimilate partitioning used in healthy plants. A brief description of assimilate partitioning in healthy plants is necessary to explore these implications.

Plants are autotrophic organisms able to synthesise complex molecules by reducing carbon, nitrogen and sulphur from simple molecules. However, not all plant tissues are autotrophic and as such need to be supplied with energy and fixed carbon (photoassimilates). Land plants possess a system for long-distance transport of photoassimilates from autotrophic (source) to heterotrophic (sink) parts. As a major translocatable product of photosynthesis, sucrose (glucose + fructose) is the main soluble component of phloem sap, and is the principle sugar transported through the phloem (see Lalonde *et al.*, 2003). Selection of sucrose as the major transport sugar in plants has been related to its non-reducing nature and relative insensitivity to metabolism (see Lemoine, 2000). Although some plants also transport other sugars (such raffinose, stachyose and verbascose) (Lemoine, 2000), experiments by Haritatos *et al.* (2000) indicated sucrose to be the major translocated sugar in Arabidopsis.

Solute flow in the phloem can be described by a mass flow hypothesis, originally proposed by Münch in 1930. This states that the production of photoassimilates in the

source tissues (mesophyll cells) increases the osmotic pressure at these points. This creates a pressure gradient between source (high osmotic pressure) and sink (lower osmotic pressure), which drives the flow of solutes to the sink(s).

The principle routes of sucrose transport through the phloem have been discussed (Lalonde *et al.*, 2003). These are: 1) the movement of sucrose from the source mesophyll cells to the companion cell of a sieve element in a mature leaf 2) movement from the companion cell to the sieve element (parts 1 and 2 are collectively known as phloem loading) 3) movement to the sink tissue by mass flow, with loss and retrieval of sucrose on the way 4) entry into the sink tissue (phloem unloading).

1.5.2.1 Phloem loading

Phloem loading can occur in two ways, symplastically through plasmodesmata between cells, or apoplastically by departure and re-entry through plasma membranes. The preferred mode of phloem loading seems to differ between plant species (Van Bel, 2003). Apoplastically, sucrose must leave the cell by transport across the plasma membrane and into the apoplast either by a facilitator or an antiporter (Lalonde *et al.*, 2003) and such efflux systems have been described biochemically (Laloi *et al.*, 1993). Sucrose must then re-enter either a companion cell or sieve element directly, such transport is thought to be mediated by a secondary transport process involving a sucrose/H^+ symporter (e.g. Riesmeier *et al.*, 1993). During the passage of sucrose from source to sink, some sucrose leaks from the phloem; this is recovered en route by sucrose/H^+ symporters (Lalonde *et al.*, 1999). In Arabidopsis, the most likely loading mechanism is carrier-mediated retrieval from the apoplast, by proton symport. Evidence to support this includes the localization of sucrose transporter AtSUC2 in companion cells (Stadler & Sauer, 1996) and the phenotypic responses of Arabidopsis mutants with disruptive insertions in AtSUC2 (Gottwald *et al.*, 2000).

1.5.2.2 Phloem unloading

In sink tissue, phloem unloading can occur apoplastically by transmembrane transport of sucrose or symplastically through plasmodesmata directly to the sink cell; both pathways may operate in the same organism, but depends on the organism concerned (Williams *et al.*, 2000). The efflux transporters involved have been postulated to be either facilitators or proton antiporters (Walker *et al.*, 1995). If sucrose has followed the apoplastic path it can be taken up directly by a sucrose/H^+ symporter or indirectly by hydrolysis to glucose and fructose by an extracellular invertase and then through hexose transporters (see Lalonde *et al.*, 2003).

1.5.2.3 Effects on invertase activity

Invertases hydrolyse sucrose into glucose and fructose and invertases have been proposed to have a role in powdery mildew infections (Hall *et al.*, 1992). In higher plants, invertases exist with optimum pH in both acidic and alkaline conditions and both forms are often present in the same tissue (Masuda *et al.*, 1988; Xu *et al.*, 1989). Acid invertase is thought to have two cellular locations, bound to the cell wall and in the vacuole, while alkaline invertase is thought to be confined to the cytoplasm (Tang *et al.*, 1996). In addition, high activities of acid invertase are thought to be associated with tissues of high sucrose turnover (i.e. sinks), while activity of alkaline invertase is thought to be predominant in tissues of low sucrose turnover (i.e. sources) (Masuda *et al.*, 1988).

Increases in the various forms of invertase activity have been reported for a variety of biotrophic infections. For a review see Hall & Williams (2000). The majority of such reports have been for acid invertase activity (Whipps & Lewis, 1981; Storr & Hall, 1992; Clark & Hall, 1998; Chou *et al.*, 2000), although increases in alkaline invertase have also been noted (Storr & Hall, 1992). In addition to this, Storr and Hall (1992) and Scholes *et al.* (1994) showed that increases in alkaline and acid invertase activity in response to infection was largely confined to the mesophyll cells.

1.5.2.4 Changes to the apoplast

Epidermal cells are heterotrophic and are therefore dependent upon the underlying mesophyll cells for photosynthates. As plasmodesmatal connections between the mesophyll and epidermis are absent or very infrequent, the movement of photosynthates between the two tissues must be via the apoplast (Hall *et al.*, 1992). The effect of powdery mildew infection on the leaf apoplast solute concentration will therefore directly affect the amount of photosynthate available to the pathogen. In addition, cell-wall invertase may contribute to sugar availability by influencing the composition of transferable carbon compounds in the apoplast.

Using the technique of intercellular washing, it was shown that apoplastic concentrations of both sucrose and glucose increased after 7 days in infected pea leaves (Aked & Hall, 1993a). These results were supported by the findings of Clark and Hall (1998). Such increases in sugar concentration in the apoplast would be advantageous to the invading pathogen as more sugar is available for uptake into the infected epidermal cell and could therefore help to maintain the flow of carbohydrate from host to pathogen. There is some evidence that such increased levels in sugar concentrations may be due to increased membrane permeability (Aked & Hall, 1993a).

1.5.3 Effects on host cell transport processes

As biotrophic fungal infections may affect the ability of normal plant sinks to compete with newly established fungal sinks and thus affect the availability of nutrients to the fungi, it is important to understand the effects of biotrophic fungal infection on host transport processes. It is well established that infection by fungal pathogens leads to increased transport capacity of the host tissue for certain solutes, although the exact nature and location of these activities is unclear. For example, Clark and Hall (1998) reported an increase in the uptake rates for sugars (particularly glucose) and amino acids in powdery mildew-infected pea leaves when compared with healthy tissue, while similar increases in uptake rates for sugars were observed in mildewed wheat leaves (Sutton *et al.*, 1999). More recently, Fotopoulos *et al.* (2003) demonstrated a significant increase in glucose uptake in Arabidopsis leaves infected with *E. cichoracearum* compared with healthy tissue; it was suggested that this was a host response rather than the fungus acting as an additional sink..

The host monosaccharide transporter proteins AtSTP4 and to a lesser extent AtSTP3, show increases in transcript expression levels in response to powdery mildew attack, (Fotopoulos *et al.*, 2003), thus supporting the previously observed increases in glucose transport activity. The role of these transporters in this interaction and the signal transduction pathways involved in their induction remain unclear at present.

1.5.4 Plant membrane transporters in biotrophic interactions

Certain types of host transporter have been implicated in response to powdery mildew interactions; a review of the structure and function of these transporters is required in order to fully discuss their roles. Two types of transporter are discussed here: 1) monosaccharide transporters, which have been implicated in biotrophic interactions and 2) the plant H^+-ATPases which provide the proton gradient used directly by symporters and antiporters. H^+-ATPases may also provide a proton gradient between the apoplast and the EHM that can drive nutrient uptake into the haustoria.

1.5.4.1 H^+-ATPase pumps

The transport of most solutes in and out of the cell is a secondary process, and plants use protons as the coupling ions with which to drive most ion and metabolite transport (Sze *et al.*, 1999; Morsomme & Boutry, 2000). The production of a proton motive force (pmf) allows otherwise energetically unfavourable processes to take place. The pmf is primarily generated by the plasma membrane (PM) H^+-ATPase (Sze *et al.*, 1999; Morsomme & Boutry, 2000; e.g. Buch-Pederson & Palmgren, 2003) which uses ATP hydrolysis to translocate protons to the cell exterior. PM H^+-ATPases are a member of the P-type ATPase family of ion pumps, so called because they form a phosphorylated intermediate during ion translocation (see Palmgren & Harper, 1999; Morsomme & Boutry,

2000). In most cases they have a single 90 -120 kDa catalytic domain (Palmgren & Harper, 1999). Plasma membrane H^+-ATPases differ substantially from the multimeric V-type and F-type H^+-ATPases (Wilkens *et al.*, 2005) in the vacuolar and mitochondrial inner membranes respectively, with respect to their biochemistry, subunit organization, mechanism of action and evolutionary origin. The Arabidopsis genome sequencing project (Arabidopsis Genome Initiative, 2000) has identified 12 members of the family, and in the *N. plumbaginifolia* genome, nine H^+-ATPase genes have been identified (Morsomme & Boutry, 2000). Within the recently sequenced rice genome, ten H^+-ATPases have been identified (Baxter *et al.*, 2003). This agrees with the hypothesis that H^+-ATPases are a multigene family in most higher plants (Arango *et al.*, 2003). The location and physiological function of some of the isoforms in Arabidopsis have been investigated; for example it has been shown that AHA3 is located in companion cells (Dewitt *et al.*, 1991; Dewitt & Sussman, 1995). Other isoforms with observed differential expression include developing seeds (Harper *et al.*, 1994, *AHA10*), pollen grains (Houlne & Boutry, 1994, *AHA9*), and perhaps root epidermis (Palmgren & Harper, 1999 *AHA2*).

1.5.4.2 Monosaccharide transporters

Monosaccharide transporters have also been described in a number of plants (see Büttner & Sauer (2000) and Williams *et al.* (2000) for references). All of these transporters belong to the major facilitator superfamily (MFS) of carrier proteins, with 12 putative transmembrane helices and a large cytoplasmic loop in the middle (Marger & Saier, 1993). The first plant sugar transporter to be cloned was the glucose transporter in *Chlorella kessler* (Sauer & Tanner, 1989), which was first studied by Komor (1973). Homology was revealed with facilitative monosaccharide transporters from mammals(Baldwin, 1993), yeast and H^+ symporters from bacteria (Baldwin, 1993; Henderson *et al.*, 1993; Kruckeberg, 1996; Büttner, 2007). Significant homology was not observed with Na^+/glucose transporters from mammals (Marger & Saier, 1993).

Detailed studies of the kinetic mechanism of monosaccharide transport have been carried out on AtSTP1 using the patch-clamp technique, suggesting the protons and sugar molecules are imported in two different steps (Boorer *et al.*, 1994). Topology models for monosaccharide transporters are largely based on evidence from non-plant sugar transporters (Baldwin, 1993; Kaback *et al.*, 1997). Given the close homology between plant and non-plant transporters, this data is relevant to plant monosaccharide transporters (Büttner & Sauer, 2000).

Monosaccharide transporters have a variety of physiological functions and in Arabidopsis they have been observed in several tissues, for example *AtSTP2*; seeds (Truernit *et al.*, 1999), *AtSTP9;* pollen (Schneidereit *et al.*, 2003) and *AtSTP4*; root tips and pollen grains (Truernit *et al.*, 1996). Most are observed in cells or tissues dependent on the import of photoassimilates, or the expression is increased under conditions of

increased metabolism (Büttner & Sauer, 2000). Monosaccharide transporters are also thought to play a role in the retrieval of monosaccharides that have been lost by passive leakage from mesophyll cells in source leaves (Büttner & Sauer, 2000). Sugar transporters could also be important within plant pathogen interactions, and recently the sugar transporter *AtSTP4* has been identified as having a role in the Arabidopsis/ *E. cichoracearum* interaction (Fotopoulos *et al.*, 2003).

1.6 Microarrays

Plant/ pathogen interactions are complex and dynamic and events occurring during the interaction have proved complicated and difficult to analyse with genetic and biochemical methods (Wan *et al.*, 2002). Knowledge of the transcription profile of both host and pathogen genes will make the identification of key molecular events in the interaction easier to achieve. Several tools are available for global analysis of gene expression, summarised by Donson *et al.* (2002) but they include Serial Analysis of Gene Expression (SAGE), Expressed Sequence Tag analysis (EST), Differential Display (DD) as well as both oligonucleotide and cDNA microarrays. Microarrays have become the standard tool for global analysis of gene expression, due to their simplicity and high throughput (Kazan *et al.*, 2001)

1.6.1 Microarray technology

DNA microarray technology was invented in Stanford University (Schena *et al.*, 1995) using a small set of Arabidopsis ESTs and has since been applied to several other model organisms including *Escherichia coli*, *Saccharomyces cerevisiae*, *Drosophila melanogaster*, *Mus musculus* and *Homo sapiens* (for references see Reymond, 2001). The two main forms of the technology are PCR fragment-based microarrays and oligonucleotide arrays.

The principle behind DNA microarrays is the hybridisation of two populations of cDNA molecules derived from mRNA (probe), to an excess of target. The target may be PCR products of cDNA libraries (cDNA arrays) or oligonucleotides (oligo arrays), or more recently from PCR products from all the predicted genes from a sequenced genome (Gene Specific Tags; GST) bound to the surface of a treated glass slide. The ratios of hybridisation between the populations (normally treated vs. untreated) can be observed by the detecting emission intensity after laser excitation of a different fluorescent marker in each population of cDNA. This marker is incorporated either during or after cDNA synthesis. Given that the amount of a labelled cDNA is proportional to the amount of an mRNA in the original mRNA population, the relative abundances of given mRNAs can be seen by the ratios of fluorescence (Figure 1.5).

The advantages and disadvantages to using either of the targets (PCR fragments or oligonucleotides) have been discussed in detail by Donson *et al.* (2002).

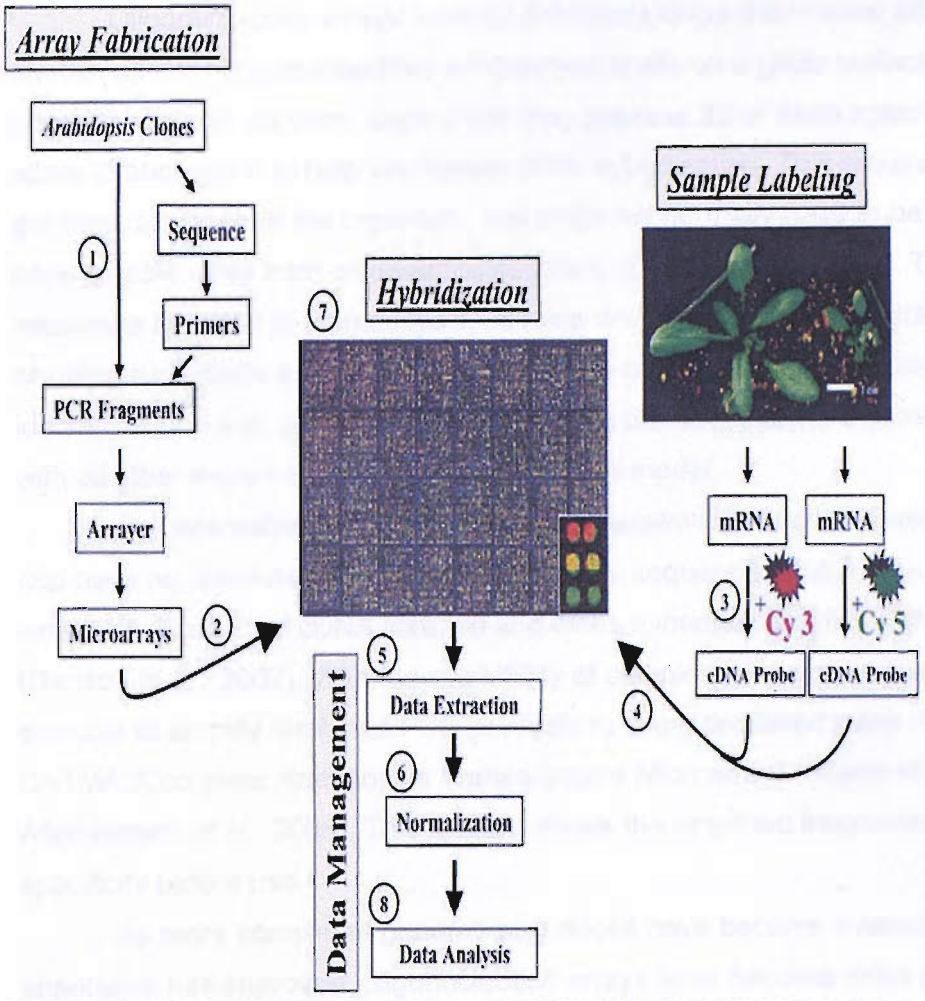

Figure 1.5 Scheme for the generation and interrogation of cDNA microarrays. 1. PCR fragments are amplified from cDNA clones. 2. PCR fragments are purified, quality control-tested on agarose gels, buffer-adjusted and spotted on to coated glass microscope slides by means of a computer-controlled X-Y-Z robot 3. Total or poly (A)+ RNA from both the test and reference sample is fluorescently labelled with either Cy3 or Cy5 nucleotides by reverse transcription. 4. The fluorescently labelled cDNAs are pooled and allowed to hybridize to the array. After hybridization, the array is washed to remove un-hybridised molecules. The amount of fluorescent cDNA that hybridizes to each of the spots is then measured by a fluorescent scanner. Through sequential exposure to excitation wavelengths specific to the two CyDyes. Spectrally characteristic emissions from these dyes are captured with a confocal sensor. Emission intensities are extracted and these are linked to the corresponding clone ID and through extensive laboratory information management systems to experimental details, array information and hybridisation conditions. 5. Data are extracted from microarrays using spot finding software. 6. Data are then normalised using specialised software such as Genespring. 8. Data are then analyzed by ratio-based data mining tools. For oligonucleotide arrays steps 1 and 2 are omitted, and oligonucleotides are synthesised onto the chip directly. Taken from (Donson *et al.*, 2002).

Oligonucleotide arrays such as Affymetrix chips (http://www.affymetrix.com) contain specific oligonucleotides synthesised *in situ* on a glass surface by a photolithographic process. Such chips may possess 20 or more specific oligonucleotide spots to each gene to help counteract cross hybridization. This requires an extensive genome database of the organism, and chips will normally have to be bought in (at considerable cost) from commercial suppliers (Donson *et al.*, 2002). This is due to the resources required to manufacture, develop and validate such an array. The advantages of using such chips are large (and increasing) coverage of the genome, the ability to identify targets that differ by just a single base pair and standardisation and comparability with all other experiments using the same chip model.

The alternative, PCR fragments microarrays are much cheaper to produce initially, and have no absolute requirement for genome sequence data. However they require extensive curation of cDNA libraries and cross-hybridisation of similar cDNAs may occur (Donson *et al.*, 2002). With the availability of complete genome sequences, it has been possible to amplify designed PCR products to every predicted gene model, such as CATMA (Complete Arabidopsis Transcriptome Microarray) (Hilson *et al.*, 2004; Allemeersch *et al.*, 2005). This method allows the amplified fragments to be tested for specificity before use.

As more completed genome sequences have become available and genome annotation has improved, oligonucleotide arrays have become more popular, due to their reliability, standardisation, sensitivity and the possession by manufacturers of a well paid sales force. As other genomes are sequenced, it will soon be possible to have the complete sets of genes of both the host and the pathogen on the same microarray, producing a unique molecular view of the coordinated interaction between the plant and its pathogen.

1.6.2 Microarray analysis

It should be pointed out that the DNA microarray techniques are prone to a number of methodological and interpretive pitfalls (Kazan *et al.*, 2001; Donson *et al.*, 2002; Wan *et al.*, 2002), and caution must be applied when drawing conclusions from microarray data. Both data verification and functional confirmation are usually needed before final conclusions can be drawn.

Microarray data must undergo statistical analysis before conclusions can be drawn, and it is only recently that commercial packages have become available to achieve this. Firstly data needs to be normalised for variations in signal intensity due to factors such as unequal label detection or incorporation (Quackenbush, 2002; Donson *et al.*, 2002). Some strategies work on the principle that overall expression (total intensity) remains constant between the two treatments, others use median values or log

transformations to minimize the intensity dependent error observed in microarray scanning (Quackenbush, 2002; Donson *et al.*, 2002; Wan *et al.*, 2002). Analysis and processing varies between researcher and its complexity is matched to the final purpose of the data, such as publication, public deposition on microarray databases or target identification (Quackenbush, 2002; Donson *et al.*, 2002).

1.6.3 Microarray databases

The enormous quantity of data produced by microarray experiments has posed challenges to the public dissemination of results. Most published microarray data has to be made available as supplementary material, and the co-coordinated analysis of different microarrays is therefore difficult. On top of this there are a variety of different data collection and analysis methods, as well as variation on the amount of information provided with each published microarray (Brazma *et al.*, 2001).

There have been several developments to overcome these problems. The most notable development was the Minimum Information About a Microarray Experiment (MIAME) standards (Brazma *et al.*, 2001) which have been adopted by most leading journals. There are also several databases which allow public access to raw data, provided in a standardised format, such as the Stanford microarray database SMD (http://genome-www5.stanford.edu) (Sherlock *et al.*, 2001) or the Nottingham stock centre (http://nasc.nott.ac.uk).

1.6.4 Applications to plant-pathogen interactions

Microarrays can be used in several ways in plant/ pathogen interactions. The most common use is to define 'regulons' or groups of co-ordinately regulated genes; this allows an inference of function to be given for many genes of unknown function. An example of this is the work carried out by Schenk *et al.* (2000), where 2375 Arabidopsis ESTs were analysed in response to treatments with salicylic acid or jasmonate. The overlap between the pathways was analysed, and two clusters of common expression patterns were observed. Microarrays can also be used as a target identification screen for genes of interest, which are then followed up by in-depth studies of function and physiology. This is useful for research groups with a particular focus on specific aspects of experimental systems; for example identification of important transcription factors. The use of microarrays from a variety of plant pathogen interactions can help distinguish which are specific to that plant/pathogen interaction, and which may be part of the plant defence response.

The availability of both known and unknown Arabidopsis mutants is extremely useful in combination with microarray analyses. An example of the use of defined mutants in plant-pathogen interactions combined with microarray analysis comes from the work of

Maleck *et al.* (2000) on SAR. This study used a cDNA microarray containing 10,000 ESTs representing approximately 7000 genes, or 25-35% of all Arabidopsis genes. Sets of SAR-specific genes could be identified by comparing wild-type plants with mutants unable to mount a SAR response.

1.7 Project aims

The close relationship formed between a biotrophic fungal pathogen and its host is thought to have a profound effect on the membrane processes in both infected epidermal tissue and possibly other tissue types. These changes are thought to result in the abnormal source-sink relationships in an infected host and subsequent loss of plant productivity (Tang *et al.*, 1996; Hall & Williams, 2000). This project aims to examine the effect of powdery mildew on the Arabidopsis transcriptome, in particular focussing on membrane transport proteins. To examine the function of membrane transporter genes which are regulated in response to powdery mildew infection, knockout mutants will be isolated in genes of interest. The phenotype of uninfected mutant plants will be examined, as will the progression of *E. cichoracearum* infection.

Chapter 2

Materials and Methods

2.1 Plant growth conditions

Plants were grown in one of three conditions: 1) a controlled environment growth room, with a 16 hour, 23°C ±1°C light cycle and an 8 hour, 20°C ± 1°C, dark cycle, average irradiance across the growing area of approximately 120 μmol $m^{-2}s^{-1}$; 2) a controlled environment growth room, with identical conditions as 1, but with 12 hour light and dark cycles; 3) a controlled glasshouse environment with a minimum 14 hour, 23°C ± 4°C light cycle and a maximum 10 hour, 18°C ± 4°C dark cycle, with seasonal variation in light intensity. Unless otherwise stated, experimental plants were grown under either condition 1 or 2, while condition 3 was used primarily for bulking seed from stock plants.

All seeds were sown in a soil mixture consisting of 1 part Vermiperl® horticultural vermiculite, 1 part Levingtons F2 compost and 1 part John Innes compost Number 2. Soil was autoclaved at 121°C and 1 bar pressure for 30 minutes. Following autoclaving 280 g of Intercept 5GR (The Scotts Company, UK) was added per m^3 of soil, for the prevention of Scarid fly and thrip attack. The active ingredient of Intercept 5GR is imidacloprid and is non-phytotoxic when used as directed.

Plants were sprayed with the insecticide Dynamec (Merck, UK) for protection against thrips as and when required, following the recommended dosage of 0.03% (v/v). The active ingredient of 'Dynamec' is abamectin, and is non-phytotoxic when used as directed.

2.2 Plant material

2.2.1 Arabidopsis

Arabidopsis seeds were sown directly into 7x7x12 cm (width, depth, and height) pots, between and 3 and 5 seeds per pot, (depending on the anticipated germination rate) and later thinned as desired. To ensure uniform germination, after sowing, the seed were stratified by covering with foil and incubating at 5°C for 48 hours. The foil was then removed and replaced with cling-film to retain humidity for up to 5 days whilst seeds germinated.

2.2.1.2 Kanamycin resistance assay

For determining resistance to kanamycin in mutants, seeds were surface sterilized in 10% (v/v) domestic bleach for 20 minutes before washing three times in sterile distilled water. These seeds were pipetted in sterile water or 0.1% (w/v) agar onto Petri dishes containing 0.8% (w/v) agar, 2.2 g/l MS (Murashige and Skoog), (Duchefa, Denmark), 100 μg/ml kanamycin and sometimes containing 1% (w/v) sucrose, under sterile conditions. The plates were covered in foil then stratified at 5°C for 48 hours. When required,

surviving seedlings were transplanted into individual pots containing autoclaved soil, covered with cling-film for up to 5 days and grown in one of the three described growth conditions. The predicted locations of the T-DNA within these genes are listed in Table 2.1.

2.2.1.3 Phenotypic characterisation of Arabidopsis mutants

Having confirmed seed lines as T-DNA insertional mutants, growth phenotypes of the progeny were studied as the plants grew to maturity. Col-8 plants, the background ecotype used in the creation of Salk T-DNA mutants (Alonso *et al.*, 2003) were grown against the mutant of interest and the segregating wild type plant identified in Chapter 5 at the same time as the mutant. This seed line is used as a control and helps to compensate for undesired point mutations that may have occurred in the original seed stock.

Measurements of the day of 1st bolt, 1st flower or 1st silique were made. A bolt was noted as present if it measured 5 mm or more above the centre of the rosette, a flower was noted as present at the first showing of white petals on developing flower buds, and a silique was noted when the 1st seed was identifiable in the developing silique (in the plant development ontology these growth stages are close to SE.00, IL.03 and FL.00 respectively) (Ilic *et al.*, 2007). On the day that a plant reached these stages, the number of leaves present was counted, the diameter of the rosettes was measured, and the height of the plant was also measured. The *aca12-1* mutant showed no statistical differences from Col-8 or the segregating wild-type in any of these measurements. Statistical significance was measured using a student's T-test ($P < 0.05$).

2.2.2 Squash (*Cucurbita maxima*)

Squash seeds (*C. maxima* L. cv. Table Ace; Moles Seeds, UK) were sown in individual pots grown under growth conditions 1 or 2. After approximately 2 weeks, seedlings were transplanted into 3L pots.

2.2.3 Spring barley (*H. vulgare*)

Spring barley seeds (*H. vulgare* L. cv. Golden Promise) were sown into 9cm pots with at least 5 seed per pot and grown under growth conditions 1 or 2.

2.3 Fungal material

2.3.1 *E. cichoracearum*

E. cichoracearum DC isolate UCSC1 innoculum was kindly provided by Dr. M. Coleman, University of East Anglia, UK. *C. maxima* rather than Arabidopsis plants were used to maintain *E. cichoracearum* as higher fungal yields can be supported. At about 3 weeks post sowing (depending on size), infection of *C. maxima* plants was carried out by

Table 2.1 Summary of T-DNA lines ordered. Potential T-DNA insertional mutants were identified by screening web-based Arabidopsis mutant databases at the NASC (http://arabidopsis.info) and T-DNA express (http://www.signal.salk.edu) against *E. cichoracearum* responsive transporter genes identified in Chapter 3.

Gene Code	Gene Name	NASC ID	Putative location of T-DNA in Gene (from start codon)	
At3g63380	*ACA12*	N598383	1st of 1 exon	718 of 3102 bp
At3g07520	*GLR1.4*	N624603	1st of 4 introns	2nd exon starts at 304 of 2586 bp
		N629955	1st of 5 exons	15 of 2586 bp
At5g46050	*PTR3*	N638430	1st of 5 exons	114 of 1749 bp
		N525186	2nd of 4 introns	3rd exon starts at 333 of 1749 bp
At3g25610	*ALA10*	N524877	3rd of 10 exons	1418 of 3609 bp
At2g18680	*At2g18680*	N501089	1st of 1 exon	151 of 864 bp
At5g40780	*LHT1*	N534566	3rd of 8 introns	258 of 1341 bp
		N536871	3rd of 8 introns	317 of 1341 bp
At3g13790	*AtcwINV1*	N591455	4th of 7 introns	1208 of 1755 bp
		N619499	7th of 7 introns	1676 of 1755 bp

removing infected leaves from a donor plant and gently contacting them with uninfected leaves on a recipient plant.

2.3.2 *B. graminis* f.sp. *hordei*

B. graminis f.sp. *hordei* isolate A6 inoculum was kindly provided by Dr. J. Scholes, University of Sheffield, UK. At between 7 and 14 days post sewing, barley was infected by gently brushing spores *of B. graminis* f.sp. *hordei* onto all leaves, using a fine brush or by shaking infected plants over healthy plants.

2.4 Infection of Arabidopsis with powdery mildew

Arabidopsis grown for infection were grown under short day conditions, to increase the time at which the first bolt was observed, in order to allow for longer time courses and obtain uniform plants with a large rosette. Plants were thinned at 1 – 2 weeks post germination to 1 plant per pot. At the 6 -7 leaf stage, 6 or 7 plants for each time point to be used were inoculated with *B. graminis* by shaking infected barley leaves onto a nylon filter with 40 µM x 40 µM mesh openings (Nitex; Sefar, UK) 2 – 3 cm above Arabidopsis plants, releasing spores, but not mycelia onto the plants below. Microscope slides were positioned under the filter alongside Arabidopsis and later inspected for a rough assessment of both spore number and the amount of hyphae that had passed through the filter. Fungal material was clearly seen landing on Arabidopsis leaves. Equally, Arabidopsis were also inoculated with *E. cichoracearum* by brushing fungal material from living, heavily infected squash leaves where the fungus appeared fresh, onto the same type of filter as described for *B. graminis,* 2 – 3 cm above 9 isolated Arabidopsis, releasing spores but not mycelia onto the plants. Microscope slides were positioned under the filter alongside Arabidopsis, and later inspected for a rough assessment of both spore number and the amount of hyphae that had passed through the filter. Untreated plants were placed in a similar area for the same length of time as was taken for infected plants. Plants were harvested after the infection and at each time point.

2.4.1 Collection of Arabidopsis for subsequent use in RNA preparations

Plants were harvested by removing the whole rosette, cut at the base, just above the soil, separating the roots from all aerial parts of the plant. This was always performed on material that had not yet produced a flowering stem. The whole rosettes were placed directly into liquid nitrogen as quickly as possible (~1 second). No leaf selection was performed.

2.5 Isolation of RNA from Arabidopsis

2.5.1 Precautions taken to limit RNAse contamination

Purified, intact RNA needs to be free of contaminating RNA-degrading enzymes (RNases) that are present within the cells from which the RNA is being extracted and are also possibly present on all equipment. It is important therefore that precautions are taken to prevent contamination by RNases, particularly at stages above 0°C and subsequent to phenol-chloroform extractions. Therefore, all glassware or ceramics used in the isolation and handling of RNA were baked, autoclaved or treated with RNAse inhibitors and sterile disposable microcentrifuge tubes were used. Additional precautions included the autoclaving of all tips, buffers and solutions, which inhibits several types of RNAse. Finally gloves were worn at all times and changed at regular intervals.

2.5.2 Isolation of total RNA

Total RNA was isolated using a phenol/SDS extraction and LiCl precipitation based on the method by Verwoerd (1989). The desired amount (up to 2.5g) of leaf material ground by mortar and pestle in liquid nitrogen to a very fine powder was transferred to a liquid nitrogen chilled centrifuge tube. 10ml/g of tissue of a 1:1 extraction buffer: phenol mix was added. The extraction buffer contained 0.1M LiCl, 0.1M Tris/HCl (pH 8.0), 10mM EDTA and 1% (w/v) SDS. The material was mixed by vortexing for about 2 minutes and 5 ml/g of tissue of pure chloroform was added. The material was again mixed by vortexing for about 30s. Samples were then centrifuged at 5000g for 10 minutes at room temperature, and the aqueous phase collected and transferred to fresh centrifuge tubes. An equal volume of 4M LiCl: collected aqueous phase was added and the solution incubated at -20°C for at least 15 hours. Extractions were then centrifuged at 12,000g for 20min at 4°C for large scale preparations, or 13,200g for 20 minutes at 4°C for small scale preparations. The supernatant was removed and the remaining pellet was then washed with 5 ml 70% ethanol. Extractions were then centrifuged at 12,000g for 5 minutes at 4°C for large scale preparation (those greater than 0.1g and unable to fit into micro-centrifuge tubes) or at 13,200g for 20 minutes at 4°C for small scale preparations. The supernatant was removed, and the wash was repeated with 100% ethanol, centrifuged as before and the supernatant removed. The pellet was then air dried for about 20 minutes. The pellet was re-suspended in 1.5 ml sterile, distilled water. 2.5 volumes 100% ethanol and 0.1 volume 3M sodium acetate (pH 5.2) were added to precipitate the nucleic acid. This was stored at –20°C for at least 2 hours. The RNA was retrieved by centrifugation 12000 x g (9500 rpm) for 20 minutes at 4°C (large scale) or at 13,200g for 20 minutes at 4°C (small scale). After removal of the supernatant the pellet was again washed as before with 70% and then 100% ethanol, before being re-suspended in either RNA storage solution (Ambion, UK), TE buffer (10mM Tris-HCl 1mM EDTA pH 8.0) or sterile distilled water as required. The volume used depended on the size of the pellet, and was typically 100-200

μl for larger preparations or 20 – 30μl for smaller preparations. This was stored at –70°C until needed.

2.6 DNase treatment of RNA

In order to be certain that PCR products were not produced from any contaminating genomic DNA that might have been carried over from RNA extractions, all RNA was treated with DNAFree or DNAFree Turbo (Ambion, UK) according to the manufacturers instructions.

2.7 DNA purification and precipitation

DNA was extracted from plant tissue using the Dnamite kit (Microzone, UK) according to the manufactures instructions.

Certain experiments required DNA to be further purified and/or concentrated. DNA was cleaned and concentrated following a slightly modified version of the method described by Sambrook et al. (1989). Two volumes of 100% ethanol and 0.1 volume of 3M sodium acetate (pH 5.2) were added and the solution was precipitated in –20°C for 1 hour. This was centrifuged at 13,200 g for 20 minutes to pellet the precipitate. The supernatant was discarded and the pellet washed with 70% ethanol, before re-suspending in a small volume (10-20μl) of TE buffer or sterile, distilled water, depending on the intended future use of the DNA.

2.8 Determination of nucleic acid concentration

The purity and concentration of samples of both DNA and RNA were normally determined by spectrophotometry using the NanoDrop (NanoDrop Technologies) which uses the Beer-Lambert law, i.e.

$$[\text{Nucleic Acid}] = OD_{260} \times \text{Extinction coefficient} \times \text{path length}$$

to determine nucleic acid concentration. The purity of nucleic acid samples is calculated using the ratio of the absorbance measurements at 230, 260 and 280nm. Where OD_{260}/OD_{230} and OD_{260}/OD_{280} = 1.8-2.0 the sample is considered pure. A lower ratio may have indicated protein, phenol or ethanol contamination (Sambrook *et al.*, 1989), although pH and ionic strength also affect these ratios (Wilfinger *et al.*, 1997).

For measuring dye incorporation in cDNA samples for use on microarrays, a traditional spectrometer was used, using a quartz micro- cuvette with a 1.0 cm path length, using the following equation

$$\frac{(OD_{550}) \times (\text{total volume})}{0.15} = \text{pMoles Cy3}$$

$$\frac{(OD_{650}) \times (\text{total volume})}{0.25} = \text{pMoles Cy5}$$

2.9 RNA gel electrophoresis

Gel electrophoresis of RNA was carried out as described by Sambrook *et al.* (1989). RNA was separated on a 1.4% (w/v) agarose gel containing 2.2M formaldehyde (from a 37% solution in water), 1 x MOPS buffer (containing 20mM MOPS (pH7.0), 8mM sodium acetate and 1mM EDTA (pH 8.0) overall pH 7.0, prepared as a 10x stock). Samples were loaded onto the gel in two volumes of sample loading buffer (50% v/v formamide, 2.2M formaldehyde (from a 37% solution in water), 1 x MOPS buffer, 25 µM ethidium bromide) and 2 µl loading buffer (50% (v/v) glycerol, 1mM EDTA, 0.5% (w/v) bromophenol blue). Electrophoresis was carried out at 80V for 1-2 hours, depending on the size of the gel. The RNA was then visualized and photographed under UV light.

2.10 DNA gel electrophoresis

Gel electrophoresis of DNA was carried out as described by Sambrook *et al.* (1989). DNA was separated on 1-2 % (w/v) agarose gels made up in 1x TAE (40mM Tris/acetate (pH 8.0) and 1mM EDTA), and 1.3 µM ethidium bromide. 1x TAE was also used as a running buffer. The DNA samples were loaded with gel loading buffer containing 0.25% (w/v) bromophenol blue and 15% (w/v) Ficoll 400 in sterile dH_2O (1µl of loading buffer per 5µl of DNA sample). Separation occurred by electrophoresis at 80 – 100 V for 1-2 hours, depending on the size of the gel and the DNA fragments. The DNA was then visualised and photographed under UV light.

2.11 Purification of DNA from agarose gels

After DNA was separated on an agarose gel as described previously, DNA fragments of interest were cut from the gel using a scalpel. The DNA was purified using the QIAquick PCR Purification Kit (QIAgen, UK) or Wizard SV Gel and PCR Clean-Up System (Promega, UK) following the manufacturers instructions.

2.12 Sequencing of PCR products

All sequencing used in this study was performed by MWG (MWG Biotech). Material was prepared as directed on to the MWG website (http://www.mwg-biotech.com/html/i_support/i_support.shtml) from purified PCR products.

2.12.1 Sequence analysis

Both the TAIR organisation (http://www.arabidopsis.org) and the Munich Arabidopsis Database (http://mips.gsf.de/proj/thal/db/index.html; MAtDB) were used as the source for specific genomic Arabidopsis DNA sequences. The intron-exon boundaries used (including those used to generate genomic organisation diagrams were provided by the MAtDB. EMBL (http://www.ebi.ac.uk/embl), GenBank (http://www.ncbi.nlm.nih.gov/BLAST) and Arabidopsis (see above) sequence databases were searched using the BLAST algorithm (Altschul *et al.*, 1990). Sequence alignments were carried out using CLUSTALW v1.8.2 (Thompson *et al.*, 1994).

2.13 Reverse Transcriptase (RT) reaction

First strand cDNA that was not for use in microarray experiments was prepared from total RNA. cDNA was made according to the instructions provided by the manufacturers of the RT enzyme 'Superscript I' (Invitrogen, UK), using 0.5 µg/µl oligo d(T)20 primers (Invitrogen, UK), 10 mM dNTPs (Promega, UK), 30 units of RNAse inhibitor ('RNAGuard'; Amersham, UK), 0.1M DTT (Invitrogen, UK) and 5x first strand buffer (Invitrogen, UK). Reactions were stored at –20°C.

2.14 Standard Polymerase Chain Reaction (PCR)

Standard PCR (i.e. not quantitative) was normally carried out using cDNA, constructed by reverse transcription reactions (RT-PCR), genomic template or previous PCR products. Reactions were carried out in 0.2 ml PCR tubes using 'BioTaq' DNA polymerase (Bioline, UK), containing 1µl 10x reaction buffer (Bioline, UK), 0.5µl 10mM dNTPs, 0.3µl 50µM $MgCl_2$, 0.2µl BioTaq (5 units/µl), 0.5 µl each of 10mM forward and reverse primers, 0.5-2 µl of template diluted as required (up to 1:50 for RT-PCR) made to a total volume of 10 µl with sterile dH_2O. Larger reaction volumes were scaled from this mix. This mix was also used ready-made 2x mix 'Biomix' (Bioline, UK) and in a standard 10 µl reaction, 5 µl of Biomix was used with 0.5 µl each of 10mM forward and reverse primers and 0.5-2 µl of template diluted as required. This reaction was then made up to a total volume of 10 µl with sterile dH_2O.(Bioline, UK) PCR on genomic DNA sources (e.g. Arabidopsis, *E. cichoracearum*) generally used 1 µl of a 1:10 dilution as template.

2.14.1 Programs

Unless otherwise stated the following PCR program was used, but the exact number of cycles performed was optimised on a sample to sample basis, depending on conditions such as template concentration, quality and thermocycler used.

Reactions were cycled at:

95°C for 5 minutes

95°C for 30 seconds
X°C for 1minute
72°C for 1minute
} 18 – 40 cycles

72°C for 10 minutes (optional)

Where X°C is the annealing temperature shown in section 2.14.2

2.14.2 Primers

Many PCR primers were used during this study (Table 2.2) Notes on specific reference gene primers are also provided.

2.14.3 Reference gene primers

To amplify actin products from Arabidopsis, oligonucleotide primers were used which had previously been designed corresponding to a highly conserved amino acid region found in (10) known actin genes identified in Arabidopsis (McDowell *et al.*, 1996), and a region unique to Actin II. Using the published sequences of actin II the predicted size of an amplified PCR fragment from spliced template with these primers is ~200bp (un-spliced 300bp).

To amplify 40S S16 component products from Arabidopsis (healthy and infected with *E. cichoracearum/B. graminis*), oligonucleotide primers were designed using the published sequences of the Arabidopsis 40S S16 component, the predicted size of an amplified PCR fragment with these primers is 435bp on either cDNA or genomic DNA as there is no splicing of this gene.

To amplify Triose Phosphate Isomerase (TPI) products from Arabidopsis (healthy ad infected with *E. cichoracearum/ B. graminis)* oligonucleotide primers previously designed for use in real-time PCR (Schiott & Palmgren, 2005) were used. The predicted size of an amplified PCR fragment with these primers is 508 bp on cDNA, but they span an intro/exon boundary, so do not produce a product on genomic DNA.

2.15 Quantitative or real-time Polymerase Chain Reaction (PCR)

Real-time PCR was normally carried out using cDNA constructed by reverse transcription reactions (RT-PCR). Reactions were composed of 6 µl SYRB green 2x mastermix (containing the modified *Thermus brockianus* DNA polymerase, nucleotides and the SYBR green fluorescent dye) 0.3 µM forwards primer, 0.3 µM reverse primer, 1- 2 µl of diluted cDNA (normally a 1:25 dilution) and made up to 12 µl total volume. Each

Table 2.2 Sequences of primers used in this study.

Primer Name	Primer Sequence (5' – 3')	Annealing Temperature used (°C)
ActinF	GGT AAC ATT GTG CTC AGT GGT GG	56
ActinR	CTC GGC CTT GGA GAT CCA CAT C	
40sF	GGC GAC TCA ACC AGC TAC TGA	56
40sR	CGG TAA CTC TTC TGG TAA CGA	
NPTIIF	GAG GAT CGT TTC GCA TGA TT	53
NPTIIR	ATA CTT TCT CGG CAG GAG CA	
Lba1	TGG TTC ACG TAG TGG GCC ATC G	66
ACA12F	AGT GCG GAT GTA TTA TCC TAG A	52
ACA12R	GGT AGA CTA CTG ACC AAG GAT G	
GluR1.4F	GGG CTA GAG AAC AAA AGC TTC A	53
GluR1.4R	CGA TTG TCC ACC AAC AAG TG	
PTR3F	AAG GCA ATC CTG TTC GAA GAT CCA TT	55
PTR3R	CCA ACG TAA GCA CCC AAG AT	
ALA10F	CTT GTC CAT GGT CAT TGG TG	52
ALA10R	GCT GGT TGT CCA GAG AAG GA	
At2g18680F	GAT TCC TTG GCA GCC AAA T	56
At2g18680R	CGA TAT GAC CAT AGA TAA GTT CCA AAC	
LHT1F	ACT CCC TTA CGC CAT GTC TC	55
LHT1R	GAT TTG CCT CCA GTG ACC AT	
TPIF	TTT CAC TGG TGA AGT GAG TG	57
TPIR	GTC GAT AAA CTC AGG CTT TA	
PR1-likeF	ACA ACC AGG CAC GAG	54
PR1-likeR	CAA GTC ACC GCT ACC CCA G	
At2g43570F	TAG CTT CGG TGC TTC CAT CT	53
At2g43570R	GCA CAT GGG AAC TCT GGT TT	
At1g26380F	GCA ATC CAA GCG GTG AGA CAG	53
At1g26380R	GAT AAA AGA GGG GGG AAT A	
GLUTSF	TCT CCT CGT TTC TTC CCA GA	54
GLUTSR	AGA CTT TGC TTG AGC TTC ACC	
At3g28270F	GAA ACT TGT AGA TAA TGC TAT TGA CCA T	54
At3g28270R	ACG GTT GAT CTT TTG CAG AAC T	
PP2CF	GCA ACC AGG AAG CTG TTG AT	53
PP2CR	GCC TTC TTA CAC CAG GAT GG	
SEN1RTF	CAT AAT CGG TTG TGA GAG CGG T	55

SEN1RTR	TTC TAC CGG CAG CTC ATT CT	
NAR2-likeF	GGT GCA CTT GAT GTC ACC AC	53
NAR2-likeR	ATC GAG CTT AGC GTC CAT GT	
At2g18690F	CCG TGG AAA ATA TCG GTT TC	53
At2g18690R	CGA TAT GAC CAT AGA TAA GTT CCA ATA	
PTR20F	AGC AAA AGG AAC AAA TGT GCT T	54
PTR20R	TCG CAA GGC GAT GGC TTC	
PTR36RTF	ACT CGG TCG CTA CAA GAT GG	53
PTR36RTR	AAC TGG TGT CCG TTT GCT TC	
At3g20300F	GGA GAG ACT CCA AGG TTA GTT GAT AG	55
At3g20300R	TTC CTG ATC TAT TGT TCT CAA AGT AAT T	
AtcwINV1F	AAA GTC GCC GTT AAA TCC TCT CAT	56
AtcwINV1R	GTT CTA TCA CAT CCG CTT TCT CCA	
AtcwINV1F1	CGA CAT ACG GAG CTT TTG TG	53
AtcwINV1R1	GAC AAC CAA ACA AAG TGG ACC	
PP2AF	TAA CGT GGC CAA AAT GAT GC	60
PP2AR	GTT CTC CAC AAC CGC TTG GT	
At5g28400F	GCT GCA GAA GGT TTT GGA AG	53
At5g28400R	CTC GAC CCC ATT TTC TGC TA	
GPI1F	GGA GTC CTC AGT GGG AAG AG	53
GPI1R	GCC ACA TGA GCT GAT GGA AT	
At2g02810	TTT CTT GTC GCT GGA GGA GT	54
At2g02810	GAC AAT GGA TTT CCG CTC AT	
DEADF	ATT TCC CGG ATT CAG CTT CT	52
DEADR	GGC ACA TCT TGT TCC GTG TA	
FBOXF	CCA CGA TGG TGG GAT TTA CT	53
FBOXR	GAC TAG GGG CAC GTC GAA TA	

biological sample was measured in triplicate on each plate to minimise technical errors. Large mastermixes of reactions were prepared and were pipetted (without template) into a 96-well plate (MJ research, USA) using a repeating pipette (Distriman, Gilson, France). Template was then added to wells individually before 0.2 ml optical grade flat caps were placed over the wells. The 96-well plate was then mixed by vortexing before spinning at 3000 RPM for 1 minute, and placed on ice. The reaction was then transferred to a real-time thermocycler (MJ research).

Reactions were cycled at:

95°C for 10 minutes

95 °C for 30 seconds
x°C for 30 seconds
72°C for 30 seconds
Data acquisition (Plate Read)
} 45 cycles

72°C for 10 minutes

50°C 10 seconds
Increment 0.2 °C
Data acquisition
} 225 cycles

Hold 4 °C

Where X°C is the primer specific annealing temperature given for each primer pair (section 2.14.2). The second set of cycles produces a melting curve, allowing the determination of the T_m of any products formed, and how many products were formed. A single T_m should be visible for each PCR, more than this indicates unwanted secondary products (e.g. PCR from genomic contamination or primer dimers).

2.15.1 Real-time PCR primer design

Primers for real-time PCR have different design requirements to those for standard PCR. Primers were designed using Primer3 (e.g. http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) to produce products under 200 bases long. Primers were blasted were checked against the whole Arabidopsis genome for potential miss-priming between family members for each gene. Primers were designed to avoid the formation of primer dimers and to have melting temperature of within 1°C of each other.

2.15.2 Calculation of Real-time PCR efficiency

One of the variables required for the estimation of gene expression is the efficiency of the PCR for each primer pair and template type (i.e. cDNA, genomic DNA, PCR product) (section 2.15.2). To calculate the efficiency of PCR dilutions of cDNA template (the same type as the sample) were prepared. The concentration of the template

should not affect the efficiency value obtained, provided the concentration is within the active range of the instrument (Opticon 2; MJ Research, USA) and there are no reaction inhibitors present in the sample that are being diluted (e.g. phenol, ethanol). If the concentration of the template is not within the active range of the instrument, it produces erroneous data that will have been noticed during the analysis stage (2.15.4) and the dilutions repeated with an appropriate template concentration.

A series of templates dilutions, 1:5, 1:10, 1:20, 1:30 and 1:60 were prepared in triplicate for each set of primers. The C_t value is the fraction value of the cycle (e.g. 17.895) at which fluorescence can just be observed by the opticon2, as determined by overlaid plots of all real-time traces for that plate. The cycle threshold (C_t) value for each of the dilutions was plotted against the Log [cDNA], and the efficiency of the primer pair could be calculated using the slope of this graph from the following equation (Pfaffl, 2001):

$$\text{Efficiency} = (e^{(-1/\text{slope})}) \text{ (where the slope is expressed as a function of ln)}$$

2.15.3 Real-time PCR analysis

To calculate the relative expression of a gene, a suitable housekeeping gene (or genes) was chosen and the C_t value was determined for both the control and treatment of interest (healthy and infected) of both the candidate gene and the housekeeping gene (reference gene). Once the C_t value and the reaction efficiency are known, then the relative expression can be calculated as follows (Muller *et al.*, 2002):

$$\text{Relative Expression} = \frac{(E_{target})^{C_t}}{(E_{reference})^{C_t}}$$

Where E is efficiency, C_t is the cycle threshold, target is the gene of interest, reference is the housekeeping gene, control is the sample that all others are being measured to (i.e. 0 day healthy) and sample is each sample in turn (i.e. this equation is repeated for all data points). This equation determines the relative expression of the candidate gene by normalisation to both the control treatment and to the reference gene. When averaging across more than 1 reference gene, the expression ratio is calculated separately for each target gene and an average taken.

2.15.4 Transgene copy number determination

The number of transgenes in a given insertional mutants was determined by real-time PCR quantification of the amount of *nptII* the kanamycin resistance gene in comparison to the amount of a gene of known copy number (Ingham *et al.*, 2001). In this

case the gene at2g02810 was used as a gene of known copy number, and the *nptII* primers described in 2.15.2 were used to amplify *nptII.*

2.16 mRNA extraction

mRNA was prepared from total RNA (Section 2.5) using the PolyATtract system (Promega, UK). Briefly the kit works by binding RNA containing a poly-A sequence, to a magnetic bead joined to a small fragment of nucleotide containing a poly-T region. Samples are then washed in SSC and are the beads are retained by a magnetic system. The protocol was used as per the manufacturers instructions, with the exception that, on the manufacturers recommendation, a 0.2x SSC wash was used instead of a 0.1x SSC wash, as plant mRNA typically contain shorter poly-A regions than mammalian mRNA, for which the system was originally designed.

2.17 Arizona Microarrays

The Arizona microarrays that were performed in Southampton were performed on a comparison of RNA from a healthy Arabidopsis vs. *E. cichoracearum* infected Arabidopsis or a comparisons of RNA from *E. cichoracearum* vs. *B. graminis.* Samples from these plants was prepared as described in 2.4.1 i.e. whole rosette from plants with no flowering stems. There was no leaf selection.

2.17.1 Production of cDNA

2μg of mRNA was prepared per sample (2 samples per array), (see section 2.16) into a 20μl volume of dH_2O. This was incubated with 1μl of random primer (0.5μg/μl), 1 μl of oligo d(T)20 (2.5 μg/μl) primer at 65°C for five minutes and chilled on ice for two minutes. 1 μl 50X aa-dNTP (25 mM dATP, 25mM dCTP, 25 mM dGTP, 10 mM TTP, and 15 mM amino-allyl dUTP (Sigma, UK)), 8 μl 5X First Strand Buffer (Invitrogen, UK), 4 μl 0.1M DTT (Invitrogen), 1 μl RNAguard (Amersham, UK), 2 μl Superscript III (Invitrogen, UK). This was incubated at 2 hours at 47 °C. After this another 1 μl of Superscript III to each sample and samples were left to incubate overnight at 47 °C. The reaction was stopped by adding 10μl 1M NaOH and incubating at 65°C for 10 minutes, and neutralized by adding 10μl 1M HCl and 200μl 1M TE buffer (pH 7.5). Unincorporated dNTPs, were removed using the QIAquick PCR Purification Kit (QIAgen, UK), as per the manufacturers instructions, except that a t the final stage purified dNTPs were eluted with 0.1 M $NaHCO_3$ (pH 9.0) rather than dH_2O. This was to obtain the correct pH for the cDNA labelling.

2.17.2 Production of CyDye labelled cDNA

Each purified cDNA sample was added to an aliquot of dried CyDye ester (Amersham, UK) one containing Cy3, the other Cy5. These were incubated at room

temperature, in the dark for 1 hour. Unincorporated CyDye, were removed using the QIAquick PCR Purification Kit (QIAgen, UK), as per the manufacturers instructions, except that before use (as per the manufactures optional instructions) 30 µl of 3M NaAc (pH 5.2) was added to each sample, to reduce the pH to a point where the column in kit is able to bind the cDNA.

2.17.3 Arizona microarray preparation

The oligonucleotide spots on Arizona microarrays must re-hydrated and cross linked to the glass slide before the array can be used. This was done (as per instructions at http://www.ag.arizona.edu/microarray) by holding the slide 1 -2 cm above a 65°C waterbath, and placing the slide on a 65°C heat block to snap dry. This was performed five times. Oligonucleotides were then cross-linked by exposing the slides to 180mJ in a UV cross-linked (Stratalinker, Stratagene, UK). The slides were then washed in 1% SDS for 5 minutes at room temperature, and 100% Ethanol for 30 seconds at room temperature, with gentle shaking. The slides were then dried by spinning at 1000 rpm for 2 minutes. Slides were then stored in a light proof box under cool, dry conditions.

2.17.4 Array hybridisation and washing

The two samples of labelled cDNA were added together and concentrated to a total volume of less than 45 µl in a vacuum centrifuge (Eppendorf, UK). Added to this was 5.5 µl 20x SSC, 3.3 µl of liquid block (Amersham, UK) and 0.5 µl 10% w/v SDS, and this was made up to 55 µl with dH_2O and mixed thoroughly. The entire mixture was denatured at 95 °C for two minutes before being chilled on ice for one minute. This mixture was then applied by pipette to the cross-linked slide, covered with a lifterslip (Eerie Scientific, USA) and placed in a watertight chamber in a waterbath (pre-heated to 55 °C) for 8 – 12 hours.

Following hybridisation, the lifterslip was released by immersing the slide in 2x SSC, 0.5% w/v SDS preheated to 55°C. The slide was then removed and immersed in 2x SSC, 0.5% w/v SDS preheated 55°C for five minutes, and then in 0.5x SSC at room temperature for five minutes and finally in 0.05x SSC @ RT for five minutes. After washing, the array was dried by spinning in a centrifuge at 500 RPM for three minutes.

2.17.5 Microarray scanning and data analysis

Hybridized arrays were scanned with an aQuire laser scanner (Genetix, UK). Spots were mapped and their intensities measured using Array-Pro (MediaCybernetics, USA). Spots are automatically flagged as present or absent by the software based on the background fluorescence intensity in the local area. The data set is normalised in Genespring using the "Locally Weighted regression and Scatterplot Smoothing" (LOEWESS) process (Cleveland, 1979), which manipulates the data on the principle

that there should be no relationship between the intensity of a spot (as measured by the product of the intensities of both channels) and the ratio of the two channels, using the Genespring program (Agilent, USA).

2.17.6 Sources of publicly available data

Two sets of publicly available microarray data were used in this study. The first, a set of data consisting of a time series of *E. orontii* infected Arabidopsis. The time points examined were 6, 12, 18, 24, 48, 72, 96, and 120 hpi. This data set is stored on the TAIR website (www.arabidopsis.org), TAIR accession: ExpressionSet:1008031468. Work from this data set has not currently been published, the outline of the experimental design is described as "responses to E. orontii infection were assayed in wild-type Col-0 plants at 6, 12, 18, 24, 48, 72, 96, and 120 hours after inoculation of adult leaves. Inoculation was done via settling tower, using 10 day old E. orontii cultures. Leaves number 7 to 10 were selected for profiling". The experimental conditions used for this experiment are shown in Table 2.3, and there wee three biological replicates for each condition i.e. 3 replicates of 6 hpi (uninfected), 3 replicated of 6 hpi (infected) etc.

The second set of data came from an experiment comparing *E. cichoracearum* infected arabidopsis Col-0 plants to callose synthase mutants at 72 hpi. This data is stored on the GEO website (http://www.ncbi.nlm.nih.gov/projects/geo) GEO accession: GSE431. Only a subset of the arrays in this experiment were used: those solely concerned with col-0, rather than callose synthase mutants. CU = Col-0 uninfected, CI = Col-8 infected (GSM6572: CU1, GSM6571: CU2, GSM6544: CU3, GSM6227: CU4, GSM6573: CI1, GSM6574: CI2, GSM6575: CI3, GSM6576: CI4). The experimental conditions used are described in the publication corresponding to this data {Nishimura, 2003 432 /id}.

2.17.7 Analysis of publicly available data

The publicly available data has already been normalised. In both cases using the MAS5.0 software. The *E. cichoracearum* and the *E. orontii* dataset had been normalised using a target intensity of 500. To further examine these datasets, a student's T-Test was performed on the datasets to determine the genes for which the control and treatment were significantly different ($P < 0.05$). Only these genes showing a significant change between experiments were compared when looking at the correlation between treatments.

2.18 Microscopy

2.18.1 Fluorescence microscopy of fungal structures

Fluorescence staining of fungal structures was performed with 3,3'-dihexyloxacarbocyanin iodide (DiO6) as described by Duckett and Read (1991). Inoculated leaves were detached and immersed for 2-3 minutes in freshly prepared stain

Table 2.3 Growth conditions used in ExpressionSet:1008031468

Condition type	Name	Value	Duration	Variable
Fungi	*Erysiphe orontii*			yes
Harvesting	Time after treatment			yes
Growth media substrate	Promix BX		4 weeks	no
Humidity	Average daily humidity	70%	4 weeks	no
Light	Light intensity	120 uE m-2 s-1	4 weeks	no
Light	Light source	Fluorescent	4 weeks	no

containing 50 μg/ml DiOC6 in 100% ethanol. The stained leaves were blotted to remove surface liquid, placed on a microscope slide with the adaxial surface uppermost, and viewed by epifluorescence (450-490nm excitation filter and a 526nm barrier filter) under a Zeiss epifluorescent microscope through a Plan10x, 20x or 40x objective lens. Fungal structures including conidia, hyphae and conidiophores stained bright green/yellow, and the presence or absence of red fluorescence from chloroplasts in underlying healthy palisade mesophyll cells indicated whether necrosis had occurred in these tissues.

2.18.2 Measurements of *E. cichoracearum* colonies

The maximum distance of hypha from the originating spore (or alternately put, the longest fungal hypha) was measured using fluorescently stained leaves and colonies as described in 2.17.1. A digital photo was taken of each colony to be measured. Colonies were selected in strict order from the leaf tip to the petiole, using a systematic visualisation of the leaf, from tip to petiole. The originating spore was identified in each image, either by eye, or occasionally by deduction from the pattern of hyphal growth. The distance between the spore and the longest hypha was measured using image analysis software (ImageJ, NCBI, USA) using the line measuring tool. A series of straight lines were drawn (using the software) along the image of the hypha, from the spore to the tip. The length of these lines (initially in pixels) was calculated using the image analysis software and calibrated to nm actual distance using a graticule. In cases where it was not initially clear which hyphal tip was the farthest from the originating spore by eye, all hyphae that might be the longest hyphae were measured and the length of only the longest used.

2.19 Source of seed stocks

All seed stocks were obtained from the NASC. Salk mutants originally came from the genome wide mutagenesis program (Alonso *et al.*, 2003).

2.20 Sources of reagents

Chemicals not indicated in the text were obtained from Sigma Chemicals, UK, Fisher Scientific, UK, and GibcoBRL Life Technologies Ltd., UK. Molecular biology enzymes were from Promega, UK and Invitrogen, UK. All primers were obtained from MWG Biotech, Germany or Sigma-Genosys, UK.

Chapter 3

Microarray Studies of Powdery Mildew Infection

3.1 Introduction

3.1.1 Microarrays in plant pathogen studies

Transcriptional regulation is considered to be the primary point of regulation in higher eukaryotes (Lewin, 2000), and plays a key role during the activation of plant defence responses (Rushton & Somssich, 1998; Eulgem, 2005; Jalali *et al.*, 2006). DNA microarrays are excellent tools to investigate the transcriptional response to powdery mildews. Although microarrays obviously fail to detect many post-transcriptional events (Kazan *et al.*, 2001), they are a standard tool (Reymond, 2001) well suited for the large scale study of plant pathogen interactions (Kazan *et al.*, 2001) and have been the subject of many reviews both in terms of technology (Donson *et al.*, 2002) and interpretation (Quackenbush, 2002) as well as applicability and limitations (Reymond, 2001; Kazan *et al.*, 2001; Wan *et al.*, 2002).

Many studies have highlighted the changes in transcription during pathogen infections, using a wide variety of pathogens, such as *Agrobacterium tumefaciens* (Ditt *et al.*, 2006), *Pseudomonas syringae* (Thilmony *et al.*, 2006), *Fusarium graminearum* (Boddu *et al.*, 2006) and the powdery mildews *B. graminis* (Both *et al.*, 2005) and *E. cichoracearum* (Zimmerli *et al.*, 2004). Other studies have used microarrays to help determine signalling pathways and co-regulated genes, using compatible versus incompatible interactions, knockout mutants, and transgenic plants which over-express transcription factors. An example of this is a study comparing virulent and non-virulent strains of *P. syringae* (Tao *et al.*, 2003). Other examples include using transgenic over-expressers to identify targets for abscisic acid (ABA) responsive transcription factors (Abe *et al.*, 2003); using *EDS1/PAD4* mutants to define defence components that are EDS1/PAD4 dependent (Bartsch *et al.*, 2006) and finally the description of the SAR transcriptome (Maleck *et al.*, 2000).

3.1.2 Microarray platforms

There are many systems that have been used for microarray analysis of plants. They include the Arabidopsis Functional Genomics Consortium microarrays (8000 genes, from PCR of cDNA of genomic DNA) (Wisman & Ohlrogge, 2000), Affymetrix GeneChips (Redman *et al.*, 2004) (the full Arabidopsis genome, oligonucleotides), CATMA arrays (Crowe *et al.*, 2003) (the full Arabidopsis genome, PCR from genomic DNA) and Arizona arrays (Pylatuik & Fobert, 2005) (full genome oligonucleotide arrays). Several comparisons of the systems have been conducted in the past (Coughlan *et al.*, 2004; Allemeersch *et al.*, 2005; Pylatuik & Fobert, 2005), with varying results. In all three cases

the authors have played a role in developing one of the tested platforms, and often the study reveals that the microarray platform they have an interest in is as good as or better than the other platforms tested. It might be concluded that the testing procedures are designed to reveal the best aspects of a given platform. This makes it difficult to make an objective decision about the platforms. However, a search of the GEO database, (the largest depository of microarray data (Barrett *et al.*, 2007) reveals that there are more Arabidopsis datasets using the Affymetrix Genechip system than any other single platform. This greatly aids comparisons between different experiments, as data from the same system can be directly compared. This is because the probes will be identical (each different platform uses a different probe or probe set for each gene), each of which will have slightly different specificity) and the process of producing GeneChip data is highly standardised.

3.1.3 Microarray analysis

There are now many web-based resources for plant (mainly Arabidopsis) expression data. Most major journals require microarray data to be made publicly available. In addition to these resources, large-scale projects such as the AtGenExpress project (http://www.uni-tuebingen.de/plantphys/AFGN/atgenex.htm) and AFGC project (http://www.arabidopsis.org/portals/masc/AFGC/RevisedAFGC/site1L.htm) have delivered data as a community resource for a wide variety of biotic, chemical, hormonal, stress, developmental and tissue-specific treatments. These data sets are available for all researchers to use. Several resources are also available for data analysis and data mining, such as the Botany Array resource (Toufighi *et al.*, 2005) which archives data and contains tools for comparing expression between treatments, looking at promoter regions and tissue specific distribution. Also available is Genevestigator (Zimmermann *et al.*, 2005) which provides some of the same functions but also provides tools for looking at many genes at once and single genes at various developmental stages.

When obtaining an expression ratio for a gene from a microarray, the signal strength of one condition (e.g. infected) is divided by the signal strength of another (e.g. healthy). However in some cases this is not possible; a gene may be totally undetectable in one condition thus giving no signal and no ratio. This may be a situation where the expression of a gene has gone from being 'on' to 'off' or *vice versa*, or it may be that it is expressed in lower than detectable amounts. This situation can arise between microarray datasets from different studies, where the sensitivity varies between the precise protocols used.

3.1.4 Real-time PCR

The advent of real-time PCR and real-time reverse transcription PCR (real-time RT-PCR) has dramatically changed the field of measuring gene expression (Wong &

Medrano, 2005). Reactions are characterised by the cycle at which the target amplicon is first detected (see Materials and Methods). This value is referred to as cycle threshold (Ct), the time at which fluorescence intensity is greater than background fluorescence. The greater the quantity of target DNA in the starting material, the faster a significant increase in fluorescent signal will appear, yielding a lower Ct (Heid *et al.*, 1996). Real-time PCR has been shown to have a lower level of variation in determining gene expression, when compared to traditional end-point methods (Schmittgen *et al.*, 2000). There are many different methods of analysing real-time data, with different methods of normalisation, or expression quantification (Pfaffl, 2001; Bustin, 2002; Muller *et al.*, 2002; Marino *et al.*, 2003; Ramakers *et al.*, 2003; Wilkening & Bader, 2004). This work uses a form of relative quantification, where the expression of a target gene is compared to one or more reference genes, thought not to change during the course of the experiment (Muller *et al.*, 2002). Determining such a gene is not a trivial process, but a recent study conducted on Arabidopsis microarray data, identified a number of genes where the expression was invariant in response to almost all experimental conditions examined in public databases (Czechowski *et al.*, 2005).

3.1.5 Chapter aims

The aim of this chapter is to describe and confirm the transcriptional response of Arabidopsis to several powdery mildews, using publicly available microarray data. Focusing on *E. cichoracearum*, the species of powdery mildew used later in this study, it will identify other treatments with similar gene expression patterns, and identify responsive genes; breaking them down into functional groups in order to better understand the physiology of *E. cichoracearum*-infected Arabidopsis. Transcription factors with likely roles in *E. cichoracearum* infection will be identified. It will also confirm the regulation of several transporter genes, which may have a role in the infection process.

3.2 Results

3.2.1 Identification of Arabidopsis genes responsive to powdery mildew infection by data mining of published microarray experiments

Arabidopsis genes changing in expression in response to powdery mildew infection were identified using a bioinformatics analysis of microarray datasets from public databases. The analysis approach is outlined in Figure 3.1. Brief descriptions of the known or putative functions as well as the abbreviations of genes that are studied in this chapter are shown in Table 3.1. This information has been drawn from TAIR (http://www.arabidopsis.org), PlantsT (http://plantst.genomics.purdue.edu) and published literature. Two datasets, both conducted on Affymetrix full genome microarrays, contained potentially useful data: a dataset from the National Science Foundation 2010 project, outlined by Ausubel (2002), and a dataset by Nishumura *et al* (2003). The first was a time series containing Arabidopsis Col-0 plants that were infected with *E. orontii*; rosette leaves were harvested at 6, 12, 18, 24, 48, 72, 96 and 120 hours post infection (hpi). The second contained Arabidopsis Col-0 plants infected with *E. cichoracearum* where rosette leaves harvested at 72 hours post infection (Nishimura *et al.*, 2003). The present study has analysed (see Materials and Methods) both datasets and calculated expression changes and compared the results (Figure 3.2). The correlation (R) between the expression ratios of genes is a measure of the strength and direction of the correlation between the patterns of expression from one treatment to another. The correlation squared (R^2) is a measure of the closeness of the data to the line of best fit (as determined by the least squares method). What constitutes a strong correlation can be a subjective judgment. For instance, in a study of infection of Arabidopsis by the fungal pathogen *Colletotrichum hugginsianum*, compared to hormone treatments, an R value of 0.513 was stated as a "large correlation" (Narusaka *et al.*, 2007). The corresponding R^2 value of 0.263 does not seem large, but it does suggest that about ¼ of the variation between the two treatments was explained by the treatment, (however, although correlation is required for proof of cause, it is not of itself proof of cause).

In the case of *E. cichoracearum*-infected plants harvested at 72 hpi and *E. orontii*-infected plants harvested at 72 hpi, the correlation between regulated (>1.5 fold-change in either treatment) genes is so low ($R^2 = 0.05$) as can be considered to be non-existent (Figure 3.2A). The R^2 value of up-regulated gene expression ratios in the same comparison is moderate $R^2 = 0.2092$ (Figure 3.2B), and for down-regulated genes is again so low as can be considered to be non-existent (Figure 3.2C) $R^2 = 0.0027$.

For *E. cichoracearum*-infected plants harvested at 72 hpi and *E. orontii*-infected plants harvested at 120 hpi, the correlation between all regulated (1.5 fold-change) genes is high enough (Figure 3.3A), $R^2 = 0.43$ that the value of X (expression ratio of a gene in *E. cichoracearum*) can be considered to be a fair predictor of Y (expression ratio of a gene in *E. orontii)*. The R^2 value of up-regulated gene expression ratios in the same comparison

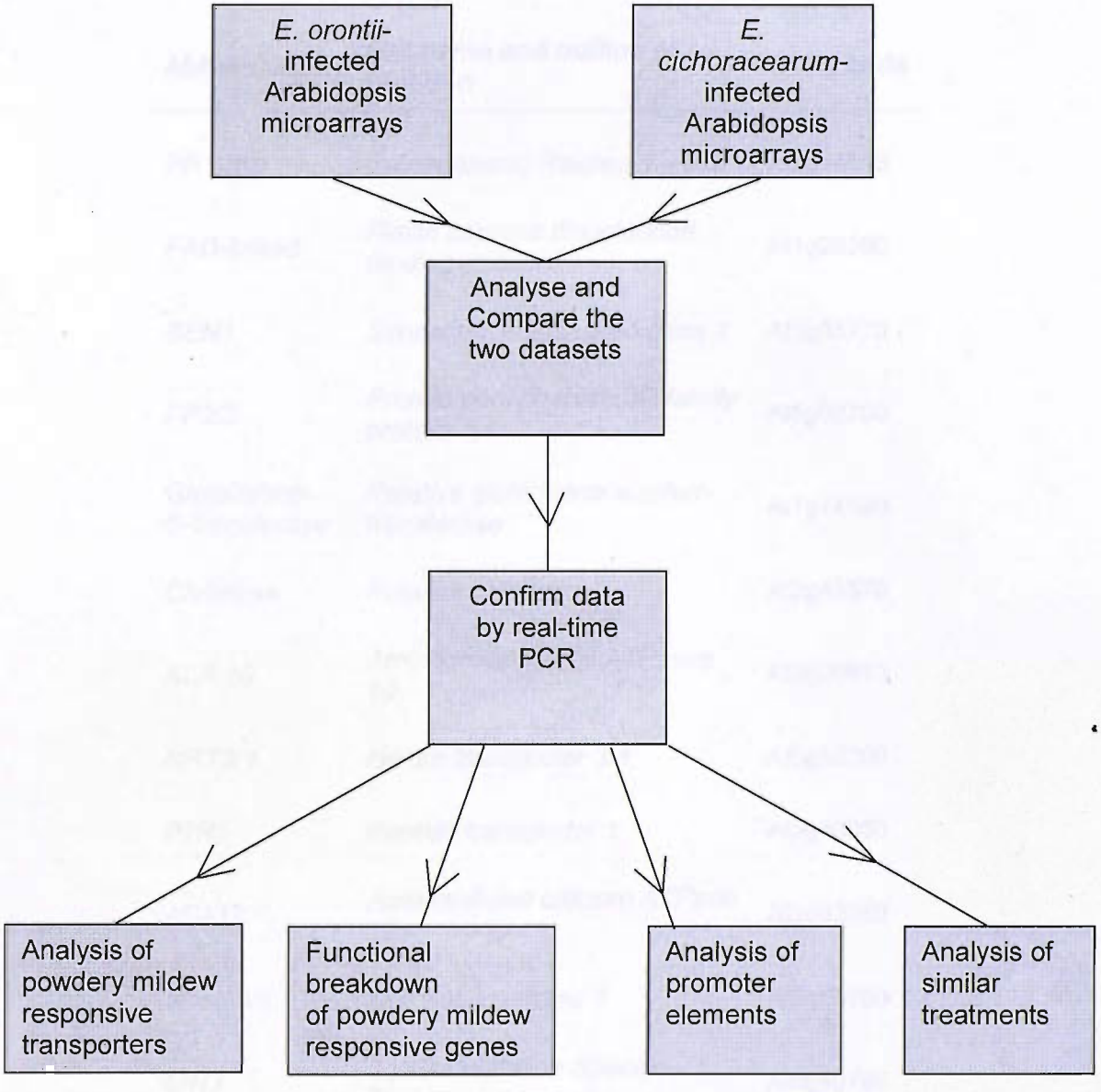

Figure 3.1 Experimental scheme for comparison and analysis of microarray data. After initial statistical processing, the two different sets of microarray data can be compared to determine how closely related the responses are to the powdery mildews *E. cichoracearum* and *E. orontii*. The microarray data will be confirmed by real-time PCR, allowing a comparison to infections established at Southampton and more detailed conclusions to be reliably drawn from the data. The data can then be analysed in a variety of ways, such as promoter analysis or functional breakdown.

Table 3.1 Abbreviations and known or predicted functions of Arabidopsis genes investigated in this study. The gene names were obtained from published records, transporter databases listed in 3.2.6 or TAIR (Rhee *et al.*, 2003).

Abbreviation	Full name and outline of function	Gene code
PR1-like	*Pathogenesis Related 1 – like*	At2g14610
FAD-linked	*Flavin adenine dinucleotide binding domain*	At1g26380
SEN1	*Senescence associate gene 1*	At5g35770
PP2C	*Protein phosphatase 2C family protein*	At5g02760
Glutathione-S-transferase	*Putative glutathione-sulphur-transferase*	At1g74590
Chitinase	*Putative chitinase*	At2g43570
ALA 10	*Aminophospholipid ATPases 10*	At3g25610
NRT3.1	*Nitrate transporter 3.1*	At5g50200
PTR3	*Peptide transporter 1*	At5g46050
ACA12	*Auto-inhibited calcium ATPase 12*	At3g63380
atcwINV1	*Cell wall invertase 1*	At3g13790
LHT1	*Lysine/Histidine Specific Transporter*	At5g40780
GLR1.4	*Glutamate Receptor 20*	At3g07520
PTR36	*Peptide Transporter 36*	At3g21670
OPT7	*Oligopeptide transporter 7*	At4g10770
PTR20	*Peptide Transporter 20r*	At1g52190
AHA11	*Arabidopsis plasma membrane H+-ATPase 11*	At5g62670

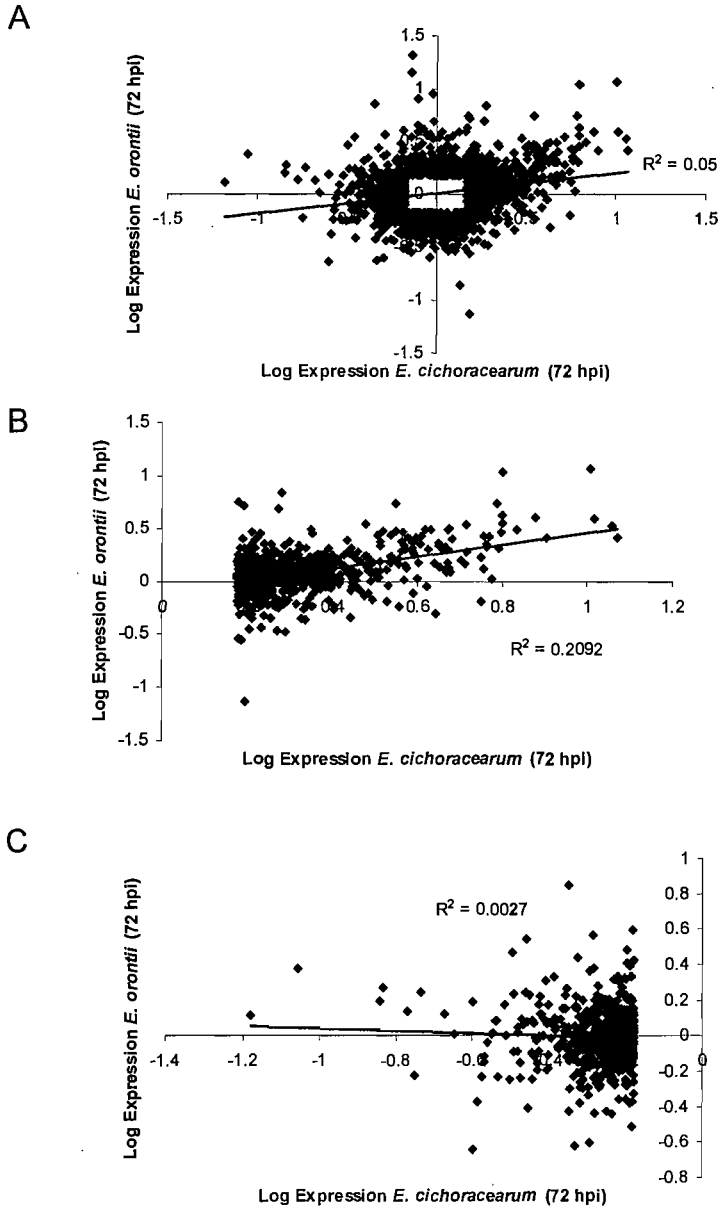

Figure 3.2 Correlation of gene expression between Arabidopsis infected with *E. orontii* or *E. cichoracearum*. Correlation between the expression of all (A), up-regulated (B), or down-regulated (C) genes in Arabidopsis when plants are infected with *E. orontii,* harvested at 72 hours post infection, and when infected with *E. cichoracearum*, harvested at 72 hours post infection. R^2 is the square of the Pearson correlation. In each case only genes with an expression ratio present in both conditions are plotted.

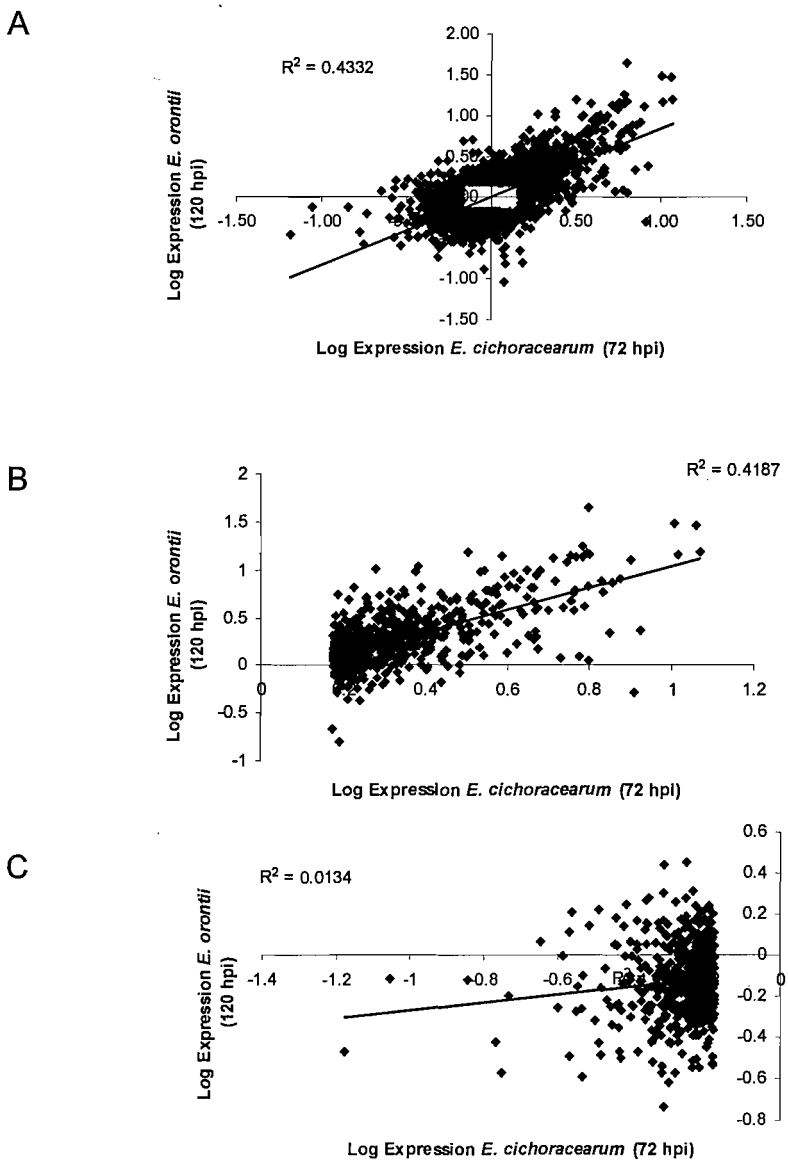

Figure 3.3 Correlation of gene expression between Arabidopsis infected with *E. orontii* or *E. cichoracearum*. Correlation between the expression of all (A), up-regulated (B) or down-regulated (C) genes in Arabidopsis when plants are infected with *E. orontii,* harvested at 120 hours post infection, and when infected with *E. cichoracearum*, harvested at 72 hours post infection. R^2 is the square of the Pearson correlation. In each case only genes with an expression ratio present in both conditions are plotted.

is equally good, R^2 = 0.4187, (Figure 3.3B) but for down-regulated genes it is again so low as can be considered to be non-existent, R^2 = 0.0134 (Figure 3.3C). Down-regulated genes normally have lower signal strengths than up-regulated genes and this leads to a greater error in the calculated ratios, but the cause of such poor correlation in down-regulated genes is difficult to pinpoint. The correlation of all regulated genes from *E. cichoracearum*-infected plants harvested at 72 hpi is much better compared with *E. orontii*-infected plants harvested at 120 hpi (Figure 3.3) than *E. orontii*-infected plants harvested at 72 hpi (Figure 3.2A and 3.3A).

3.2.2 Confirmation of microarray data by real-time PCR

Real-time PCR was used to confirm the expression ratios of selected genes from the microarray data, using infections established in Southampton. This allowed an assessment of how reliably any conclusions from the microarray data could be applied to our infection procedures and also gave an indication of biological variation. This method was also used to explore how the genes responded over time during *E. cichoracearum* infection which is information was previously unknown.

An initial assessment of both the technical variation associated with real-time PCR and the biological variation associated with infection was made. Figure 3.4 shows three repeated measurements of the *40S* reference gene over time from the same cDNA sample (see section 3.2.3 for the selection of reference genes). The expression of genes that have not been compared to a reference gene 'raw expression' are shown by determining E^{Ct} for each point. The details of this are contained in the Materials and Methods chapter, but it is important to recognise that the raw E^{Ct} of rarer transcripts have a higher E^{Ct} value than more common ones. This is not the case when looking at expression ratios using reference genes. Figure 3.5 shows the expression of the *PR1-like* gene, over time for three biological replicates (based against reference genes (see section 3.2.3). This data show that the technical variation in expression measurements made by real-time PCR is extremely low, but the biological variation in the expression of genes in response to *E. cichoracearum* is considerably higher. Although it can be seen that the expression of *PR1-like* does not peak at the same time or magnitude in all biological replicates, the trend in expression during infection with *E. cichoracearum* is clear; *PR1-like* is up-regulated during *E. cichoracearum* infection. The assessment that biological variability does not alter the general conclusions, means that for clarity and conciseness, results can be shown from a representative infection rather than from the three investigated, provided the three were similar. Results from here on are taken from a representative infection. Where variation in the trend was observed, all data will be shown. Biological repeat 2 has been used for display purposes, as when all the real-time PCR expression data is taken as a whole it generally represented the middle-ground of the three biological repeats.

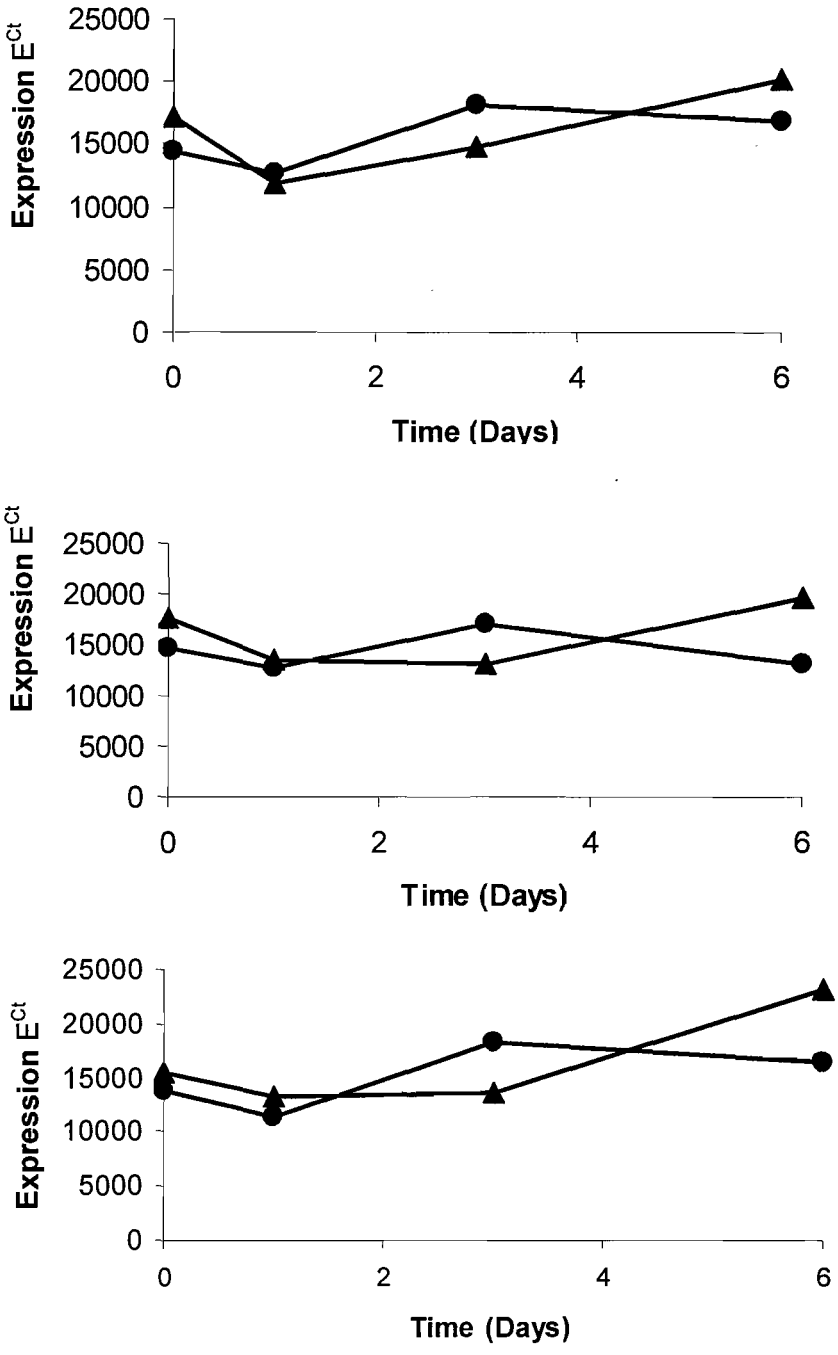

Figure 3.4 Three technical repeats using real-time PCR to measure the expression of the *40S Subunit 16B* (*At3g04230*). The same samples of cDNA prepared from a single infection series were measured three times. Each real-time PCR experiment uses three repeated measurements, so nine measurements were performed on each cDNA in this case. Standard deviation for the nine samples was always below 1%, but was on average 0.5%.

▲: Expression rations for healthy Col-0. •: Expression ratios for Col-0 infected with *E. cichoracearum*

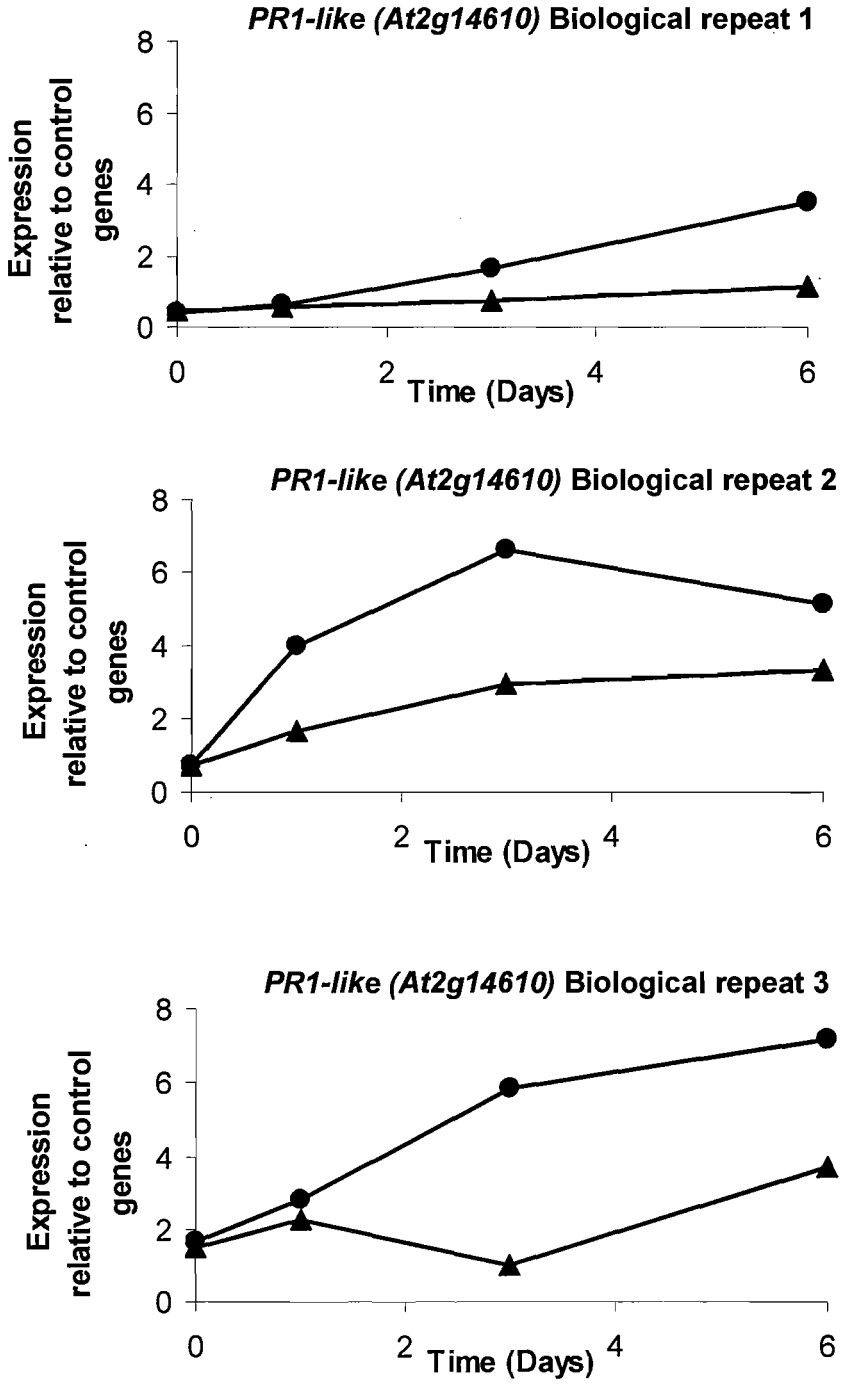

Figure 3.5 The *PR1-like* gene, *At2g14610* is up-regulated during *E. cichoracearum* infection, but shows a considerable degree of biological variation. The expression of *At2g14610* was measured by real-time PCR for three biological replicates of *E. cichoracearum* infection.

▲: Expression ratios for healthy Col-0. ●: Expression ratios for Col-0 infected with *E. cichoracearum*

3.2.3 Selection and confirmation of reference genes

In order to determine gene-expression changes with real-time PCR, it is necessary to compare the levels of a transcript of interest to a control transcript thought to be unresponsive to the treatment. A selection of genes were considered for use and then screened against the microarray datasets for expression changes. The genes eventually selected and used here are the *40S subunit S16C (At3g04230)*, *triose phosphate isomerase (TPI) (At3g55440)* and *PP2A (At1g13320)*. The *40S* subunit was chosen originally because of the historical use of ribosomal subunits as control genes in Arabidopsis literature (Kolukisaoglu *et al.*, 2002; Boldt *et al.*, 2005; Ishibashi *et al.*, 2005). *TPI* was chosen because it had been used previously and optimised real-time primers are already available (Schiott & Palmgren, 2005). Finally, the *PP2A* subunit was chosen because it had been highlighted by a comprehensive genome-wide search of microarray databases for good reference genes (Czechowski *et al.*, 2005). By looking at the microarray datasets it can be seen that the genes show no change in expression during the treatments (Figure 3.6), which makes them excellent candidates for reference genes. These genes were then investigated by real-time PCR on material prepared at Southampton (Figure 3.7) and the expression patterns of the three showed excellent agreement with each other. Reference genes are expected to show no overall response to infection, but levels of transcripts may vary from sample to sample depending on other biological factors such as age, as well as technical factors such as the efficiency at which the template cDNA was made from the starting RNA. The most important feature about a selection of reference genes therefore is that they should share the same expression patterns over time, which they clearly do.

3.2.4 Confirmation of powdery mildew responsive genes by real-time PCR

A number of up- and down- regulated genes were selected for confirmation by real-time PCR, using Arabidopsis infected with *E. cichoracearum* in conditions in Southampton (see Materials and Methods). This would confirm the relevance of the datasets as a whole to *E. cichoracearum* infections conducted with our isolate. The criteria by which the genes were selected were: 1) a statistical estimate of reliability for the data from the microarrays 2) having a high fold-change in expression, but not having a low signal strength 3) having a DNA sequence that allowed for suitable gene-specific real-time primers 4) whether they were regarded as 'marker' genes for the progress of the infection or whether these genes were not known to be involved in the powdery mildew/ Arabidopsis interaction.

The dataset describing *E. orontii*-infected plants was made public some time before the *E. cichoracearum* dataset. Initially, therefore, genes were selected based on

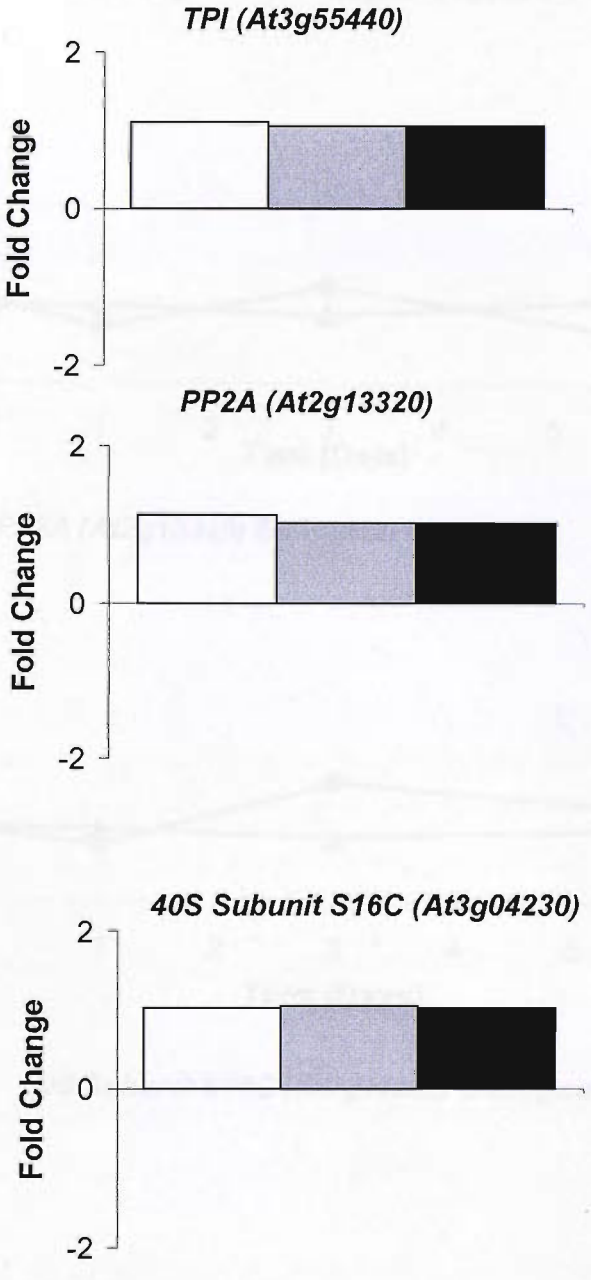

Figure 3.6 Expression changes in selected reference genes for powdery mildew infection. Fold-changes for individual genes were obtained from publicly available microarray datasets. White: Arabidopsis Col-0 infected with *E. cichoracearum* harvested at 72 hpi. Grey: Col-0 infected with *E. orontii* harvested at 72 hpi. Black: Col-0 infected with *E. orontii* harvested at 120 hpi.

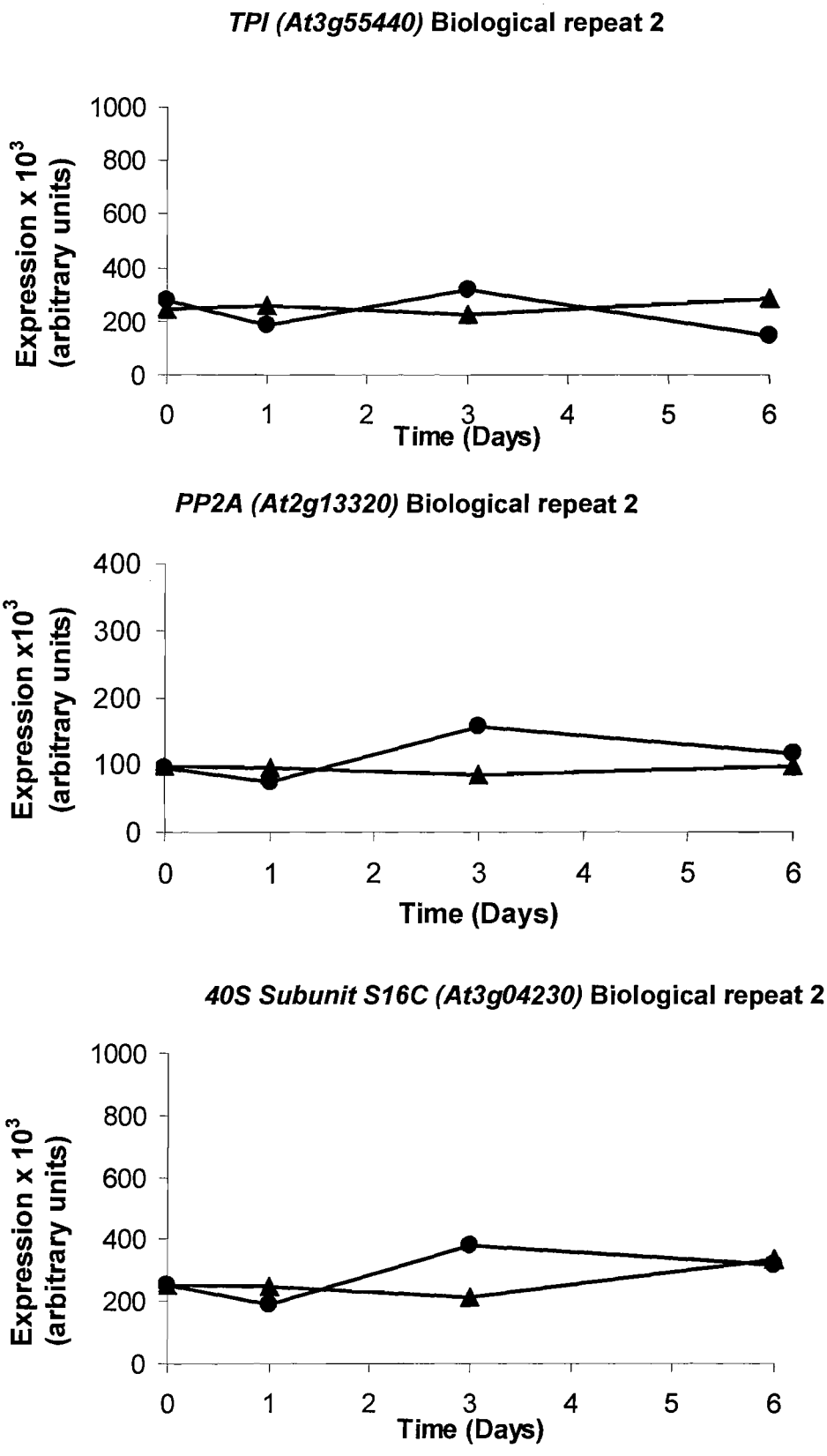

Figure 3.7 Real-time PCR of reference genes for the powdery mildew infection. ▲: E^{Ct} values (a measure of expression) for healthy Col-0. •: E^{Ct} values for Col-0 infected with *E. cichoracearum*. The absolute value of E^{Ct} is related to the level of transcript; rare transcripts have a higher value than more common ones.

their response to *E. orontii,* and another selection of genes was added once their response to *E. cichoracearum* at (72 hpi) was known. Figure 3.8 shows the expression ratios of the selected genes (Table 3.2) and how the data varies between the different microarray datasets.

In Figure 3.8 clearly marked 'assumed' values have been used where a gene has an expression ratio in one experiment, but not another. This value was calculated by using the average signal strength of the lowest single percentile of detected signals on that array. This has the effect of assuming approximately the lowest signal strength detectable. Other more complex methods are available (Troyanskaya *et al.*, 2001), but are more appropriate for data where assumed values are used later in data analysis. In this case the assumed values are used only for display purposes and are not included in numerical analysis such as the assessment of correlation. Therefore it is appropriate to use a less time consuming method, provided that the actual values are treated with caution. The selection of up-regulated genes shows a greater up-regulation in *E. orontii*-infected Arabidopsis (harvested at 120 hpi) than in *E. cichoracearum*-infected Arabidopsis, and the most variable results are from *E. orontii*-infected Arabidopsis harvested at 72 hpi. The most up-regulated gene was the *PR1-like* gene, which is over 40 times up-regulated. The expression patterns of these genes were checked by real-time PCR using *E. cichoracearum*-infected material prepared at Southampton, Figure 3.9 shows the real-time PCR data for the genes shown in Figure 3.8 and shows that each of the genes has a different pattern over time. Some genes, such as *PR1-like* and the putative chitinase *At2g43570*, increase in expression over time irrespective of treatment with powdery mildew, but others decreased (such as *At3g28270*). The maximum difference in expression in relation to the control genes was highest for both PR1-like and the putative chitinase *At2g43570* at 3 days or 72 hpi. However, the FAD domain-containing protein *At2g26380* was most up-regulated at 6 days/ 144 hpi.

3.2.5 Comparison of microarray datasets to real-time PCR data

A comparison of the expression ratios on the genes selected in 3.2.3 and 3.2.4 determined by real-time PCR (on infected material from this study) and microarray data from infected material obtained by other laboratories shows that they correlate well (Table 3.2 and Figure 3.10). It is clear from the data that the real-time PCR data presented here show less extreme expression ratios than the microarray data. This could be the result of a systematic overestimation of gene expression by microarrays, systematic under representation by real-time PCR, a reflection that Arabidopsis has not responded as vigorously to *E. cichoracearum* at Southampton, or most likely a combination of any of the three. The closest relationship between gene expression data from the microarrays and real-time PCR is when *E. cichoracearum*-infected Arabidopsis (from this study) were

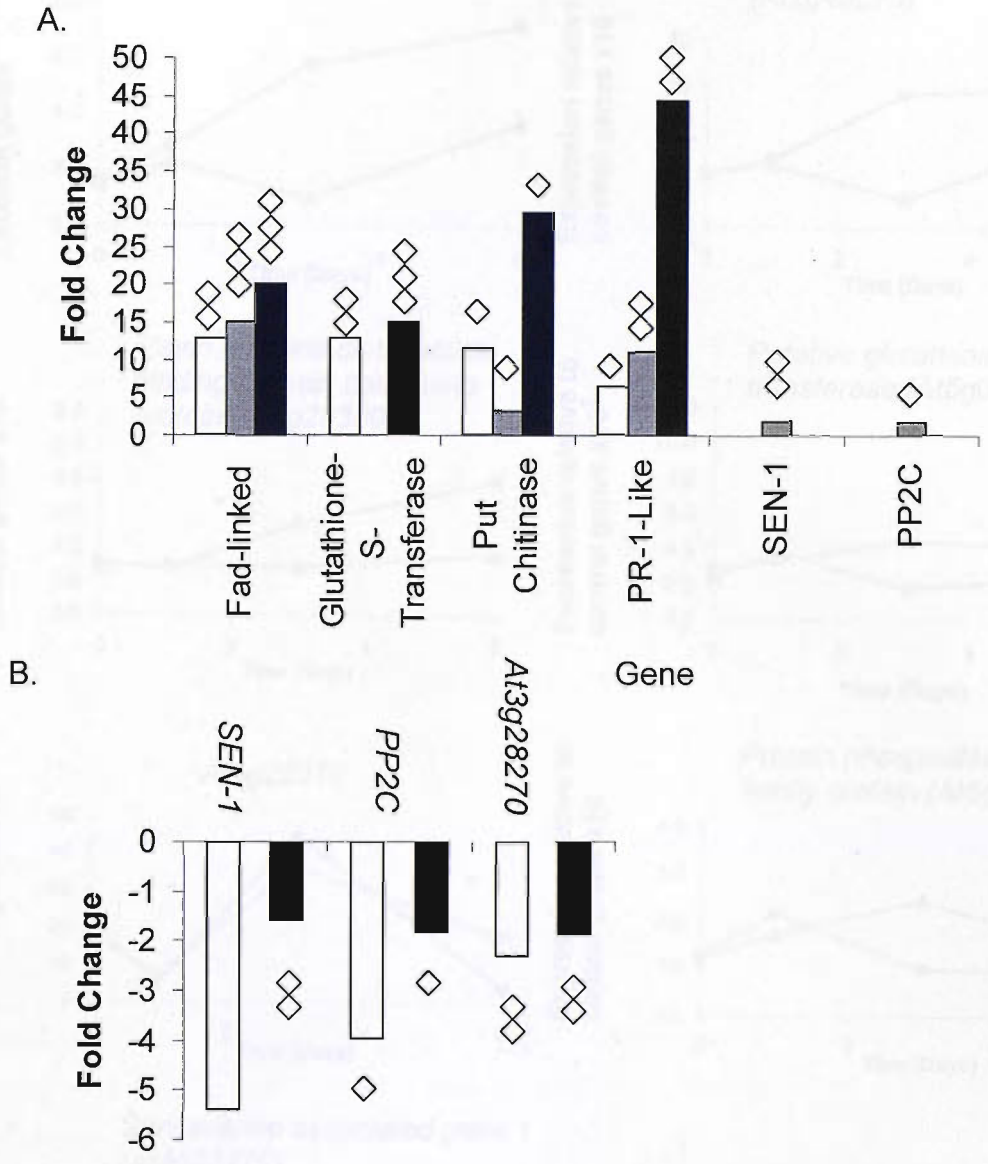

Figure 3.8 Selected regulated genes in response to powdery mildew infection. A, Up-regulated genes, B. Down regulated genes. Fold-changes for individual genes were obtained from publicly available microarray datasets. White: Arabidopsis Col-0 infected with *E. cichoracearum* harvested at 72 hpi. Grey: Col-0 infected with *E. orontii* harvested at 72 hpi. Black: Col-0 infected with *E. orontii* harvested at 120 hpi. ◊ represents a qualitative estimate of the reliability of the data. ◊ indicated an error of 5 – 10%; ◊◊ 10 – 20% and ◊◊◊ represents an assumed value, No ◊ indicates a likely error of less than 5%. This is based on the standard deviation of the two sets of data compared (infected and uninfected).

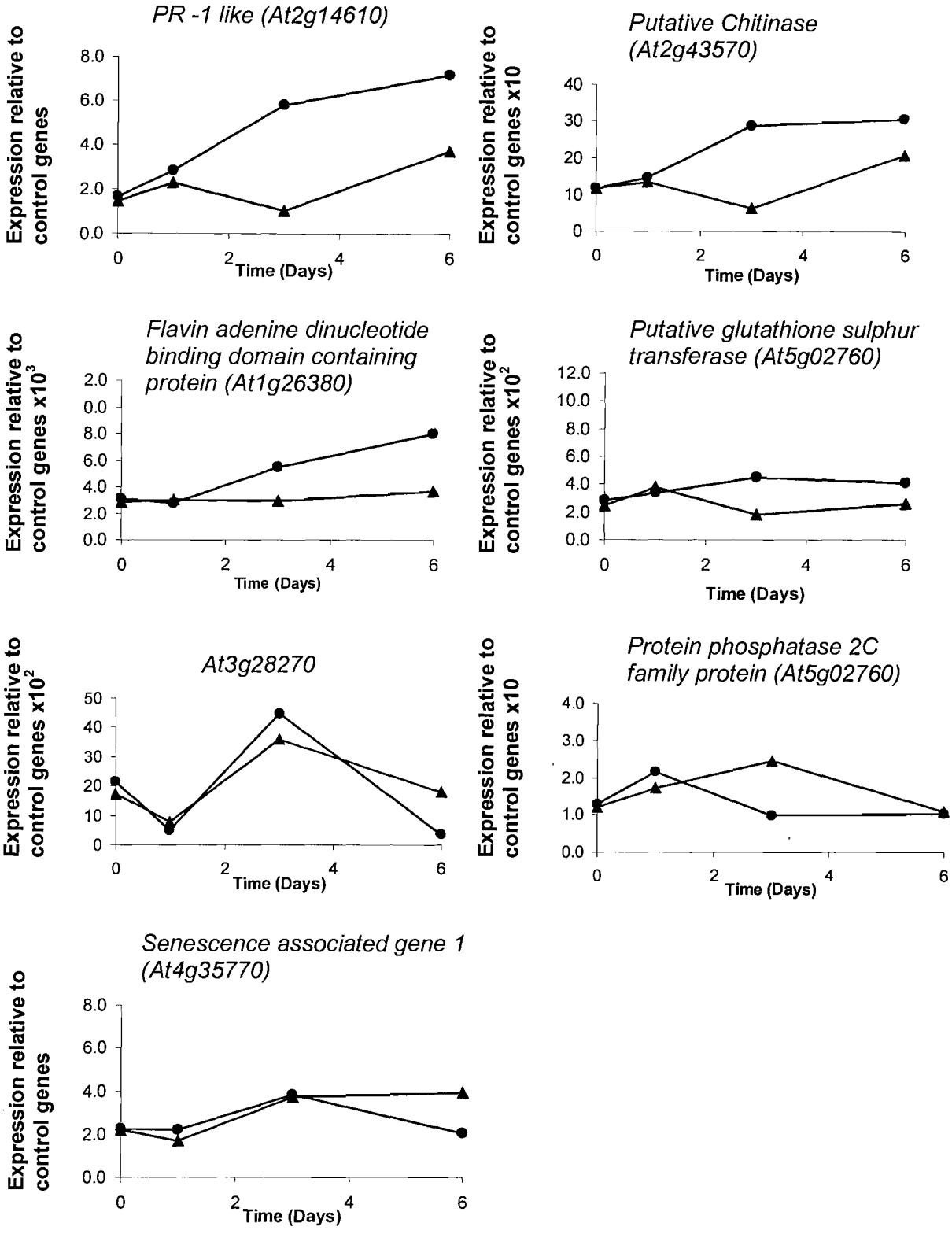

Figure 3.9 Real-time PCR of powdery mildew responsive genes. ▲: Average expression relative to three reference genes for healthy Col-0. ●: Average expression relative to three reference genes for Col-0 infected with *E. cichoracearum*. The absolute value of the expression ratio is related to the level of transcript compared to the reference genes, the more common transcripts have a higher value than the rarer ones.

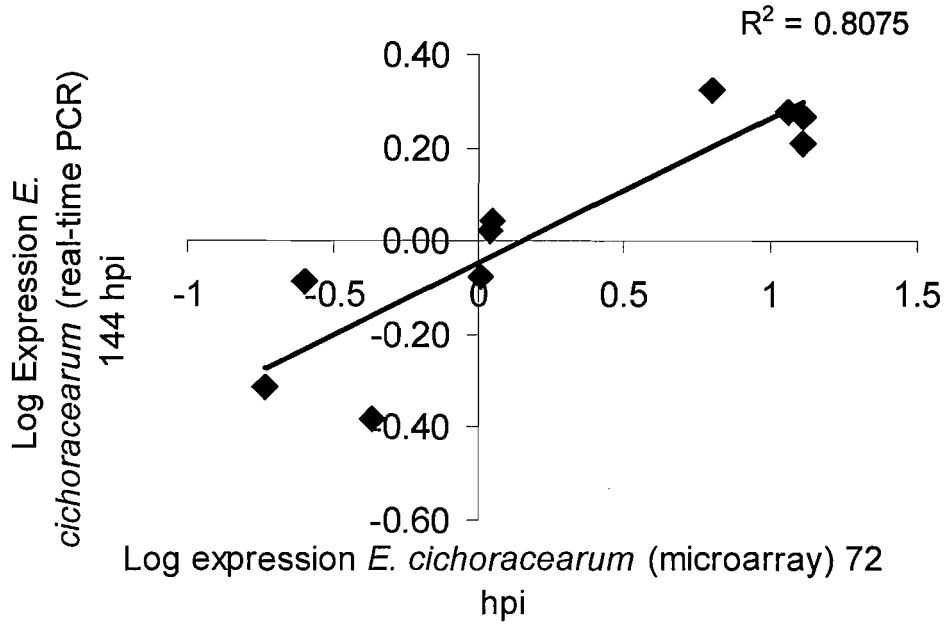

Figure 3.10 Correlation between gene expression as measured by real-time PCR and microarrays. The correlation between the log expression ratios of selected genes as measured by microarray when infected with *E. cichoracearum* and harvested at 72 hpi (Nishimura *et al.*, 2003), and by real-time PCR when infected with *E. cichoracearum* and harvested at 144 hpi.

Table 3.2 The Pearson correlation between gene expression as measured by real-time PCR and different microarray datasets. The values for gene expression obtained by real-time PCR (3.2.4) were compared to the other publicly available powdery mildew microarray datasets, as in Figure 3.10.

		Real-Time PCR data (*E. cichoracearum*)	
Microarray data		72 hpi	144 hpi
E. cichoracearum	72 hpi	0.307	0.808 (shown in detail in Figure 3.10)
E. orontii	72 hpi	0.329	0.510
E. orontii	120 hpi	0.486	0.796

harvested at 144 hpi and *E. cichoracearum*-infected Arabidopsis (from other laboratories) were analysed by microarray at 72 hpi. Real-time data from 144 hpi was always more highly correlated to any given microarray dataset than real time data from 72 hpi. Of all the microarray datasets, *E. orontii*-infected Arabidopsis harvested at 72 hpi correlates least with real-time data (Table 3.2). From this comparison it was decided that the publicly available microarray datasets of powdery mildew would be generally comparable to the infection procedures, strains and time points used in this study.

3.2.6 Selection and confirmation of transporters genes regulated in response to powdery mildew infection

As the information from the publicly available microarray datasets was shown to be relevant to this study (section 3.2.5), the datasets were investigated to identify Arabidopsis transporter genes that were regulated by powdery mildew infection. The datasets were screened by a list of genes containing all known and putative membrane-spanning proteins. This list was originally published in Maathuis *et al.* (2003) and was supplemented over time with fresh annotations from the Arabidopsis Membrane Protein Library (Ward, 2001), the PlantsT database (http://plantst.genomics.purdue.edu) and the transporter listings of ARAMEMNON (van der Graaff *et al.*, 2006). In addition to this, genes encoding both known and putative invertases were included. Previous work has shown invertases to be induced in response to *E. cichoracearum* infection (Fotopoulos *et al.*, 2003) and some resources for looking at invertases were already available in Southampton. From this dataset of expression responses to powdery mildew for known and putative membrane transporters, a group of genes were selected for further study. The criteria by which genes were selected were: 1) a statistical estimate of reliability for the data from the microarrays 2) having a high fold-change in expression, but not having a low signal strength 3) having a DNA sequence that allowed for suitable gene-specific real-time primers 4) whether a T-DNA knockout line was available for that gene. The microarray data showed most transporters to have a greater response to *E. orontii* at 120 hpi than to *E. cichoracearum* at 72 hpi (Figure 3.11). Although the datasets as a whole have been confirmed as accurate (section 3.2.5) and large-scale conclusions can be drawn with confidence, the measurement of any one individual gene in response to *E. cichoracearum* could still be erroneous. As preparing T-DNA knockout plants in a given gene represents a considerable investment it is crucial to confirm that the gene does indeed respond to infection. Therefore the response of each individual transporter gene selected was investigated by real-time PCR to confirm its response (Figure 3.12). For the putative transporter genes investigated, there is a good correlation between the data from real-time PCR (harvested at 144 hpi on material from Southampton) to 72 hpi microarray data (Figure 3.13). The Pearson correlation is

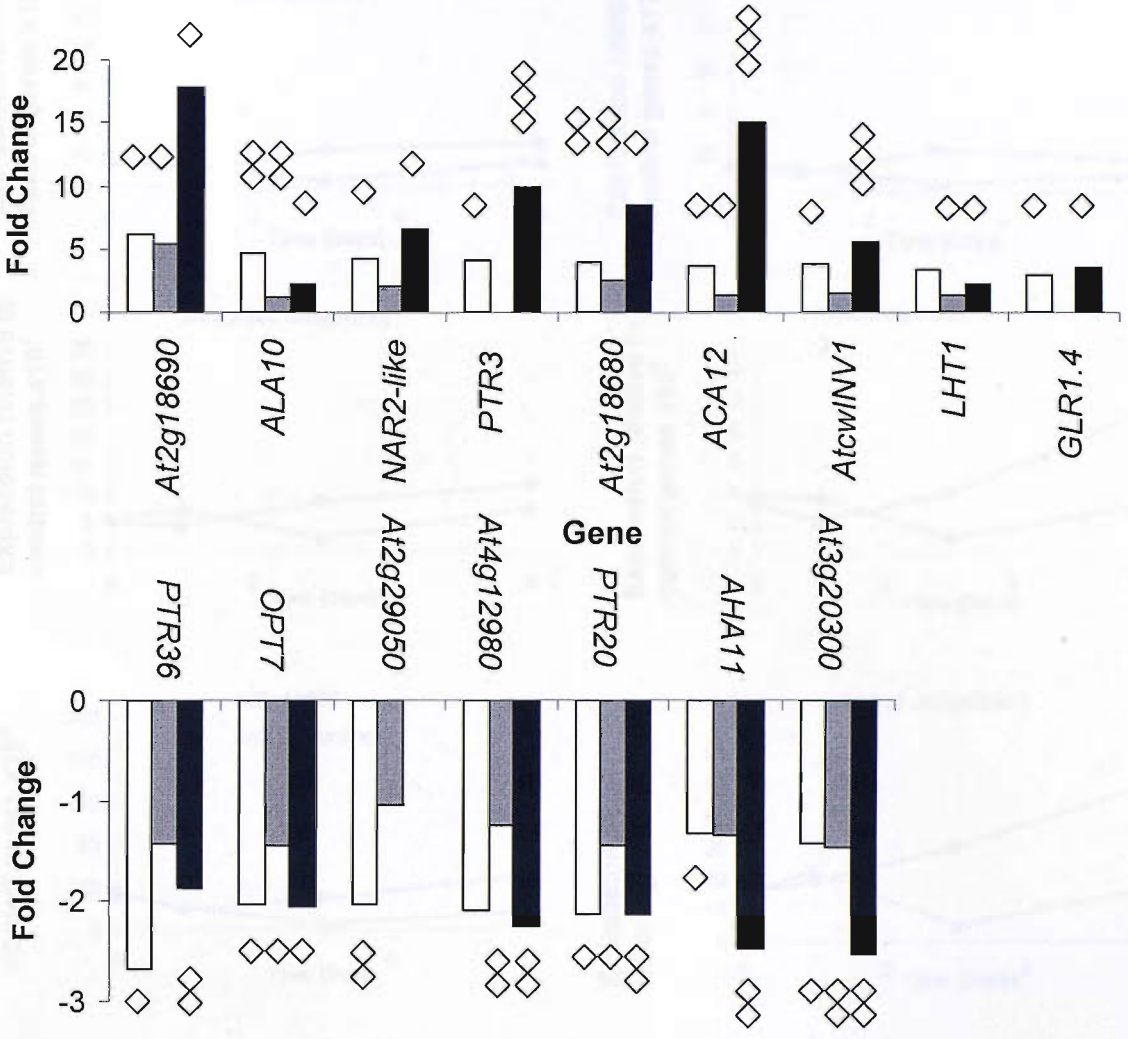

Figure 3.11 Selected transporter genes regulated in response to powdery mildew infection. A. Up-regulated B. Down-regulated genes. Fold-changes for individual genes were obtained from publicly available microarray datasets. White: Arabidopsis Col-0 infected with *E. cichoracearum* harvested at 72 hpi. Grey: Col-0 infected with *E. orontii* harvested at 72 hpi. Black: Col-0 infected with *E. orontii* harvested at 120 hpi. ◊ indicates an error of 5 – 10%, ◊◊ indicates an error of 10 – 20% and ◊◊◊ represents an assumed value, zero ◊'s indicates a likely error of less than 5%. This is based on the standard deviation of the two sets of data compared (infected and uninfected).

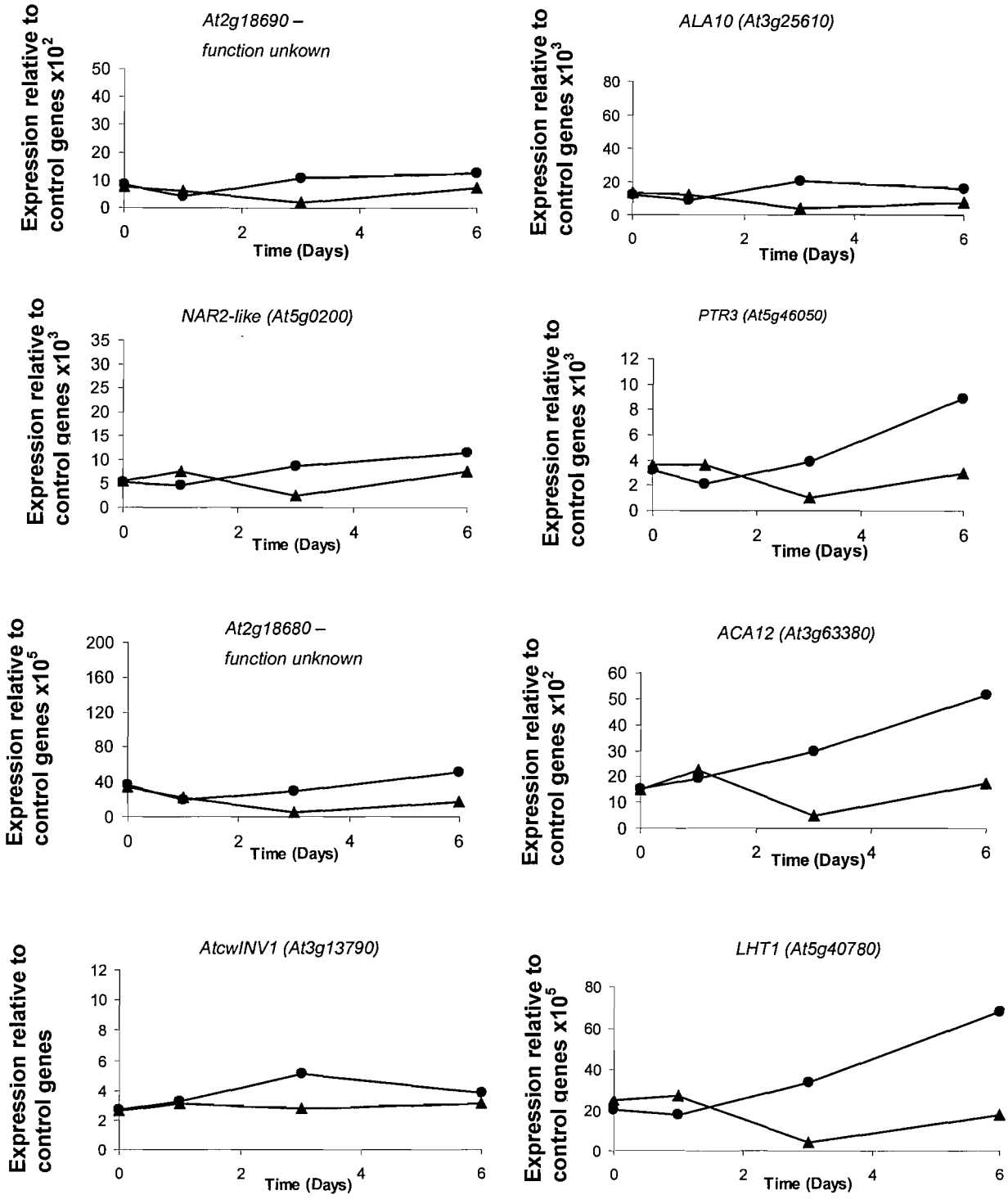

Figure 3.12 Expression ratios of genes measured by both microarrays and real-time PCR. Correlation between the log expression of selected genes as measured by microarray when infected with *E. cichoracearum* and harvested at 72 hpi (Nishimura *et al.*, 2003), and by real-time PCR when infected with *E. cichoracearum* and harvested at 72 hpi (this study). ▲: Expression relative to an average of three reference genes for healthy Col-0. ●: Expression relative to an average of three reference genes for Col-0 infected with *E. cichoracearum*.

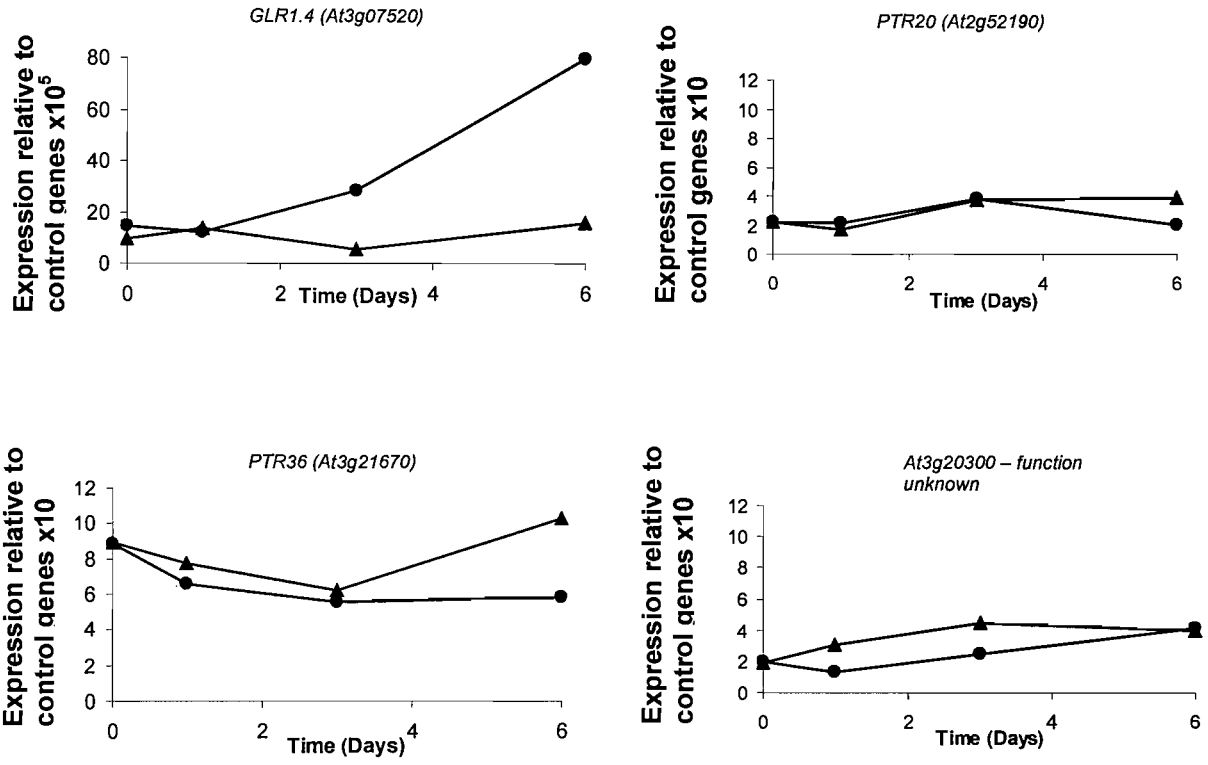

Figure 3.12 continued

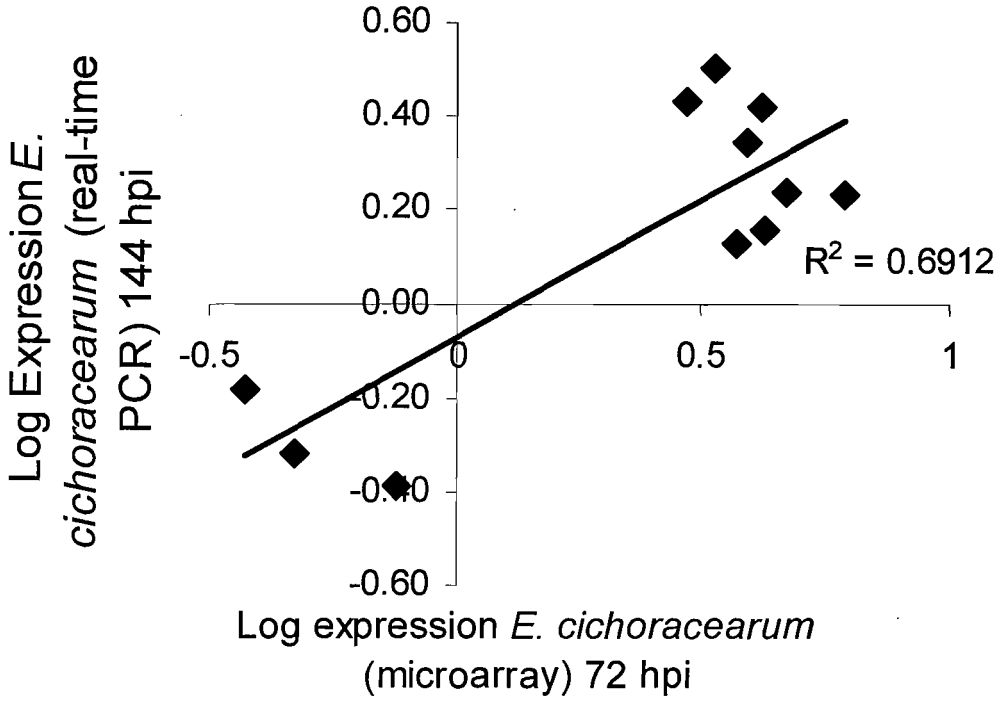

Figure 3.13 Expression ratios of transporter genes measured by both microarrays and real-time PCR. Correlation between the log expression of selected transporter genes as measured by microarray when infected with *E. cichoracearum* and harvested at 72 hpi (Nishimura *et al.*, 2003), and by real-time PCR when infected with *E. cichoracearum* and harvested at 144 hpi (this study).

however slightly lower than for the selection of general genes, as described in 3.2.5 (0.729 compared to 0.807).

3.2.7 Functional breakdown of genes regulated in response to powdery mildew infection

Having determined that as a whole, data from the publicly available microarrays of *E. cichoracearum* were reliable, the data could be studied in more detail. Analysed data was ascribed to broad biological or molecular functions according to GO:slim categories. GO:slim is a cut-down version of the Gene Ontology system (Ashburner *et al.*, 2000) particularly useful for giving a summary of the results of microarrays. There are three parallel systems of classification: classification by location (mitochondria, nucleus, etc.); classification by molecular function (DNA binding, hydrolase, transferase, etc); or classification by biological function (development, cell organisation, stress response etc.). Each gene has a location classification, a molecular classification and a biological function classification. Within these, a gene may appear more than once. For example a gene that is capable of both a transferase and hydrolase function will appear in both molecular classifications; a gene product that is involved in signal transduction and transcription would appear in both biological function categories. The proportions of the genes responding to powdery mildew (>1.5 fold expression change) that fitted into any given category were compared to the proportions for the genome as a whole (Figure 3.14). It can be seen that the largest grouping in both biological and molecular classifications is 'function unknown'. However, a considerably greater percentage of genes are of unknown molecular function rather than unknown biological function. This is likely due to the large amount of microarray datasets now available, which can ascribe a potential biological function to responsive genes, but give no clue to molecular function. The most notable changes in proportions for biological function were increases in the number of genes in: the 'other' biological functions category, response to abiotic/ biotic stimulus, response to stress, signal transduction, transcription and transport. Also notable are the decreases in the 'other' cellular, metabolic and physiological processes. The most notable changes in the proportions of molecular function were the increases in 'other' molecular functions, DNA or RNA binding, protein binding, transcription factor activity, structural molecule activity and receptor binding or activity. This was mirrored by a drop in the proportion of genes of unknown molecular function (compared to the entire genome) and a substantial drop in the enzyme activities, 'other', transferase and hydrolase as well as 'other' binding functions.

3.2.8 Promoter analysis of genes responding to powdery mildew infection

In 3.2.7 it was shown that the proportion of genes responding to powdery mildew infection having transcription factor activity was much higher than the proportion of genes in the

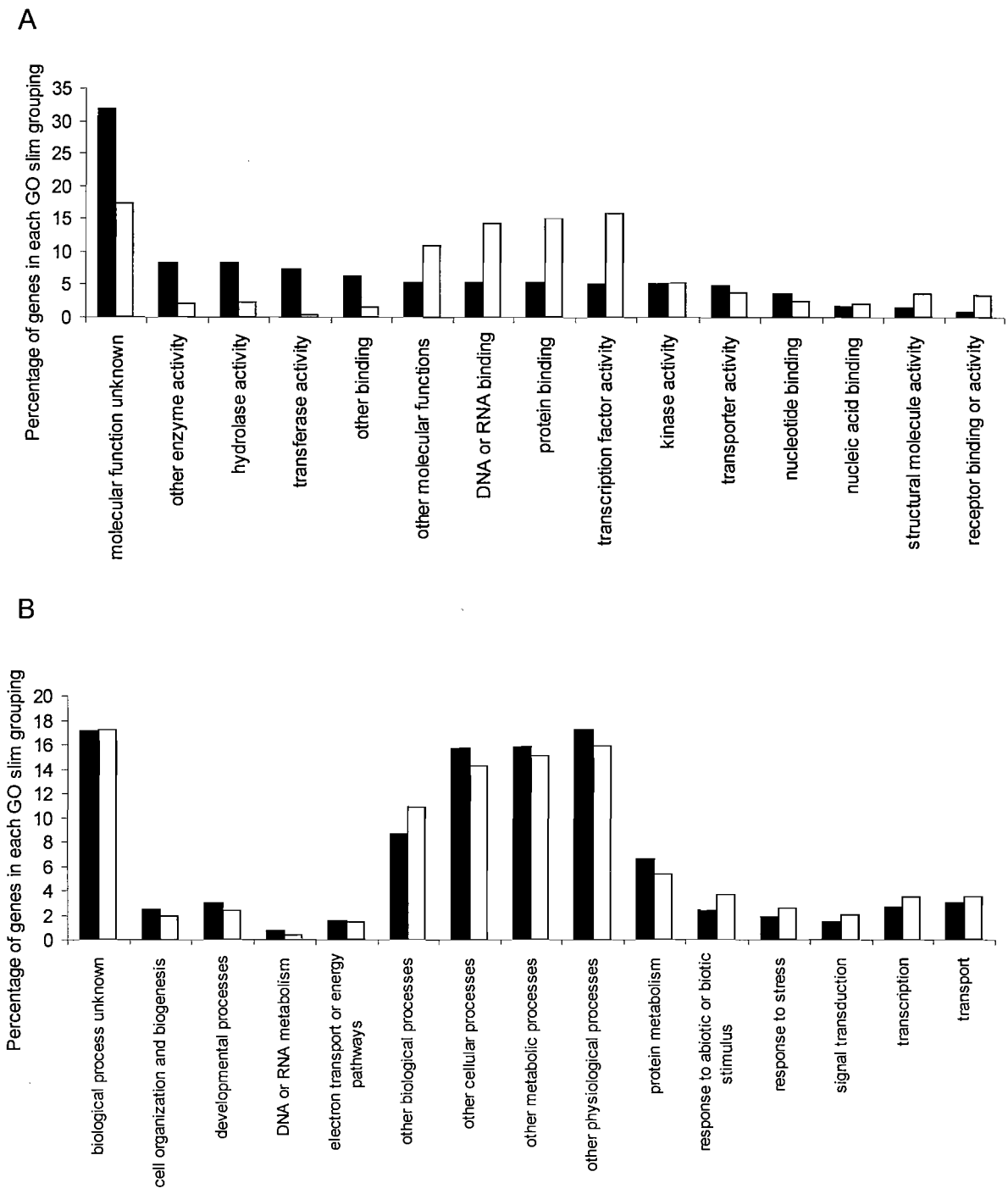

Figure 3.14 The breakdown of biological and molecular function for genes responsive to *E. cichoracearum at 72 hpi.* Black bars: the percentage of the genes from the entire Arabidopsis genome in each GO slim function. White bars: the percentage of genes showing a 1.5 fold or greater change in expression in response to *E. cichoracearum*. A: Using GO:slim molecular categories, B: using GO:slim biological categories.

genome having transcription factor activity. To examine this further a promoter analysis of genes responding to powdery mildew infection was undertaken. The Athena database (O'Connor *et al.*, 2005) contains 105 characterised TF binding sites, and is able to assess whether any of those sites are over-represented in the promoters of a group of genes, compared to all genes. It does not contain any binding sites determined by *in silico* approaches only. The program returns a P-value to determine reliability, but due to the testing of multiple hypothesis (105 tests for 105 transcription factors) the Bonferroni correction has been applied, the effect of which (simply speaking) is to reduce the P-value threshold for determination of promoter enrichment to 0.0001, rather than 0.05. All genes showing a fold-change of greater than 1.5-fold (4092) were analysed together, followed by up-regulated (2,114) and then down-regulated (1,978) and the results are displayed in Table 3.3. The separation of genes into groups was based on the principle that up- and down-regulated genes may contain different promoters, which would reduce the chances of seeing a statistically relevant enrichment of promoters. The standard TATA box and the CW_8G motif were the most over-represented binding sites. It can be seen that T-box, W-box and GARE binding sites are over-represented only in up-regulated genes, while I-box, ARF and G-box binding sites are over represented only in down-regulated genes. The GARE and MYB4 binding sites are not statistically over-represented in the up- or down-regulated gene listed, but when both are combined, they become so. This indicates that these elements are not by nature related to the direction of regulation, but are involved in it.

3.2.9 Similarities to other biological treatments

The Genevestigator database (Zimmermann *et al.*, 2005) is a tool for analysing microarray datasets. At the time of analysis, there were datasets from full genome Affymetrix chips for 82 treatments, covering biotic, chemical, hormone, light, nutrient and stress. It also contains datasets from developmental time series and tissue-specific experiments. As this database uses only datasets from Affymetrix Arabidopsis microarrays, gene-wise comparisons between the treatments are now much easier than in the past. The 100 most responsive genes were compared to other treatments using the square of the Pearson correlation. Table 3.4 shows the highest and lowest correlations of all the treatments on Genevestigator. The most similar treatment to infection with *E. cichoracearum* was infection with *E. orontii*, while infection with *P. infestans* was also closely related. Non-biotic treatments that were similar were ozone and nitrogen starvation. Interestingly the two lowest correlations of all the 83 stress treatments listed were treatment with the hormones methyl jasmonate and auxin.

Table 3.3 Over-represented transcription factor binding sites in *E. cichoracearum* responsive genes. Statistically over-represented transcription binding factor sites in genes showing any 1.5 fold-change, 1.5 fold up-regulated and 1.5 fold down-regulation in response to *E. cichoracearum* out of the 105 examined. P-values above the cut-off of 10^{-4} are shown underlined.

TF Binding Site	P-Value All responsive genes	P-Value up-regulated genes	P-Value down-regulated genes	Reference
TATA-box	$\underline{<10^{-10}}$	$\underline{<10^{-10}}$	$\underline{<10^{-10}}$	(Taiz & Zeiger, 2002)
CW_8G	$\underline{<10^{-10}}$	$\underline{<10^{-6}}$	$\underline{<10^{-8}}$	(Tang & Perry, 2003)
AtMYC2	$\underline{<10^{-9}}$	$>10^{-4}$	$\underline{<10^{-9}}$	(Abe *et al.*, 1997)
T-box	$\underline{<10^{-9}}$	$\underline{<10^{-9}}$	$>10^{-4}$	(Chan *et al.*, 2001)
W-box (WKRY)	$\underline{<10^{-9}}$	$\underline{<10^{-9}}$	$>10^{-4}$	(Rushton *et al.*, 1996)
MYB1	$\underline{<10^{-8}}$	$\underline{<10^{-4}}$	$\underline{<10^{-5}}$	(Abe *et al.*, 2003)
GARE	$\underline{<10^{-7}}$	$\underline{<10^{-6}}$	$>10^{-4}$	(Skriver *et al.*, 1991)
I-box	$\underline{<10^{-5}}$	$>10^{-4}$	$\underline{<10^{-5}}$	(Giuliano *et al.*, 1988)
MYB4	$\underline{<10^{-5}}$	$>10^{-4}$	$>10^{-4}$	(Molina & Grotewold, 2005)
ABRE-like	$\underline{<10^{-5}}$	$>10^{-4}$	$>10^{-4}$	(Chaudhary & Crossland, 1996)
ARF	$\underline{<10^{-5}}$	$>10^{-4}$	$\underline{<10^{-5}}$	(Guilfoyle *et al.*, 1998)
G-box	$\underline{<10^{-4}}$	$>10^{-4}$	$\underline{<10^{-8}}$	(Giuliano *et al.*, 1988)

Table 3.4 Most similar treatments on Genevestigator. The Pearson correlation to the linear line of best fit (not shown) between the 100 genes most responsive to *E. cichoracearum* infection and various treatments.

Top 10 Correlations	R^2	Bottom 10 Correlations	R^2
E. orontii	0.479	Uniconaxole	0.001
Ozone	0.351	ABA	0.001
P. infestans	0.340	brz91	0.0008
Low Nitrogen, glucose	0.285	Daminozide	0.0006
Cold	0.265	Sulphur deprivation	0.0004
Low K	0.258	Zearalenone	0.0003
B. cinerea	0.250	Red light	0.0003
High CO_2	0.208	Zeatin	0.0002
Cycloheximide	0.207	Methyl jasmonate	0.0001
$AgNO_3$	0.206	Auxin	0.0001

3.2.10 Functional breakdown of *E. cichoracearum* responsive transporters

In order to investigate the potential functions of transporter genes shown to be involved in the response to *E. cichoracearum*, responsive transporters were grouped in broad functional categories bases on the transporter classification, and gene annotation by TAIR. Gene annotation by TAIR contains information derived from protein or sequence homology and experimental data. Table 3.5 shows this breakdown, in comparison to the number of genes falling into that category in the genome as a whole. Categories such as the F- or V-type pumps or pyrophosphatases were not represented at all in the responsive transporters. Some groups showed a marked increase in representation compared to the genome. By proportion, almost twice as many sugar transporters responded to infection as compared to the genome. The proportions of ammonium, nitrate and peptide/ protein transporters were all increased in comparison to their proportional representation in the genome.

Table 3.5 Breakdown of the function of Arabidopsis transporters that are responsive to *E. cichoracearum* infection. Transporters which showed a 2 fold or greater change in expression in response to *E. cichoracearum* infection of Arabidopsis were sorted into categories based on the transporter functions ascribed by TAIR. Dark grey indicates a sizeable or increase or decrease in proportion compared to the genome.

	Percentage of genes	
Function	In responding genes	In genome
ABC	10.6	12.3
Amino Acid Transporter	4.3	6.6
Ammonium transporter	2.1	0.6
Anion transporter	2.1	2.7
Aquaporin	5.3	3.9
Auxin Transporter	2.1	1.2
Cation transport	5.3	11.2
CNGC	1.1	2.0
F- or V- type Pump	0.0	3.1
Glutamate Receptor	3.2	2.0
Ion Channel	2.1	2.8
MATE	2.1	6.0
Metal transport	2.1	3.3
Nitrate transport	2.1	0.9
Other Metabolite transporter	1.1	2.2
Other Transport	2.1	3.1
Peptide/ Protein transport	8.5	5.9
Pyrophosphorylase	0.0	1.0
P-type Pump	7.4	4.7
Sugar Transporter	13.8	6.5
Sulphate	1.1	1.1
Unknown Function	21.3	17.1

3.3 Discussion

The transcriptional response of plants to pathogen challenge is complex (Jalali *et al.*, 2006). Via defence signalling (as described in Chapter 1), pathogen attack co-ordinates massive transcriptional reprogramming (Katagiri, 2004; Jones & Dangl, 2006). These changes are multi-purposed (Schulze-Lefert & Panstruga, 2003): transcriptional changes responsible for initial defence against the pathogen are required (such as penetration resistance (Hardham *et al.*, 2007), followed by defence response against the establishment of biotrophy (Schulze-Lefert & Panstruga, 2003). Equally plants must cope with the nutritional changes that occur in powdery mildew infections, not just the additional demand for nutrients to mount defence responses, but also the drain on nutrients that a compatible biotrophic pathogen imposes (Chou *et al.*, 2000; Both *et al.*, 2005; Swarbrick *et al.*, 2006). There may also be transcriptional changes induced by and for the benefit of the pathogen, that manipulate the host plant's response (Chou *et al.*, 2000; Vogel & Somerville, 2000; Schulze-Lefert & Panstruga, 2003; Both *et al.*, 2005).

3.3.1 Correlation in gene expression profiles induced by two powdery mildews

This study has shown a good correlation between microarray data obtained from Arabidopsis infected with different powdery mildews (*E. cichoracearum* and *E. orontii*) using different experimental conditions. This data has also been shown to correlate well with real-time data from material prepared in Southampton. It can be seen that any differences in experimental methods that do exist between material prepared in other studies and material prepared in Southampton do not significantly affect the outcome of investigations into transcriptional responses. The data from the two pathogens has thus been verified and enables data from these experiments to be used with confidence when drawing conclusions.

A global comparison of powdery mildew responsive genes produces a moderate to high correlation between genes regulated in response to *E. cichoracearum* and *E. orontii* (R^2 = 0.41 and 0.43), and it can be seen that this correlation is far stronger for up-regulated genes than for down-regulated genes (Figure 3.3). A correlation of R^2 of ~0.4 may not at first seem high, but this value is 1/3 higher than any other treatment in the Genevestigator database (Table 3.4). The quantitative transcriptional response of each gene to any given response is complex and is potentially influenced by many factors, such at the exact physiological status of the plant, the age of the plant, the environmental conditions and epigenetic modifications. Given this and the fact that these are two different species of pathogens (albeit with the same lifestyle) an R^2 value of ~0.4 can be considered high. It can therefore be seen that the differences between the two will likely focus on species-specific defence responses (*E. cichoracearum* might trigger different R genes to *E. orontii*) and potentially reflect the difference in virulence between them. The

transcriptional response of Arabidopsis to two compatible powdery mildews is therefore broadly similar. The commonalities in the transcriptional response are likely to be related to common defences activated by slightly different signalling pathways, and the response of plants to the nutritional changes that accompanies biotrophy. Despite this, it has been known for some time that there are differences in the genetic components involved in the defence response of Arabidopsis to the two pathogens. At least two studies involving enhanced resistance or enhanced susceptibility mutants have shown a different response between the two powdery mildews (Dewdney *et al.*, 2000; Vogel & Somerville, 2000). The *pmr1* mutant (Vogel & Somerville, 2000) (for which mutations are currently unmapped) is resistant to *E. cichoracearum* yet retains susceptibility to *E. orontii*. Equally, the *eds15* (Dewdney *et al.*, 2000) mutant (for which mutations are also currently unmapped) which is more susceptible to *E. orontii*, is not more susceptible to *E. cichoracearum*. There is relatively little work in this area, and this data set can be used to identify genes showing a differential response between *E. cichoracearum* and *E. orontii*. If a strain of *E. orontii* was obtained and the practical difficulties of keeping two highly related powdery mildews in the same area without any cross contamination could be overcome, future work could examine the consequences of knockouts in differentially expressed genes.

This study has shown that changes in the transcriptome of *E. orontii*--infected Arabidopsis lag behind the changes in the transcriptome of *E. cichoracearum*- infected Arabidopsis. As measured by whole genome microarrays, the transcriptome of *E. cichoracearum*-infected Arabidopsis at 72 hpi is more closely related to the transcriptome of *E. orontii*-infected Arabidopsis at 120 hpi than it is to 72 hpi *E. orontii* infected Arabidopsis (Figures 3.2 and 3.3). Previous work has strongly indicated that *E. orontii* is more virulent than *E. cichoracearum* on Arabidopsis (Plotnikova *et al.*, 1998; Vogel & Somerville, 2002), and this study has observed that most gene expression changes are more severe in *E. orontii*-infected Arabidopsis than in *E. cichoracearum*-infected Arabidopsis at the same time points. The fact that *E. cichoracearum* is less virulent than *E. orontii* could be an explanation for the different expression profiles over time. An explanation for the increased virulence of *E. orontii* could be that plant defence responses against it are delayed in comparison to those against *E. cichoracearum*, thus enabling biotrophy to become more quickly established. This explanation would fit well with the data shown in this study, as described above and shown in Figures 3.2 and 3.3. The successful establishment of biotrophy involves a large amount of transcriptional reprogramming. It might be expected then, that a subset of genes will be more strongly regulated by a more virulent pathogen, whilst the majority might be less regulated. This also fits well with the data shown in this study which as noted above, show that the gene expression changes that do occur in *E. orontii*-infected Arabidopsis are generally more extreme than those observed in *E. cichoracearum* at the same time point following infection. Real-time data (where the exact fold-change values should be more accurate

than those obtained from microarray studies) from *E. cichoracearum*-infected Arabidopsis, which uses material prepared in Southampton, show some differences in the timings of transcriptional changes compared to microarray data obtained in other studies. The microarray data from *E. cichoracearum*-infected Arabidopsis harvested at 72 hpi are far more closely correlated to real-time data from *E. cichoracearum*-infected Arabidopsis harvested at 144 hpi than they are to real-time data from *E. cichoracearum*-infected Arabidopsis harvested at 72 hpi (both of which were prepared in Southampton) (Table 3.2). This is a different situation to the comparisons of microarray data, as the real-time data are analysing only a highly regulated subset of genes, and are not representative of the genome-wide transcription response. The difference between the two sets of data does however, warrant an explanation. As both studies are using the same isolate of *E. cichoracearum* (UCSC1) on the same host (Col-0) it is expected that there are no genetic differences in the host or pathogen. It should be noted that as an obligate biotroph, the *E. cichoracearum* isolates must be constantly maintained on a living host, and cannot be stored until use. The two isolates have therefore been genetically separated for tens, possibly hundreds of generations, although as described in Chapter 1, reproduction will have been almost exclusively asexual. This situation is very difficult to avoid, when using obligate pathogens with generation times of approximately one week. Equally, differences in the growth conditions of Arabidopsis, and therefore the physiological status of the host plant, might affect the strength of the defence response to *E. cichoracearum*. Differences in humidity (humidity levels were not controlled in Southampton) and differences in the density and physiological status of the infecting conidia might also affect the germination rates of conidia and the subsequent development of powdery mildew. Whatever the underlying cause, the difference in the host plant response is likely to be due to one of two reasons: *E. cichoracearum* used in Southampton might develop more slowly than *E. cichoracearum* used in the microarray studies; or the *E. cichoracearum* used in Southampton is more virulent and is evading detection by the host for longer. As the gene expression changes observed in Southampton are less extreme than those observed in the microarray data (Figures 3.10 and 3.13) (in contrast to the situation between *E. cichoracearum* and *E. orontii*), this suggests that the *E. cichoracearum* used in Southampton is developing more slowly. The real-time data obtained from material prepared in Southampton are also more closely correlated to microarray data from *E. orontii*-infected Arabidopsis harvested at 120 hpi, than they are to rnicroarray data from *E. orontii*-infected Arabidopsis harvested at 72 hpi (Table 3.2). The magnitude of the expression changes in the examined genes is reduced further in comparison to the microarray data from *E. orontii*. These lines of evidence, taken together, suggest that the *E. cichoracearum* used in Southampton is developing more slowly on Arabidopsis, and eliciting smaller changes in gene expression than have been measured in the microarray studies, but changes in these genes are still elicited.

3.3.2 Functional breakdown of genes responsive to *E. cichoracearum* infection

When looking at the function (as defined by the Go:slim category annotations) of genes regulated in response to *E. cichoracearum* infection, 10% or more are in each of the following categories: DNA or RNA binding proteins, proteins with protein binding activity, proteins with transcription factor activity and proteins with other molecular functions (Figure 3.14). The large proportion of genes in the 'other molecular functions' category is potentially due to the large number of proteins with anti-pathogen activities that do not fall into the standard categories of the Go:slim groupings. Between 5 and 10% of genes are in each of the following categories: structural proteins; receptor binding or activity; or kinase activity. This is interesting as genes involved in structural molecule activity and receptor binding activity represent a very small proportion (>2%) of the total genome. It should be remembered though, that a large proportion (>30%) of genes currently have no known molecular function – some of the genes may have structural molecular activity, or receptor binding or activity but are merely un-annotated. The spread of gene functions fits well with evidence from previous studies which show changes to transcription (DNA or RNA binding activity and transcription factor activity) (Katagiri, 2004; Jones & Dangl, 2006), signal transduction (protein binding activity) (Jalali *et al.*, 2006; Bruce & Pickett, 2007) and MAMP and intracellular protein detection (receptor binding or activity) (Bittel & Robatzek, 2007; Bent & Mackey, 2007). Similarly, when looking at the biological function of regulated genes, a relatively large proportion of genes is known to be responsive to biotic or abiotic stimuli or responsive to stress. It can be seen though, that the majority of genes are still involved in 'other cellular', 'other metabolic' and 'other physiological' processes.

3.3.3 Transcription factor binding sites in *E. cichoracearum* responsive genes

This study has show that several transcription factor binding sites are over-represented in genes which response to *E. cichoracearum*. The CW_8G, MYC2, T-Box, W-Box, MYB, GARE, I-Box, ABRE-like, ARF and G-box sites are all over-represented (Table 3.3). Although the relevance of some of these transcription factors might at first seem questionable, many of the transcription factors or transcription factor families that bind the listed sites have been implicated in plant defence responses or plant stress responses before (Mahalingam *et al.*, 2005). The transcriptional response to pathogen attack has similarities with other types of stress condition; for example, many of the transcription factor families listed above are involved in the response of Arabidopsis to oxidative stress (Mahalingam *et al.*, 2005). This is not surprising as one of the plant responses against pathogen attack is the induction of an oxidative burst (Lamb & Dixon, 1997).

The CW_8G motif is bound by MADS family transcription factors, which function in the development of flowers and reproductive organs, and have a role in controlling flowering time (Garcia-Maroto *et al.*, 2003). So why do genes responsive to *E. cichoracearum* infection possess binding sites for MADS-box transcription factors? It has been shown that certain compatible interactions (*P. syringae*, *X. campestris* and *P. parasitica*) can cause accelerated flowering in Arabidopsis (Korves & Bergelson, 2003). Although this has not yet been proved for any fungal pathogen (*P. parasitica* is an oomycete, not a true fungi), the presence of MADS boxes in *E. cichoracearum* responsive genes strongly suggests that flowering time is also affected by *E. cichoracearum* infection. This has not been investigated during this study, and could be an avenue for further work. Indeed the entire issue of developmental changes and fitness costs to infected and resistant plants during the course of powdery mildew infection would benefit from further study. Interesting work has been performed on this subject for other host-pathogen interactions, with counterintuitive results that indicated a reduced fitness for resistant plants in certain circumstances (Korves & Bergelson, 2004).

The MYB family of transcription factors is a large and diverse group; a few are involved in defence responses and stress responses (Jalali *et al.*, 2006). A member of the MY2B family was shown to be involved in stress and abscisic acid signalling (Abe *et al.*, 1997), while a member of the MYB family was shown to have roles in both biotic and abiotic stress signalling (Mengiste *et al.*, 2003). Further research could identify the transcription factor which potentially binds to these domains, and which genes are regulated by the factor, in order to clarify the role it plays in plant defence responses. Given the varied nature of the MYB group of transcription factors, there may only be a limited amount of information that can be inferred by the study of homologous genes.

The T-box motif was identified from the GAPDH gene as a light-regulated response element (Chan *et al.*, 2001), but was heavily over-represented in a list of genes regulated in response to oxidative stress (Mahalingam *et al.*, 2005). This transcription factor binding site may represent a link between the control of photosynthetic pathways and the generation of a ROS burst. This is consistent with the observation that ROS signals emanating from chloroplasts are a critical component of the initial physiological response to certain kinds of stress response (Mahalingam *et al.*, 2005).

W-box binding sites are highly over-represented in genes responsive to *E. cichoracearum* infection (Table 3.3). W-boxes are bound by the WRKY family of transcription factor that are unique to plants (Jalali *et al.*, 2006). W-boxes are present in the promoters of many plant defence genes and clusters of W-boxes are present in a single promoter region, indicating that WKRY proteins may act synergistically (Jalali *et al.*, 2006).

Several families of transcription factors are known to be involved in the response of plants to pathogens; some like the WKRY family function almost exclusively in this role,

others such as those which bind to the ABRE-like motif function in a more general response to plant hormones. Yet other families still are far more diverse (such as the MYB family) and only a small proportion of transcription factors in the family are thought to be involved in plant defence responses. The role of these transcription factors is often less well studied. Future research into these may cast light into the connections between ordinarily non-defence activities and induced plant defences. Examples of this are transcription factors involved in the control of flowering time and the production of ROS.

3.3.4 Treatments with similar gene expression profiles to *E. cichoracearum* infection of Arabidopsis

This study has also compared microarray data from powdery mildew-infected Arabidopsis to other results available in public microarray databases. In particular, the Genevestigator database was used as it provides an excellent interface for comparing different treatments. As it was not possible at the time to compare extremely large numbers of genes across the hundreds of datasets available (due to computing resource limitations) the 50 most up-regulated and 50 most down-regulated genes were compared across treatments. As expected, the treatment producing gene expression changes that most closely correlate to the changes in genes regulated by *E. cichoracearum* infection is *E. orontii* infection (Table 3.4). Following this is a number of other stress related conditions and it is interesting to note that the second highest correlation is not with another pathogen but is with ozone treatment. Several environmental stress conditions (low nitrogen and low glucose, low potassium, and high carbon dioxide) are also closely related to *E. cichoracearum* infection. This highlights the importance of the physiological changes induced by biotrophy which profoundly alter photoassimilate partitioning, relative to purely defence related changes. Equally, treatments with a very poor correlation to *E. cichoracearum* infection can be informative. Treatments with auxin and in particular MeJA have a low correlation to *E. cichoracearum* infection. This supports the argument that JA is antagonistic with SA – see Glazebrook (2005) and Rojo (2003) for a discussion of the interplay between JA and SA. SA is known to be heavily involved in defence against biotrophs including *E. cichoracearum* (Glazebrook, 2005). JA and ET are known to be involved in the defence against hemi-biotrophic and necrotrophic pathogens (Glazebrook, 2005). Auxin is a growth hormone (Taiz & Zeiger, 2002) and whilst plants are under severe nutrient stress, processes related to enhanced growth will probably be reduced.

Looking in detail into the function of transporters responsive to *E. cichoracearum* infection, Table 3.5 shows that sugar transporters, glutamate receptors, peptide transporters, P-type pumps and nitrate transporters represent a high proportion of responsive genes. By proportion, all of these groups are over-represented in responsive genes as compared to their proportion of the entire genome. Specific (putative) regulated sugar transporters were STP4 (At3g19930), STP1 (At1g11260) and STP27 (At4g04750).

Specific (putative) regulated glutamate receptors were GR7 (At2g29110), GR8 (At2g29110) and GR20 (At3g07520). Specific regulated P-type pumps were ACA12 (At3g63380), ALA10 (At3g25610), ALA1 (At5g04930), ACA1/PEA1 (At1g11260), ALA7 (At3g13900). This is consistent with a hypothesis that host membrane transporters play a crucial role in the redistribution of nutrients within an infected plant. It is not clear whether the expression changes in the transporters responsible for the distribution of the primary forms of nutrients within plants (sugars, peptides and other nitrogenous compounds) alter nutrient distribution primarily for host defences or nutrient transfer to the powdery mildew. The role of P-type pumps and glutamate receptors within plant defences is as yet unknown. Further work is required to establish those roles and the true function of changes in expression for sugar, nitrate and peptide transporters.

Chapter 4

Full-Genome Transcriptional Studies Conducted in Southampton

4.1 Introduction

Although the results of full-genome microarrays on both *E. cichoracearum* and *E. orontii* are available for public use, the ability to perform in-house transcriptional studies allows great flexibility in the types of experiments which can be conducted. Researchers can perform the exact experiment desired; using the time points, genotypes and countless other variables required, rather than having to make do with those provided in previously available data. It is also difficult to predict what data will become publicly available in the future, so researchers often choose to pursue their own transcriptomic experiments, rather than wait for data from other (unknown) experiments to become publicly available.

The physiology of the host plant once biotrophy is well-established, is an area of active research. Much effort is focused on determining the exact methods of nutrient transfer between host and pathogen and the response of the plant to this nutrient transfer. Most research has however, focused on the initial response of the plant to powdery mildew infection, in excellent and highly successful attempts to elucidate the mechanisms behind the host defence response. However, a longer time-frame (in terms of hours post infection) is required to look at the physiology of a diseased host plant, compared to those that have been used in publicly available data. By using later time points, this should have the effect of reducing the impact on the transcriptome of the initial stages of plant defence against the pathogen, relative to changes in the host physiology induced by biotrophy.

4.1.1 Chapter Aims

This chapter aims to outline the transcriptional response of Arabidopsis to *E. cichoracearum* at 144 hpi, a time point for which no publicly available data are available. This should allow changes which occur in well-developed powdery mildew colonies in transcription to be monitored. This should allow a greater understanding of the physiology of the host plant response to well-established biotrophy.

4.2 Results

4.2.1 Development of the microarray protocol

A variety of microarray technologies and protocols have been used during the course of this project. Previously, custom made AMT (Arabidopsis Membrane Transporter) oligonucleotide arrays were used at Southampton. As technology has developed, other types of microarray have become appropriate and these were discussed in Chapter 3. Although Affymetrix GeneChip arrays are currently regarded as the highest quality microarrays available, they are prohibitively expensive in many cases. For this project, it was decided that it would be appropriate to use some of the other full genome microarray technologies available. To this end 'Arizona' microarrays (printed by the University of Arizona, but using the Operon probe set). A measure of the technical success of the microarrays is the number of spots called as 'present' by the microarray analysis software; although at appropriate magnification the spots are normally distinguishable by eye. The number of expressed genes will vary from tissue to tissue and treatment to treatment. For instance, on the Affymetrix arrays conducted using *E. orontii* shown in Chapter 3, the average number of genes called as 'present' per array was 13,347 compared to the approximately 24,000 probes on the array. Initially, microarrays run here at Southampton were not successful, but results improved dramatically over time (Figure 4.1). A succession of modifications of the initial microarray protocol were made and all had a positive incremental effect on the results. In summary the changes introduced were:

1) The starting template for cDNA synthesis was changed from total RNA to mRNA purified from total RNA

2) The cDNA synthesis duration and temperature were changed, as was the reverse transcriptase enzyme used

3) The procedure for purifying cDNA and labelled cDNA was changed, using a different kit supplier

4) The procedure for labelling cDNA was changed from dye incorporation during cDNA synthesis to post synthesis labelling

5) The hybridisation equipment was changed, using raised cover-slips and hybridisation chambers which maintain humidity

As modifications were made to the protocol, the data produced was of increasing quality, although success was only achieved using Arizona arrays. Printed oligonucleotide microarrays are normally regarded as being less sensitive than Affymetrix GeneChips, so fewer genes are expected to be detected by Arizona microarrays. The samples investigated and the success of the arrays on which they were run (provided they worked even partially) is shown in Table 4.1. Some arrays are of excellent quality, with around 10,000 genes having a 'present' call in both channels. This is about 80% of the number of genes observed using the more sensitive Affymetrix GeneChips. Some arrays are of

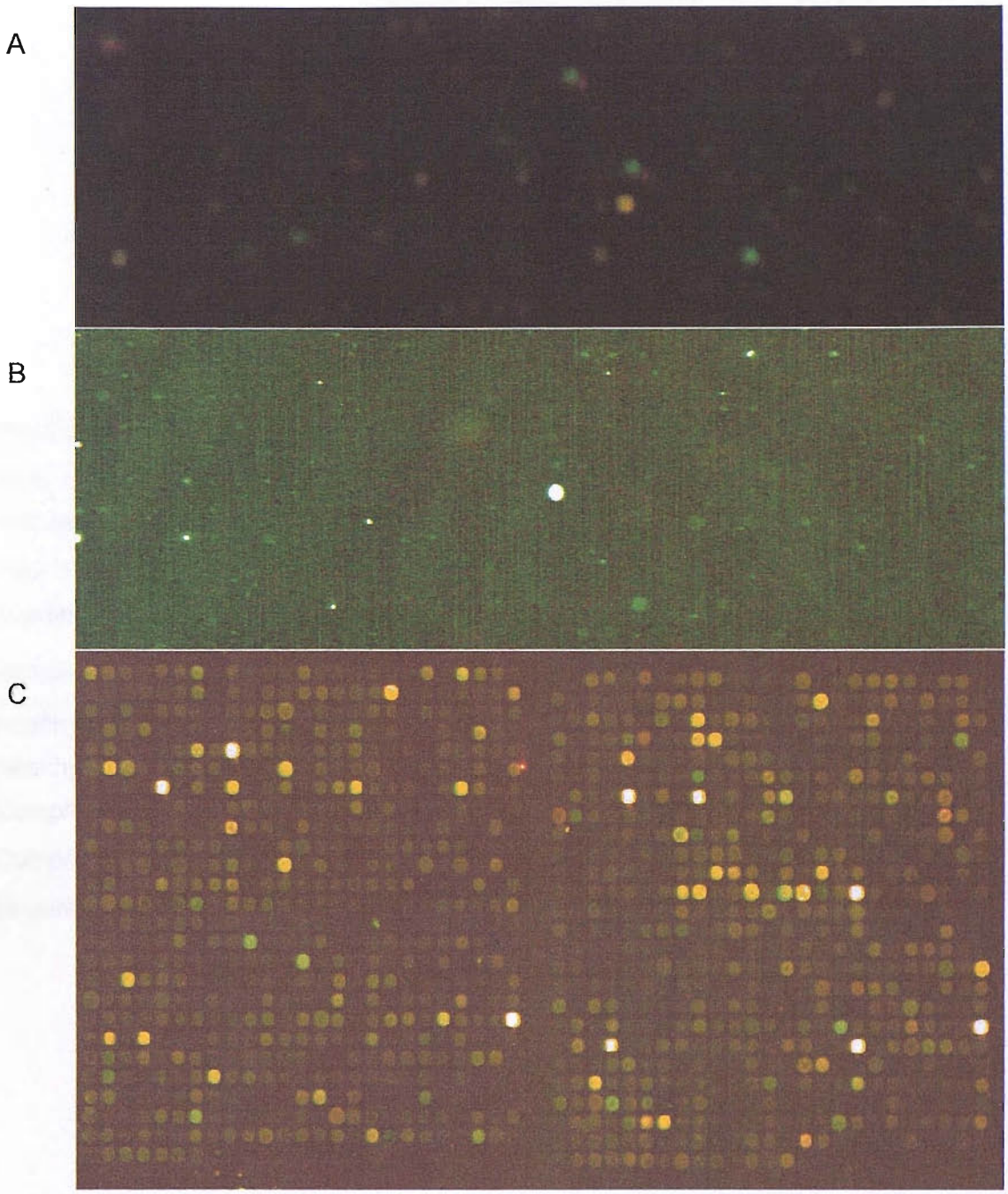

Figure 4.1 Examples of successful and unsuccessful microarrays. Several types of microarray were used during the project, and results steadily improved. A: An oligonucleotide array printed by the University of Arizona, B: A CATMA microarray based on PCR amplified sequences specific to most of the Arabidopsis genome, C: An example of a successful microarray; the array displayed is an 'Arizona' microarray. Due to the density of target spotting on full genome arrays, it is not possible to display the entire array at a resolution where spots are visually distinguishable.

The 4.1 Arrays performed in this study and a measure of the technical success. Several Arizona microarrays were performed on RNA prepared from healthy plants, plants infected with E. cichoracearum or plants infected with B. graminis. In all cases samples were harvested at 144 hours (6 days) post infection. Each sample was prepared from a separate biological sample.

Identifier	Channel 1	Channel 2	Genes present
Healthy/Comp1	Uninfected	*E. cichoracearum* infected	1,240
Healthy/Comp2	Uninfected	*E. cichoracearum* infected	2,923
Comp/Incomp1	*E. cichoracearum* infected	*B. graminis* infected	7,306
Comp/Incomp2	*B. graminis* infected	*E. cichoracearum* infected	10,012
Healthy/Comp3	*E. cichoracearum* infected	Uninfected	10,776

acceptable quality, yielding a good amount of data but less than could have been achieved (5,000 – 7,000 genes 'present'). Some arrays were of low quality, with the number of genes called 'present' close to the levels at which microarray normalisation becomes unreliable (1,000 – 5,000 genes 'present'). Exactly which arrays are used in each analysis will be clearly indicated.

4.2.2 Analysis of compatible interaction microarrays

To study time points in the Arabidopsis/ *E. cichoracearum* interaction that are later after infection than had been used in the publicly available data, a separate microarray analysis was conducted at Southampton. Several Arizona microarrays were conducted using material prepared at Southampton (see Table 4.1). The raw data from these microarrays was analysed as described in Chapter 2. The LOWESS algorithm was applied in all cases. Although the algorithm is known to be unreliable on microarrays with less than 1000 genes called present there were enough genes called in each for this array to be used. The results of these arrays were compared to each other and very little correlation was found between them (Table 4.2). This is most likely due to the low number of genes called as 'present' on microarrays A and B, and strongly indicates that data extracted from these microarrays would not be reliable. It was therefore decided to proceed in analysing the results of the last and successful microarray, microarray E, which had 10,796 genes called as 'present'. With only 1 data point per probeset, it was not possible to use a student's T-Test to select only genes with statistically significant expression changes.

The five most up- and down-regulated genes in response to *E. cichoracearum* as measured by Arizona microarray are shown in Figure 4.2. Four out of the five most up-regulated genes are known to be defence related (the defensins and PR-1). The data from this microarray were compared to the real-time PCR data described in Chapter 3 (Figure 4.3), both from biological replicate 3 (the replicate used in the microarray) and the average real-time data from all biological replicates (as used in Chapter 3). The correlation is greatest between real-time data drawn from the same biological repeat ($R^2 = 0.44$), but is not as high as expected for a comparison of what should ideally be identical data.

Figure 4.4 shows the result of some additional real-time PCR which was carried out on genes identified as being strongly up- or down-regulated in response to *E. cichoracearum* at 144 hpi in the Arizona microarray (rather than the genes used in Chapter 3 which were originally identified from publicly available microarray data). Although the amount of data were limited due to time constraints, the correlation between the microarray data and real-time data is excellent ($R^2 = 0.97$).

Comparing the data from this Arizona array to the publicly available Affymetrix datasets revealed relatively low levels of agreement (Table 4.3). It can be seen not only that the correlation is low, but also that the Arizona microarray results are slightly closer to

Table 4.2 The correlation between results of three Arizona microarrays conducted at Southampton. Three Arizona microarrays were conducted on RNA from healthy Arabidopsis and Arabidopsis infected with *E. cichoracearum* harvested at 144 hpi. The correlation was determined by calculating the Pearson correlation of genes showing greater than a 1.5 fold change in response to one or more of the treatments.

Identifier	Healthy/Comp2	Healthy/Comp3
Healthy/Comp1	0.20	0.03
Healthy/Comp2		0.18

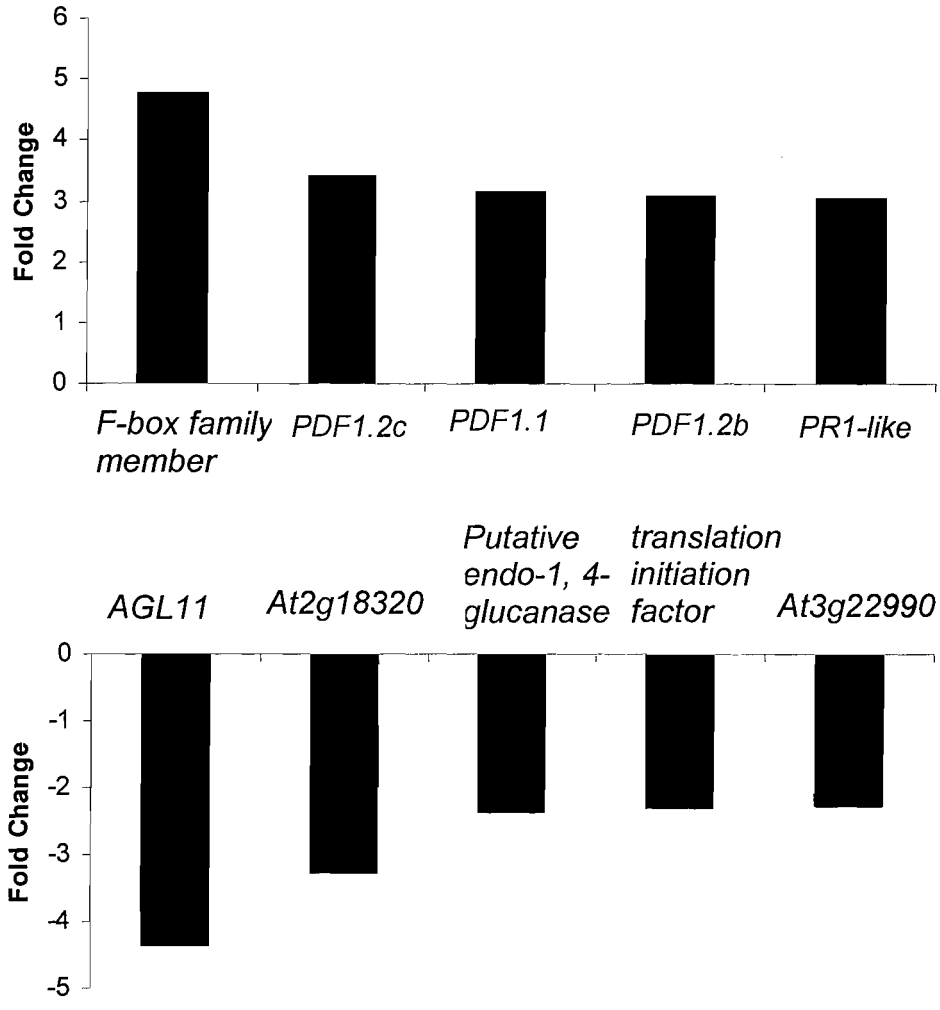

Figure 4.2 The five most up- and down- regulated genes in response to *E. cichoracearum* at 144 hpi, as measured by Arizona microarray. The microarray was carried out on material from healthy Arabidopsis and Arabidopsis infected with *E. cichoracearum* and harvested at 144 hpi. Abbreviations *PDF*: *Plant Defensin*, *PR-1 like*: *pathogenesis responsive 1 like*, *AGL11*: *AGAMOUS-LIKE 11*

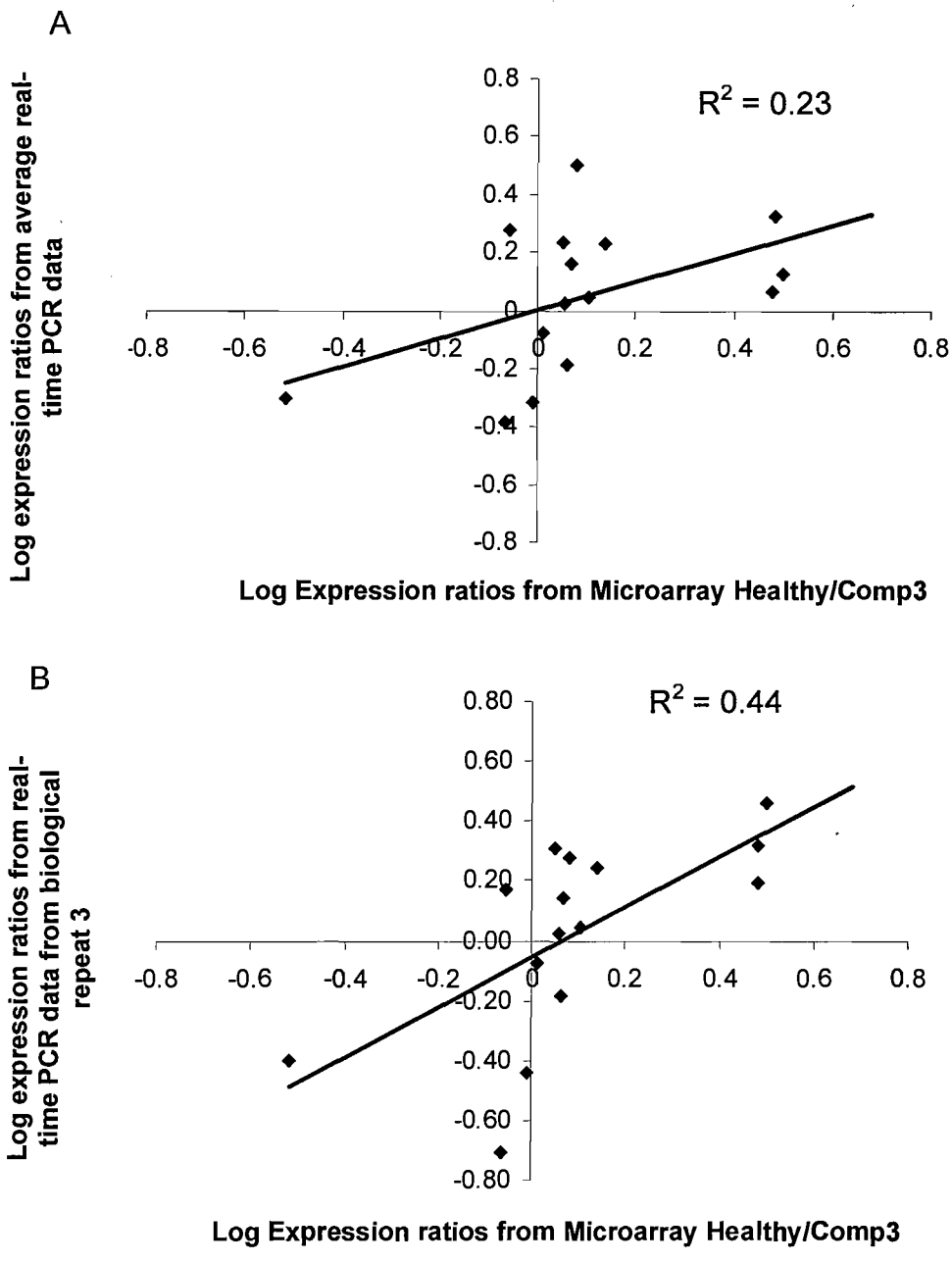

Figure 4.3 Comparison between microarray data and real-time PCR data. The log of expression ratios for genes already investigated by real-time PCR (in Chapter 3) as determined by the Arizona microarray 'E' plotted against: A. Using average expression ratios from real-time PCR from 3 biological repeats; B. The log of expression ratios for real-time data drawn only from the biological repeat used in the microarray.

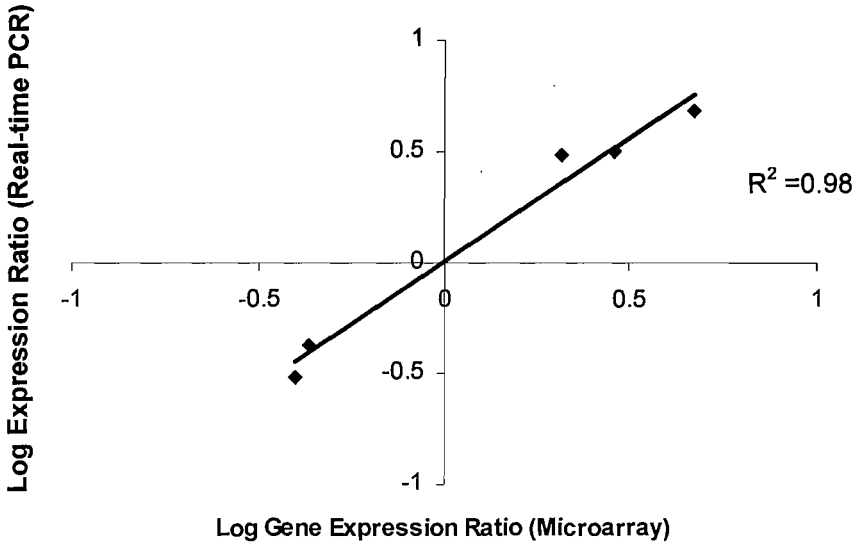

Figure 4.4 Comparison of gene expression ratios determined by real-time PCR and microarray data. A selection of genes was identified as being up- or down-regulated from the Arizona microarray results, and real-time PCR was conducted to test the response of these. The real-time data was obtained from the same sample as the microarray data; harvested at 144 hpi.

Table 4.3 Comparison of Arizona microarray data and publicly available microarray data. The correlation was calculated using the Pearson correlation for genes showing a greater than 1.5 fold change in either treatment.

Array	Correlation
E. cichoracearum 72 hpi	0.233
E. orontii 72 hpi	0.254
E. orontii 144 hpi	0.255

E. orontii microarrays than they are to the *E. cichoracearum*. The results of a 144 hpi microarray might not be closely related to earlier time points; in fact part of the reason behind the experiment was to identify further genes regulated in the infection process. The responses of genes showing a greater than 1.5 fold change in expression in response to *E. cichoracearum* infection were compared with other treatments using Genevestigator.

The highest correlations were observed with: *P. infestans* infection, cold stress, *B. cinerea*, ozone treatment and *E. cichoracearum* infection. The five least correlated treatments were: uniconaxole (an inhibitor of gibberellin synthesis), MG13 (a proteasome inhibitor), oxidative stress, ibuprofen (a lipoxygenase inhibitor) and hypoxia.

As in Chapter 3, the promoters of genes showing a more than 1.5 fold change in expression were analysed to examine if any promoters were over-represented, compared to their representation in the entire Arabidopsis genome. Again, the Athena database was used, and again the P-Value for determining enrichment was 0.0001 because of the problems testing multiple hypotheses. Two ABA related promoters and the G-box binding site are over represented (Table 4.4).

4.2.3 Analysis of compatible and incompatible infection microarrays

To investigate the differences in the response of Arabidopsis to compatible and incompatible powdery mildew infections, two full genome microarrays comparing Arabidopsis infected with *E. cichoracearum* to Arabidopsis infected with *B. graminis* were successfully completed (see Table 4.1). Each array was conducted on material prepared from a different biological repeat. These arrays represent a biological repeat of the infection process. Following data extraction and normalisation, the response of genes to compatible and incompatible powdery mildew infections could be seen. The 5 most up- and 5 most down-regulated genes are shown in Figure 4.5. With a few exceptions, there is relatively little functional data on the 10 genes shown in Figure 4.5; most information comes from homology-based annotation. Most do not currently possess gene symbols, other than the gene numbers assigned by the genome annotation project. Noteable exceptions are *PSBW* (the gene encoding the phtotosystem II reaction centre), *PER42* (*peroxidase 42*) and *EXPA15* (an alpha expansin).

A comparison of the two sets of data revealed that there was very little correlation between the two microarrays. The use of arbitrary cut-off values for fold expression is a crude method of analysis, but it can be a legitimate part of a preliminary analysis. When genes changing in expression by more than 1.5 fold are compared, only 17 genes in total showed the same regulation (either both up-regulated or both down- regulated). Using a higher cut-off of 2-fold, no genes showed the same pattern of responsiveness. Using the same method to measure correlation as used in Chapter 3, the Pearson correlation between the two sets of data was less than 0.01 (data not shown). Although a high level of variation between biological repeats has been observed in publicly available microarray data, a higher degree of correlation would have been expected. Evidence from Chapter 3

indicates a correlation of up to $R^2 = 0.4$ between infections by different powdery mildews, so a higher correlation would be expected between identical biological treatments.

A limited selection of responsive genes from each microarray was chosen. If the limited selection of genes showed a correlation to the microarray data, the selection of genes could be expanded. Unfortunately, the real-time data for the selection of genes did not match the microarray data (Figure 4.6). No observable relationship could be seen between the two sets of data. Following this, it was decided not to continue checking these arrays by real-time PCR and pursue more promising lines of research.

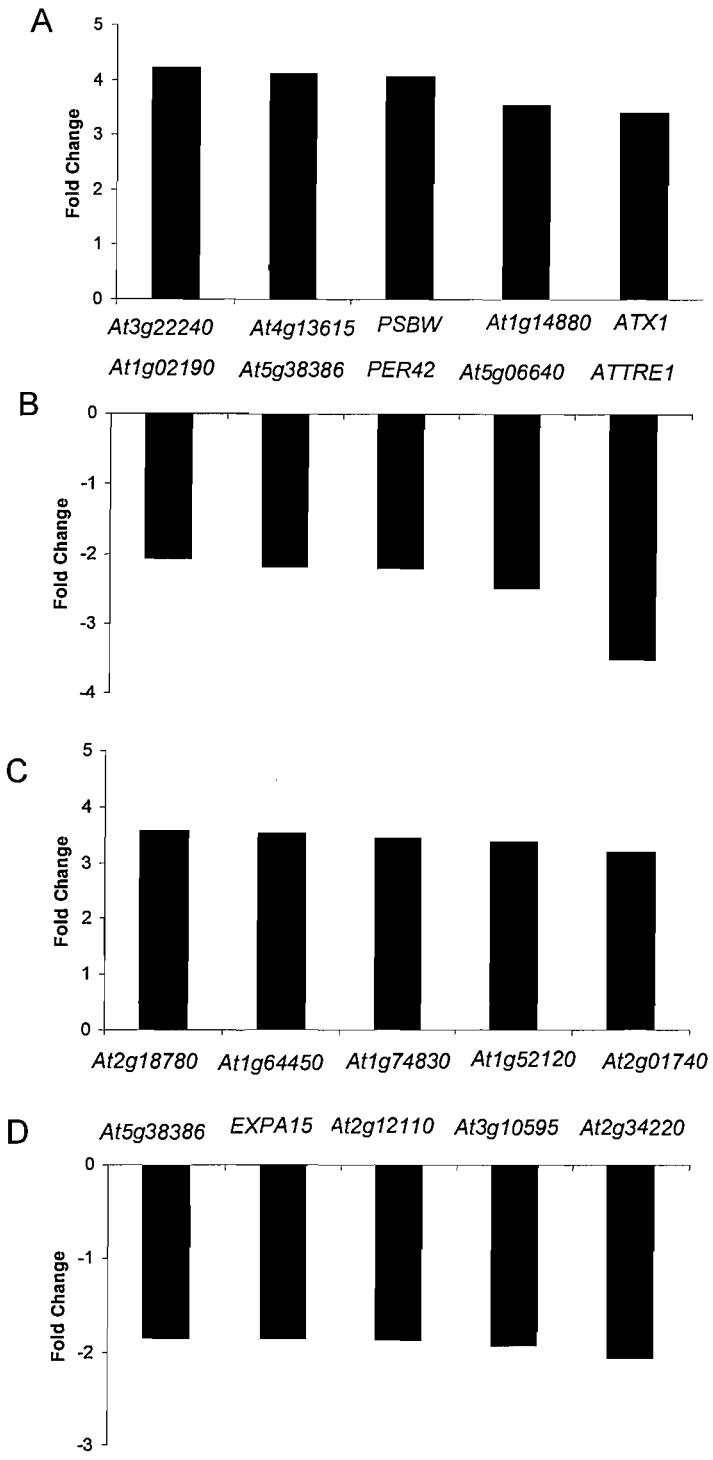

Figure 4.5 The five most up-regulated and down-regulated genes from two microarrays comparing *E. cichoracearum* infected Arabidopsis with *B. graminis* inoculated Arabidopsis. Data from microarrays Comp/Incomp1 and Comp/Incomp2 were analysed using the Genespring software to give the expression ratios between incompatible and compatible infections. A: the five most up-regulated genes from microarray Comp/Incomp1, B: the five most down-regulated genes from microarray Comp/Incomp1, C: the five most up-regulated genes from microarray Comp/Incomp2, D: the five most down-regulated genes from microarray Comp/Incomp2. A fold change of 2 indicates that the expression of that gene is 2 fold greater in the incompatible infection compared to the compatible infection.

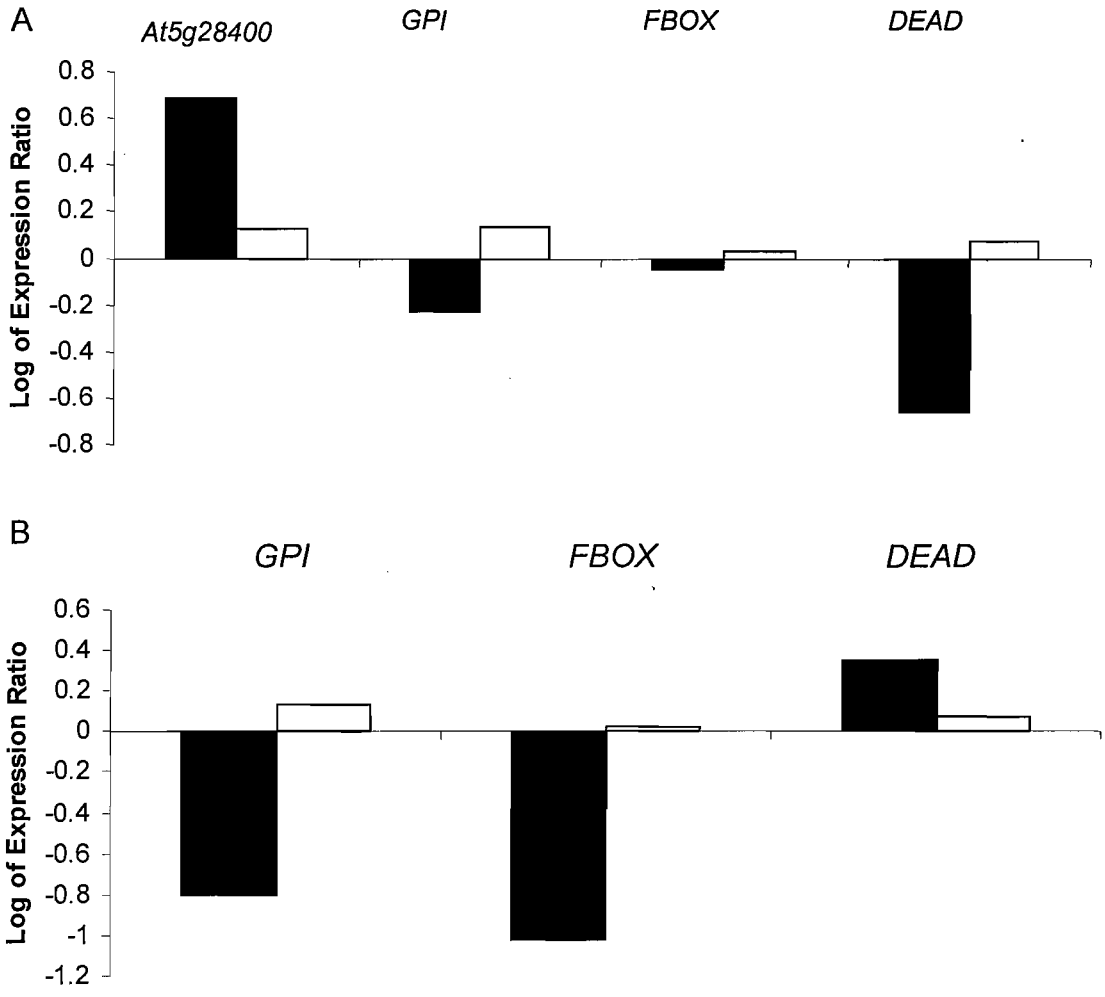

Figure 4.6 Comparison of gene expression ratios determined by real-time PCR and microarray data. White bars: expression ratios of selected genes as determined by real-time PCR compared to microarray data (Black Bars). A. expression ratios of the same selected genes drawn from microarray D (Arabidopsis infected with a compatible powdery mildew *E. cichoracearum*, and an incompatible powdery mildew, *B. graminis* at 144 hpi). B. expression ratios of the same selected genes drawn from microarray E, a biological repeat of Arabidopsis infected with a compatible powdery mildew *E. cichoracearum*, and an incompatible powdery mildew, *B. graminis* at 144 hpi.

4.3 Discussion

This study has conducted a series of microarrays on *E. cichoracearum* infected Arabidopsis. Initially these were unsuccessful but a series of protocol developments resulted in successful microarrays (Figure 4.1). Due to time constraints only a limited number of microarrays were conducted after the development of a successful protocol. Comparisons between the data from successful microarrays and the little data that could be obtained from unsuccessful microarrays produced very poor correlations, indicating that even the limited amount of data produced from an unsuccessful microarray is not reliable. However, in terms of microarrays comparing the transcriptome of healthy Arabidopsis to *E. cichoracearum* infected Arabidopsis, data from genes showing a high fold-change in expression were extremely reproducible by real-time PCR on the same sample ($R^2 = 0.98$). Further microarrays on several biological repeats would: improve the number of genes for which there was available data; reduce the effect of biological variability; and give an indication of the statistical reliability of the microarray data. Regardless, the microarray successfully conducted provides important information on some genes that are regulated in response to *E. cichoracearum* infection of Arabidopsis (at 144 hpi), but it is important to note that there will be other genes that are regulated in this response which are not picked up on this array (false negatives).

Microarrays comparing a compatible (*E. cichoracearum*) to an incompatible (*B. graminis*) interaction were not successful. Although both of the conducted microarrays produced a high number of expression ratios (7,306 and 10,012), it was not possible to confirm any of the microarray data by real-time PCR for either of the microarrays. When all of the data from these arrays were compared, the arrays were found to be very poorly correlated ($R^2 < 0.01$). Due to time constraints, the cause of this was not investigated further but a potential cause of this is the heavy bias towards signal from only one of the two channels (dyes) in the microarrays. This makes the normalisation process less reliable as data from weaker channels require more manipulation. Other potential causes might be protocol errors or contamination of the microarray sample, non-specific hybridisation or mis-annotation of the spot IDs. As there was no further investigation into possible causes of this, it is not clear why this should occur only in these two microarrays, while data from the healthy/comp3 array were highly reproducible by real-time PCR.

4.3.1 Analysis of *an E. cichoracearum* infected Arabidopsis

The most up-regulated gene observed *is At5g25290*, a gene and protein with no known prior publications. Current gene annotations describe it simply as an 'F-box family protein' (http://www.arabidopsis.org) and has no molecular, biological or cellular function currently ascribed to it using the gene ontology system (Ashburner *et al.*, 2000). Using the Genevestigator database it is clear that Affymetrix microarrays do not normally detect the expression of this gene; only a handful of treatments have a detectable level of

expression. Treatments which up-regulate this gene are (in order): programmed cell death; hypoxia; high light intensities and treatment with chitin. The F-box gene displays no significant regulation in either of the publicly available data sets for *E. cichoracearum* or *E. orontii* infected Arabidopsis, as the signal for this gene was below the detection limit in both experiments. There are no studies publicly available on the Genevestigator database that show down-regulation of this gene and this is probably due to the low number of studies with detectable signals for the gene.

Several of the remaining top five most up-regulated genes are plant defensins (PDFs). No specific work has been performed on the gene *PDF1.2c* (the second most up-regulated gene), but the protein product is identical to *PDF1.2a* and *PDF1.2b*. *PDF1.2a* was the originally identified member of the *PDF1.2* group and is sometime known only as *PDF1.2.* The plant protein *PDF1.2a* is a JA, SA-independent anti-fungal protein (Penninckx *et al.*, 1996; Manners *et al.*, 1998). The mode of action of plant defensins is not entirely clear at present, but current evidence suggests that although that they induce membrane permeabilisation, they do not function as pore forming proteins (Theis & Stahl, 2004) as unlike defensins from insect and animal kingdoms, they are not capable of forming such pores in artificial lipid bilayers (Theis & Stahl, 2004). A current hypothesis for the method of action for plant defensins is that they might bind a specific receptor which would activate endogenous signal transduction components, which may affect the activity of endogenous ion channels or ion transporters (Theis & Stahl, 2004). Equally plant defensins may allow selective Ca^{2+} uptake into pathogens through activated ion channels, as it is believed that a gradient in cytosolic Ca^{2+} concentration, generated by tip-localised Ca^{2+} channels, is essential for driving polarised growth of fungal hyphae (Theis & Stahl, 2004). Although the protein sequences of the PDF1.2 group are identical, the transcripts are not and this allows the 70-mer OPERON probes used in Arizona arrays to differentiate between the transcripts of the three members of the group. The extent to which past work on *PDF1.2(a)* transcript levels (by RT-PCR for example) is specific to any one of the three will differ between experiments, given that the existence of the other two nearly identical copies of *PDF1.2*(a) in the genome remained unknown until 2002 (Thomma *et al.*, 2002). Given that the gene products of *PDF1.2a*, *b* and *c* are identical, it is not surprising that they show almost identical levels of regulation. Although *PDF1.2b* and *PDF1.2c* are in the top five most up-regulated genes, *PDF1.2a* is the sixth most up-regulated gene.

Looking at the Genevestigator database, *PDF1.2* is mildly up-regulated in response to *E. orontii* at 120 hpi (1.46 fold for *PDF1.2a* and 1.75 fold for *PDF1.2b*, PDF1.2c is not detected on Affymetrix arrays) but less so for *E. cichoracearum* at 72 hpi (1.17 fold for *PDF1.2a* and 1.16 fold for *PDF1.2c*).

The most down-regulated gene is *AGL11*, a transcription factor thought to be found only in ovules and carpels (http://www.arabidopsis.org). Further investigation on Genevestigator using a series of microarrays for specific plant organs confirmed this and

no treatment currently in the Genevestigator database substantially alters the levels of *AGL11* transcripts. Real-time PCR conducted to confirm the expression of the transcripts was not successful – no transcript could be detected (data not shown). False positive results do occur in microarray experiments (Quackenbush, 2002), due to many factors including non-specific hybridisation (potentially due to poor post-hybridisation washing in a (very) localised area of the slide) or the presence of fluorescent material close to the area of a spot that is not removed by the automatic spot checking software. To apply an algorithm systematically over a microarray image reduces experimenter bias. To retrospectively check individual gene spots and disqualify them if data are difficult to explain would introduce experimenter bias, so it is not appropriate to simply 'remove' a particular gene from the output. The best way to counter false-positive results is through repetition.

Analysis using Genevestigator with the other 4 most down-regulated genes shows that other treatments inducing down-regulation were: senescence; the application of syringolin; infection with *P. infestans* and treatment with SA. This supports the role of these genes in the response of Arabidopsis to *E. cichoracearum* infection as all of the treatments listed above are related to defence responses.

This study has shown some of the responses of Arabidopsis to *E. cichoracearum* at 144 hpi. The importance of defensins in this interaction at the 144 hpi time point has been highlighted. It would not be possible to tell this from the publicly available microarray studies currently available. Due to the limited amounts of replication available it is not sensible to pursue a more in-depth analysis of the transcriptional response of Arabidopsis to *E. cichoracearum*. Now that a successful protocol has been developed, more microarrays are needed in order to reduce false-positive and false-negative results and this will provide a sound base for more advanced analysis of the transcriptional response of Arabidopsis to *E. cichoracearum* during established biotrophy.

Chapter 5

The Generation and Confirmation of Homozygous T-DNA Insertional Mutants

5.1 Introduction

The inactivation of a gene is normally the most direct way to understand its function (Parinov & Sundaresan, 2000), and understanding gene function is a critical challenge in plant biology with approximately 30 % of the Arabidopsis genome encoding genes of no known or predicted function (Arabidopsis Genome Initiative, 2000). Functional genomics uses both forwards and reverse genetics approaches to determine gene function, but reverse genetics has more potential on a genome wide scale (Holtorf *et al.*, 2002). To this end Arabidopsis has been the subject of several genome-wide mutagenesis studies, by both T-DNA and transposon insertions (Raina *et al.*, 2002; e.g. Alonso *et al.*, 2003; Kuromori *et al.*, 2004; Radhamony *et al.*, 2005; Nishal *et al.*, 2005). This has been completed so successfully that seed stocks for plants with a T-DNA or transposon insertion are available for almost all Arabidopsis genes (Radhamony *et al.*, 2005). Some resources have characterised the insertion site by direct sequencing (Liu *et al.*, 1995; Alonso *et al.*, 2003; Radhamony *et al.*, 2005), and this information has been placed on interrogatable databases (http://signal.salk.edu/cgi-bin/tdnaexpress; http://arabidopsis.info). These non-targeted approaches have been complimented by methods for targeted gene disruptions, such as TILLING (Targeting Induced Local Lesions IN Genomes) (McCallum *et al.*, 2000; Till *et al.*, 2003).

5.1.2 Obtaining insertional mutants

The original source of all of the seed stocks from which homozygous insertion mutants were isolated in this study was the genome-wide insertional mutagenesis program conducted by the Salk Institute (Alonso *et al.*, 2003); therefore, it is helpful to discuss the methods for obtaining insertional mutants here. The Salk lines contain T-DNA insertions and are available as seed stocks through the NASC (Nottingham Arabidopsis Seed Centre, http://arabidopsis.info) as an unknown mixture of plants that are wild-type, heterozygous or homozygous for the T-DNA insert. Only homozygous insert lines are suitable for mutant studies, as heterozygous plants may continue to produce full-length mRNA. Stocks from the Salk institute require not only the determination of the zygosity of the seed stock line but also confirmation of the insert location and a determination of the number of inserts. The determination of the number of different T-DNA insertion events is important as it has been estimated that the original plant lines carry an average of 1.5 T-DNA insertions (Alonso *et al.*, 2003).

5.1.2.1 Kanamycin-based selection of mutants

The T-DNA used in this study contains a dominant kanamycin resistance gene *NPTII* (Neomycin Phosphotransferase) (Alonso *et al.*, 2003). In theory, as kanamycin resistance from the *NPTII* gene is a dominant trait, seeds from a wild-type parent (no progeny containing any alleles of *NPTII)* plated onto kanamycin-containing plates will die; seeds from a homozygous parent will all survive (all progeny will contain an allele of *NPTII*). Seeds from a heterozygous parent will show a 3:1 survival: death ratio, as the Mendelian inheritance of the *NPTII* gene will result on average, with one plant in every four containing no alleles of *NPTII* (and therefore dying) with the remaining three plants inheriting at least 1 allele of *NPTII* (and therefore surviving). Plants containing a T-DNA in two or more separate locations in the genome will show altered ratios depending on the number of insert locations, the zygosity of the T-DNA at those locations and whether any two separate insertions are on the same chromosome.

In practice this situation is complicated by the phenomenon of gene silencing. The *NPTII* gene from Salk-T-DNA lines experiences frequent silencing (see http://signal.salk.edu) which means that plants containing T-DNA inserts may still die on kanamycin plates. In the case of lines with more than 1 insert, it is possible that the T-DNA insert of interest is silenced, but another independent insert is not. This could give results that would directly contradict PCR screening (described below). For these reasons screening by kanamycin resistance can only be seen as an indication of the number of T-DNA inserts and zygosity, and serves to confirm data determined by PCR (see below).

5.1.2.2 PCR-based selection of mutants

Although the antibiotic resistance conferred by the T-DNA is sometimes unreliable, the presence of conserved sequences (in particular the left border of the T-DNA) on the edge of the T-DNA allows a PCR-based screen to be effective. Zygosity determined by PCR relies on the determination of the presence or absence of wild-type and mutant copies of the gene by separate PCRs. This is done on the basis that a wild-type plant will contain wild-type copies of the gene, but no mutant copies; a homozygous insertion mutant *vice versa*. A heterozygous plant will have both wild-type and mutant copies present. In practice this is determined by two separate PCRs on genomic DNA extracted from candidate plants. A pair of primers (forwards and reverse) is designed by the experimenter to be specific to the gene of interest and located either side of the suspected insert site. A Lb primer specific to the left border of the T-DNA has already been designed and tested by the Salk institute. The presence of wild-type copies of the gene is checked with the F and R primer. The large size of the T-DNA (several kb) would prevent a PCR using the F and R primers from forming a product if the T-DNA is indeed located between the F and R primers. The T-DNA can insert in two orientations with respect of the gene: the left border of the T-DNA can be towards the 5' end of the gene (with the right border

nearer to the 3' end); or *vice versa*. Depending on the orientation (which has been putatively determined by the Salk Institute) either the F or the R primer is used in combination with the Lb primer, and a product will only be produced if the T-DNA is indeed present.

5.1.2.3 Determining T-DNA copy number

There are several possible methods of determining exactly how many inserts are in a given seed line. Originally the Salk Institute intended the kanamycin selection to be the predominant method, but as discussed earlier this has proved unreliable. The other most commonly used method for determining the number of inserts is by Southern blot. In summary, this involves digesting a large quantity of genomic DNA from a putative insertional mutant with restriction endonucleases, separating the result of this by size on an agarose gel and probing a blot made from this with a T-DNA specific DNA probe (Sambrook *et al.*, 1989). Aside from being time consuming, this can lead to the underestimation of copy number of T-DNA insertions, if several fragments of restricted DNA do not resolve on an agarose gel or more likely, if two T-DNAs are present so close together that they are both on a single fragment. Southern analysis with samples digested separately with different enzymes can reduce, but not eliminate these problems. In response to these concerns, alternative protocols have been suggested. In particular the estimation of copy-number by real-time PCR has been suggested (Ingham *et al.*, 2001), and used by several investigators (Okamoto *et al.*, 2006; Barroco *et al.*, 2006). For the purpose of this work, the term copy number refers to the number of inserts per haploid genome; a heterozygous two-copy plant contains two given transgenic sequences, a homozygous two-copy plant four. The principle behind the method is that real-time PCR can be used to quantify the number of given T-DNA sequences. In this situation the quantity of transgenic sequences is compared to the quantity of sequence for a known single copy endogenous gene (Ingham *et al.*, 2001). The process and theory of quantifying sequences in DNA is not any different to quantifying cDNA when performing an expression analysis. If a suspected homozygous insertional mutant has only one T-DNA insert location, the quantity of T-DNA sequences and a single copy genes will be equal, if there is more than one location, the quantity of T-DNA sequences will be greater (Bubner & Baldwin, 2004). In this study the gene *At2g02810* was used as efficient real-time primers were available, and it is known to be a single copy gene (Arabidopsis Genome Initiative, 2000). To quantify exactly how many T-DNA inserts are present would require prior knowledge of the zygosity of the suspected DNA. Put simply, a T-DNA line with 4 T-DNA sequences could contain two homozygous T-DNA locations or four heterozygous ones. This method also has difficulty in determining the exact number of inserts above one T-DNA insert (or two T-DNA sequences) (Bubner & Baldwin, 2004). This is due to the technical limitations of real-time PCR; while four T-DNA copies are two

times greater than two T-DNA copies, six T-DNA copies are only 0.20 times greater than five. Fold changes of 0.2 are not normally within the range of accuracy for real-time PCR. This is not a problem however for studies of insertional mutants – the purpose of determining copy number in these cases is normally to confirm that there is only one insert location – if there is more than one, a program of backcrossing and possibly mapping would normally need to be started, regardless of the copy number.

5.1.3 Chapter aims

The aim of this chapter is to identify and isolate homozygous insertional mutants for genes shown to be regulated in response to powdery mildew infection in Chapters 3 and 4. Following identification they will be analysed to determine the exact insert location, copy number and absence of full-length cDNA, so that subsequently a phenotypic analysis can be performed.

5.2 Results

5.2.1 Isolation of homozygous insertional mutants

To investigate the physiological function of the transporters identified in Chapter 3 as responsive to *E. cichoracearum* infection, this study has identified plants homozygous for T-DNA insertions in those genes. The original source of the seed stocks from which homozygous insertion mutants were isolated was the genome-wide insertional mutagenesis program conducted by the Salk Institute (Alonso *et al.*, 2003). The selection of lines for study was based on the initial assessment by the Salk Institute, indicating that the T-DNA fell within a transporter gene of interest. Stocks with inserts putatively located in *ACA12, GLR1.4, PTR3*, *ALA10, At2g18680*, *LHT1* and *AtcwINV1* were obtained. The predicted locations of the T-DNA within these genes are listed in Chapter 2. The process of insertional mutant confirmation was undertaken with all the seven genes of interest, but as the process is essentially identical for each one, it will be described in detail for one line – *ACA12*, and summarised for the others.

5.2.2 Isolation of *aca12* insertional mutants

The seed stock N598383, which putatively contained an insert in *ACA12*, was obtained and the original seed received from the NASC were grown to maturity. The zygosity of these individual lines was determined by PCR, and the number of T-DNA inserts determined by real-time PCR. The zygosity of the progeny of some of these plants, as well as an indication of number of T-DNA inserts, were determined by the survival ratios of seedlings on kanamycin-containing plates.

Zygosity was determined by a series PCRs on genomic DNA extracted from candidate plants using a combination of three primers ACA12F, ACA12R and Lb. The locations of the primers and T-DNA in the genomic and mRNA sequence are shown in Figure 5.1. The primers ACA12F and ACA12R are specific to *ACA12* and will produce a PCR product of 880 bp on a wild-type copy of *ACA12*. A PCR using the gene-specific ACA12F primer and the T-DNA specific Lb primer would be expected to produce a product of about 850 bp. This is only approximate as re-arrangement at the T-DNA borders make this region variable in size and the initial Salk sequencing may not be accurate (Alonso *et al.*, 2003). Figure 5.2 shows that plant N598383-1 is wild type, (producing only the ~900 bp wild-type gene product), plants N598383-2 and 3 are homozygous, (containing only the ~850 bp gene-T- DNA product). Plant N598383-4 was identified as being heterozygous, (containing the wild type gene product and the gene-T-DNA product).

For a confirmation that plants homozygous for the T-DNA insert no longer produced a full-length mRNA for the gene of interest, a homozygous plant was grown to maturity, and harvested. RNA was extracted from this and cDNA was

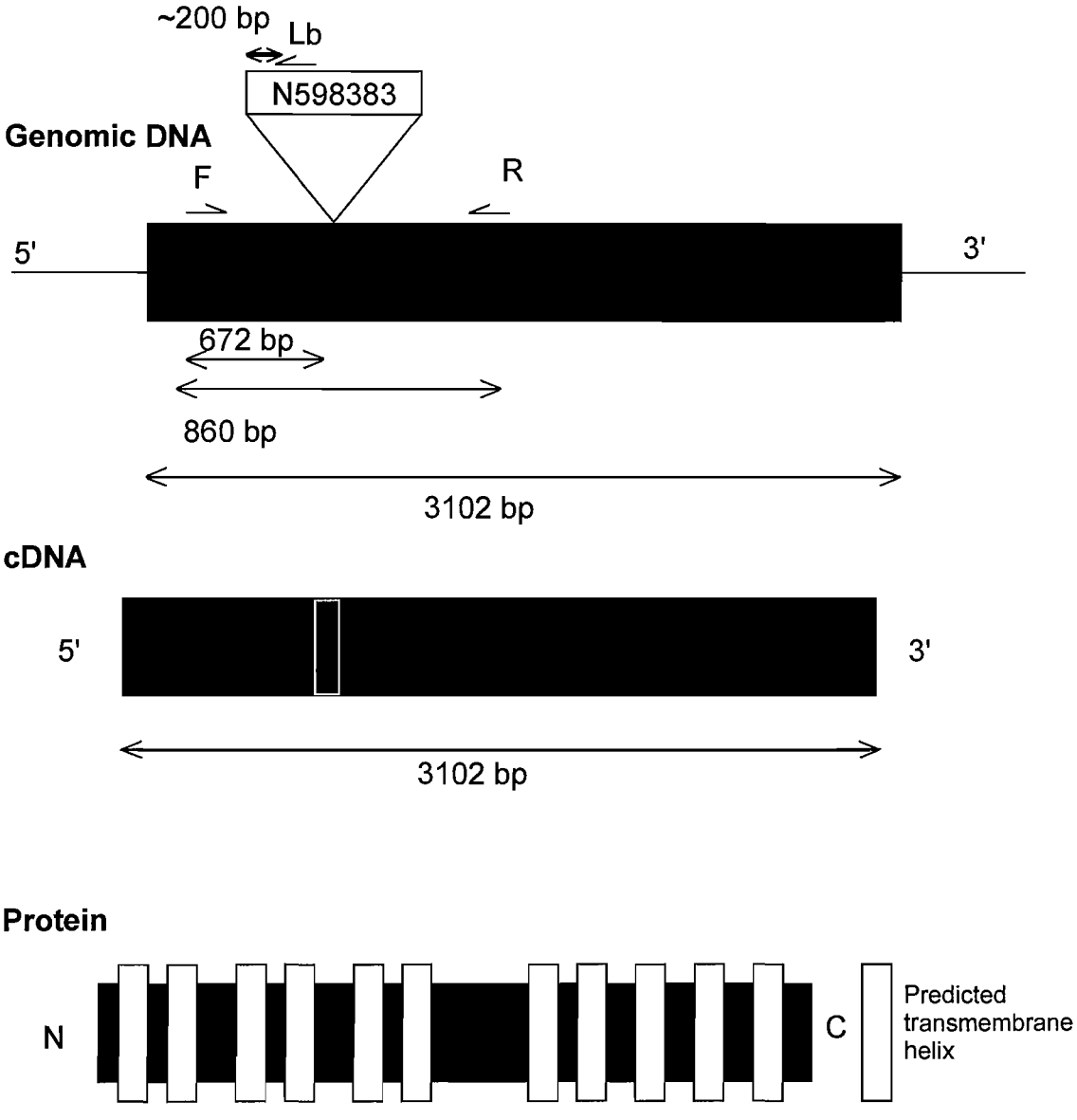

Figure 5.1 Representation of the genomic, cDNA and protein sequence of *ACA12*. The T-DNA in line N598383 is in the single exon of *ACA12*. The white box in the cDNA indicates the location of the T-DNA. Single headed arrows indicate the direction of the gene-specific forwards (F, ACA12F) and reverse (R, ACA12R) primers, as well as the Lb primer specific to the left border of the T-DNA. Double-headed arrows indicate the size of the respective DNA sequences.

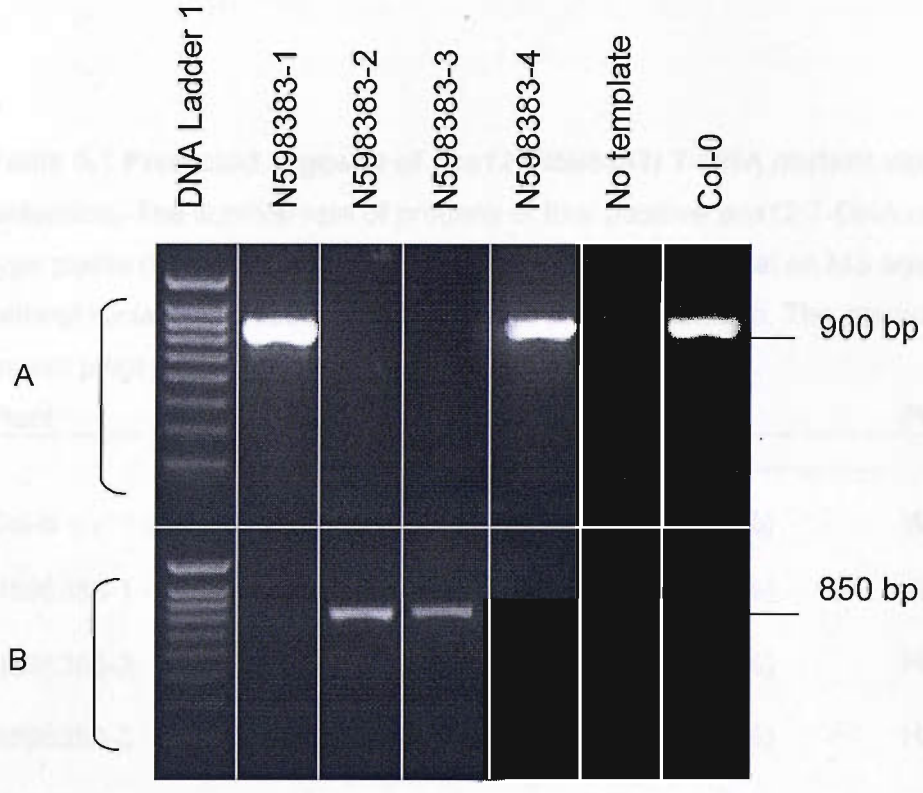

Figure 5.2 Identification of a homozygous *aca12* insertional mutant PCR on genomic DNA extracted from Col-0 and four potential T-DNA insertional mutants. A: PCR using gene-specific primers ACA12F and ACA12R; B: PCR using the gene-specific primer ACA12F and the T-DNA primer Lb. Refer to Figure 5.1 for the location of the primers. Using the logic described in the text, plants N598383-2 and N598383-3 were identified as being homozygous for the T-DNA insert. The DNA ladder used was Q-step1 (Yorkshire Biosciences, UK).

Table 5.1 Predicted zygosity of *aca12* (N598383) T-DNA mutant using antibiotic selection. The survival rate of progeny of four putative *aca12* T-DNA mutants and wild-type plants (Col-8) visually assessed by percentage survival on MS agar plates, with and without kanamycin (100 µg/ml) at 10 days post germination. The predicted zygosity of the parent plant is given.

Plant	Number and Percentage survival		Predicted Zygosity
	Kan+	Kan-	
Col-8	0/64 (0%)	65/65 (100%)	Wild-type
N598383-1	0/68 (0%)	70/70 (100%)	Wild-type
N598383-2	72/72 (100%)	68/68 (100%)	Homozygote
N598383-3	74/74 (100%)	68/68 (100%)	Homozygote
N598383-4	55/75 (73%)	70/70 (100%)	Heterozygote

subsequently prepared from the RNA. Figure 5.3 shows that no product corresponding to the full-length *ACA12* could be amplified from a homozygous plant. Positive controls showed that this band could be amplified from wild-type plants and that as a positive control full-length Actin could be amplified from cDNA prepared from the plant homozygous for the insert.

In order to check the number of T-DNA inserts present, and to confirm the zygosity of the stocks, seed from these plants was plated on kanamycin-containing plates (Table 5.1). These results confirmed the PCR results, as N598383-1 was identified as wild-type, N598383-2 and 3 as homozygous and N598383-4 as heterozygous. The 73% survival rate (approximately equal to a 3:1 survival: death ratio) of the heterozygote plant indicates that this seed line does not contain any other T-DNA inserts. This is only an indication however, as other T-DNAs may be silenced or the parent plant may have contained more than 1 T-DNA insert, which did not segregate into the particular plants tested, but has into other plants, or the parent plant may have contained another closely-linked T-DNA insert on the same chromosome.

In order to confirm that only one T-DNA insert was present, a real-time PCR approach was used to determine the number of T-DNA inserts. As described in 5.1, a T-DNA specific sequence is quantified against an endogenous sequence. In this case the T-DNA specific sequence was part of the *NPTII* gene, and the endogenous gene was *at1g18610*. This analysis determined that as expected, the wild-type plant N598383-1 contained no *NPTII* and the heterozygous plant N598383-4 contained half as much *NPTII* per *At2g18610* as the homozygous plants N598383-2 and N598383-3 (Figure 5.4). This is exactly what is expected when comparing a single insert homozygous plant to a single insert heterozygote plant and strongly indicates there is only one T-DNA insertion site in the homozygous plants N598383-2 and N598383-3.

In order to confirm the status of the insertional mutant, the insert site was sequenced from both the F and Lb primers, which showed the exact insert location in *ACA12* (Figure 5.5), which was at 718 bp out of 3102bp in the cDNA. Following this N598383-2 was re-designated *aca12-1.*

5.2.3 Isolation of *glr1.4* insertional mutants

The seed stock N629955, which putatively contains a T-DNA insert in *GLR1.4,* was obtained and the original seed received from the NASC were grown to maturity. In outline, the zygosity of these was confirmed by PCR and antibiotic selection, the insert location was sequenced, the number of other T-DNA inserts was quantified by real-time PCR and the resultant insertional mutant was checked for the presence of *GLR1.4* mRNA, all using the same methods described for *aca12-1.* The locations of the primers used to determine zygosity and the location of T-DNA in the genomic and cDNA sequences are shown in Figure 5.6.

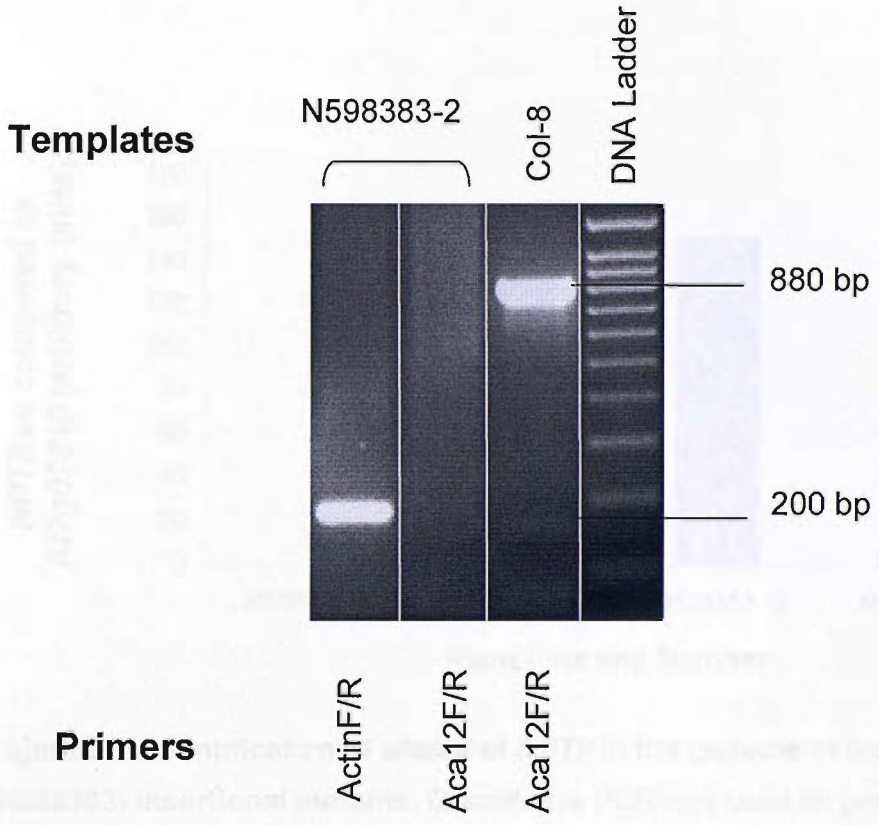

Figure 5.3 No full-length cDNA corresponding to *ACA12* can be amplified from an *aca12* insertional mutant. cDNA was prepared from RNA extracted from plant N598383-2. PCR using ActinF and ActinR primers was used to detect full-length Actin cDNA. The gene-specific primers ACA12F and ACA12R were used to detect *ACA12* cDNA. The DNA ladder used was Q-step1 (Yorkshire Biosciences, UK).

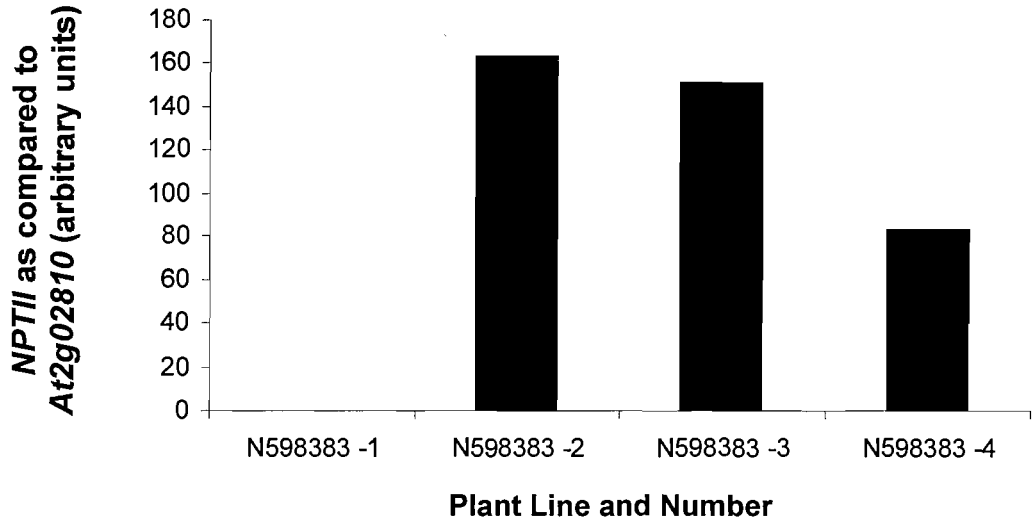

Figure 5.4 Quantification of alleles of *NPTII* in the genome of four potential *aca12* (N598383) insertional mutants. Quantitative PCR was used on genomic DNA extracts from four potential T-DNA mutants. Using Q-RT-PCR an assessment of the amount of *NPTII* relative to the amount of gene *At2g02810* was made for each sample. The wild-type plant N598383-1 contains no alleles of *NPTII*; the homozygous plants N598383-2 and N598383-3 contain twice as much *NPTII* per *At2g02810* as the heterozygous plant N598383-4.

ATGAGGGACCTCAAGGAATATGATTACAGTGCACTTTTGCTCAACCTTACCACCTCCAG
CCTCAACAAGGCCCAGAGGCGTTGGCGTTTTGCCTACGCTGCCATCTACTCTATGAGGG
CTATGCTCTCTCTTGTTAAGGAGATAGTTCCCGCAAGGATTGATCCCAAAACCTCAGAT
GCCTCTCTCTCTCTCCTACACAGCCCTCGAGTCCGGTGAGGGAGCAAAGATCAACTC
TATGCCTCTCTCTTACGTTCCTGCCATTGATCAAGAACAACTTGTGGAGATCATGAAGG
GTAAGGACTTACCGGGCATCCAAGCGCTGGGCGGCGTGGAGGGTGTCGCCGCTTCCCTT
AGGACTAACCCCACCAAAGGGATCCACGGGAATGAGCAAGAAGTCAGTAGACGCCGTGA
CCTCTTTGGCTCTAACACCTACCATAAGCCACCGCCTAAAGGACTTCTCTTCTTTGTGT
ATGAAGCTTTCAAAGACCTAACCATCTTGATCTTGTTGGTCTGCGCTATTTTCTCCCTT
GGCTTCGGTATCAAAGAACATGGCATCAAAGAAGGTTGGTATGAAGGCGGAAGCATCTT
TGTAGCAGTCTTCTTGGTCATAGTTGTCTCTGCTCTCAGCAACTTTAGGCAGGAGAGAC
AGTTCGACAAGCTGTCCAAGATAAGCAATAACATCAAAGTGGAAGTCCTTCGGGACAGC
AGGCGGCAACTTAATAACACATTGCGGACGCTTAGACAAATTAATAACACATTGCGGAC
GTTTTTAATGTACTGGGGTGGTTTTTCTTTTCACCAGTGAGACGGGCAACAGCTGATTG
CCCTTCACCGCCTGGCCCTGAGAGAGTTGCAGCAAGCGGTCCACGCTGGTTTGCCCCAG
CAGGCGAAAATCCTGTTTGATGGTGGTTCCGAAATCGGCAAAATCCCTTATAAATCAAA
AGAATAGCCCGAGATAGGGTTGAGTGTTGTTCCAGTTTGGAACAAGAGTCCACTATTAA
AGAACGTGGACTCCAACGTCAAAXXX$_x$ GCATGGCCCACTACGTGAACCA

Figure 5.5 Sequence analysis of *aca12-1*. PCR between *aca12* and the T-DNA was performed on genomic DNA (see Figure 5.1 for primer locations) and the product sequenced from both ACA12F and Lb primers, to confirm the presence of the T-DNA insert in *ACA12*. Grey sequence represents the gene-specific forwards primer. Clear sequence represents sequence from the genome sequencing project. Yellow sequence represents gene-specific sequence obtained in this study. Blue sequence represents T-DNA specific sequence obtained in this study. Green sequence represents T-DNA sequence of unknown length that was unreadable between the known T-DNA sequence and the Lb. Pink represents the Lb primer. The join between the yellow and blue bases represents the insertion site of the T-DNA confirming the presence of the T-DNA in *ACA12*.

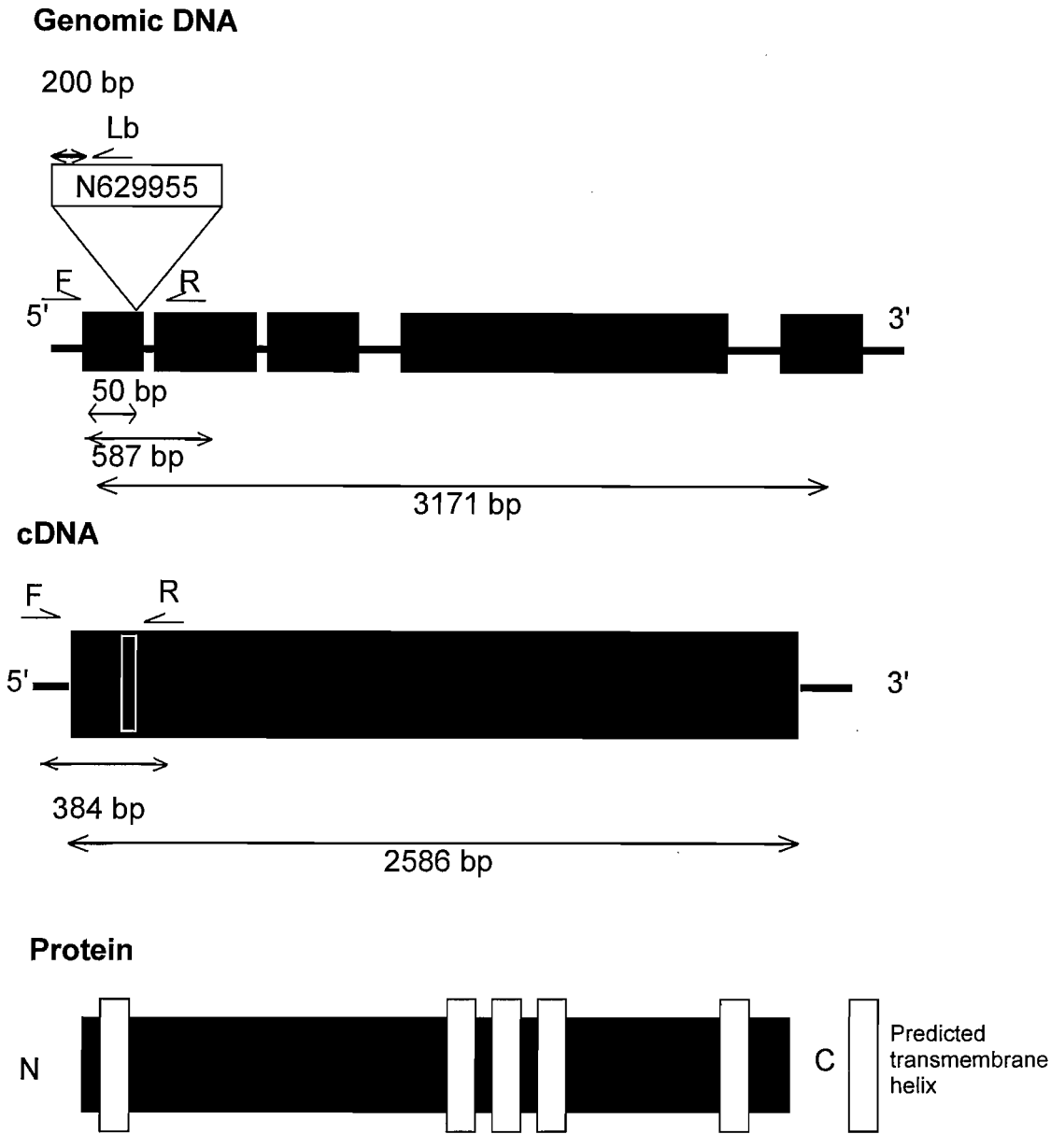

Figure 5.6 Representation of the genomic, cDNA and protein sequence of *GLR1.4*. The T-DNA in line N629955 is in the 1st of the 5 exons in *GLR1.4*. The white box in the cDNA indicated the location of the T-DNA. Single headed arrows indicate the direction of the gene-specific forwards (F, GLR1.4F) and reverse (R, GLR1.4R) primers, as well as the Lb primer specific to the left border of the T-DNA. Double-headed arrows indicated the size of the respective DNA sequences.

The primers GLR1.4F and GLR1.4R are specific to *GLR1.4*, and PCR using them will produce a product of 587 bp on a wild-type copy of *GLR1.4*.A PCR using the gene-specific F primer and the T-DNA specific Lb primer would be expected to produce a product of about 250 bp, on copy of *GLR1.4* containing a T-DNA in the expected position. In Figure 5.7, it can be seen that in reality a 450 bp product is produced. Sequencing of this (Figure 5.8) revealed that the gene/ T-DNA border is 15bp from the start ATG codon, but the Lb primer site is 200 bp further from the edge of the T-DNA/ gene boundary than would be expected. This 200 bp distance is filled by DNA from the T-DNA, which indicates that an unexpected DNA re-arrangement may have taken place during T-DNA insertion. While this is unexpected, DNA re-arrangements at the borders of T-DNA insertion sites are relatively common, and are unlikely to affect the consequences of T-DNA insertion. Using the same logic as described in 5.2.2, Figure 5.7 shows that N629955-1 is homozygous for the T-DNA, N629955- 2 and 3 are wild-type and N629955-4 is heterozygous. Following this N629955-1 was re-designated as *glr1.4-1*. This result was confirmed by an identical PCR performed on a separate occasion. For a final confirmation that plants homozygous for the T-DNA insert no longer produced a full-length cDNA for the gene of interest, a homozygous plant was grown to maturity, and harvested. RNA was extracted from this material and cDNA was subsequently prepared from the RNA. Figure 5.9 shows that no product corresponding to the full-length *GLR1.4* could be amplified from a homozygous insertional mutant. Positive controls showed that this band could be amplified from wild-type plants and that full-length cDNAs other than *GLR1.4* could be amplified from cDNA prepared from the homozygous insert plant. Real-time analysis of the number of alleles of *NPTII* showed that the genome of homozygous plant N619955-1 contained twice as many *NPTII* alleles as the heterozygous plant, a strong indication that there is only one T-DNA insert in this line (data not shown).

5.2.4 Isolation of *ptr3 insertional mutants*

The seed stock N638430, which putatively contains a T-DNA insert in *PTR3,* was obtained and the original seed received from the NASC were grown to maturity. The zygosity of these was confirmed by PCR and antibiotic selection, the insert location was sequenced, the number of other T-DNA inserts quantified by real-time PCR and the resultant insertional mutant was checked for the presence of *PTR3* mRNA, all using the same methods described for *aca12-1*. The locations of the primers used to determine zygosity and the location of the T-DNA in the genomic and cDNA sequences are shown in Figure 5.10. The primers PTR3F and PTR3R are specific to *PTR3* and PCR on a wild-type copy of the *PTR3* gene using them will produce a 900 bp product. When using the gene-specific reverse primer and the T-DNA specific Lb primer on a copy of *PTR3* containing a T-DNA in the expected position, PCR would be expected to produce a product of around 1200 bp. In Figure 5.11 it can be seen that PCR gene-T-DNA PCR

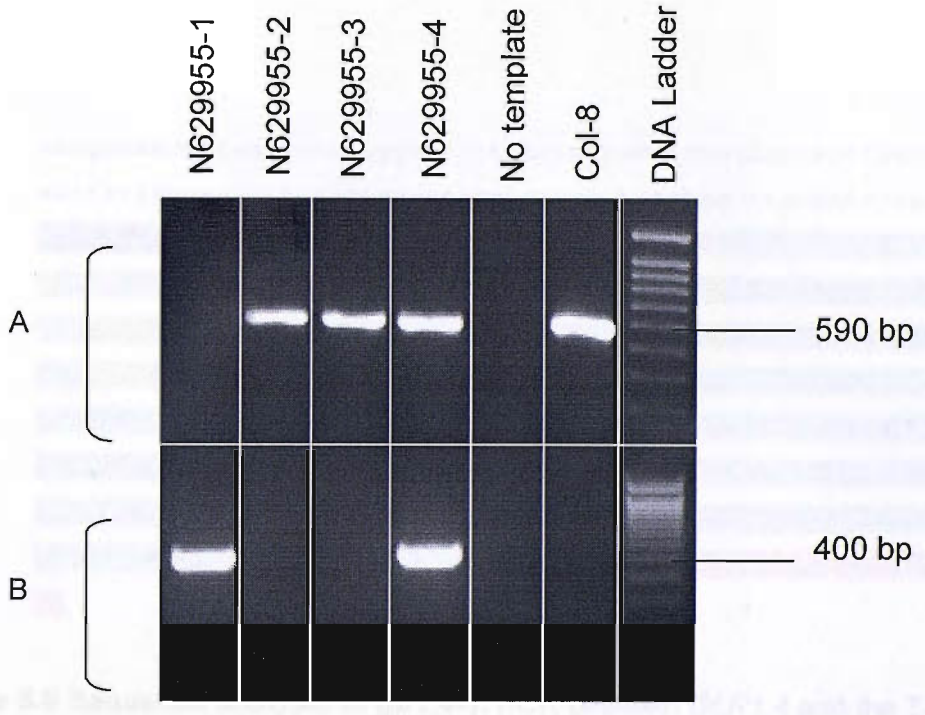

Figure 5.7 Identification of a homozygous *glr1.4* mutant PCR on genomic DNA extracted from Col-0 and four potential T-DNA insertional mutants. A: PCR using gene-specific primers GLR1.4F and GLR1.4R, B: PCR using the gene-specific primer GLR1.4F and the T-DNA primer Lb. Refer to Figure 5.6 for the location of the primers. Using the logic described in the text, plants 2 and 3 were identified as being homozygous for the T-DNA insert. The DNA ladder used was Q-step1 (Yorkshire Biosciences, UK).

aaagacaaccttaatgaccttggaatcttaattagtacctgaagagtaaattagtttaa
aattatttggaatcttgaactaaattagatgtcagatatatagcttataatacaaatag
gggctagagaacaaaagcttcaccaagATGGAGAATTGTATGTAACATTGACGTTTAGA
TGGAGAATAACCAGGATATATTGTGGTGTAAACAAATTGACGTCCGCAAAATGGTTAGA
AAATAACCAGGATATATTGTGGTGTAAACAAATTGACGCTTAGACAACTTAATAACACA
TTGCGGACGTTTTTAATGTACTGGGGTGGTTTTTCTTTTCACCAGTGAGACGGGCAACA
GCTGATTGCCCTTCACCGCCTGGCCCTGAGAGAGTTGCAGCAAGCGGTCCACGCTGGTT
TGCCCCAGCAGGCGAAAATCCTGTTTGATGGTGGTTCCGAAATCGGCAAAATCCCTTAT
AAATCAAAAGAATAGCCCGAGATAGGGTTGAGTGTTGTTCCAGTTTGGAACAAGAGTCC
ACTATTAAAGAACGTGGACTCCAACGTCAAAGCGAXXX$_{x}$GCATGGCCCACTACGTGAAC
CA

Figure 5.8 Sequence analysis of *glr1.4-1*. PCR between *GLR1.4* and the T-DNA was performed on genomic DNA (see Figure 5.6 for primer locations), and the product sequenced from Lb primer to confirm the presence and exact location of the T-DNA insert in *GLR1.4*. The GLR1.4F primer was not used for sequencing as the priming site was too close to the expected insert site to obtain an accurate sequence read of the join. The grey sequence represents the gene-specific forwards primer, the clear sequence represents sequence from the genome sequencing project, yellow sequence represents gene-specific sequence obtained in this study, the blue sequence represents T-DNA specific sequence obtained in this study, the green sequence represents a sequence of unknown length that was unreadable between the known T-DNA sequence and the Lb primer in pink. The join between the yellow and blue bases represents the insertion site of the T-DNA confirming the presence of the T-DNA in *GLR1.4*. Uppercase letters represent coding sequence, lowercase indicate non-coding sequence.

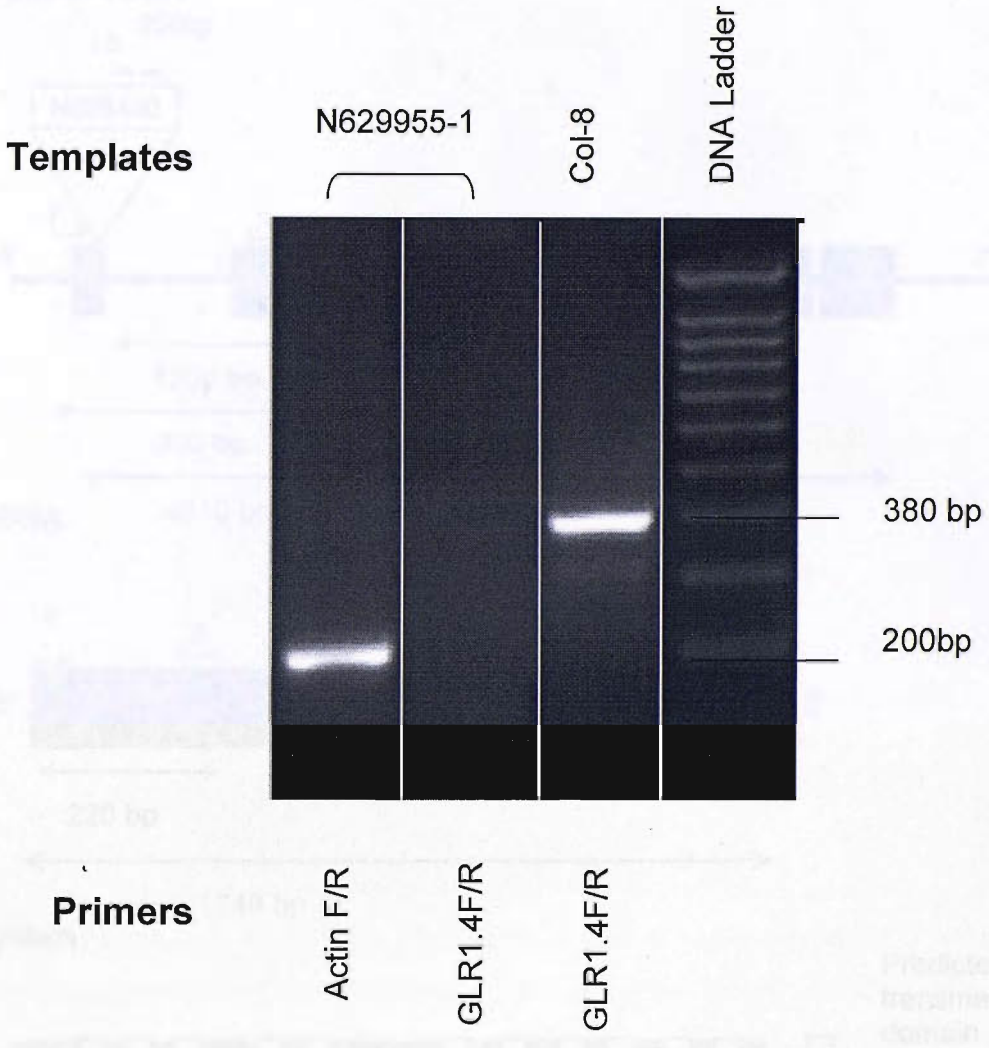

Figure 5.9 No full-length cDNA corresponding to *GLR1.4* can be amplified from a *glr1.4* insertional mutant. cDNA was prepared from RNA extracted from plant N629955-1. PCR using ActinF and ActinR was used to detect full-length Actin cDNA. The gene specific primers GLR1.4F and GLR1.4R were used to detect *GLR1.4* cDNA, see Figure 5.6 for primer locations. The DNA ladder used was Q-step1 (Yorkshire Biosciences, UK).

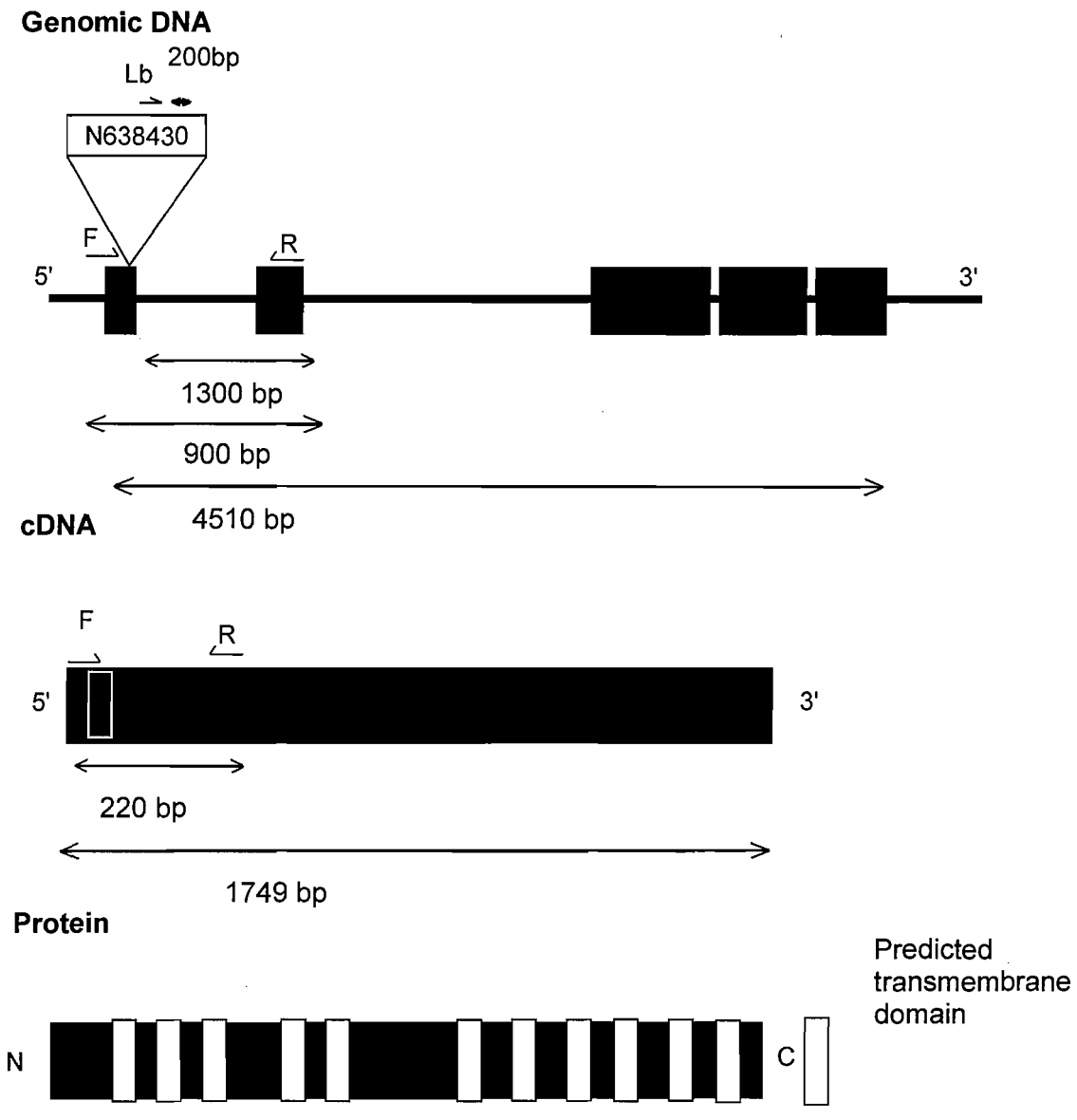

Figure 5.10 Representation of the genomic, cDNA and protein sequence of *PTR3*. The T-DNA in line N638430 is in the 1st of the 5 exons in *PTR3*. Single headed arrows indicate the direction of the gene-specific forwards (F, PTR3F) and reverse (R, PTR3R) primers, as well as the Lb primer specific to the left border of the T-DNA.

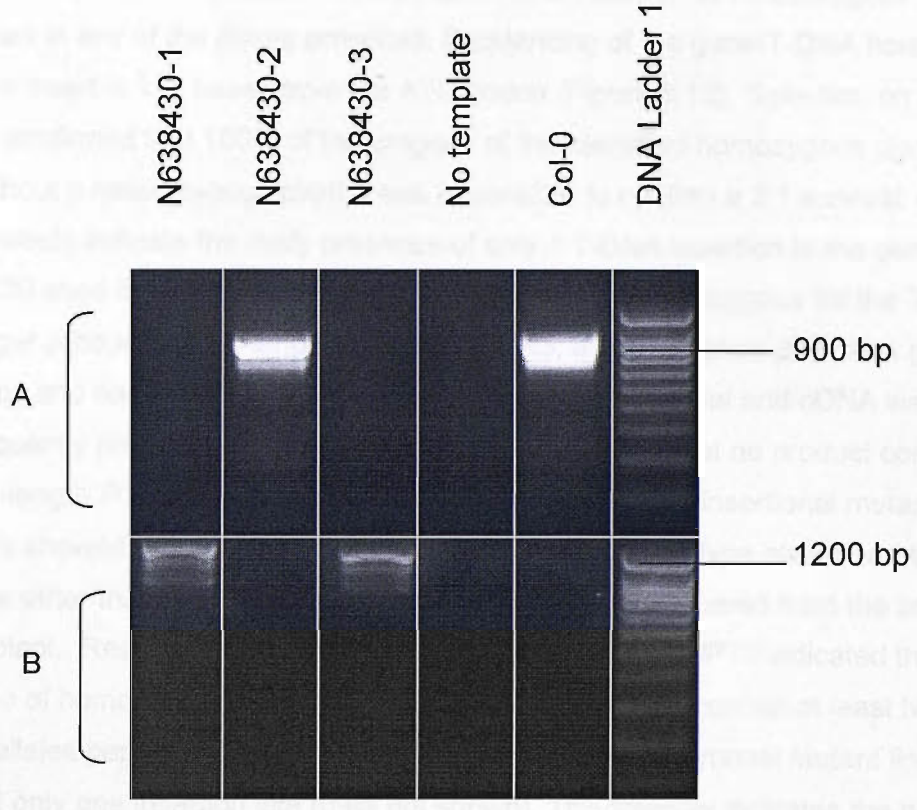

Figure 5.11 Identification of a homozygous *ptr3* insertional mutant PCR on genomic DNA extracted from Col-0 and four potential T-DNA insertional mutants. A: PCR using gene-specific primers PTR3F and PTR3R; B: PCR using the gene-specific primer PTR3 and the T-DNA primer Lb. Refer to Figure 5.10 for the location of the primers. Using the logic described in the text, plants N638430-1 and 3 were identified as being homozygous for the T-DNA insert. The DNA ladder used was Q-step1 (Yorkshire Biosciences, UK).

produces several products which are close in size. The larger and more prominent of these products is the expected; the smaller products are possibly due to mis-priming within the expected product. Sequencing was performed on the band corresponding to the predicted size, after extraction from the agarose gel. Using the same logic as described in 5.2.1, Figure 5.11 shows that N638430-1 and 3 are homozygous for the T-DNA insert and N638430-2 is wild-type. This result was confirmed by a repeat of the PCR on a separate occasion. Following this N638430-1 was re-designated *ptr3-3* as two non allelic mutants have already been identified in *PTR3* (Karim *et al.*, 2007). No heterozygous plants were identified in any of the plants screened. Sequencing of the gene/T-DNA border revealed that the insert is 111 bases from the ATG codon (Figure 5.12). Selection on antibiotic media confirmed that 100% of the progeny of the identified homozygous plants survived, but without a heterozygous plant it was impossible to confirm a 3:1 survival: death ratio, which would indicate the likely presence of only 1 T-DNA insertion in the genome for the N638430 seed line. For a final confirmation that plants homozygous for the T-DNA insert no longer produced a full-length mRNA for *PTR3*, a homozygous plant was grown to maturity, and harvested. RNA was extracted from this material and cDNA was subsequently prepared from the RNA. Figure 5.13 shows that no product corresponding to the full-length *PTR3* could be amplified from a homozygous insertional mutant. Positive controls showed that this band could be amplified from wild-type plants and that full-length mRNAs other than *PTR3* could be amplified from cDNA prepared from the homozygous insert plant. Real-time analysis of the number of alleles of *NPTII* indicated that the genome of homozygous plants N638430-1 and N638430-3 contain at least twice as many *NPTII* alleles per genome as other homozygous T-DNA insertional mutant lines thought to contain only one insertion site (data not shown). This strongly indicates the presence of more than one T-DNA insertion site in these plants. The surest way of removing unwanted T-DNA insertion sites is to repeatedly backcross the homozygous line to a wild-type plant (three generations is recommended), using only plants containing the T-DNA inserted into *PTR3* from each generation. As this is extremely time consuming, it was decided to check the phenotype of the insertional mutant lines, and only perform the backcrosses if a phenotype as observed.

5.2.5 Isolation of *ala10* insertional mutants

The seed stock N524877, which putatively contains a T-DNA insert in *ALA10*, was obtained and the original seed received from the NASC were grown to maturity. In outline, the zygosity of these was confirmed by PCR and antibiotic selection, the insert location was sequenced, the number of other T-DNA inserts was quantified by teal-time PCR and the resultant insertional mutant was checked for the presence of *ALA10* mRNA, all using the same methods as described for *aca12-1*. The locations of the primers used to determine zygosity and the location T-DNA in the genomic and cDNA sequences are

TGACAGTAGAAGAGGTAGGAGACGATTACACAAAAGATGGAACAGTTGATCTTCAAGGC
AATCCTGTTCGAAGATCCATTAGAGGTCGTTGGAAAGCTTGCTCTTTCGXXX$_x$GCATGG
CCCACTACGTGAACCAXXX$_x$ATTGACGCTTAGACAACTTAATAACACATTGTGGTGTAA
ACAAATTGACGCTTAGACAACTTAATAACACATTGCGGACGTTTTTAATGTACTGGGGT
GGTTTTTCTTTTCACCAGTGAGACGGGCAACAGCTGATTGCCCTTCACCGCCTGGCCCT
GAGAGAGTTGCAGCAAGCGGTCCACGCTGGTTTGCCCCAGCAGGCGAAAATCCTGTTTG
ATGGTGGTTCCGAAATCGGCAAAATCCCTTATAAATCAAAAGAATAGCCCGAGATAGGG
TTGAGTGTTGTTCCAGTTTGGAACAAGAGTCCACTATTAAAGAACGTGGACTCCAACGT
CAAAGACGAAAGCCGCTGACGTGTTGTTGgtatttattctttcactaatttagtttcct
ttatattttatattctttatcatagagtttaattacaattaaaatgattattaacagtc
ttttttcgaagtttttaagatctatcttatatcttaaaagttgccatgagtcttggaaa
cttttcaatggttacgacagaactaaaagt

Figure 5.12 Sequence analysis of *ptr3-3*. PCR between *PTR3* and the T-DNA was performed on genomic DNA (see Figure 5.10 for primer locations), and the product sequenced from the Lb primer to confirm the presence and exact location of the T-DNA insert in *PTR3*. The PTR3F primer was not used for sequencing as the priming site was too close to the expected insert site to obtain an accurate sequence read of the join. The clear sequence represents sequence from the genome sequencing project, yellow sequence represents gene-specific sequence obtained in this study, the blue sequence represents T-DNA specific sequence obtained in this study, the green sequence represents the bulk of the T-DNA, which has not been sequenced here, and the gap between the sequencing primer and the first accurate sequence, while the Lb primer is shown in pink. The join between the yellow and blue bases represents the insertion site of the T-DNA confirming the presence of the T-DNA in *PTR3*. Uppercase letters represent coding sequence, lowercase indicate non-coding sequence.

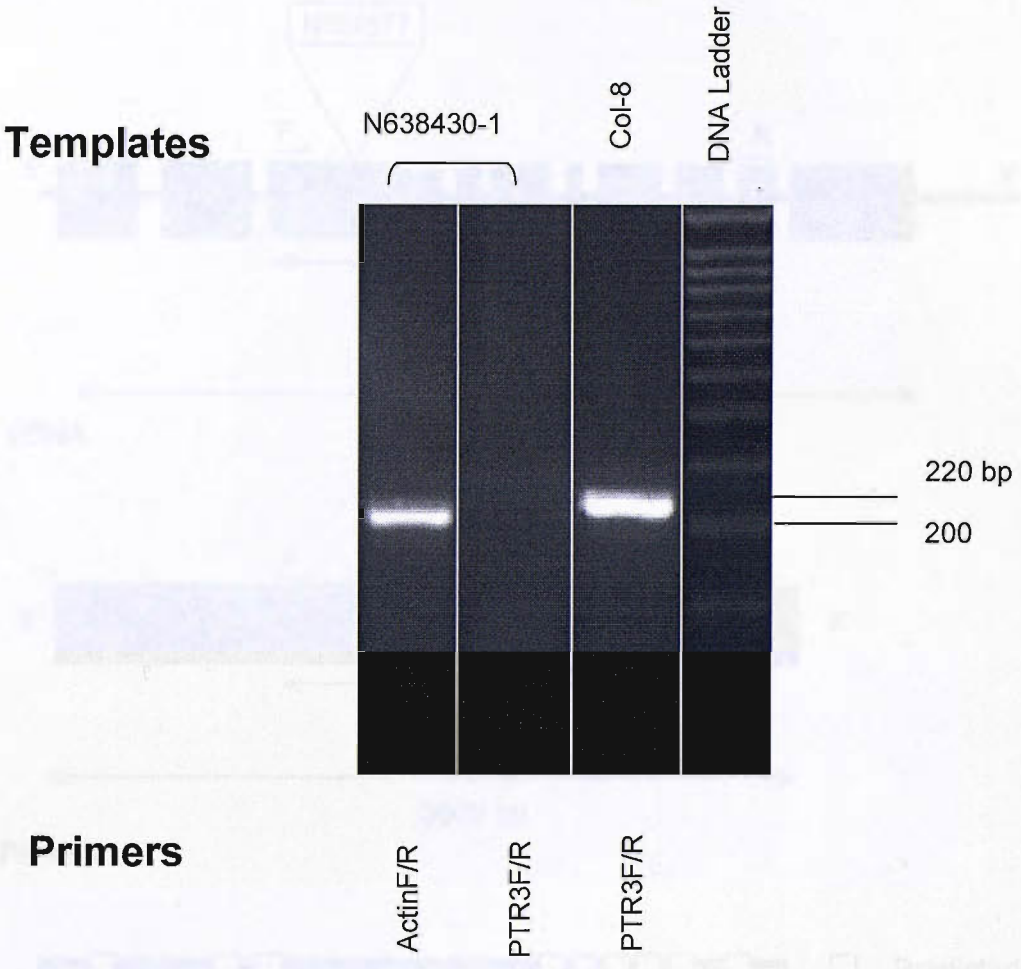

Figure 5.13 No full-length cDNA corresponding to *PTR3* can be amplified from the *ptr3-3* mutant. cDNA was prepared from RNA extracted from the plant N638430-1. PCR using ActinF and ActinR primers was used to detect full-length Actin cDNA. The gene-specific primers PTR3F and PTR3R were used to detect *PTR3* cDNA. The DNA ladder used was Q-step1 (Yorkshire Biosciences, UK).

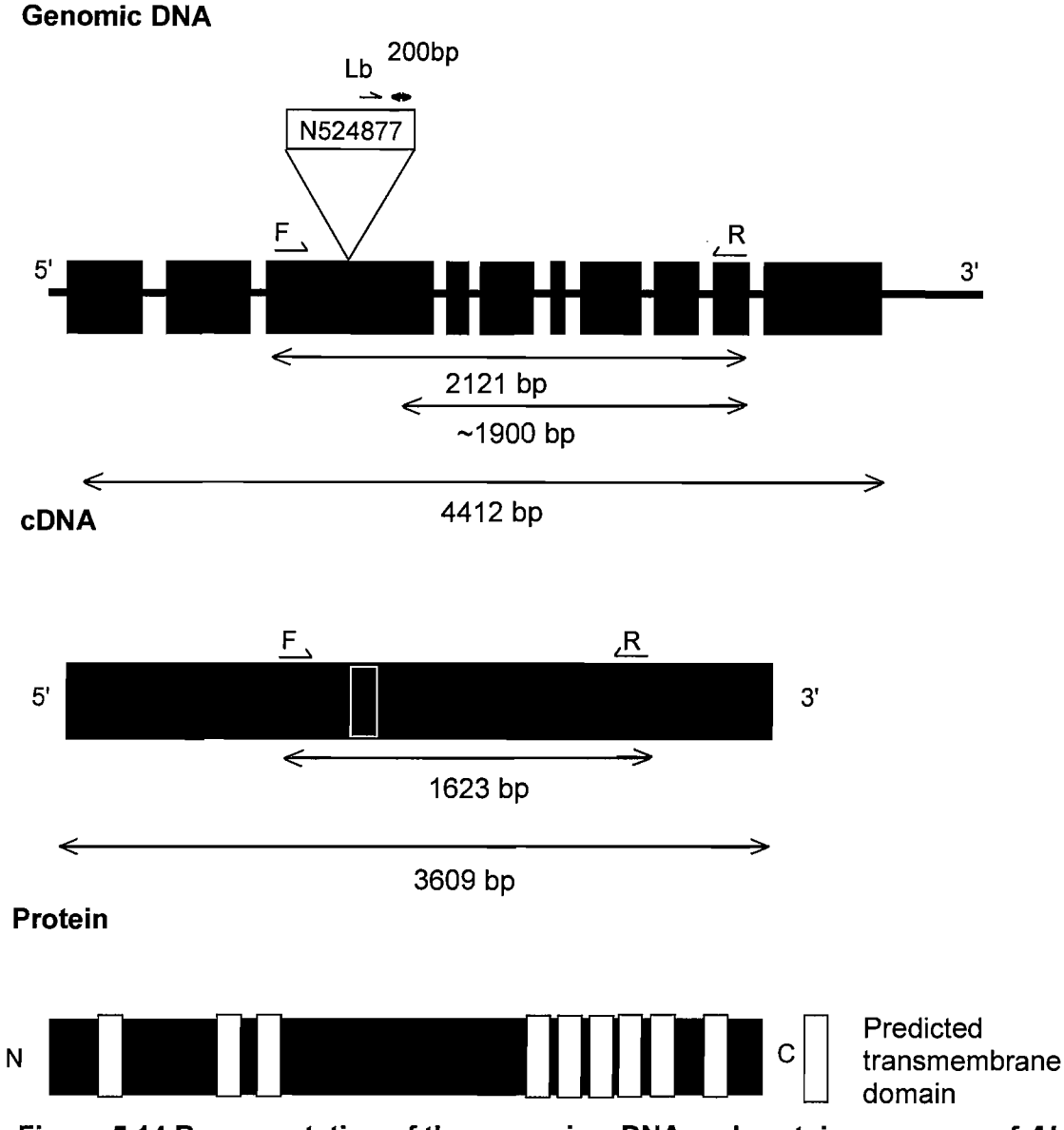

Figure 5.14 Representation of the genomic, cDNA and protein sequence of *ALA10*. The T-DNA in line N524877 is in the 3rd of the 10 exons in *ALA10*. Single headed arrows indicate the direction of the gene-specific forwards (F, ALA10F) and reverse (R, ALA10R) primers, as well as the Lb primer specific to the left border of the T-DNA. Double-headed arrows indicate the size of the respective DNA sequences.

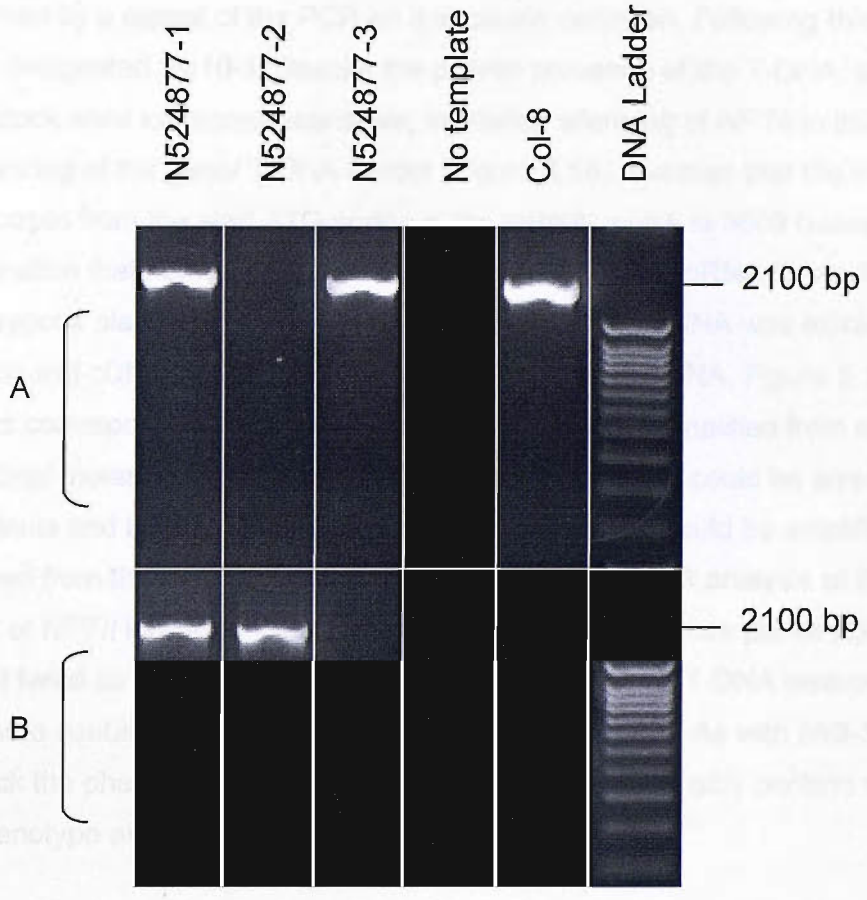

Figure 5.15 Identification of a homozygous *ALA10* mutant PCR on genomic DNA extracted from Col-0 and four potential T-DNA insertional mutants. A: PCR using gene-specific primers ALA10F and ALA10R; B: PCR using the gene-specific primer ALA10F and the T-DNA primer Lb. Refer to Figure 5.14 for the location of the primers. Using the logic described in the text, plant N524877-2 was identified as being homozygous for the T-DNA insert. The DNA ladder used was Q-step1 (Yorkshire Biosciences, UK).

shown in Figure 5.14. The primers ALA10F and ALA10R are specific to *ALA10* and PCR on a wild type copy of the *ALA10* gene using them should produce a 2100 bp product. When using the gene-specific reverse primer and the T-DNA specific Lb primer on a copy of *ALA10* containing a T-DNA in the expected location, another 2100 bp product should also be produced. Using the logic described in 5.2.1, Figure 5.15 shows that N524877-3 is wild-type (this plant was used for the segregating wild-type), N524877-2 is homozygous for the T-DNA insert and N524877-1 is heterozygous for the T-DNA insert. This result was confirmed by a repeat of the PCR on a separate occasion. Following this, plant N524877-2 was designated *ala10-1.* Despite the proven presence of the T-DNA, all plants from this seed stock were kanamycin-sensitive, indicating silencing of *NPTII* in this line. Sequencing of the gene/ T-DNA border (Figure 5.16) revealed that the insert is located 1388 bases from the start ATG codon in the mRNA, which is 3609 bases long. For a final confirmation that homozygous plants no longer produced mRNA for *ALA10*, a homozygous plant was grown to maturity and harvested. RNA was extracted from this material and cDNA was subsequently prepared from the RNA. Figure 5.17 shows that no product corresponding to the full-length *ALA10* could be amplified from a homozygous insertional mutant. Positive controls showed that this band could be amplified from wild-type plants and that full-length cDNAs other than *ALA10* could be amplified from cDNA prepared from the homozygous insert plant. Real-time PCR analysis of the number of alleles of *NPTII* indicated that that the genome of homozygous plants N524877-2 contains at least twice as many *NPTII* alleles as other homozygous T-DNA insertional mutant lines thought to contain only one insertion site (data not shown). As with *ptr3-3*, it was decided to check the phenotype of the insertional mutant lines, and only perform the backcrosses if a phenotype as observed.

5.2.6 Isolation of a*t2g18680* insertional mutants

The seed stock N501089, which putatively contains a T-DNA insert in *At2g18680,* was obtained and the original seed received from the NASC were grown to maturity. The zygosity of these was confirmed by PCR and antibiotic selection, the insert location was sequenced, the number of other T-DNA inserts quantified by real-time PCR and the resultant insertional mutant was checked for the presence *of At2g18680* cDNA, all using the same methods as described for *aca12-1.* The locations of the primers used to determine zygosity and of the T-DNA in the genomic and cDNA sequences are shown in Figure 5.18. The primers At2g18680F and At2g18680R are specific to *At2g18680* and PCR on a wild-type copy of the At*2g18680* gene using them should produce a 517 bp product. When using the gene-specific At2g18680R primer and the T-DNA specific Lb primer on a copy of *At2g18680* containing a T-DNA in the expected location, a 700 bp product should be produced. Using the logic described in 5.2.1, Figure 5.19 shows that N501089-1 and N501089-3 are wild-type (N501089-1 was used as segregating wild-type

AGCATTTTCATCAATAGAGACATTCATATGTACTATGAAGAAACGGATAAACCCGCGCA
GGCACGGACTTCAAATTTGAATGAAGAACTCGGTATGGTTGATACGATTCTATCCGATA
AAACGGGGACTTTAACGTGCAATTCAATGGAGTTTATCAAGTGTTCTATCGCGGGTAAG
GCTTATGGACGCGGTATAXXXxGCATGGCCCACTACGTGAACCAXXXxCACGGGAGCGC
TTTCGCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGGAA
CAACACTCAACCCTATCTCGGGCTATTCTTTTGATTTATAAGGGATTTTGCCGATTTCG
GAACCACCATCAAACAGGATTTTCGCCTGCTGGGGCAAACCAGCGTGGACCGCTTGCTG
CAACTCTCTCAGGGCCAGGCGGTGAAGGGCAATCAGCTGTTGCCCGTCTCACTGGTGAA
AAGAAAAACCACCCCAGTACATTAAAAACGTCCGCAATGTGTTATTAAGTTGTCTAAGC
GTCAATTTGTTTACACCACAATATATCAGACTGAAGTTGAAAGAGCTATGGCTGTGAGA
AGCGGTGGTTCGCCTTTGGTTAATGAAGATTTGGATGTTGTTGTGGATCAGTCTGGTCC
TAAGGTTAAAGGGTTTAACTTTGAGGATGAAAGGGTTATGAATGGGAATTGGGTGAGGC
AGCCTGAGGCTGCCGTGTTGCAGAAGTTTTTCAGATTGCTTGCTGTGTGTCACACGGCA
ATACCTGAGACCGATGAAGAGTCCGGGAATGTCTCTTATGAAGCTGAGTCTCCGGATGA
AGCTGCGTTTGTTGTTGCAGCCCGAGAGTTCGGGTTCGAGTTCTTTAACCGGACACAAA
ATGGGATCTCTTTTCGTGAACTGGATCTTGTATCGGGAGAGAAAGTTGA

Figure 5.16 Sequence analysis of *ala10-1*. PCR between *ALA10* and the T-DNA was performed on genomic DNA (see Figure 5.14 for primer locations), and the product sequenced from the Lb primer to confirm the presence and exact location of the T-DNA insert in *ALA10*. The ALA10F primer was not used for sequencing as the priming site was too far from the expected insert site to obtain an accurate sequence read of the join. Clear represents sequence from the genome sequencing project. Yellow sequence represents gene-specific sequence obtained in this study. Blue sequence represents T-DNA specific sequence obtained in this study. Green sequence represents the bulk of the T-DNA, which has not been sequenced here, and the gap between the sequencing primer and the first accurate sequence. Pink represents the Lb primer. The join between the yellow and blue bases represents the insertion site of the T-DNA confirming the presence of the T-DNA in *ALA10*.

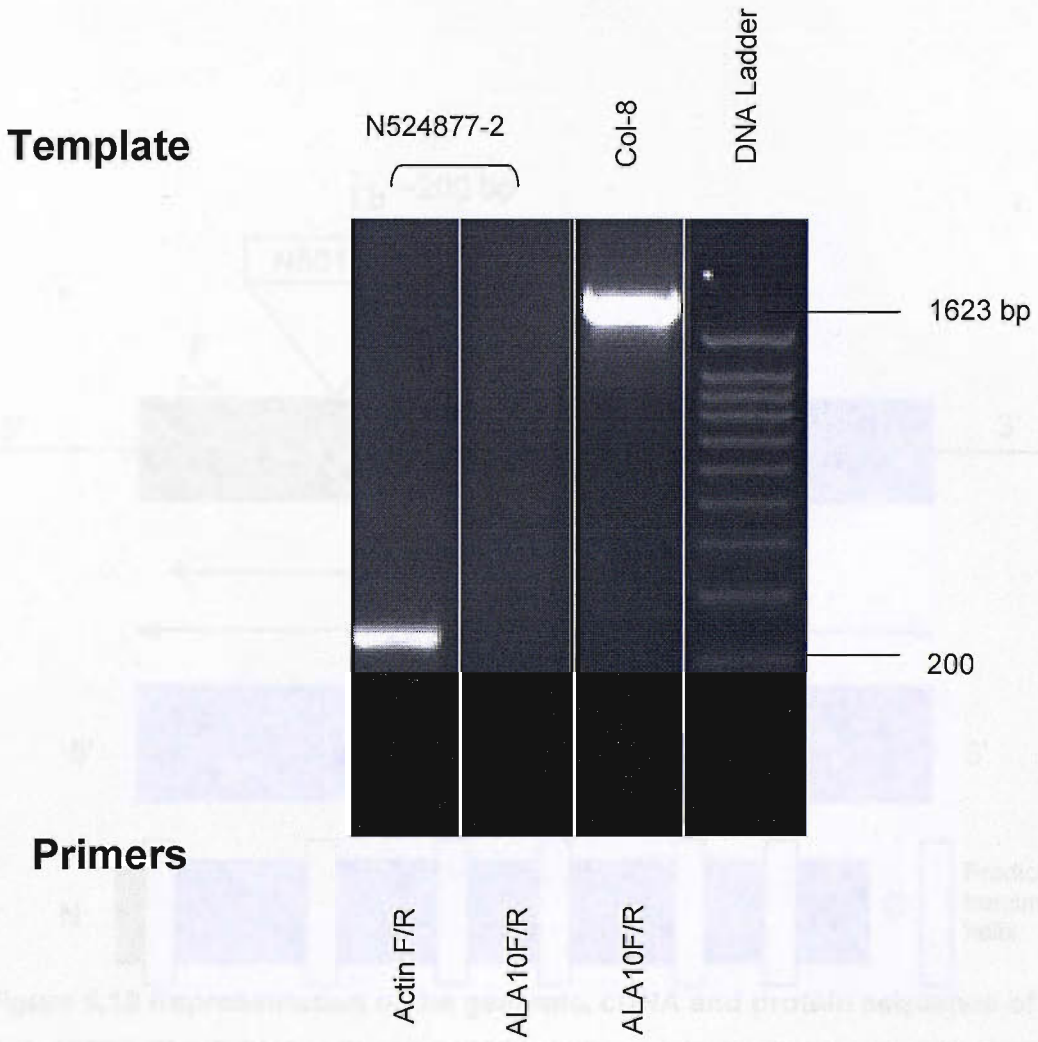

Figure 5.17 No full-length cDNA corresponding to *ALA10* can be amplified from the *ala10-1* mutant. cDNA was prepared from RNA extracted from plant N638430-1. PCR using ActinF and ActinR was used to detect full-length Actin cDNA. The gene-specific primers ALA10F and ALA10R were used to detect ALA10 cDNA. The DNA ladder used was Q-step1 (Yorkshire Biosciences, UK).

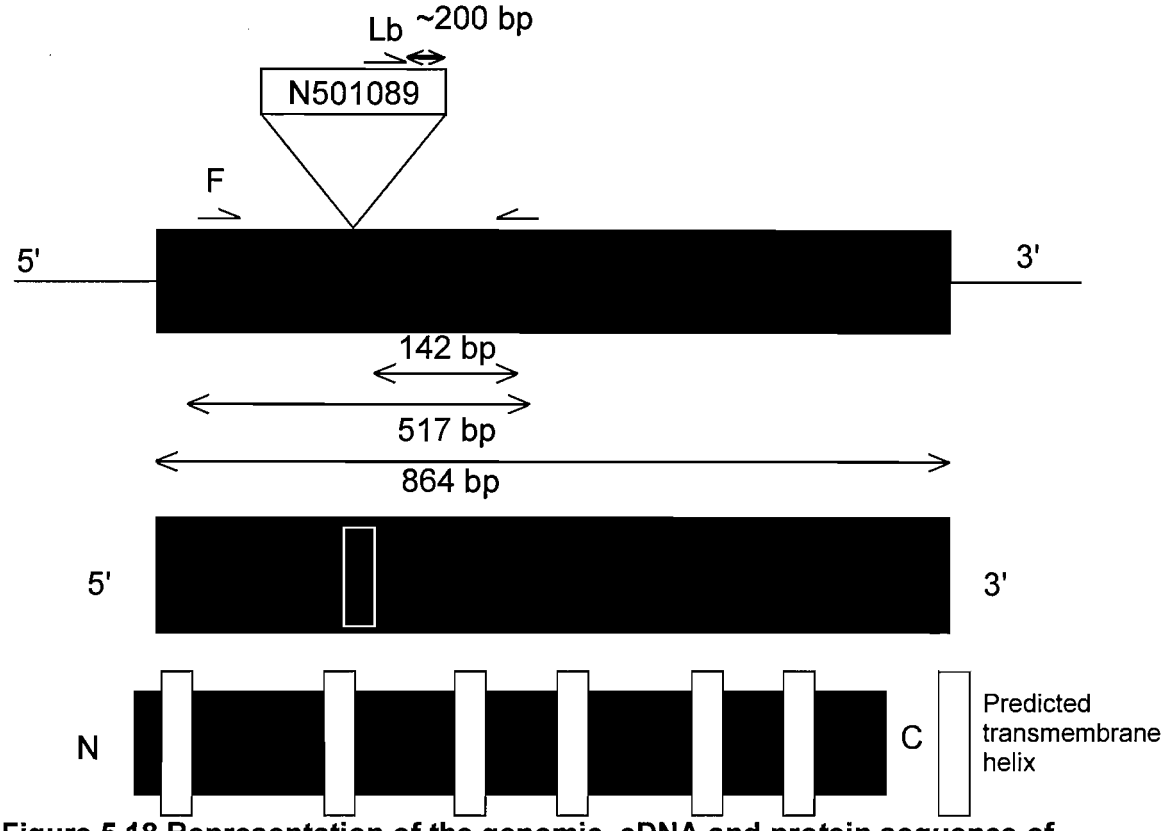

Figure 5.18 Representation of the genomic, cDNA and protein sequence of *At2g18680*. The T-DNA in line N501089 is in the 1st and only exon in *At2g18680*. Single headed arrows indicate the direction of the gene-specific forwards (F, At2g18680F) and reverse (R. At2g18680R) primers, as well as the Lb primer specific to the left border of the T-DNA. Double-headed arrows indicate the size of the respective DNA sequences.

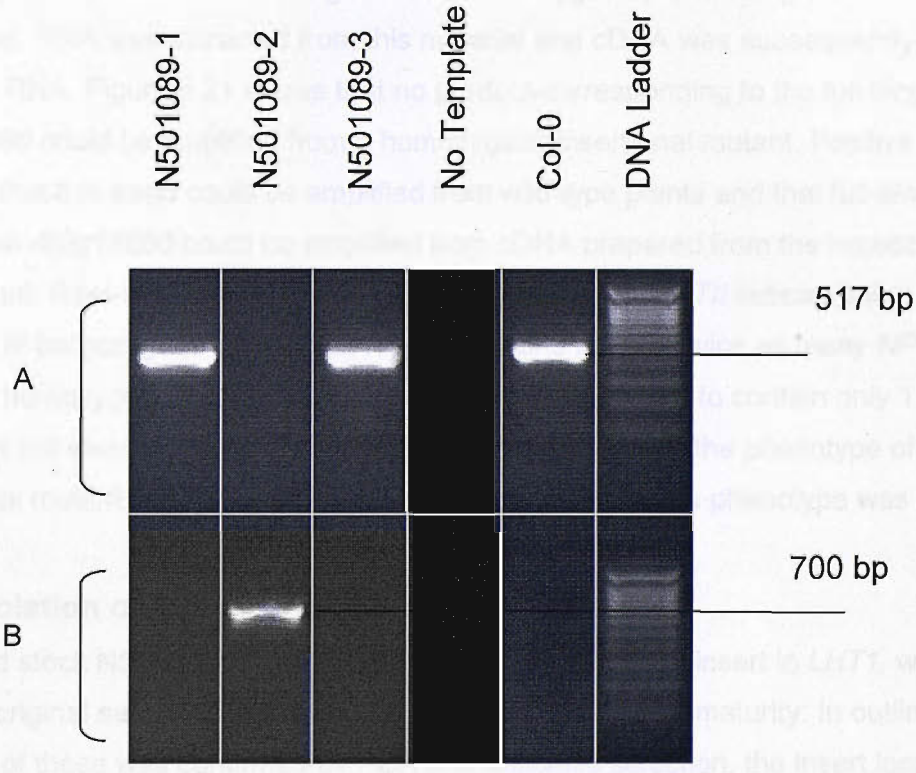

Figure 5.19 Identification of a homozygous *at2g18680* insertional mutant. PCR on genomic DNA extracted from Col-0 and four potential T-DNA insertional mutants. A: PCR using gene-specific primers At2g18680F and At2g18680R; B: PCR using the gene-specific primer F and the T-DNA primer Lb. Refer to Figure 5.18 for the location of the primers. Using the logic described in 5.2.1, plant N501089-2 was identified as being homozygous for the T-DNA insert. The DNA ladder used was Q-step1 (Yorkshire Biosciences, UK).

in later experiments) and N591089-2 is homozygous for the T-DNA insert. This result was confirmed by a repeat of the PCR on a separate occasion and following this N501089-2 was re-designated *at2g18680-1*. Progeny from N501089-1 and N501089-2 were plated onto kanamycin media (not shown). Progeny of N501089-1 all died on kanamycin-containing media, confirming the wild-type status, and indicating that there are probably no other T-DNA insertion sites. Progeny of N501089-2 all survived, confirming the insertional mutant status. Sequencing of the mutant (Figure 5.20) showed that the T-DNA gene border is at 102 bp from the start ATG rather than the 135 bp predicted by the Salk institute sequencing. For a final confirmation that homozygous plants no longer produced a wild-type copy of mRNA for *At2g18680*, a homozygous plant was grown to maturity and harvested. RNA was extracted from this material and cDNA was subsequently prepared from the RNA. Figure 5.21 shows that no product corresponding to the full-length *At2g18680* could be amplified from a homozygous insertional mutant. Positive controls showed that this band could be amplified from wild-type plants and that full-length cDNAs other than *At2g18680* could be amplified from cDNA prepared from the homozygous insert plant. Real-time analysis of the number of alleles of *NPTII* indicated that the genome of homozygous plants N524877-2 contains at least twice as many *NPTII* alleles as other homozygous T-DNA insertional mutant lines thought to contain only 1 insertion site (data not shown). As with *ptr3-3*, it was decided to check the phenotype of the insertional mutant lines, and only perform the backcrosses if a phenotype was observed.

5.2.7 Isolation of *lht1* insertional mutants

The seed stock N536871, which putatively contains a T-DNA insert in *LHT1,* was obtained and the original seed received from the NASC were grown to maturity. In outline, the zygosity of these was confirmed by PCR and antibiotic selection, the insert location sequenced, the number of other T-DNA inserts quantified by real-time PCR and finally checked for the presence of *LHT1* mRNA in identified insertional mutants. This was done using the same methods as described for *aca12-1*. The locations of the primers used to determine zygosity and of the T-DNA in the genomic and cDNA sequences are shown in Figure 5.22. The primers LHTF and LHTR are specific to *LHT1* and PCR on a wild-type copy of the *LHT1* gene using them should produce a 341 bp product. When using the gene-specific R primer and the T-DNA specific Lb primer on a copy of *LHT1* containing a T-DNA in the expected location, a 420 bp product should be produced. Using the logic described in 5.2.1. Figure 5.23 shows that N536871-1 is wild-type (N526871-1 was used as a segregating wild-type in subsequent experiments) and N536871-2 is homozygous for the T-DNA insert, while N536871-3 was heterozygous for the T-DNA insert. Following this, N536871-2 was re-designated as *lht-1* as it is allelic to a mutant already identified in *LHT1* (Hirner *et al.*, 2006). However it can be seen in Figure 5.23 that the PCR using primers R and Lb produces a product of 500 bp, not 420 bp. Sequencing of *lht-1* revealed that the T-

ttcttgaatgttataatggaggttgcgggtattctaaatgaatcccgcaaactctcctc
aagaacaaaaagttgATGTTCTCAGTCTTGGTATTCCCTCTCTTACTCAATTGTTTAGT
TTACTTGTTCAACGCCATTGCCATCAAACCCGAGATAACAAACTTGATCCTTGAATCAX
XX_xGCATGGCCCACTACGTGAACCAXXX_xCTTTGACGTTGGAGTCCACGTTCTTTAATA
GTGGACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGGCTATTCTTTTGAT
TTATAAGGGATTTTGCCGATTTCGGAACCACCATCAAACAGGATTTTCGCCTGCTGGGG
CAAACCAGCGTGGACCGCTTGCTGCAACTCTCTCAGGGCCAGGCGGTGAAGGGCAATCA
GCTGTTGCCCGTCTCACTGGTGAAAAGAAAAACCACCCCAGTACATTAAAAACGTCCGC
AATGTGTTATTAAGTTGTCTAAGCGTCAATTTGTAGTTTGTTACCTATGACAGATCCAA
ACACCCCGGAATTTGCTGCCCATCTTATGAGAGTCTTTGTAGATTTTCGCCAGTTTGTC
AATTCTTTATACATCTTCATCGCCGTCTCCTCCATCATCAACCTTTTATCCACTCTTGT
CATGGTTCATGCATCGGCTCTTACTCATAAAGATGATAGCTTCGAGATCAAAGATTTTC
CGATTCTGACGCTTAAATATTGGAAGGGACCTCTTGTGACCAATTTCTATATTGTTCTC
TTCAGTCTTGGCTATTGGTTCCTATTCTTTATAGTCCTATTTTCTATCGTTTTCTTCTC
TACAAAACTCGATTCCTTGGCAGCCAAATCTCGCGCTTTATTCATTGTATTTGCAGTCT
TTGAGTCTTACTTAGCTATCGTTTGGAACTTATCTATGG

Figure 5.20 Sequence analysis of *at2g18680-1*. PCR between *At2g18680* and the T-DNA was performed on genomic DNA (see Figure 5.18 or primer locations), and the product sequenced from the Lb primer to confirm the presence and exact location of the T-DNA insert in *At2g18680*. The grey sequence represents the gene-specific reverse primer At2g18680R, the clear sequence represents sequence from the genome sequencing project, yellow sequence represents gene-specific sequence obtained in this study, the blue sequence represents T-DNA specific sequence obtained in this study, the green sequence represents the bulk of the T-DNA, which has not been sequenced here, and the gap between the sequencing primer and the first accurate sequence, while the Lb primer is shown in pink. The join between the yellow and blue bases represents the insertion site of the T-DNA confirming the presence of the T-DNA in *At2g18680*. Uppercase letters represent coding sequence, lowercase indicate non-coding sequence.

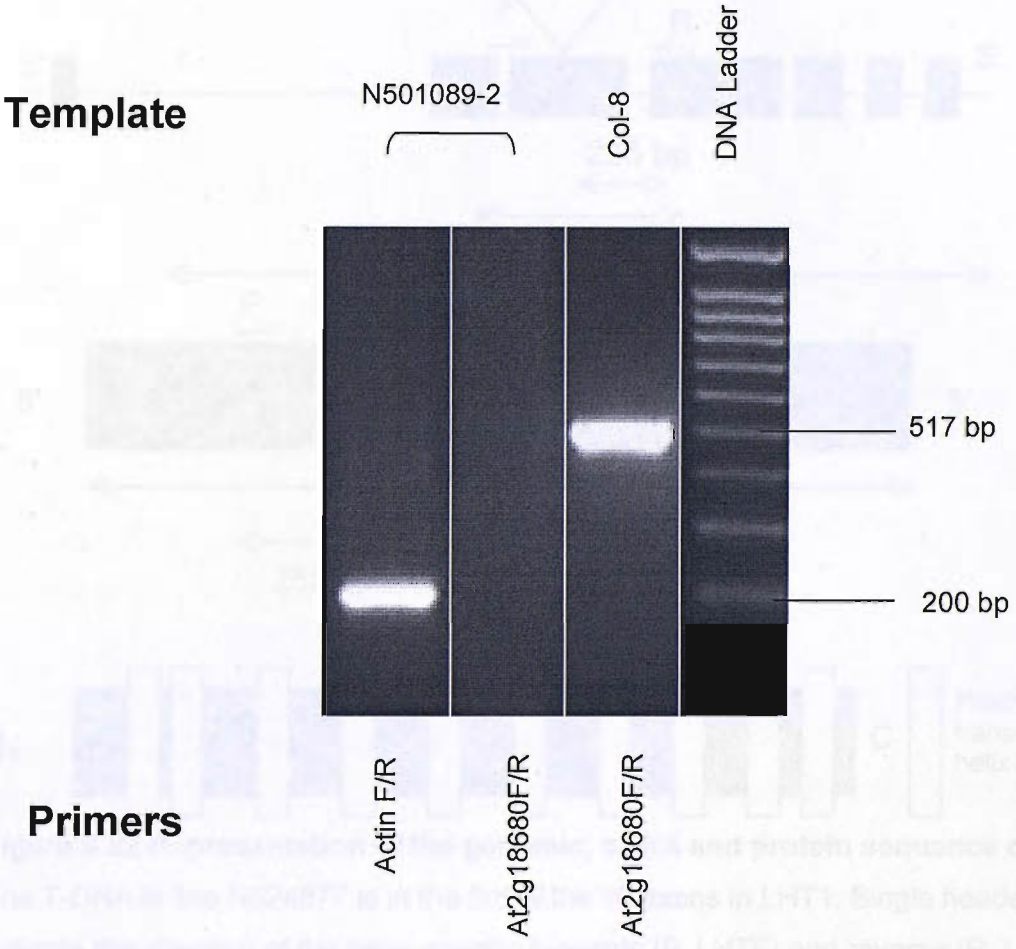

Figure 5.21 No full-length cDNA corresponding to *At2g18680* can be amplified from an *at2g1860* insertional mutant. cDNA was prepared from RNA extracted from plant N501089-2. PCR using ActinF and ActinR primers was used to detect full-length Actin cDNA. The gene specific primers At2g18680F and At2g18680R.were used to detect *At2g18680* cDNA. The DNA ladder used was Q-step1 (Yorkshire Biosciences, UK).

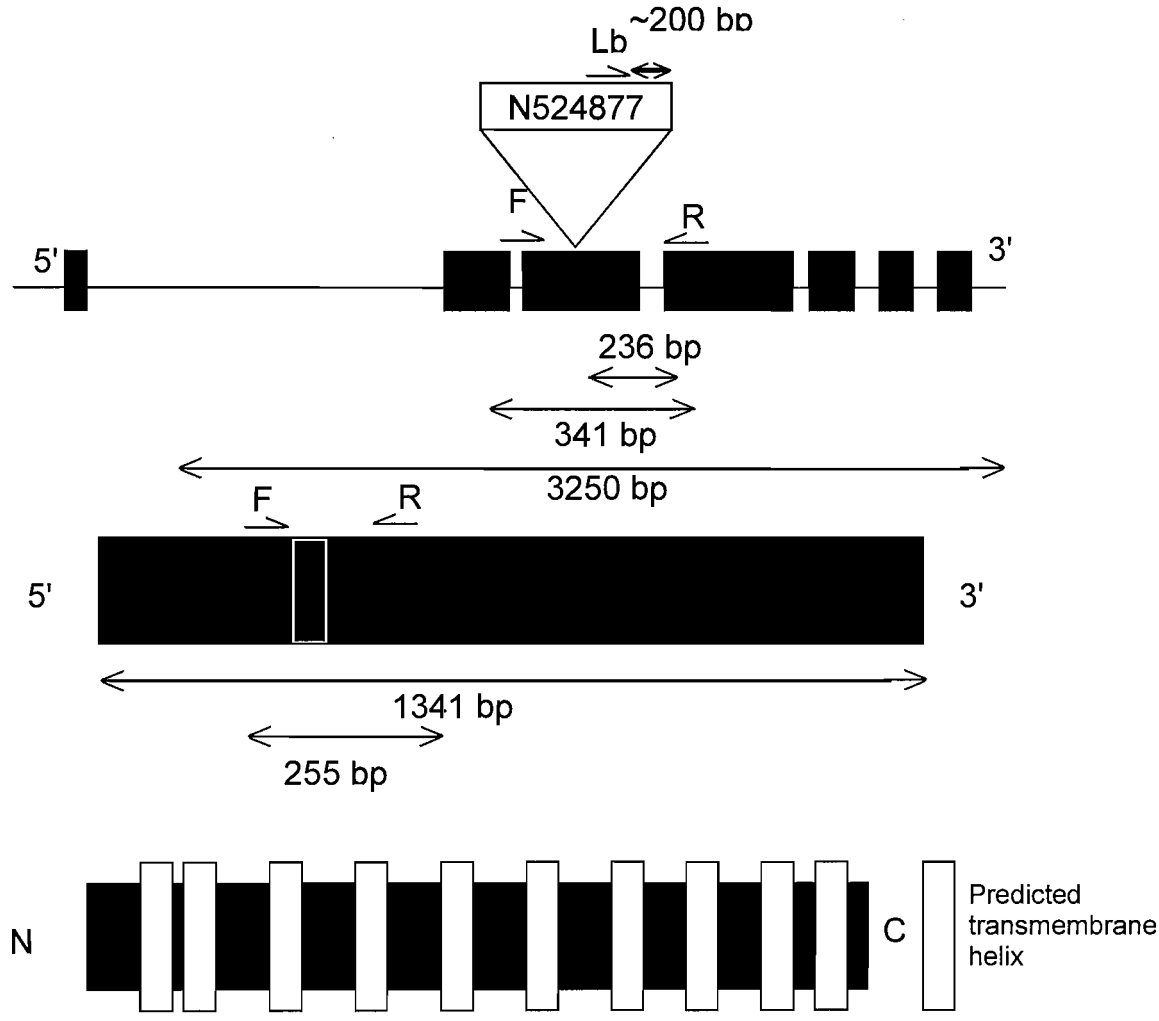

Figure 5.22 Representation of the genomic, cDNA and protein sequence of *LHT1.*
The T-DNA in line N524877 is in the 3rd of the 10 exons in LHT1. Single headed arrows indicate the direction of the gene-specific forwards (F, LHTF) and reverse (R, LHTR) primers, as well as the Lb primer specific to the left border of the T-DNA. Double-headed arrows indicate the size of the respective DNA sequence.

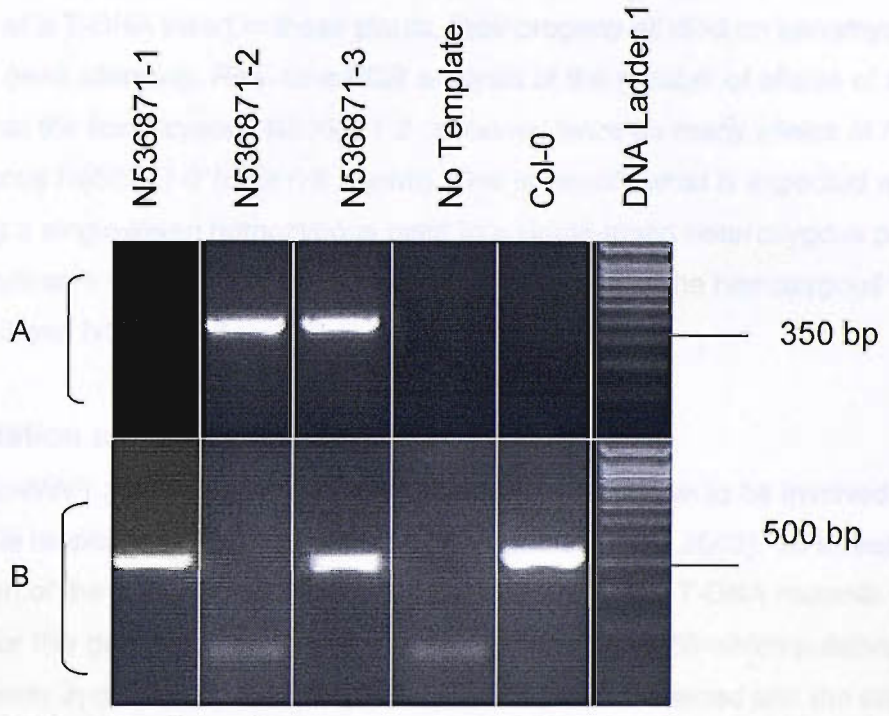

Figure 5.23 Identification of a homozygous *lht1* insertional mutant. PCR on genomic DNA extracted from both Col-0 four potential T-DNA insertional mutants. A: PCR using gene-specific primers LHT1F and LHTR; B: PCR using the gene-specific primer LHT1F and the T-DNA primer Lb. Refer to Figure 5.22 for the location of the primers. Using the logic described in the text, plant N536871-1 was identified as being homozygous for the T-DNA insert. The DNA ladder used was Q-step1 (Yorkshire Biosciences, UK).

DNA gene border for line N536871 is 250 bp from the start ATG codon, not 304 bp as predicted from the Salk Institute (Figure 5.24). For a final confirmation that plants homozygous for the T-DNA no longer produced a wild-type copy of cDNA for *LHT1*, a homozygous plant was grown to maturity and harvested. RNA was extracted from this material and cDNA was subsequently prepared from the RNA. Figure 5.25 shows that no product corresponding to the full-length *LHT* mRNA could be amplified from a homozygous insertional mutant. Positive controls showed that this band could be amplified from wild-type plants and that full-length mRNAs other than *LHT1* could be amplified from cDNA prepared from the homozygous insert plant. Despite the proven existence of a T-DNA insert in these plants, their progeny all died on kanamycin media, indicating gene silencing. Real-time PCR analysis of the number of alleles of *NPTII* showed that the homozygous N536871-2 contained twice as many alleles of *NPTII* as the heterozygous N536871-3 (data not shown). This is exactly what is expected when comparing a single-insert homozygous plant to a single-insert heterozygous plant and strongly indicates that there is only 1 T-DNA insertion site in the homozygous plants N536871-2 and N536871-3.

5.2.8 Isolation of *atcwINV1* insertional mutants

AtcwINV1 (previously known as fruct1) has been shown to be involved in the Arabidopsis response to *E. cichoracearum* (Fotopoulos *et al.*, 2003). To investigate further the function of the cell wall invertase AtcwINV1, homozygous T-DNA mutants were obtained for this gene. The seed stocks N619499 and N591455 which putatively contain T-DNA inserts in different locations within *AtcwINV1* were obtained and the original seed received from the NASC were grown to maturity. In outline, the zygosity of these was confirmed by PCR and antibiotic selection, the insert location sequenced, the number of other T-DNA inserts quantified by real-time PCR and finally checked for the presence of *AtcwINV1* cDNA in identified insertional mutants. This was done using the same methods as described for *aca12-1*. The locations of the primers used to determine zygosity and location of the T-DNA in the genomic and cDNA sequences are shown in figure 5.26.The primers F and R are specific to *AtcwINV1* and are either side of the suspected T-DNA location in the seed line N591455. The primers F1 and R1 are also specific to *AtcwINV1*, but are located either side of the suspected insert location in the seed line N619499. PCR on a wild-type copy of the *AtcwINV1* gene using the F and R primers should produce a 955 bp product, while PCR using the F1 and R1 primers should produce a product of 593 bp. PCR using the F primer and Lb primer should produce a product of about 1100 bp on T-DNA containing plants from the seed line N591455, while the R1 and Lb primer should produce a product of about 400 bp on T-DNA containing plants from the seed line N619499. Using the logic described in 5.2.1, Figure 5.27 shows that N619499-2 is wild-

tcccctaaaccaaaaccggtttatttacctaaaccggatttatcaaatcttaaccggt
tttaatttgttttcttttcagGGGACCGGGAATTGCAGTGTTGGTTTTGTCATGGGTXX
X_2GCATGGCCCACTACGTGAACCAXXX$_2$CTTTGACGTTGGAGTCCACGTTCTTTAATAG
TGGACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGGCTATTCTTTTGATT
TATAAGGGATTTTGCCGATTTCGGAACCACCATCAAACAGGATTTTCGCCTGCTGGGGC
AAACCAGCGTGGACCGCTTGCTGCAACTCTCTCAGGGCCAGGCGGTGAAGGGCAATCAG
CTGTTGCCCGTCTCACTGGTGAAAAGAAAAACCACCCCAGTACATTAAAAACGTCCGCA
ATGTGTTATTAAGTTGTCTAAGCGTCAATTTGATACATAACACTATACACATTATGGCA
AATGGTGGAAATGCATGAAATGGTTCCTGGAAAGCGTTTTGATCGTTACCATGAGCTCG
GACAACACGCGTTTGGAGAAAAACTCGGTCTTTATATCGTTGTGCCGCAACAATTGATC
GTTGAAATCGGTGTTTGCATCGTTTATATGGTCACTGGAGGCAAATCTTTAAAGAAATT
TCATGAGCTTGTTTGTGATGATTGTAAACCAATCAAGCTTACTTATTTCATCATGATCT
TTGCTTCGGTTCACTTCGTCCTCTC

Figure 5.24 Sequence analysis of *lht1-1*. PCR between *LHT1* and the T-DNA was performed on genomic DNA (see Figure 5.22 for primer locations), and the product sequenced from the Lb primer to confirm the presence and exact location of the T-DNA insert in *LHT1*. Grey sequence represents the gene-specific reverse primer LHT1R. Clear sequence represents sequence from the genome sequencing project. Yellow sequence represents gene-specific sequence obtained in this study. Blue sequence represents T-DNA specific sequence obtained in this study. Green sequence represents the bulk of the T-DNA, which has not been sequenced here and the gap between the sequencing primer and the first accurate sequence. Pink represents the Lb primer. The join between the yellow and blue bases represents the insertion site of the T-DNA confirming the presence of the T-DNA in *LHT1*.

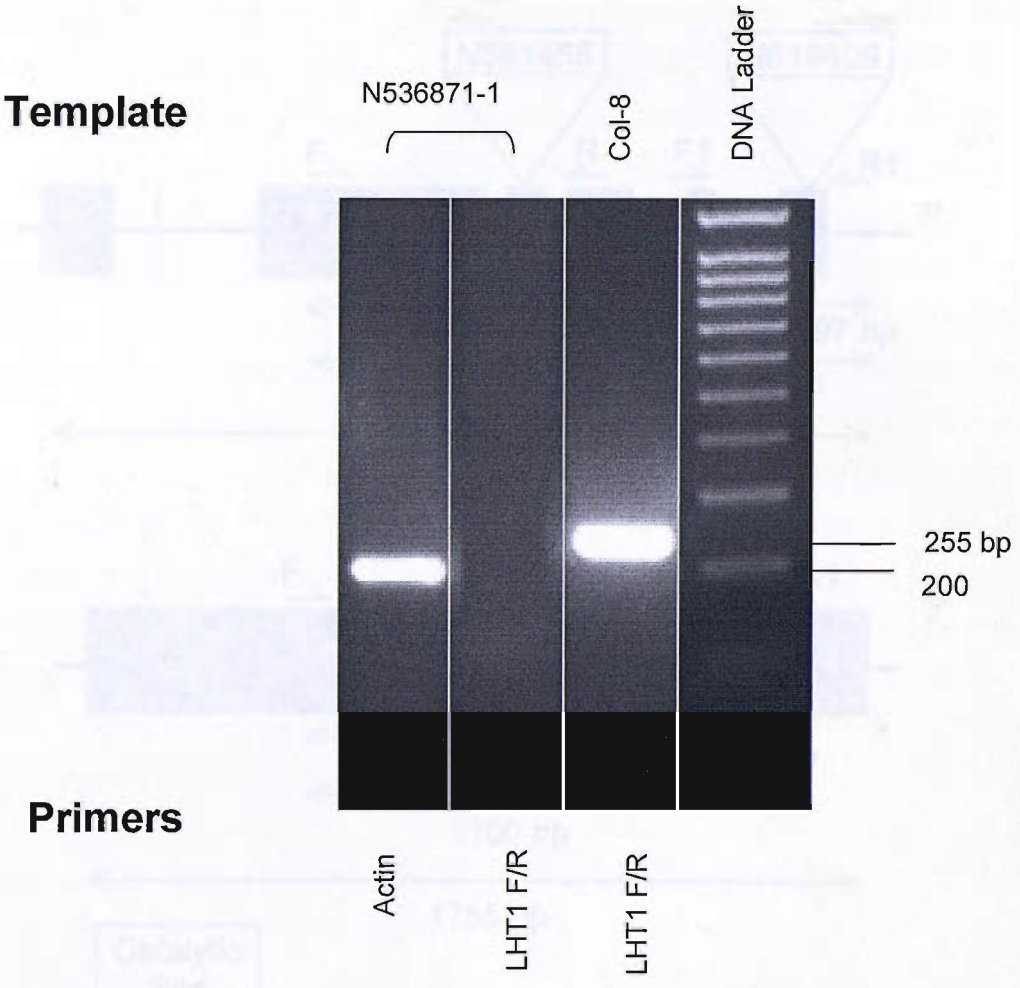

Figure 5.25 No full-length cDNA corresponding to *LHT1* can be amplified from *LHT1* insertional mutants. cDNA was prepared from RNA extracted from plant N536871-2. PCR using ActinF and ActinR primers was used to detect full-length Actin cDNA. The gene-specific primers LHT1F and LHT1R were used to detect *LHT1* cDNA. The DNA ladder used was Q-step1 (Yorkshire Biosciences, UK).

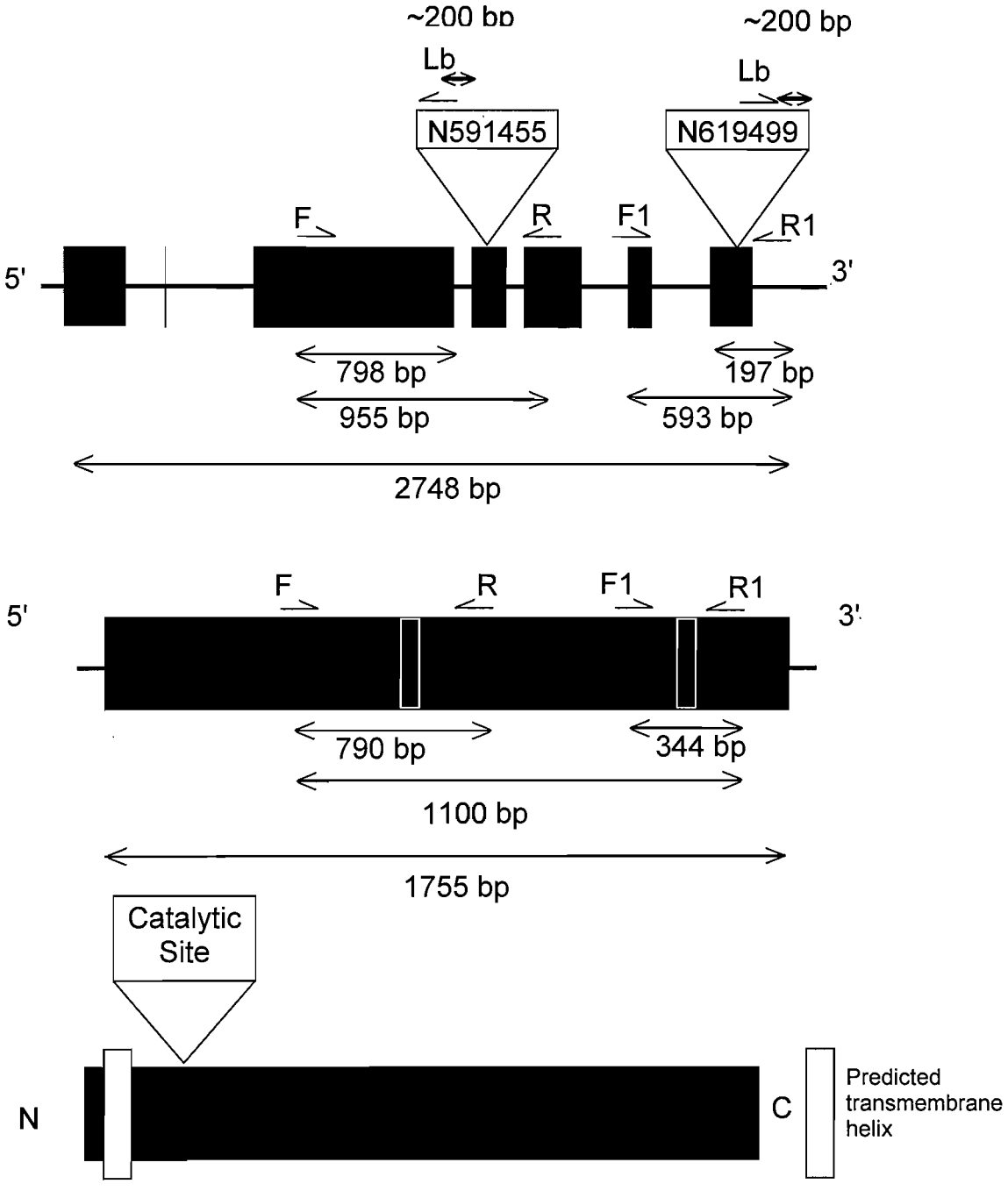

Figure 5.26 Representation of the genomic, cDNA and protein sequence of *AtcwINV1*. The T-DNA in line N591455 is in the 4th of the 7 exons in *AtcwINV1* while the T-DNA in line N619499 is in the 7th exon. Single headed arrows indicate the direction of the gene-specific forwards (F, AtcwINV1F/F1) and reverse (R, AtcwINVR/R1) primers, as well as the Lb primer specific to the left border of the T-DNA.

type and N619499-1 is homozygous for the T-DNA insert, while N619499-3 is heterozygous for the T-DNA insert. All of the progeny of N619499-1 survive when plated onto kanamycin-containing plates, while progeny of N619499-3 showed a 3:1 survival: death ratio (data not shown). This indicates that this line is likely to contain only 1 T-DNA insert. Thus, N619499-1 was re-designated *atcwinv1-2* as a non allelic mutant has already been identified in *AtcwINV1* (Quilliam *et al.*, 2006). Using the logic described in 5.2.1, Figure 5.28 shows that N591455-2 is wild-type and N591455-1 is homozygous for the T-DNA insert, while N591455-3 is heterozygous for the T-DNA insert. All of the progeny of N591455-1 survive when plated onto kanamycin- containing plates, while progeny of N591455-3 showed a 3:1 survival: death ratio (data not shown). This indicates that this line is likely to contain only 1 T-DNA insert. Following this, N591455-1 was re-designated *atcwinv1-3*. Both *atcwinv1-2* and *atcwinv1-3* were sequenced to determine the exact insert location (Figure 5.29), which was at 1662 of 1755 bp (full-length) of cDNA for *atcwinv1-2* (N619499) and 1206 bp of 1755 bp (full-length) for *atcwinv1-3* (N591455).

For a final confirmation that plants homozygous for the T-DNA insert no longer produced a full-length cDNA for the gene of interest, a homozygous plant from each line was grown to maturity and harvested. RNA was extracted from this material and cDNA was subsequently prepared from the RNA. Figure 5.30 shows that no product corresponding to the full-length *AtcwINV1* could be amplified from either a N591455 or N619499 homozygous insertional mutant, while full-length Actin cDNA could.

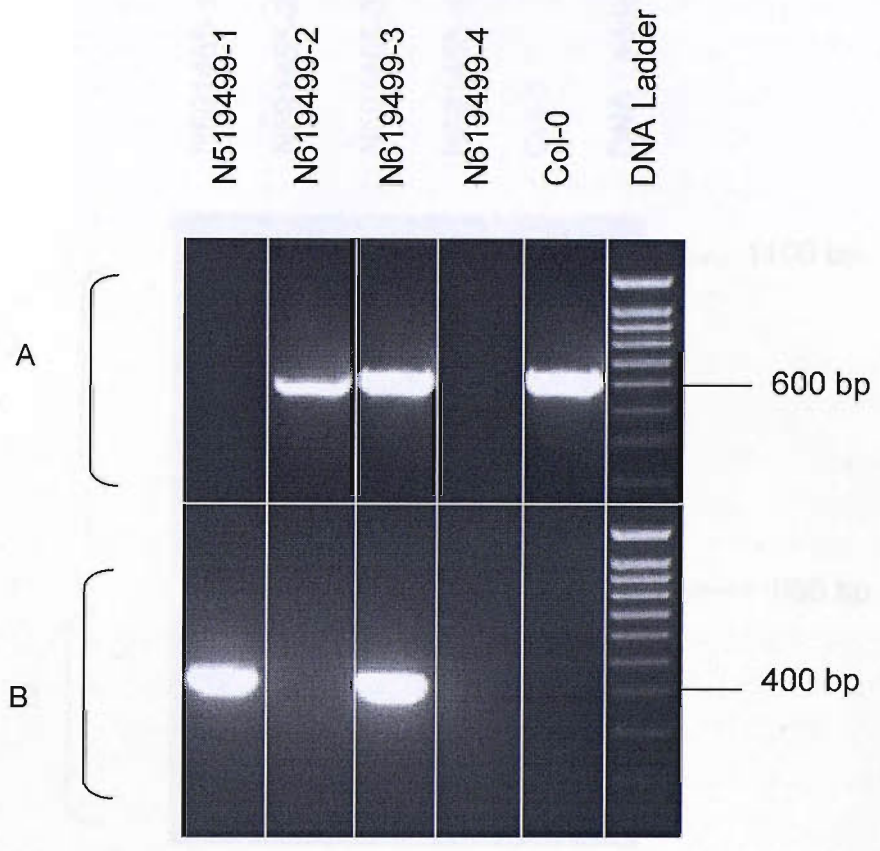

Figure 5.27 Identification of a homozygous *atcwINV1* mutant. PCR on genomic DNA extracted from Col-0 and three potential T-DNA insertional mutants. A: PCR using gene-specific primers AtcwINV1F1 and AtcwINVR1; B: PCR using the gene-specific primer AtcwINVR1 and the T-DNA primer Lb. Refer to Figure 5.26 for the location of the primers. Using the logic described in the text, plant N619499-1 was identified as being homozygous for the T-DNA insert. The DNA ladder used was Q-step1 (Yorkshire Biosciences, UK).

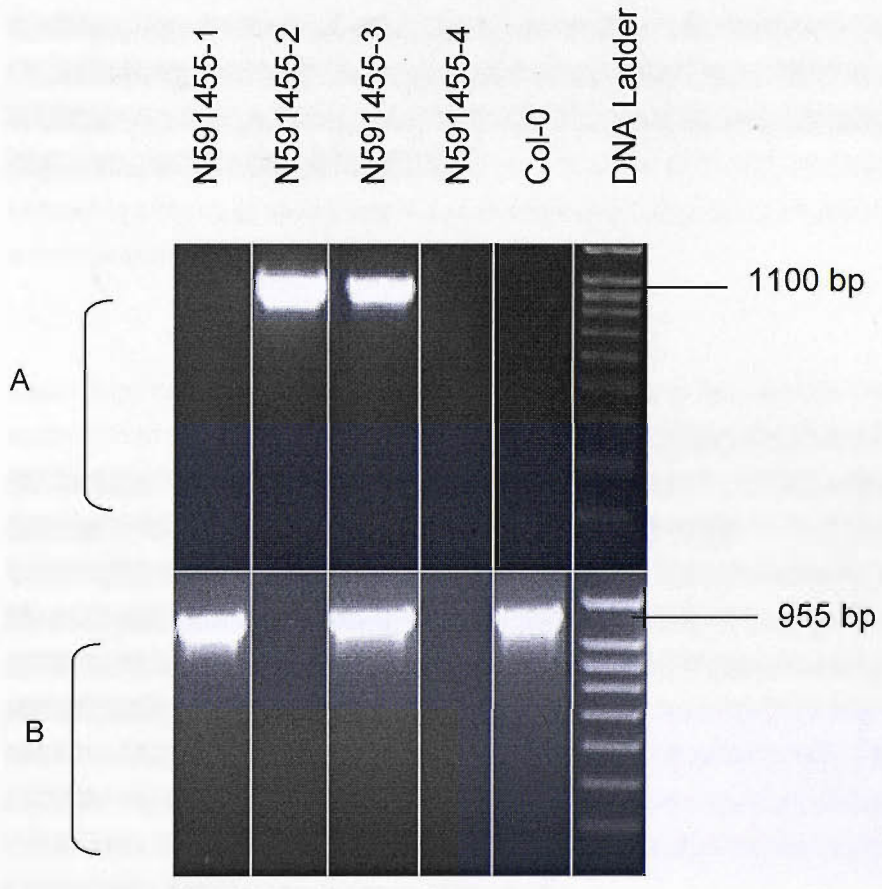

Figure 5.28 Identification of a homozygous *atcwINV1* mutant. PCR on genomic DNA extracted from Col-0 and three potential T-DNA insertional mutants. A: PCR using gene-specific primers AtcwINV1F and AtcwINV1R; B: PCR using the gene-specific primer AtcwINV1F and the T-DNA primer Lb. Refer to Figure 5.26 for the location of the primers. Using the logic described in the text, plant N519455-1 was identified as being homozygous for the T-DNA insert. The DNA ladder used was Q-step1 (Yorkshire Biosciences, UK).

A

gtaattagttacgcttaactgaaactaataatgtgaatataattgttagagattgaatc
aatttttttgtttctttcttgattagACGATTCCAAGGAAAATATGGCTTGATAGATCAG
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gatttgtacag

B

tagATTGATCATTCAGTAGTGGAGAGTTTCGGTGGAAAGGGAAGAGCATGCATTACCTC
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AATTACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGT
TCCAAACTGGAACAACACTCAACCCTATCTCGGGCTATTCTTTTGATTTATAAGGGATT
TTGCCGATTTCGGAACCACCATCAAACAGGATTTTCGCCTGCTGGGGCAAACCAGCGTG
GACCGCTTGCTGCAACTCTCTCAGGGCCAGGCGGTGAAGGGCAATCAGCTGTTGCCCGT
CTCACTGGTGAAAAGAAAAACCACCCCAGTACATTAAAAACGTCCGCAATGTGTTATTA
AGTTGTCTAAGCGTCAATTTGTTTACACCACAGTTCACATCTCTTTGCTTTTAATTATG
GATATCAAAGTGTTGATGTCTTAAACTTAAATGCTTGGAGCATGAACTCTGCCCAAATC
AGTTGAtcacgtaaagatacacacatgtaaacacattacatctccattgatttagcatt
tatatgagttgattttggagagatcttatatggtccactttgtttggttgtcaataaaa
ttgtccaatttgtttgtttgaaataattctaaaag

Figure 5.29 Sequence analyses of *atcwinv1-2 and atcwinv1-3*. PCR between *atcwINV1* and the T-DNA was performed on genomic DNA (see Figure 5.22 for primer locations) for A. *atcwinv1-2* (N619499) and B. *atcwinv1-3* (N5915455). The insert location was sequenced from the Lb primer in both cases and confirms the presence and exact location of the T-DNA inserts in *AtcwINV1*. The clear sequence represents sequence from the genome sequencing project, yellow sequence represents gene-specific sequence obtained in this study, the blue sequence represents T-DNA specific sequence obtained in this study, the green sequence represents the bulk of the T-DNA, which has not been sequenced here and the gap between the sequencing primer and the first accurate sequence, while the Lb primer is shown in pink. The join between the yellow and blue bases represents the insertion site of the T-DNA confirming the presence of the T-DNA in *atcwINV1-1.* Uppercase letters represent coding sequence, while lowercase represents non-coding sequence.

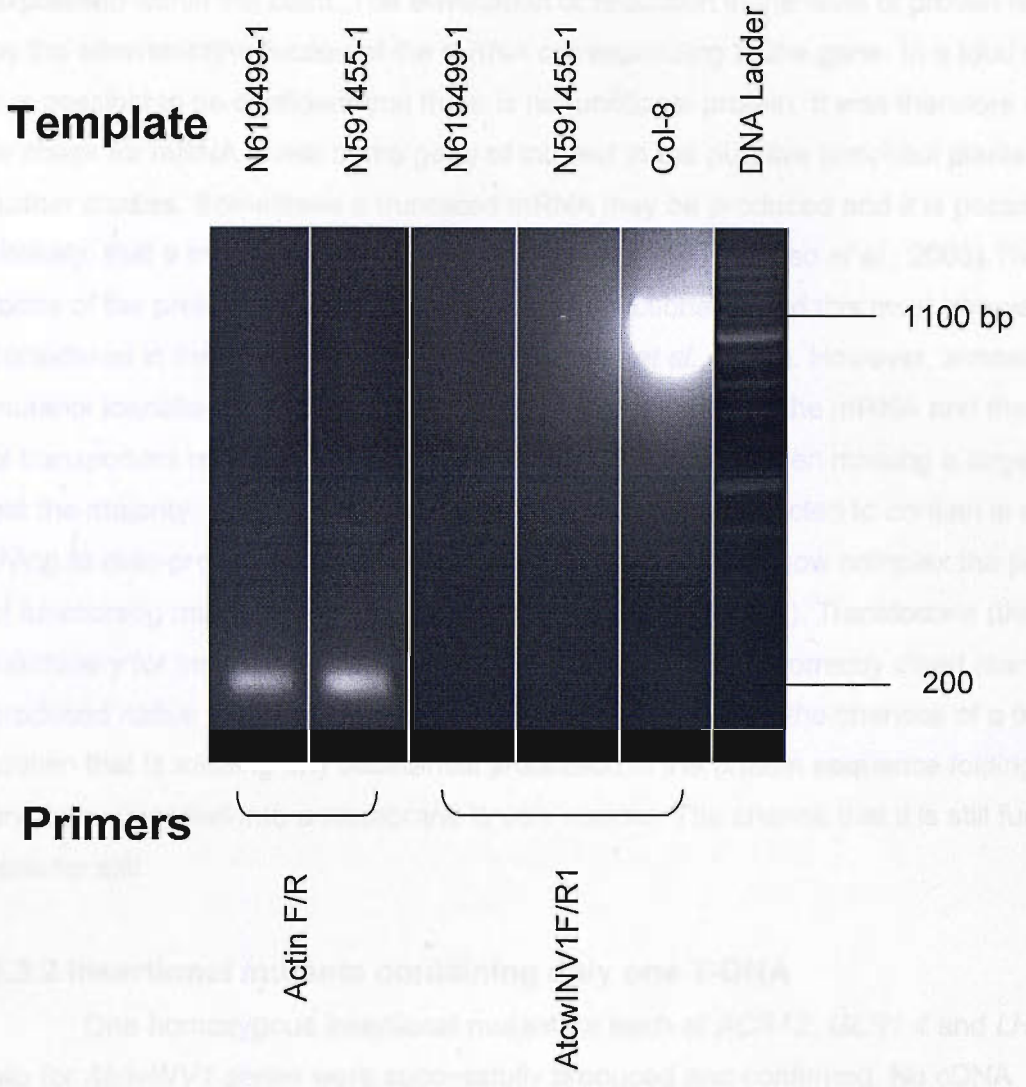


Figure 5.30 No full-length cDNA corresponding to *AtcwINV1* can be amplified from *atcwinv1-2* or *atcwinv1-3*. cDNA was prepared from RNA extracted from plants N591455-1 and N619499-1.PCR using ActinF and ActinR primers was used to detect full-length Actin. The PCR used to detect full-length potential T-DNA insertional mutants uses the gene-specific primers AtcwINV1F and AtcwINV1R, see Figure 5.26 for primer locations. The DNA ladder used was DNA Molecular Weight Ladder XIV (Roche, UK).

5.3 Discussion

5.3.1 Confirmation of homozygous insertional mutants

Homozygous mutants have been generated and confirmed for a selection of *E. cichoracearum*-responsive genes identified in Chapter 3 (Table 5.2). The purpose of the insertional mutants is to eliminate or greatly reduce the amount of functional protein expressed within the plant. The elimination or reduction in the level of protein is achieved by the elimination/reduction of the mRNA corresponding to the gene. In a total null mutant it is possible to be confident that there is no functional protein. It was therefore necessary to check for mRNA levels of the gene of interest in the putative knockout plants before further studies. Sometimes a truncated mRNA may be produced and it is possible but unlikely, that a truncated protein may also be produced (Alonso *et al.*, 2003).Truncated forms of the protein may retain some residual functionality and this must always be considered in the analysis of any mutant (Alonso *et al.*, 2003). However, almost all of the mutants identified here have a T-DNA within the first third of the mRNA and the likelihood of transporters retaining any significant degree of function when missing a large number, if not the majority, of the transmembrane helices they are predicted to contain is slim. Work trying to over-produce functional membrane proteins shows how complex the production of functioning membrane transporters is (Grisshammer, 2006). Translocons (the machinery for inserting proteins into cell membranes) fail to correctly insert many over-produced native transporter proteins (Grisshammer, 2006) – the chances of a transport protein that is missing any substantial proportion of the protein sequence folding correctly and being inserted into a membrane is very remote. The chance that it is still functional is remoter still.

5.3.2 Insertional mutants containing only one T-DNA

One homozygous insertional mutant for each of *ACA12*, *GLR1.4* and *LHT1* and two for *AtcwINV1* genes were successfully produced and confirmed. No cDNA corresponding to the respective mRNAs could be identified in any mutant line. Real-time PCR and kanamycin segregation analysis indicate that these lines contain only 1 independent T-DNA insertion. *aca12* insertional mutants are likely to be missing eight out of the twelve predicted transmembrane helices; *glr1.4* insertional mutants are likely to be missing four of the five predicted transmembrane helices, while *lht1* insertional mutants are likely to be missing seven out of the ten predicted transmembrane helices. AtcwINV1 is not predicted to contain any transmembrane helices, but is membrane bound. The insert locations are about 19/20 of the way into the cDNA, in the 7th of 7 exons for *atcwinv1-2* and about 6/10 of the way into the mRNA, in the 4th of the 7 exons of *AtcwINV1* for *atcwinv1-3*.

5.3.3 Insertional mutants likely to contain more than one T-DNA

Homozygous insertional mutants were identified and confirmed for *PTR3, ALA10* and *At2g18680*, the insert location was sequenced and no cDNA corresponding to the respective mRNAs could be identified. However, using a real-time PCR analysis, it was shown that all of these lines are likely to contain more than one T-DNA.

Both the wild-type PCR and the gene-to-T-DNA PCR on *PTR3* produced multiple products, probably due to mis-priming from regions within the *PTR3* with similar sequences to the primer site. It was however, easy to tell specific bands from non-specific bands by their size, and sequencing confirmed the location of the T-DNA as within *PTR3*.

PTR3 mutants are likely to lack all of the eleven predicted transmembrane helices and would be unlikely to be a functional protein, *ALA10* mutants are likely to lack six of the nine predicted transmembrane and *At2g18680* mutants are predicted to lack four of the six transmembrane helices.

In all of these mutants it is possible that any phenotypic effects observed are from a second insert. It is also possible, (but highly unlikely) that a second mutation could compensate a phenotypic effect of the mutated gene of interest. The possibility of other insert locations impacts on the status of the segregating wild-type plant. Although this plant contains no T-DNA within the gene of interest, it may contain a T-DNA in another location. Phenotypic analysis of these mutants using the segregating wild-type plants should take this into account and rely more heavily on the slightly more distantly related Col-8 wild-type (from which the Salk mutants were originally prepared). Given the time consuming nature of backcrossing plants with wild-type plants for a sufficient number of generations to be confident that any other T-DNAs have been removed (if indeed they can be removed) it was decided to analyse the insertional mutants and only perform backcrosses if a phenotype was observed.

Table 5.2 Summary of mutants identified. All the mutants and segregating wild-type plants confirmed in this chapter are summarised and listed with their identifiers (old and new). The sequenced location of the insert and the expected number of inserts are also listed. The segregating wild-type plants are used as controls in experiments in later chapters.

Line Identifier	Gene Code and name	Status	Number of likely separate T-DNA insertions	Sequenced T-DNA location (in the cDNA)
N598383-2 now *aca12-1*	At3g63380 *ACA12*	Homozygous insertional mutant	1	718 of 3102 bp
N598383-1	NA	Segregating wild-type	NA	NA
N629955-1 now *GLR1.4-1*	At3g07520 *GLR1.4*	Homozygous insertional mutant	1	15 of 2586 bp
N629955-2	NA	Segregating wild-type	NA	NA
N638430-1 now *ptr3-3*	At5g46050 *PTR3*	Homozygous insertional mutant	>2	111 of 1749 bp
N638430-2	NA	Segregating wild-type	NA*	NA
N524877-2 now *ala10-1*	At3g25610 *ALA10*	Homozygous insertional mutant	>2	1388 of 3609 bp
N524877-3	NA	Segregating wild-type	NA*	NA
N501089-2 now *at2g18680-1*	At2g18680 *At2g18680*	Homozygous insertional mutant	>2	102 of 864 bp
N501089-1	NA	Segregating wild-type	NA*	NA
N535871-2 now *lht1-1*	At5g40780 *LHT1*	Homozygous insertional mutant	1	250
N536871-1	NA	Segregating wild-type	NA	NA
N619499-1 now *atcwinv1-2*	At3g13790 *AtcwINV1*	Homozygous insertional mutant	1	1220 of 1755 bp
N619499-2	NA	Segregating wild-type	NA	1676 of 1755 bp
N591455-1 now *atcwinv1-3*	At3g13790 *AtcwINV1*	Homozygous insertional mutant	1	
N591455-2	NA	Segregating wild-type	NA	

Chapter 6

Phenotypic Characterisation of Insertional Mutants

6.1 Introduction

6.1.1 Transporter proteins in Arabidopsis

The complete sequencing of the Arabidopsis genome has broadened and accelerated plant biology research (Arabidopsis Genome Initiative, 2000), and one of the main focuses of current Arabidopsis functional genomics is assigning functions to newly identified genes (Radhamony *et al.*, 2005). The functions of approximately 69% of the genes were classified according to sequence similarity to proteins of known functions in a variety of organisms (Arabidopsis Genome Initiative, 2000). Information about the hypothesised role of an unknown gene may be deduced from its sequence using already known functions of similar genes as the basis for comparison (Holtorf *et al.*, 2002).

A large proportion of proteins in Arabidopsis are involved in the transport of many compounds including phytohormones, photoassimilates and signalling molecules. Membrane transport processes in Arabidopsis have been compared to those of fungi, animals and prokaryotes, and over 1700 predicted membrane transport proteins (with 5 or more transmembrane helices) have been identified in Arabidopsis (Maathuis *et al.*, 2003). A brief description of the transporter families and membrane proteins studied in this chapter will be given.

6.1.2 Amino acid transporters

Amino acids are the currency of nitrogen exchange in plants, so amino acid transport is a fundamental activity in plant growth (Rentsch *et al.*, 2007). The majority of amino acid transporters in plants studied to date are proton-amino acid symporters (Bush, 1993). These transporters couple amino acid uptake to the proton electrochemical potential gradient that is maintained across the plasma membrane of plant cells by a P-type proton pumping ATPase (Bush, 1993). Molecular cloning of amino acid transporters by functional complementation in yeast has revealed that there are multiple gene families, encoding different classes of amino acid transporters in plants (Bush *et al.*, 1996). Recent studies have shown there are more than 50 amino acid transporter genes in the Arabidopsis genome (Liu & Bush, 2006). Two superfamilies of amino acid transporters have been defined; the amino acid, polyamine and choline transporters superfamily (APC) and the amino acid transporter (ATF) superfamily (Fischer *et al.*, 1998; Williams & Miller, 2001). There are five known sub-classes of transporters within the ATF superfamily: the amino acid permeases (AAPs), the lysine, histidine transporters (LHTs), the proline transporters (ProTs), the putative auxin transporters (AUXs) and the aromatic and neutral amino acid transporter (ANT) family (Ortiz-Lopez *et al.*, 2000).

The amino acid transporter (LHT1) investigated in this study is part of the LHT sub-class of plant amino acid transporters. Relatively little is known about the structure of the LHT sub-class. The original identifiers of the class (Chen & Bush, 1997) showed that is likely to be different in structure to other members of the ATF superfamily as it differs in the number of transmembrane helices. There is however, a domain in the first TMH which is highly conserved amongst many eukaryotic amino acid transporters (Chen & Bush, 1997).

LHT1 itself has been the subject of a number of interesting studies. The first characterisation through functional complementation of a yeast mutant (Chen & Bush, 1997) demonstrated amino acid transport ability and described the substrate specificity in yeast (lysine and histidine). Two recent papers (Hirner *et al.*, 2006; Svennerstam *et al.*, 2007) have focused on the physiological role of LHT1. LHT1 was identified as being involved in the uptake of amino acids from the soil, and uptake into leaf mesophyll cells (Hirner *et al.*, 2006) and was reported at the same time as a phenotypic analysis of *lht1* mutants was being carried out in this study. *LHT1* was shown to be expressed in leaves and roots (Hirner *et al.*, 2006) and *lht1* mutants were shown to have reduced growth compared to wild-type plants, but this was not quantified at the time as other phenotypic traits were investigated (Hirner *et al.*, 2006). The study showed an important role for amino acid uptake in roots by LHT1; *lht1* mutants were shown to have a reduced rate of amino acid uptake and were substantially smaller in size on media/ soil where inorganic nitrogen was limiting (Hirner *et al.*, 2006). Interestingly *lht1* mutants were also smaller on soil where inorganic nitrogen was not limiting (Hirner *et al.*, 2006); this was surprising as LHT1 was shown to be able to transport inorganic nitrogen (Chen & Bush, 1997) and the supply of nitrogen should not be limiting growth. Re-expression of *LHT1* using a leaf-specific promoter enabled plants to grow normally (Hirner *et al.*, 2006). A root specific role was also indicated by experiments on the growth of seedlings. *lht1* mutants do not grown on medium containing Asp as the only nitrogen source, although Col-0 plants do (Hirner *et al.*, 2006). Re-expression of *LHT1* in *lht1* mutants only in leaves did not complement this phenotype, while re-expression using a systemic cauliflower mosaic virus 35S promoter did (Hirner *et al.*, 2006). This suggests that LHT1 has a role in amino acid uptake in both roots and leaves. The amino acid uptake specificities suggested by this study were noticeably different to the ones suggested by the original yeast experiments (Chen & Bush, 1997; Hirner *et al.*, 2006). Subsequently, using a forwards genetic approach *LHT1* was identified as being involved in the uptake of Ala from soil by roots (Svennerstam *et al.*, 2007), and the study also identified a substantially different substrate specificity for LHT1 than had been shown originally (Chen & Bush, 1997), but agreed with the other recent study of *LHT1* (Hirner *et al.*, 2006). This is probably due to *in planta* vs. *in vitro* differences). Selection for this mutant was based on the principle that Ala transporter mutants would be resistant to the toxic D-enantiomer of Alanine (Svennerstam *et al.*,

2007). Interestingly, *lht1* is grown in soil containing inorganic nitrogen display reduced growth compared to wild-type plants (Hirner *et al.*, 2006), but plants grown on artificial media containing inorganic nitrogen do not (Svennerstam *et al.*, 2007). This difference is difficult to explain without further experiments, and was not commented on by the authors of the latter paper.

6.1.3 P-type pumps

Two P-type pumps are investigated in this study, the *ACA12* putative calcium pump and the *ALA10,* a potential flipase. Arabidopsis has been shown to have 46 P-type pumps, but only the type P_{2B} (ACA – Autoinhibited Ca^{2+} ATPase) and the type P_4 (ALA, aminophospholipid translocase ATPase) pumps studied are discussed here. There are 14 Ca^{2+} ATPases in Arabidopsis and these form two distinct groups, type P_{2A} (or ECA for ER-type Ca^{2+} ATPases) and type P_{2B} (or ACA for autoinhibited Ca^{2+} ATPase). ACA2 is an example of a calmodulin-regulated Ca^{2+} ATPase and has been localised to the endoplasmic reticulum using a GFP fusion protein (Hong *et al.*, 1999). In comparison to animal homologues the distinct feature of ACA2 and the other ACA ATPases is the absence of a long C-terminal regulatory domain. Instead there is a unique, relatively long N-terminal domain of 160 residues which contains a calmodulin-binding domain (Harper *et al.*, 1999). The catalytic activity of ACA2 can be modulated by cytoplasmic concentration either through activation upon binding calmodulin or by inhibition following phosphorylation by Ca^{2+}-dependent protein kinases (Hwang *et al.*, 2000). ACA2 has been shown to contain an autoinhibitory domain sequence located between amino acids 20 and 44, and it is thought that an interaction between this sequence and a site in the Ca^{2+} pump core keeps the pump inactivated (Curran *et al.*, 2000).

The N-terminal domain also contains a calmodulin-binding sequence, as shown by calmodulin binding assays (Harper *et al.*, 1999). The calmodulin/Ca^{2+} ATPase interaction at the plasma membrane was shown to be dependent on calmodulin isoforms showing isoform-specific Ca^{2+} dependencies (Luoni *et al.*, 2006). In the absence of calmodulin the regulatory domain interacts with the sites in the cytoplasmic loops hampering the catalytic activity of the enzyme. This may provide a Ca^{2+} regulated feedback system to control Ca^{2+} levels in the cytoplasm. The activation of ACA2 occurs as a result of calmodulin binding to a site overlapping or immediately adjacent to the autoinhibitory domain, and thereby displacing its inhibitory interactions (Hwang *et al.*, 2000). Calcium ATPase specificity and function has been studied using the generation of insertional mutants. For example, ACA9 has been shown to be required for normal pollen tube growth and fertilisation, as disruption of *ACA9* resulted in partial male sterility (Schiott *et al.*, 2004). See also Baegaard *et al.* (2006) for more detail on the calmodulin binding of these pumps

6.1.4 Glutamate Receptors

Glutamate receptors in plants were identified by homology to the ionotropic glutamate receptors (iGluRs) in animals (Lacombe *et al.*, 2001). In animals iGluRs are non-selective cation channels that function predominately as glutamate-gated Na^+ and Ca^{2+} influx pathways (Davenport, 2002). Currently, the Arabidopsis genome is thought to encode 20 such receptors which split into 3 distinct phylogenetic groups, upon which the nomenclature is based: AtGLR1.x for the 1st clade, AtGLR2.x for the second and AtGLR3.x for the third (Lacombe *et al.*, 2001; Chiu *et al.*, 2002).

Plants are predicted to have a high degree of structural similarity with animal iGluRs (Davenport, 2002). There are 4 transmembrane domains in animal iGluRs, although one is not predicted to actually cross the membrane (Chiu *et al.*, 1999). In Chapter 5 it was shown that a hydropathy plot indicated 5 transmembrane domains in *AtGlr1.4* (the one investigated in this study). However in cornparison with animal iGluRs this is likely to be a hydrophobic plasma membrane signal sequence. The third hydrophobic region identified in Chapter 5 is the non-membrane spanning- hydrophobic loop region which lines the pore and confers ion selectivity on the channel (Davenport, 2002). The channel structure is very similar between plants and animals (Lam *et al.*, 1998; Davenport, 2002) although, interestingly, there are differences in the region of the third transmembrane helix which is thought to control ion selectivity (Lam *et al.*, 1998; Davenport, 2002).

The defining feature of glutamate gating by such channels has not yet been demonstrated in plants. Glutamate gating of calcium fluxes has been observed in plant roots, but a link to AtGLRs was not established (Dennison & Spalding, 2000). However, several experiments have shown AtGLRs to be involved in physiological processes: over-expression of AtGLR3.2 causes Ca^{2+} and Na^+ hypersensitivity (Kim *et al.*, 2001) and unspecified AtGLRs have a role in light signalling transduction (Lam *et al.*, 1998; Davenport, 2002). Other than these transporters, only a few of the GLR genes have been studied directly: AtGLR3.7 has been expressed in Xenopus oocytes and appears to function as a constitutively active, Ca^{2+} permeable, but non-selective cation channel with no evidence of glutamate gating (Davenport, 2002). AtGLR3.4 has been expressed in oocytes and was reported to mediate Ca^{2+} influx (Davenport, 2002).

6.1.5 PTR transporters

Peptide transporters are important in Arabidopsis: in 2002 there were 10 times more peptide transporter genes encoded by the Arabidopsis genome than by any other genome sequenced at the time (Stacey *et al.*, 2002; Rentsch *et al.*, 2007). Peptide transporters transport peptides of between 2 and 6 amino acids, exhibiting broad side-chain specificity, but highly specific stereoisomer specificity and avoid competition with free amino acids (Stacey *et al.*, 2002). There are 3 families of peptide transporters; the

ABC family which hydrolyses ATP as the energy source (Detmers *et al.*, 2001); the PTR family which uses the proton gradient (Steiner *et al.*, 1995) and the OPT family (Koh *et al.*, 2002) which probably uses the proton gradient.

The PTR family (a member of which is studied in this chapter) is predicted to have 12 transmembrane helices, with a unique (FYxxINxGSL) domain in the fourth or fifth hydrophobic region (Steiner *et al.*, 1995). Further experiments on human PTR group transporters indicate that this motif is essential for transport activity (Stacey *et al.*, 2002). The PTR transporter studied here (*PTR3*) has been the subject of several previous works (Karim *et al.*, 2005; Karim *et al.*, 2007). Molecular modelling of *PTR3* suggested that it has 12 transmembrane helices (as expected for a member of the PTR family) and a large cytoplasmic loop (Karim *et al.*, 2005). Upstream elements suggest that *PTR3* is involved in abiotic stress. This was proved to be the case as *PTR3* is indeed induced in response to salt stress and *ptr3* T-DNA insertional mutants have reduced germination rates on media containing high levels of salt. *PTR3* was also induced by mechanical wounding and high levels of amino acids in the media (Karim *et al.*, 2005).

A later study used complementation of a yeast mutant defective in di- and tri-peptide transport to prove that *PTR3* could mediate the transport of peptides (Karim *et al.*, 2007). In the same study it was also shown that *PTR3* was up-regulated when the hormones SA, MeJA and ABA were added to the growth media. Mutants preventing the accumulation/ production of SA, and mutants preventing the accumulation/ production of MeJA, did not show *PTR3* induction following wounding, indicating their involvement in the signalling of wounding by PTR3 (Karim *et al.*, 2007).

Interestingly, *ptr3* T-DNA insertion mutants were more susceptible to the necrotroph *E. carotovora*. *E. carotovora* is a compatible interaction, so *PTR3* is involved in the response of Arabidopsis to compatible infections (Karim *et al.*, 2007). However, this increased susceptibility was not seen when Arabidopsis were infected with another necrotrophic pathogen, the bacterial *Xanthomonas campestris* pv. *campestris*. *ptr3* mutants were also found to more susceptible to the hemibiotrophic pathogen *P. syringae* pv. tomato (Karim *et al.*, 2007). Real-time PCR analysis on Col-0 plants showed that *PTR3* was induced in response to *P. syringae*, but was induced to higher levels by *P. syringae* missing type III insertion systems, indicating that fully virulent *P. syringae* can suppress the induction of *PTR3*. It was hypothesised that *PTR3* could lead to increased susceptibility due to reduced concentrations of amino acids and thus lower levels of defence-related peptides, proteins or amino acid derivatives in infected tissue (Karim *et al.*, 2007). However it was also noted that *PTR3* plants accumulate more ROS on oxidative stress inducing media (Karim *et al.*, 2007) and was hypothesised that this could affect SA signalling which is under redox regulation, although it was clear that further experiments are needed in order to further understand the mechanism by which *PTR3* affects plant defence (Karim *et al.*, 2007)

6.1.6 Invertases

Plant invertases (β-fructofuranosidase E.C. 3.2.1.26) are a family of enzymes that hydrolyse sucrose into fructose and glucose (Taiz & Zeiger, 2002). Although they are not transporters, they have shown to be up-regulated in response to *E. cichoracearum*. There are four possible groups of plant invertase thought to exist; insoluble cell-wall bound invertase and soluble vacuolar, cytoplasmic and free apoplastic invertases (Tymowska-Lalanne & Kreis, 1998; Kim *et al.*, 2000; Hirose *et al.*, 2002), with all except the cytoplasmic forms having acidic pH optima. The existence of an apoplastic group of invertases is putative and is based on the predicted ioselectric point of certain cell-wall-type invertases, such as INCW4 from maize and INV1 from rice.

Cell-wall and vacuolar invertases show a high degree of sequence homology to each other and show similar enzymatic and biochemical properties, such as the ability to hydrolyse other similar fructose-glucose saccharides (Roitsch & Gonzalez, 2004) and the glycosylation of both enzymes is similar (Pagny *et al.*, 2003).

The cytoplasmic invertases are a highly homologous group of invertases, but have low sequence homology to both vacuolar and cell-wall invertases. Relatively little is known about this class of invertase, as the low enzyme activity is rapidly lost after tissue homogenisation (Sturm, 1999; Roitsch & Gonzalez, 2004). They have a much higher preference, sometimes a requirement, for sucrose as a substrate when compared to the vacuolar and cell-wall invertases (Lee & Sturm, 1996; Sturm *et al.*, 1999).

The physiological functions of invertase are complex, with four potentially distinct forms, and multiple members of some forms. Many invertases show differential regulation depending on tissue type, cell type, subcellular location and developmental stage (e.g. Roitsch *et al.*, 2000). There are three main interwoven functions of invertases, all of which have been the subject of recent reviews; the hydrolysis of sucrose to produce hexoses for the varied needs of: metabolism (Sturm, 1999; Sturm & Tang, 1999; Roitsch *et al.*, 2000; Sherson *et al.*, 2003; Roitsch *et al.*, 2003; Roitsch & Gonzalez, 2004); signalling (e.g. Roitsch *et al.*, 2000) and osmoregulation (e.g. Sturm & Tang, 1999).

6.1.7 Chapter aims

The aim of this chapter is to describe the phenotype under standard growth conditions of all of the insertional mutants isolated in Chapter 5, in comparison to both the segregating wild-type plants also isolated in Chapter 5 and the Col-8 wild-type.

6.2 Results

6.2.1 Phenotypic characterisation of *aca12-1*

Having confirmed *aca12-1* as a T-DNA insertional mutant, growth phenotypes of the progeny were studied as described in Chapter 2, as were all mutants analysed below. Measurements of the day of 1st bolt, 1st flower or 1st silique were made (Figure 6.1A). On the day that a plant reached these stages, the number of leaves present was counted (Figure 6.1B), the diameter of the rosettes was measured, (Figure 6.1C) and the height of the plant was also measured (Figure 6.1D). The *aca12-1* mutant showed no statistical differences from Col-8 or the segregating wild-type in any of these measurements.

6.2.2 Phenotypic characterisation of *glr1.4-1*

The *glr1.4-1* mutant showed significantly delayed bolting (Figure 6.2A) compared to Col-8 (2.7 days or 6.4% slower, P = 0.010) and the segregating wild-type (2.3 days or 5.3% slower, P = 0.032). Compared to Col-8, *glr1.4-1* also showed significantly delayed flowering (2.9 days or 6.0% slower, P = 0.012) and delayed appearance of siliques (3.3 days or 6.4% slower, P = 0.009). Although flowering and appearance of first silique were delayed when compared to the segregating wild type, this was above (although close to) the level of significance (P = 0.053 and P = 0.061). When repeated (not shown), a similar pattern of delayed bolting (5.3% slower), flowering (3.7% slower) and appearance of silique (2.4 %) was observed, but only delayed bolting was significantly different.

The *glr1.4-1* mutant showed significantly more leaves at 1st bolt, compared to Col-8 (1.87 or 12.2% more leaves, P = 0.012) and the segregating wild-type (1.73 or 11.23% more leaves, P =0.018), but no significant changes in leaf number at any other growth stage (Figure 6.2B). This was representative of other repeats.

The rosette diameter of *glr1.4-1* was larger than both Col-8 and the segregating wild-type at every growth stage (Figure 6.2C).

The height of *glr1.4-1* was only significantly different to Col-8 and the segregating wild-type at the appearance of the 1st silique (Figure 6.2D). This was representative of other repeats.

6.2.3 Phenotypic characterisation of *ptr3-3*

There was a slight (~2%) delay in the appearance of the 1st bolt, 1st flower or 1st silique of Col-8 or the N638430-2 compared to *ptr3-3* (Figure 6.3A). However, this was not statistically significant (P > 0.05). No consistent pattern in the number of leaves of *ptr3-3* compared to Col-8 or N638430-2 was observable (Figure 6.3B), and there were no statistical differences between the genotypes.

There was no significant difference in the rosette diameter at any growth stage (Figure 6.3C) between Col-8 and *ptr3-3*, although N638430-2 had a significantly reduced rosette diameter at 1st bolt compared to both Col-8 (7.5 mm or 7.1 % smaller, P = 0.006)

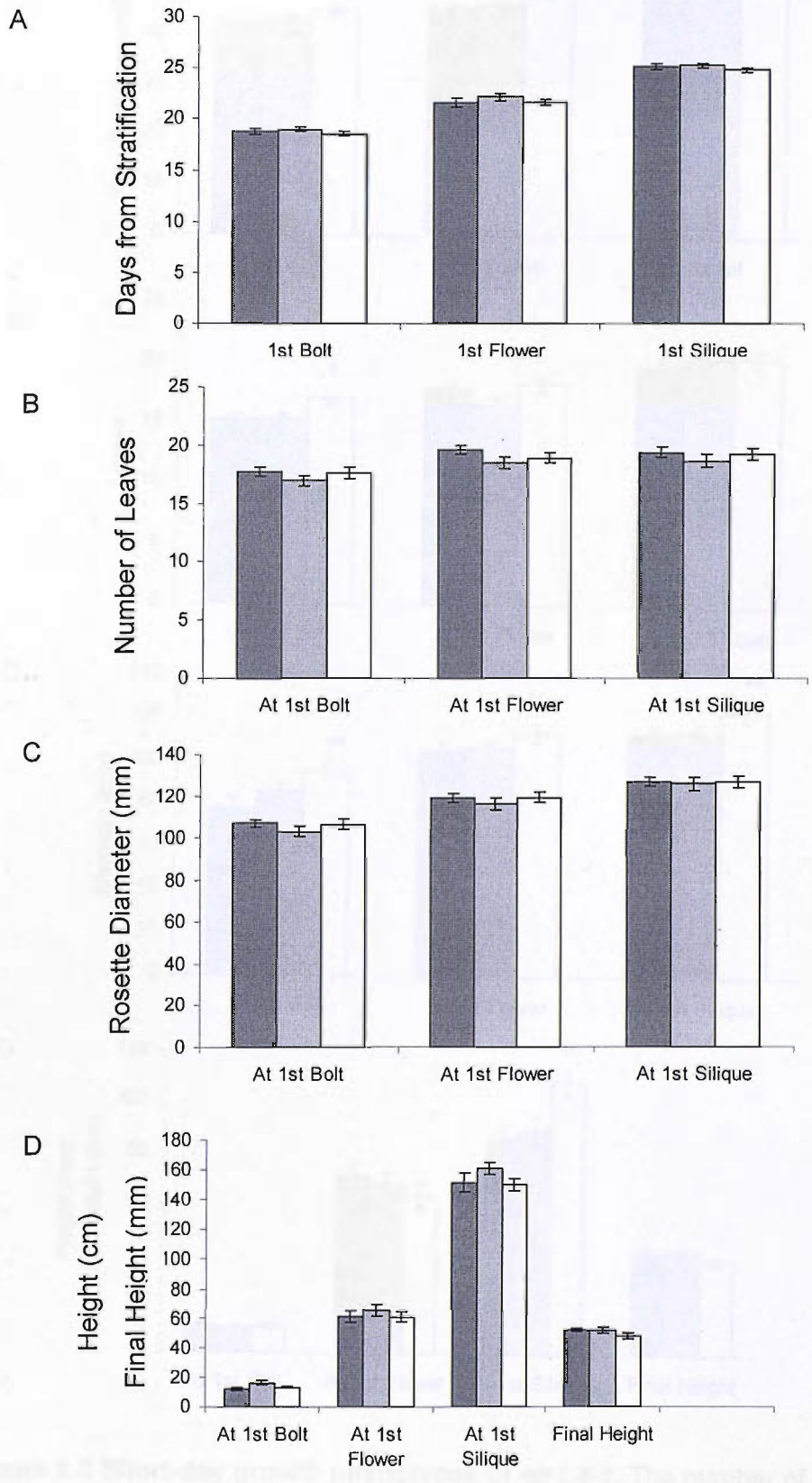


Figure 6.1 Short-day growth phenotypes of *aca12-1*. The number of days after stratification for the production on the 1st bolt, flower and silique (A), number of leaves (B), diameter of rosette (C), and height of plant (D) at each growth stage. Dark grey, Col-8; light grey, segregating wild-type (N629955-2); white, *aca12-1*. All measurements were and *ptr-1* (10.3 mm or 9.4% smaller, P = 0.003) and at 1st flower, compared to Col-8 (8.1

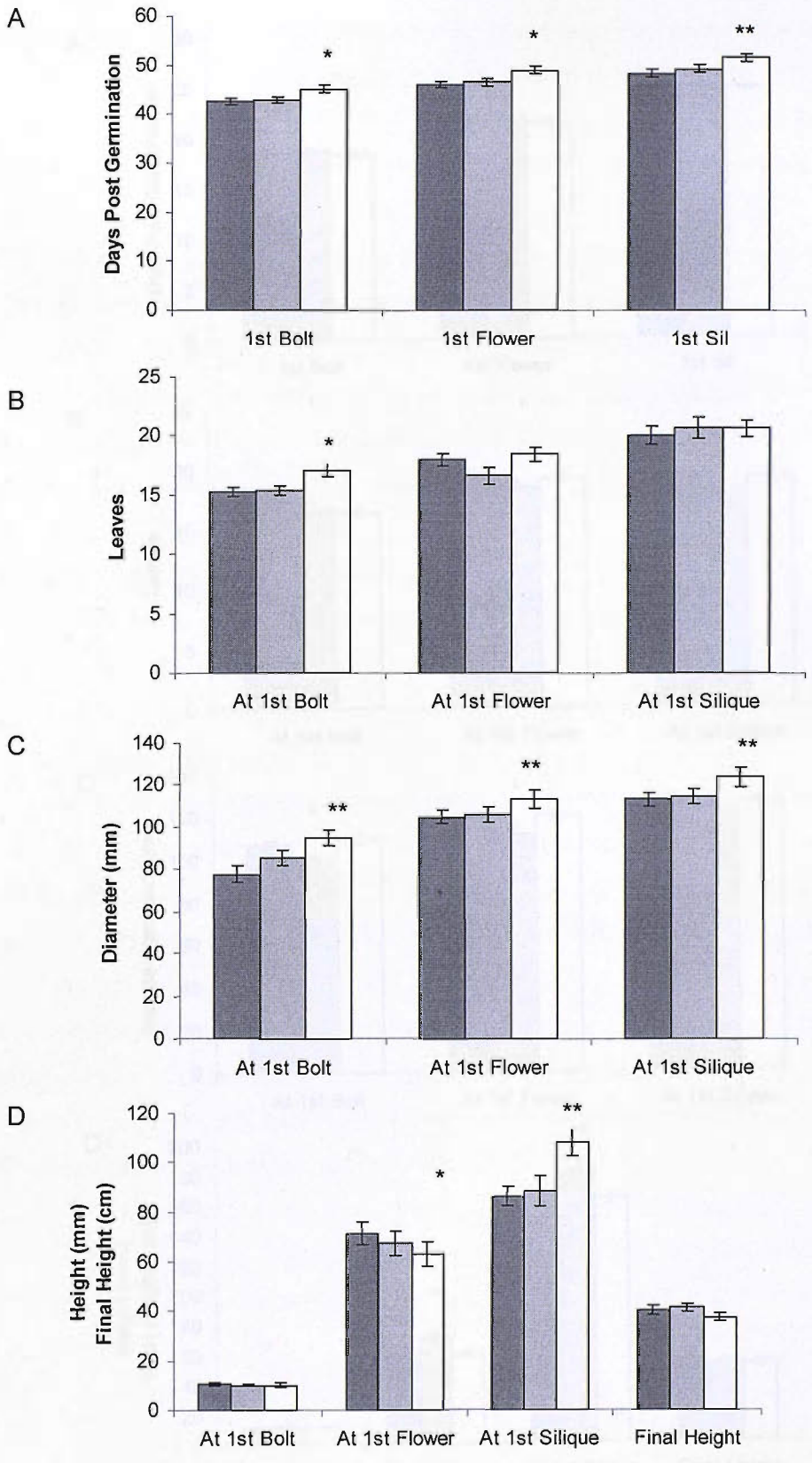


Figure 6.2 Short-day growth phenotypes of *glr1.4-1*. The number of days after stratification for the production on the day of 1st bolt, flower or silique (A), number of leaves (B), diameter of rosette (C), and height (D) at each growth stage. Dark grey, Col-8; light grey, segregating wild-type (N629955-2); white, *glr1.4-1*. All measurements were taken on the day a plant reached a 1st bolt, 1st flower or 1st silique. n = 30. Standard error is shown. *, $P < 0.05$; **, $P < 0.001$.

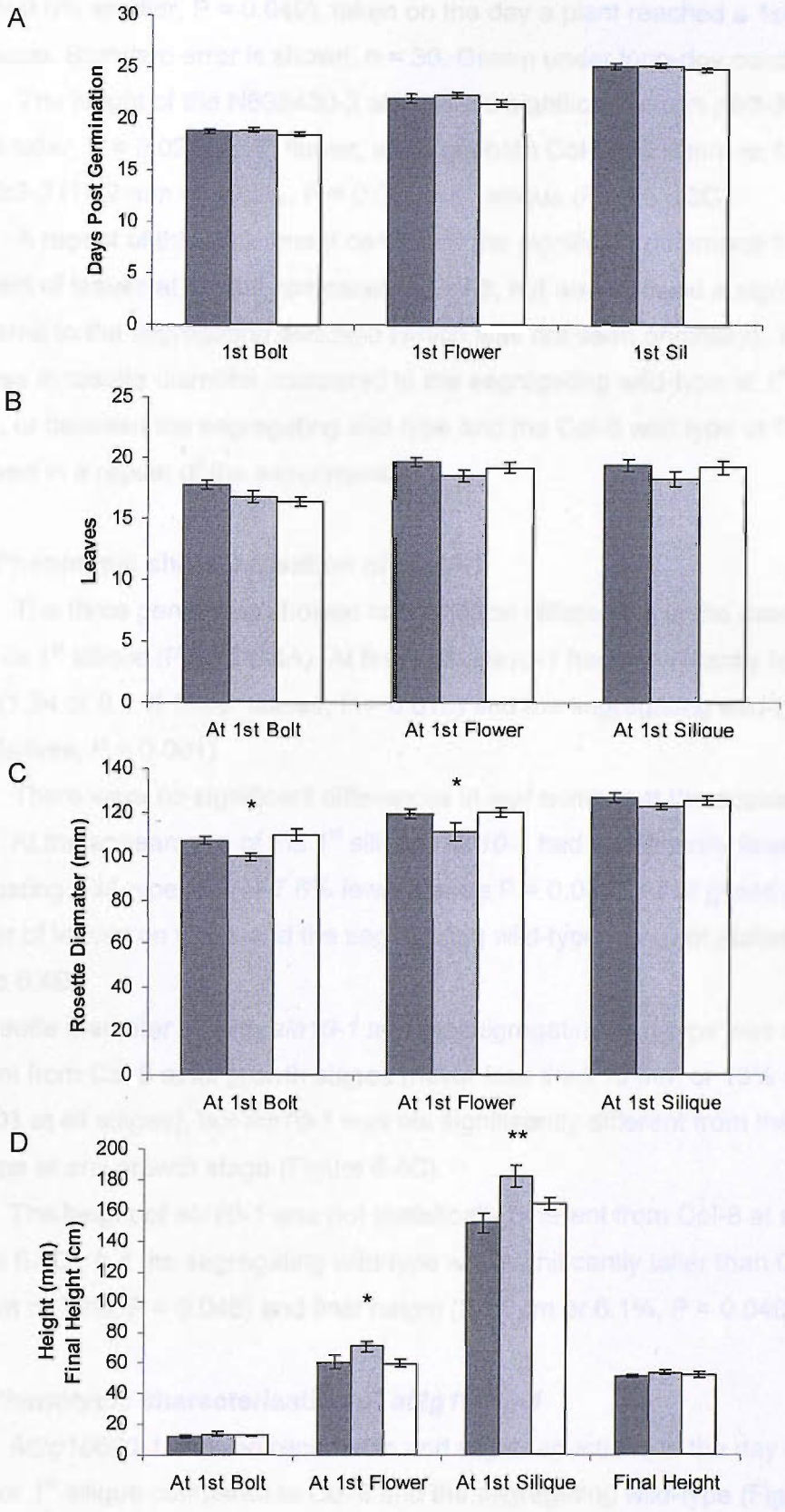


Figure 6.3 Short-day growth phenotypes of *ptr3-3*. The number of days after stratification for the production on the 1st bolt, flower or silique (A), number of leaves (B), diameter of rosette (C), and height (D) at each growth stage. Dark grey, Col-8; light grey segregating wild-type, N638430-2; white, *ptr3-3,* All measurements were taken on the day a plant reached a 1st bolt, 1st flower and 1st silique. n = 30. Standard error is shown. *, $P < 0.05$; **, $P < 0.001$.

mm or 9.0% smaller, P = 0.049). taken on the day a plant reached a 1st bolt, 1st flower or 1st silique. Standard error is shown. n = 30. Grown under long-day conditions.

The height of the N638430-2 also differs significantly from *ptr3-3* (11.3 mm or 15.9% taller, P = 0.021) at 1st flower, and from both Col-8 (30.4 mm or 16.72%, P = 0.003) and *ptr3-3* (18.2 mm or 10.0%, P = 0.03) at 1st silique (Figure 6.3D).

A repeat of this experiment confirmed the significant difference between the numbers of leaves at 1st bolt compared to Col-8, but also showed a significant difference compared to the segregating wild type (which was not seen originally). The significant increase in rosette diameter compared to the segregating wild-type at 1st bolt and 1st flower, or between the segregating wild type and the Col-8 wild type at 1st bolt was not observed in a repeat of the experiment.

6.2.4 Phenotypic characterisation of *ala10-1*

The three genotypes showed no statistical differences in the date of 1st bolt, 1st flower or 1st silique (Figure 6.4A). At first bolt, *ala10-1* had significantly fewer leaves than Col-8 (1.34 or 8.1 % fewer leaves, P = 0.010) and the segregating wild-type (1.7 or 10.4% fewer leaves, P = 0.001).

There were no significant differences in leaf number at the appearance of the 1st flower. At the appearance of the 1st silique, *ala10-1* had significantly fewer leaves than the segregating wild-type (1.4 or 7.6% fewer leaves P = 0.048). At all growth stages, the number of leaves on Col-8 and the segregating wild-type were not statistically different (Figure 6.4B).

The rosette diameter of both *ala10-1* and the segregating wild-type was significantly different from Col-8 at all growth stages (never less than 13 mm or 13% smaller diameter P < 0.01 at all stages), but *ala10-1* was not significantly different from the segregating wild-type at any growth stage (Figure 6.4C).

The height of *ala10-1* was not statistically different from Col-8 at any growth stage (Figure 6.4D), but the segregating wild-type was significantly taller than Col-8 at 1st bolt (2.8 mm or 23%, P = 0.048) and final height (3.15 cm or 6.1%, P = 0.040) (Figure 6.4D).

6.2.5 Phenotypic characterisation of *at2g18680-1*

At2g18680-1 showed repeatable and slight reductions in the day of 1st bolt, 1st flower or 1st silique compared to Col-8 and the segregating wild-type (Figure 6.5A). However these differences were very slight (never greater than 2.5%) and were not statistically significant.

At2g18680-1 showed a pronounced decrease in the number of leaves at 1st bolt compared to Col-8 (1.34 leaves or 8.2 %, P = 0.002) and the segregating wild-type (1.7 or 10.4% fewer leaves, P = 0.010). The segregating wild-type and Col-8 were not

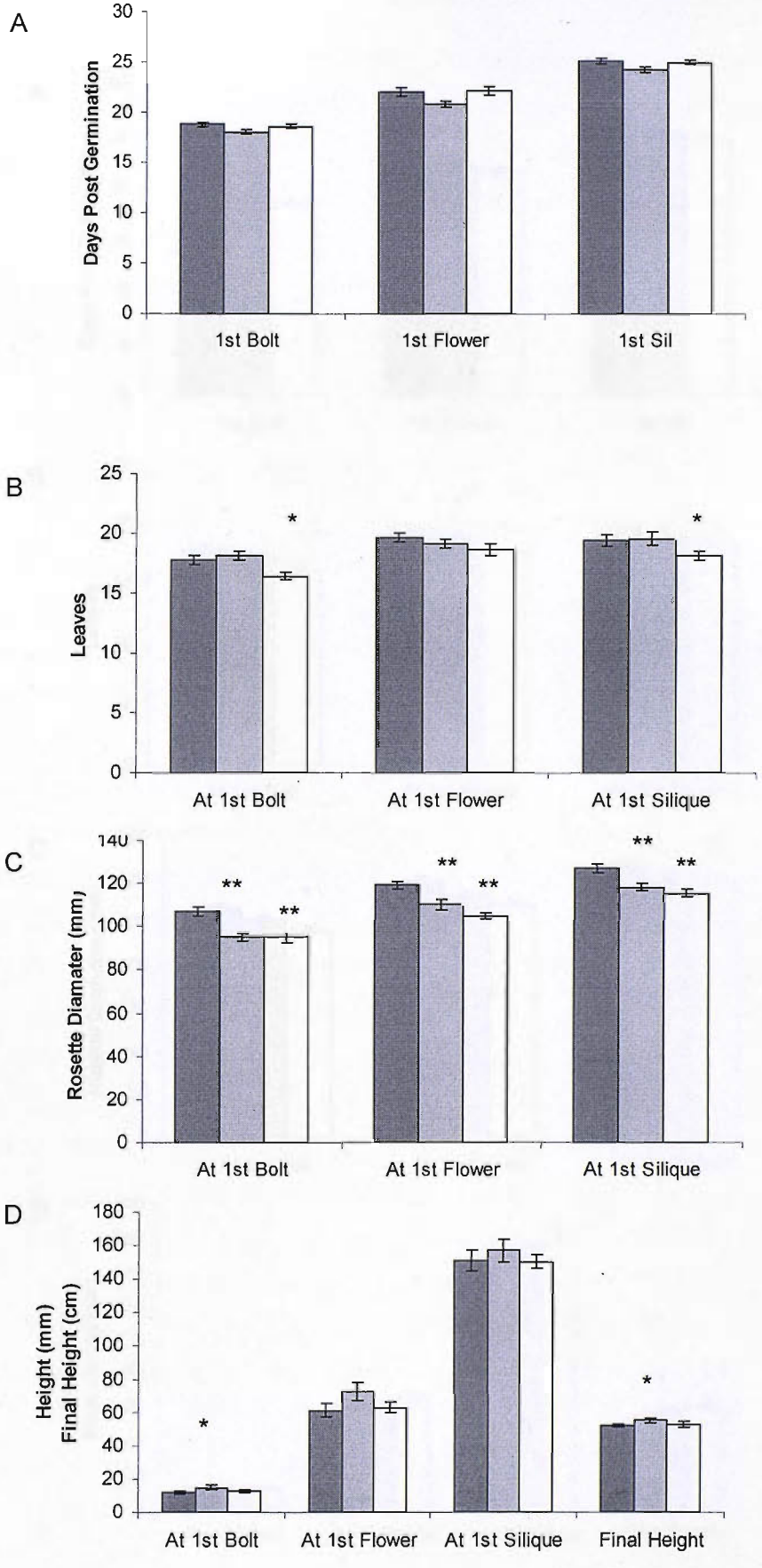


Figure 6.4 Short-day growth phenotypes of ala10-1. Day of 1st bolt, flower or silique (A), number of leaves (B), diameter of rosette (C), and height (D) at each growth stage. Dark grey, Col-8; light grey, segregating-wild-type N534788-3; white, *ala10-1*, all measurements were taken on the day a plant reached a 1st bolt, 1st flower or 1st silique. n = 30. Standard error is shown. *, $P < 0.05$; **, $P < 0.001$.

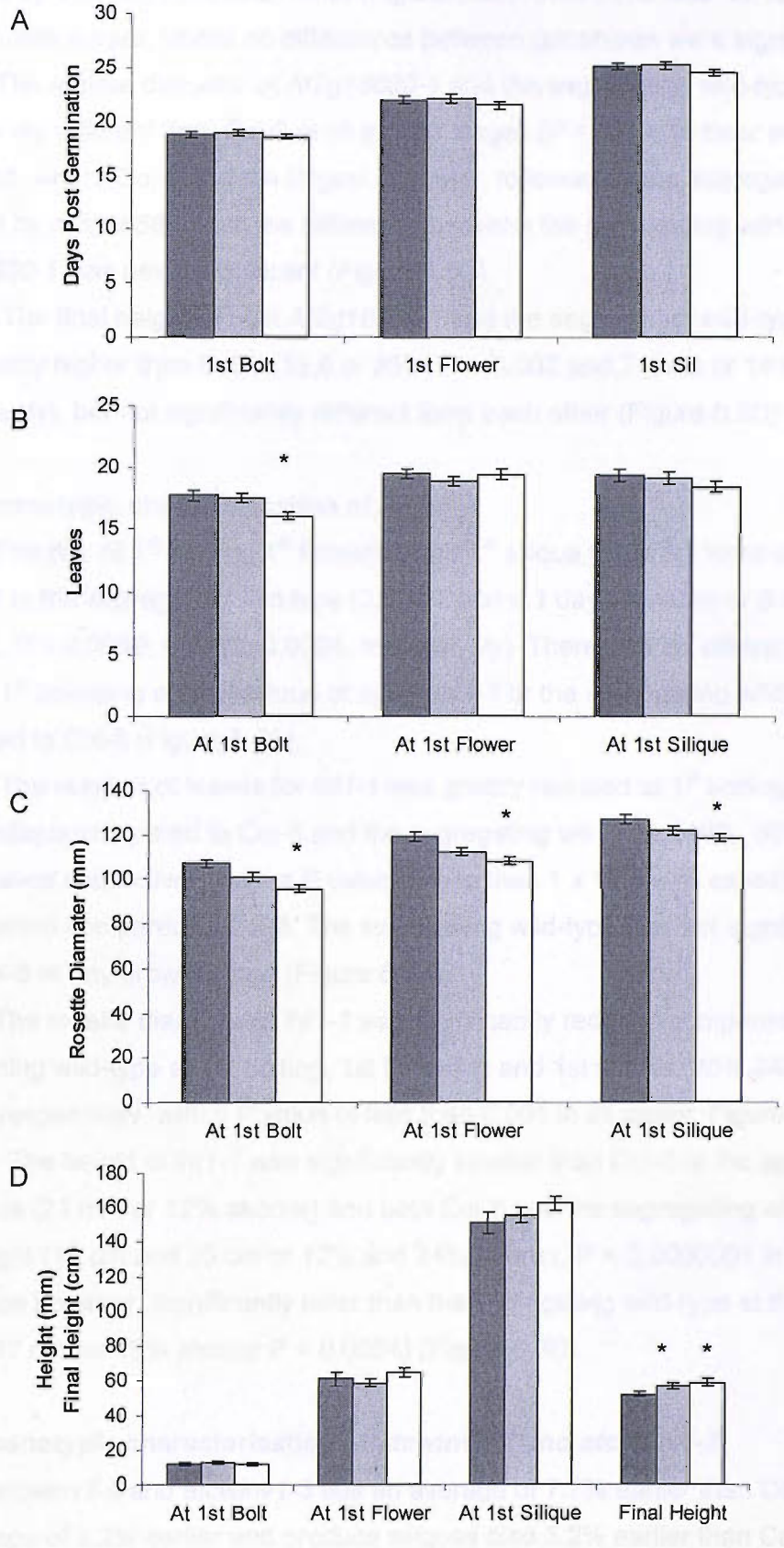


Figure 6.5 Short-day growth phenotypes of *at2g18680-1*. Day of 1st bolt, flower or silique (A), number of leaves (B), diameter of rosette (C), and height (D) of at each growth stage. Dark grey, Col-8; light grey, segregating wild-type (N501089-1); white, *at2g18680-1*. All measurements were taken on the day a plant reached a 1st bolt, 1st flower or 1st silique. n = 30. Standard error is shown. *, $P < 0.05$.

significantly different from each other (Figure 6.5B). This trend was not repeated in the other growth stages, where no differences between genotypes were significant.

The rosette diameter of *At2g18680-1* and the segregating wild-type was significantly different from Col-8 at all growth stages ($P < 0.05$). A clear trend can be observed, where Col-8 had the largest diameter, followed by the segregating wild-type, followed by *at2g18680-1* but the difference between the segregating wild-type and *at2g18680-1* was never significant (Figure 6.5C).

The final height of both *At2g18680-1* and the segregating wild-type was significantly higher than Col-8 (18.0 or 35%, $P = 0.002$ and 7.4 cm or 14%, $P = 0.03$ respectively), but not significantly different form each other (Figure 6.5D).

6.2.6 Phenotypic characterisation of *lht1-1*

The day of 1st bolting, 1st flowering and 1st silique for *lht1-1* were significantly different to the segregating wild type (2.5, 4.1 and 4.1 days delayed or 6.1%, 9.4%, 8.8% delayed, $P = 0.0059$, 0.0002, 0.0004, respectively). There was no difference in day of 1st bolting, 1st flowering and 1st silique of either *lht1-1* or the segregating wild-type when compared to Col-8 (Figure 6.6A).

The number of leaves for *lht1-1* was greatly reduced at 1st bolting, 1st flowering and 1st silique compared to Col-8 and the segregating wild-type (49%, 36% and 48% fewer leaves respectively, with a P value of less than 1×10^{-9} in all cases) with very similar figures when compared to Col-8. The segregating wild-type was not significantly different from Col-8 at any growth stage (Figure 6.6B).

The rosette diameter of *lht1-1* was significantly reduced compared to the segregating wild-type at 1st bolting, 1st flowering and 1st silique (26% 24% and 32% smaller respectively, with a P value of less than 0.001 in all cases. Figure 6.6C).

The height of *lht1-1* was significantly smaller than Col-8 at the appearance of the 1st silique (21 mm or 12% shorter) and both Col-8 and the segregating wild-type at the final height (19 cm and 26 cm or 12% and 24% shorter, $P < 0.0000001$ in both cases). *lht1-1* was however, significantly taller than the segregating wild-type at the day of 1st flower (17 mm or 19% shorter $P = 0.0024$) (Figure 6.6D).

6.2.7 Phenotypic characterisation of *atcwinv1-2 and atcwinv1-3*

atcwinv1-2 and *atcwinv1-3* bolt an average of 7.7% earlier than Col-8 and flower an average of 5.2% earlier and produce siliques also 5.2% earlier than Col-8 plants (Figure 6.7A). All measurements were statistically significant with $P < 0.05$.

The number of leaves at each growth stage was generally reduced in invertase mutants (Figure 6.7B). *atcwinv1-2* had a large decrease in the number of leaves at all growth stages compared to Col-8, with 20.71 % fewer leaves at first bolt, 24.34% fewer leaves at first flower and 19.51% fewer leaves at first silique. *AtcwINV1-1* had a slight

increase in the average number of leaves at first bolt with 2.64% more leaves than Col-8, but is not significantly different from the wild-type. In other repeats, however, this was not the case, and AtcwINV1 had fewer leaves at first bolting, and was significantly different from Col-8. *AtcwINV1-1* shows a significant decrease in the number of leaves at flowering, with 9.54 % fewer leaves with P >0.05. A similar trend was seen at first silique, where there were 9.21 % fewer leaves, but this was not significant in the experiment shown, although it was in other repeats.

The rosette diameter of *AtcwINV1-1* and *atcwinv1-2* was significantly smaller that the Col-8 (P >0.05) at all time points (Figure 6.7C). *AtcwINV1-1* was 15.6%, 18.3% and 7.4% smaller at first bolt, 1st flower and 1st silique respectively. *atcwinv1-2* was 35.6%, 34.1% and 8.0% smaller at first bolt, 1st flower and 1st silique respectively.
The height of *AtcwINV1-1* and atcwinv1-2 was significantly smaller than Col-8 (P >0.05) at flowering, first silique and final height, but not at 1st bolt (Figure 6.7D). *AtcwINV1-1* was 13.88, 17.67, and 13.40 % smaller at flowering, first silique and final height, respectively. *atcwinv1-2* was 4.98, 42.99 and 16.29% smaller at flowering, first silique and final height respectively.

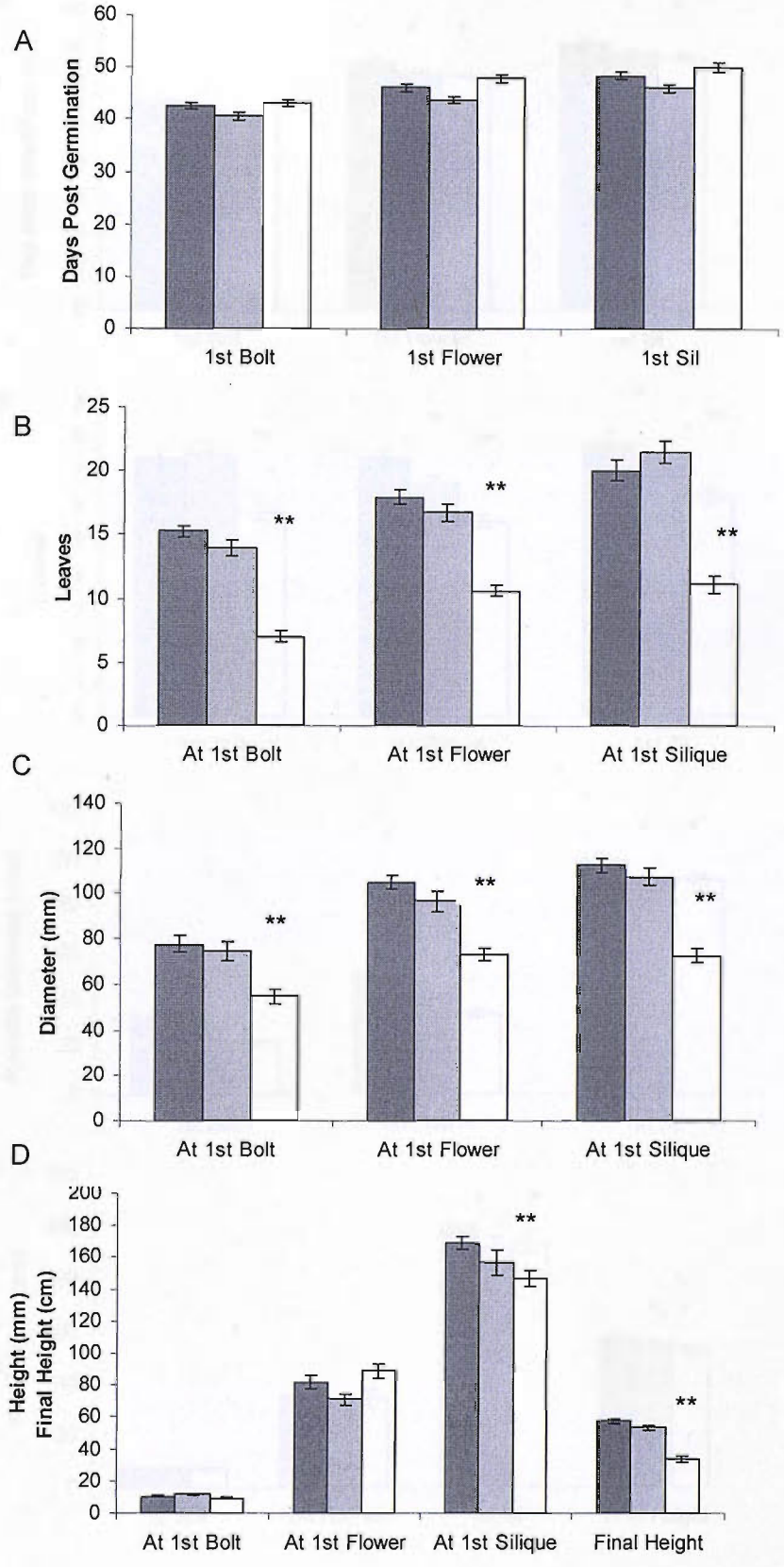


Figure 6.6 Short-day growth phenotypes of *lht1-1*. Day of 1st bolt, flower or silique (A), number of leaves (B), diameter of rosette (C), and height (D) at each growth stage. Dark grey, Col-8; light grey, segregating wild-type (N536871-1); white, *lht1-1*, All measurements were taken on the day a plant reached a 1st bolt, 1st flower or 1st silique. n = 30. Standard error is shown. *, $P < 0.05$; **, $P < 0.001$.

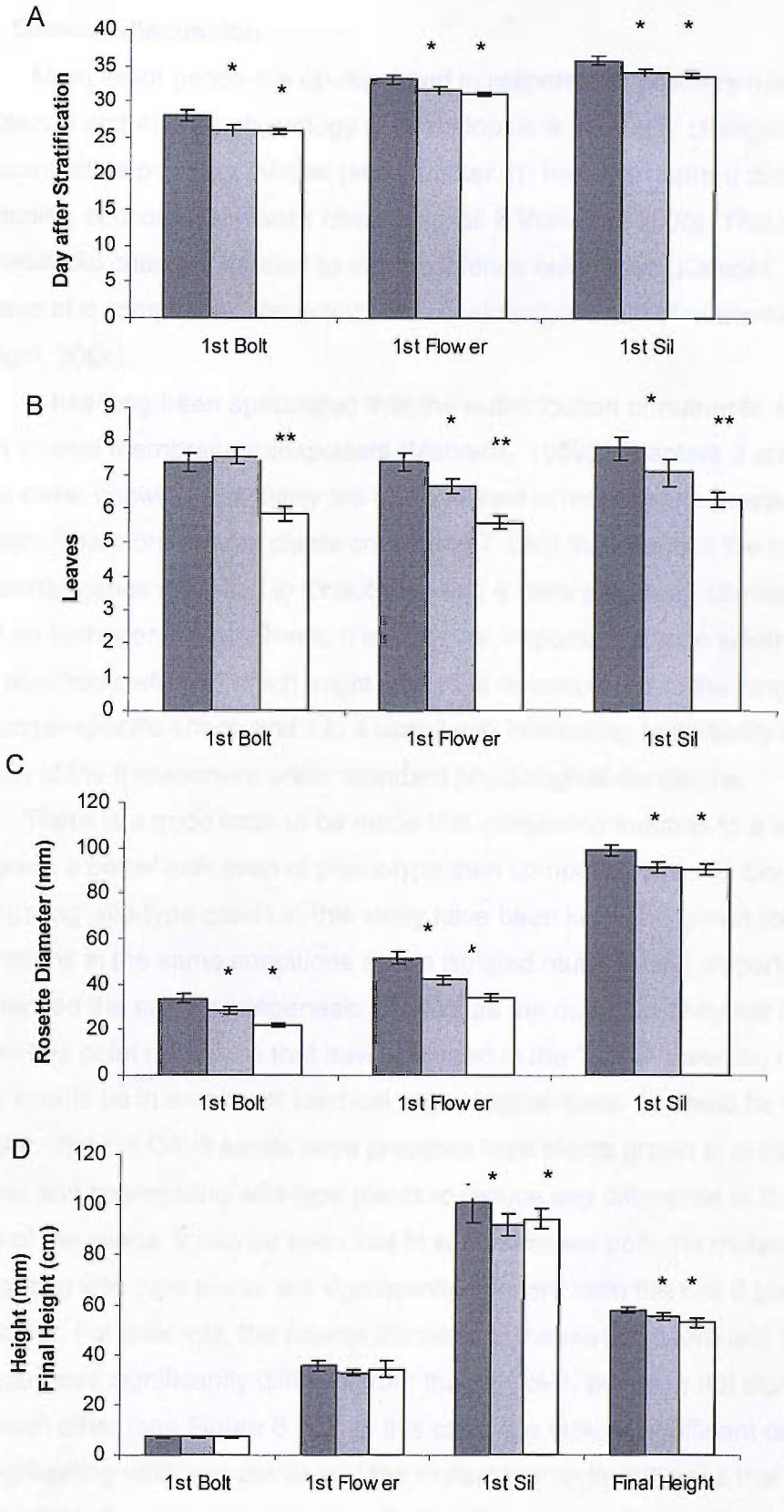


Figure 6.7 Short-day growth phenotypes of *atwinv1-2 and atcwinv1-3.* Day after stratification of 1st bolt, flower and silique (A), number of leaves (B), diameter of rosette (C) and height (D) at each growth stage. Dark grey, Col-8; light grey, *atcwinv1-2*; white *atcwinv1-3*. All measurements were taken on the day a plant reached 1st bolt, 1st flower or 1st silique. Standard error is shown. n= 30. *, $P < 0.05$; **, $P < 0.001$.

6.3 Discussion

6.3.1 General discussion

Many plant genes are up-regulated in response to powdery mildew infection (see Chapters 3 and 4). The physiology of Arabidopsis is markedly changed following infection with compatible powdery mildew (see Chapter 1); both the nutrient distribution and partitioning of photoassimilates changes (Hall & Williams, 2000). This is partially due to the metabolic changes needed to mount defence responses (Katagiri, 2004), but also in the case of a compatible interaction, the considerable drain of nutrients to the pathogen (Katagiri, 2004).

It has long been speculated that the redistribution of nutrients within the plant would involve membrane transporters (Manners, 1989). Chapters 3 and 4 suggest this to be the case, showing that many are up-regulated in response to powdery mildew infection. Therefore mutant plants containing T-DNA insertions in the most up-regulated transporter genes identified in Chapters 3 and 4 were prepared, ultimately, to see the effect on pathogen development. It is however, important to note whether these mutants have pleiotropic effects, which might affect the development of the fungus independently of a fungal-specific effect, and it is a useful and interesting opportunity to the examine function of the transporters under standard physiological conditions.

There is a good case to be made that comparing mutants to a segregating wild-type gives a better indication of phenotype than comparing them to Col-8 wild-type plants. Segregating wild-type plants in this study have been kept and grown for several generations in the same conditions as the isolated mutants and, importantly, have experienced the same mutagenesis protocol as the mutants. They are more likely to contain any point mutations that have occurred in the T-DNA insertion mutant, and the seeds should be in an almost identical physiological state. It should be made clear however, that the Col-8 seeds were prepared from plants grown in similar conditions to mutants and segregating wild-type plants to reduce any difference in the physiological status of the seeds. It can be seen that in several cases both the mutants and the segregating wild-type plants are significantly different from the Col-8 plants, but not from each other. For example, the rosette diameter of the *ala10* mutant and the segregating wild type were significantly different from that of Col-8, but were not significantly different from each other (see Figure 6.4C). In this case, the lack of significant differences between the segregating wild-type plants and the mutants strongly indicates that the absence of *ALA10* cDNA does not affect the rosette diameter of Arabidopsis. The situation is slightly less clear when mutant lines are suspected as having more than one independent T-DNA insertion. This is because a segregating wild-type plant may also carry the same independent T-DNA insertion, which may be either hetero- or homozygous. If a seed line contains more than 1 T-DNA insertion, and a significant difference in growth and

development between Col-8 and the mutant is seen, the mutant line would need backcrossing to eliminate the independent T-DNA.

An important point regarding the significance of differences in measurements is that if the threshold of significance is set at 0.05 (or a 1 in 20 chance that observed differences are due to chance alone), in any given set of 20 measurements with a P value of around 0.05, one should appear significant just by chance. In this study 56 measurements were deemed significant (although many at P values lower than 0.05). Thus any individual differences deemed significant in one experiment but not in a repeat of the same experiment are not necessarily experimental errors, but could easily be due entirely to chance.

6.3.2 *lht1* insertional mutants

lht1 mutants have a profoundly different phenotype to wild type plants with delayed bolting, flowering and appearance of 1st silique, with fewer leaves, smaller rosette diameter and shorter height at all measured growth stages compared to Col-8 and segregating wild-type mutants.

During the course of this study, two papers were published regarding mutants in *LHT1,* one of which was published shortly before measurements of growth and development were to be performed. This study adds to the information known about *LHT1*. Detailed observations of the mutant phenotype were not made in either of the two existing studies, although the existence of a phenotype was noted (Hirner *et al.*, 2006). It was described briefly that, at 24 days post germination and beyond (roughly at the point of bolting) *lht1* mutants "displayed growth inhibition and older leaves became yellow earlier than for wild-type plants" (Hirner *et al.*, 2006). It was also shown that *lht1* mutants show significantly reduced dry weights by and after 24 days post germination. Another study observed statistically significant differences in growth between *lht1* mutants and Col-0 only after 31 days (Svennerstam *et al.*, 2007).

The data that this present study contributes are more detailed in the number of metrics measured and the number of developmental stages over which they were taken. This should allow future studies in the physiological function of *LHT1* to be interpreted more accurately, as other data can be related more precisely to the effect of *lht1* insertional mutants on whole plant growth.

It seems that *LHT1* reduces growth of Arabidopsis on normal soil probably by reduced uptake of amino acids from the apoplast into the cytoplasm of mesophyll cells not by reduced uptake of amino acids from the soil (Hirner *et al.*, 2006). This was indicated by higher levels of amino acids in the apoplast of *lht1* mutants. In plants, nitrogen must cycle from the roots to nitrogen sinks via the xylem, and importantly, back again via the phloem (Williams & Miller, 2001). This process provides roots with nitrogen, signals nitrogen use by sinks and helps drive solute flow (Hirner *et al.*, 2006). Thus it is expected that inhibition

of uptake from the apoplast reduces cell-to-cell transport and the cycling of amino acids within the plant. A defect in amino acid cycling is expected to limit nitrogen use and thus growth (Hirner *et al.*, 2006).

6.3.3 *atcwinv1* insertional mutants

The *atcwinv1* mutants display a small but significant phenotype. Both *atcwinv1* mutants bolt, flower and produce the 1st silique earlier than Col-8 and both mutants display fewer leaves and have a smaller rosette diameter at all growth stages. This is contrary to previously published findings which stated that "inactivation of the *AtcwINV1* gene produced no difference in growth phenotype (either during the vegetative or reproductive phases)" (Quilliam *et al.*, 2006). In that study *AtcwINV1* was also inactivated by a T-DNA insertion (Line 258_A01 from the SAIL insertion program) and the inactive state was confirmed by real-time PCR.

Studies in other plants had indicated that cell-wall invertases were vital in photoassimilate partitioning and that mutants might produce an observable phenotype: anti-sense repression of cell-wall invertase in maize led to infertility (Goetz *et al.*, 2001) and anti-sense repression of cell-wall invertase in carrots caused massive pleiotropic effects (Tang *et al.*, 1999). It should be noted however, that cultivated plants such as carrots and maize often have massively different photoassimilate partitioning compared to the virtually wild Arabidopsis. Interestingly, over-expression of a yeast invertase fused to either an apoplastic or cytoplasmic targeting sequence and expressed specifically in meristems, gives rise to phenotypic differences (Heyer *et al.*, 2004). Apoplastic location of the invertase (making it akin to cell-wall invertase) accelerated floral development and increased seed yield, while location in the cytoplasm had the opposite effect (Heyer *et al.*, 2004). The proposed explanation was that the invertase targeted to the apoplast increased sink strength, (which is thought to cause the shoot atypical meristem to undergo floral transition), while expression in the cytoplasm caused a decrease in sink strength (Heyer *et al.*, 2004). It was noted that the accelerated floral development only occurred when apoplastic targeted invertase plants were grown in long-day conditions (Heyer *et al.*, 2004). Results not shown in this study (due to insufficient repetition) gave a preliminary indication that the differences in growth and development measured here were more pronounced when a*tcwinv1* mutants were grown under long-day conditions.

The most likely explanation for statistically significant differences between two a*tcwinv1* mutants and wild-type plants found here but not observed before (Quilliam *et al.*, 2006) is that these differences are only observable under detailed investigation. No obvious differences between the cell-wall invertases and the Col-8 wild type can be observed by eye. The level of detail involved in the original study was not clear (Quilliam *et al.*, 2006).

The probable explanation for why over-expression of invertase in the cytoplasm accelerates flowering, while inactivation through a T-DNA insertion has a similar effect, is that in the over-expression study, the invertase was placed under the control of a meristem specific promoter, while cell-wall invertase inactivation in the mutants used in this study will be plant-wide. The potential changing of sink strength in the leaves may predominate over changes elsewhere, causing any effect on flower sink strength to be negated. This might make any phenotype observed primarily related to sink strengths in leaves or other organs, rather than flowers.

6.3.4 *glr.4* insertional mutants

glr1.4 insertional mutants show delayed production of the 1st bolt, 1st flower and 1st silique and show a larger number of leaves and a significantly larger rosette diameter at bolting compared to wild-type plants, although this effect was not seen at other growth stages. There is very limited evidence on the role of glutamate receptors in plants, other than they may be involved in the broad categories of nutrient uptake, signalling and intra-plant transport (Davenport, 2002). This study has indicated that *glr1.4* is up-regulated in response to powdery mildew infection and that the loss of g*lr1.4* causes a mild growth reduction.

6.3.5 Other T-DNA insertional mutants

The *aca12-1* mutant and segregating wild-type were unique in this study, in that there were no significantly different measurements between them or the Col-8 wild-type, at any time point. While *ala10-1* did show some differences to the Col-8 wild type, so did the segregating wild-type. This leads to the conclusion that *ALA10* does not affect the growth and the development of the plant significantly during standard growth conditions.

ptr3-3 showed few differences compared to either control. Differences in rosette diameter between *ptr3-3* and the segregating wild-type observed in the original experiment were not repeatable, although a significant difference between the numbers of leaves at first bolt was confirmed. This led to the conclusion that *PTR3* has no major effect on plant growth and development under standard conditions, with the exception of one repeatable measurement. This implies that *PTR3* could be involved in a highly specific part of plant growth and development around the development of the bolt. There is no evidence currently to suggest that the expression of *PTR3* is specific to a particular time point at the level of whole organs but it could be expressed at a specific time in a group of cells. It is also possible that the effects of *PTR3* loss on the whole plant can normally be overcome by other peptide transporters, but that at a specific point in time (perhaps due to a very specific need for peptides) cannot be overcome. That this decrease in the number of leaves does not affect later growth, indicates that other genes do not just compensate

for *PTR3* loss, but can overcome the negative effects quickly to produce plants which do not continue to have fewer leaves through the rest of their development.

At2g18680 mutants showed a significant decrease in the number of leaves at 1st bolt, as well as a decrease in rosette diameter compared to Col-8. However, the rosette diameter was not different to the segregating wild-type, and the segregating wild-type was significantly different to Col-8. This indicates that A*t2g18680* does not influence rosette diameter, but that another genetic (or epigenetic) difference between the mutant seed line and Col-8 does. Chapter 5 indicated that there may be more than one insert in this seed line, and it is possible that this insert is located in the segregating wild-type plant. This leaves the possibility that a second insert in an unknown gene is causing the phenotype. Backcrossing of this line could be performed to try and remove a secondary insert, or the location of a potential second insert could be determined to investigate whether it is likely to lead to the loss of function for a gene product, and if that is the case, further investigations could reveal if it affected plant growth.

This study has shown that while some of the mutants examined have obvious phenotypes, many have very limited or no obvious phenotypes, and the question remains as to why this might be the case. Although functional redundancy is one obvious answer it also appears that many phenotypes are conditional (Bouche & Bouchez, 2001). The genes investigated here were shown to be up-regulated in response to powdery mildew, and this chapter has investigated the growth and development of Arabidopsis under standard growth conditions. However these mutants may show different phenotypes when challenged with the pathogen that causes their up-regulation.

Chapter 7

Fungal growth on transporter mutants

7.1 Introduction

Pathogen growth must be monitored accurately if differences in the growth between treatments and/or genetic backgrounds are to be investigated. Several previous studies have looked at the growth of powdery mildew on Arabidopsis, and a wide variety of methods have been used to measure growth. At least nine different variations on methods to measure fungal growth have been used relatively recently (Adam & Somerville, 1996; Adam *et al.*, 1999; Wilson *et al.*, 2001; Vogel *et al.*, 2002; Maleck *et al.*, 2002; Vogel *et al.*, 2004; Liu *et al.*, 2005; Ramonell *et al.*, 2005; Stein *et al.*, 2006; Moriura *et al.*, 2006). Broadly speaking, they fit into two categories: firstly, disease severity ratings, where severity is visually assessed into discrete categories; secondly, more quantitative measurements of individual powdery mildew features. Visual assessments are often based on macroscopic symptoms such as the percentage of the leaf covered by powdery mildew or the number of infected leaves, but they can also be performed on microscope observations such as the number of colonies producing conidia. Quantitative measures are normally based on features such as hyphal length or conidia counts.

7.1.1 *npr1-1* mutants

The *npr1-1* mutant used in this chapter is a non-producer of *PR1* (Cao *et al.*, 1994). As discussed in Chapter 1 NPR1 interacts with transcription factors in defence response pathways. *npr1-1* mutants do not exhibit SAR (Dong, 2004) and are more susceptible to *E. cichoracearum* (Xiao *et al.*, 2005). That *npr1-1* mutants have been shown to allow more growth of *E. cichoracearum* on Arabidopsis (Xiao *et al.*, 2005) makes them useful a control plant when using new techniques to measure fungal growth. This Chapter demonstrates the development of such a technique and uses *npr1-1* mutants as a known effect seed line in order to confirm the accuracy of the technique.

7.1.2 Chapter aims

The aim of this chapter is to measure fungal growth and development on the insertional mutants derived in Chapter 5, using an accurate and precise method. The mutants identified all lack full-length cDNA for genes which are highly regulated in response to powdery mildew infection. By observing the effect of these insertional mutants, it is hoped that an insight will be gained into the function of these genes and into the development of biotrophy of Arabidopsis.

7.2 Results

7.2.1 Improvements in fluorescent microscopy

During the course of this project, equipment that was increasingly suitable for high-throughput measurement of powdery mildew colonies became available. Figure 7.1 shows visualisations of fungal colonies on Arabidopsis that were made using the various technologies. These advances have facilitated the development of techniques for measuring fungal growth using image analysis; techniques which were previously too impractical for use. All of these technologies use images of fluorescently stained of fungal colonies (see Materials and Methods). At the start of the project, a high specification epifluorescent microscope containing a highly sensitive black and white camera was used to visualise fungal colonies. The high cost of using this equipment and slow throughput of images meant that the number of images, and thus the number of measurements from individual colonies that could be taken, was limited. Although the camera attached was highly sensitive, being black and white it was not able to record the most striking aspects of fluorescent staining; that the colonies are bright green, and the surroundings are relatively unstained or even red (Figure 7.1A). Subsequently, a lower specification epifluorescent microscope became available, with lower grade-optics and a less sensitive, but colour, camera attached. The images that resulted (Figure 7.1B) were sufficient for making measurements of fungal colonies, largely due to the greater contrast resulting from the use of a colour camera. The lower cost of using this equipment allowed the equipment to be used for longer, and more measurements to be taken. The usefulness of this equipment was limited, however, by the time consuming nature of obtaining images. The final development in microscopy equipment was the availability of a fluorescent dissecting microscope, with a standard consumer digital camera attached through the eyepiece. The quality of the optics and digital camera were more than sufficient to visualise individual colonies on the leaf surface and take measurements from them. This allowed more rapid collection of data, albeit with a loss in image quality (Figure 7.1C). A real benefit of using this system is that it produces relatively high resolution, but low magnification images, and it is able to photograph the entire surface of an infected Arabidopsis leaf in sufficient detail to take measurements from in just a few images (Figure 7.1D).

7.2.2 Creation of a microscope-based fungal growth assay

Initially a PCR based approach was investigated as a method of quantifying fungal growth. Other studies have used similar methods (Mercado-Blanco *et al.*, 2003; Alkan *et al.*, 2004; Stummer *et al.*, 2006), where real-time PCR was used to quantify the amount of fungal DNA present. Relating the severity of infection to fungal DNA levels has significant difficulties however. Measurements of the absolute amount of fungal DNA per leaf or per

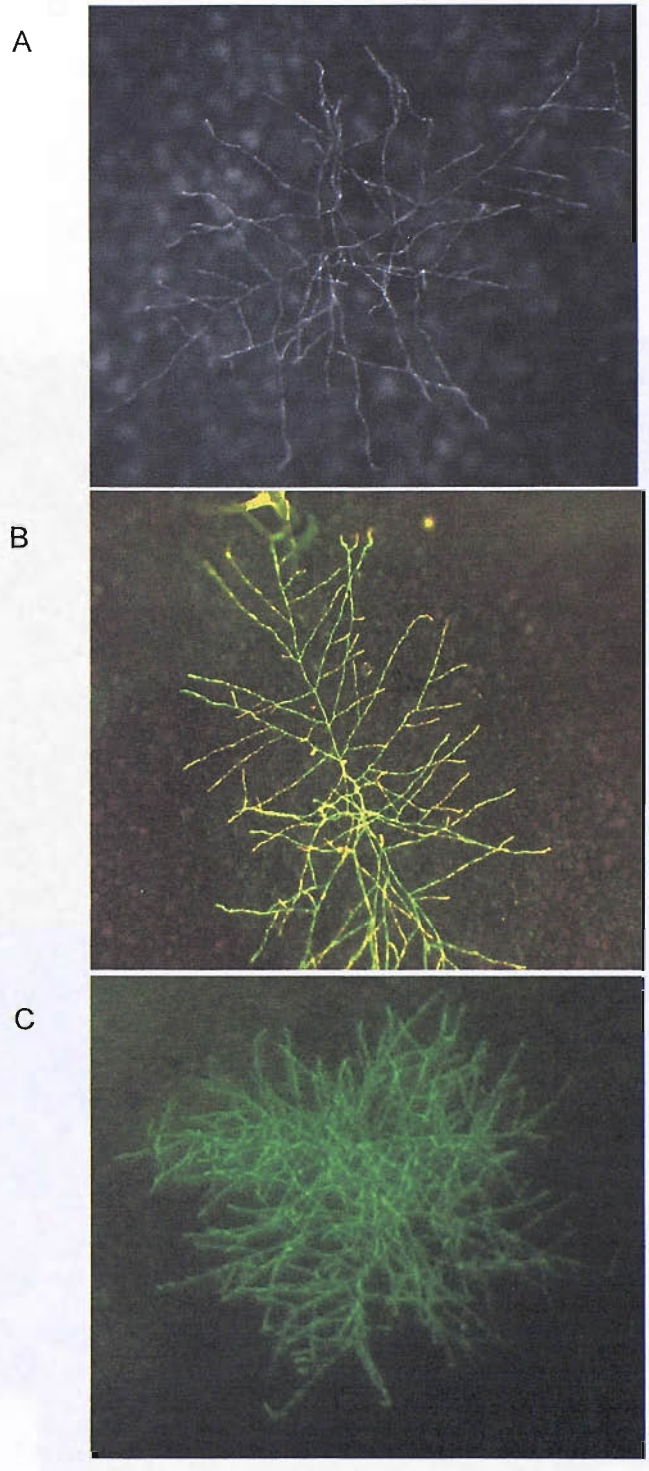


Figure 7.1 Developments in visualisation technologies. A. An image of an *E. cichoracearum* colony, using a highly sensitive black and white camera. B. An image of an *E. cichoracearum* colony, using a lower specification epifluorescent microscope with a colour camera. C. An image of an *E. cichoracearum* colony, using a fluorescent dissecting microscope, taken with a conventional digital camera with a specially adapted eyepiece. D. Images from a fluorescent dissecting microscope are overlaid to show the entire surface of an Arabidopsis leaf.

D

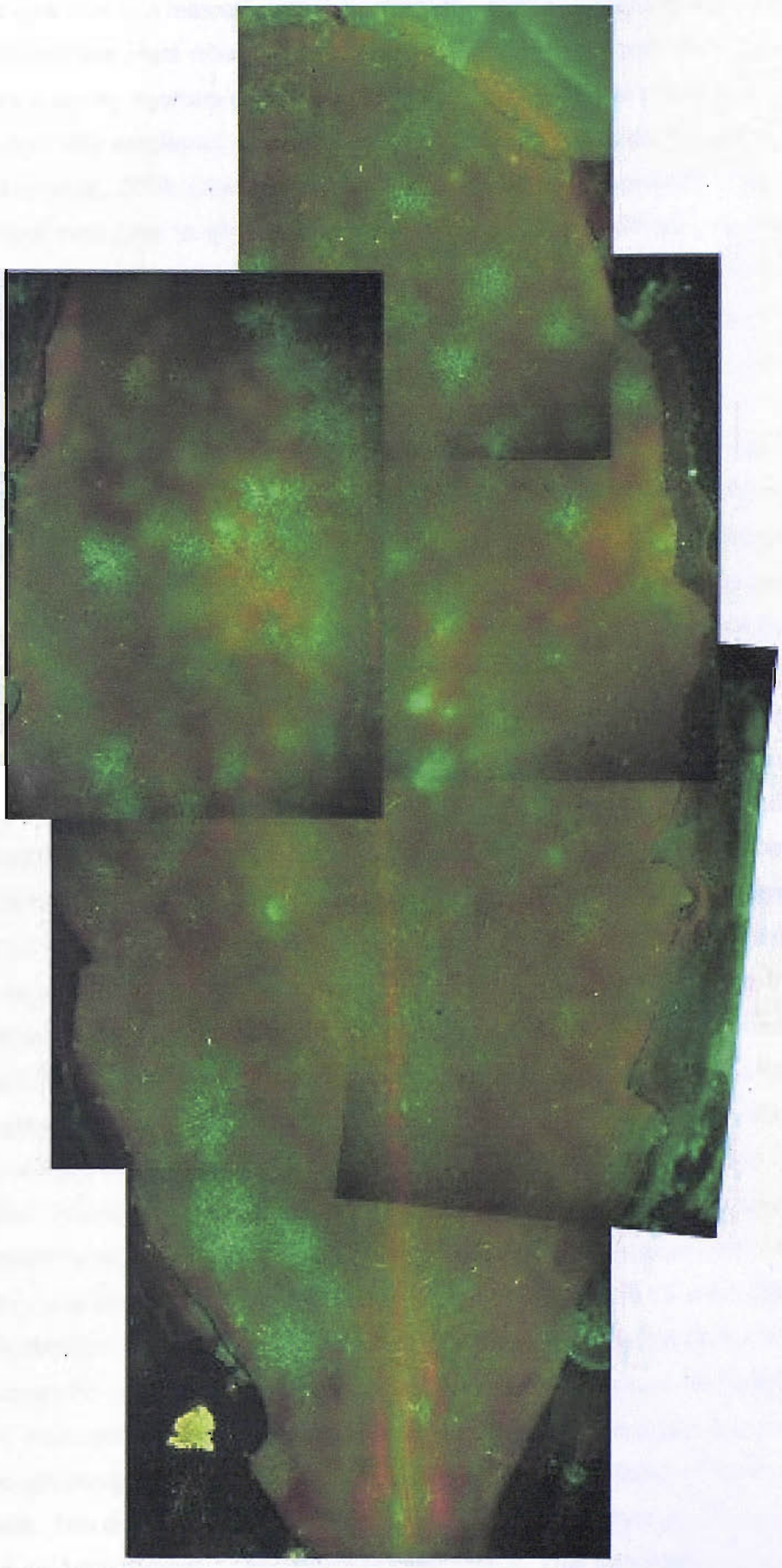


Figure 7.1 continued

plant are not necessarily valid as not all DNA extractions are equally efficient. If 5% less DNA was extracted from one plant/fungal sample compared to an identically infected sample, (perhaps due to a less efficient extraction) the absolute amount of fungal DNA extracted from the first plant would be 5% less than that of the second. This could only be corrected by extremely rigorous calibrations of the extraction and processing techniques. The method normally employed, and used in several previous studies (Mercado-Blanco *et al.*, 2003; Alkan *et al.*, 2004; Stummer *et al.*, 2006), compared the amount of fungal DNA to the amount of host DNA in order to counter different extraction efficiencies. However this was not suitable in this situation. Unlike the situations in previous studies, the host plant (Arabidopsis) is itself growing fast during the course of the pathogen infection. The growth in the amount of Arabidopsis DNA during the course of infection may actually exceed the increase in fungal DNA during the same period. When comparing the proportion of fungal DNA to Arabidopsis DNA in the above situation, a decrease in the amount of fungal DNA compared to the amount of Arabidopsis DNA would be observed. This very real possibility would make it difficult to interpret data without extremely rigorous and time consuming calibrations. In order to overcome the limitations of DNA based assays, a protocol based on the visual measurements of multiple fungal colonies was developed.

Initially, fungal growth was measured by determining the total hyphal area of each colony (Figure 7.2). However, this was extremely time consuming and therefore only a limited number of measurements could be made, which made the technique unsuitable for use in large experiments. Measurements of total hyphal length also proved to be time consuming to make in a sufficient number to prove that small differences in fungal development between host genotypes were significant. Colony diameter was also considered as a measurement of fungal growth, but was disregarded as it is extremely difficult to measure, due to the high asymmetry of most powdery mildew colonies. Instead, the distance from the initial spore of a powdery mildew colony to the tip of the longest hyphal branch was used as a measure of fungal growth. While this measurement might have a high level of variation, a high degree of replication could easily be used to counter this weakness. Two techniques were investigated and compared: 1) measurement of the maximum distance of hypha from the originating spore; or 2) an assessment of individual colony size on a scale of 1-5. In outline (see Materials and Methods for a full description of the method used) the measurement of the maximum distance of hypha from the originating spore (or alternately put, the longest fungal hypha) requires an image of each colony to be measured. The originating spore can normally be easily identified in the image, although occasionally the location of the spore has to be deduced by the pattern of hyphal growth. The distance between the spore and the longest hypha can be measured using image analysis software using line measuring tools. This involves creating a series of straight lines along the image of the hypha, from the spore to the tip. The length of

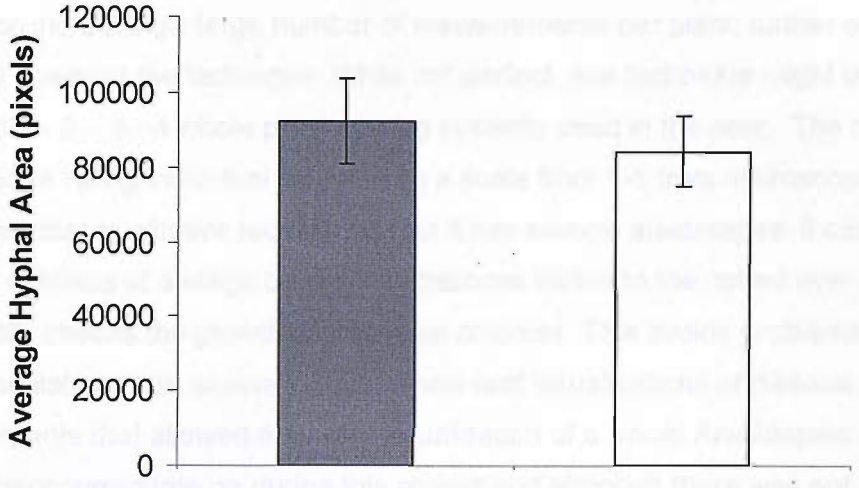


Figure 7.2 Measurement of total hyphal area on Col-8 and *npr1-1*. Dark grey, *npr1-1*; clear, Col-8. The average hyphal area as measured by fluorescent microscopy for *npr1-1* and Col-8. ImageJ was used to measure the area of each colony. n = 10.

these lines (initially in pixels) is calculated using image analysis software, and can be calibrated to nm actual distance. In cases where it is not entirely clear which hyphal tip is the farthest from the originating spore by eye, all hyphae that might be the longest hyphae would be measured and the length of only the longest used. To avoid any unintentional sampling bias in colony selection (such as taking photos only of colonies that were easy to visualise), a desired or target number of colonies to measure per leaf would be selected before the start of the experiment. That number of colonies per leaf would then be measured, with colony selection working in strict order from the leaf tip to the petiole. Some leaves may have less then the target number of colonies and thus not all leaves will have the same number of colony measurements taken from them.

As more appropriate microscope equipment became available, it became possible to take an increasingly large number of measurements per plant, further enhancing the potential power of the technique. While not perfect, this technique might be superior to the standard 1 - 2 – 3 - 4 whole plant scoring systems used in the past. The other potential technique of rating individual colonies on a scale from 1-5 from microscope images is similar to other qualitative techniques, but it has several advantages: it can be used to observe colonies at a stage before they become visible to the naked eye; and it specifically checks the growth of individual colonies. This avoids problems where unequal initial inoculations can severely effect whole-leaf visualisations of disease severity. Developments that allowed the rapid visualisation of a whole Arabidopsis leaf at high resolution occurred late on during this project and although there was not time to use this approach more thoroughly, it would facilitate a rapid and efficient colony counting system on a large number of plants.

The quantitative analysis itself takes approximately 3-4 times longer to perform than the qualitative analysis, but as the majority of time spent in obtaining results is actually in obtaining the fluorescent images, not in measuring them, thus increase in overall time investment is relatively small.

7.2.3 Validation of a microscope-based fungal growth assay

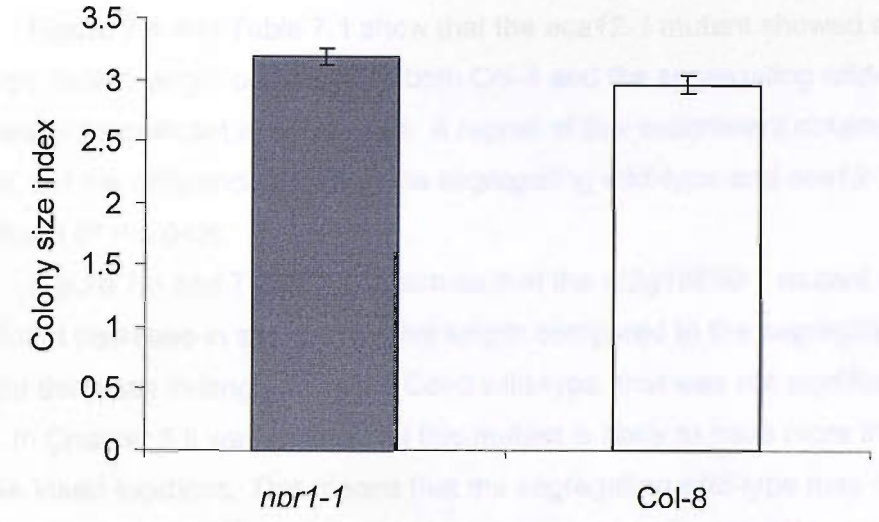
To investigate whether this approach would be valid, plants with a known defect in susceptibility to powdery mildew infection (*npr1-1* mutants) were compared to Col-8. Figure 7.3A shows the measurement of maximum hyphal length on 5 dpi *E. cichoracearum* infected *npr1-1* and Col-8 plants, while 7.3B shows the results of a 5 level qualitative test on the same microscopy study (see Chapter 2 for details). It can be seen that, as expected, both methods show that at 120 hpi, the average size of an *E. cichoracearum* colony is larger on *npr1-1* than on Col-8. This difference is greatest when using the quantitative methodology, which shows that the average maximum hyphal distance on *npr1-1* is 20% larger than on Col-8, while the qualitative test shows only a 7.4% increase on *npr1-1*. The P-value for the quantitative study ($P = 4.68 \times 10^{-10}$) was far

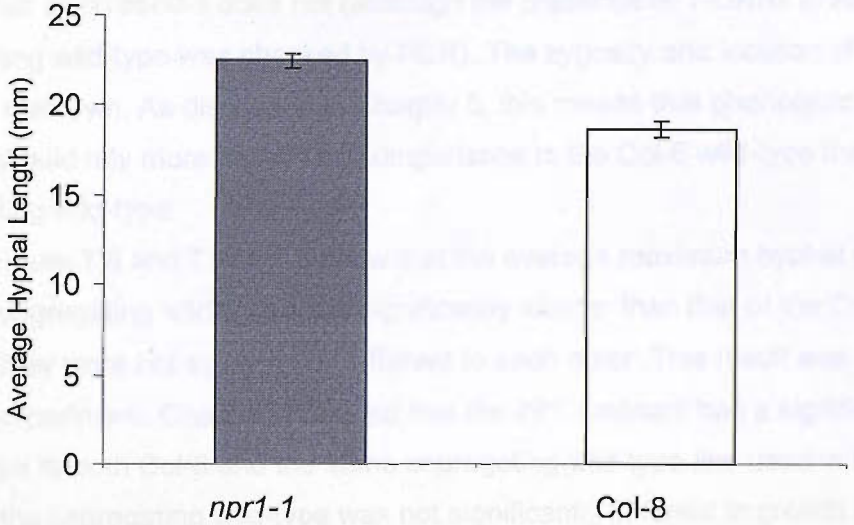
lower than that in the qualitative study (P = 0.03). This result was repeated in a replicate of this experiment, although the average length of the longest hyphae on both genotypes was almost 10% greater. This might be due to growth conditions that were not controlled, such as humidity, or the vitality of the original spores.

Figure 7.3C shows the measurement of maximum hyphal length over time, on a Col-8 plant. This shows that during the period measured, powdery mildew grew fastest between 96 and 144 hpi. After approximately 6 days post infection (144 hpi), the measurement of fungal length becomes unreliable as the colonies start to become confluent. It becomes very difficult to determine which hypha belongs to which colony and the distance between colonies may well alter the size of colonies, due to the availability of nutrients/ response of plant defences. The large differences in P-Values showed that the quantitative method was far more useful and would be far more sensitive in determining differences in powdery mildew growth on different genotypes. The method should also provide greater confidence in any given result than a qualitative result.

7.2.4 Fungal growth on knockout mutants

ACA12, *GLR1.4*, *PTR3*, *ALA10*, *LHT1*, *At2g18680* and *AtcwINV1* have all been shown to be up-regulated in response to powdery mildew infection. T-DNA insertion mutants have been identified in each of the genes (Chapter 5) and the majority produced no phenotype under standard growth conditions (Chapter 6). The mutants are likely to greatly reduce the amount of, or possibly knock-out the protein that was encoded by the genes. The growth of fungal colonies was monitored on all of these mutants, using the method described and validated above, i.e. the average length of the longest hyphae measured on infected plants. Ten leaves (each from a separate plant), per genotype were investigated, and only the first fifteen colonies from the tip of each leaf were measured. This theoretically makes 150 measurements per genotype, but as described in 7.2.2, in practice there were not always15 colonies growing on every leaf examined. Each experiment was conducted twice and each of the mutants was tested in turn and this was repeated, (rather than one mutant tested twice and then another mutant tested twice, etc.). The average number of measurements per genotype was 125 for the first run of measurements and 130 for the second. The results for these mutants were highly repeatable, with the trends being very similar. As described in 2.2.3, although the trends between the genotypes remained the same, the variation in actual magnitude of fugal hyphal length was approximately 10%. Given this, the P-value for the differences between genotypes did change between experiments. In some cases this tipped a non-significant result into significance (at the $P < 0.05$ level) and vice versa. Where this is the case, it has been clearly indicated in the text, but unless otherwise stated the figures display the results of the first experiment.





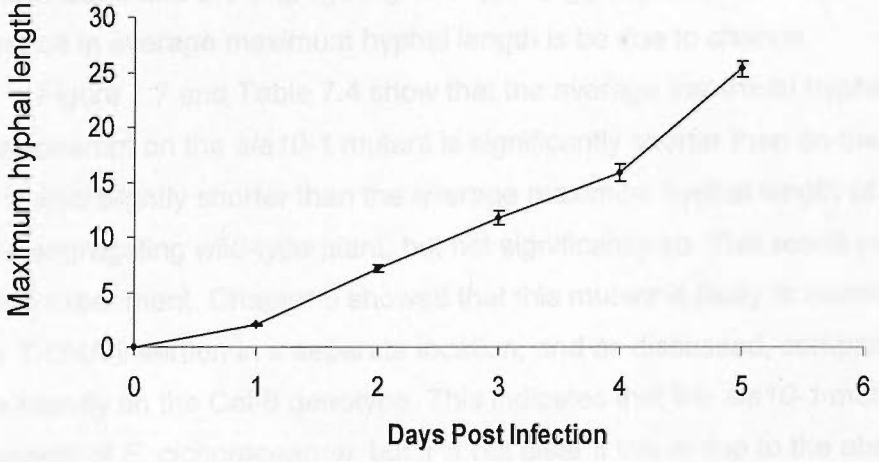


Figure 7.3 Measurements of the *E. cichoracearum* infection progress. For A and B: Dark grey, *npr1-1*; clear, Col-8. A. The average hyphal length as measured by fluorescent microscopy for *npr1-1* and Col-8 at 5 dpi. B. The average of qualitative rankings of *E. cichoracearum* infection severity per colony on a 1-5 scale for *npr1-1* and Col-8. C. The average hyphal length of *E. cichoracearum* colonies growing on Col-8 over time. For A and B: n =195 and 159 for *npr1-1* and Col-8 respectively. For C: n = 90 for each time point. Standard error is shown is all cases.

Figure 7.4 and Table 7.1 show that the *aca12-1* mutant showed a slightly reduced average hyphal length compared to both Col-8 and the segregating wild-type plant, but this was not significant in either case. A repeat of this experiment obtained the same trends, but the difference between the segregating wild-type and *aca12-1* results was just significant ($P = 0.049$).

Figure 7.5 and Table 7.2 illustrates that the a*t2g18680-1* mutant showed a significant decrease in average hyphal length compared to the segregating wild-type, and a slight decrease in length from the Col-8 wild-type, that was not significant at the $P < 0.05$ level. In Chapter 5 it was shown that this mutant is likely to have more than two2 separate T-DNA insert locations. This means that the segregating wild-type may contain T-DNA inserts that *at2g18680-1* does not (although the presence of T-DNAs in *At2g18680* of the segregating wild-type was checked by PCR). The zygosity and location of such inserts remains unknown. As discussed in Chapter 5, this means that phenotypic analysis of this mutant should rely more heavily on comparisons to the Col-8 wild-type than the segregating wild-type.

Figure 7.6 and Table 7.3 show that the average maximum hyphal lengths for *lht1-1* and the segregating wild-type were significantly shorter than that of the Col-8 wild-type, but that they were not significantly different to each other. This result was confirmed in a second experiment. Chapter 5 showed that the *lht1-1* mutant has a significantly different phenotype to both Col-8 and the same segregating wild-type line used in this experiment, but that the segregating wild-type was not significantly different in growth phenotype to Col-8. The low P value ($P < 0.0001$) for the difference in average maximum hyphal length between Col-8 and the segregating wild-type suggests that it is very unlikely that the difference in average maximum hyphal length is be due to chance.

Figure 7.7 and Table 7.4 show that the average maximum hyphal length of *E. cichoracearum* on the *ala10-1* mutant is significantly shorter than on the Col-8 wild-type. This is also slightly shorter than the average maximum hyphal length of *E. cichoracearum* on the segregating wild-type plant, but not significantly so. This result was confirmed in a second experiment. Chapter 5 showed that this mutant is likely to contain at least one other T-DNA insertion in a separate location, and as discussed, comparisons should rely more heavily on the Col-8 genotype. This indicates that the *ala10-1* mutant does retard the growth of *E. cichoracearum*, but it is not clear if this is due to the absence of *ALA10* or to the presence of a T-DNA elsewhere in the genome of the *ala10-1* mutant. In order to determine this, this plant would need to be backcrossed with a wild-type plant, preferably three times. There was not time for this in this study, but it would be useful to do this in the future.

Figure 7.8 and Table 7.5 shows that the average maximum hyphal length of *E. cichoracearum* is significantly longer on the *glr1.4-1* mutant than on the segregating wild-type plant and significantly shorter than on the Col-8 mutant, and that the segregating

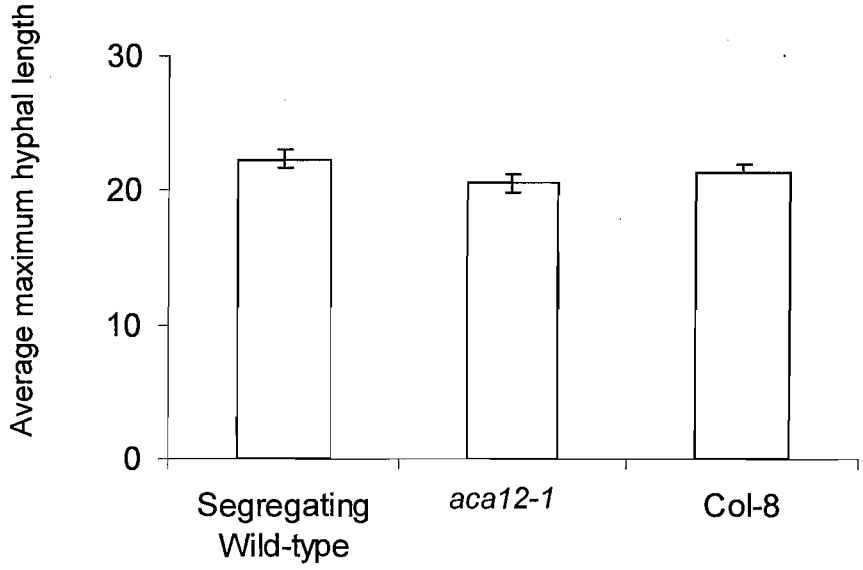


Figure 7.4 The average length of the longest fungal hypha on the *aca12-1* mutant as compared to wild-type plants. The centre of the colony was determined by eye, and length of the longest hypha was determined by measuring the length of a series of straight lines to the end of the longest hypha, using the ImageJ program. Segregating wild-type n = 110 from 10 plants, *aca12-1* n = 112 from 10 plants, Col-8 n = 123 from 10 plants.

Table 7.1 Table of significances using an unpaired T-test for the measurements displayed in Figure 7.3. Although the average length of the longest hypha was slightly shorter for aca12-1 than for either of the other genotypes used, this was not significant at the $P < 0.05$ level.

	Segregating Wild-type	*aca12-1*	Col-8
Segregating Wild-type		0.08	0.34
aca12-1	0.08		0.31
Col-8	0.34	0.31	

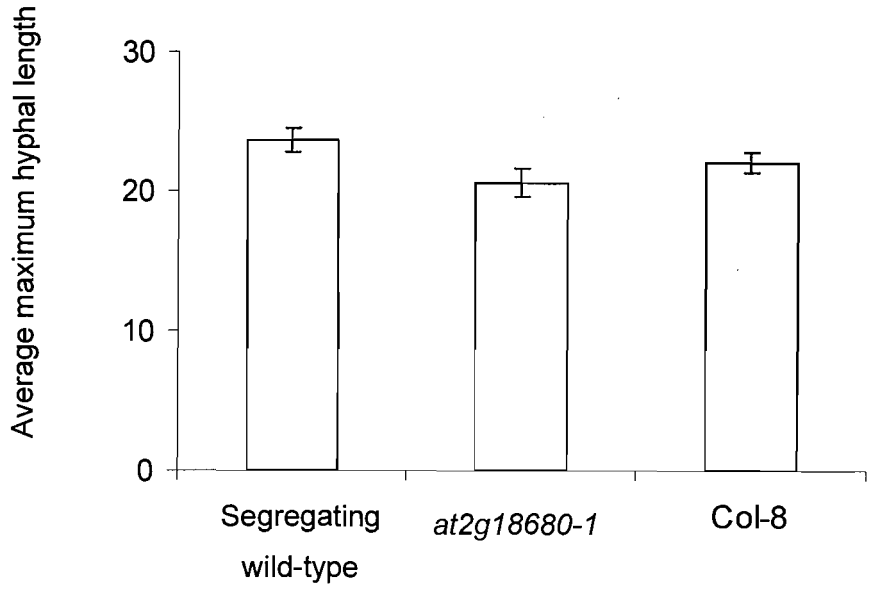


Figure 7.5 The average length of the longest fungal hypha on the *at2g18680-1* mutant as compared to wild-type plants. The centre of the colony was determined by eye, and length of the longest hypha was determined by measuring the length of a series of straight lines to the end of the longest hypha, using the ImageJ program. Segregating wild-type n = 108, *at2g18680-1* n = 114, Col-8 n = 100.

Table 7.2 Table of significances using an unpaired T-test for the measurements displayed in Figure 7.4. The average length of the longest hypha of *at2g18680-1* was significantly shorter than the segregating wild-type, but not the Col-8 wild-type (at the P < 0.05 level). Significant results are highlighted.

	Segregating Wild-type	*at2g18680-1*	Col-8
Segregating Wild-type		0.026	0.165
at2g18680-1	0.026		0.242
Col-8	0.165	0.242	

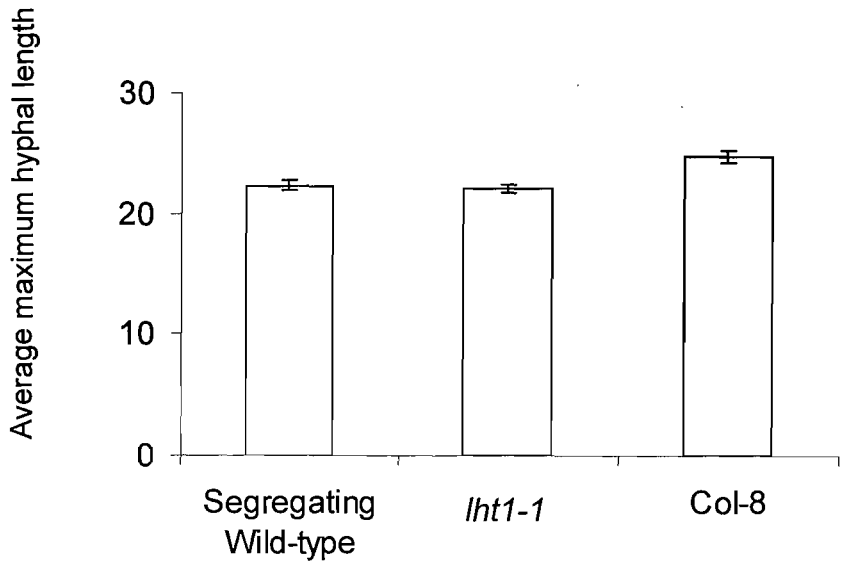


Figure 7.6 The average length of the longest fungal hypha on the *lht1-1* mutant as compared to wild-type plants. The centre of the colony was determined by eye, and length of the longest hypha was determined by measuring the length of a series of straight lines to the end of the longest hypha, using the ImageJ program. Segregating wild-type n = 137, *lht1-1* n = 123, Col-8 n = 127.

Table 7.3 Table of significances using an unpaired T-test for the measurements displayed in Figure 7.5. The average length of the longest hypha of both *lht1-1* and the segregating wild-type was significantly shorter than the Col-8 wild-type, but not from each other. Significant results are highlighted.

	Segregating Wild-type	*lht1-1*	Col-8
Segregating Wild-type		0.668	$< 1 \times 10^{-4}$
lth1-1	0.668		$< 1 \times 10^{-3}$
Col-8	$< 1 \times 10^{-4}$	$< 1 \times 10^{-3}$	

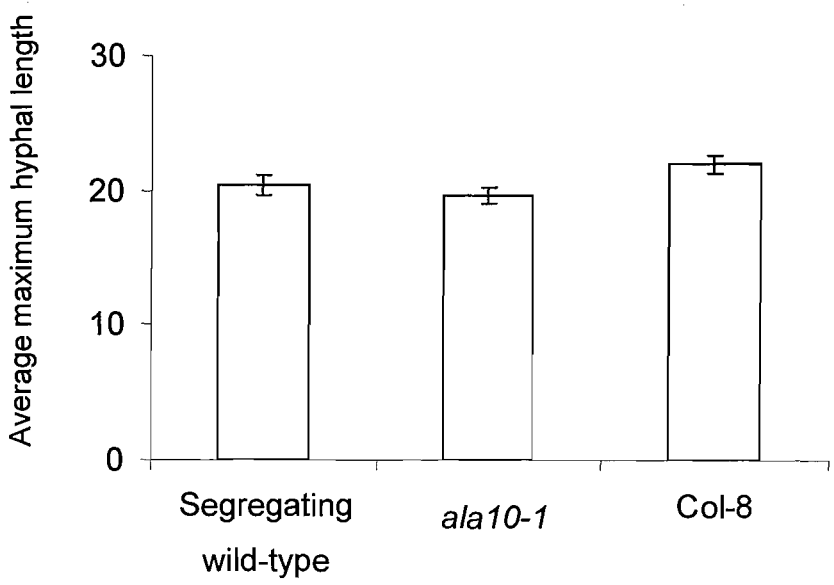


Figure 7.7 The average length of the longest fungal hypha on the *ala10-1* mutant as compared to wild-type plants. The centre of the colony was determined by eye, and length of the longest hypha was determined by measuring the length of a series of straight lines to the end of the longest hypha, using the ImageJ program. Segregating wild-type n = 144, *ala10-1* n = 150, Col-8 n = 123.

Table 7.4 Table of significances using an unpaired T-test for the measurements displayed in Figure 7.6. The average length of the longest hypha of *ala10-1* was significantly shorter than that of the Col-8 wild-type (at the $P < 0.05$ level), but was not significantly shorter than the segregating wild-type plant. Significant results are highlighted.

	Segregating Wild-type	*ala10-1*	Col-8
Segregating Wild-type		0.485	0.124
ala1-1	0.485		0.016
Col-8	0.124	0.016	

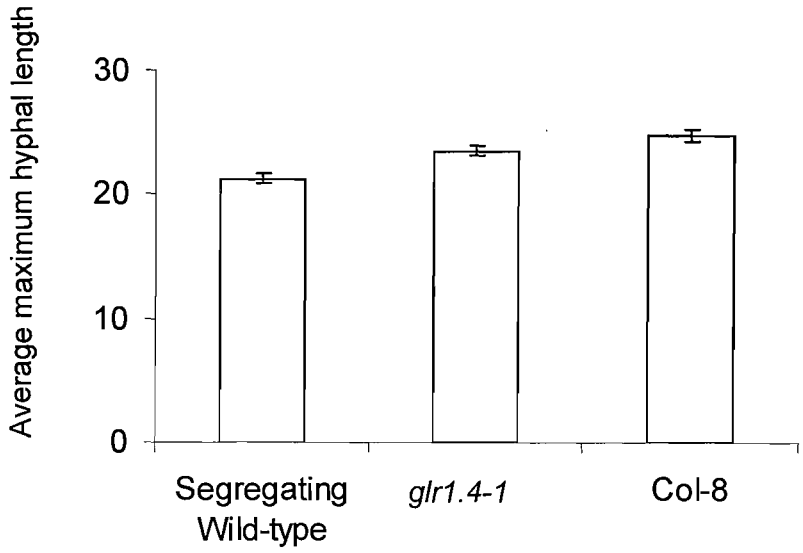


Figure 7.8 The average length of the longest fungal hypha on the *glr1.4-1* mutant as compared to wild-type plants. The centre of the colony was determined by eye, and length of the longest hypha was determined by measuring the length of a series of straight lines to the end of the longest hypha, using the ImageJ program. Segregating wild-type n = 148, *glr1.4-1* n = 120, Col-8 n = 127.

Table 7.5 Table of significances using an unpaired T-test for the measurements displayed in Figure 7.7. The average length of both the longest hypha of *glr1.4-1* and the segregating wild-type were significantly shorter than that of the Col-8 wild-type (at the $P < 0.05$ level), but were not significantly different from each other. Significant results are highlighted.

	Segregating Wild-type	*glr1.4*-1	Col-8
Segregating Wild-type		0.0004	1.0×10^{-10}
glr1.4-1	0.0004		0.047
Col-8	1.0×10^{-10}	0.047	

wild-type was significantly shorter than the Col-8 wild-type. When this experiment was repeated, a similar trend was observed, but the average maximum hyphal length on the segregating wild-type was closer in length to *glr1.4-1* and the difference between them was not significant.

Figure 7.9 and Table 7.6 show a similar situation for the *ptr3-3* mutant, in that there are no significant differences between the genotypes. The maximum hyphal length of the mutant *ptr3-3* was slightly smaller than that if the segregating wild-type, but slightly larger than that of the Col-8 wild-type. A repeat of this experiment confirmed this result.

7.2.5 Fungal growth on *atcwINV1* mutants

In contrast to the measurement of the maximum hyphal length on the other insertional mutants tested, experiments on the two *atcwinv1* mutants proved difficult, with contradictory result between the four experiments conducted (Figure 7.10 and Table 7.7). In the first of the experiments, the average hyphal length of *atcwinv1*-3 was greater than Col-8 and *atcwinv1*-2 ($P= 2.67 \times 10^{-8}$ and 6.32×10^{-8} respectively). In the second experiment, the average hyphal length of Col-8 was greater than both *atcwinv-2* and *atcwinv1-3* (P=0.0011 x 10 and 0.000237 respectively). This result was repeated in experiment three (P = 0.00048 and 0.0025 respectively). However, in experiment four the average hyphal length of *atcwinv1-3* was once again larger than Col-8 and *atcwinv1-2* ($P = 1.65 \times 10^{-8}$ and 5.72 x 10-5).

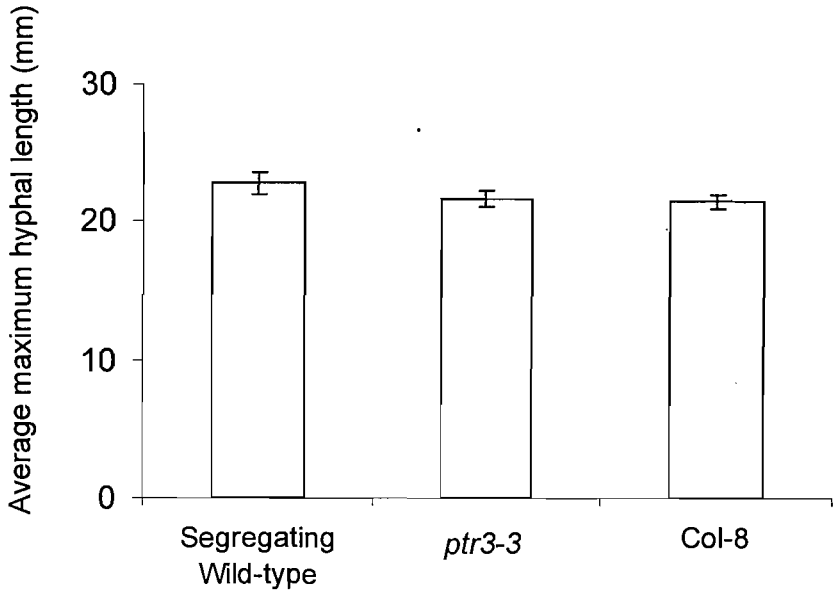


Figure 7.9 The average length of the longest fungal hypha on the *ptr3-3* mutant as compared to wild-type plants. The centre of the colony was determined by eye, and length of the longest hypha was determined by measuring the length of a series of straight lines to the end of the longest hypha, using the ImageJ program. Segregating wild-type n = 121, *ptr3-3* n = 124, Col-8 n = 123.

Table 7.6 Table of significances using an unpaired T-test for the measurements displayed in Figure 7.7. The average length of the longest hypha of *ptr3-3* was not different between Col-8, *ptr3-3* or the segregating wild-type (at the $P < 0.05$ level).

	Segregating Wild-type	*ptr3-3*	Col-8
Segregating Wild-type		0.22	0.16
ptr3-3	0.22		0.79
Col-8	0.16	0.79	

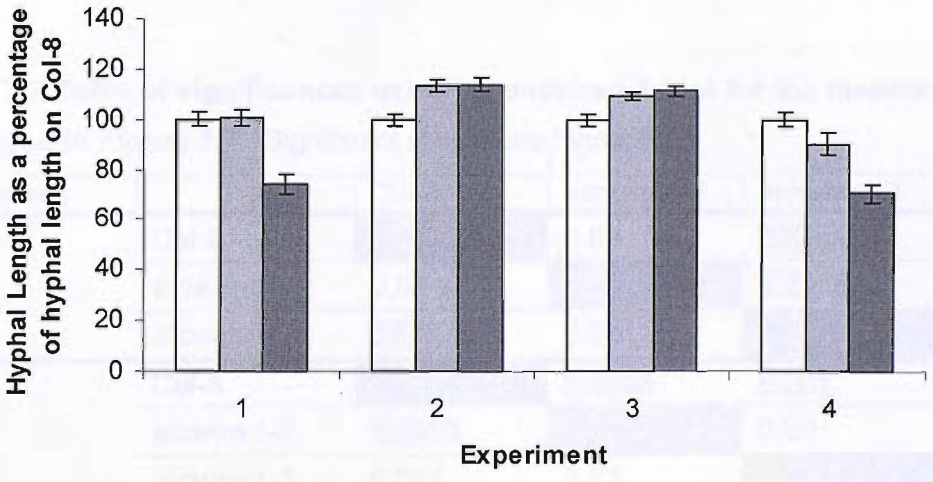


Figure 7.10 Average lengths of longest fungal hypha for *atcwinv1-3 and atcwinv1-3* compared to Col-8. The length of the longest fungal hypha was measured using ImageJ at 5 days post infection and is displayed as a proportion of the average longest hyphal length of Col-8. Clear. Col-8; light grey *atcwinv1-2*; dark grey *atcwinv1-3*.
In experiment 1 n = 30, 20 and 20 for Col-8 *atcwinv1-2* and *atcwinv1-3* respectively, for experiment 2 n = 90, 90 and 90 for Col-8 *atcwinv1-2* and *atcwinv1-3* respectively, for experiment 3 n = 90, 90 and 90 for Col-8, *atcwinv1-2* and *atcwinv1-3* respectively; and for experiment 4 n = 90, 90 and 40 for Col-8, *atcwinv1-2* and *atcwinv1-3* respectively.

Table 7.7 Table of significances using an unpaired T-test for the measurements displayed in Figure 7.7. Significant results are highlighted.

Experiment		Col-8	*atcwinv1-2*	*atcwinv1-3*
1	Col-8		0.89	2.6×10^{-8}
	atcwinv1-2	0.89		6.2×10^{-8}
	atcwinv1-3	2.6×10^{-8}	6.2×10^{-8}	
2	Col-8		0.0003	0.001
	atcwinv1-2	0.0003		0.93
	atcwinv1-3	0.001	0.93	
3	Col-8		0.0025	0.00048
	atcwinv1-2	0.0025		0.49
	atcwinv1-3	0.00048	0.49	
4	Col-8		0.012	1.6×10^{-8}
	atcwinv1-2	0.012		5.2×10^{-5}
	atcwinv1-3	1.6×10^{-8}	5.2×10^{-5}	

7.3 Discussion

7.3.1 General Discussion

Broadly speaking, two types of method exist for monitoring powdery mildew growth on Arabidopsis: disease severity ratings, where severity is visually assessed into discrete categories; or more quantitative measurements of individual powdery mildew features. Disease severity ratings have obvious advantages. They are often simple and rapid, which enables a large sample of plants to be studied (Stummer *et al.*, 2006). The disadvantages are equally obvious, in that such methods are subjective even with the best of experimenters and procedures. Such methods may well fail to pick up small differences in fungal development without an extremely large number of measurements being taken (Nutter *et al.*, 1993). Despite this, these techniques have been used in several studies to investigate powdery mildew pathogenesis on Arabidopsis (Adam & Somerville, 1996; Adam *et al.*, 1999; Wilson *et al.*, 2001; Maleck *et al.*, 2002). The second group of methods also has obvious advantages; such methods are directly quantitative and can measure a variety of growth indicators. The disadvantages are equally clear; methods may require specialised microscopy equipment and taking measurements from the resultant images is often time consuming. This competes against the desire for a high degree of replication when using metrics which show a high variation. Many studies have used total hyphal length or conidia counts as indicators of fungal growth (Frye & Innes, 1998; Vogel & Somerville, 2000; Vogel *et al.*, 2002; Vogel *et al.*, 2004; Liu *et al.*, 2005; Ramonell *et al.*, 2005; Stein *et al.*, 2006). None of these studies took more than 30 measurements in any given experiment, and the smallest difference in fungal length or conidia count shown to be significant was 20%.

An interesting exception to the two groups of methods is a technique that directly counted total conidia production from *B. graminis* by attracting spores to electrically charged plates. The number of conidia attracted to the plates were counted (via sampling) to give an estimate of the total number of spores released (Moriura *et al.*, 2006).

As powdery mildews are biotrophic, these pathogens must strike a delicate balance between extracting sufficient resources from the plant to complete their life cycle, but not so much that the plant is killed prematurely (Vogel & Somerville, 2000). Disease severity is often measured by the effect of powdery mildew on a plant, such as how many leaves are covered by mildew, or what proportion of a given leaf is covered (Wilson *et al.*, 2001; Maleck *et al.*, 2002). However, the relationship that a direct measurement of a powdery mildew colony (such as hyphal length) might have to disease severity is complex. For example, measurements of hyphal length or conidia count will not record any effects on the rate of spore germination, which is also something that would affect disease severity as observed at the level of the whole plant.

There is no single best way of measuring powdery mildew growth and development, as the method will be based around the exact requirements of the study, normally to prove the involvement or the effect of the involvement of a gene, protein or treatment.

Maleck *et al.* (2002) compared a number of different mutants to wild-type plants and used a disease severity rating methodology based on the number of leaves of Arabidopsis that had fungal hyphae present. Using 10 plants per geneotype, although differences in disease severity between genotypes were shown, there was no indication of which of these, (if any) were significant.

Wilson *et al.* (2001) also assessed fungal growth by disease severity ratings, using 30 measurements per sample. Their assessment was based on the percentage of leaf covered by powdery mildew, on a scale of 1–4. Although actual values were not given, the smallest difference in disease severity shown by the technique to be statistically significant was approximately 40%.

The most frequent assessments of fungal growth, as indicated by direct measurement of powdery mildews, are the number of conidia (per colony, per hyphal length or per area) and hyphal length. The number of conidia per mm^2 and per hyphal length was deemed a suitable indicator of fungal growth by Frye and Innes (1998). The study was aimed at identifying powdery mildew resistant Arabidopsis and used an unequal number of measurements (15 to 20) between genotypes. The smallest observed difference between control plants and mutants was 7%, but no estimate of statistical significance was made. The study used only microscope fields of view where there was an approximately equal density of powdery mildew colonies on each sample, in order to ensure equal sampling (Frye & Innes, 1998). Another study using conidia counts as a measure of fungal development was conducted using only colonies in the central portion of the upper surface of four leaves, for a total of between 13 and 24 colonies. The smallest statistically significantly difference measured between genotypes was 51% (Liu *et al.*, 2005). Ramonell *et al.* (2005) measured conidia per colony and conidia per mm^2 as indicators of fungal growth. This was the most sensitive of the studies compared here, measuring a difference of 20% in conidia count per colony between two genotypes as being significantly different ($P < 0.05$) and used an unequal number of measurements of between 20 and 30 per genotype.

Other studies have placed more emphasis on the measurement of hyphal length as an indicator of fungal growth and development; Stein *et al.* (2006) used hyphal length per colony to determine fungal growth. Although exact values were not given, the smallest difference between genotypes that was detected as being significant was approximately 45%, although this was using an unusually high threshold of significance ($P < 0.001$). Several studies conducted by the same experimenters (Vogel & Somerville, 2000; Vogel *et al.*, 2002; Vogel *et al.*, 2004) have also used total hyphal length as a measurement of

fungal growth. This was based on 15 colony measurements, although the entire experiment was repeated once. Although exact values were not given, the smallest difference detected that was classified as significant was approximately 50%.

7.3.2 Validation of a method for measuring powdery mildew growth

The growth and development of powdery mildew has been investigated on seven insertional T-DNA mutants. The growth of powdery mildew on these mutants has been investigated using a microscope-based image analysis method which has been validated during the course of this study. The chief advantage of this method is that it is possible to take a large number of measurements (greater than 100) per genotype. This is significantly more measurements than in previous studies, which should allow smaller differences in powdery mildew growth between genotypes to be determined as statistically significant. This also normally lowers the P value of any given measurement, potentially increasing the confidence in the result.

This study has compared the average maximum hyphal length of powdery mildew on insertional mutants to both Col-8 (the original genetic background of the insertional mutants used in this study) and to segregating wild-type plants. This should provide greater confidence in any observed differences between genotypes, as any alterations to the genetic background since the genetic departure of the mutants from Col-8 should be present in the respective segregating wild-type plant. In theory there should not be a significant difference between segregating wild-type plants and Col-8, however in two cases during this study differences have indeed been observed. This makes the analysis of fungal growth complex, as it is not entirely clear why these differences arise, making it difficult to determine the true result.

During the course of this study some significant differences in powdery mildew growth and development on Arabidopsis have been seen. However the magnitudes of these differences are not as great as have been observed in previous studies; the largest difference observed in this study was 16.7%.

In a study similar in concept to this one, Ramonell *et al.* (2005) examined the effect on powdery mildew growth and development of T-DNA insertional mutants in chitin responsive genes. The function of nine genes were examined in this way, and despite being highly responsive to chitin, only three knockouts produced any effect on fungal growth that could be determined as significant. It was speculated that this was due to functional redundancy within the analysed genes. However, it is also possible that the differences measured would have been statistically significant if a more precise analysis of fungal growth had been made. In fact, most of the mutants analysed showed a difference in conidia per mm^2 of 10% or more.

7.3.3 *lht1 insertional* mutants

Chapter 6 showed that *lht1* insertional mutants have a profound growth defect. Powdery mildew must rely on the host plants for their supply of nitrogen, and biotrophic pathogens are known to metabolise host amino acids (Burrell & Lewis, 1977). It has been shown that some biotrophic pathogens possess amino acid transporters in their haustoria (Struck *et al.*, 2004). It has been suggested that the system which plants possess for the retrieval of amino acids from the apoplast may act as an important passive defence mechanism; keeping the levels of amino acids in the apoplast low (Struck *et al.*, 2004; Hirner *et al.*, 2006). It has been shown that the loss of LHT1 increases the level of amino acids in the apoplast (Hirner *et al.*, 2006), in particular levels of Ser, Val, Ile, Leu, Phe and Lys are present at more than double normal concentrations. Given this, it is surprising that *lht1 insertional* mutants do not appear to greatly affect the growth of powdery mildew. Results from this study show that the average maximum hyphal length of powdery mildew is 12% smaller on *lht1* insertional mutants than on Col-8 plants. Unfortunately there was no difference in the average maximum hyphal length of *lht1* insertional mutants compared to the segregating wild-type line. It is unclear why this is the case but the situation was repeatable. In either case an initial hypothesis might have been that powdery mildew growth on *lht1* insertional mutants would be enhanced due to a potential loss of control over amino acid distribution within plant leaves. It is important to remember however, that powdery mildew haustoria are not in contact with the plant apoplast, but are instead in contact with the extra-haustorial matrix that surrounds them. As discussed in Chapter 1 all nutrients must pass through epidermal cells into the extra-haustorial matrix. It has been shown that the concentrations of amino acids in the apoplast increase in *lht1 insertional* mutants, but the effects on amino acid content of epidermal cell cytoplasm are not known. The amino acid content of entire leaves in *lht1* insertional mutants has been examined and there was no significant difference in overall amino acid levels (although the level of some individual amino acids was increased). Obviously, leaf structure is complex and the redistribution of amino acids within leaves (both mature and developing) of *lht1 insertional* mutants will probably vary between cell types within leaves. Taking this into account, it may well be that amino acid concentrations in epidermal cells (and potentially therefore the extra-haustorial matrix) are reduced in *lht1 insertional* mutants. In this context it is important to note that the biotrophic pathogen *U. viciae-fabae*, from which the amino acid transporter discussed in Chapter 1 was identified (Struck *et al.*, 2004), possesses intracellular hyphae which would be exposed to the apoplasm (although the transporter itself was identified in *U. viciae-fabae* haustoria, which are not exposed to the apoplast). It is therefore possible that pathogens such as epiphytic fungi and bacteria able to utilise apoplastic amino acids may show enhanced growth on *lht1 insertional* mutants. This would be an interesting direction for further study. Equally important is investigation into

the differences in fungal growth and development on the segregating wild-type plant as compared to Col-8. A possible avenue of further study would be to isolate a second segregating wild-type plant line, and repeat the experiment.

7.3.4 a*t2g18680* insertional mutants

This study has shown that the growth of *E. cichoracearum* on a*t2g18680* insertional mutants is significantly decreased compared to Col-8, and decreased compared to the segregating wild-type (but not significantly P = 0.242). However, in order to confirm this result, it would be necessary to backcross the mutant line with Col-8 several times, as this line is likely to contain more than one T-DNA insertion. Irrespective of this, it would be interesting to study this further. Very little is known about the function or possible function of At2g18680. It contains an N-glycosylation site, four protein kinase C phosphorylation sites and four casein kinase II phosphorylation sites (www.expasy.ch/prosite) and 6 likely transmembrane domains. A protein-protein BLAST search only returns significant homology to other hypothetical genes or genes of unknown function. All of these proteins come solely from the plant kingdom (http://www.ncbi.nlm.nih.gov/BLAST). Although the effect on fungal growth of the absence of *At2g18680* remains an open question, the lack of any substantial information on this gene, and the large change in expression in response to powdery mildew infection, make it a fascinating target for further study.

7.3.5 *ala10* and *glr*1.4 insertional mutants

This study has shown that the average maximum hyphal length of *E. cichoracearum* on *ala10* insertional mutants was significantly reduced in comparison to Col-8 ($P < 0.05$) and was reduced in comparison to the segregating wild-type, but this was not significant. Chapter 5 showed that this seed line contains more than one independent T-DNA insertion. Therefore, in order to investigate the effect that the loss of ALA10 has on fungal growth and development, it would be necessary to perform a backcross with a wild-type plant (ideally Col-8).

This study has shown that the average maximum hyphal length is significantly smaller on *glrR1.4* insertional mutants compared to Col-8. It has also shown that the average maximum hyphal length on *glr1.4* insertional mutants is significantly larger than on the segregating wild-type. The segregating wild-type is itself significantly smaller than Col-8. This result was repeatable. This difference between the segregating wild-type and Col-8 makes this result difficult to interpret. Future study on the role of *glr1.4* on the growth and development of *E. cichoracearum* would be to prepare a second segregating wild-type plant and repeat the experiment.

7.3.6 *ptr3* insertional mutants

PTR3 is an SA and JA responsive, wound-inducible protein that has proven ability to transport peptides *in vitro* (Karim *et al.*, 2007). *ptr3* insertional mutants are more susceptible to the necrotrophic pathogen *E. carotovora* and the hemibiotrophic *P. syringae*, but there is no difference in susceptibility to the virulent necrotroph *X. campestris* pv. *campestris* (Karim *et al.*, 2007). *PTR3* is highly induced in response to several pathogens: *E. cichoracearum* (as shown in this study), *B. cinerea*, *E. orontii* and *P. infestans*. It has been hypothesised that *ptr3* insertional mutants may have lower amino acid availability and that this could reduce the production of defence-related peptides, proteins or amino acid derivatives (Karim *et al.*, 2007). The levels of amino acid in *ptr3* insertional mutants have not been investigated, but levels of two defence-related compounds (glucosinolate and camalexin), were shown to be unaffected by T-DNA insertions in the *PTR3* gene. This has led to presumption that *PTR3* insertional mutants do not have a general reduction in the amounts of amino acid-derived defence molecules (Karim *et al.*, 2007). Interestingly it has been shown that *ptr3* insertional mutants accumulate increased levels of oxidative compounds when grown on oxidative stress-inducing media (containing paraquat and rose bengal) (Karim *et al.*, 2007). This was hypothesised to affect SA signalling (which is under redox regulation) (Dong, 2004; Fobert & Despres, 2005). Induction of SAR is initiated by an oxidative burst after which the cellular redox state of the cell recovers and returns to a reduced state, conditions known to cause NPR1 monomers to travel to the nucleus where they activate SAR genes. When formation of the reducing state is blocked, NPR1 stays as inactive multimers in the cytoplasm (Dong, 2004; Fobert & Despres, 2005). Therefore in *ptr3* insertional mutants the excessive oxidative environment may prevent the monomerisation of NPR1 and thus the induction of the SAR response. This could cause the observed increased susceptibility to pathogens (Karim *et al.*, 2007). It is not clear why increased susceptibility is observed in response to only certain pathogens.

Results presented here clearly show that there are no significant differences in the average maximum hyphal length between *ptr3* insertional mutants and either Col-8 or the segregating wild-type plant, despite the fact that the severity of SAR induction is known to influence Arabidopsis resistance to *E. cichoracearum*. This implies that the method of *PTR3* related enhanced susceptibility might not be related to NPR1 activation. Noticeably the only pathogens to which *ptr3* insertional mutants have been shown to be more susceptible are bacterial and possess a necrotrophic stage in their lifestyle (Karim *et al.*, 2007). Further studies using a wide range of fungal pathogens, including those with a necrotrophic stage in their life cycle, might illuminate the role of PTR3 in controlling susceptibility.

The large number of measurements taken in this study, coupled with the negligible difference (<1%) in average maximal hyphal length between *ptr3* insertional mutants and

Col-8, allows a confident statement that the absence of *PTR3* does not impact on the growth of *E. cichoracearum* to any significant degree. The possibility that *ptr3* insertional mutants have an extremely small impact on fungal development still remains, but if so the contribution which *PTR*3 makes that is not replaceable through the action of another process (such as a homologous gene) must be very small. Given that *PTR3* is strongly induced in response to *E. cichoracearum*, it is likely that *PTR3* is functionally redundant during powdery mildew pathogenesis of Arabidopsis.

7.3.7 *aca12* insertional mutants

This study did not identify any differences in the growth of *E. cichoracearum* on *aca12* insertional mutants, compared to either Col-8 or the segregating wild-type plant. This was also the case when the phenotype *of aca12* insertional mutants were monitored under standard growth conditions. *ACA12* itself has been the subject of little study, in comparison to some other members of the *ACA* gene family of Arabidopsis. The most likely explanation for the absence of any phenotype for *ACA12* insertional mutants under any conditions examined is that there is a degree of functional redundancy within the calcium pumps of Arabidopsis, or at least sufficient functional redundancy to compensate for the loss of ACA12. An interesting avenue of further study would be to examine the expression of the other *ACA* genes in Arabidopsis in *aca12* insertional mutants, both under standard growth conditions and in response to powdery mildew infection. It might be hypothesised that one or more of the remaining *ACA genes* might increase in expression in compensation for the loss of ACA12. If double mutants were prepared from any such *ACA* members and *ACA12*, a phenotype might then be observed. The removal of multiple ACA transporters might however, produce pleiotropic effects that would make a detailed analysis of such mutants difficult.

7.3.8 *atcwlNV1* insertional mutants

Chapter 6 showed that *atcwlNV1* insertional mutants display a small but significant growth phenotype under standard growth conditions, and that this was repeatable. However, when the average maximum hyphal length was measured on *atcwlNV1* mutants, no repeatable pattern was observable. Four experiments were carried out, due to the contradictory findings from the first two experiments. Of these experiments, the final three were the more robust; they contained more measurements of individual colonies, on more separate leaves, from more individual plants. This was due to the increasingly advanced microscope equipment that became available during the course of this study, which allowed a larger number of measurements to be taken. In all of these experiments the differences observed were highly significant. In the three most robust experiments, the two *atcwlNV1* insertional mutants produced a very similar effect on the average maximum hyphal length. This gives an indication that the observed differences are indeed due to the

absence of *atcwINV1*. Practically, the seeds used in these experiments all came from the same batch and the experiments were conducted in a seemingly identical manner. It is likely though that an experimental variable that affects the growth of *E. cichoracearum* on *atcwINV1* mutants was not properly controlled during the course of this study. It is unclear what this variable might be, all of the standard growth conditions (light intensity, day length, night length, daytime temperature, night-time temperature and soil composition) were almost identical between experiments and plants were grown in the same highly controlled growth facilities. It should be noted that humidity levels are not controlled in these facilities and it might be that humidity or some other as yet unidentified variable differed between the experiments. The observed contradictions between experiments could result from this. Further work on the effect of *AtcwINV1* on the growth of *E. cichoracearum* should try to identify the source of variation and obtain repeatable results.

Chapter 8
General Discussion

Plants are constantly challenged by microbes and viruses, both above and below ground and plants are resistant to almost all of these. A small number however, evade the plant defences and cause significant diseases (Heath, 2000). These diseases harm the reproductive potential of the plant, but in agricultural situations they can cause dramatic and potentially devastating decreases in crop yield (Oerke *et al.*, 1994). Powdery mildews are biotrophs and obligate pathogens of plants. They develop an intimate relationship with the host plant, upon which they depend entirely for nutrients. Powdery mildews alone cause billions of dollars of damage in lost agricultural yield every year (Oerke *et al.*, 1994), and much research has been done on the response of plants not only to pathogen attack in general, but in response to powdery mildews in particular. Although many key features of the plant defence response to powdery mildews have been elucidated, the exact mechanism by which nutrients are transferred from host to pathogen still remain elusive, and the effect that the drain on nutrients has on the host plant physiology is also relatively unknown.

The aim of the research presented here is to expand on the knowledge of the physiology of the host under biotrophy and the role that transporters play during a compatible infection. Public microarray datasets were mined for target transporter genes, while microarrays of different time points were attempted here in Southampton. These target genes were examined by real-time PCR to confirm changes and expression, and seed lines potentially containing T-DNA in genes of interest were obtained. Homozygous mutants of these seed lines were obtained and confirmed. These seed lines were grown and examined for the presence of phenotype in the absence of the *E. cichoracearum* infection. The progress of infection on these lines was then examined to see if the growth *of E. cichoracearum* was affected.

The response of Arabidopsis to *E. cichoracearum* and to *E. orontii* was found to be related. *E. orontii* infection is the most closely related treatment to *E. cichoracearum* in the Genevestigator database), but there were important differences between the two. Data from *E. cichoracearum* infected Arabidopsis is more related to *E. orontii*-infected Arabidopsis at later time points, and the magnitude of gene expression changes induced by *E. orontii* are higher on average.

Of the proteins encoded by genes which are regulated in response to *E. cichoracearum* infection, most fall into the categories of DNA- and RNA-binding proteins, proteins with protein-binding activity, proteins with transcription factor activity and proteins

with receptor binding or kinase activity. The predominance of these categories (as opposed to hydrolase or transferase activities for example) suggests that signal transductions pathways are being regulated. This shows that even at three days post infection, signal transduction events dominate the transcriptional response of Arabidopsis. The precise function of many of the proteins remains unknown, in terms of their DNA, RNA or protein targets, however the fact that many proteins are transcription factors clearly indicates the transcriptome of Arabidopsis is still undergoing 'reprogramming'. This study has also shown that *E. cichoracearum* infection induces gene expression changes which are similar to changes induced by other pathogen infections, as well as by low levels of glucose and nitrogen. Now that a successful protocol has been developed for conducting microarrays at Southampton it will be possible to add more information to the microarray study initiated in Chapter 2.

This study has highlighted the prominence of some of the developmental responses to *E. cichoracearum* infection. Binding sites for transcription factors of the MADS family, which function in the development of flowers and reproductive organs (Garcia-Maroto *et al.*, 2003), were over-represented in the promoters of regulated genes. The relationship between infection and flowering time is area is worthy of further study. Seeds formed from the reproductive organs of a plant are often the most agriculturally important parts of a plant, and further study may result in a deeper understanding of how stresses, such as disease, relate to flowering and therefore seed production.

Of the transporters regulated in response to *E. cichoracearum* infection, most are part of groups thought to be responsible for the transport of primary nutrients within Arabidopsis (sugars and nitrogenous compounds). It is presumed that the regulation of these transporters produces the changes observed in response to powdery mildew infection (Aked & Hall, 1993), however further work would need to be carried out to confirm that this is the case. Equally, it is not clear to what extent changes in nutrient distribution within plants is primarily for use in defence responses and as a reaction primarily by the plant to nutrient drain by the pathogen as opposed to being induced by the pathogen to enable nutrient uptake. The expression changes of transporter genes that were identified as being regulated in response to *E. cichoracearum* using microarray studies were investigated further by real-time PCR on a separate set of material prepared in Southampton. There was good agreement between the two techniques, with all transporter genes examined showing the same (strong up- or down- regulation) between the two experiments. While there were many genes of interest which could have been investigated further, a subset of potential transporter genes and an invertase gene were selected because of the strength of regulation.

Seed lines with T-DNA insertions in the genes for *PTR1*, *ACA12*, *GR20*, *ALA10*, *AT2G18680*, *LHT1* and *AtcwINV1* were obtained and homozygous mutants were prepared. These homozygous mutants were sequenced and confirmed as no longer possessing cDNA in the gene of interest. Some seed lines displayed no phenotypic differences from wild-type plants at all, while others such as *lht1* mutants, *atcwinv1* mutants and *glr1.4* mutants displayed an abnormal phenotype. *lht1* mutants displayed greatly reduced growth in comparison to wild-type plants: possessing fewer leaves, smaller rosette diameter and shorter heights at the appearance of the first bolt, flower and first silique. All of these three growth stages were also delayed in comparison to wild-type plants. Invertase mutants also displayed a growth phenotype, but the difference between wild-type and mutants was much reduced in comparison to the phenotype displayed by *lht1* mutants. *Atcwin1* mutants bolt, flower, and produce the first silique earlier than wild-type plants. Both *Atcwinv1* mutants isolated in this study also produced fewer leaves and had a smaller rosette diameter at all growth stages. *glr1.4* mutants also show an abnormal phenotype: a delayed production of the first bolt, flower and silique, and show a larger number of leaves and a larger rosette at bolting.

Work published during the course of this study also showed the reduced growth phenotype of *lht1* mutants (Hirner *et al.*, 2006; Svennerstam *et al.*, 2007), but this study measured the phenotype of *lht1* mutants in more detail. *LHT1* has been shown to be a broad specificity, high affinity amino acid transporter (Hirner *et al.*, 2006). The apoplast of *lht1* mutants were shown to have a higher level of amino acids than wild-type plants, which is expected to reduce amino acid cycling within a plant, reducing nitrogen use and plant growth (Hirner *et al.*, 2006). It has been suggested that levels of amino acids in the apoplast are kept low as a passive defence mechanism; reducing the availability of nitrogen to would-be pathogen (Struck *et al.*, 2004). Results from this study show that *lht1* mutants do not permit greater growth of *E. cichoracearum*. *E. cichoracearum* does not come into contact with the apoplasm, but forms haustoria within epidermal cells. In order for enhanced amino acid levels to be directly relevant to *E. cichoracearum* infection, the amino acid levels of epidermal cells would need to increase. Future work could examine whether this is actually the case in *lht1* mutants, and if not, why not. Equally, future work could monitor the progress of pathogens that have direct contact with the apoplasm, such as *P. syringae*. The work presented here shows that simply increasing the amino acid content of the apoplast does not affect the growth of *E. cichoracearum*. The assay used to monitor *E. cichoracearum* in this study is particularly suited to measuring this; an increased availability of nutrients to the fungus might have a negligible impact of the germination of powdery mildew conidia, but would impact on the rate of growth of established colonies.

Previous studies have indicated that *AtcwINV1* mutants do not display a different phenotype compared to wild-type plants (Quilliam *et al.*, 2006). This study, however, found a small but repeatable growth difference between *AtcwINV1* mutants and wild-type plants. This is probably due to the large number of plants used, and the level of detail of measurements used in investigating the growth of plants used in this study. However, no observable change in the development of *E. cichoracearum* on Arabidopsis was repeatable; a priority for further research is to repeat these experiments, perhaps in growth conditions where the humidity of the growth chamber is controlled.

Although many of the T-DNA insertional knockouts used in this study did not show a growth phenotype, this is not necessarily unexpected (Cutler & McCourt, 2005; Quilliam *et al.*, 2006). Many phenotypes are expected to be conditional (Bouche & Bouchez, 2001) and functional redundancy in genes, and potentially in whole pathways of genes (Cutler & McCourt, 2005), also contributes to a lack of observable phenotypes. Given that these genes show regulation in response to *E. cichoracearum*, it might be expected that a phenotype only emerges under *E. cichoracearum*, or related pathogen, infection. A study of gene expression in T-DNA mutant lines might indicate if the expression of other genes has changed in order to compensate for the loss of a gene. It might then be possible to prepare lines with T-DNA in any compensating genes, and create seed lines with multiple T-DNA insertions. Some of the T-DNA insertional mutant lines examined here are likely to contain more than one T-DNA insert location. If further expression studies are carried out, it will be necessary to backcross these lines with Col-8 to remove any other T-DNA insertions (if possible). Equally, plants containing knockouts in more than one gene of interest should not be prepared from parent plants potentially containing other inserts.

None of the mutant plants examined showed any significant change in the development of *E. cichoracearum* compared to wild-type plants and segregating wild-type plants. A study similar in structure to this one, used microarrays examining gene expression patterns following chitin treatment of Arabidopsis leaves to identify highly regulated genes (Ramonell *et al.*, 2005). Insertional mutants were then prepared in genes of interest. In that study, nine mutants were investigated and three were found to more susceptible to *E. cichoracearum*, while the remaining six were observed to have no difference in susceptibility to *E. cichoracearum* (Ramonell *et al.*, 2005). There was no indication as to whether there were any phenotypic differences observable compared to wild-type plants under standard growth conditions. The study did not focus on one type of gene (such as transporters) over another. Investigations using purified elicitors might produce a higher proportion of mutants with observable differences in powdery mildew growth, because the effect of the elicitor on the host should be more precise. Genes responding to the treatment should be specifically part of the reception of the signal, part

of the signal transduction cascade or part of the defence response to it. The elicitor in itself will not cause disease and other signal cascades (such as in those in response to effectors) should not be induced. Most of the transcriptional response should be related to signal transduction, with the defence responses themselves being much more limited than when using a live pathogen. As such, responding genes are much more likely to be directly involved in the sensing of chitin, and insertional mutants in the cascade stand a higher chance of seriously impairing the transduction or response to the signal. Despite all of these factors, two thirds of the insertional mutants investigated showed no reduction to *E. cichoracearum*.

This study has set a good base for further research, having isolated a number of mutants which can be used in further studies, and highlighted a number of avenues for further research. However it is clear that the web of functional redundancy that surrounds the response of Arabidopsis to *E. cichoracearum* and in particular to the response of transporters to *E. cichoracearum* infection, presents experimental challenges. Trying to understand the physiology of established biotrophy is likely to prove more complicated than understanding the defence responses to the initiation of biotrophy. The availability of public microarray data showing the response of Arabidopsis to two species of powdery mildew, and a detailed bioinformatics analysis (in greater depth than it has been possible to do here) into the response of Arabidopsis should reveal further insights into powdery mildew pathogenesis. Now that a successful protocol for conducting microarrays has been established at Southampton, future research will be able to combine genetic approaches with transcriptome analysis in an affordable and rapid manner. This should allow the analysis of the transcriptome of the insertional mutants examined here, to be examined both under standard growth conditions and during infection. This should continue to allow progress into the study of powdery mildew infection of Arabidopsis.

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