IDENTIFICATION OF CANDIDATE DEFENCE RESPONSE GENES ASSOCIATED WITH THE BARLEY-*PYRENOPHORA TERES* INCOMPATIBLE INTERACTION

by

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DECLARATION BY CANDIDATE

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ABSTRACT

Barley net- and spot-form of net blotch, caused by two *formae* of the hemibiotrophic fungus *Pyrenophora teres*, are two of the major diseases affecting barley crops worldwide. In this study, the method of suppression subtractive hybridisation was used to isolate barley epidermal genes that were differentially expressed in the early stages of both net blotch incompatible compared to compatible interactions. As a result, two subtracted libraries of cDNA clones comprising mainly of gene transcripts of low abundance were generated. Quantitative real-time PCR was employed to verify and profile the differential expression of forty-five subtracted transcripts during the first 48 hours of infection, resulting in the identification of twenty-eight clones that were pathogen-induced and differentially expressed. These clones were grouped into one of eight clusters depending on the kinetics of their expression, and they included groups of genes that were up-regulated early (within 3 hai) and later (24 hai) in both barley-*P. teres* incompatible interactions. Among the differentially expressed clones were those with sequence homology to genes that encode proteins involved in calcium signal perception (e.g. a calcineurin B-like protein), detoxification (e.g. multidrug transporters), carbohydrate metabolism (e.g. an invertase), and signal transduction (e.g. protein kinases). Furthermore, the expression profiles generated for each individual gene cluster were similar for both net- and spot-form interactions, indicating that the resistance-associated defence response against both pathogens may be mediated by the same molecular mechanism. The differentially expressed genes are discussed with respect to their potential functional role in contributing to net blotch disease resistance. In addition, a model detailing early events that may take place in the barley-*P. teres* incompatible interaction is presented.

CONFERENCE PROCEEDINGS

Bogacki P, Oldach K, Knogge W, and Williams KJ (2004). Profiling of gene expression in the incompatible interaction between barley and the fungus *Pyrenophora teres* (f. *teres* and f. *maculata*). *9th International Barley Genetics Symposium*, Brno, Czech Republic.

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CHAPTER I

Plant-pathogen interactions: a review

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1.1 INTRODUCTION

Plants, like animals, are constantly being challenged by a diverse range of pathogenic organisms such as viruses, mycoplasma, bacteria, fungi, nematodes, protozoa, and parasites. However, unlike animals, plants do not have a circulatory system and are unable to produce antibodies and thus have evolved a unique immune system of their own. Plants defend themselves by utilising a combination of physiological and biochemical barriers. Their defence mechanisms are so efficient that a certain plant species can only be successfully infected by a limited number of pathogens. However, when disease does occur, crop plants in particular are extremely vulnerable because they are usually grown as genetically uniform monocultures. As a consequence farmers are continually battling to protect their crops from pathogens that can cause considerable losses in yield and quality.

A number of strategies based on chemical control and classical breeding are in place for controlling plant diseases. However, as this chapter will point out, they have their limitations, and alternative strategies may need to be implemented to more efficiently control diseases affecting crop plants in the future. An alternative strategy is to genetically manipulate plants so that they can utilise key components of their own defence repertoire to thwart pathogens. The challenge is to identify these key components. In the past 10-15 years, much effort has been devoted to unravel the earliest responses that occur in plants following pathogen attack. These studies have primarily focused on the identification of signal transduction components that relay information following pathogen recognition, as well as the identification of the induction of genes transcribed that code for defence responses. From these studies, it is clear that the nature of the plant defence response is complex and sophisticated. This chapter will attempt to give a brief introduction to the basic concepts concerning molecular plant-microbe interactions and review the state of knowledge in this research area up to and including the year 2002 when this project began.

1.2 PLANT DEFENCE MECHANISMS AGAINST FUNGAL PATHOGENS

1.2.1 Fungal infection of plants

Among the diverse range of pathogenic organisms that are capable of colonising crop plants, it is the fungi that are predominantly responsible for inflicting disease on susceptible hosts (Knogge, 1996). Plants come into contact with three types of fungal pathogens with contrasting modes of attack. Necrotrophs produce toxic enzymes and metabolites that kill plant tissue directly upon invasion whereas biotrophs and hemibiotrophs initially keep the cells in infected plant tissue alive to enable their infection cycle to continue (Stuiver and Custers, 2001). In the case of hemibiotrophs, the initial biotrophic phase is followed by a subsequent necrotrophic mode of existence during the later stages of infection.

Most pathogenic fungi have evolved mechanisms to penetrate the two outer plant structural barriers – the cuticle and the epidermal cell wall. This is facilitated by the secretion of hydrolytic enzymes such as cutinases and cellulases which can degrade these structural components, as well as the formation of specialised penetration organs called appressoria (Kolattukudy, 1985; Mendgen and Deising, 1993). After successful penetration the fungus then often secretes toxins or plant hormone-like compounds that manipulate the physiology of the host to the benefit of the pathogen (Walton, 1996). In the vast majority of cases however, fungal penetration fails. Of the approximately 100,000 known fungal species, less than 10% are able to colonise plants and an even smaller fraction of these are actually capable of causing disease (Knogge, 1996). This inefficiency can be attributed to an efficient plant defence system that can detect pathogenic organisms early and activate defence components to arrest further fungal development.

1.2.2 General plant defence mechanisms

Plants can utilise a combination of physiological and biochemical barriers to protect themselves from fungi and other types of pathogens. Plant resistance to pathogens predominates for three reasons: (1) in most cases pathogens are unable to penetrate the host's natural preformed defences, (2) the plant does not support the niche

requirements of a potential pathogen and is thus considered a non-host, or (3) certain pathogens induce race-specific plant defence responses following their recognition by the plant.

The natural preformed defence barriers include the cuticle and the epidermal cell wall (Knogge, 1996), as well as some natural products of secondary metabolism called phytoalexins that have antimicrobial properties (VanEtten et al., 1994; Dixon, 2001). For example, saponins are widely occurring, constitutively expressed, glycosylated steroids, steroidal alkaloids or triterpenes, many of which have antimicrobial activity *in vitro* (Dixon, 2001).

Non-host resistance, referred to as the resistance shown by a plant species towards pathogens for which it is not considered a host, is the most common and durable form of plant resistance to pathogenic organisms. It is assumed that pathogens target and subsequently overcome non-host resistance in order to colonise and multiply on a plant species. To date, little progress has been made in our understanding of the mechanisms underlying non-host resistance. However, the emerging picture is that it could be controlled by a combination of processes that mediate generally occurring (i.e. preformed) and induced race-specific defence responses that take place following pathogen recognition (Heath, 2001).

The induced race-specific defence response is dependent on a recognition event that occurs between plant and pathogen. For this to happen, specific elicitors (or effectors) encoded by pathogen avirulence genes interact directly or indirectly with corresponding host receptors encoded by plant resistance genes (Staskawicz et al., 1995; Dangl and Jones, 2001; Luderer and Joosten, 2001). This perception of elicitor molecules by the plant leads to the induction of a large array of biochemical changes that form an integral part in conferring resistance to plant pathogens.

Therefore, susceptibility ensues when: (1) the preformed plant defence mechanisms are inefficient, (2) the plant is not able to detect the pathogen, or (3) the induced plant defence responses are ineffective.

1.3 RECOGNITION AND INDUCED PLANT DEFENCE RESPONSES

1.3.1 The "gene-for-gene" *versus* the "guard" hypothesis

The biochemical basis of host-specific resistance follows the gene-for-gene concept first postulated by Flor (1971) who showed that flax resistance to the flax rust fungus, *Melampsora lini*, resulted from the interaction of paired cognate genes from the host and pathogen. This led to the notion that for each gene conferring resistance (*R*) in the plant there is a corresponding avirulence (*Avr*) gene in the pathogen that determines pathogenicity. If both genes are present in the interaction in an active form, then the plant becomes resistant – inhibiting pathogen growth, whereas, if either one or both genes are absent then the pathogen is able to progress through its normal life and susceptibility ensues. In biological terms, the resistant interaction is referred to as "incompatible", whereas the susceptible interaction is "compatible". The direct physical interaction between *R* and *Avr* gene products may follow a receptor-ligand model (Figure 1A). However, only the report of Jia et al. (2000) provides evidence in support of this theory. In that study, the avirulence gene *Avr-Pita* from the rice blast fungus, *Magnaporthe grisea*, was found to confer resistance on rice cultivars containing resistance gene *Pi-ta*, and subsequent experimentation showed that the interaction between protein products of both genes was direct.

It is now becoming apparent that the interaction between *R* and *Avr* gene products often may be indirect and the involvement of at least a third component has been shown for a number of fungal and bacterial plant-pathogen interactions (Bisgrove et al., 1994; Salmeron et al., 1996; Joosten et al., 1997; Kooman-Gersmann et al., 1998). The identification of an additional R protein required for *avrPto*/*Pto*-mediated resistance in the tomato-*Pseudomonas syringae* incompatible interaction (Salmeron et al., 1996) led to the guard hypothesis to explain Avr-R protein interactions (Van der Biezen and Jones, 1998). The guard hypothesis (Figure 1B) implies that the third component could be the virulence target of an Avr molecule and that the role of the R protein is to "guard" or protect the virulence target. In the presence of the "guarding" R protein, binding of the Avr protein to its virulence target will initiate the defence response leading to pathogen resistance, whereas in the absence of the R protein this binding will result in susceptibility.

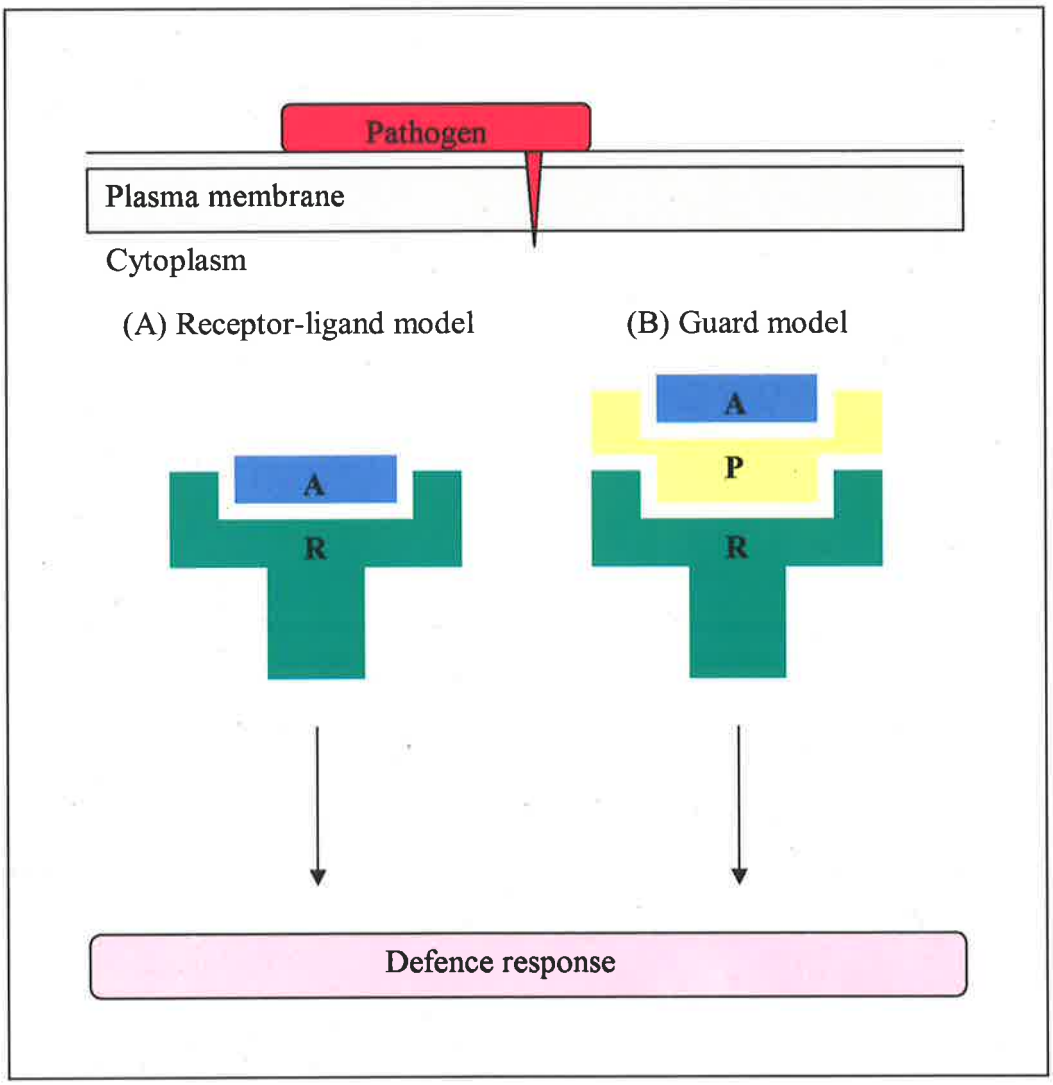

Figure 1: Biochemical interpretations of the gene-for-gene model [modified from Bonas and Lahaye (2002)]. (A) The classical receptor-ligand model predicts that direct interaction between an Avr protein from the pathogen (A; blue) and a matching R protein from the plant (R; green) initiates the defence reaction. (B) In the conceptual framework of the guard model, the resistance protein safeguards a matching pathogenicity target (P; yellow).

1.3.2 Features of R and Avr proteins

A variety of *R* and *Avr* genes have been cloned in recent years. *R* genes encode only five classes of proteins that are categorised according to their structural features and cellular localisation (Takken and Joosten, 2000; Dangl and Jones, 2001). The largest class of R proteins have an intracellular leucine-rich repeat (LRR) domain and a predicted nucleotide binding site (NBS). It is the LRR domain that is believed to mediate protein-protein interactions or determine specific recognition of elicitors by plant receptor molecules (Kobe and Deisenhofer, 1994; Jones and Jones, 1996). The NBS-LRR class of R proteins is further subdivided into those containing a coiled-coil (CC) or a Toll/interleukin 1 receptor-like (TIR) N-terminal domain. Examples of NBS-LRR type R proteins include Pi-ta from rice (resistance to *M. grisea*) (Bryan et al., 2000) and Mla1 and Mla6 from barley (resistance to *Blumeria graminis* f. sp. *hordei*) (Halterman et al., 2001; Zhou et al., 2001).

The other four classes of plant R proteins are structurally diverse. The rice *Xa21* gene (resistance to *Xanthomonas oryzae*) and tomato *Cf-x* genes (resistance to *Cladosporium fulvum*) encode two of these remaining four classes. *Xa21* encodes a transmembrane receptor carrying a large extracellular LRR domain and an intracellular protein kinase domain (Song et al., 1995), and *Cf-x* genes encode single pass membrane proteins with extracellular LRRs (Jones et al., 1994). *Pto* from tomato (resistance to *P. syringae* strains carrying *avrPto*) and *RPW8* from *Arabidopsis* (broad-spectrum resistance to fungal strains causing powdery mildew) encode the last two R protein classes. *Pto* encodes a serine/threonine protein kinase (Martin et al., 1993), and *RPW8* encodes a small, probable membrane protein with a possible CC domain and essentially no other homology to known proteins. As Pto and RPW8 do not have LRR domains, it has been suggested that they may possibly be the third components required for Avr protein perception rather than being true R proteins themselves (Luderer and Joosten, 2001).

In contrast, Avr proteins show little or no homology to one another, and with the exception of some virally encoded proteins, they have no deduced or experimentally defined functions (Luderer and Joosten, 2001). AvrPto (from *P. syringae*), for example, is a mostly hydrophilic protein of 164 amino acids with no homology to

protein sequences in GenBank or EMBL databases (Salmeron and Staskawicz, 1993). Avr-Pita (from *M. grisea*) encodes a predicted 223 amino acid pro-protein, which is processed into a putative 176 amino acid mature protein with homology to zinc-dependent metalloproteases (Jia et al., 2000; Orbach et al., 2000). Although the number of Avr proteins for which a clear function for the pathogen has been demonstrated is limited, it is now generally accepted that these proteins are bifunctional and also have a role in the virulence of the pathogen (White et al., 2000; Luderer and Joosten, 2001).

1.3.3 The hypersensitive response (HR)

A multitude of plant resistance-associated reactions are initiated following the interaction between *Avr* and *R* gene products. For a biotrophic pathogen, *R* gene mediated disease resistance is normally characterised by a form of programmed cell death called the hypersensitive response (HR) which leads to the death of plant cells within a few hours of contact thereby depriving the pathogen access to further nutrients. The HR is often associated with an "oxidative burst" which is characterised by the accumulation of highly reactive oxygen species (ROS) including the superoxide anion (O_2^-) and its dismutation product, hydrogen peroxide (H_2O_2) (Lamb and Dixon, 1997; Bolwell, 1999). It is generally accepted that ROS are involved in triggering and/or executing the HR (Heath, 2000) and the "oxidative burst" has been implicated in the plant defence response to both specific and non-specific elicitors (May et al., 1996; Lamb and Dixon, 1997; Higgins et al., 1998).

The HR is further associated with Ca^{2+} and K^+ ion fluxes across the plant plasma membrane (Heath, 2000). The concentration of cytosolic Ca^{2+} is an obligate intracellular messenger coordinating responses to a diverse range of developmental cues and environmental challenges (White, 2000; Sanders et al., 2002) and cytosolic Ca^{2+} perturbations have been observed in response to several pathogen-derived elicitors (Mithöfer et al., 1999; Blume et al., 2000; Grant et al., 2000; Lecourieux et al., 2002).

The plant signal molecule salicylic acid (SA) also accumulates in plant tissues undergoing the HR (Heath, 2000). SA is a key signalling molecule for activation of

defence responses and resistance to various pathogens in many host plant species (Dempsey et al., 1999) and the requirement for SA in *R* gene mediated resistance has been demonstrated by showing that transgenic plants engineered to degrade SA with the bacterial *NahG* gene are unable to induce cell death after pathogen attack (Gaffney et al., 1993; Delaney et al., 1994; 1995; Kachroo et al., 2000).

Additional features of the HR include oxidative cross-linking of the plant cell wall (Brisson et al., 1994), as well as the accumulation of phytoalexins and pathogenesis-related (PR) proteins (Van Loon and Van Strien, 1999). In many instances the HR is also associated with an enhancement in resistance to a broad spectrum of pathogens throughout the plant known as systemic acquired resistance (SAR) (Sticher et al., 1997). SAR occurs upon attempted infection by many necrosis-causing avirulent pathogens and provides protection against subsequent infections by a wide range of unrelated pathogens. For example, treatment of cucumber plants with a proteinaceous elicitor derived from *P. syringae* induced SAR against diverse pathogens, including the anthracnose fungus (*Colletotrichum lagenarium*), tobacco necrosis virus, and the bacterial angular leaf spot bacterium (*P. syringae* pv. *lachrymans*) (Strobel et al., 1996).

Although the HR alone is a feasible defence mechanism against biotrophic pathogens that require living host cells for their survival, exceptions have been reported. For example, Yu et al. (1998) identified an *Arabidopsis* mutant line in which effective gene-for-gene resistance occurred despite the virtual absence of HR cell death. The mutant line was defective in HR cell death but retained characteristic responses to avirulent *P. syringae* such as induced PR gene expression and strong restriction of pathogen growth. Therefore, it has been suggested that the cell death component of the HR may function more as a signalling system rather than as a direct defence mechanism (Graham and Graham, 1999; Heath, 2000).

1.3.4 The HR against biotrophic *versus* necrotrophic pathogens

In contrast to biotrophic pathogens, the HR is not a feasible plant defence mechanism against necrotrophic pathogens, which can exploit the resulting cell death for their own survival. ROS are thought to play a dual role in plant resistance to pathogens. On

one hand they are involved in the induction of several defence reactions such as the HR and *PR* gene expression, and on the other their accumulation may be involved in successful pathogenesis (Heath, 2000; Kumar et al., 2001). In the case of necrotrophic pathogens, ROS accumulation has been shown to be an indicator of successful pathogenesis (von Gönner and Schlösser, 1993; von Tiedemann, 1997; Govrin and Levine, 2000; Kumar et al., 2001). For example, the production of H_2O_2 which was linked to a HR did not protect *Arabidopsis* plants against infection by *Botrytis cinerea* (Govrin and Levine, 2000), and likewise, an increase in H_2O_2 levels in barley *mlo* genotypes infected with *Bipolaris sorokiniana* was associated with an increase in disease susceptibility towards this necrotrophic pathogen (Kumar et al., 2001). Furthermore, it has been proposed that necrotrophic pathogens are able to protect themselves from the toxic effects of ROS during their colonisation of host tissue by secreting anti-oxidant enzymes like catalases, peroxidases, and superoxide dismutases that can remove or inactivate ROS (Mayer et al., 2001). Hence, although the association between ROS production and HR-related cell death is widely accepted (Lamb and Dixon, 1997; Bolwell, 1999), its benefit for resistant strategies varies according to the type of pathogen that is encountered.

1.3.5 Signalling molecules involved in coordinating plant defence responses

The involvement of SA in the plant defence response and its association with the HR has already been mentioned. SA-dependent signalling is important for some gene-for-gene resistance responses, for local responses that limit growth of virulent pathogens, and for SAR (Glazebrook, 2001). In addition to SA, incompatible pathogens are also capable of inducing the production of two more key endogenous secondary signals, ethylene (ET) and jasmonic acid (JA), which participate in the activation and/or potential of plant defence responses (McDowell and Dangl, 2000; Wang et al., 2002).

In response to certain necrotrophic pathogens it has been shown that ET and JA signalling pathways interact with each other, co-regulating the expression of some genes in plant defence (Penninckx et al., 1998; Thomma et al., 1998). In addition, it was found that ET-JA-dependent responses are utilised differentially against pathogens with contrasting modes of attack. *NahG* plants or *npr1* mutants, which are impaired in SA-signalling and which have increased susceptibility to the biotrophic

fungus *Peronospora parasitica* and the bacterium *P. syringae* (Delaney et al., 1994; 1995) were not affected in their defence response against the necrotrophic fungi *Alternaria brassicicola* and *B. cinerea* (Thomma et al., 1998; 1999). The generalisation is that ET-JA-dependent defence responses are initiated by necrotrophic pathogens, whereas SA-dependent responses are triggered by biotrophic pathogens. Furthermore, colonisation of *Arabidopsis* roots by certain rhizosphere bacteria confers another form of disease resistance called induced systemic resistance (ISR), which is also dependent on the ET-JA response pathway (Pieterse et al., 1996; 1998). Therefore, it is apparent that plants can activate distinct defence responses depending on which pathogens they come into contact with and that a considerable amount of "cross-talk" occurs between the SA, ET, and JA signalling pathways. Schenk et al. (2000) studied the changes in expression levels of 2375 selected genes upon pathogen infection or SA, ET, and JA treatment and found that although some genes were affected only by one signal, many responded to two or more defence signals. Thus, it seems that a substantial network of regulatory interaction and coordination does exist between the different signalling pathways for selective defence gene deployment.

1.3.6 *R* gene signal transduction components

A number of recent studies utilising disease-susceptible plant mutants have identified several genes that are required for the function of specific plant *R* genes. Most of this work has focused on *Arabidopsis* where it appears that at least three *R* gene signal transduction pathways exist (Glazebrook, 2001). The first is dependent on *NDR1* (for non-race specific disease resistance) gene function and is based on the finding that a mutation of *NDR1* resulted in loss of resistance governed by several resistance genes against the bacterial pathogen *P. syringae* and the fungal pathogen *P. parasitica* (Century et al., 1995; 1997). The second pathway was defined by mutations in the *EDS1* (for enhanced disease susceptibility) gene, which also suppressed resistance to *P. parasitica* specified by several different *RPP* (for resistance to *P. parasitica*) genes (Parker et al., 1996). This study showed clear functional differences between *EDS1* and *NDR1*. Although some examples to the contrary have been found, it has been proposed that CC-NBS-LRR-type *R* genes signal through NDR1 whereas TIR-NBS-LRR-type *R* genes signal through EDS1 (Aarts et al., 1998). Furthermore, it seems that *R* genes requiring *EDS1* also require the *PAD4* (for phytoalexin-deficient) gene

(Glazebrook et al., 1997) as shown by extensive similarities between *eds1* and *pad4* mutants (Falk et al., 1999; Jirage et al., 1999; Clarke et al., 2001). EDS1 and PAD4 act upstream of SA to promote SA accumulation (Glazebrook, 2001). The genetic components of the other pathways are not known but the finding that two *R* genes, *RPP7* and *RPP8*, require neither *NDR1* or *EDS1* suggests that there is at least a third pathway in *Arabidopsis* for the transduction of *R* gene signals (McDowell et al., 2000).

Additional genes that act in either the SA or ET-JA signalling pathways have also been identified in *Arabidopsis*. Using various genetic screens, the *NPR1* (for nonexpresser of *PR* genes; also called *NIM1* for nonimmunity) gene was identified as a key regulator acting downstream of SA to promote the expression of SAR and local acquired resistance (Cao et al., 1994; Delaney et al., 1995). Other genes such as *EDS5* and *SID2* (Nawrath and Métraux, 1999; Dewdney et al., 2000) and *EDR1* (for enhanced disease resistance) (Frye et al., 2001) have been shown to function upstream of SA accumulation. The role of ET and JA in the activation of disease resistance mechanisms was shown by their regulation of the *pdf1-2* gene which encodes a plant defensin induced by JA and necrotrophic pathogen infection (Penninckx et al., 1996; 1998). It was demonstrated that ET and JA seem to be required simultaneously for *pdf1-2* expression as the gene was not activated in either of the ET- and JA-insensitive mutants, *ein2* and *coi1*, respectively. Furthermore, resistance to necrotrophic pathogen infection has been shown to be compromised in *ein2* (Thomma et al., 1999) and *coi1* (Thomma et al., 1998) plants.

In barley, identifying components of *R* gene signalling has primarily focused on powdery mildew resistance governed by the biotrophic fungus, *Blumeria graminis* f. sp. *hordei*. Race-specific disease resistance is triggered by a multitude of powdery mildew *R* genes (*Mlx*) and it has been shown that two additional genes, *Rar1* and *Rar2*, are required for many but not all *R* gene mediated resistance to *B. graminis* (Freialdenhoven et al., 1994; Peterhänsel et al., 1997). While the product of *Rar2* is still unknown, *Rar1* encodes a small zinc-binding protein and both genes function up-stream of an oxidative burst which is closely linked to the HR in barley and is absent in susceptible *Rar1* and *Rar2* mutants (Thordal-Christensen et al., 1997; Shirasu et al., 1999; Hückelhoven et al., 2000). In addition, a separate genetic pathway based on

recessive loss-of-function alleles of the *Mlo* gene has been shown to mediate durable and race-non-specific resistance to *B. graminis* (Jørgensen, 1992; Peterhänsel et al., 1997). Freialdenhoven et al. (1996) showed that two genes, *Ror1* and *Ror2*, are required for *mlo*-controlled powdery mildew resistance, and that mutations in these genes restore successful penetration of host cells and reduce the frequency of spontaneous cell wall appositions in the absence of the pathogen. Resistance based on *mlo* is not associated with a HR in the region of pathogen attack but is associated with a spontaneous cell death response correlated with H_2O_2 accumulation (Peterhänsel et al., 1997). In their study it was shown that a different genetic pathway than the one leading to the HR controls this form of plant cell death. *Mlo* encodes a novel class of integral membrane proteins anchored by seven trans-membrane helices (Büschges et al., 1997), and although its role is unclear, it has been proposed to function as a suppressor of defence responses as barley lines carrying *mlo*-resistance alleles have been shown to induce spontaneous defence responses such as the formation of cell wall appositions (Wolter et al., 1993).

1.3.7 Differential gene expression in compatible *versus* incompatible interactions

A comparison in the expression of defence related genes between incompatible and compatible interactions has only been made in a small number of studies. Lamb et al. (1992) proposed that cellular responses are accelerated in incompatible interactions compared to compatible ones. This was based on studies that revealed marked differences in the temporal and spatial expression patterns of three defence genes in race-cultivar-specific interactions between bean and *Colletotrichum lindemuthianum*, the causal agent of anthracnose (Bell et al., 1984; Bell et al., 1986; Hedrick et al., 1988). Earlier and stronger accumulation of *PR* gene transcripts in resistant compared to susceptible interactions has also been observed in barley leaves challenged by the powdery mildew fungus (Freialdenhoven et al., 1994; Peterhänsel et al., 1997; Hückelhoven et al., 2000). Boyd et al. (1994) found that although avirulent and virulent isolates of the powdery mildew fungus were able to induce expression of three *PR* genes at the same time, their transcripts levels were maintained at higher levels during infection with the avirulent isolate only. Therefore, based on current evidence, the incompatible interaction appears to be characterised by a more rapid and intense up-regulation of some *PR* genes at the very least. Whether this pattern of

differential expression holds true for other defence related genes remains to be determined.

1.3.8 Non-host resistance

The genetic and molecular basis of non-host resistance is poorly understood. Several lines of evidence suggest that this form of resistance may be based on general preformed or passive defence mechanisms (Heath, 2001). These include plant actin microfilaments whose disruption has led to the loss of non-host resistance in several plants against a range of non-host fungi (Kobayashi et al., 1997), as well as a number of secondary metabolites with antimicrobial activity (Dixon, 2001). For example, Papadopoulou et al. (1999) showed that oat mutants deficient in the synthesis of anti-fungal saponins became susceptible to the non-host take-all wheat pathogen *Gaeumannomyces graminis* var. *tritici*. In addition, some forms of non-host resistance may involve induced defence responses following attempted pathogen invasion that are similar to those observed in animal innate immunity. These responses are based on the recognition of general pathogen associated molecular patterns (PAMPs) such as bacterial lipopolysaccharides and flagellin (Kopp and Medzhitov, 1999; Medzhitov and Janeway, 2000). Gómez-Gómez and Boller (2002) reported that, just like in animals, plants also contain transmembrane proteins with extracellular LRR domains that recognise general elicitors such as bacterial flagellins and activate defence responses when stimulated by them.

It has also been proposed that non-host resistance defence responses may be similar to those induced during gene-for-gene or host resistance (Heath, 2001). For example, non-host resistance has been associated with both the HR (Kamoun et al., 1998; Laugé et al., 2000; Vleeshouwers et al., 2000) and ROS production (Hückelhoven et al., 2001), and there is genetic evidence implicating gene-for-gene interactions controlling the non-host resistance of some cereals to non-specific races of the powdery mildew fungi (Matsumura and Tosa, 1995). In that study, the authors showed that the rye mildew fungus, *B. graminis* f. sp. *secalis*, which is a non-host pathogen on wheat, carries *Avr* genes corresponding to *R* genes effective against races of the wheat mildew fungus, *B. graminis* f. sp. *tritici*. Therefore, it seems that non-specific general defence mechanisms together with specific mechanisms based on gene-for-gene

interactions can contribute to non-host resistance, with the importance of either resistance mechanism varying between different pathosystems.

1.4 ENGINEERING DISEASE RESISTANCE IN PLANTS

The deployment of *R* genes in new cultivars is presently the most common way for breeders to introduce resistance in their crops to plant pathogens. However, in many cases, newly integrated *R* genes have been shown to lack durability in the field (Pink and Puddephat, 1999). Pathogens are usually able to overcome *R* gene-mediated recognition by accumulating mutations in their corresponding *Avr* genes. A good example is the barley leaf scald pathogen, *Rhynchosporium secalis*, whose highly variable pathogenicity has been well documented (McDonald et al., 1999; Salamati et al., 2000). In the long term, the use of single *R* genes is therefore not expected to provide good durable disease resistance. Furthermore, specific *R* genes may not always be present in crop plant species of commercial interest. Using genetic engineering to transfer *R* genes between different plant species is possible but there is no guarantee that this approach will work – especially if the species are not closely related (Rommens and Kishore, 2000).

1.4.1 Manipulation of genes encoding antimicrobial proteins

The discovery of some of the molecules and genes involved in plant disease resistance has provided the basis for genetic engineering strategies aimed at producing durable disease resistance. The traditional approach has involved constitutive over-expression of at least one gene encoding an antimicrobial compound such as a PR protein or synthesising a phytoalexin. Ever since the first report of a constitutively expressed chitinase (PR-3 protein family) conferring increased disease resistance in tobacco and canola plants to *Rhizoctonia solani* (Broglie et al., 1991), numerous studies have shown that up-regulating the expression of various PR proteins can result in plants having decreased disease severity after infection by fungal pathogens (Zhu et al., 1994; Jach et al., 1995; Tabei et al., 1998). Similarly, increased disease resistance has been reported in transgenic tobacco and alfalfa plants that constitutively express certain phytoalexins (Hain et al., 1993; He and Dixon, 2000; Hipskind and Paiva, 2000). At the same time, these strategies have not always proved to be successful. For

example, transgenic tobacco plants producing PR-5 did not prevent disease development when infected with *Phytophthora parasitica*, despite *in vitro* assays showing that PR-5 was effective against this fungus (Liu et al., 1994). Furthermore, reduced plant size and fertility has been observed in mutant plants which constitutively express *PR* genes (Bowling et al., 1994; 1997). In some cases, it has also been shown that resistance relies on the synergistic effects of different PR proteins (Jach et al., 1995; Jongedijk et al., 1995). In transgenic tobacco, co-expression of three barley *PR* genes that encode products with anti-fungal properties (a class II chitinase, a class II β-1,3-glucanase, and a type I ribosome-inactivating protein) led to significantly higher resistance to the fungus *Fusarium oxysporum* f. sp. *lycopersici* compared with the expression of each of these three genes alone (Jongedijk et al., 1995).

1.4.2 Manipulation of genes encoding key regulators of the defence response

An alternative, and perhaps more attractive approach to engineer disease resistance in plants involves activating the whole array of defence responses associated with *R* gene mediated resistance by over-expressing key components of the signal transduction pathway (Stuiver and Custers, 2001). Because the molecular events that occur after the specific interaction between *R* and *Avr* gene products appear to be non-specific, this approach does not dependent on a successful recognition event between plant and pathogen. Furthermore, the activation of a full-fledged defence response is expected to be far more effective than over-expressing single genes encoding antimicrobial compounds (Cao et al., 1998; Honée, 1999; Stuiver and Custers, 2001). Although the majority of the key regulators associated with *R* gene mediated resistance remains unknown, the report of Cao et al. (1998) demonstrates the potential of using this approach. In this study, a 2-3-fold over-expression of the *NIM1/NPR1* gene provided complete resistance to both the bacterial pathogen *P. syringae* and the oomycete pathogen *P. parasitica* – two very different pathogens that are virulent on wild-type *Arabidopsis*. Furthermore, NPR1 over-expressing plants did not show any significant difference in growth rate, morphology, or developmental timing compared to wild-type plants.

1.5 NET BLOTCH DISEASE OF BARLEY

1.5.1 The causal agent and forms of net blotch

Net blotch disease of barley (*Hordeum vulgare* L.) is caused by the hemibiotrophic fungus *Pyrenophora teres*. The disease has a worldwide distribution and is found wherever the crop is grown in temperate and humid conditions (Shipton, 1973). Relatively recent outbreaks of net blotch have been reported in Australia, New Zealand, Canada, Scandinavia, France, Germany, South Africa, and the Middle East, with reported grain yield losses ranging from 10 to 40% (McDonald and Buchannon, 1964; Jordan, 1981; Khan, 1987). Net blotch disease is associated with two different leaf symptoms: (1) a net-form (NF), and (2) a spot-form (SF) lesion. Smedegård-Petersen (1971) described how the causative pathogens were in fact derived from two existing *formae* of *P. teres*, for which he proposed the names *P. teres* f. *teres* (*Ptt*; NF symptoms) and *P. teres* f. *maculata* (*Ptf*; SF symptoms). The NF pathogen causes typical net blotch lesions that have dark brown striations extending longitudinally and transversely within a lesion to form a net-like pattern (Figure 2A), whereas the SF pathogen produces dark brown circular or elliptical lesions without netting (Figure 2B).

1.5.2 Fungal penetration and colonisation

P. teres is predominantly a seed-borne pathogen (Hampton, 1980; Jordan, 1981) but crop debris can also be a source of inoculum (Taylor et al., 2001). Under humid conditions the pathogen generates conidia (asexual spores) that are dispersed in moist air and that germinate following adhesion to the leaf surface at temperatures between 10 and 25°C (Keon and Hargreaves, 1983).

Differences in successful and abortive attempts by *P. teres* to penetrate and infect barley leaves were first described by Keon and Hargreaves (1983). In successful penetration and infection, conidia of *P. teres* germinated, formed appressoria and penetrated host epidermal cells within 24 hours of inoculation. Following penetration of the epidermal cell wall, the fungus developed intracellular infection vesicles. Once the epidermal cell layer had been breached, colonising hyphae from the fungus

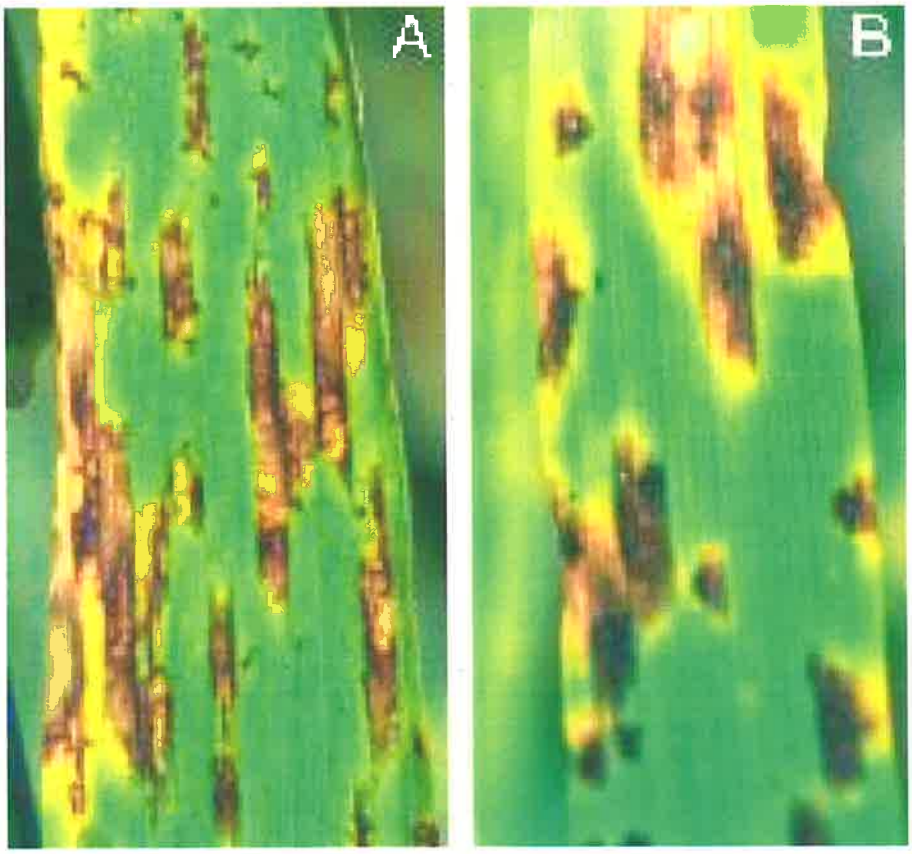

Figure 2: Barley leaves showing typical lesions of (A) net form of net blotch (caused by *Pyrenophora teres* f. *teres*), and (B) spot form of net blotch (caused by *Pyrenophora teres* f. *maculata*). [Figure adapted from Williams et al. (2001)].

developed exclusively in the apoplast of the mesophyll tissue. Lesion development was noticeable two days after inoculation. Schäfer (2000) confirmed these findings by monitoring the progress of fungal infection within the plant using transformed *P. teres* expressing the green fluorescent protein. It was reported that the infection cycle took approximately 10 days under controlled conditions.

In contrast, failure by the fungus to successfully penetrate the epidermal cell wall was associated with the deposition of appositions (or papillae) against the inner surface of the cell wall at the site of penetration (Keon and Hargreaves, 1983). This observation was made 24 hours after inoculation. In other studies, the formation of epidermal cell wall appositions in barley cells has also been linked to penetration resistance against *B. graminis* (von Röpenack et al., 1998) and *R. secalis* (Xi et al., 2000).

1.5.3 The genetics of net blotch disease resistance

Schaller (1955) discovered the first major *R* gene, *Pt1*, conferring net blotch resistance in barley. Since then, a number of genetic studies on the inheritance of net blotch disease resistance have shown that resistance is controlled by one or a few genes that confer a distinctly low (resistant) infection response (Mode and Schaller, 1958; Khan and Boyd, 1969; Bockelmann et al., 1977; Graner et al., 1996; Williams et al., 1999; Molnar et al., 2000; Manninen et al., 2000). It was also found that resistance to NF and SF is inherited independently (Ho et al., 1996). In the most recent studies, single dominant genes for net blotch resistance in barley have been identified using molecular marker mapping. For NF resistance, Graner et al. (1996) mapped a single dominant *R* gene from the cultivar "Igri" to the proximal region of chromosome 3HL – a region also characterised by the presence of additional *R* genes against barley yellow dwarf virus (*yd2*, Collins et al., 1996) and *R. secalis* (*Rhy*, Barua et al., 1993; *Rh*, Graner and Tekauz, 1996). Additionally, Manninen et al. (2000) identified and mapped another major *R* gene from accession "CI9819" to the centromeric region of the short arm of chromosome 6H. For SF resistance, a single major gene, *Rpt4*, was identified from "Galleon" and mapped to the long arm of chromosome 7H (Williams et al., 1999) while another resistance locus was mapped to chromosome 2H (Molnar et al., 2000). The quantitative inheritance of net blotch disease resistance has also been described (Arabi et al., 1990; Steffenson and Webster, 1992; Steffenson et al., 1996).

Steffenson et al. (1996) identified quantitative trait loci (QTLs) for seedling and adult plant resistance from the barley variety "Steptoe". For seedling resistance three QTLs were identified – one on chromosome 4H and the other two on chromosome 6H. For adult plant resistance seven QTLs were found – one QTL on each of the following chromosomes 2H, 4H, 5H, 6H, and 7H, and two QTLs on chromosome 3H. These results suggest that different sets of *R* genes are deployed depending on the developmental stage of the plant.

1.5.4 Molecular aspects of the barley defence response

The study of molecular events that take place during the interaction of *P. teres* and barley is an area that has received little attention. On the fungal side, Ruiz-Roldán et al. (2001) identified a mitogen-activated protein kinase gene from *P. teres* named *PTK1* that is required for conidiation, appressorium formation, and pathogenicity of *P. teres* on barley. The only known plant molecular event is that PR proteins accumulate in barley leaves infected with the net blotch pathogen. The PR proteins induced by *P. teres* include peroxidases, β-1,3-glucanases, chitinases, and thaumatin-like proteins (Reiss and Bryngelsson, 1996; Reiss et al., 2000; Reiss and Horstmann, 2001). Reiss and Bryngelsson (1996) made the observation that the same PR proteins induced following *P. teres* infection were also induced in barley leaves infected with *B. graminis* and *Puccinia hordei* - suggesting that barley may utilise the same general defence mechanisms against different pathogens. In addition, Reiss et al. (2000) also cloned and sequenced some cDNAs from barley leaves infected with *P. teres* that were not classified as genes encoding for PR proteins. These cDNAs were shown to be infection related and they had sequence homologies to a glutathione-S-transferase, a glycolate oxidase, wheat analogue like WIR- (for wheat induced resistance), WCI- (for wheat chemical induction), and WALI- (for wheat aluminum-induced) proteins, and a superoxide dismutase (SOD).

Due to the limited knowledge of plant genes involved in the barley defence response to *P. teres*, this project employed a strategy to compare net blotch resistant and susceptible interactions in order to better understand the barley molecular mechanisms leading to net blotch disease resistance.

1.6 PROJECT AIMS AND SIGNIFICANCE

Barley is the second most important cereal crop grown in Australia with an average annual production of approximately 5 million tonnes. The 3 million tonnes exported by Australia accounts for almost 20% of total world exports and is a significant source of revenue. To maintain this level of production, research is needed to implement effective strategies to manage diseases affecting yield and quality.

Although fungicides are commonly used to control barley net blotch diseases, several cases of *P. teres* isolates developing resistance to them have been reported (Sheridan and Grbavac, 1985; Peever and Milgroom, 1992, Campbell and Crous, 2002). This, combined with increased public concerns about fungicide use on food crops, makes the use of resistant barley varieties the most effective and economical method of controlling net blotch diseases. At present, the deployment of *R* genes in new cultivars provides good net blotch control, however, as a long term option for control of leaf diseases, alternative strategies may need to be developed to account for high pathogen diversity and mutation rates which can overcome the effectiveness of deploying *R* genes in new cultivars. Because *P. teres* has a mixed (sexual and asexual) reproductive system, it constitutes a high evolutionary risk and thus has the potential to overcome the deployment of major *R* genes (McDonald and Linde, 2002a&b). Therefore, it is necessary to investigate new strategies that can confer broad spectrum resistance to net blotch isolates, either independent of, or in tandem with, the current methodology of *R* gene deployment.

Potentially, a more effective way by which net blotch resistance can be introduced into barley breeding varieties is by genetically manipulating the plant so that it is always able to use it's own defence armoury to combat pathogen attack – irrespective of whether *R* gene mediated plant defence is engaged or not. The aim of this study was to identify and profile the expression of genes that are differentially expressed in the barley resistant response to both net blotch-causing pathogens. More specifically, the focus was on identifying genes that were differentially expressed in epidermal tissue within 24 hours of infection. As the epidermis is the closest point of contact between plant and pathogen, it represents the most likely source of finding early expressed defence response genes associated with net blotch resistance. Furthermore,

the epidermal cell layer has been reported to be the site of penetration when *P. teres* attempts to infect the host plant (Keon and Hargreaves, 1983; Schäfer, 2000).

The outcome of this study may provide breeders with information on genes critical to the defence response, which may provide the basis for breeding barley with more durable resistance to the net blotch pathogens, either by traditional or transgenic means.

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CHAPTER II

Identification of net blotch resistant and susceptible barley genotypes for differential screening

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2.1 INTRODUCTION

In the last two decades, many research groups have documented the reactions that occur between *P. teres* isolates (*Ptt* in most instances) and various barley lines. Barley lines range from being highly resistant to highly susceptible to both pathogenic forms of *P. teres* (Harrabi and Kamel, 1990; Douiyssi et al., 1998; Williams et al., 1999) and pathotype diversity has been shown to exist within populations of the fungus (Steffenson and Webster, 1992; Robinson and Jalli, 1996). With the aim of identifying the plant genes that may be involved in conferring resistance to both virulent *Ptm* and *Ptt* isolates, it was considered advantageous to work with one barley line that is resistant to both forms of net blotch, and a second one that is susceptible to both forms. For this purpose, the lines CI9214 and B87/14 were chosen. CI9214 is a resistant line identified in Canada that has resistance to some but not all *P. teres* isolates (Tekauz and Buchannon, 1977; Tekauz, 1990) and is being used in breeding programs, whereas B87/14, a South African line, was chosen on the basis of a previous seedling test which demonstrated its susceptibility to certain barley net blotch isolates (Dr. K. Williams, personal communication). This chapter outlines the pathological and molecular tests that were undertaken to (a) confirm the suitability of using these lines for differential screening, and (b) verify that a plant defence response had been initiated within 24 hours of infection.

2.2 MATERIALS AND METHOD

2.2.1 Plant material and pathogen inoculation

2.2.1.1 Plant and fungal material used

Seeds of barley cultivars CI9214 and B87/14 were provided by Dr. Hugh Wallwork (South Australian Research and Development Institute [SARDI], Adelaide, Australia). Australian field isolates of *Ptt* (19/98) and *Ptm* (43/96) were also provided by Dr. Wallwork. The isolates were originally obtained from barley leaves showing NF and SF symptoms that had been collected from fields in South Australia.

Species	Isolate code	Collection site	Collection year	Symptoms
Ptt	19/98	Warooka, S.A.	1998	Net form
Ptm	43/96	Waitchie, S.A.	1996	Spot form

2.2.1.2 Inoculum preparation

Freeze-dried symptomatic leaves infected with the *Ptm* and *Ptt* isolates were surface-sterilised by immersion for 30 s in anti-bacterial solution (Milton, Carole Park, Qld, Australia) and rinsed with sterile water. This sterilisation procedure was repeated 3 times under aseptic conditions in a laminar flow hood. Dried leaf sections were placed in a moist container and incubated at room temperature under continuous ultraviolet (UV) light for 2-3 days to induce sporulation. Single spores (conidia) were scraped from the leaf, transferred onto 3.9% potato dextrose agar (Becton Dickinson, Sparks, MD, USA) and left to germinate at room temperature for 2 days in the dark. Mycelial plugs (5 mm diameter) from a single germinated spore were then placed at the centre of 2% water agar (Becton Dickinson, USA) plates containing sterile barley leaf strips arranged in a circle around the central mycelial plug, and incubated at room temperature under UV light. All agar was supplemented with 0.01% streptomycin. To determine if the mycelia were generating spores, plates were periodically examined under a microscope. After 14 days, the plates were flooded with sterile water and gently scraped with a sterile brush to strip conidia from the agar surface. The conidial suspension was then filtered through a test sieve with a mean aperture size of 500

microns and adjusted to a concentration of 1 x 10^4 conidia/ml with the aid of a haemocytometer. Polyoxyethylenesorbitan monolaurate (Tween-20) was added to a final concentration of 0.01% and the suspension was sprayed onto 10 day old seedlings using a fine mist and applied at a rate of approximately 0.8 ml inoculum per plant.

2.2.1.3 Inoculations and sampling

Seeds of CI9214 and B87/14 were sown in University of California potting mix at a rate of 3 to 4 seeds per pot (cones 4 cm in diameter by 20 cm deep) and grown at 17°C under fluorescent light with a 12 h light/12 h dark photoperiod in a controlled-environment chamber. Once the plants had reached the first leaf stage (also referred to as the seedling stage), approximately 10 days after germination, they were sprayed with conidial suspensions (1 x 10^4 spores/ml in 0.01% Tween-20) of the virulent *Ptm* and *Ptt* isolates. Mock-inoculated plants were sprayed using the same conditions, but with a water solution containing only 0.01% Tween-20. After inoculation, the plants were kept at 19°C in the dark for 24 h at 100% relative humidity before being subjected to a 12 h light (at 24°C)/12 h dark (at 19°C) photoperiod once more. The abaxial side of the epidermis from infected and control leaves was peeled at 1, 3, 6, 12, 24, and 48 hours after inoculation (hai), and immediately frozen in liquid nitrogen. To reduce the impact of biological variation, ten epidermal samples per time point were pooled and snapped frozen. Some plants from each cultivar were kept in the controlled-environment chamber for 14 days as positive controls to confirm the expected resistant and susceptible phenotypes. Control plants were assessed for symptoms and rated according to a numerical lesion-type scale developed by Tekauz (1985). This numerical scale facilitates the assessment of resistance based on lesion morphology and was designed for use with inoculated plants grown under controlled conditions. Phenotypic scores range from 1 to 10, with 1 indicating the most resistant reaction type and 10 the most susceptible reaction type. For practical purposes of breeding for net blotch resistance, the transition between desirable resistant and undesirable susceptible reactions is in the lesion type 5-6 range. Lesions rated below type 5 remain restricted in size whereas those greater than type 5 expand over time. Therefore, for both forms of net blotch, resistant and susceptible phenotypes are broadly defined by lesion type scores of 1-5 and 6-10, respectively (Tekauz, 1985).

2.2.2 Molecular analysis

2.2.2.1 Fungal DNA extraction

DNA from the *Ptm* and *Ptt* isolates that were used for the inoculation experiments was isolated using the method of Campbell et al. (1999), with minor modifications. Briefly, *Ptm* and *Ptt* single spore isolates were grown on PDA for 7 days after which five mycelial plugs from these cultures were transferred to 2 L flasks containing 100 ml of a yeast and glucose medium (8 g/l yeast extract and 5 g/l glucose). Flasks were incubated on a rotary shaker at 150 rpm at room temperature to enable the fungus to grow. After 2 d, mycelia were removed from the liquid culture, blot dried on sterile filter paper, then transferred to 50 ml tubes and stored at -80°C until required. To extract the DNA, mycelia were crushed in liquid nitrogen to a fine white powder using a mortar and pestle, and approximately 250 µg was transferred to a 2 ml Eppendorf tube containing 500 µl fungal DNA extraction buffer of 50 mM Tris (pH 7.2), 50 mM NaCl, 50 mM EDTA, and 3% (w/v) SDS. Then, 350 µl phenol was added followed by 150 µl chloroform/isoamylalcohol (24:1, v/v). The suspension was shaken vigorously by hand, incubated at room temperature for 15 min while being further mixed on an orbital rotor, and subsequently centrifuged at 13,000 rpm for 60 mins. The aqueous phase was transferred to a clean Eppendorf tube and an equal volume of chloroform was added. After the suspension was mixed and centrifuged at 13,000 rpm for 10 min, the aqueous phase was once more transferred to a clean tube and subjected to two more chloroform extraction procedures. DNA was precipitated by adding 0.54 volumes isopropanol and incubating the tube at -20°C for 2 h, pelleted by centrifugation at 13,000 rpm for 5 min, washed twice in 70% ethanol, then dried and resuspended in 50 µl H_2O and stored at -20°C for future use.

2.2.2.2 Isolate-specific PCR

A PCR-based assay using the primers PTT-F/R and PTM-F/R developed by Williams et al. (2001) was used to confirm the identity of the isolates used for inoculations.

Primer	Sequence
PTT-F	CTCTGGCGAACCGTTC
PTT-R	ATGATGGAAAAGTAATTTGTA
PTM-F	TGCTGAAGCGTAAGTTTC
PTM-R	ATGATGGAAAAGTAATTTGTG

The PCR was conducted in 25 µl reactions containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.2 mM of each dNTP, 2.2 mM $MgCl_2$, 1 U *Taq* polymerase and 10 pmol of each primer. 1 µl of diluted (1:10) fungal DNA was used as template. The PCR cycling parameters followed a touchdown protocol that began with a denaturation step of 94°C for 30 s, an annealing step of 65°C reducing by 1°C per cycle to 56°C, and an extension step of 72°C for 30 s. There were 35 cycles in total, followed by an extra extension at 72°C for 10 mins. Amplifications were performed in a PTC-100 MJ Research thermocycler (Waltham, MA, USA) and products were separated through 1.5% agarose (Scientifix, Clayton, VIC, Australia) gels in TAE buffer (40 mM Tris base, 1 mM EDTA, 20 mM acetic acid) before being stained with 0.5 mg/L ethidium bromide and visualised under UV light.

2.2.2.3 Expression analysis of a defence-associated gene

RNA was extracted from inoculated and mock-inoculated whole leaf tissue harvested at 1, 3, 6, 12, and 24 hai as outlined in section 3.2.1. Northern blots were made as described in section 3.2.5. A cDNA clone encoding a barley PR-5 gene (Gregersen et al., 1997) was provided by Dr. Klaus Oldach (University of Adelaide) and labelled with [α-^{32}P] as outlined in section 3.2.5. Blots were hybridised with labelled probe and washed according to standard procedures (Sambrook et al., 1989).

2.3 RESULTS

2.3.1 Phenotypic analysis

Seedling tests for net- and spot-form of net blotch resistance and susceptibility were carried out using the barley lines CI9214 and B87/14. Plants were assessed for

phenotype 14 days after inoculation and scored using the numerical lesion-type scale developed by Tekauz (1985). Figure 3 shows a typical result that was obtained following inoculation with the *Ptt* isolate, 19/98. CI9214 seedlings showed strong resistance to this isolate and were given a reaction score of 1.5 whereas B87/14 seedlings showed clear net-form symptoms and were given a score of 7.0. For the inoculation experiment with the *Ptm* isolate 43/96, CI9214 and B87/14 seedlings responded in a similar fashion with respect to disease phenotype (data not shown). CI9214 showed minimal spot-form symptoms, achieving a reaction score of 3.0, whereas B87/14 showed more prominent disease symptoms characteristic of a susceptible response and was given a score of 6.0.

2.3.2 Molecular analysis of re-isolated *P. teres* isolates

In addition to using symptom expression as a means of identifying pathogen type and because the spore morphology of both *formae* of *P. teres* is almost identical, the PCR assay developed by Williams et al. (2000) was used to confirm that *Ptm* and *Ptt* isolates were responsible for the net blotch disease symptoms observed in the B87/14 cultivar. The assay was performed with fungal DNA that had been extracted from cultures of four single spores isolated from leaf tissue displaying prominent net- or spot-form lesions. The results of this assay show that the *formae*-specific primers amplified the diagnostic bands that are used to distinguish one isolate type from the other (Figure 4). Primer set PTM amplified the *Ptm*-diagnostic 411 bp band from leaves showing spot-form symptoms only, whereas primer set PTT only amplified the *Ptt*-diagnostic 378 bp band from leaves displaying net-form symptoms. No amplification products were generated when PTM and PTT primers were used to analyse leaves with net- and spot-form lesions, respectively. These results confirmed that the disease symptoms observed in the net blotch susceptible cultivar, B87/14, were attributed to infection from either a *Ptm* or *Ptt* isolate.

2.3.3 *PR-5* defence gene induction

To establish if the barley cultivars B87/14 and CI9214 activate a defence response within 24 hours of inoculation with either the *Ptm* or *Ptt* isolate, the expression of a *PR-5* gene was analysed in leaf tissue isolated from infected seedlings at 1, 3, 6, 12,

Figure 3: CI9214 and B87/14 seedling reactions 14 days after inoculation with *Ptt* isolate, 19/98. Plants were scored according to the numerical lesion-type scale developed by Tekauz (1985). CI9214 and B87/14 were given seedling reaction scores of 1.5 (resistant) and 7.0 (susceptible), respectively.

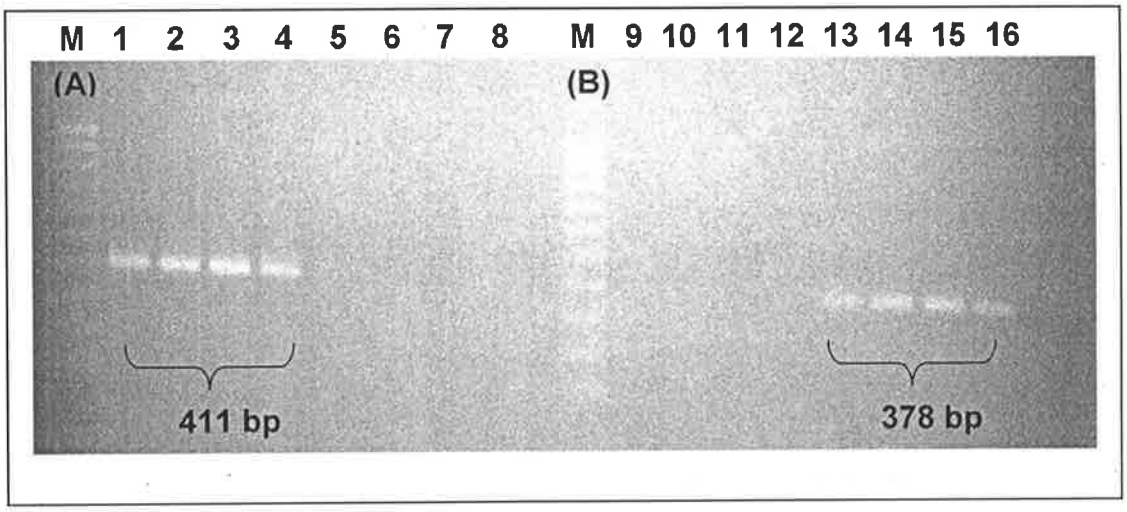

Figure 4: Agarose gel showing amplification products from *P. teres* isolate-specific PCR using DNA template from single spore isolates derived from **(A)** the *Ptm* inoculation, and **(B)** the *Ptt* inoculation. Lanes 1-4 and 9-12: amplification products using the PTM primers. Lanes 5-8 and 13-16: amplification products using the PTT primers. Lane M: 1 kb DNA ladder (in 100 bp increments).

and 24 hai. In both NF and SF incompatible interactions, *PR-5* was expressed weakly at 12 hai and then more strongly at 24 hai. In contrast, although *PR-5* appeared to be induced to a similar extent 24 hai in both compatible interactions, no expression signal was detected at the earlier time point of 12 hai (data not shown).

2.4 DISCUSSION

In this chapter, seedlings of the barley cultivars CI9214 and B87/14 were assessed for their reaction to challenge by South Australian *Ptm* and *Ptt* isolates. As anticipated, there were clear differences in the way these two cultivars responded to infection by the two isolates. CI9214, a Canadian breeding line previously associated with net blotch resistance (Tekauz and Buchannon, 1977; Tekauz, 1990; Williams et al., 1999), showed resistance to both isolates used, whereas B87/14 was susceptible to both. Also, CI9214 appeared to be more resistant to the *Ptt* isolate (reaction score: 1.5) than the *Ptm* isolate (reaction score: 3.0). The seedling reaction score to the *Ptm* isolate is in accordance with Williams et al. (2003) who reported a score of 3.5 in the CI9214 seedling reaction to a mixture of five *Ptm* isolates, which included *Ptm* isolate 43/96 used in this study. In addition, using a combination of candidate markers and F_2 crosses, the authors were able to show that CI9214 SF seedling resistance was attributed to *Rpt4*, the SF *R* gene originally identified in the cultivar Galleon (Williams et al., 1999). In contrast, the genetic source of NF resistance in CI9214 is unknown. Based on the findings of Ho et al. (1996) who reported that resistance to NF and SF is inherited independently, it is not likely that *Rpt4* contributes to NF resistance in CI9214.

Because NF and SF lesions on barley can be confused with each other, it was important to correctly identify isolates from infected leaf samples to make sure that the observed symptoms were not caused by a mixed infection. By using the PCR-based assay developed by Williams et al. (2001), it was conclusively shown that the NF and SF symptoms on B87/14 seedlings were indeed caused by isolates of *Ptt* and *Ptm*, respectively. Combined with the phenotypic analysis, these results confirmed that CI9214 and B87/14 were suitable cultivars to conduct a screen for genes that are differentially expressed in the incompatible interaction between barley and both *formae* of *P. teres*.

When exposed to pathogens, many plants accumulate several groups of PR proteins (Van Loon and Van Strien, 1999). To verify that a defence response had been initiated by the plant within 24 hours of infection by the *P. teres* isolates used, the expression of a *PR-5* gene was monitored in infected leaf tissue at 1, 3, 6, 12, and 24 hai. It has previously been reported that the same *PR-5* gene is induced early in barley plants that are resistant to scald, with transcript accumulation peaking at 24 hai. In contrast, expression of this gene is weaker and occurs later in a susceptible cultivar (Hahn et al., 1993; Steiner-Lange et al., 2003). A similar induction behaviour for *PR-5* was observed in the present study, with transcripts accumulating earlier and more strongly in leaves of the net blotch resistant cultivar, CI9214. Gene expression was seen as early as 12 hai and peaked at 24 hai. In contrast, although a comparably high level of *PR-5* gene induction was observed at 24 hai in the susceptible cultivar, B87/14, the gene was not expressed at 12 hai. Combining these observations with those made by Keon and Hargreaves (1983), who showed that *P. teres* was capable of penetrating the barley epidermal cell layer within 24 hai, it is evident that defence responses are initiated by the plant within hours of pathogen contact. More significantly, expression profiling of *PR-5* in the barley-*P. teres* interaction revealed that defence-related gene transcripts may also accumulate differentially in the early stages of incompatible and compatible interactions. Therefore, in this study, the 24 hai time point was selected to look for genes that were differentially expressed in the barley-*P. teres* incompatible interaction.

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CHAPTER III

Differential accumulation of plant defence response genes in the barley-*P. teres* incompatible interaction

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3.1 INTRODUCTION

The study of host genes whose expression patterns are altered after encountering a particular stimulus is commonplace as the products of these genes often play an important functional role in regulating the host response. Differential gene expression analysis involves comprehensive sampling of the cDNA of two or more cDNA populations together with the sensitive detection of differences in mRNA abundance for both known and novel genes. Differential display of mRNA (Liang and Pardee, 1992), cDNA-AFLP (Bachem et al., 1996), and suppression subtractive hybridization (SSH) (Diatchenko et al., 1996) are the three main PCR-based techniques that have been used to study differential gene expression in a range of biological systems including plant-pathogen interactions (Vercauteren et al., 2001; Xiong et al., 2001). Based on a number of studies that have analysed the efficiency of each PCR-based technique the general consensus points to SSH as being the most reliable method for isolating differentially expressed gene transcripts. The inclusion of a normalisation step in the subtraction procedure generates high level enrichment of rare transcripts thereby making the concentrations of high and low abundance cDNAs roughly equal and thus giving this protocol a distinct advantage over differential display and cDNA-AFLP which generally enrich for more abundant transcripts (Bertioli et al., 1995). The inability to equalise concentrations of high and low abundance cDNAs can contribute to the repeated isolation of abundant genes as shown by Kuang et al. (1998) who used differential display and reported that they isolated cytokeratin 18, a gene differentially expressed in a breast carcinoma cell line, 110 times. It has also been shown that the short primers and low annealing temperatures used in differential display PCR may produce non-specific background products leading to problems in cloning and analysis of the PCR products which first need to be excised from high resolution gels (Jones and Harrower, 1998). Dilger et al. (2003) recently used both cDNA-AFLP and SSH to identify differentially expressed genes during conidial germination in *P. teres* and they

reported that the procedure for cloning SSH fragments was far superior to that of cDNA-AFLP. Only 3 of the 35 differentially expressed transcripts they examined were derived from cDNA-AFLPs. Therefore, of the available PCR-based methods for differential gene expression analysis, SSH was chosen for this study because it facilitates the isolation of differentially expressed transcripts of low abundance and does not rely on gels for analysis and cloning of PCR products.

3.1.1 Suppression subtractive hybridisation (SSH) overview

A schematic representation of the SSH method derived essentially from Diatchenko et al. (1996) is shown in figure 5. Firstly, two double-stranded (ds) cDNA populations are defined – "tester" cDNA, which contains the differentially expressed cDNAs (target), and "driver" cDNA in which the target transcripts are absent or present at lower levels. The tester and driver cDNA populations are digested with a four-base cutting restriction enzyme (usually *Rsa*I) and the tester cDNA fragments are divided into two samples (1 and 2) and ligated with two different adapters (adapter 1 and adapter 2). Two hybridisation steps then follow. In the first, an excess of driver is added to each sample of the tester. Following heat-denaturation and annealing, the single-stranded (ss) cDNA fraction (a) is normalised, resulting in concentrations of high and low abundance cDNAs becoming roughly equal. This is a result of the reannealing process generating homo-hybrid cDNAs (b) being faster for the more abundant molecules. Furthermore, the ss cDNA fraction (a) is significantly enriched in cDNAs for differentially expressed genes, as "common" non-target cDNAs form heterohybrids (c) with the driver.

In the second hybridisation the two samples from the first hybridisation are mixed together. As well as the formation of (b) and (c) hybrids from the reassociation of the remaining normalised and subtracted ss tester cDNAs, new hybrids (e) are formed that have different adapter sequences (one from sample 1 and the other from sample 2) at their 5' ends. Addition of more denatured driver at this stage further enriches fraction (e) for differentially expressed genes. Because they have different adapter sequences, the subtracted and normalised (e) molecules can be preferentially amplified by PCR using a pair of primers corresponding to the outer parts of adapters 1 and 2. For this selective amplification to occur, an extension reaction needs to be performed first to

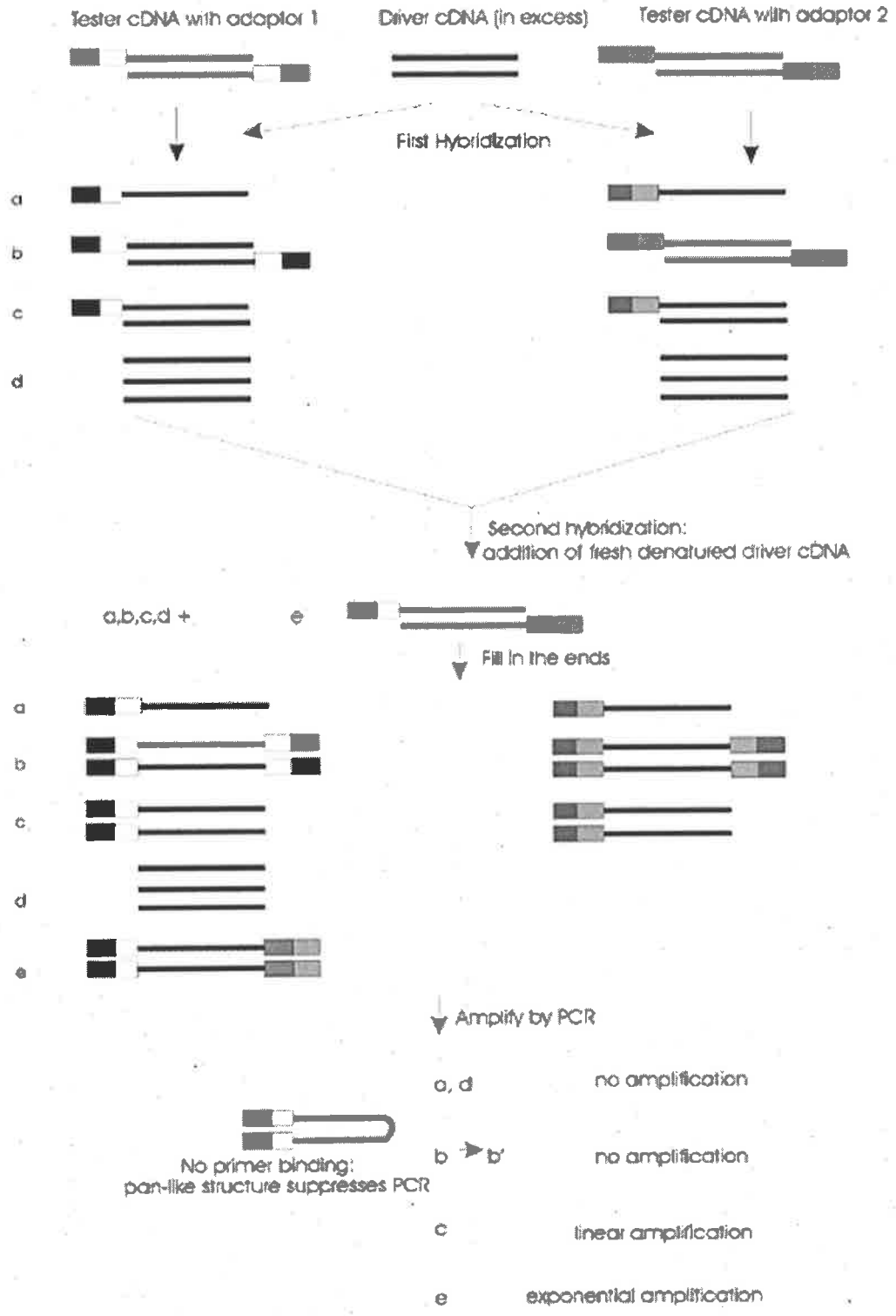

Figure 5: Schematic diagram of the SSH procedure described by Diatchenko et al. (1996). Boxes represent the inner and outer portions of adaptors 1 and 2. Solid lines represent the *Rsa*I-digested tester or driver cDNA.

fill in the sticky ends of the molecules for primer annealing. The nature of the PCR means that exponential amplification can only occur with type (e) molecules. This is the suppression PCR effect. Type (b) molecules contain long inverted repeats on the ends and form stable "panhandle-like" structures that cannot serve as templates for exponential PCR. Type (a) and (d) molecules do not contain primer-binding sites, and type (c) molecules can only be amplified at a linear rate. In a subsequent PCR, further amplification of type (e) molecules is achieved using nested primers. In this PCR step, background PCR products that were amplified from the first round of PCR are further reduced in proportion. The enriched differentially expressed PCR products can then be inserted directly into a T/A vector and sub-cloned.

3.2 MATERIALS AND METHODS

3.2.1 RNA extraction

Before starting RNA extraction, the workbench surface and apparatus used for extraction were thoroughly rinsed with RNase*Zap*™ (Ambion, Austin, TX, USA) to prevent RNase contamination as specified in the manufacturer's user manual. Epidermal peels from infected and control leaves (see section 2.2.1.3) were ground in liquid nitrogen and total RNA was extracted using Tri Reagent (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's instructions. To reduce the impact of biological variation, total RNA was extracted from ten epidermal samples per time point. Typical yields were 0.3 µg RNA per epidermal peel.

3.2.2 Suppression subtractive hybridisation

3.2.2.1 Tester and driver double-stranded cDNA preparation

The BD SMART™ PCR cDNA synthesis kit (BD Biosciences Clontech, Palo Alto, CA, USA) was used to generate tester and driver ds cDNA for SSH according to the manufacturer's instructions. In this protocol, approximately 0.5 µg of total RNA was used for first-strand cDNA synthesis. Two populations of tester and driver cDNA were prepared, one for the NF associated SSH and one for the SF. In both

subtractions, total epidermal RNA from inoculated CI9214 (resistant) and B87/14 (susceptible) seedlings at 24 hai was the source of tester and driver cDNA, respectively. Following synthesis, the cDNAs were digested with *Rsa*I and purified.

3.2.2.2 Subtractive hybridisation

SSH was performed using the Clontech PCR-Select cDNA subtraction kit (BD Biosciences Clontech, USA) according to the manufacturer's protocol beginning with the ligation of adapters to the digested tester cDNA population. Two hybridisations were carried out to obtain differentially expressed cDNAs. The first hybridisation was to achieve equalisation and enrichment of differentially expressed sequences, while the second hybridisation was to form ds cDNA tester molecules with different adapters on each end, that correspond to differentially expressed cDNAs.

3.2.2.3 Suppression PCR amplification

Two PCR amplifications were performed as described in the protocol. The first was to amplify exponentially ds cDNAs with different adaptor sequences on each end, and the second was to enrich the differentially expressed cDNAs, and further reduce background.

3.2.3 Subtracted cDNA library synthesis

Products of the second PCR from both net and spot form associated subtractions were directly inserted into the pCR®2.1-TOPO® plasmid vector (Invitrogen, Carlsbad, CA, USA) and transformed into chemically competent *Eschericia coli* strain DH5α cells. All recombinant clones were grown at 37°C overnight in 96-well plates in 200 µl Luria-Bertani (LB) media containing Ampicillin (100 mg/L). The cDNA inserts were then amplified by colony PCR using a PTC-100 thermocycler (MJ Research, USA) and M13 forward and reverse primers, which were complementary to vector sequences flanking both sides of the insert. The final reaction volume of 25 µl was comprised of a mixture containing 1X reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.15 µM of each primer, 0.5 units of *Taq* DNA polymerase, and 1 µl of

bacterial culture. The PCR was effected using the following cycling conditions: 1 min at 95°C, 40 s at 94°C, 35 s at 57°C, 1 min at 72°C, then 35 cycles of 40 s at 94°C, 35 s at 52°C, 1 min at 72°C, and a final extension step of 10 min at 72°C. PCR products were electrophoretically separated on a 1.2% agarose gel, stained with ethidium bromide, then visualised and photographed under UV light.

3.2.4 Differential screening of the cDNA libraries using macroarrays

3.2.4.1 Preparation of cDNA macroarrays

To prepare cDNA macroarrays, 5 µl of the PCR reaction used to amplify cDNA inserts from the subtracted libraries was combined with an equal volume of freshly made 0.6 M NaOH to denature the DNA. Two microlitres of denatured product was then spotted in duplicate onto Hybond-N^+ nylon membranes (Amersham Biosciences, Little Chalfont, BUCKS, England). The membranes were neutralised in 0.5 M Tris-HCl (pH 7.5) for 4 min and the DNA was cross-linked to the membrane under 120 mJ of UV light. Two identical membranes were prepared for each of the subtracted libraries.

3.2.4.2 Hybridisation and autoradiography

Macroarrays were hybridised with [α-^{32}P] dCTP-labelled probes from tester and driver cDNA as well as the subtracted cDNA in hybridisation buffer containing 5X SSC, 5X Denhardt's solution, 0.5% (w/v) SDS, and 100 µg/ml salmon sperm DNA. Hybridisation was performed at 72°C overnight with continuous agitation. The membranes were then washed four times with a low-stringency washing solution (2X SSC/0.5% SDS) at 68°C, two times with a high-stringency washing solution (0.2X SSC/0.5% SDS) at 68°C, and placed in contact with phosphor imaging plates (BAS-MP 2040S; Fujifilm, Minato, Tokyo, Japan) in cassettes (BAS cassette 2040; Fujifilm, Japan) at room temperature for 24 h. The imaging plates were scanned using a STORM 860 phosphorimager (GE Healthcare, Little Chalfont, BUCKS, England) and the resulting hybridisation signals were inspected visually. The membranes were stripped for re-use by washing them in 0.5% SDS at 100°C for 7 min.

3.2.5 Differential screening of selected clones using Northern blots

Total RNA was extracted from inoculated and mock-inoculated whole leaf tissue harvested at 24 hai using Tri reagent as outlined in section 3.2.1. To check RNA integrity, 10 µg RNA was electrophoresed on a 1.2% (w/v) agarose-formaldehyde gel. For Northern blot analysis, 10 µg of total RNA was transferred to Hybond N^+ nylon membranes (Amersham-Pharmacia, UK) according to standard procedures (Sambrook et al., 1989). Probes were made from PCR-amplified fragments of selected SSH clones, purified with QIAquick PCR purification columns (Qiagen, Valencia, CA, USA), and labelled with [α-^{32}P] using the *redi*prime^{TM} random prime labelling system (Amersham-Pharmacia, UK) as per the manufacturer's instructions. Incorporation of [α-^{32}P] into the probe was confirmed using a geiger counter. Only probes with radioactivity >1kcps were used for hybridisation. Blots were hybridised with labelled probes and washed according to standard protocols (Sambrook et al., 1989).

3.2.6 cDNA sequencing and analysis

3.2.6.1 Template preparation and sequencing

cDNA inserts to be sequenced were amplified by colony PCR as described in section 3.2.3. The PCR products were purified using QIAquick PCR purification columns (Qiagen, USA) following the product manual and quantified using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). Cycle sequencing reactions were prepared using the Big Dye Terminator Version 3.1 labelling kit (PE Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocol but modified so that one-eighth of the recommended reaction mix was used to conserve reagents. DNA sequencing was performed on an ABI 3700 DNA Sequencer (PE Applied Biosystems, USA).

3.2.6.2 cDNA sequence analysis

cDNA sequences were edited using Vector NTI software (Invitrogen, USA) to remove sequencing ambiguities and vector sequences. The edited sequences were used to

query the public nucleotide and amino acid sequence databases available from the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov). Sequence comparisons were carried out using the basic local alignment search tool (BLAST) algorithms, BLASTn and BLASTx (Altschul et al., 1990).

3.3 RESULTS

3.3.1 cDNA library construction

Two libraries of putative differentially expressed cDNA transcripts arising from the barley-*Ptt* and barley-*Ptm* incompatible interactions were generated following SSH. In all, more than 1000 colonies were picked from both transformations and screened by colony PCR for positive incorporation of SSH transcripts into the plasmid vector. The NF cDNA library consisted of 335 positive clones whereas 367 positive clones made up the SF cDNA library (barley-*Ptm* interaction).

3.3.2 Macroarray analysis

The efficiency of the SSH procedure was evaluated by spotting each clone in duplicate onto a nylon membrane which was then probed with the tester and driver pool of cDNA fragments as well as the subtracted fragments from the respective library. A representative differential screen is shown in figure 6. In these experiments almost all of the 335 NF and 367 SF spotted clones emitted a strong signal following hybridisation with the subtracted cDNA probe (Figure 6C). In contrast, when these same clones were screened with either tester or driver cDNA probes the hybridisation signals were weak or non-existent for 242 NF (72%) and 302 SF (82%) clones (Figures 6A and 6B). These were classified as category (1) clones and were thought to represent low abundance transcripts that had been enriched during subtraction.

The relatively small percentage of clones that did produce a signal following hybridisation with tester and driver cDNA probes were grouped into three different

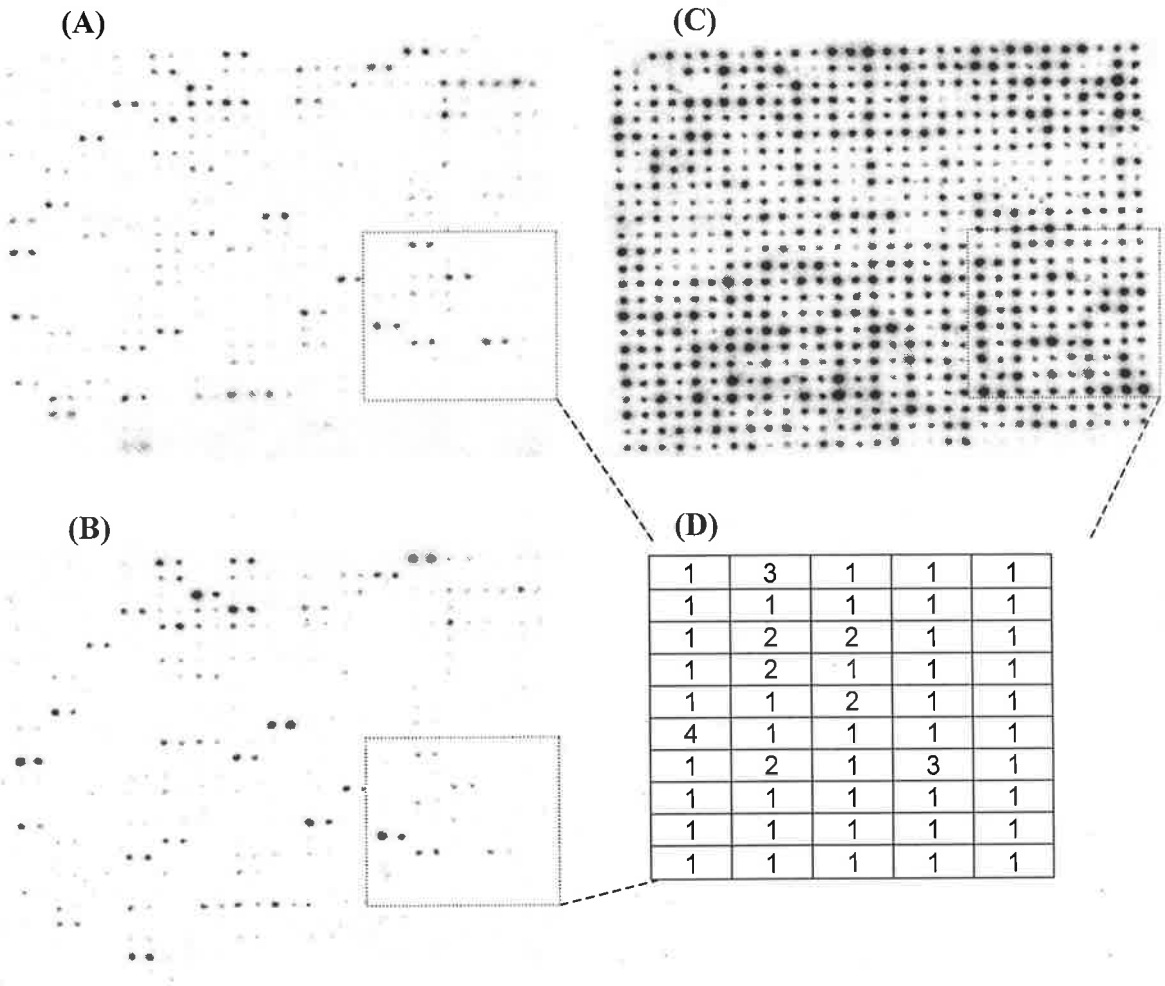

Figure 6: Representative hybridisation profiles corresponding to barley-net blotch resistant **(A)** and susceptible **(B)** interactions. Macroarray filters containing duplicates of 367 cDNAs from the SF SSH library were hybridised with ^{32}P-labelled cDNA prepared with epidermal RNA from the net blotch resistant, CI9214 [tester] **(A)**, and susceptible, B87/14 [driver] **(B)**, barley cultivars. Diagram **(C)** represents the same filter hybridised with ^{32}P-labelled subtracted [tester - driver] cDNA derived from the SSH. Based on their hybridisation profiles, the clones were categorised into the following groups: **Category 1** clones were of low abundance and potentially differentially expressed; **Category 2** clones were not differentially expressed; **Category 3** clones were up-regulated in the net blotch resistant interaction; **Category 4** clones were down-regulated in the net blotch resistant interaction. Diagram **(D)** shows examples of each category from a portion of the macroarray. Category 1 clones are clearly most numerous.

categories based on their hybridisation profiles. The most numerous of these were clones that were found to hybridise to subtracted probes and equally to both tester and driver probes and this group consisted of 48 NF (20%) and 30 SF (10%) clones. These were classified as category (2) clones and were discarded as false positives because they appeared not to be differentially expressed. Category (3) and (4) clones displayed a hybridisation signal that was either stronger or weaker (difference in signal intensity > 2-fold judged by visual inspection) with the tester than with the driver cDNA probe and these clones were thought to represent differentially expressed transcripts of high abundance. Category (3) consisted of 3 NF (<1%) and 15 SF (4%) transcripts that appeared to be up-regulated in the resistant genotype whereas category (4) was comprised of 12 NF (4%) and 11 SF (3%) clones that seemed to be down-regulated. Figure 7 summarises the distribution of the SSH clones into the four categories described based on their macroarray hybridisation profiles.

3.3.3 Northern blot analysis

Multiple Northern blots of whole leaf RNA were used to screen a number of category (1) SSH clones selected at random to confirm that they were (a) pathogen-induced and (b) differentially expressed between the resistant and susceptible net blotch cultivars at 24 hai.

The SSH procedure was performed using tester and driver cDNA generated from leaf tissue that had been inoculated with either the NF- or SF-causing pathogen. As no mock-inoculated leaf tissue was used for SSH it was necessary to confirm that the isolated clones were in fact infection-related as well as being differentially expressed. This was achieved by comparing expression profiles between pathogen-inoculated and mock-inoculated leaf tissue sampled at 24 hai by visual inspection.

In total, 73 random category (1) NF and SF clones that were representative of low abundance transcripts that had been enriched during subtraction were selected for Northern blot analysis. From this group, no hybridisation signal was detected for 26 of the probes used. From the remaining 47 clones for which a hybridisation signal was observed, 23 appeared to be pathogen-induced as indicated by differences in

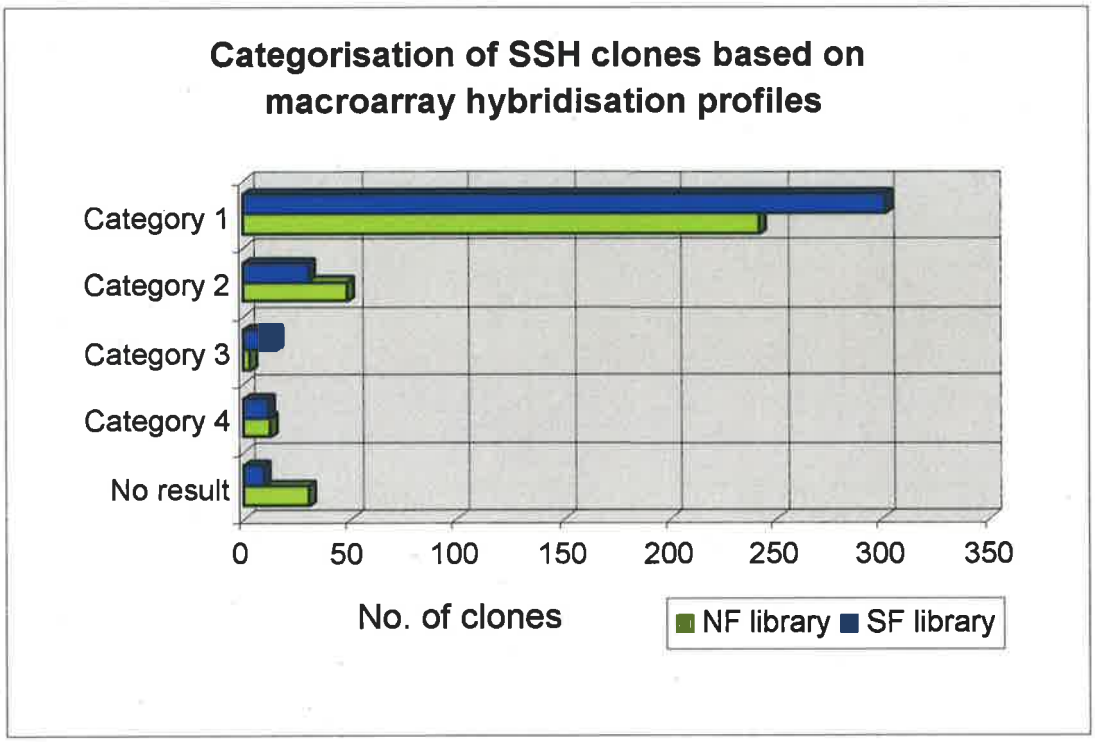

Figure 7: Categorisation of SSH clones from the NF (green) and SF (blue) libraries based on their macroarray hybridisation profiles. **Category 1**: clones which hybridised to probes derived from subtracted cDNA but not from tester or driver cDNA; **Category 2**: clones which hybridised to the subtracted cDNA probe, and equally to both tester and driver cDNA probes; **Category 3**: clones which hybridised to subtracted, tester, and driver cDNA probes, but with the tester cDNA probe showing a signal intensity >2 times that of the driver; **Category 4**: clones which hybridised to subtracted, tester, and driver cDNA probes, but with the driver cDNA probe showing a signal intensity >2 times that of the tester; **No result**: Clones which did not hybridise to the subtracted, tester, or driver cDNA probes.

expression between pathogen- and mock-inoculated samples. There was no change in expression for the other 24 clones i.e. their corresponding genes were expressed at the same level across all experimental treatments at the 24 h time point.

To verify differential expression, and hence identify putative net blotch resistance-associated SSH clones, the expression levels of the 23 pathogen-induced clones were compared in the resistant (CI9214) and susceptible (B87/14) genotypes. This analysis revealed that 12 clones were differentially expressed. Six of these clones (NF267, NF434, NF528, NF579, SF310, SF432) were up-regulated with the level of expression greater in the resistant genotype while the other six (NF229, NF294, NF472, NF717, SF606, SF641) were down-regulated with a level of expression greater in the susceptible genotype. Based on these results, the overall efficiency of the SSH procedure in isolating differentially expressed transcripts as determined by Northern blot-mediated expression profiling of whole leaf RNA was calculated to be 26% i.e. there were 12 differentially expressed clones among the 47 for which a hybridisation signal was detected. These results are summarised in figure 8A.

Representative hybridisation profiles for eight clones that were used to probe NF and SF Northern blots either simultaneously or individually are shown in figures 8B and 8C, respectively. All of these clones appeared to be pathogen-induced 24 hai as shown by their differential expression in pathogen- compared to mock-inoculated leaf tissue. NF434, NF528, and SF310 are examples of clones whose expression was observed to be up-regulated in the incompatible compared to compatible interaction whereas NF472, SF606, and SF641 appeared to be slightly down-regulated. NF353 and NF717 were pathogen-induced but not differentially expressed. The full list of 73 clones subjected to Northern blot analysis together with their differential classification based on their hybridisation profiles is shown in appendix A. Another interesting observation was that when clones isolated from either the NF or SF interaction were used to probe both NF- and SF-derived Northern blot membranes, the resulting hybridisation profiles from the two different interactions were very similar – if not the same (Figure 8B).

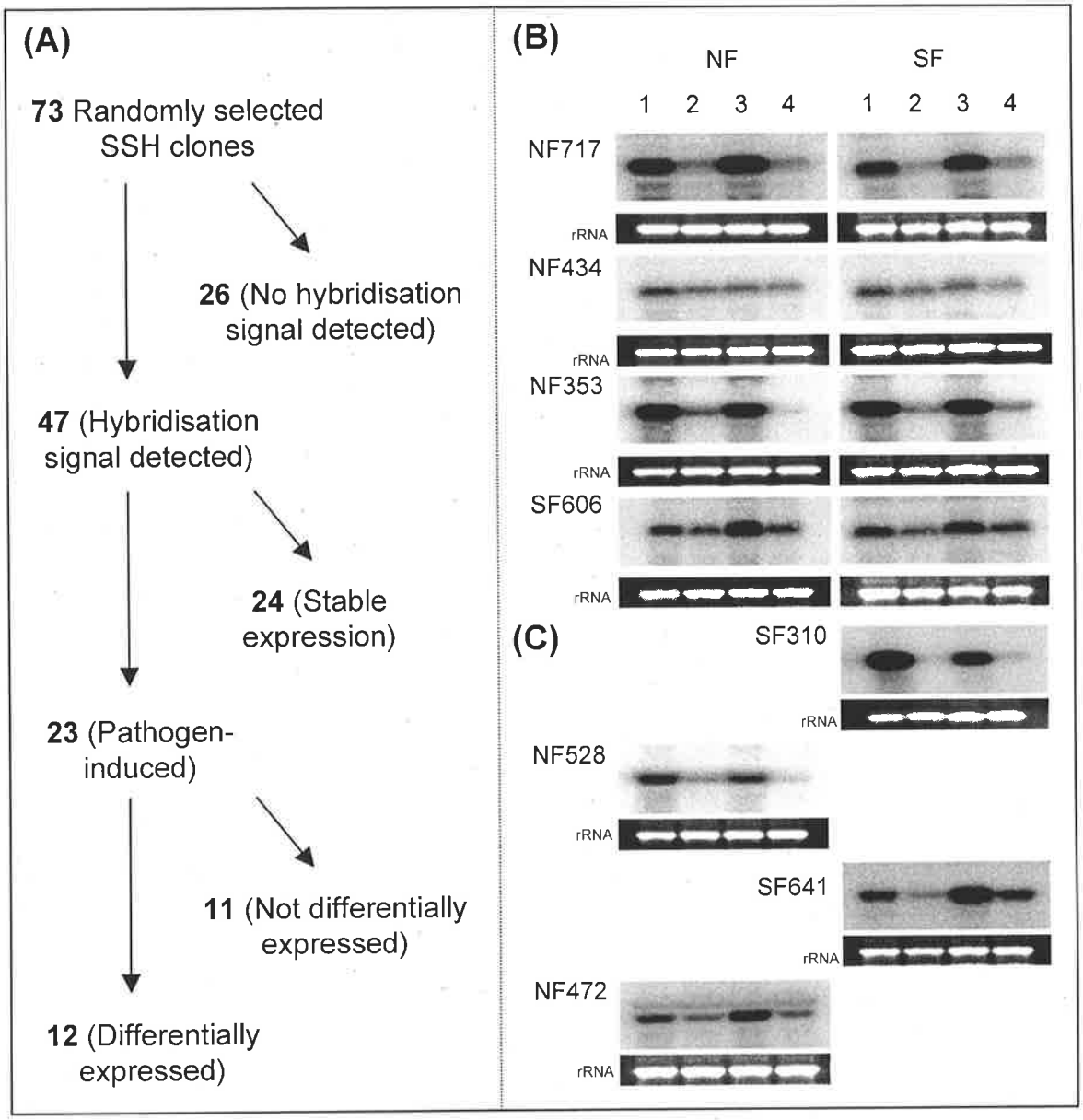

Figure 8: Summary of Northern blot analysis and representative hybridisation profiles for selected SSH clones. **(A)** Clones whose corresponding probes gave a hybridisation signal were broken down to identify the ones that were both pathogen-induced and differentially expressed. **(B** and **C)** Representative hybridisation profiles for clones in either NF or SF interactions - or both. RNA from whole leaf tissue was used for the blots and the leaves were harvested 24 hai. Lane 1: CI9214 inoculated with appropriate *P. teres* isolate; Lane 2: CI9214 mock-inoculation; Lane 3: B87/14 inoculated with appropriate *P. teres* isolate; Lane 4: B87/14 mock-inoculation. Equal quantities of RNA loading is shown by comparable intensities of ethidium bromide-stained bands of rRNA.

3.3.4 Sequence analysis of the SSH clones

A total of 440 cDNA clones (199 SF + 241 NF) were randomly selected for sequencing from among the 702 clones in the two cDNA libraries. The initial sequencing of some category (2) and all 12 category (3) and (4) clones, which according to the macroarray analysis were differentially expressed, revealed a high proportion of clones having sequence homology with genes encoding either ribulose-1,5-bisphosphate carboxylase (RUBISCO), the photosystem II (PSII) protein D1 or a subtilisin-chymotrypsin inhibitor 2 (CI2). This accounted for much of the redundancy observed in the two cDNA libraries and consequently 75 clones (17%) were sequenced that represented these proteins (42 RUBISCO, 19 CI2, and 14 PSII protein D1). The vast majority of clones that were selected for sequencing comprised low abundance category (1) transcripts. Although the frequency with which identical fragments were isolated and sequenced was low, some redundancy resulting from the sequencing of two or more different fragments representing a single transcript did occur. This form of redundancy was observed for 191 clones (43%) and resulted in the formation of 58 different contigs. Thus of the 440 cDNA clones that were sequenced, 307 aligned to individual genes of which 249 were represented by one copy in the library.

3.3.5 Categorisation of the SSH clones into functional classes

The 307 individual gene transcripts were categorised according to probable function based on their alignments with known sequences in the protein database using the BLASTx algorithm (Altschul et al., 1990) (Appendix B). A similarity to a known sequence was considered as significant if the obtained BLAST score had an E-value of less than $1x10^{-5}$. Because many of the clones were short in length (222 bp average) and likely to have originated from the 3' end of the gene, there was insufficient coding information from the insert sequence alone to predict their functions using BLAST analysis. Therefore, in many cases the amino acid sequence encoded by the best barley expressed sequence tag (EST) match was used to probe the protein database to determine putative function. From the 307 gene transcripts, 282 showed significant similarities to barley ESTs and thus it was assumed that they were derived from the host and not the pathogen. The remaining 25 transcripts that did not show significant

homology with barley ESTs were classified as "novel" genes – representing either fungal genes or previously undiscovered barley genes.

The distribution of clones from both SSH libraries into their functional categories is illustrated in figures 9A and 9B. It is clearly evident that both SSH libraries show a similar distribution pattern, with genes encoding unknown and metabolism-related proteins comprising almost 50% of all categorised transcripts. In addition, the Venn diagram in figure 9C shows that 25 (8%) of the 307 individual gene transcripts were common to both SSH libraries. These are listed in table 1. Thirteen of the clones in table 1 represent single unique genes (CON2, 4, 6, 16, 25, 27, 29, 30, 36, 46, 47, 49, 51), whereas the remaining twelve correspond to multiple gene transcripts for RUBISCO (CON1, 43), PSII protein D1 (CON32, 53), CI2 (CON7, 48, 52), a translation initiation factor (CON3, 15, 38), or a subunit of adenosine triphosphate (ATP) synthase (CON8, 22).

To help describe the observed distribution patterns, the NF and SF SSH libraries were combined (Figure 10). Together, novel genes, and those encoding unknown proteins and proteins of unknown function were represented by 129 (41%) clones in the combined library. The second and third largest groups were attributed to clones encoding proteins involved in cell metabolism and stress/defence responses. They were represented by 67 (22%) and 34 (11%) clones, respectively. Individually, the remaining five functional groups were not represented by more than 21 (7%) clones. 21 (7%) clones showed homology with genes that code for proteins associated with gene expression/regulation, 17 (6%) were similar to signal transduction components, 15 (5%) with proteins involved in proteolysis, and 12 (4%) were represented by clones encoding either transporters or proteins associated with cell division/growth.

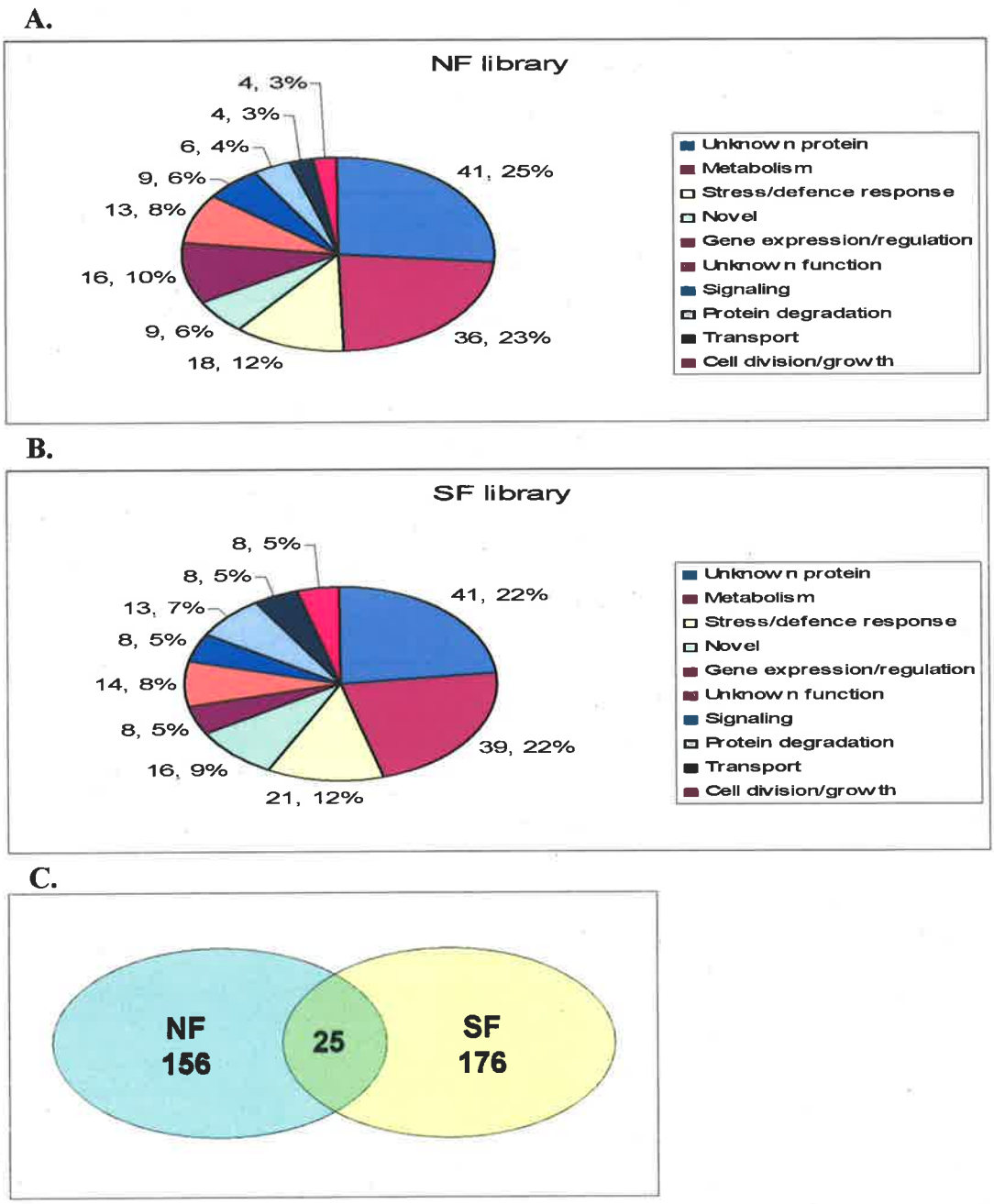

Figure 9: Distribution of SSH clones from the NF **(A)** and SF **(B)** subtraction libraries into functional groups based on their sequence alignments using BLASTx. Diagram **(C)** shows the number of SSH clones that were common to both NF and SF subtraction libraries.

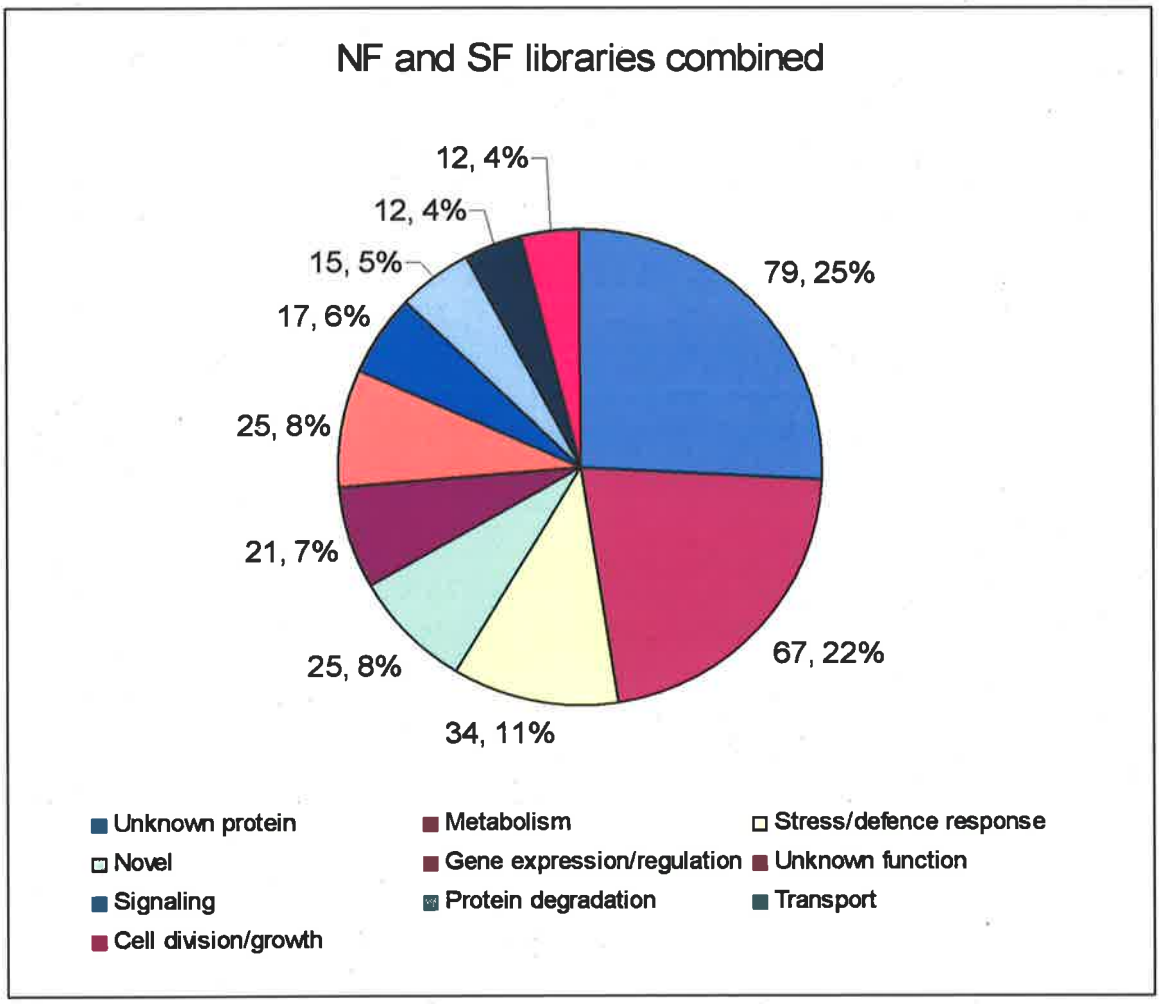

Figure 10: Distribution of SSH clones from the NF and SF subtraction libraries combined into functional groups based on their sequence alignments using BLASTx.

Table 1: Functional categories and sequence similarities of the 25 SSH clones that were common to both NF and SF subtraction libraries.

Clone	Size (bp)	Protein similarity Identity	Origin	BLASTx Acc. No.	Similarity
Stress or defence response					
CON2	134	Pathogenesis-related protein 1a ^ (NF382) (SF432)	*H. vulgare*	S37166	5e-27 (67%, 89aa)
CON4	316	Catalase 1 ^ (NF209) (SF606)	*H. vulgare*	AAC17729	6e-47 (100%, 88aa)
CON6	290	Mitochondrial aldehyde dehydrogenase, putative (ALDH) ^ (NF680,696,697) (SF512)	*A. thaliana*	NP_190383	6e-11 (65%, 55aa)
CON27	196	Pathogenesis-related protein 4 ^ (NF129,213) (SF33,270)	*H. vulgare*	T06169	e-10 (96%, 30aa)
CON36	181	Superoxide dismutase ^ (NF187) (SF641)	*Z. mays*	P23345	3e-74 (89%, 152aa)
Metabolism					
CON1	286	Ribulose-1,5-bisphosphate Carboxylase ^ (NF12,29,56,67,334,484,487,490,735) (SF314,395)	*W. microscopica*	AAK72538	e-45 (100%, 86aa)
CON8	243	Vacuolar ATP synthase subunit G 1 ^ (NF87,827) (SF556)	*N. tabacum*	O82702	e-18 (47%, 110aa)
CON16	341	Glutamate dehydrogenase A ^ (NF717) (SF333)	*N. plumbaginifolia*	O04937	2e-23 (79%, 63aa)
CON22	329	ATP synthase beta chain ^ (NF157,158) (SF182)	*H. vulgare*	P00828	2e-53 (100%, 105aa)
CON29	217	Probable alpha-galactosidase ^ (NF224,552) (SF222)	*H. Vulgare*	T04423	6e-22 (100%, 48aa)
CON32	229	Photosystem II protein D1 ^ (NF828,842) (SF34,249)	*H. vulgare*	P05337	e-29 (100%, 62aa)
CON43	296	Ribulose bisphosphate Carboxylase ^ (NF36,110,182,273,277,305,388,397,407,479,715,770) (SF16,54,291,335)	*L. Chinensis*	CAA90004	3e-52 (98%, 98aa)
CON53	339	Herbicide binding protein D1 ^ (NF106,249,265,324,410,678,766) (SF123,261)	*L. perenne*	AAK53381	4e-61 (100%, 113aa)
Protein degradation					
CON7	269	Subtilisin-chymotrypsin inhibitor 2 (CI2E) ^ (NF676) (SF189,272,361,548,620)	*H. vulgare*	T06181	9e-19 (67%, 68aa)
CON47	353	Cysteine proteinase ^ (NF108) (SF113)	*Z. mays*	S60456	4e-47 (92%, 91aa)
CON48	315	CI2E ^ (NF210,369) (SF81,192,219,316)	*H. vulgare*	T06181	2e-32 (98%, 68aa)
CON52	335	CI2E ^ (NF84,113,445,495,518) (SF38,97)	*H. vulgare*	AAM22827	3e-16 (60%, 73aa)

Note: The prefix CON (for contig) was used to name 2 or more clones sharing matching/overlapping sequences. ^ Shows the individual NF and SF clones representing each contig.

Table 1: (Continued)

Clone Identity	Size (bp)	Protein similarity	Origin	BLASTx Acc. No.	BLASTx Similarity
Gene expression/regulation					
CON3	181	Protein translation factor SUI1 homolog (GOS2 protein) ^ (NF43) (SF470)	*O. sativa*	P33278	e-43 (88%, 96aa)
CON15	172	Translation initiation factor 5A ^ (NF551) (SF247)	*O. sativa*	AAC67555	8e-35 (79%, 74aa)
CON38	398	Putative translation initiation factor SUI1 ^ (NF229,799) (SF529)	*O. sativa*	BAB89060	e-31 (87%, 72aa)
Unknown function					
CON46	413	Ethylene-forming-enzyme-like dioxygenase-like protein ^ (NF353,537,640) (SF73,87,148)	*O. sativa*	BAC22234	e-27 (58%, 99aa)
CON51	214	Auxin-regulated protein ^ (NF357,438) (SF258)	*Z. elegans*	AAM12952	5e-24 (73%, 69aa)
Unknown protein					
CON25	149	Hypothetical protein p85RF ^ (NF712) (SF202)	*P. armeniaca*	T51098	8e-28 (70%, 79aa)
CON49	163	Unknown protein ^ (NF480) (SF240)	*A. thaliana*	AAK28636	e-27 (70%, 86aa)
CON30	274	(No significant homology) ^ (NF698) (SF216)			

3.4 DISCUSSION

3.4.1 Differential screening of SSH clones using macroarrays and Northern blots reveals limitations in detecting clones of low abundance

The method of SSH (Diatchenko et al., 1996) was used in an attempt to identify genes that are differentially expressed in the incompatible interaction between barley and two *P. teres* isolates that can induce net- or spot-form of net blotch disease symptoms. The cloning of SSH transcripts yielded two EST libraries corresponding to inoculations with either the NF or SF causing isolate. The NF library contained 335 clones whereas the SF library consisted of 367 clones. Macroarrays of each library were probed with subtracted, tester, and driver cDNAs and the clones were grouped into four different categories depending on their hybridisation profiles. It was anticipated that differentially expressed transcripts would be identified by looking at differences in the signal intensity of each clone when probed with either tester or driver cDNA. However, it was found that the majority of clones only hybridised to the subtracted probes and not (or only very weakly) to the tester or driver probes. This group of clones made up category (1), which was represented by 242 (72%) and 302 (82%) of all NF and SF clones, respectively. These figures are consistent with the study of Grenier et al. (2002) who also used macroarrays to analyse SSH-derived transcripts and found that the majority of clones hybridised only to subtracted probes. Thus although it was not possible to determine if category (1) clones were differentially expressed by macroarray analysis they were still selected for further analysis because they potentially correspond to low abundance transcripts enriched during SSH as a direct consequence of differential expression.

Category (2) clones appeared to hybridise to subtracted cDNA probes and equally to both tester and driver probes and therefore represented non-differentially expressed ESTs. Collectively, they represented 48 (14%) and 30 (8%) of all NF and SF clones respectively, however, it should be noted that the actual percentages are likely to be higher as some category (1) clones are also expected to be non-differentially expressed. SSH efficiency has been shown to vary from study to study (Kuang et al., 1998; Birch et al., 1999; Grenier et al., 2002) so these figures are not surprising. All category (2) clones were discarded as false positives.

Thus, only a relatively small proportion of clones (5% NF and 7% SF) were grouped into the differentially expressed categories (3) (up-regulated) or (4) (down-regulated). The large number of category (1) clones indicated that the macroarrays were not sensitive enough to detect lowly abundant transcripts and consequently this method proved to be largely ineffective in identifying clones that are differentially expressed in the two barley-*P. teres* incompatible interactions studied. Although the production of microarrays was not part of this project, their enhanced sensitivity may have facilitated the identification of more differentially expressed clones. Becker et al. (2004) recently compared differential gene expression results obtained using micro and macroarrays and found that twice as many potentially differentially expressed genes were detected using the former method. In addition, most of the genes they identified using macroarrays corresponded to highly expressed genes.

One of the limitations with verifying the differential character of SSH clones is that detection of rare cDNAs in Northern analysis is difficult, requiring high concentrations of RNA for the blots and some times up to 2 weeks of film exposure (Diatchenko et al., 1996). In this study, a high proportion of cDNAs were not detected following Northern analysis – even though phosphor imaging plates were used in place of x-ray film to maximise sensitivity in most cases. The lack of sensitivity with using Northern analysis for the study of SSH clones has been encountered previously (e.g. Grenier et al., 2002; Avrova et al., 2004; Guilleroux and Osbourn, 2004). Trying to identify differentially expressed genes in this way proved to be laborious and time consuming. Due to the large amount of epidermal tissue (approximately 30 peels) needed to extract just 10 μg of RNA for Northern analysis it was not practical to perform a large-scale differential screen using multiple blots of epidermal RNA as probe. Instead, a large-scale screen was performed with multiple Northern blots of whole leaf RNA and this may have contributed to the problems encountered in detecting some of the clones – especially if they were epidermis specific as the abaxial epidermis from which RNA was initially extracted for SSH comprises only about 1/10 of the whole leaf (Gregersen et al., 1997).

The functional classification of 307 individual gene transcripts from the NF and SF cDNA libraries was achieved by aligning the translated sequences of individual clones (in most cases the best matching barley EST) with known translated sequences using

the BLASTx algorithm. Genes from a number of functional categories were represented in both libraries, and although further experimentation is needed to verify their functional assignment, which is based on sequence homology alone, it nevertheless provides a measure of the diversity of genes putatively involved in the defence response against both net blotch-causing pathogens. The multiple sequencing of transcripts encoding RUBISCO, PSII protein D1, or CI2 among the putatively differentially expressed category (3) and (4) clones was disappointing as all three are highly abundant proteins that have been characterised extensively in other studies.

RUBISCO and PSII protein D1 are important components of photosynthesis, with the former being the first and key enzyme in the Calvin-Benson cycle of photosynthetic fixation of CO_2 (Griffin and Seemann, 1996), and the latter being a major subunit of the oxygen-evolving complex in PSII where water is oxidised to produce atmospheric oxygen (Ferreira et al., 2004). It is likely that the abundant nature of the mRNA encoding these proteins, in particular RUBISCO, which constitutes up to 65% of total leaf protein (Ellis, 1979), played a role in the repeated isolation of their corresponding clones in both subtraction libraries. It is widely accepted that photosynthesis is repressed in pathogen-infected plants and the coincidental down-regulation of RUBISCO-encoding genes accompanying this phenomenon has been reported (Mouly and Roby, 1988; Berger et al., 2004; Swarbrick et al., 2006). In addition, pathogen infection has been shown to reduce the levels of some PSII subunits culminating in the reduction of photosynthetic electron transport (Rahoutei et al., 2000). CI2, a protein inhibitor of serine proteinases, has previously been shown to inhibit the proteinase activities of the Fusarium head blight-causing fungus in barley grain (Pekkarinen and Jones, 2003). Proteinase inhibitors are one of the most abundant classes of proteins found in microorganisms, plants, and animals (Kassell, 1970), and their role as antifungal proteins involved in plant defence responses has been well documented (Theis and Stahl, 2004).

It was a little surprising that none of the SSH clones showed sequence homology with any fungal genes as both plant and fungal RNAs would have been present in the infected epidermal tissue from which RNA was extracted for SSH. Since *P. teres*, like the yeast *Saccharomyces cerevisiae*, is an ascomycete and the entire genome of *S. cerevisiae* has been sequenced (Mewes et al., 1997), many *P. teres* genes should have

homologs in the yeast genome, and therefore would be expected to match yeast genes preferentially to plant genes. A low proportion of fungal genes amongst plant genes has also been reported in other cDNA libraries generated from pathogen-inoculated plant tissue (Fristensky et al., 1999; Rauyaree et al., 2001; Asiegbu et al., 2005). Contributing factors could be that (a) fungal biomass is small compared to plant biomass, and (b) the SSH libraries were enriched for sequences involved in the net blotch resistant interaction, in which fungal proliferation is by definition inhibited. It is also possible that the tester and driver fractions used for SSH had similar fungal genes expressed at 24 hai that were subtracted during SSH. However, it cannot be ruled out that some of the SSH clones assigned as novel were in fact derived from *P. teres*.

3.4.2 Candidate genes involved in the barley-*P. teres* incompatible interaction

3.4.2.1 Genes with unknown function or non-significant homology

Novel genes or genes encoding proteins of unknown function constituted the largest group of transcripts in both SSH libraries. This can be attributed to (a) the SSH normalisation step which equalises the abundance of cDNAs within the target population – thereby enriching lowly abundant transcripts that may have previously gone undetected, and (b) the nature of the SSH protocol itself which facilitates the isolation of transcripts originating from the 3' end of genes which are less likely to show sequence homology with known genes.

3.4.2.2 Genes involved in metabolism

Clones in the second largest category showed homology with genes encoding proteins involved in primary metabolism. This group was represented by 23% and 22% of all NF and SF clones, respectively, and included a number of photosynthesis-related transcripts (e.g. RUBISCO subunits, components of photosystems I and II), transcripts involved in cellular respiration (e.g. a putative acetyl-coenzyme A synthetase, a glyceraldehyde-3-phosphate dehydrogenase, a malate dehydrogenase, ATP synthase subunits), and sink metabolism (e.g. several types of invertases). It is known that pathogen infection affects primary metabolism in the plant and the isolation of these

clones probably reflects the switch from normal to "defence" metabolism. A common aspect of defence metabolism appears to be the repression of photosynthesis in the infected plant and this has been reported in plants challenged by biotrophic (Berger et al., 2004; Swarbrick et al., 2006), necrotrophic (Berger et al., 2004), and hemibiotrophic (Scharte et al., 2005) pathogens. Conversely, due to an increased demand for energy needed to mount a defence response, cellular respiration is enhanced in pathogen-infected plants and the differential expression of several transcripts encoding enzymes involved in glycolysis, the Krebs cycle, and the pentose phosphate pathway, was reported in the interaction between *Arabidopsis* and the bacterial pathogen, *P. syringae* pv. *tomato* (Scheideler et al., 2002).

Defence metabolism in pathogen-infected plants also appears to involve a coordinated regulation between the repression of photosynthesis and the induction of sink metabolism leading to dramatic changes in carbohydrate partitioning. Central to sink metabolism is the host apoplastic (cell wall-bound) invertase whose activity at the protein and/or transcript level has been reported to increase in plant-pathogen interactions resulting in the accumulation of hexose sugars (Roitsch et al., 2003; Berger et al., 2004; Scharte et al., 2005; Swarbrick et al., 2006). The differential induction of invertase activity between an incompatible and compatible plant-pathogen interaction has previously been reported for both race-specific (*Mla12*) and broad-spectrum (*mlo*) resistance in barley leaves infected with *B. graminis* (Swarbrick et al., 2006), and in tomato roots infected with the necrotrophic fungal pathogen, *F. oxysporum* (Benhamou et al., 1991).

3.4.2.3 Genes with similarities to defence- and stress-related genes

The next most prevalent group, making up 12% of both NF and SF SSH clones, showed similarity to genes encoding proteins involved in various stress and defence responses. Among this group were transcripts derived from the anti-oxidant response (e.g. germin-like protein, glutathione *S*-transferase, SOD, catalase), PR proteins (e.g. PR-1, PR-4, chitinase, peroxidase), as well as those corresponding to genes induced in response to stresses such as phosphate (Pi) starvation, aluminum stress, and heat shock (e.g. Pit2 protein, WALI proteins, heat shock protein).

From the anti-oxidant response proteins, a higher level of SOD activity was recently reported in a barley-*P. teres* resistant compared to susceptible interaction over a 48 h period (Able, 2003). In this study, ROS were only detected in the net blotch susceptible response and total SOD activity in the resistant response 24 hai was more than twice that of the susceptible response. The identification of a SOD-encoding clone in the present work further emphasises the potential importance of anti-oxidant enzymes in determining the outcome of this plant-pathogen interaction. Plant SOD activity was also shown to be higher in downy mildew resistant compared to susceptible genotypes in cucumber (Yun et al., 1995) and pearl millet (Babitha et al., 2002). The germin-like protein (GLP)-encoding clone isolated in the subtraction library shares sequence homology with a GLP-encoding gene (*HvGLP4*, previously known as *HvOxOLP*) that is predominantly expressed in the epidermis of barley leaves infected with *B. graminis* and that also exhibits SOD activity (Wei et al., 1998; Christensen et al., 2004). Furthermore, the transient over-expression of *GLP4* in wheat and barley enhanced their resistance to *B. graminis* (Christensen et al., 2004). The increased expression of glutathione *S*-transferase (GST) genes in plants following infection by pathogens has also been shown (Mauch and Dudler, 1993; Hahn and Strittmatter, 1994; Dean et al., 2005). In tobacco plants challenged by the hemibiotrophic fungal pathogens, *Colletotrichum destructivum* and *Colletotrichum orbiculare*, expression of two GST-encoding genes increased progressively during the course of infection and silencing one of these resulted in a significant increase in susceptibility of tobacco to infection (Dean et al., 2005). The principle H_2O_2-scavenging enzyme in plants is catalase and the importance of this enzyme in oxidative stress tolerance was highlighted in transgenic tobacco in which catalase-deficient plants showed enhanced H_2O_2 levels and increased susceptibility to paraquat, ozone, and salt stress (Willekens et al., 1997).

PR proteins are known to play an important role in plant defence mechanisms against a variety of biotic and abiotic stresses. In barley, the differential expression of *PR-1* gene transcripts has previously been reported in the powdery mildew pathosystem (Peterhänsel et al., 1997; Hückelhoven et al., 2000; Schultheiss et al., 2003). In these studies, *PR-1* was induced earlier and stronger in powdery mildew resistant compared to susceptible lines. Schultheiss et al. (2003) showed that silencing *PR-1* expression resulted in *B. graminis* penetrating the cell wall more frequently – indicating that PR-1

contributes to penetration resistance against this fungus. Furthermore, it has been reported that chitinases and peroxidases also play a significant role in this pathosystem. Chitinous cell walls of filamentous fungal pathogens are a target of enzymatic digestion to suppress infection, and in powdery mildew-infected barley, the early induction of chitinase transcripts was correlated with resistance to *B. graminis* (Kogel et al., 1994), and exogenous application of chitinase was effective in degrading the infection structures of this pathogen (Toyoda et al., 1991; Ikeda et al., 1996). Likewise, the early induction of peroxidase transcripts was also observed in powdery mildew resistant barley lines (Thordal-Christensen et al., 1992; Kogel et al., 1994). Additionally, the differential expression of these PR protein-encoding genes has also been reported in incompatible compared to compatible plant-pathogen interactions involving both hemibiotrophic and necrotrophic fungi (Sasaki et al., 2004; Desmond et al., 2006). For example, in the study conducted by Desmond et al. (2006) in which wheat plants were inoculated with the necrotrophic fungal pathogen, *Fusarium pseudograminearum*, induction of chitinase, PR-1, and peroxidase genes (among others) was consistently greater in the partially resistant genotype compared to the susceptible genotype. The finding of PR protein-encoding clones in the barley-*P. teres* subtraction libraries is therefore not surprising. Instead, it further corroborates the notion that the early induction of *PR* genes is a common plant resistance-associated defence mechanism against plant pathogens.

The isolation of several clones with sequence similarity to gene transcripts previously identified in plants undergoing abiotic stress supports the idea that, for some stimuli at least, biotic and abiotic stress signalling pathways may be inter-connected. Evidence for crosstalk in the plant response to biotic and abiotic stresses was recently reported in *Arabidopsis* plants responding to various treatments, including heavy metal stress and high salinity, and infection by two necrotrophic fungal pathogens (Narusaka et al., 2004). The authors analysed the expression of 49 *cytochrome P450* genes and were able to show that, in some cases, the same genes were expressed in response to the different biotic and abiotic stimuli that the plants were exposed to. Genes encoding putative components of biotic stress responses have also been identified in *Arabidopsis* following wounding (Cheong et al., 2002) and in rice plants undergoing salt stress (Chao et al., 2005). Three of the stress-related clones share sequence homology with genes that were found to be up-regulated in wheat upon exposure to

Aluminum (Al). These genes encode putative proteinase inhibitors (*wali5* and *wali6*), and a cysteine-rich protein of unknown function (*wali7*) (Snowden and Gardner, 1993; Richards et al., 1994). Hamel et al. (1998) also identified a number of genes in wheat that were differentially expressed by Al stress. The authors reported that most of the genes found to be up-regulated by Al shared homologies with genes induced by pathogens. Again, this reinforces the idea that plants respond to certain biotic and abiotic stresses via common signal transduction pathways. The finding of a putative heat shock protein (HSP)-encoding clone in the subtraction library is also consistent with reports implicating members of this protein family in disease resistance. It has been shown that one family member in particular, HSP90, interacts with and is essential for resistance mediated by several R proteins in *Arabidopsis* (Hubert et al., 2003; Lu et al., 2003; Takahashi et al., 2003; Liu et al., 2004) and tomato (van Bentem et al., 2005). Interestingly, heat treatment of powdery mildew susceptible barley seedlings was also shown to reduce subsequent infection by the fungus responsible for the disease (Schweizer et al., 1995).

3.4.2.4 Genes involved in gene expression, signal transduction, and protein degradation

Individually, the remaining five functional groups did not contribute more than 10% of clones in either library. Those classified as proteins controlling gene expression (e.g. transcription factors), signal transduction (e.g. several types of protein kinases, LRR proteins), and protein degradation (e.g. 26S proteasome subunits) comprised only 10% and 5%, 6% and 5%, and, 4% and 7% of all NF and SF clones, respectively.

The identification of cDNA clones encoding putative transcription factors suggests that proteins involved in regulating the transcriptional activation of defence-related genes may also play a role in the barley-*P. teres* incompatible interaction. The MYB family is one of the largest families of transcription factors characterised in plants, and the isolation of a clone encoding a putative MYB family member is in accordance with a recent study in which a cDNA transcript for a MYB transcription factor was found to be up-regulated in sunflower plants within 48 hours of infection by the necrotrophic fungus, *Phoma macdonaldii* (Alignan et al., 2006). More significantly, functional analysis of plant MYB transcription factors has shown that they regulate

disease resistance (Yang and Klessig, 1996; Lee et al., 2001; Vailleau et al., 2002). Five clones encoding putative zinc-finger proteins and one for a KOW domain-containing transcription factor were also identified in the subtraction library. Zinc-finger motifs function as components of DNA binding and protein-protein interaction domains (Takatsuji, 1998), and research to date has implicated several zinc-finger protein family members in mediating plant defence responses (Dietrich et al., 1997; Robatzek and Somssich, 2002; Serrano and Guzmán, 2004; Oh et al., 2005). For example, Oh et al. (2005) identified a zinc-finger protein-encoding gene, *CaPIF1*, which was specifically induced in the incompatible interaction between pepper plants and three bacterial pathogens. Using transgenic tobacco plants in which *CaPIF1* was over-expressed or silenced, the authors showed that the corresponding protein plays a crucial role in activating common defence responses, such as *PR* gene expression, induced by both basal and *R* gene signalling pathways. In contrast, no previous reports have implicated KOW domain-containing transcription factors as constituents of plant defence responses. However, a recent study in which a putative RNA binding protein containing KOW motifs was shown to be a critical component of resistance pathways shared by two *R* gene types (Zhang et al., 2005), shows that KOW domain-containing proteins also have the potential to regulate the barley-*P. teres* defence response.

It is widely accepted that kinases play important roles in disease resistance and signal transduction in plants by modifying target proteins through the phosphorylation of serine, threonine, or tyrosine residues (Romeis, 2001). Receptor-like kinases, for example, are characterised by an extracellular domain that is probably involved in signal perception, a transmembrane domain, and a cytoplasmic kinase domain, which may initiate a signal transduction cascade into the plant cell. Some receptor-like kinases have been identified as *R* gene products (Song et al., 1995; Feuillet et al., 1997) while others have been associated with defence responses based on their differential expression in plants responding to pathogen attack or treatment with elicitors (Czernic et al., 1999; Gómez-Gómez and Boller, 2000). Other kinase subfamily members identified in the SSH library include a putative mitogen-activated protein kinase (MAPK) and a casein kinase, and both have previously been implicated in the regulation of plant disease resistance. Zhang and Klessig (1998) identified two tobacco MAPKs that were activated following the infection of resistant tobacco plants carrying the *N* resistance gene with tobacco mosaic virus, and more recently,

Brodersen et al. (2006) reported an *Arabidopsis* MAPK that appears to be central to the induction of the ET/JA defence pathway. Studies involving casein kinases in regulating plant disease resistance have not been as extensive but their up-regulation has been reported during the rice-*M. grisea* (Rao et al., 2002) and olive-*Spilocaea oleagina* (Benitez et al., 2005) interactions. LRR proteins function in a number of signal transduction pathways and play an important role in mediating protein-protein interactions (Kobe and Deisenhofer, 1994). The LRR domain is a structural feature of four out of the seven major classes of plant R proteins (Kruijt et al., 2005), and genes encoding other proteins with LRR regions have also been found to be induced in plants following pathogen infection (Hipskind et al., 1996; Jung et al., 2004). Recently, Jacques et al. (2006) identified an LRR protein in tobacco that was induced early during the HR initiated by elicitins, *Ralstonia solanacearum*, and Tobacco Mosaic Virus. The authors were able to show through functional analysis that this protein was a potential modulator of the HR.

Genes encoding proteins involved in protein degradation constituted up to 7% of the net blotch resistance-associated transcriptome. Nearly half of the SSH clones in this category belong to the ubiquitin/26S proteasome pathway, including four that encode proteasome subunits and one that encodes a ubiquitin conjugating enzyme. Proteolysis of important regulatory proteins is a key aspect of cellular regulation and recent discoveries have shown that various ubiquitin/26S proteasome pathway components may control key aspects of plant defence (Tör, 2003; Smalle and Vierstra, 2004). For example, in tobacco plants treated with an elicitor of plant defence reactions, three genes encoding proteasome subunits were up-regulated and their differential expression was correlated with plants developing a systemic acquired resistance (Dahan et al., 2001; Suty et al., 2003).

The barley-*P. teres* defence transcriptome also consisted of two clones encoding cysteine proteinases (CPs) which have been shown to be involved in a variety of plant processes, including proprotein processing, plant cell death, and protein turnover (Shimada et al., 1994; Solomon et al., 1999; Palma et al., 2002). Changes in the expression of CP genes have also been described in plants challenged by pathogens (Avrova et al., 1999; Kruger et al., 2001; Hao et al., 2006). In the most recent of these reports, a role for CPs in limiting pathogen infection was suggested after it was found

that silencing two novel CP genes, *NbCYP1* and *NbCYP2*, in tobacco resulted in the corresponding plants showing increased susceptibility to the hemibiotrophic fungus, *C. destructivum* (Hao et al., 2006). The fact that both genes were shown to be involved in limiting disease spread shows that CPs have the potential to regulate host defence responses. Van der Hoorn and Jones (2004) proposed that CPs may play a role in defence execution by helping to degrade pathogen virulence molecules or by activating defensive enzymes from proproteins, and it is possible that the CPs encoded by the SSH clones in the library have one of these functions.

3.4.2.5 Genes involved in transport and protein synthesis

SSH clones representing proteins involved in cellular transport (e.g. ion channels, multidrug transporters) and protein synthesis (e.g. ribosomal proteins) were the least prevalent.

The identification of SSH clones encoding ion channels is probably not unexpected considering their perceived importance in regulating signal transduction in plants (Ward et al., 1995). The putative differential induction of chloride and potassium channels observed in the barley-*P. teres* incompatible interaction is consistent with previous studies in which K^+/Cl^- effluxes have been activated in plant cells responding to challenge by fungal elicitors (Nürnberger et al., 1994; Zimmermann et al., 1998; Xie and Chen, 2000; El-Maarouf et al., 2001). For example, compared to control conditions, early Cl^- effluxes of different intensities were detected in tobacco suspension cells treated with the elicitors cryptogein, pectolyase, and oligogalacturonides (Zimmermann et al., 1998). Furthermore, anion channel blockers have been shown to inhibit not only Cl^- efflux, but also K^+ efflux, H_2O_2 production, and phytoalexin accumulation (Jabs et al., 1997), suggesting that anion channel activation represents an upstream step in the defence response pathway.

The transporter functional category also contains three SSH clones that encode multidrug transporter proteins, namely a multidrug resistance-like ATP binding cassette (ABC) transporter, a multidrug and toxin efflux (MATE) transporter, and a major facilitator superfamily (MFS) antiporter. These and other classes of multidrug efflux proteins have previously been implicated in plant defence responses. The

possible involvement of multidrug transporters in the export of plant-produced antimicrobial toxins was recently described by Jasiński et al. (2001), who identified an ABC transporter gene, *NpABC1*, whose expression was induced by the diterpenoid sclareol in the leaf epidermis of tobacco plants. The authors showed that the encoded protein may participate in the extrusion of this antimicrobial terpenoid. A subsequent study showed that transgenic plants in which NpABC1 (renamed to NpPDR1) was prevented showed increased sensitivity to sclareol and reduced resistance to the necrotrophic fungus, *B. cinerea* (Stukkens et al., 2005).

The potential importance of MATE transporters in disease resistance was highlighted by Nawrath et al. (2002), who showed that EDS5, which is an important component of salicylic acid-dependent *R* gene-mediated signalling in *Arabidopsis*, belongs to the MATE transporter family. In a previous study, *eds5 Arabidopsis* mutants did not accumulate SA after pathogen inoculation and showed enhanced susceptibility to both virulent and avirulent forms of *P. syringae* and *Perenospora parasitica* (Nawrath and Métraux, 1999). Although reports implicating MFS antiporters in plant defence responses have not been as extensive, the finding of a defence-inducible MFS antiporter-encoding gene in maize plants challenged by two fungal pathogens (Simmons et al., 2003), shows that this family of multidrug transporters also has the potential to regulate responses to biotic stress. Because *P. teres* produces at least three phytotoxins that incite many of the prominent symptoms associated with net blotch disease in barley (Weiergang et al., 2002), it is possible that the successful induction of a plant detoxification system regulated by multidrug transporters may play a significant role in determining the outcome of this interaction.

It is probable that the transcriptional up-regulation of various defence-related genes also requires a corresponding increase in translational machinery and in previous studies ribosomal proteins have been shown to be differentially expressed in plants in which a defence response has been activated (Chakravarthy et al., 2003; Gabriëls et al., 2006). Ribosomal proteins are major components of the ribosome and may represent up to 15% of all cellular proteins (Mager, 1988). Their potential importance in regulating a successful defence response has been highlighted in recent studies in which the silencing of certain genes encoding ribosomal proteins was shown to

suppress the HR in tomato plants challenged by *Pto* (Lu et al., 2003) and *Cladosporium fulvum* (Gabriëls et al., 2006).

In addition, it was found that 25 of the 307 clones (8%) were common to both SSH libraries, suggesting that there is some degree of overlap between the two different defence responses. Given the close genetic relationship between *Ptt* and *Ptm*, this is perhaps not surprising. Indeed, one may have expected more common clones to be identified. The small number observed may be an indication that the SSH procedure used in this study was not entirely comprehensive.

3.4.3 Selection of clones for detailed expression analysis

Although the SSH procedure enabled the identification of over 300 genes that are potentially differentially expressed and involved in the resistance-associated defence response, the limitation is that it is difficult to analyse and interpret such a large amount of data in a short time frame. Therefore, based on a review of the SSH clones identified in this study, a small subset consisting of 45 clones was selected for expression profiling in the leaf epidermis (Table 2). This subset consisted of clones whose corresponding gene products are involved in a diverse range of cellular functions that could be necessary for net blotch resistance to occur. It included three of the six category (3) clones (NF434, NF579, SF310) identified earlier by macroarray analysis as being up-regulated in the barley-*P. teres* incompatible interaction, as well as five clones (NF353, NF552, NF717, SF113, SF512) that were identified by sequencing as being common to both NF and SF subtraction libraries (see table 1).

As genes encoding putative signal transduction components may act as "master switches", eleven SSH clones from this functional class (NF66, NF69, NF205, NF472, NF579, SF45, SF232, SF449, SF474, SF618, SF623) were selected for further analysis. Some may encode receptors that interact directly or indirectly with *P. teres*-derived elicitors while others may transduce defence signals and/or regulate gene networks that could boost defence signalling and thereby lead to an increase in disease resistance. Eight clones associated with cellular detoxification mechanisms (NF313, NF390, NF423, SF475, SF512, SF522, SF595, SF613) were chosen because they are good candidates for genes involved in protecting plant cells from *P. teres* toxins or

Table 2: Functional categories and sequence similarities of SSH clones selected for detailed Q-PCR expression analysis.

Clone Identity	Size (bp)	Protein similarity	Origin	BLASTx Acc. No.	BLASTx Similarity
Signalling and regulatory proteins					
NF66	366	Putative receptor-like protein kinase	*O. sativa*	AAP68881	7e-07 (69%, 42aa)
NF69	246	High mobility group box protein 2	*O. sativa*	BAB85204	2e-17 (63%, 71aa)
NF205	159	Protein kinase family	*A. thaliana*	NP_1953035	e-10 (66%, 45aa)
NF472	193	Putative LRR protein	*O. sativa*	AAO23085	2e-24 (91%, 58aa)
NF579	327	CONSTANS B-box zinc finger family protein	*A. thaliana*	NP_195607	e-25 (42%, 147aa)
SF45	164	LRR protein family	*A. thaliana*	NP_187250	e-24 (52%, 109aa)
SF232	152	Putative casein kinase I	*O. sativa*	BAB92346	5e-21 (96%, 50aa)
SF449	172	Putative PHD-type zinc finger protein	*A. thaliana*	AAM65374	e-24 (84%, 57aa)
SF474	118	Serine/threonine protein kinase 1, nonphototropic hypocotyl protein 1-like	*A. sativa*	T08033	e-28 (85%, 68aa)
SF618	469	Receptor-like protein kinase	*A. thaliana*	NP_200898	9e-17 (49%, 79aa)
SF623	392	14-3-3-like protein A	*H. vulgare*	P29305	3e-53 (100%, 103aa)
Detoxification					
NF313	393	Sterol delta-7-reductase	*A. thaliana*	NP_175460	2e-53 (74%, 130aa)
NF390	342	ARP protein NADPH oxidoreductase homolog	*A. thaliana*	S57614	5e-11 (68%, 45aa)
NF423	129	MFS antiporter	*Z. mays*	AAN33180	7e-08 (61%, 44aa)
SF475	278	Putative cytochrome P450	*L. rigidum*	AAK38091	e-45 (91%, 92aa)
SF512	290	Putative mitochondrial aldehyde dehydrogenase	*A. thaliana*	NP_190383	6e-11 (65%, 55aa)
SF522	257	Aldehyde dehydrogenase	*O. sativa*	AAG43027	e-29 (75%, 82aa)
SF595	176	Flavin monooxygenase	*A. thaliana*	CAD39838	5e-23 (82%, 58aa)
SF613	275	MATE efflux protein family	*A. thaliana*	NP_188997	7e-25 (41%, 146aa)
Stress or defence response					
NF408	123	Hypersensitive-induced reaction protein 1	*H. vulgare*	AAN17462	4e-19 (100%, 46aa)
NF422	166	Chitinase	*O. sativa*	JC5844	3e-64 (69%, 172aa)
SF260	106	Wheat aluminum induced protein wali 5	*T. aestivum*	JQ2361	e-35 (75%, 90aa)
SF310	134	Pathogenesis-related protein 1	*H. vulgare*	S37166	5e-27 (67%, 89aa)
Protein degradation					
SF113	353	Cysteine proteinase	*Z. mays*	S60456	4e-47 (92%, 91aa)
SF387	252	Ubiquitin conjugating enzyme	*H. vulgare*	AAP04430	6e-82 (97%, 148aa)
SF400	130	20S proteasome alpha subunit F	*O. sativa*	BAA28276	2e-76 (93%, 148aa)
SF427	105	20S proteasome alpha subunit E	*O. sativa*	Q9LSU1	4e-66 (96%, 130aa)
SF575	135	26S proteasome regulatory particle triple-A ATPase subunit 6	*O. sativa*	BAB17626	5e-57 (97%, 90aa)

Table 1: (Continued)

Clone	Size (bp)	Protein similarity	Origin	BLASTx Acc. No.	BLASTx Similarity
Calcium perception					
SF372	507	Calcineurin B-like protein	*P. sativum*	AAM91028	e-22 (90%, 53aa)
SF593	260	Copine-related	*A. thaliana*	NP_196946	e-10 (59%, 44aa)
Carbohydrate metabolism					
SF66	203	Sorbitol transporter	*P. cerasus*	AAO39267	5e-30 (69%, 88aa)
SF111	424	Putative invertase	*A. thaliana*	AAM65926	4e-44 (87%, 96aa)
SF532	237	UDP-glucose 4-epimerase	*O. sativa*	BAC02925	5e-33 (78%, 88aa)
Transporters					
SF77	298	Putative integral membrane protein NRAMP	*H. vulgare*	CAD55951	4e-48 (100%, 99aa)
SF464	221	Chloride channel	*O. sativa*	BAB972667	3e-23 (90%, 60aa)
Unknown function					
NF353	413	Ethylene-forming enzyme-like dioxygenase-like protein	*O. sativa*	BAC22234	e-27 (58%, 99aa)
NF434	322	Deoxymugineic acid synthase 2	*H. vulgare*	BAC10595	2e-28 (98%, 64aa)
NF468	237	Probable α-galactosidase	*H. vulgare*	T04423	2e-37 (97%, 78aa)
NF717	341	Glutamate dehydrogenase A	*N. plumbaginifolia*	O04937	2e-23 (79%, 63aa)
NF804	180	Putative ABA-responsive protein	*O. sativa*	AAL31061	9e-47 (60%, 138aa)
SF265	178	Oxysterol-binding family protein	*A. thaliana*	NP_200750	5e-20 (66%, 59aa)
SF324	124	Putative ubiquinone binding protein	*O. sativa*	BAA95821	e-33 (91%, 72aa)
SF468	166	CBS domain containing protein	*A. thaliana*	NP_190422	e-16 (74%, 54aa)
SF528	327	Adenine nucleotide translocator 2	*T. aestivum*	Q41630	5e-16 (100%, 41aa)
SF551	183	L-allo-threonine aldolase	*A. thaliana*	NP_566228	7e-06 (44%, 50aa)

ROS which have been shown to accumulate in the barley-*P. teres* interaction. To confirm that defence responses had in fact been initiated in the plant after inoculation, four previously described stress/defence related SSH clones (NF408, NF422, SF260, SF310) were selected for expression analysis to serve as positive internal controls. Five clones homologous with genes that encode proteins involved in proteolysis (SF113, SF387, SF400, SF427, SF575) were selected. Their isolation and hence putative up-regulation in the SSH library may indicate that the induction of a proteolytic pathway involved in degrading proteins originating from *P. teres* could be a critical factor in determining disease outcome. In plants, the calcium ion is a ubiquitous intracellular messenger involved in diverse signalling pathways, and to determine if Ca^{2+} signalling may play a role in the barley-*P. teres* incompatible interaction, two clones associated with Ca^{2+} signal perception (SF372, SF593) were selected for detailed expression analysis. One of the kinase clones (SF474) may be involved in regulating Ca^{2+} transients as well. Pathogen inoculation has also been shown to induce sink metabolism in infected tissue and three clones (SF66, SF111, and SF532) whose gene products may regulate carbohydrate partitioning were chosen on the basis that their induction may supply cells with the energy they need to mount a defence response. Two clones implicated in regulating the transport of chloride and metal ions (SF77, SF464) were selected to determine if these molecules may play a role in the net blotch resistant interaction. Lastly, ten clones (NF353, NF434, NF468, NF717, NF804, SF265, SF324, SF468, SF528, SF551) were selected at random. Some of their corresponding gene products may be associated with phytohormone signalling (NF353, NF804), some may be signal transducing proteins (SF265, SF468), while others have uncertain putative functions.

In anticipation of the resulting expression profiles, a number of questions were raised:

(1) How efficient was the SSH procedure used in this study?

Expression profiling using cDNA generated from epidermal RNA combined with the increased sensitivity of Q-PCR should give a more accurate picture of SSH efficiency than the one obtained using Northern blot analysis.

(2) To what extent, if any, are the selected clones differentially regulated in both NF and SF incompatible compared to compatible interactions? And, is the incompatible interaction characterised by a stronger and earlier induction of key defence genes as reported in some previous studies?

Monitoring the expression of selected clones at 1, 3, 6, 12, 24, and 48 hai in both interactions is expected to shed light on their expression kinetics and reveal any differences in the timing of their induction/repression.

(3) How do expression profiles for individual clones compare between the NF and SF interactions?

It is apparent that NF and SF resistance in barley is governed by different resistance and quantitative trait loci. Expression profiling of individual SSH clones in both interactions should provide information on whether the same or different pathogen-induced genes control NF and SF resistance-related defence responses.

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CHAPTER IV

Expression profiling of SSH transcripts reveals novel insights into the early induction of genes associated with the barley-*P. teres* incompatible interaction

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4.1 INTRODUCTION

The profiling of mRNA transcription has developed into an important tool for studying patterns of expressed genes that are induced by particular events during the life of an organism. In plants, the ability to quantify mRNA transcript levels has led to the identification of genes involved in important biological processes such as development and plant defence. This research has been made possible by RNA detection techniques such as Northern blot analysis, *in situ* hybridisation, reverse transcription (RT)-PCR, RNAse protection assays, and cDNA arrays, yet of the current technologies that are available, it is the method of quantitative real-time PCR (Q-PCR) that has become the most powerful way in which mRNA transcription levels are able to be quantified.

4.1.1 Gene expression profiling by quantitative real-time PCR (Q-PCR)

The basic principles of Q-PCR stem from conventional RT-PCR with the difference being that Q-PCR utilises fluorescent techniques together with suitable instrumentation that enables the amplification and detection of a cDNA target to be monitored in real-time during the PCR. These features make Q-PCR the most sensitive method available for the detection and quantification of gene expression levels (Bustin 2000). Q-PCR has two main advantages over Northern blot analysis which for a long time has been the most commonly used technique for RNA quantification; (1) it facilitates gene expression studies in tissues from which it is difficult to obtain sufficient quantities of RNA for Northern blot analysis, and (2) it can discriminate between closely related mRNAs which is sometimes not the case with Northern blots.

The simplest method for monitoring amplified products involves the detection of the binding of a fluorescent dye called SYBR green to ds DNA (Morrison et al., 1998). Because SYBR green binds specifically to ds DNA, a Q-PCR can be monitored in real-time by measuring the amount of fluorescence produced at the end of the elongation step of every PCR cycle.

4.1.2 Q-PCR normalisation

It is generally accepted that gene expression levels should be normalised by carefully selected internal reference genes, sometimes referred to as a housekeeping genes, whose expression does not vary in response to experimental treatment. Due to the difficulty in determining whether a given gene has the constitutive property of a housekeeper, most studies use one of a common set of housekeeping genes without any further validation of its presumed level of stable expression. Some of the commonly used housekeeping genes include glyceraldehyde 3-phosphate dehydrogenase (GAPDH), albumin, actins, tubulins, cyclophilin, and 18S or 28S rRNAs. However, a number of studies have shown that expression of these genes varies under certain experimental conditions and consequently they may be unsuitable references for RNA transcription analysis (Radonic et al., 2004; Schmittgen and Zakrajsek, 2000; Zhong and Simons, 1999). One argument against the use of rRNA genes for example comes from a recent study that described an imbalance between rRNA and mRNA fractions in total RNA samples from the same tissue (Solanas et al., 2001). In addition, it has been suggested that the use of rRNAs as standards is unsuitable because rRNA synthesis is regulated independently from mRNA synthesis (Radonic et al., 2004). Therefore, even in Northern blot analyses, where normalising gene expression by means of fluorescence staining of rRNA or using 18S- and 28S-specific antisense probes is common practice, the conclusions drawn from normalised expression data may be questionable. To address the problems associated with using only one housekeeping gene whose stable expression has not been validated, Vandesompele et al. (2002) described a method whereby the use of multiple control genes can be used to generate a normalisation factor based on their level of expression relative to each other. Significantly, this method was found to be more accurate than using a single gene for normalisation, which led to relatively large errors in 10-25% of samples they tested.

4.2 MATERIALS AND METHODS

4.2.1 Expression profiling by Q-PCR

4.2.1.1 Preparation of cDNA template

RNA preparations were quantified using a spectrophotometer and stored at -80°C until required. To prepare cDNA template for Q-PCR, RNA preparations were treated with RNase-free *DNase* I (Ambion, USA) to remove residual DNA before first-strand synthesis. First-strand cDNA was prepared from 0.5 µg of epidermal RNA, using 500 ng universal Oligo$(dT)_{18}$ primer and 200 units of SuperScript III reverse transcriptase (Invitrogen, USA), at 50°C for 1 h in a 20 µl reaction that also contained 1X first-strand buffer, 5 mM DTT, and 40 units of RNaseOUT (Invitrogen, USA).

4.2.1.2 Experimental design

Q-PCR was performed in a RG 3000 Rotor-Gene Real Time Thermal Cycler (Corbett Research, Mortlake, NSW, Australia). The final reaction volume of 12 µl was comprised of a mixture containing 1X QuantiTect SYBR Green PCR reagent (Qiagen, USA), 7.2 pmol of each forward and reverse primer, and 1 µl of a 1:10 dilution of transcribed cDNA. The PCR was effected using the following cycling conditions: 15 min at 95°C, followed by 35 cycles of 20 s at 95°C, 30 s at 55°C, 30 s at 72°C, and 15 s at the optimal data acquisition temperature for each gene specific product (approximately 2°C below the T_m of the specific PCR product), which was determined in a previous PCR by heating the reaction products from 70°C to 99°C and analysing the resulting melt curve. For each gene, PCR reactions were set up in duplicate to increase the accuracy and reliability of the procedure and melt curve analysis was always performed at the end of each run to confirm that there was no signal from non-specific binding products. PCR primers were designed using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). They were specific to the nucleic acid sequence of each respective SSH clone or contig out of SSH clone and homologous EST fragments. Individual gene specific primers and acquisition

temperatures for all SSH clones subjected to Q-PCR expression analysis are shown in appendix C.

4.2.1.3 Data analysis

The Rotor-Gene 6 software (Corbett Research, Australia) was used to establish a standard curve that was generated from the PCR of serial ten-fold dilutions (10^7-10^2 copies/µl) of a purified 122 bp amplicon derived from the cDNA of a barley cyclophilin gene (Burton et al., 2003). The standard curve was formed by plotting copy number (expressed in logarithmic form) *versus* values of threshold cycle (C_T) and gene specific transcript quantities were automatically calculated by the supporting software by factoring in the C_T value for each target transcript into the equation of the line of best fit. A fixed fluorescence threshold of 0.1 was used to determine all C_T values. The standard curve derived from the cyclophilin gene transcript was imported into subsequent Q-PCR runs to determine quantities of target gene transcripts. In each independent run, one experimental sample was defined as a standard to calibrate the imported curve. To account for differences in the amount of RNA present in each sample, the geometric averaging of eight internal reference gene transcripts by the geNorm program (Vandesompele et al., 2002) was used to generate normalisation factors (NormFs). The NormFs were derived from the average expression stability of the three most stably expressed transcripts as determined by geNorm. The fold changes of differentially expressed gene candidates were calculated by dividing the normalised expression data of the inoculated samples by the corresponding data obtained for the mock inoculations. Differentially expressed genes were grouped into clusters based on similar expression profiles by visual inspection.

4.3 RESULTS

4.3.1 Primer specificity

Each gene-specific primer set designed to amplify a particular SSH clone successfully amplified a unique product of expected size when cDNA was used as template in a Q-PCR run. In all cases, this was verified by melting curve analysis, which showed that

single products with specific melting temperatures had been amplified. A typical example of the analysis that was used to verify the specificity of Q-PCR products is shown in figure 11. In these examples, agarose gel and melt profiles are illustrated for gene specific Q-PCR products corresponding to four SSH clones (SF623, NF422, SF468, and NF353). The melt curves (A, C, E, and G) clearly show single definitive peaks for each product and these peaks correlate to the single bands of expected size observed on the corresponding gel pictures (B, D, F, and H). Individual melt profiles for all clones subjected to Q-PCR expression analysis are shown in appendix D.

4.3.2 Normalisation

The geometric averaging of eight internal control gene transcripts by the geNorm program (Vandesompele et al., 2002) was used to select the best three reference genes for accurate normalisation of mRNA levels for individual SSH clones in epidermal leaf tissue. Three of these control transcripts, GAPDH, α-tubulin, and cyclophilin, were selected on the basis of a previous study that showed their stable expression in a range of barley tissues (Burton et al., 2004). The other five clones were subtracted clones isolated from this study that showed a low level of variation in C_T values across all experimental samples following Q-PCR (SF324, SF387, SF400, SF427, and SF575). As the same amount of RNA was reversely transcribed for all samples, this was an indication that these genes were not differentially expressed. These five novel reference transcripts were initially selected for expression analysis because they encode for proteins involved in ubiquitin-mediated proteolysis. SF324 has homology with a ubiquinol-cytochrome C reductase, SF387 is homologous with a ubiquitin conjugating enzyme, SF400 and SF427 are similar to genes encoding 20S proteasome alpha subunits F and E, respectively, and SF575 has sequence similarity with a 26S proteasome subunit. The geNorm program calculates the average expression stability for individual control genes as an *M* value – the lower the *M* value, the greater the level of stability, and hence the more a particular gene is suited to act as a reference. The stepwise exclusion of the gene with the highest *M* value resulted in a clear decrease of *M* for the remaining control genes (Figure 12), therefore demonstrating their greater suitability as references compared to the excluded gene/s. The order of elimination of control genes was cyclophilin, GAPDH, α-tubulin, SF324, and SF387, and the normalisation factor was generated from the three most stably expressed

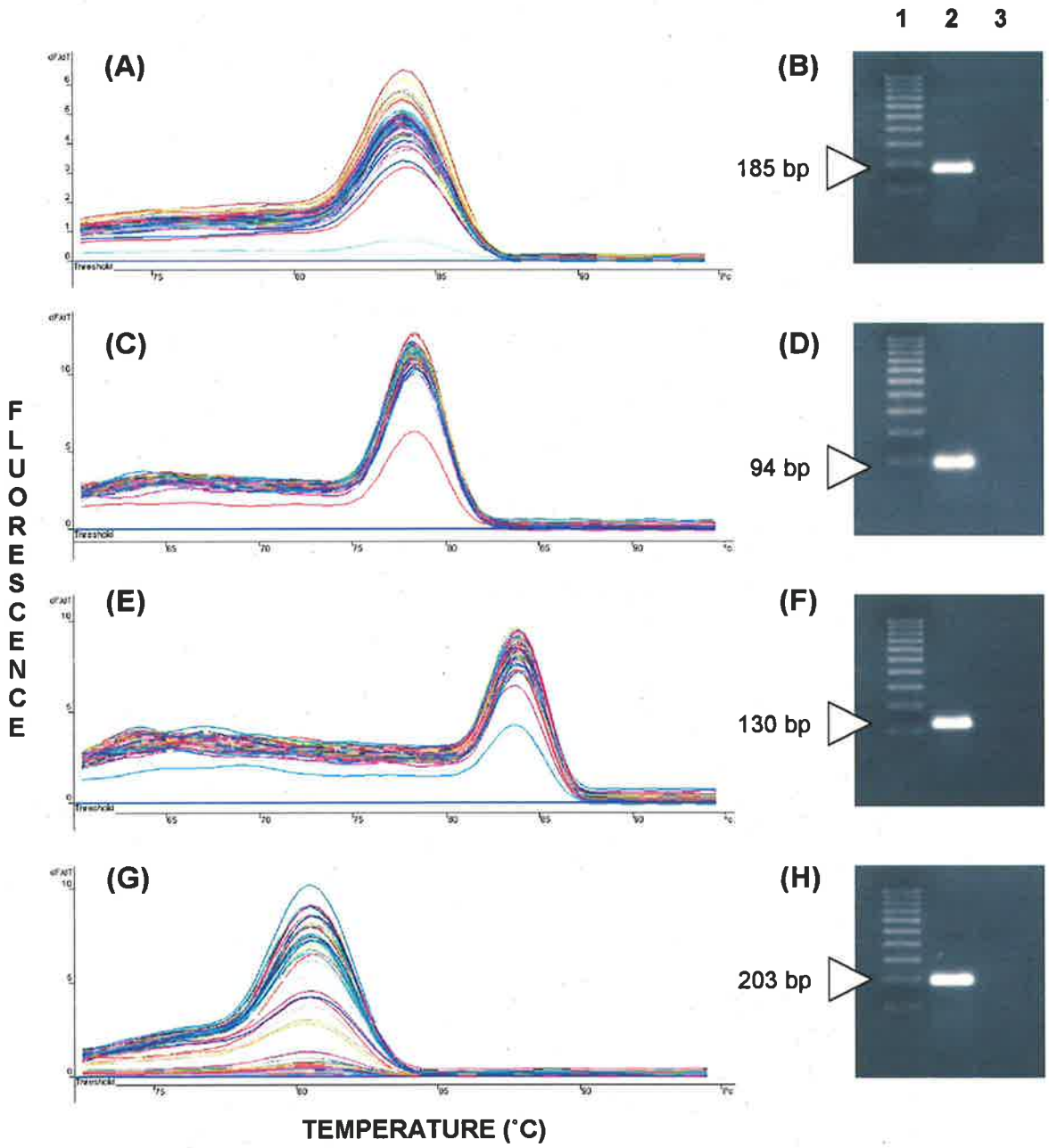


Figure 11: Example of analysis of specificity of Q-PCR products amplified for the following defence response genes: **(A)** and **(B)** SF623 (HvPtr3); **(C)** and **(D)** NF422 (HvPtr4); **(E)** and **(F)** SF468 (HvPtr16); **(G)** and **(H)** NF353 (HvPtr28), using the designed primers listed in appendix C. **(A)**, **(C)**, **(E)**, and **(G)**; Melting profiles of the corresponding gene transcripts amplified from cDNA derived from all experimental treatments. **(B)**, **(D)**, **(F)**, and **(H)**; Agarose gel electrophoresis of corresponding PCR product. Lanes: 1 = 1 kb DNA ladder; 2 = product amplified from barley leaf cDNA; 3 = negative (water) control.

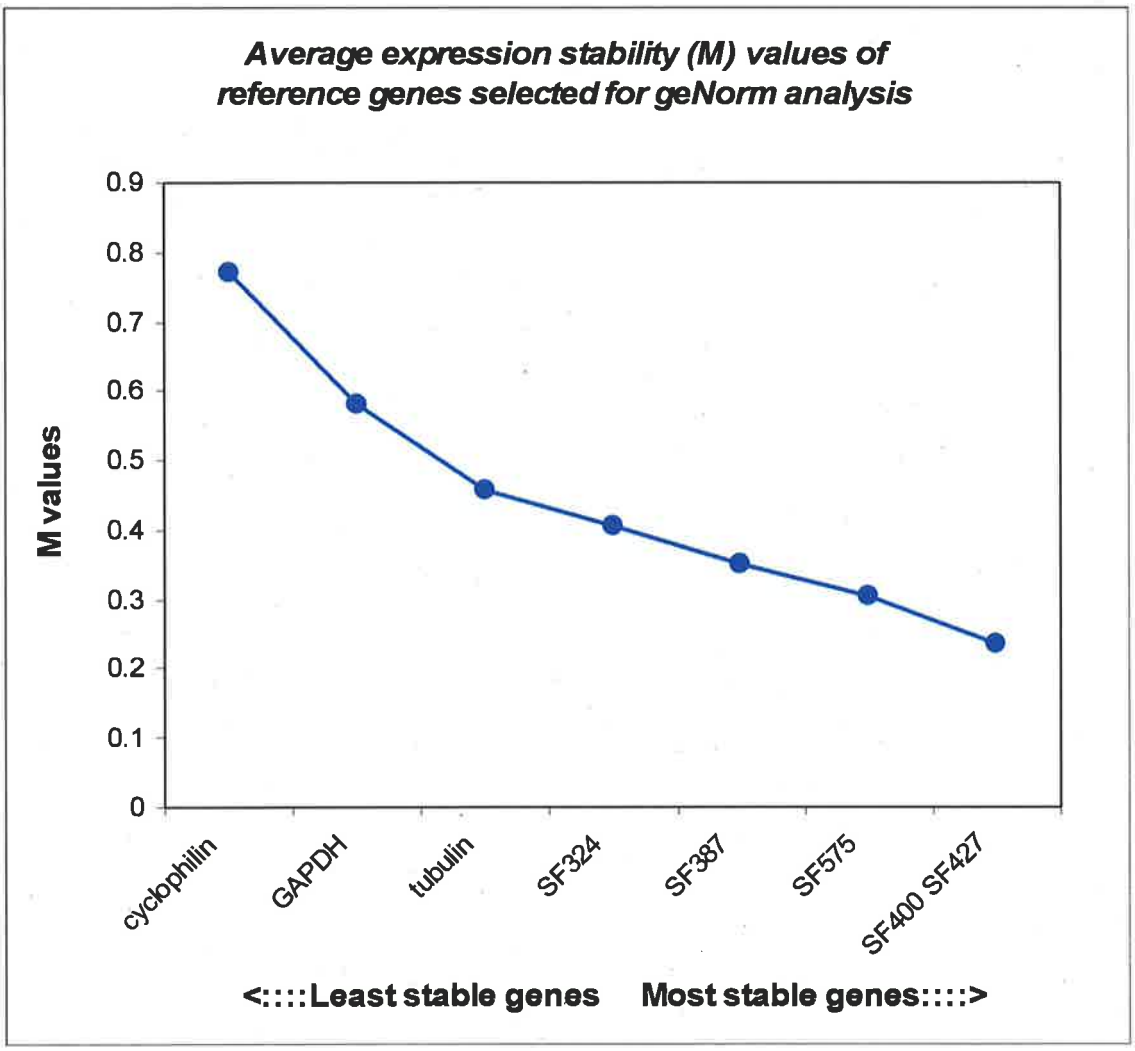


Figure 12: Chart indicating the average expression stability (M) value of remaining reference genes at each step during stepwise exclusion of the least stable reference gene. Starting from the least stable gene at the left, the genes are ranked according to increasing expression stability, ending with the two most stable genes at the right (which cannot be further ranked).

genes, SF575, SF427, and SF400. Appendix E shows the normalisation factors that were subsequently used to normalise Q-PCR-generated expression data for all experimental samples.

4.3.3 SSH efficiency

Q-PCR generated expression profiles of 45 SSH clones revealed that 28 clones (62%) were infection-related and differentially expressed in both NF and SF incompatible interactions at different time points following *P. teres* inoculation. The remaining 17 clones (38%) were expressed at the same levels as the control samples (discussed below). To establish the efficiency of differential gene isolation using the SSH method the level of fold induction for all 45 clones was calculated in both NF and SF incompatible interactions by dividing the normalised expression value for each clone at 24 hai in the resistant genotype by the corresponding value in the susceptible genotype. Based on a 1.5 fold cut-off value used to assign genes as being differentially expressed, the 45 SSH clones were distributed among four categories depending on their level of fold induction (Figure 13). In the NF interaction, 24 clones were differentially expressed (18 up-regulated, 6 down-regulated), whereas in the SF interaction, 29 were differentially expressed (21 up-regulated, 8 down-regulated). Thus, out of 90 expression profiles from both NF and SF interactions combined, 53 were either up- or down-regulated by a factor of 1.5 at the 24 hai time point. Therefore the overall efficiency of the SSH procedure to isolate differentially expressed genes as determined by Q-PCR was 59%.

4.3.4 Transcript profiling of selected SSH clones

The expression profiles of 45 selected SSH clones were analysed at 1, 3, 6, 12, 24, and 48 hai in the net blotch resistant and susceptible genotypes by Q-PCR, using a 1.5 fold cut-off value (relative to expression in the mock-inoculated controls) to assign genes as being differentially expressed. The corresponding genes were grouped into eight clusters depending on the kinetics of their expression in infected tissue relative to water-inoculated controls (Figures 14.1 – 14.8). Expression profiles for each gene cluster are shown individually for both NF and SF interactions and a consensus profile is shown depicting the mean expression pattern generated when the individual profiles

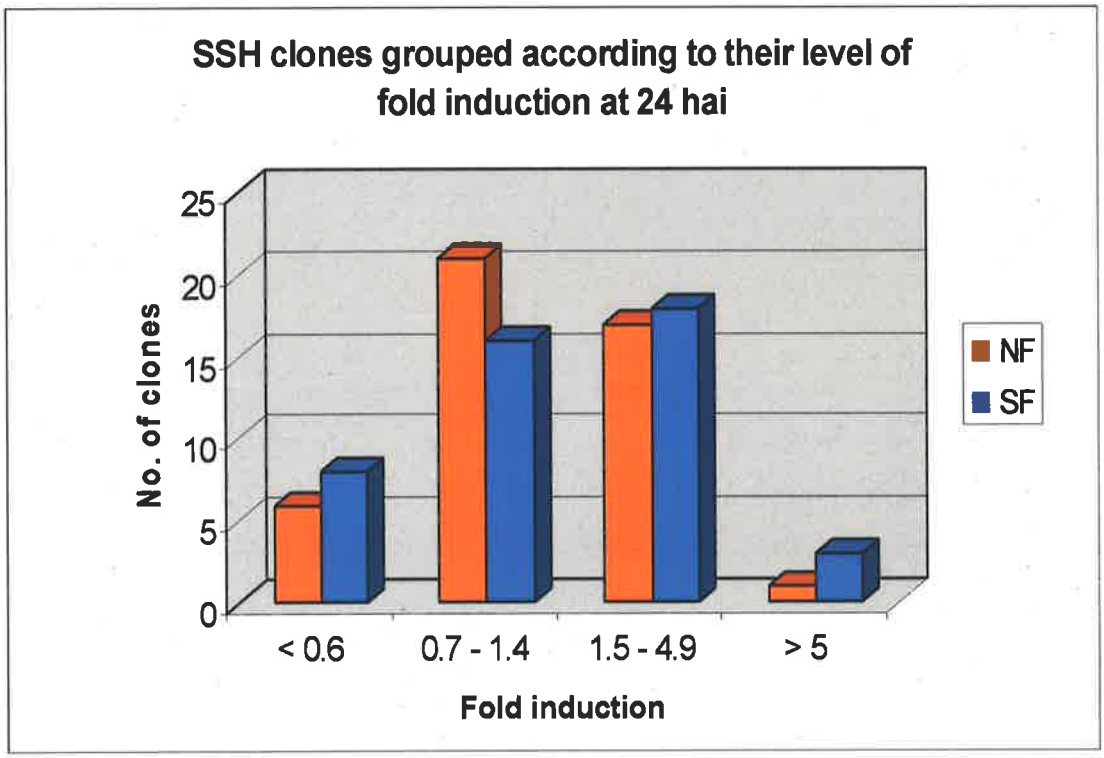


Figure 13: Q-PCR expression analysis reveals varying levels of differential expression for 45 SSH clones in the NF (orange) and SF (blue) incompatible interactions at 24 hai. A 1.5-fold cut-off value was used to consider genes as being significantly differentially expressed or not. A fold induction value less than 0.6 signifies a gene that was down-regulated by more than 1.5-fold in the incompatible interaction; values from 0.7 to 1.4 correspond to genes that were not significantly differentially expressed; a value of 1.5 or more means that a gene was up-regulated in the incompatible interaction.

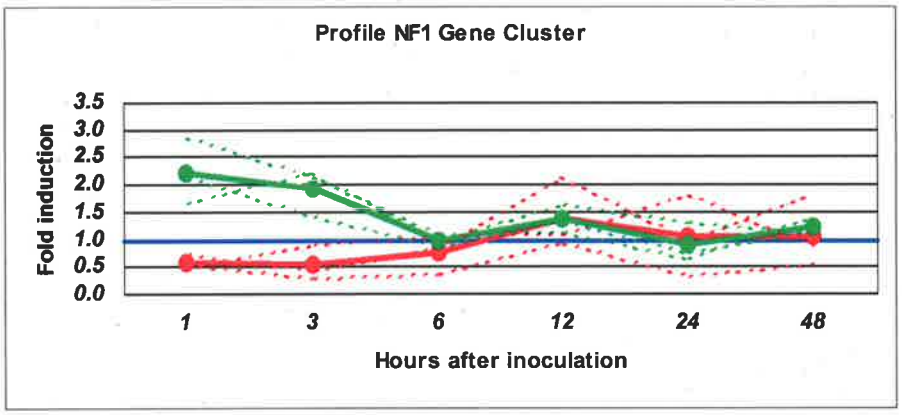
from both interactions were combined. Table 3 lists all of the differentially expressed genes that were grouped into clusters sharing similar expression profiles. These genes were renamed with the prefix HvPtr (for *Hordeum vulgare P. teres* resistance).

The first cluster (Figure 14.1) contained three genes (*HvPtr1* to *HvPtr3*) that were up-regulated by a factor of 2 in the incompatible interaction very early after infection (1 and 3 hai). In the compatible interaction, the same genes were found to be down-regulated by a factor of 2 at the corresponding time points. The combined expression profiles of seven genes (*HvPtr4* to *HvPtr10*) made up cluster 2 (Figure 14.2) which was characterised by a 4-fold induction in gene expression at 12 hai in the resistant response compared to both susceptible and control responses. A delayed induction of these genes was observed in the susceptible genotype at 48 hai. The expression profile of clones included in cluster 3 (Figure 14.3) shows that this group of genes (*HvPtr11* to *HvPtr17*) was induced later and stronger in the incompatible interaction. The genes appear to be initially down-regulated from 1 to 6 hai, and then up-regulated from 6 to 24 hai where there is a 2.5-fold increase in transcript levels relative to controls. In contrast, the compatible interaction shows an inverse mode of expression for this cluster. The mean expression profiles of two genes (*HvPtr18* and *HvPtr19*) form cluster 4 (Figure 14.4) which is highlighted by an early and late up-regulation of these genes at 3 and 24 hai in the resistant genotype only. The same genes do not appear to be differentially expressed in the susceptible genotype. Clusters 5 and 6 (Figures 14.5 and 14.6) have a similar expression profile with their representative genes (*HvPtr20* to *HvPtr27*) being strongly up-regulated at 24 hai in the incompatible interaction. The difference between the two clusters is in the level of up-regulation observed at this time point, with cluster 5 genes being up-regulated 40-fold and cluster 6 genes 1500-fold. The genes grouped into clusters 5 and 6 are also up-regulated at 12 hai in both resistant and susceptible genotypes, however, in the resistant response the gene transcripts are further induced at 24 hai whereas in the susceptible response they return to control levels. Cluster 7 (Figure 14.7) was unique to one gene (*HvPtr28*) that appeared to be differentially expressed in the incompatible interaction only. The gene was up-regulated 15-fold in the resistant genotype within 1 hai before returning to control levels at 6 hai. It was then induced again and continued to be up-regulated 15-fold from 12 to 48 hai. Cluster 8 (Figure 14.8) was made up of the remaining 17 genes that were found not to be differentially expressed i.e. mean fold induction was <1.5

Table 3: Differentially expressed genes identified in the early stages of the barley-*P. teres* incompatible interaction were clustered into groups of genes sharing similar Q-PCR expression profiles.

Gene Identity		Protein similarity	Origin	BLASTx Acc. No.	E-value
Profile 1 Gene Cluster					
HvPtr1	(SF474)	Serine/threonine protein kinase	Oat	T08033	e-28
HvPtr2	(NF66)	Putative receptor-like protein kinase	Rice	AAP68881	7e-07
HvPtr3	(SF623)	14-3-3-like protein A	Barley	P29305	3e-53
Profile 2 Gene Cluster					
HvPtr4	(NF422)	Chitinase	Rice	JC5844	3e-64
HvPtr5	(SF618)	Receptor-like protein kinase	Arabidopsis	NP_200898	9e-17
HvPtr6	(SF66)	Sorbitol transporter	Cherry	AAO39267	5e-30
HvPtr7	(NF423)	Major facilitator superfamily antiporter	Maize	AAN33180	7e-08
HvPtr8	(NF313)	Sterol delta-7-reductase	Arabidopsis	NP_175460	2e-53
HvPtr9	(NF579)	CONSTANS B-box zinc finger protein	Arabidopsis	NP_195607	e-25
HvPtr10	(SF372)	Calcineurin B-like protein	Pea	AAM91028	e-22
Profile 3 Gene Cluster					
HvPtr11	(SF111)	Putative invertase	Arabidopsis	AAM65926	4e-44
HvPtr12	(SF512)	Mitochondrial aldehyde dehydrogenase	Arabidopsis	NP_190383	6e-11
HvPtr13	(NF468)	Probable α-galactosidase	Barley	T04423	6e-22
HvPtr14	(NF205)	Protein kinase family	Arabidopsis	NP_195303	5e-10
HvPtr15	(SF522)	Aldehyde dehydrogenase	Rice	AAG43027	e-29
HvPtr16	(SF468)	CBS domain containing protein	Arabidopsis	NP_190422	e-16
HvPtr17	(SF475)	Putative cytochrome P450	Lolium	AAK38091	e-45
Profile 4 Gene Cluster					
HvPtr18	(SF45)	LRR protein family	Arabidopsis	NP_187250	e-24
HvPtr19	(SF232)	Putative casein kinase I	Rice	BAB92346	5e-21
Profile 5 Gene Cluster					
HvPtr20	(SF551)	Threonine aldolase	Arabidopsis	NP_566228	7e-06
HvPtr21	(NF717)	Glutamate dehydrogenase A	Tobacco	O04937	2e-23
HvPtr22	(NF804)	Putative ABA responsive protein	Rice	AAL31061	9e-47
HvPtr23	(SF613)	MATE efflux protein	Arabidopsis	NP_188997	7e-25
HvPtr24	(SF593)	Copine-related	Arabidopsis	NP_196946	e-10
HvPtr25	(SF260)	Wheat aluminium induced 5 protein	Wheat	JQ2361	e-35
Profile 6 Gene Cluster					
HvPtr26	(SF310)	PR protein 1a	Barley	S37166	5e-27
HvPtr27	(SF595)	Flavin monooxygenase	Arabidopsis	CAD39838	5e-23
Profile 7 Gene Cluster					
HvPtr28	(NF353)	Ethylene-forming enzyme-like protein	Rice	BAC22234	e-27

A.



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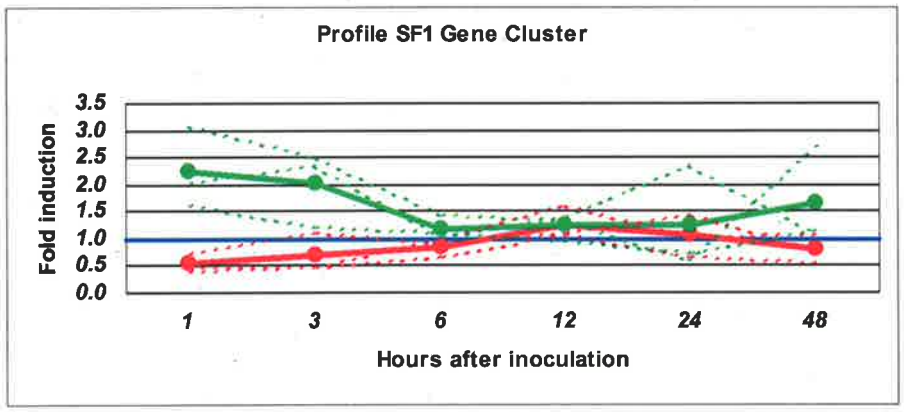


C.

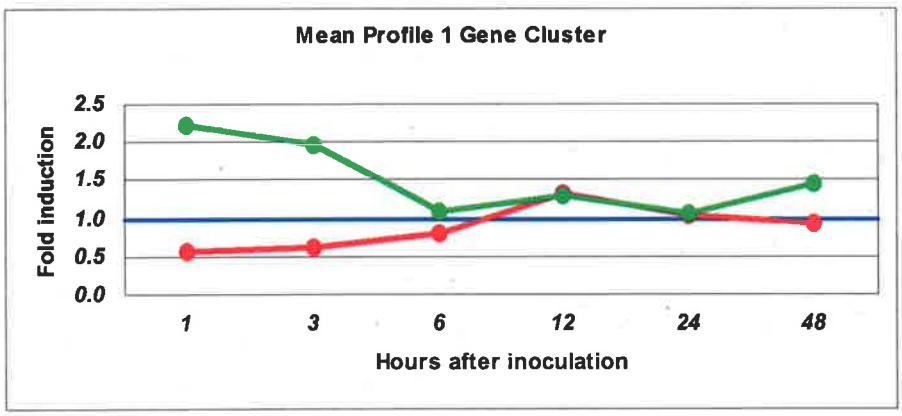
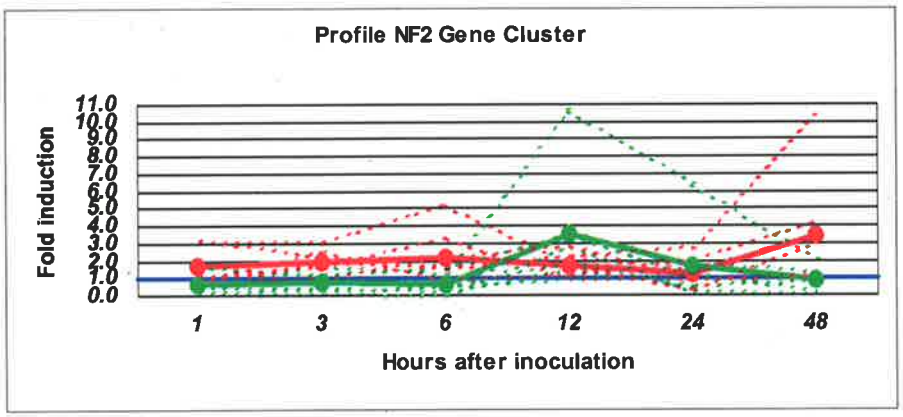


Figure 14.1: Q-PCR generated expression profiles of gene cluster 1 (3 genes) in the epidermis of barley leaves inoculated with *Ptt* (NF; Box A) and *Ptm* (SF; Box B). Broken lines represent expression profiles for individual members of the gene cluster and solid lines represent mean expression profiles of the individuals within the gene cluster. Green line = incompatible interaction (CI9214); Red line = compatible interaction (B87/14). The *y* axis indicates the fold induction for all of the genes in the cluster relative to their expression in mock-inoculated control plants. Hence, the value 1 (blue line) is an indicator of no differential expression. Box C shows the mean expression profile generated for this gene cluster from the NF and SF interactions combined.

D.



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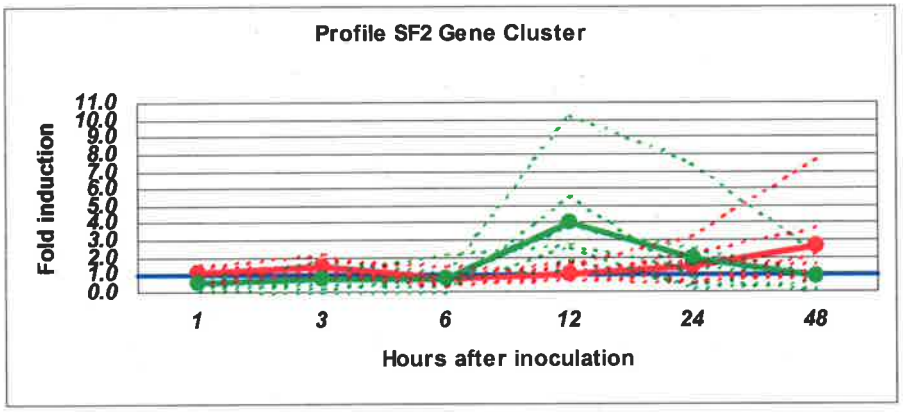


F.

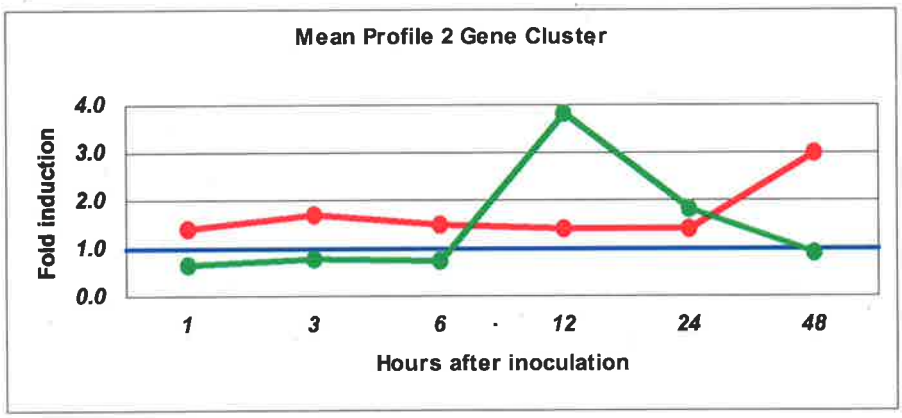
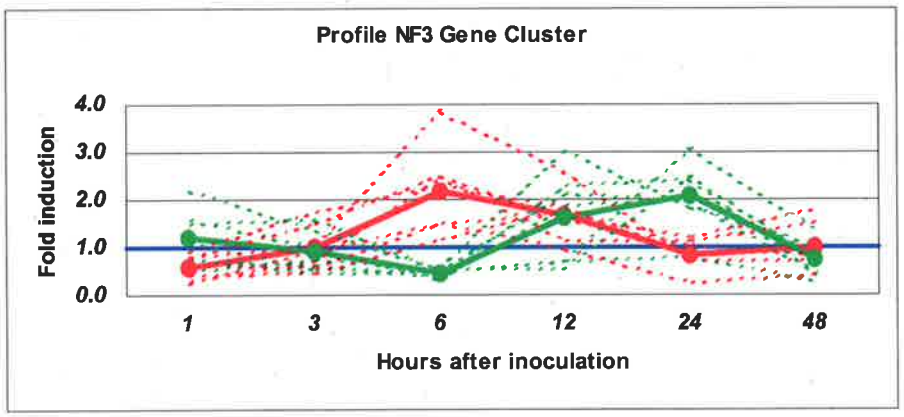
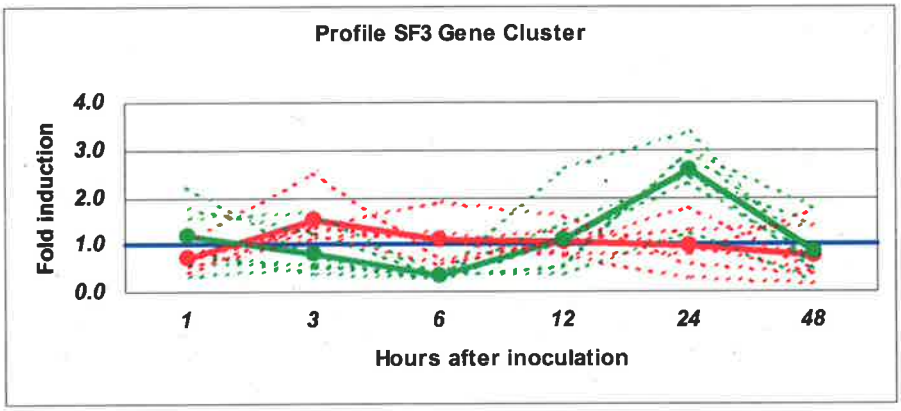


Figure 14.2: Q-PCR generated expression profiles of gene cluster 2 (7 genes) in the epidermis of barley leaves inoculated with *Ptt* (NF; Box D) and *Ptm* (SF; Box E). Broken lines represent expression profiles for individual members of the gene cluster and solid lines represent mean expression profiles of the individuals within the gene cluster. Green line = incompatible interaction (CI9214); Red line = compatible interaction (B87/14). The *y* axis indicates the fold induction for all of the genes in the cluster relative to their expression in mock-inoculated control plants. Hence, the value 1 (blue line) is an indicator of no differential expression. Box F shows the mean expression profile generated for this gene cluster from the NF and SF interactions combined.

G.



H.



I.

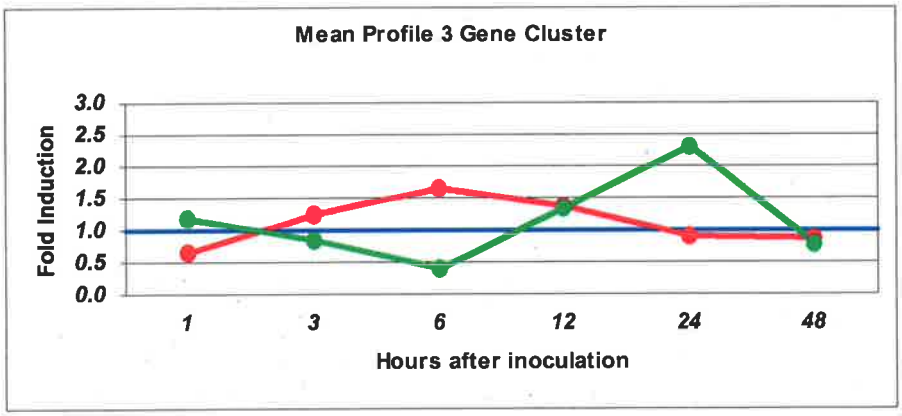
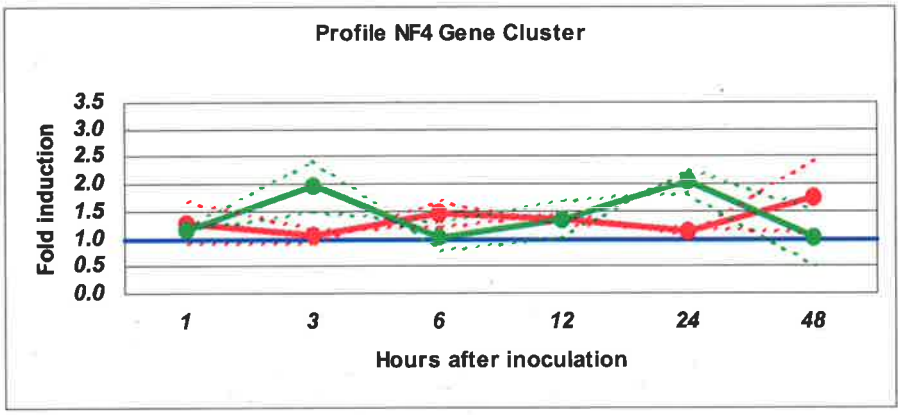
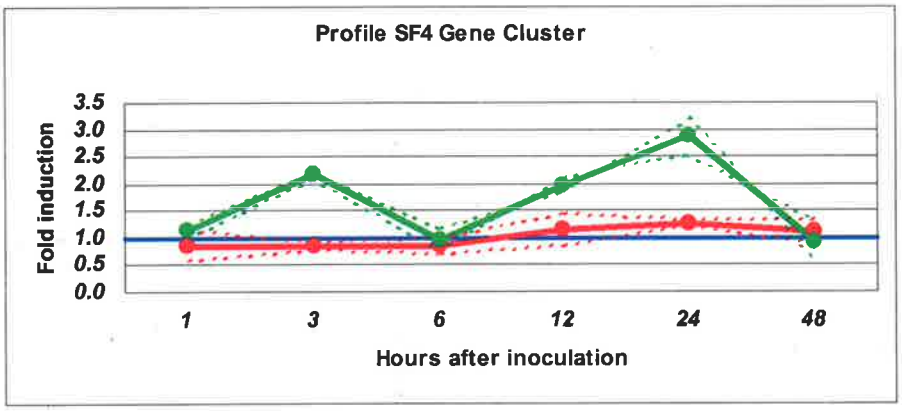


Figure 14.3: Q-PCR generated expression profiles of gene cluster 3 (7 genes) in the epidermis of barley leaves inoculated with *Ptt* (NF; Box G) and *Ptm* (SF; Box H). Broken lines represent expression profiles for individual members of the gene cluster and solid lines represent mean expression profiles of the individuals within the gene cluster. Green line = incompatible interaction (CI9214); Red line = compatible interaction (B87/14). The *y* axis indicates the fold induction for all of the genes in the cluster relative to their expression in mock-inoculated control plants. Hence, the value 1 (blue line) is an indicator of no differential expression. Box I shows the mean expression profile generated for this gene cluster from the NF and SF interactions combined.

J.



K.



L.

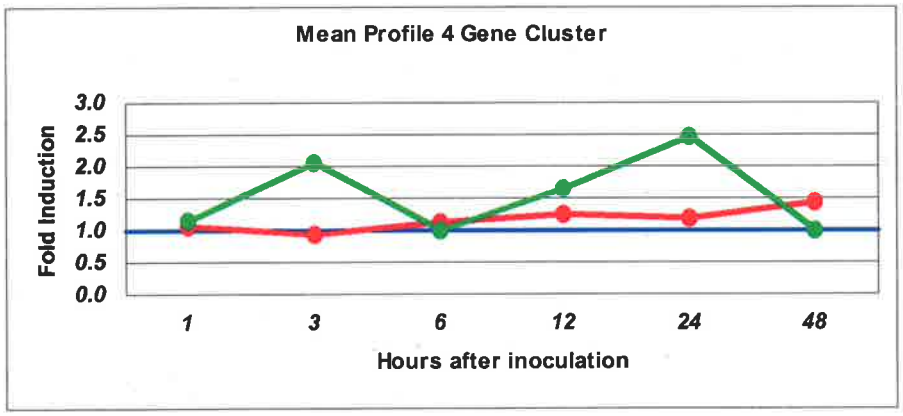
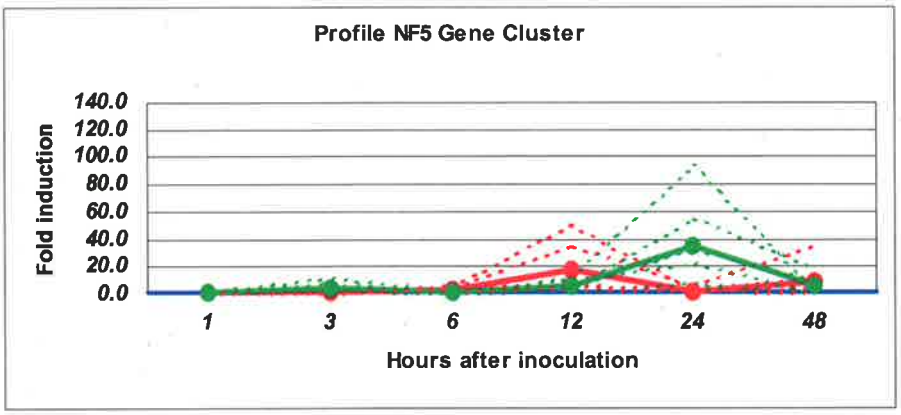
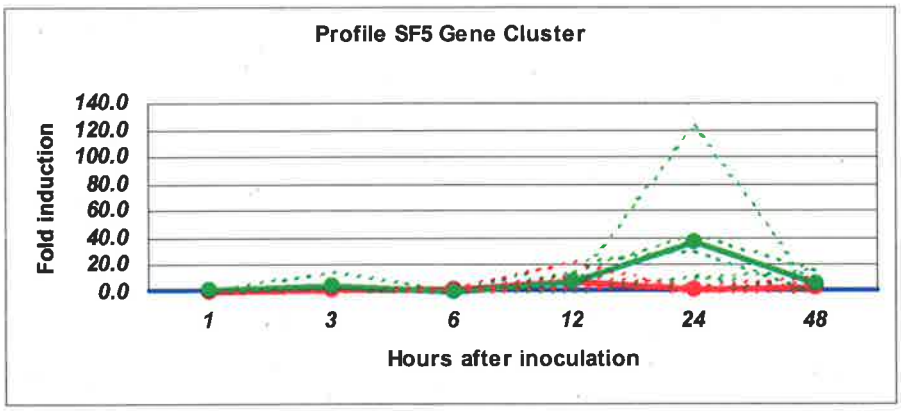


Figure 14.4: Q-PCR generated expression profiles of gene cluster 4 (2 genes) in the epidermis of barley leaves inoculated with *Ptt* (NF; Box J) and *Ptm* (SF; Box K). Broken lines represent expression profiles for individual members of the gene cluster and solid lines represent mean expression profiles of the individuals within the gene cluster. Green line = incompatible interaction (CI9214); Red line = compatible interaction (B87/14). The *y* axis indicates the fold induction for all of the genes in the cluster relative to their expression in mock-inoculated control plants. Hence, the value 1 (blue line) is an indicator of no differential expression. Box L shows the mean expression profile generated for this gene cluster from the NF and SF interactions combined.

M.



N.



O.

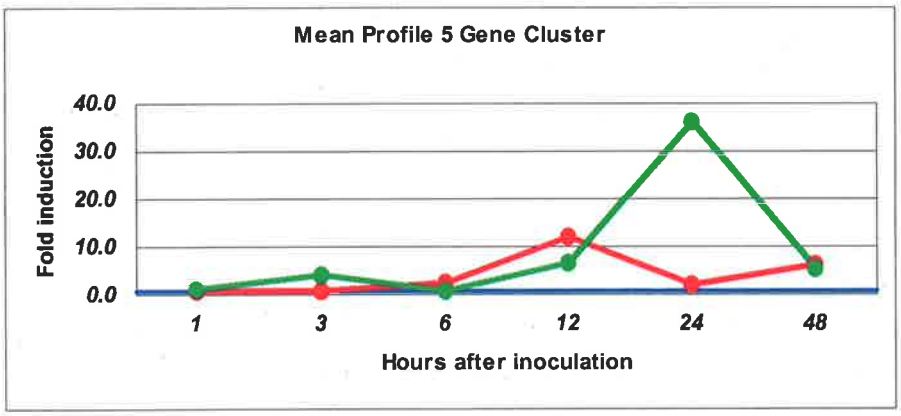
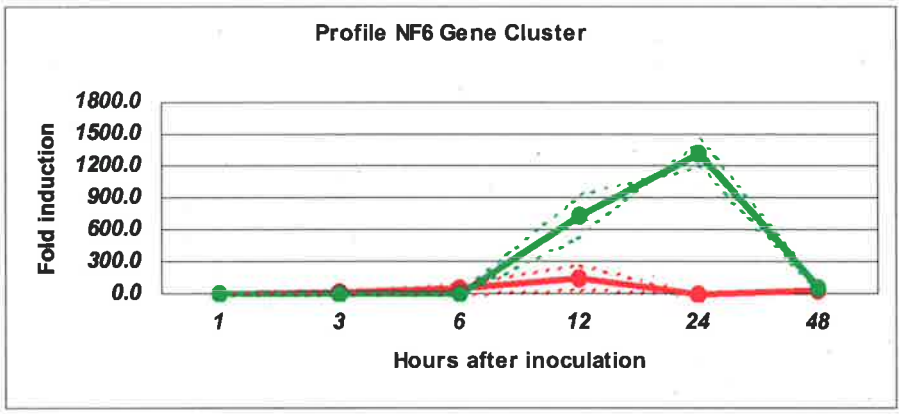
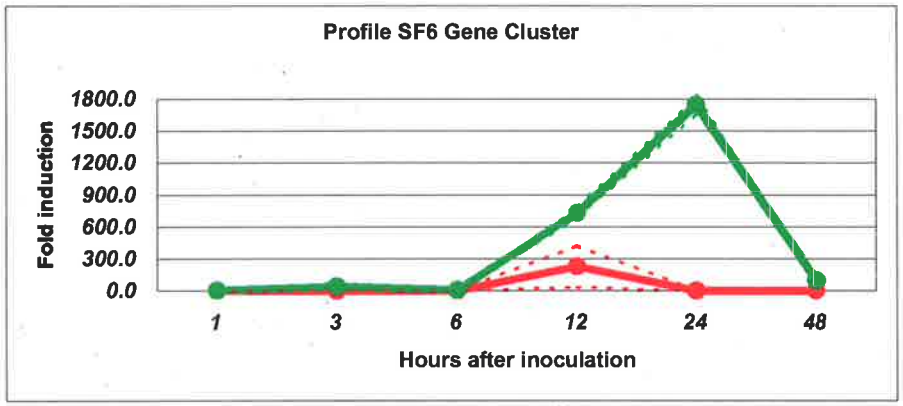


Figure 14.5: Q-PCR generated expression profiles of gene cluster 5 (6 genes) in the epidermis of barley leaves inoculated with *Ptt* (NF; Box M) and *Ptm* (SF; Box N). Broken lines represent expression profiles for individual members of the gene cluster and solid lines represent mean expression profiles of the individuals within the gene cluster. Green line = incompatible interaction (CI9214); Red line = compatible interaction (B87/14). The *y* axis indicates the fold induction for all of the genes in the cluster relative to their expression in mock-inoculated control plants. Hence, the value 1 (blue line) is an indicator of no differential expression. Box O shows the mean expression profile generated for this gene cluster from the NF and SF interactions combined.

P.



Q.



R.

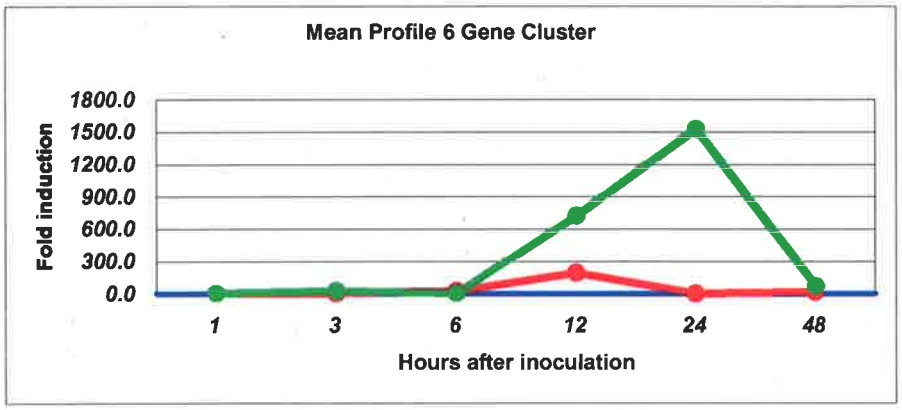
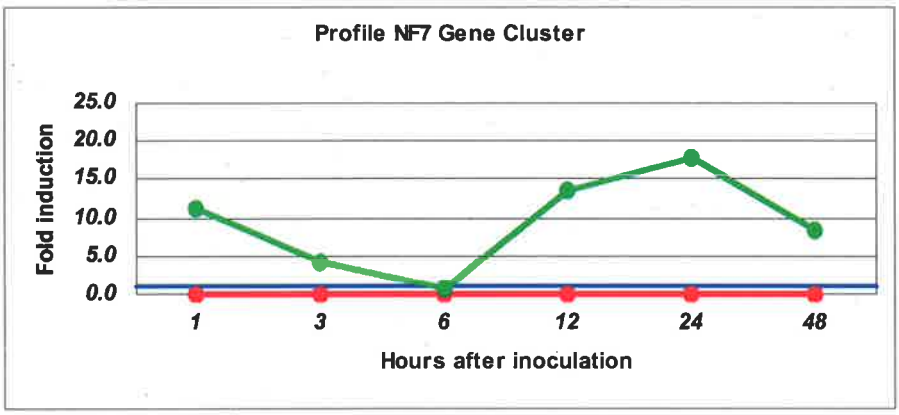
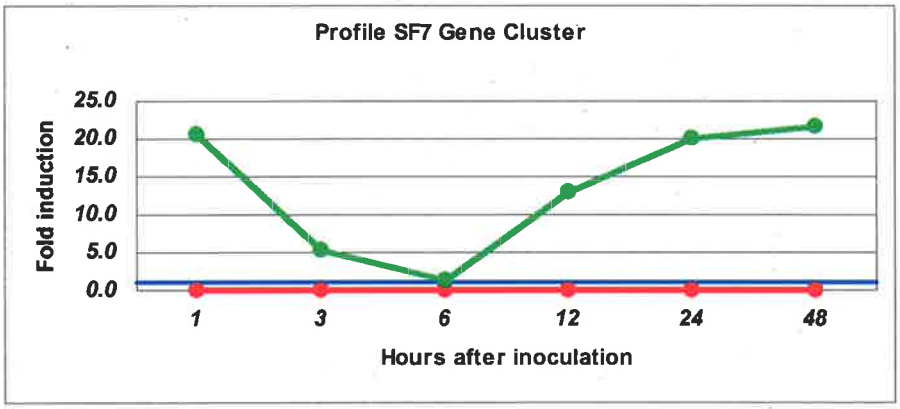


Figure 14.6: Q-PCR generated expression profiles of gene cluster 6 (2 genes) in the epidermis of barley leaves inoculated with *Ptt* (NF; Box P) and *Ptm* (SF; Box Q). Broken lines represent expression profiles for individual members of the gene cluster and solid lines represent mean expression profiles of the individuals within the gene cluster. Green line = incompatible interaction (CI9214); Red line = compatible interaction (B87/14). The *y* axis indicates the fold induction for all of the genes in the cluster relative to their expression in mock-inoculated control plants. Hence, the value 1 (blue line) is an indicator of no differential expression. Box R shows the mean expression profile generated for this gene cluster from the NF and SF interactions combined.

S.



T.



U.

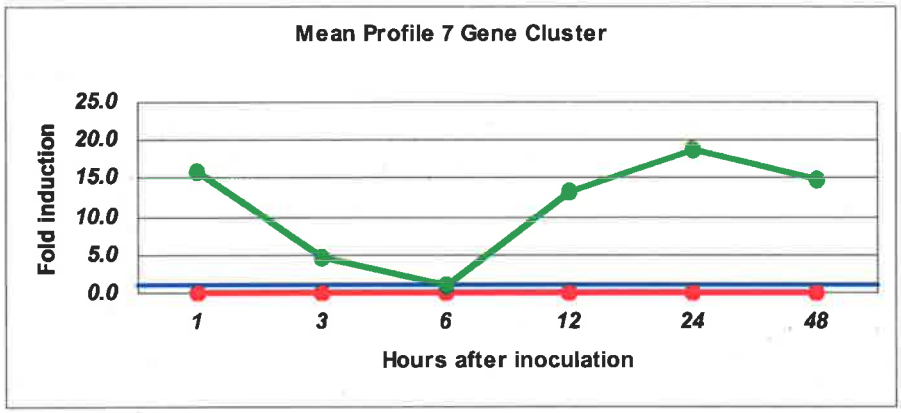
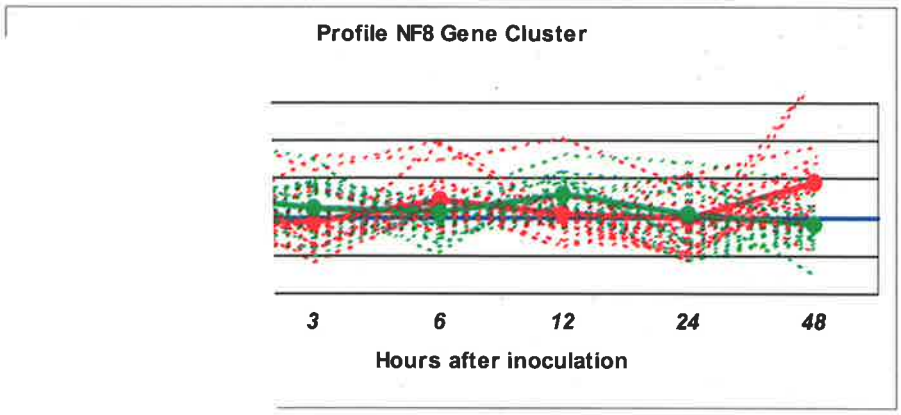
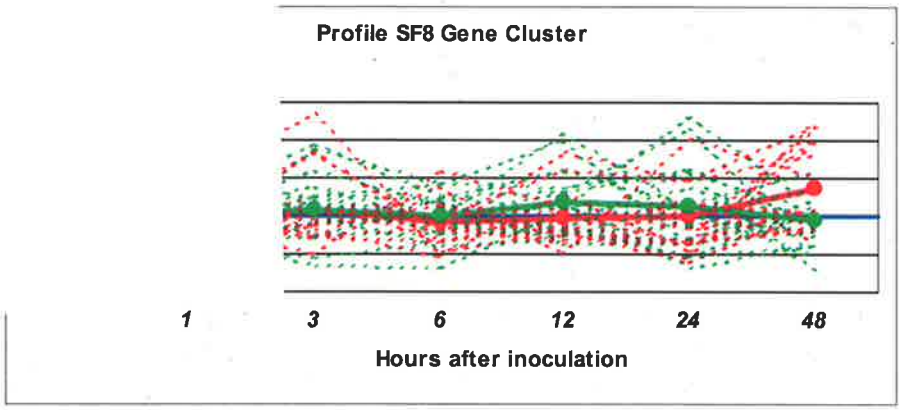


Figure 14.7: Q-PCR generated expression profiles of gene cluster 7 (1 gene) in the epidermis of barley leaves inoculated with *Ptt* (NF; Box S) and *Ptm* (SF; Box T). Broken lines represent expression profiles for individual members of the gene cluster and solid lines represent mean expression profiles of the individuals within the gene cluster. Green line = incompatible interaction (CI9214); Red line = compatible interaction (B87/14). The *y* axis indicates the fold induction for all of the genes in the cluster relative to their expression in mock-inoculated control plants. Hence, the value 1 (blue line) is an indicator of no differential expression. Box U shows the mean expression profile generated for this gene cluster from the NF and SF interactions combined.

V.





X.

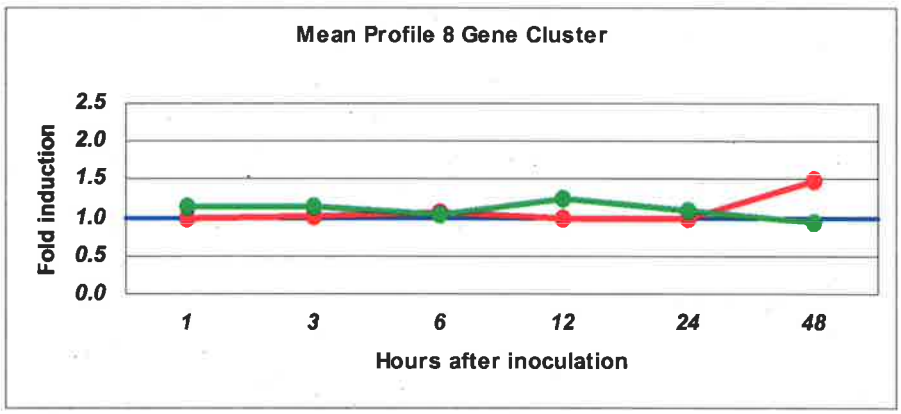


Figure 14.8: Q-PCR generated expression profiles of gene cluster 8 (17 genes) in the epidermis of barley leaves inoculated with *Ptt* (NF; Box V) and *Ptm* (SF; Box W). Broken lines represent expression profiles for individual members of the gene cluster and solid lines represent mean expression profiles of the individuals within the gene cluster. Green line = incompatible interaction (CI9214); Red line = compatible interaction (B87/14). The *y* axis indicates the fold induction for all of the genes in the cluster relative to their expression in mock-inoculated control plants. Hence, the value 1 (blue line) is an indicator of no differential expression. Box X shows the mean expression profile generated for this gene cluster from the NF and SF interactions combined.

across all experimental time points. Their expression is graphically represented by an almost straight line that does not deviate much from the value of 1, which signifies no change in gene expression between inoculated and mock-inoculated samples.

4.4 DISCUSSION

4.4.1 Normalisation

The geNorm program (Vandesompele et al., 2002) proved to be very useful in identifying which genes were best suited to act as internal controls for the normalisation of Q-PCR generated expression data. The addition of five newly identified reference transcripts – SF324, SF387, SF400, SF427, and SF575 – to the three commonly used housekeeping genes – cyclophilin, α-tubulin, and GAPDH – was invaluable. Not only did it help generate more accurate normalisation factors, but it also highlighted the danger associated with selecting just one housekeeping gene without first testing its presumed constant level of expression following experimental treatment. As it turned out, the least stable genes as calculated by geNorm were in fact the commonly used housekeepers, cyclophilin, α-tubulin, and GAPDH – in that order. Because their *M* values never exceeded 1.5, it can be argued that they are stably expressed to some degree, however, the fact that the *M* values of the newly identified control transcripts dropped significantly following their stepwise exclusion shows that they were better normalisation controls.

Four of these five SSH clones were initially selected for expression analysis because they share sequence homology with genes involved in the ubiquitin/26S proteasome-dependent proteolytic pathway. SF387 shares homology with a gene encoding a ubiquitin conjugating enzyme, SF400 and SF427 are similar to proteins for the 20S proteasome alpha subunits F and E, respectively, and clone SF575 encodes a 26S proteasome regulatory particle triple-A ATPase subunit 6 protein. These clones were initially selected for expression analysis because it was thought that induction of the ubiquitin-mediated proteolytic pathway may be one strategy that the plant may use to combat the introduction of foreign proteins derived from the net blotch causing fungi. In previous studies, rapid degradation of the disease resistance gene RPM1 during the HR (Boyes et al., 1998) was indicative of increased proteolytic activity in plant

defence responses, and the up-regulation of some proteasome subunits has been correlated with plants developing SAR (Dahan et al., 2001; Suty et al., 2003). In the barley-net blotch pathosystem, it appears that ubiquitin-mediated proteolysis does not play a major role in the early stages of both barley-*P. teres* incompatible interactions. Instead of the ubiquitin/26S proteasome complex, it is possible that the autophagy pathway may degrade some of the foreign proteins associated with the pathogen. Autophagy is the other major proteolytic pathway in cells (Klionsky and Emr, 2000) that can be induced in plants by multiple stress factors, including pathogen attack (Liu et al., 2005).

4.4.2 Cluster analysis

A key step in analysing gene expression studies is the grouping of expression data into clusters that contain members more similar to each other than to the remainder of the data. In this study, the approach of direct visual inspection was used to group together genes with similar expression patterns. This method was used by Cho et al. (1998) to cluster genes whose expression correlated with particular phases of the cell cycle and was deemed appropriate because of the relatively small data set. For larger-scale gene expression studies, computational techniques based on complex algorithms are commonly used, however, the various algorithms have been shown to differ from one another and typically deliver only partly concordant results when applied to a given data set. In a recent report, it was demonstrated that 20-40% of genes from a high-quality data set were classified differently by two algorithms (Hart et al., 2005). Hence, there is no scientific evidence to suggest that using an algorithm to analyse the expression data would have generated more accurate clusters than the ones obtained via direct visual inspection.

In total, the expression profiles of 45 SSH clones were determined by Q-PCR using a 1.5 fold cut-off value to assign genes as being differentially expressed. Although the commonly used threshold value is 2 fold (Cheong et al., 2002; Scheideler et al., 2002), several studies have shown that a lower cut-off ranging from 1.2 to 2 can be used reliably (Jin et al., 2001; Mysore et al., 2002; Zik and Irish, 2003; Gibly et al., 2004, Schweizer et al., 2005). It is also important to note that the expression data presented in this study was generated from single biological experiments which meant that the

significance of the expression data was not verified by statistical analysis. Although this is unlikely to effect the results for gene clusters showing obvious differences in gene expression (i.e. gene clusters 5-7) it is acknowledged that caution must be taken when interpreting the significance of the remaining gene clusters for which a lower level of differential gene expression was observed, particularly those showing differences between 1.5 and 2 fold.

4.4.3 SSH efficiency

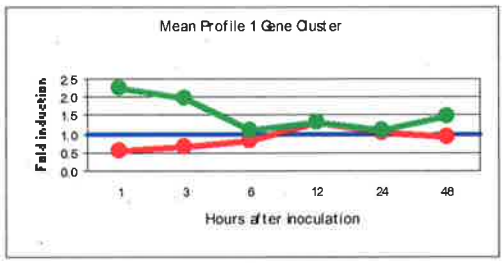
From the resulting expression analysis at 24 hai only, the efficiency with which the SSH procedure isolated differentially expressed genes was 59% which is far greater than the 26% value established by expression profiling at the same time point using Northern blots. The Q-PCR value is likely to be the more accurate of the two because (a) expression signals were detected for all clones using Q-PCR which was not the case with Northern blots and (b) Q-PCR expression profiling was conducted using RNA from the epidermis (the tissue from which RNA was initially extracted for SSH) and not whole leaf RNA as was the case with the Northern blots. In other SSH studies the percentage of clones representing differentially expressed genes has ranged from approximately 70% (Kuang et al., 1998; Birch et al., 1999; Grenier et al., 2002) to as high as 97% (Ros et al., 2004). Regardless of the SSH efficiency it is clear that the entire repertoire of differentially expressed genes in the barley-*P. teres* incompatible interaction was not isolated from one subtraction alone and the fact that clones isolated from one of the net blotch incompatible interactions were also differentially expressed but not identified in the other also suggests that SSH is not a comprehensive approach.

4.4.4 Putative functions of the analysed genes

The major outcome of the detailed expression analysis was the identification of 28 SSH clones whose corresponding genes appeared to be differentially expressed at various time points in the early stages of the barley-*P. teres* incompatible interaction. Although the expression profiling alone does not guarantee that these genes are components of the signal transduction pathway leading to net blotch disease resistance, it has provided a framework by which candidate genes can be selected for

further detailed studies based on their expression patterns. In the next part of the discussion, each individual clone/group of clones is discussed with respect to the putative functional roles their corresponding proteins may play in the barley-*P. teres* defence response. Because these putative functional roles are based on the alignments of short cDNA sequences with sequences posted in GenBank, it is acknowledged that more sequence information would be necessary to verify the gene identity given to a particular clone.

4.4.4.1 Profile 1 gene cluster



[Green = incompatible interaction]
[Red = compatible interaction]

Characteristics:

Very early up-regulation of genes in the incompatible interaction (from 1 to 3 hai). Genes down-regulated during the same time period in the compatible interaction.

The expression profile for clone **HvPtr1** suggests that induction of the corresponding gene as early as 1 hai is important in the resistance-associated defence response of the barley-*P. teres* interaction. A BLAST search with the best EST match revealed that clone HvPtr1 has high sequence homology with a nonphototropic hypocotyl 1 (NPH1) protein from *Arabidopsis*. Huala et al. (1997) showed that *Arabidopsis* NPH1 is a plasma membrane-associated serine/threonine protein kinase with a putative redox-sensing domain and further studies showed that this NPH1 protein, which has since been renamed to PHOT1, becomes heavily phosphorylated on irradiation with blue light and is one of the major photoreceptors of the signal transduction pathway for phototropism (Briggs and Christie, 2002). This thesis describes for the first time the identification of a gene encoding a putative phototropin from barley, and although phototropins have never been implicated with plant defence responses, the finding that *Arabidopsis* PHOT1 is involved in the downstream activation of calcium-permeable channels (Baum et al., 1999; Stoelzle et al., 2003) makes clone HvPtr1 an interesting candidate for further study as calcium signalling is known to initiate cellular responses to a diverse range of developmental cues and environmental challenges, including many plant-pathogen interactions (White and Broadley, 2003). For example, an

increase in calcium influx was recently shown to be associated with the Flax-*F. oxysporum* incompatible interaction (Olivain et al., 2003). Therefore an interesting experiment would be to determine if there is any correlation between *HvPtr1* expression and calcium levels in the cells of barley leaves infected with *P. teres* as any difference in calcium influx could influence the different signalling cascades leading to net blotch resistance or susceptibility.

Clones **HvPtr2** and **HvPtr5** share sequence homology with genes encoding putative receptor-like protein kinases (RLKs) from rice and *Arabidopsis*, respectively. RLKs comprise the largest gene family of receptors in plants, with over 600 RLKs in *Arabidopsis* and more than 1100 in rice (Shiu et al., 2004). Studies of pathogen responses in plants suggest that RLKs could play an important role in recognising pathogen-derived elicitors and in activating defence responses. For example, several *R* genes have been shown to encode RLKs. These include *Xa21* and *Xa26* from rice, which are required for resistance to the bacteria *Xanthomonas oryzae* pv. *oryzae* (Song et al., 1995; Sun et al., 2004), and the barley stem-rust resistance gene, *Rpg1* (Brueggeman et al., 2002). Additionally, it has been proposed that RLKs are involved in the specific recognition of PAMPs and therefore they may be key components of signal transduction pathways associated with non-host resistance (Nürnberger and Brunner, 2002). These include the *Arabidopsis* FLS2 (Gómez-Gómez and Boller, 2000) and EFR (Zipfel et al., 2006) RLKs, which are involved in recognising the bacterial PAMPs flagellin and EF-Tu, respectively.

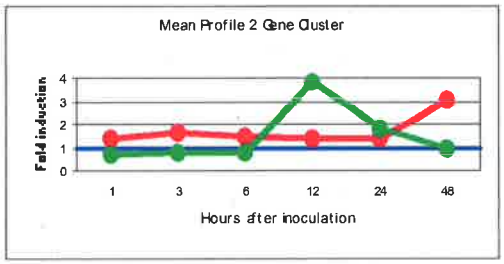
The early differential expression of RLK-encoding genes in incompatible compared to compatible plant-pathogen interactions has also been demonstrated. For instance, Czernic et al. (1999) showed that, like *HvPtr5*, an RLK-encoding gene, *AtRLK3*, was up-regulated within 12 h in the incompatible interaction between *Arabidopsis* and the bacterial pathogen, *R. solanacearum*, but not differentially expressed in the compatible interaction. Clones HvPr2 and HvPtr5 are therefore interesting candidates for further study because the putative RLKs they encode may be integral components of the signal transduction pathway leading to net blotch resistance. In particular, the early up-regulation of clone HvPtr2 within 3 hai in the barley-*P. teres* incompatible interaction suggests that its corresponding RLK may be one of the first molecules involved in *P. teres* elicitor perception and/or induction of defence components. In

light of the current evidence, using a transgenic approach to over-express RLK-encoding genes, such as *HvPtr2* and *HvPtr5*, may prove to be a good strategy for generating barley breeding lines with net blotch resistance. In support of this strategy, Godiard et al. (2003) showed that an *Arabidopsis* cultivar normally susceptible to bacterial wilt disease showed increased resistance to the causal pathogen when transformed with a gene, *ERECTA*, that encodes an RLK.

Clone **HvPtr3** had the highest sequence homology with a 14-3-3-like protein A from barley that was isolated in a subtractive cDNA library screen for transcripts accumulating in barley leaves following infection by the non-host wheat powdery mildew fungus, *B. graminis* f. sp. *tritici* (Brandt et al., 1992). However, in contrast to the barley-*P. teres* interaction, Gregersen et al. (1997) showed that although this 14-3-3 transcript accumulated early (within 6 hours) and weakly in the defence response, no difference was noted between incompatible and compatible interactions with the barley powdery mildew fungus. 14-3-3 proteins function as regulators of a wide range of target proteins and because they do this via direct protein-protein interactions it is widely accepted that they have the potential to regulate plant signalling pathways in response to abiotic and biotic stress (Roberts et al., 2002; Roberts, 2003). In addition to the barley-powdery mildew pathosystem, 14-3-3 transcript accumulation has also been reported in soybean inoculated with *P. syringae* pv. *glycinea* (Seehaus and Tenhaken, 1998), cotton roots inoculated with the wilt pathogen *Verticillium dahliae* (Hill et al., 1999), and tomato challenged with the avirulence elicitor Avr9 from the fungal pathogen *C. fulvum* (Roberts and Bowles, 1999). In support of the work presented here, Roberts and Knowles (1999) identified three 14-3-3 genes that were found to be up-regulated in the incompatible compared to compatible response, including one that was up-regulated very early in the interaction (within 4 hours). Further evidence for the involvement of 14-3-3 proteins in disease resistance comes from the *Arabidopsis AKR2* gene whose product interacts with a 14-3-3 protein and negatively regulates a number of pathogen resistance responses (Yan et al., 2002). However, exactly what role 14-3-3 proteins play in disease resistance remains unknown. They have been associated with the activation of the plasma membrane H^+-ATPase (Oecking and Hagemann, 1999; Finnie et al., 2002), which has been reported to be involved in defence, wound and general stress signalling in plants via pH regulation (Vera-Estrella et al., 1994). They have also been shown to regulate a

number of other signalling proteins, including transcription factors and kinases (Roberts, 2003). Interestingly, Kinoshita et al. (2003) showed that phototropins (see HvPtr1 discussion) are among the kinases that 14-3-3 proteins interact with.

4.4.4.2 Profile 2 gene cluster



Characteristics:

Genes up-regulated 12 hai in the incompatible interaction. Possible delayed induction of the same genes 24 hai in the compatible interaction.

Clone **HvPtr4** was selected as a positive control for expression analysis as it shares homology with a gene from rice that codes for a chitinase enzyme. Chitinases [EC 3.2.1.1.4] belong to the PR3 family of PR proteins and are characterised by their ability to degrade chitin – a β-1,4-linked polymer of *N*-acetyl-glucosamine found in many fungal cell walls (Van Loon and Van Strien, 1999). It has been shown that chitinases, either alone or in combination with β-1,3-glucanases, can inhibit the growth of different fungi in vitro (Mauch et al., 1988; Zareie et al., 2002). In addition, transgenic plants producing elevated levels of chitinase have displayed improved resistance to fungal pathogens (Melchers and Stuiver, 2000). Because chitinase protein activity and transcript levels have been shown to increase in a number of plant-pathogen interactions (e.g. Li et al., 2001; McFadden et al., 2001; Wang et al., 2005), clone HvPtr4 was selected as a positive indicator of defence responses being initiated in the barley-*P. teres* interaction. In the aforementioned studies, chitinase activity/transcript levels were also shown to be induced earlier in the resistant compared to susceptible interactions, and this is in accordance with the expression pattern observed for clone HvPtr24 which was up-regulated within 12 hai in the net blotch resistant cultivar but not until 24 to 48 hai in the susceptible cultivar. In addition, it is evident from the barley-*P. teres* incompatible interaction that the induction of chitinase-encoding genes may happen as early as 6 to 12 h after pathogen infection and not 24 h and later as indicated by previous studies (Li et al., 2001; McFadden et al., 2001; Wang et al., 2005). In those reports, expression analysis was

conducted using Northern blots of RNA extracted from whole leaf tissue. In this study, expression profiling using epidermal RNA shows that the activation of certain defence gene transcripts may occur significantly earlier in the epidermis than the mesophyll. In any case, it appears that the early induction of chitinase-encoding genes may be associated with the development of an incompatible interaction in the barley-*P. teres* pathosystem.

Clone **HvPtr6** has sequence homology with one of two sorbitol transporter genes that were recently cloned from sour cherry (*Prunus cerasus*) fruit tissues that accumulate large quantities of sorbitol (Gao et al., 2003). Sorbitol is an acyclic polyol and although it is a primary photosynthetic product and one of the principal sources of sugar for sink tissues in plants, very little is known about the molecular regulation of this compound. Further evidence implicating the involvement of sorbitol transporters in disease resistance comes from the study of Zierold et al. (2005) who also identified a clone with sequence homology to a sorbitol transporter that was found to be associated with *mlo*-mediated powdery mildew resistance in barley. In accordance with the findings presented here, this clone was also up-regulated by a similar level (4.1 fold) in a resistant compared to susceptible line within 24 hai. Different sugar transporters have been reported to be induced in other plants following pathogen infection (Williams et al., 2000). An increase in sugar levels has been reported for several pathosystems, including tobacco infected with potato virus Y (Herbers et al., 2000) and *Arabidopsis* infected with *Albugo candida* (Chou et al., 2000). It is possible that these pathogens (along with *P. teres*) withdraw carbohydrates from the plant, thereby creating a metabolic sink around the infection site which then needs to be replenished with sugars so that the surrounding cells have an energy source to mount an efficient defence response. Sugar transporters are likely to play an important role in providing this energy source as it has been shown that photosynthesis and photosynthetic gene expression is repressed in plants following pathogen attack (Herbers et al., 2000; Berger et al., 2004). The fact that a putative invertase was found to be up-regulated in the barley-*P. teres* incompatible interaction at 24 hai (see clone HvPtr11 discussion) provides further evidence that an increase in sugar levels at the 12-24 hai time point could be critical for the induction of a successful defence response. Monitoring the levels of prime carbon sources such as sorbitol and sucrose

in the barley-net blotch pathosystem would help to shed light on any link between elevated sugar levels and disease resistance.

One of the clones selected for expression analysis based on its potential association with cellular detoxification mechanisms was **HvPtr7** whose nearest gene homolog encodes a major facilitator superfamily (MFS) antiporter. Simmons et al. (2003) also reported the induction of an MFS gene, *Zm-mfs1*, within 12 h in maize challenged by two fungal pathogens. However, in that study the authors reported that there was no difference in *Zm-mfs1* transcript accumulation in resistant compared to susceptible interactions and that *Zm-mfs1* appears to respond as part of a general defence mechanism. This makes *HvPtr7* the first putative MFS gene to be differentially induced in a resistant plant-pathogen interaction. Although plant MFS transporters have not been extensively studied, a number of reports have shown that plant pathogenic fungi utilise MFS antiporters to export their own toxins, thus rendering themselves resistant, while delivering toxins to the plant (Alexander et al., 1999; Callahan et al., 1999; Pitkin et al., 2000). Therefore it is possible that the HvPtr7 protein functions in a similar way in the plant by exporting plant-produced toxins that can be used to thwart pathogen attack. Interestingly, toxin efflux pumps are believed to assist other detoxification systems of eukaryotes, such as the cytochrome P450 system, by reducing the toxin load on this degradation process (Van Bambeke et al., 2000). The differential induction of a putative cytochrome P450 gene in this study (*HvPtr17*) suggests that certain genes encoding multidrug efflux proteins may play a role in determining the outcome of the barley-*P. teres* interaction.

Clone **HvPtr8** displays high sequence homology with a gene from *Arabidopsis* that encodes a putative sterol delta-7-reductase (S7R) – an important enzyme in the plant sterol biosynthetic pathway. *S7R* genes have not previously been described in barley and their involvement in plant-pathogen interactions has also yet to be demonstrated. Sterols play at least two critical roles in plants: as bulk components of membranes regulating stability and permeability (Bach and Beneviste, 1997) and as precursors of growth-promoting brassinosteroids (BRs) (Fujioka and Sakurai, 1997). In a previous study, loss-of-function mutations in the *Arabidopsis S7R* gene to which *HvPtr8* shows significant homology, resulted in mutant plants displaying a dwarf phenotype and significantly reduced levels of BRs in their tissues (Choe et al., 2000). The authors

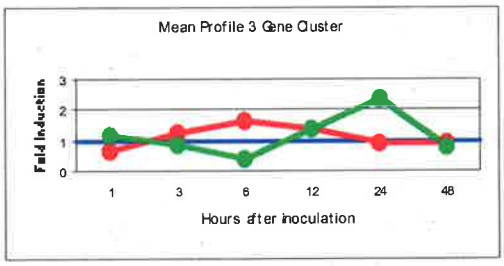
showed that altered BR biosynthesis was the primary cause of the dwarf morphology. It is therefore conceivable that, due to the induction of a putative S7R encoded by *HvPtr8*, a signal transduction pathway leading to the early synthesis of BRs may also be a key factor in determining the outcome of the barley-*P. teres* interaction. In support of this idea, it was recently reported that treatment with brassinolide, the most biologically active BR, induced resistance in tobacco plants against tobacco mosaic virus, the bacterial pathogen *P. syringae*, and the fungal pathogen *Oidium* sp., and in rice against *M. grisea* and *X. oryzae*, which cause rice blast and bacterial blight, respectively (Nakashita et al., 2003). Furthermore, a number of studies have indicated that there is cross-talk between BRs and other plant hormones, including those that regulate plant defence responses. For example, BRs have been shown to induce the expression of key enzymes involved in the ET (Yi et al., 1999) and JA (Muessig et al., 2000) biosynthetic pathways.

The sequence of clone **HvPtr9** is mostly similar to a member of the *Arabidopsis CONSTANS* family – a group of genes that encode transcription factors with B-box zinc-binding domains that have been implicated in promoting flowering by inducing the expression of the direct downstream genes (Kardailsky et al., 1999). Transcription factors of this type have not previously been reported as components of plant defence responses. However, the fact that mutations or rearrangements in some B-box family members have been associated with human diseases and cancers (Torok and Etkin, 2000) suggests that members of this gene family may play important roles in fundamental biological processes. Therefore, it is possible that the putative transcription factor encoded by *HvPtr9* may regulate the expression of a gene whose product is required for net blotch resistance to take place. This could involve one of the genes co-regulated with *HvPtr9* in cluster 2 or another gene that is found to be induced around 12 h after pathogen infection.

The nearest homolog to clone **HvPtr10** is a gene that encodes a putative calcineurin B-like protein (CBL) from pea. CBLs are Ca^{2+}-binding proteins with structural characteristics that suggest they have the potential to regulate stress and hormone signalling in plants by transducing Ca^{2+} signals (Luan et al., 2002). This thesis describes for the first time the potential involvement of a plant *CBL* gene in conferring resistance to a microbial pathogen. Although the expression of *CBL* genes has never

been monitored in plants challenged by pathogens, expression studies of *CBL1*, the founding member of the *CBL* gene family in *Arabidopsis*, have shown that it is strongly induced by wounding, drought, high salt, and cold stress (Kudla et al., 1999; Piao et al., 2001). Furthermore, Albrecht et al. (2003) showed that disruption of *CBL1* gene function renders mutant plants hypersensitive to drought and cold stress. Conversely, constitutive over-expression of *CBL1* induced the expression of known stress-regulated genes and enhanced stress tolerance. In light of these observations, it is evident that CBLs have the potential to regulate stress signalling cascades in plants - a process that is also likely to involve their respective kinase effectors, the CBL-interacting protein kinases (CIPKs) (Shi et al., 1999; Batistic and Kudla, 2004). Interestingly, *HvPtr10* appears to be induced downstream of *HvPtr1* in the barley-*P. teres* incompatible interaction. The *HvPtr1* gene product may be involved in activating Ca^{2+}-permeable ion channels in the plasma membrane, and because early changes in cytosolic Ca^{2+} have been reported in plants challenged by microbial pathogens (White and Broadley, 2003), net blotch resistance could involve Ca^{2+} signalling and the signal could be transmitted via the putative CBL encoded by *HvPtr10* and its effector CIPK to components of the signal transduction pathway for disease resistance. This pathway may also involve *HvPtr24* which is induced downstream of both *HvPtr1* and *HvPtr10* and whose gene product is also putatively associated with Ca^{2+} signal transduction.

4.4.4.3 Profile 3 gene cluster



Characteristics:

Initial period of down-regulation (until 6 hai) followed by the genes being up-regulated 24 hai in the incompatible interaction. Possible inverse mode of expression observed in the compatible interaction.

Clone **HvPtr11** has sequence homology to a putative plant invertase [EC 3.2.2.26] from *Arabidopsis* that is similar to a neutral invertase identified in carrot (Sturm et al., 1999). Invertases are involved in sucrose metabolism and exist in different isoforms with varying pH optima. Neutral or alkaline invertases are thought to be located in the

cytosol, whereas acid invertases are found either in the vacuole or cell wall (Sturm et al., 1999). Although all isoforms have essentially the same function in catalysing the cleavage of sucrose to glucose and fructose, neutral or alkaline invertases have not received the same attention as their acidic counterparts, and in fact, clone HvPtr11 represents the first identification of a gene encoding a putative neutral invertase in barley.

In general, previous studies have shown that physiological treatments which increase metabolic demand within the leaf tend to lead to increases in invertase activity at the protein and/or transcript level, and this has been reported in plant-pathogen interactions involving both biotrophic (Scholes et al., 1994; Tang et al., 1996; Fotopoulos et al., 2003; Swarbrick et al., 2006) and necrotrophic (Benhamou et al., 1991; Berger et al., 2004) fungi. Among these reports, differences in the timing of induction of invertase activity has been observed between resistant and susceptible interactions in tomato roots infected with *F. oxysporum* (Benhamou et al., 1991) and in barley leaves challenged by *B. graminis* (Swarbrick et al., 2006). In both cases, the resistant interaction was characterised by an earlier (detected within 48 hai) and stronger accumulation of invertase. At the gene level, the differential expression of mRNA invertase-encoding transcripts has been shown for resistant *versus* control interactions in carrot roots and leaves infected with the bacterial pathogen, *Erwinia carotovora* (Sturm and Chrispeels, 1990), and in tobacco leaves infected with the oomycete, *Phytophthora nicotianae* (Scharte et al., 2005). A common finding in both studies was the early up-regulation (within 24 hai) of invertase gene expression in the resistant interaction.

The only study in which detailed invertase transcript accumulation has been monitored within the first 24 hours of a plant-pathogen interaction was performed by Sturm and Chrispeels (1990). The resulting expression profile in carrot roots and leaves, was very similar to the one observed in the barley-*P. teres* compatible interaction. In carrots, there was an increase in invertase transcript levels at 1, 3, and 6 hai, followed by a dramatic reduction at 12, 18, and 24 hai - where no transcript was detected. In barley, the putative neutral invertase transcript was up-regulated at 3 hai for the SF interaction and at 3 and 6 hai for the NF interaction, after which transcript accumulation in both interactions returned to control levels. An increase in invertase

activity may result in a greater accumulation of glucose and fructose, and although it has been shown previously that defence response genes are induced by elevated sugar levels (Ehness et al., 1997; Herbers et al., 2000), in the plant-pathogen compatible interaction this may not to be the case. It is more likely that an increase in invertase gene induction contributes in part to the breakdown of signalling components involved in photosynthesis - and ultimately cell death. Jang and Sheen (1994) showed that elevated levels of some hexoses, including glucose and fructose, exerted specific repression of the promoter activity of three maize photosynthetic genes. Scholes et al. (1994) also showed that the rate of photosynthesis in barley was inhibited by higher concentrations of glucose and fructose. Although this was most noticeable 5 days after inoculation with the powdery mildew fungus, it cannot be ruled out that the signalling mechanisms leading to this decrease occur much earlier in the interaction.

Clones **HvPtr12** and **HvPtr15** share sequence similarity with genes encoding aldehyde dehydrogenases (ALDHs) from rice and barley, respectively. ALDHs constitute a diverse protein family found in various organisms and although at present there is no published evidence implicating these proteins in plant defence responses to microbial pathogens, their involvement in the oxidation of aldehydes (Yoshida et al., 1998) implies that *HvPtr12* and *HvPtr15* may act to detoxify the cellular environment if indeed these toxic compounds do accumulate in plant cells following *P. teres* infection. Two lines of evidence suggest that they do. The isolation of several clones associated with the anti-oxidant response and the fact that ROS accumulation has previously been reported in this pathosystem (Able, 2003) could lead to an increase in aldehyde levels as this compound is a by-product of the lipid peroxidation chain reaction caused by ROS (Witz, 1989; Esterbauer et al., 1991). In addition, the up-regulation of clone HvPtr20 may be indicative of aldehyde accumulation as the corresponding protein catalyses a reaction in which acetaldehyde is also produced as a by-product.

The potential involvement of plant ALDHs as components of defence responses to biotic stress is highlighted by studies which have shown that ALDH gene transcripts accumulate differentially in plants responding to various abiotic stress treatments (Kirch et al., 2001; Chen et al., 2002; Ozturk et al., 2002). In fact, the nearest homolog to HvPtr12 is a putative mitochondrial ALDH-encoding gene from barley that was

down-regulated in plants under submerged conditions (Meguro et al., 2001). Mitochondrial ALDHs belong to the class 2 family of ALDHs and their involvement in the cellular detoxification of ethanol-derived acetaldehyde has been documented (Wang et al., 1998). An interesting experiment would be to monitor aldehyde formation in the resistant and susceptible interactions to get a better understanding of how the resistant genotype copes with the toxic by-products associated with oxidative stress. Altering ALDH activity may represent one good defence strategy, and one study has already shown that transgenic *Arabidopsis* plants over-expressing an *ALDH* gene displayed improved tolerance to dehydration, salt, H_2O_2, and heavy metal stress - most likely by scavenging toxic aldehydes and thus reducing lipid peroxidation (Sunkar et al., 2003).

Clone **HvPtr13** shows sequence homology with a probable α-galactosidase (α-D-galactoside galactohydrolase [EC 3.2.1.22]). In plants, α-galactosidases are known to catalyse the hydrolysis of various storage compounds like galacto-oligosaccharides, -lipids, and -proteins, and because seeds contain large quantities of these substances, work on α-galactosidases has mainly focused on seed tissue (Feurtado et al., 2001; Guimarães et al., 2001). The induction of α-galactosidase genes following pathogen attack has not been reported previously, however, recent findings suggest that they are induced under different stress conditions such as dark-induced and natural senescence of barley leaves (Chrost et al., 2004; Chrost and Krupinska, 2000) and low temperature deacclimation in petunia (Pennycooke et al., 2004). The nearest known homolog to clone HvPtr13 is the barley cDNA clone named HvSF11. HvSF11 shows high homology to known α-galactosidases and its transcript level was shown to decrease in flag leaves during natural senescence and inversely increase in primary foliage leaves induced to senesce by darkness (Chrost and Krupinska, 2000). Furthermore, Chrost et al. (2004) showed a correlation between *HvSF11* gene expression and α-galactosidase activity in primary foliage leaves, and that *HvSF11* transcript levels decreased after exogenous application of sucrose and glucose. Sugar starvation in plant senescence is a direct consequence of a decline in photosynthesis, and the authors have hypothesised that because of their ability to hydrolyse terminal α-1,6-linked α-galactose residues from glycolipids, glycoproteins, or oligosaccharides, α-galactosidases may provide an alternative energy source to

photosynthetic assimilates - thereby contributing to leaf survival. The expression profile of clone HvPtr13 supports this hypothesis. *P. teres* needs to induce cell death to survive and the up-regulation of *HvPtr13* gene induction in the incompatible interaction at the same time that there is a down-regulation in the compatible interaction, may be a reflection of the successful activation of a pathway that contributes to preventing leaf senescence in the *P. teres* resistant genotype.

Clone **HvPtr14** has the highest sequence homology with a protein kinase from *Arabidopsis* that is similar to dual specificity serine/threonine/tyrosine (STY) protein kinases identified in soybean and peanut (Feng et al., 1993; Rudrabhatla and Rajasekharan, 2002). It is well known that protein kinases are key components in signalling mechanisms critical for responses to environmental stresses and attack by pathogens. Although there is no previous report implicating STY protein kinases in plant-pathogen interactions, their expression has been shown to be regulated in peanut and cucumber by abiotic stress treatments like drought, salt, and cold (Rudrabhatla and Rajasekharan, 2002; Jang et al., 2004). At present, the biochemical function of all previously reported STY protein kinases remains unknown, however their reported involvement in the tyrosine phosphorylation of target proteins is interesting as this reaction has previously been implicated in plant defence signalling leading to the HR (Adam et al., 1997; Rajasekhar et al., 1999). In addition, Shih et al. (2004) recently showed for the first time in plants that protein tyrosine phosphorylation may also be involved in programmed cell death. It is therefore possible that the early up-regulation of HvPtr14 transcripts in the barley-*P. teres* compatible interaction could be associated with STY protein kinase-mediated tyrosine phosphorylation of proteins that initiate the early induction of signals leading to HR-associated cell death. Zhang et al. (2000) also demonstrated that the timing for fungal elicitin-treated tobacco cells to commit to a death program correlated with the appearance of high levels of gene expression for a protein kinase, WIPK, in the first eight hours after treatment. After 24 hours, *WIPK* gene expression was not detected. Based on this evidence, the late or altered induction of a putative STY protein kinase involved in the cell death signalling pathway may play an important role in the resistance of barley to *P. teres*.

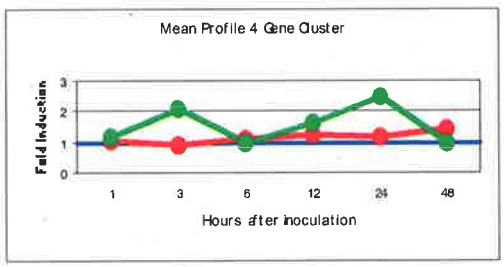
Clone **HvPtr16** shares sequence homology with a gene that encodes a cystathionine-β-synthase (CBS) domain-containing protein of unknown function. Genes that encode

proteins with CBS domains have not been functionally characterised in plants, and in fact, only one such gene has previously been implicated in resistance responses in these organisms. This gene, called *OsBi1*, was found to be specifically induced within 24 h in rice plants interacting with the brown planthopper insect (Wang et al., 2004). The authors also showed that *OsBi1* was induced in response to ethylene treatment but not to infection with the rice blast fungus. However, it must be noted that *OsBi1* expression analysis was only carried out 48 h after pathogen inoculation, so it is possible that the gene may have been expressed earlier. Although it is unclear whether any functional similarities exist between *OsBi1* and *HvPtr16*, the fact that plant responses to herbivore feeding behaviour and defensive reactions to pathogens are similar (Taylor et al., 2004) suggests that the CBS domain may also be an important regulatory component in the barley-*P. teres* interaction. Interestingly, because *HvPtr16* appears to be co-regulated with *HvPtr28* in the incompatible interaction, it is possible that like OsBi1, the CBS domain of the corresponding HvPtr16 protein may be associated with the ethylene signal transduction pathway. Other putative functions come from animal studies where CBS domains have been shown to be involved in the intracellular trafficking and function of chloride channel proteins (Carr et al., 2003; Estevez et al., 2004). Their role as metabolite sensors (Scott et al., 2004; Ignoul and Eggermont, 2005) and their involvement in mediating protein-protein interactions and protein regulation (Milan et al., 2000; Shan et al., 2001) has also been described. The physiological importance of CBS domains is emphasised by the observation that point mutations in CBS domains can affect specific protein function and cause several hereditary diseases in humans (Ignoul and Eggermont, 2005).

Clone **HvPtr17** has sequence homology with a cytochrome P450 monooxygenase (P450) enzyme. Plant systems are known to utilise a diverse array of P450s, and because of their role in so many biosynthetic and detoxificative pathways, it is not surprising that a clone encoding a P450 protein was identified in the SSH library. Pathogen induced and stress response P450 genes have previously been described in tobacco (Takemoto et al., 1999), maize (Persans et al., 2001), and barley (Hess et al., 1998; Ueda et al., 2002), and although the specific biological function of these genes remains unknown, other P450 genes have been implicated in numerous defence-related pathways, including the biosynthesis or catabolism of signalling molecules such as auxin and abscisic acid (ABA) (Hull et al., 2000; Krochko et al., 1998), and

the synthesis of antimicrobial toxins such as 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) (Frey et al., 1997). Although clone SF475 shares the highest sequence similarity with a P450 from *Lolium rigidum* (Fischer et al., 2001), it is also homologous with a P450, CYP72A5, that was induced in maize seedlings and roots pre-treated with the herbicide protectants, naphtalic anhydride and phenobarbital (Persans et al., 2001). CYP72A5 continued to be induced following treatment with the herbicide triasulfuron, but was not induced when the herbicide was applied without prior treatment with either one of the protectants. In a similar way, it is possible that the putative P450 encoded by clone SF475 may itself function as a plant-made "protectant" and that its expression may lead to the induction of *P. teres* detoxification enzymes, possibly in association with other detoxification systems mediated by FMO and MATE proteins (discussed earlier).

4.4.4.4 Profile 4 gene cluster



Characteristics:

Genes up-regulated early (3 hai) and later (24 hai) in the incompatible interaction. Genes expressed at control levels in the compatible interaction.

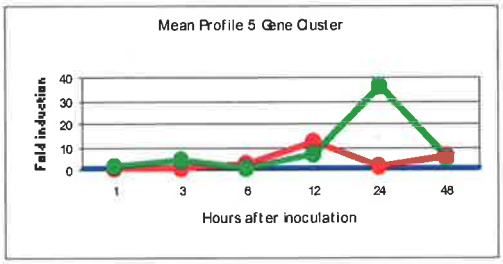
The deduced amino acid sequence of clone **HvPtr18** indicates that the corresponding gene encodes an LRR protein. The importance of LRR proteins in signal transduction pathways and their role as mediators of protein-protein interactions is well known and has been briefly discussed earlier. In plant-pathogen interactions, the structural LRR domains can function as the binding sites of plant-derived or pathogen-induced ligands and specific recognition of pathogen-derived elicitors or products encoded by Avr genes (Jones and Jones, 1996). Members of the LRR superfamily include R proteins that contain both extracellular (e.g. Xa21 from rice (Song et al., 1995)) and cytosolic (e.g. RPS2 from *Arabidopsis* (Bent et al., 1994)) domains, as well as cell surface receptors like the plant transmembrane receptor proteins TMK1 (Chang et al., 1992) and receptor-like kinase proteins RLK5 (Walker, 1993), that can bind to specific ligands. The potential involvement of a putative LRR protein encoded by

HvPtr18 in the barley-*P. teres* incompatible interaction is thus not surprising and is consistent with a number of recent studies in which proteins containing LRR domains have been reported to be induced in plants following pathogen infection (Hipskind et al., 1996; Jung et al., 2004; Jacques et al., 2006).

The second clone in this cluster, **HvPtr19**, shares homology with a gene that encodes a putative casein kinase I (CKI) from rice. CKI is a member of the serine/threonine protein kinase family, and in mammals, this protein has been associated with a diverse range of cellular processes including membrane trafficking, DNA-repair pathways, and cellular differentiation (Gross and Anderson, 1998). In plants, the physiological functions of CKI remain largely unknown. Although CKI-encoding genes from barley have not previously been described, they have been isolated from several other plant species, and interestingly, one was shown to be up-regulated in the incompatible interaction between olive tree and the biotrophic fungus responsible for peacock spot disease, *S. oleagina* (Benitez et al., 2005). In that study, the olive CKI-encoding gene, *Olest57*, was up-regulated by approximately 7-fold within 8 hai in the peacock spot resistant cultivar, whereas in the susceptible cultivar it was not significantly up-regulated until 48 hai and later. The authors also showed that *Olest57* was induced in the resistant olive cultivar 8 h after treatment with SA, methyl jasmonate, and ethephon – an inducer of ET. Because the time points differ at which *Olest57* and *HvPtr19* expression was monitored, it is difficult to make a clear comparison between their corresponding expression profiles. The fact that *HvPtr19* was up-regulated 3 hai and again at 24 hai suggests that, like *HvPtr18*, it may play a dual role in the plant defence response. It appears that it may be involved in both early signalling events that occur directly downstream of pathogen recognition and later events that may be involved in defining the defence response. In accordance with a previous study in which a putative CKI gene, *OsCKI*1, was found to be up-regulated in rice following treatments with BR and ABA (Liu et al., 2003), it is conceivable that the later events in the barley-*P. teres* interaction may well involve these two plant hormones as two clones associated with their production or presence, HvPtr8 and HvPtr22, were up-regulated in the net blotch resistant cultivar at 12 and 24 hai, respectively. It is also possible that proteins that mediate, or are regulated by, the function of BR, ABA, and other plant hormones, may serve as substrates for CKI, allowing integration of the various hormonal responses. In support of this, a related SA-inducible casein kinase,

casein kinase II, was shown to be involved in the transcriptional activation of plant defence response genes via the phosphorylation of members of the TGA family of transcription factors (Hidalgo et al., 2001; Kang and Klessig, 2005).

4.4.4.5 Profile 5 gene cluster



Characteristics:

Genes up-regulated by almost 40-fold 24 hai in the incompatible interaction. Induction of corresponding genes in the compatible interaction suppressed after 12 hai.

Clone **HvPtr20** has sequence homology to a threonine aldolase (TA) protein that is responsible for catalysing the reversible cleavage of the amino acid threonine to glycine and acetaldehyde (Liu et al., 1997). This is the first description of a TA in any plant study since the enzyme was first isolated from maize and mung bean in the early 1980s (Masuda et al., 1980; 1982). In a recent report, levels of threonine were shown to increase in cell cultures of the legume *Medicago truncatula* upon elicitation with methyl jasmonate (MJ) over a 48 h period (Broeckling et al., 2005). This finding gave indirect evidence that TA may be induced upon pathogen infection as MJ – a derivative of JA – is a well known inducer of plant defence responses (Pozo et al., 2005). The present study gives more direct evidence that this may be the case. Current knowledge of animal and microbe TA activity provides few clues as to why this enzyme may be important in plant disease resistance. One possible explanation could be that TA plays a role in the reduction of acetaldehyde which is toxic to cells and has been shown to accumulate under several stress conditions (Clements et al., 2002; Kürsteiner et al., 2003; Tsuji et al., 2003; Fujishige et al., 2004). Interestingly, Tadege et al. (1998) made the observation that an over-production of pyruvate decarboxylase, which converts pyruvate to acetaldehyde, in potato leaves resulted in a higher level of acetaldehyde accumulation and HR-associated lesion formation following *Phytophthora infestans* infection. The differential induction of clones HvPtr12 and 15 suggests that acetaldehyde may indeed accumulate in barley leaves following *P. teres* infection. If it does, then it is possible that the putative TA encoded

by *HvPtr20* may help in its reduction, thereby reducing necrosis development and arresting fungal growth as *P. teres* switches to its necrotrophic phase.

Clone **HvPtr21** has sequence homology to a glutamate dehydrogenase (GDH) enzyme. The exact role of GDH in plants has been redefined in recent years ever since it was discovered that GDH is not the primary enzyme involved in ammonium assimilation. Instead, it is now more commonly associated with the recycling of carbon molecules by supplying the organic molecule 2-oxoglutarate to tissues that are deprived of carbon (Robinson et al., 1992; Aubert et al., 2001). The finding from this study that GDH is potentially a defence-related enzyme supports at least one claim that GDH is involved in sensing the redox status of the plant and as such may represent a stress monitoring protein (Dubois et al., 2003). An increase in GDH levels was also observed during the first 24 hai of *Linum* cotyledons infected with the flax rust fungus (Sadler and Shaw, 1979), and more recently, Osuji and Madu (1996) reported that treatment of maize with the fungal elicitor chitosan induced GDH to isomerise, thus ensuring the rapid salvage of the ammonium released during the plant-pathogen interaction. An increase in GDH expression could therefore be a direct result of the inactivation by stress condition of the prominent ammonium assimilatory pathway - a situation that imposes the necessity for a complementary pathway for the plant to survive.

Clone **HvPtr22** has sequence homology to a putative abscisic acid (ABA) responsive protein that has not been identified in any previous plant-pathogen interactions. ABA itself is a plant hormone and has been the subject of extensive research because of its perceived role in the regulation of several abiotic stress responses such as low temperature, salinity, and drought (Shinozaki and Yamaguchi-Shinozaki, 2000; Xiong et al., 2002). In addition, there is also evidence that ABA is involved in the regulation of plant responses to pathogen attack. Studies in which ABA synthesis has been blocked or where exogenous ABA has been applied prior to inoculation have shown that ABA synthesis/application is correlated with the susceptibility of several plant species to various pathogens (Edwards, 1983; Audenaert et al., 2002; Mohr and Cahill, 2003). The fact that a putative ABA responsive protein is differentially expressed in the barley-*P. teres* incompatible interaction may suggest that the ABA signalling pathway is also involved in the barley defence response against *P. teres*.

However, with no definitive information regarding sequence homology with known genes, it is impossible to speculate as to what role the corresponding gene for clone NF804 may have in disease resistance.

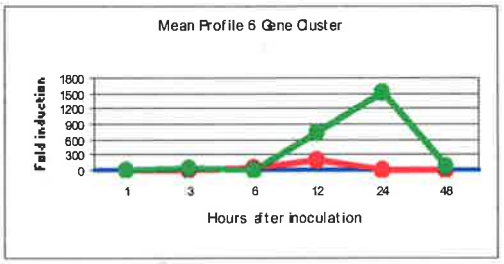
Clone **HvPtr23** has sequence homology with the multidrug and toxin efflux (MATE) family of transporters which form an extensive group of membrane proteins of largely unknown function. The *NorM* gene from *Vibrio parahaemolyticus* is the only biochemically characterised MATE family member to date and is responsible for pumping antimicrobial agents out of bacterial cells in exchange for sodium (Morita et al., 1998, 2000). Although never identified in barley up until now, MATE genes have been discovered in other plant species, and interestingly, three family members have previously been implicated in the plant defence response. *ALF5* renders *Arabidopsis* resistant to toxins (Diener et al., 2001), *EDS5* expression is induced in *Arabidopsis* following *P. syringae* infection (Nawrath et al., 2002), and most recently, *Zm-mfs1* was found to be induced in maize following inoculation with the fungal pathogens *Cochliobolus heterostrophus* and *Cochliobolus carbonum* (Simmons et al., 2003). The discovery of a putative defence-inducible barley MATE gene in this study provides further evidence that MATE proteins are involved in the plant defence response. Nawrath et al. (2002) showed that *EDS5* was induced earlier and stronger in *Arabidopsis* leaves inoculated with an avirulent strain of *P. syringae* compared to an isogenic virulent strain, and that this expression was strongly correlated with an increase in SA and PR-1 transcript accumulation. Although the *EDS5* and *HvPtr23* expression profiles differ, it is clear that *HvPtr23* is also induced more strongly in the barley-*P. teres* incompatible interaction and that this expression could be co-regulated with PR-1 transcript accumulation as seen with clone HvPtr26. Furthermore, the strong induction of *HvPtr23* transcripts in the barley leaf epidermis is also consistent with the observation that *ALF5* gene expression in *Arabidopsis* was strongest in the root epidermis (Diener et al., 2001). In contrast, there was no apparent difference in the expression of *Zm-mfs1* in response to *C. heterostrophus* and *C. carbonum* in both resistant and susceptible maize genotypes, however, it was noted that the *C. heterostrophus* resistance response in particular may be atypical of plant resistance responses as it is not associated with *PR* gene induction (Simmons et al., 2001). *P. teres* is known to produce at least three phytotoxins that incite many of the prominent symptoms caused by the fungus (Weiergang et al., 2002), so in the same way that the

NorM gene from *V. parahaemolyticus* functions to expel antimicrobial agents out of bacterial cells, it is possible that the *HvPtr23* gene may function to export *P. teres* toxins out of plant cells. It is also interesting that clone HvPtr23 appears to be co-regulated with clone HvPtr27 which is homologous with the FMO family of proteins. Along with the differential expression of clone HvPtr17 (discussed earlier in this chapter) which encodes a cytochrome P450 enzyme, it is likely that successful activation of the plant detoxification system plays a major role in determining resistance or susceptibility to *P. teres* infection in barley.

Clone **HvPtr24** shares sequence homology with a gene that encodes a copine-related protein. Although their exact biochemical function remains unknown, studies in animals have shown that copines can mediate the Ca^{2+}-dependent association of target proteins with membrane phospholipids, a phenomenon that could influence intracellular localisation and the activities of the target proteins (Tomsig and Creutz, 2002). In plants, the recent identification of an *Arabidopsis* copine mutant, *cpn1-1*, which showed dramatically increased resistance to a bacterial and an oomyceteous pathogen, constitutive *PR* gene expression, and accelerated HR-related cell death compared to wild-type plants, supported a role for CPN1 as a negative regulator of plant defence responses, including the HR (Jambunathan et al., 2001). In a subsequent study, the *CPN1* transcript was also shown to accumulate earlier and stronger in incompatible compared to compatible *Arabidopsis-P. syringae* pv *tomato* (*P. s. t.*) interactions (Jambunathan and McNellis, 2003). Avirulent *P. s. t.* bacteria triggered *CPN1* transcript accumulation as early as 4 hai and reached a peak at 6 hai whereas the virulent strain induced transcript accumulation only 24 hai, with peak levels observed at 36 hai. Although *HvPtr24* showed a different pattern of expression in the barley-*P. teres* interaction, its expression profile in relation to those of clones HvPtr1 and HvPtr10, which also encode proteins putatively involved in Ca^{2+} signal perception, supports the idea put forward by Jambunathan and McNellis (2003) that copines may be involved in channelling Ca^{2+} signals specifically for defence purposes. In the barley-*P. teres* incompatible interaction, this pathway may involve the early induction of Ca^{2+}-permeable channels by a protein kinase (HvPtr1) directly after pathogen recognition, the subsequent transmission of Ca^{2+} signals by a CBL protein (HvPtr2), and later, the specific channelling of Ca^{2+} signals into an appropriate defence pathway mediated by a copine-related protein (HvPtr24). If, like *CPN1*,

HvPtr24 can also act as a negative regulator of plant cell death responses, its observed up-regulation in the barley leaf epidermis may be critical in depriving hemibiotrophic pathogens like *P. teres* with the essential nutrients they need to survive.

4.4.4.6 Profile 6 gene cluster



Characteristics:

Similar to Profile 5 but significantly higher level of up-regulation (1500-fold) in the incompatible interaction.

The nearest homologs to clone **HvPtr25** are a gene from wheat, *WALI5*, that was induced in plants following Al treatment (Snowden and Gardner, 1993), and a gene from barley, *bsi*1, whose transcript levels increased significantly within 8 h during the barley-*Stagonospora nodorum* incompatible interaction (Stevens et al., 1996). The proteins encoded by all these genes are related to the Bowman-Birk-type family of serine proteinase inhibitors (PIs). These proteins play an important role in inhibiting trypsin and chymotrypsin of external origin, and the induction of their corresponding genes has been documented in plants challenged by both necrotrophic (Cordero et al., 1994; Stevens et al., 1996) and hemibiotrophic (Mur et al., 2004) fungal pathogens. In addition, in vitro experiments have shown that some plant PIs can directly inhibit fungal growth (Joshi et al., 1998; Pernas et al., 1999). The significant up-regulation of *HvPtr25* in the barley-*P. teres* incompatible interaction suggests that a higher concentration of PIs may be required to inhibit growth and multiplication of *P. teres*. It is possible that the putative PI encoded by *HvPtr25* may be involved in inhibiting the functions of proteinases secreted by *P. teres* which contribute in manipulating the plant's physiology for the benefit of the pathogen. In a recent report, Qu et al. (2003) for the first time provided evidence that PIs can arrest fungal invasion and inhibit the growth of fungi not only in vitro, but also in vivo. In that study, transgenic over-expression of a Bowman-Birk PI gene, *RBBI2-3*, in rice resulted in strong resistance in the seedling period to the fungal pathogen responsible for rice fungus blast disease, *Pyricularia oryzae*. The differential induction of *HvPtr25* in the net blotch resistant

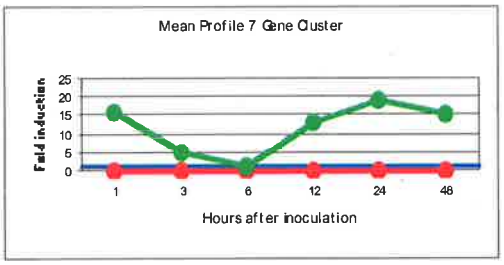
interaction suggests that it may play a similar role to *RBBI2-3* in conferring disease resistance.

Clone **HvPtr26** has sequence homology to the same PR protein (PR-1b) as identified by Muradov et al. (1993) and Bryngelsson et al. (1994) who both studied the interaction between barley and the powdery mildew causing fungus, *B. graminis* (a biotroph). Previous expression profiles for this PR-1b clone have generally shown an earlier and stronger accumulation of transcripts in powdery mildew resistant barley lines compared to susceptible lines (Freialdenhoven et al., 1994; Peterhänsel et al., 1997; Hückelhoven et al., 2000; Schultheiss et al., 2003). Clone HvPtr26 was thus selected as an obvious candidate to act as a positive control to confirm induction of defence responses in barley against *P. teres*. The findings presented here show that PR-1b transcripts also accumulate earlier and stronger in the *P. teres* resistant line than in the susceptible line suggesting similarities in PR-1b expression against hemibiotrophic and biotrophic fungi. Schultheiss et al. (2003) demonstrated that over-expression of PR-1b limited penetration of *B. graminis* on barley, so it is possible that enhanced PR-1b expression, especially in the epidermal cell layer, may play a vital role in arresting *P. teres* development.

Clone **HvPtr27** has high sequence homology to the flavin containing monooxygenase (FMO) family of proteins. Only clone HvPtr26, which encodes a PR-1 protein, matches its high level of up-regulation in the barley leaf epidermis following *P. teres* infection. Research into plant FMO-like proteins has not been extensive and certainly there is no previous report of the involvement of FMOs in plant defence. In animals, FMOs are one of the two main microsomal enzyme systems involved in the oxidation of xenobiotics, especially nitrogen-, sulfur-, selenium-, and phosphorus-containing compounds (Damani and Nnane, 1996; Ziegler, 1993). Along with the cytochrome P450 family of proteins it has been shown that they play an important role in the metabolism of several therapeutical drugs (Ohmi et al., 2003). In plants, FMOs have not been well characterised and to date there is only evidence that some family members are involved in auxin biosynthesis. Zhao et al. (2001) identified an FMO-like enzyme from *Arabidopsis* called YUCCA that catalyses a key step in tryptophan-dependent auxin biosynthesis. The over-expression of YUCCA in these plants resulted in elevated levels of free indole-3-acetic acid (IAA), which is the major auxin found in

plants. There is no significant similarity in amino acid sequence between YUCCA and HvPtr27, so it is difficult to draw any conclusions regarding what role the *HvPtr27* gene may have in barley net blotch resistance. However, a YUCCA-like function would make this clone an interesting candidate for further study as IAA treatment of potato plants was recently shown to reduce disease severity by 50% when the plants were inoculated with a compatible race of the late blight-causing fungus, *P. infestans* (Noël et al., 2001).

4.4.4.7 Profile 7 gene cluster



Characteristics:

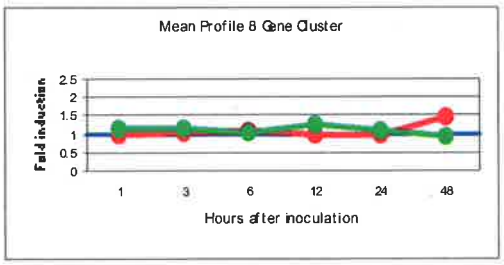
Gene up-regulated as early as 1 hai in the incompatible interaction and then maintained level of up-regulation from 12 to 48 hai. Expressed at very low levels in the compatible interaction.

The only clone with a unique expression profile was **HvPtr28** whose nearest homolog is a gene that encodes an ethylene-forming-enzyme-like dioxygenase. This putative redox enzyme is a member of the 2OG-FE(II) oxygenase superfamily and may catalyse the final step of the ethylene biosynthetic pathway in which 1-aminocyclopropane-1-carboxylic acid is converted to the plant hormone ethylene (Kende, 1993). The most intriguing aspect of this clone was that expression of its corresponding gene was by enlarge not detected at all in the net blotch susceptible genotype. Conversely, a strong and differential level of expression was observed in the resistant genotype. The fact that HvPtr28 was first up-regulated within 1 hai in the incompatible interaction suggests that, much like the three clones encoding putative signal transduction components in cluster 1, it too may encode a gene whose early induction is critical in switching on components of the resistance-associated defence pathway. It is conceivable that this pathway could be compromised in the susceptible genotype because it is unable to express the corresponding gene at the level required to initiate the signalling cascade.

The ethylene-forming-enzyme is more commonly known as 1-aminocyclopropane-1-carboxylate (ACC) oxidase and accumulation of ACC oxidase transcripts has previously been reported in plants challenged by microbial pathogens (Jia and Martin, 1999; Zanetti et al., 2002). In the report of Jia and Martin (1999), this accumulation was also correlated with an incompatible interaction (involving *Pto*-containing tomato plants and the bacterial pathogen *P. syringae* pv. *tomato* expressing *avrPto*). It is also interesting to note that the nearest barley homolog to HvPtr28 was found to encode a putative redox enzyme that interacts with the *Brome mosaic virus* (*Bmv*) coat protein (Okinaka et al., 2003). This protein shared sequence homology with an ACC oxidase from, among other related proteins, and although its interaction with the *Bmv* coat protein was found to aid viral infection in this case, it nevertheless provides evidence that related proteins may have the capacity to interact directly with pathogen elicitors and affect the outcome of a defence response.

Ethylene has a highly diverse role in plant growth and development as well as in various plant disease resistance pathways. The possible involvement of ethylene in conferring resistance to *P. teres* is interesting as this hormone has been shown to regulate resistance responses against other necrotrophic fungi. Pre-treatment of tomato plants with ethylene resulted in an increased resistance to *B. cinerea* (Diaz et al., 2002) and over-expression of the ethylene-response-factor1 gene in *Arabidopsis* was sufficient to confer resistance to *B. cinerea* and *Plectosphaerella cucumerina* (Berrocal-Lobo et al., 2002). More recently, Chagué et al. (2006) reported that exposure of *B. cinerea* to ethylene inhibited mycelium growth in vitro and caused transcriptional changes in a large number of fungal genes, therefore indicating that ethylene-induced plant resistance may involve effects of plant ethylene on both the plant and the fungus. Ethylene has also been reported to be responsible for the induction of proteinase inhibitor (O'Donnell et al., 1996) and PR protein (Ryals et al., 1996) genes, and the up-regulation of two clones putatively encoding these genes (HvPtr25 and HvPtr26) 24 hai appears to coincide with the up-regulation of HvPtr28. Although it is tempting to implicate the corresponding gene as having a role in ethylene biosynthesis, further study is required to establish if the accumulation of HvPtr28 transcript is correlated with an increase in ethylene production during the barley-*P. teres* interaction.

4.4.4.8 Profile 8 gene cluster



Characteristics:

Genes expressed at or near control levels in both incompatible and compatible interactions (no differential expression).

The 17 clones that made up cluster 8 were classified as not differentially expressed because their level of induction/repression was below the 1.5 fold cut-off value that was used in this study to define differential expression. Two things are possible in relation to these clones, (1) they may be among the false positives isolated during SSH, or (2) their lower fold changes in transcript abundance may still be of biological significance – in which case it is likely that the total number of candidate defence genes associated with net blotch resistance has been underestimated. Indeed, the putative involvement of some cluster 8 genes in plant defence has been demonstrated in other pathosystems. For example, *NF408* encodes a putative NRAMP (for natural resistance-associated macrophage protein), and recently, two *NRAMP* genes were found to be induced stronger in the rice-*M. grisea* resistant compared to susceptible interaction (Zhou and Yang, 2004). SF77 shares homology with hypersensitive-induced reaction genes which have been implicated in the induction of the HR in barley (Rostoks et al., 2003) and SF265 shows significant similarity to oxysterol binding proteins, whose genes have been shown to be up-regulated within 5 hai in barley plants resistant to powdery mildew (Hein et al., 2004).

For expression clusters 1, 3, and 4, it should be noted that the fold induction of each respective gene cluster in the incompatible interaction is not greatly elevated compared to the compatible interaction and/or the water control inoculation. However, this may not be unusual for resistance-associated defence transcripts. The *Npr1* transcript, for example, was also shown to accumulate by about two-fold in *Arabidopsis* following pathogen attack, yet a two-fold constitutive elevation of the transcript resulted in the up-regulation of a number of antimicrobial proteins and enhanced resistance to several pathogens (Cao et al., 1998).

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CHAPTER V
General discussion

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The aim of this work was to begin to elucidate the molecular events that take place when barley is challenged by the two *formae* of *Pyrenophora teres* responsible for net form and spot form of net blotch disease. Considering that the net blotches are among the major diseases affecting barley crops in Australia and worldwide, it continues to be surprising how relatively few research publications have focused specifically on this pathosystem. Since the end of 2001 when this project began, the search for major QTLs for resistance to the net blotches has continued, and additional QTLs have been identified from different sources, mainly on chromosome 6H, for NF resistance (Cakir et al., 2003; Ma et al., 2004; Emebiri et al., 2005; Friesen et al., 2006). However, it is unclear from these studies if the resistance is governed by the same or closely linked genes. For SF resistance, additional QTLs have been identified on chromosomes 4H, 5H, and 7H (Williams et al., 2003; Friesen et al., 2006). The small number of known resistance loci combined with the ability of *P. teres* to reproduce sexually increases the chances of the pathogen overcoming new sources of resistance and therefore the introgression of these sources into breeding varieties is not expected to be an effective strategy for durable resistance. In addition, most of the aforementioned studies have dealt with seedling resistance to *P. teres* isolates, which does not necessarily equate to adult plant resistance. For instance, *Rpt4*-positive adult lines were shown to have a low heritability of SF resistance in the field (Williams et al., 2003).

In this study, a search was conducted for plant defence response genes involved in the signal transduction pathway leading to NF and SF resistance. The aim was to identify candidate genes that could serve as targets for genetic manipulation to improve net blotch resistance in barley and hence provide breeders with an alternative strategy for breeding disease resistant crops. By conducting an SSH-based differential screen to isolate differentially expressed genes in both NF and SF incompatible compared to compatible interactions, a pool of perhaps tens of thousands of genes was narrowed down to 307 candidates that defined the barley-*P. teres* resistance-related defence transcriptome. The challenge was to then further narrow down the field of potential

defence gene candidates and this was done by analysing their expression during the first 48 h of both NF and SF incompatible and compatible interactions. Because most of the gene transcripts were found to be of low abundance, the method of Q-PCR was chosen for the analysis as it is the most sensitive method available for detecting gene expression levels.

Due to time constraints, it was not possible to analyse all of the 307 gene transcripts that made up the barley-*P. teres* defence transcriptome. Therefore, 45 genes were chosen for expression analysis based on their putative functional roles in a range of cellular processes that could contribute to disease resistance. Not only did this form of analysis reveal 28 candidate defence response genes that were differentially expressed in the two barley-*P. teres* incompatible interactions, it also revealed some interesting insights into how common genes may be involved in conferring resistance to both *formae* of *P. teres*. Furthermore, the detailed expression analysis that was conducted enabled the formation of a hypothetical model for net blotch resistance. This model is based on a defence pathway in which key components are amplified following early pathogen recognition.

5.1 Resistance to the barley net blotch pathogens may be governed by a common set of defence genes and signal transduction pathways

Perhaps the most interesting observation made in this study was that the candidate genes involved in resistance to *P. teres* shared largely similar, if not the same, expression patterns in both barley-*P. teres* interactions. This observation suggests that a common group of genes regulates defence responses against *Ptm* and *Ptt* in the barley leaf epidermis. Therefore, probably not the recognition but the signal transduction pathway leading to NF and SF resistance is also postulated to be the same. Thus although resistance to NF and SF is governed by different resistance loci, the genes that they harbour may essentially trigger the biosynthesis of a common set of resistance-related defence components. This may have significant implications for breeders if a candidate defence response gene is shown to confer net blotch resistance by functional analysis. To generate elite lines with durable resistance against both net blotch-causing pathogens, breeders may have the option of incorporating transgenic

lines over-expressing one key gene associated with net blotch resistance into their programs rather than using traditional means to introgress multiple sources of *R* gene-based resistance which the pathogens may eventually overcome. Alternatively, breeders may consider a pyramiding approach whereby lines containing major net blotch *R* genes are transformed with resistance-related defence response genes to generate elite cultivars with both durable and broad-spectrum disease resistance.

5.2 The barley-*P. teres* incompatible interaction: Is it controlled by a non-host signal transduction pathway whose components are "amplified" following the recognition of avirulence effectors by the plant?

The barley-*P. teres* incompatible interaction is associated with a coordinated regulation of a diverse range of pathogen-induced genes. The defence response is well orchestrated and appears to involve groups of genes that are co-regulated. The observed differential expression of three genes within 3 hai indicates that defence reactions against *P. teres* are initiated at the first physical contact between fungal conidiospores and the barley leaf and probably before the pathogen has had the chance to develop further and penetrate the leaf surface. In the barley-powdery mildew pathosystem, the differential induction of defence genes in cultivars associated with *Mla*-mediated resistance occurred only after 16 hai, at which point the fungus had already penetrated the host cell (Halterman et al., 2003). This led the authors to postulate that delivery and recognition of the corresponding pathogen avirulence effectors most likely occurred after penetration and during early haustorial development. Exactly when recognition of *P. teres* avirulence effectors by the plant takes place is unknown, however, despite *P. teres* having a different mode of infection, information gained from the barley-*B. graminis* interaction suggests that this is unlikely to occur within 3 hai. In contrast, clear differences in host defence gene expression in the barley leaf epidermis were observed in *mlo*-mediated resistant cultivars within 6 h of *B. graminis* infection, at which point the fungus is still in its pre-penetration stage (Zierold et al., 2005). It is therefore possible that cluster 1 genes may be involved in the recognition of non-specific PAMPs derived from *P. teres* which may in turn trigger the biosynthetic pathway for non-host resistance. Interestingly, two of the three putative proteins encoded by these genes are kinases,

which is consistent with current models on plant innate immunity which propose that PAMPs are recognised by receptor-like protein kinases (Jones and Takemoto, 2004).

At the beginning of this project, it was assumed that resistance to both forms of net blotch in the cultivar CI9214 was governed by *R* genes as a result of the host's innate defence response being ineffective against the causal pathogens. While *R* genes are still thought to play a crucial role in the barley-net blotch incompatible interaction, it is now evident that the resistant host's innate defence response may not have been bypassed after all and that it may in fact work in conjunction with, and perhaps even share the same components as, the *R* gene-mediated signal transduction pathway for net blotch resistance. Indeed, genetic overlap between specific and innate defence responses has been reported (van Wees et al., 2003; Narusaka et al., 2005) and it was previously suggested that one function of R-mediated signalling is to more effectively activate defence mechanisms that are shared by both pathways and to accelerate and amplify defence responses (Dangl and Jones, 2001). Evidence for potential genetic overlap between specific and innate defence responses occurring in the barley-*P. teres* incompatible interaction comes from the up-regulation of genes in clusters 4 and 7, first within 3 hai, and then again from 12 hai and beyond. A possible explanation for the differential induction of the same genes at two different stages is that the proteins they encode may be components of signal transduction pathways common to both specific and basal defence. The same genes may be induced early and later following perception of *P. teres* PAMPs and avirulence effectors, respectively. Again, as with the genes in cluster 1, the two cluster 4 genes encode proteins involved in signal perception, and thus may be key genes involved in transducing defence signals directly after the pathogen is recognised by the plant.

The role of ethylene in plant defence has been well documented, and the up-regulation of a gene potentially involved in its synthesis early and late in the barley-*P. teres* interaction provides indirect evidence that it too may positively regulate basal as well as specific defence responses. Recently, the ethylene signalling pathway was clearly associated with non-host penetration resistance to *Alternaria alternata* in *Arabidopsis* (Narusaka et al., 2005). In addition, plant hormones in the form of abscisic acid and brassinosteroids, have also been implicated in the net blotch resistant interaction.

Evidence for potential amplification of defence responses in the net blotch resistant cultivar, possibly associated with the switching on of the *R* gene signal transduction pathway, comes from the expression profiles of gene clusters 5 and 6. In both clusters, there is an initial peak 3 hai at which the corresponding genes are up-regulated in the incompatible interaction, followed by a much larger "amplified" peak at 24 hai. Again, could it be that these genes are marginally induced as part of the innate defence response and more significantly induced once specific recognition of the pathogen has occurred? In this scenario, based on the differential expression of gene clusters 5 and 6 early in the interaction, it appears that the induction of genes involved in the defence response pathway associated with non-host resistance may also play a role in conferring net blotch resistance in barley. This leads to a further question: Would the induction of basal defence mechanisms in the resistant cultivar alone be sufficient to confer net blotch resistance, or is the "amplified" response an absolute requirement?

Calcium transients also potentially play an integral role in determining the outcome of the barley-*P. teres* interaction, with evidence from this study suggesting that the opening of Ca^{2+}-permeable channels may be one of the first events that take place following the attachment of fungal spores onto the leaf surface. Candidate genes from clusters 2 and 5 have been identified that may be involved in channelling defence-specific Ca^{2+} "signatures" into the appropriate signal transduction pathways. The differential accumulation of gene transcripts controlling carbohydrate metabolism is also a feature of the net blotch resistant interaction, occurring most prominently from 12 to 24 hai. The likely importance of replenishing sites of infection with an energy source caused by the pathogen withdrawing nutrients from the host, thereby creating a metabolic sink, has been discussed. But exactly what effect is this having on the pathogen? Following inoculation, it was observed that small necrotic lesions began to appear in both resistant and susceptible cultivars at 24 hai. Yet it was only in the susceptible cultivar that the lesions spread and became more prominent, eventually giving rise to the susceptible phenotype observed. One explanation could be that it is around this time point that *P. teres*, being a hemibiotrophic pathogen, is switching from a biotrophic to a necrotrophic mode of infection. If, as a biotroph, *P. teres* had triggered a HR-like cell death around the 24 hai time point then this would become beneficial if it then switched modes of infection. The HR has already been found to accommodate several necrotrophic pathogens (Govrin and Levine, 2000; Kumar et al.,

2001). Therefore, increasing energy sources for cells affected by low metabolism preventing cell death may be a good defence strategy against this type of pathogen.

It was also presumed that detoxification mechanisms would play an integral role in the barley-*P. teres* incompatible interaction. *P. teres* is known to produce toxins and the antioxidant response has been associated with resistance to the pathogen. The potential pumping out of toxins by designated transporters and control of ROS production is highlighted by the up-regulation of genes in clusters 2, 3, 5, and 6. Based on the differential expression of two aldehyde dehydrogenases in cluster 3, and considering their possible role as antioxidants, there is an indication that ROS are being produced at around the 24 hai time point, which is consistent with a possible HR induced by *P. teres* as shown by the small necrotic lesions observed on infected plants. Expression studies on genes encoding proteins more commonly associated with the antioxidant response may reveal additional trends on ROS generation during the interaction. On the other hand, genes potentially involved in the transport of toxins appear to be induced in the time course from 3 hai onwards, suggesting that toxins may be being secreted by the pathogen throughout the interaction.

Based on these observations, a model has been developed detailing the events that may occur during the first 48 h of the barley-*P. teres* incompatible interaction (Figure 15). The model is split into two stages and shows the functional gene clusters that may regulate the defence response before and after the pathogen has penetrated the leaf surface. Before penetration, and during the presumed biotrophic phase of its life cycle, *P. teres*-derived PAMPs are recognised by RLKs within 3 hai. RLKs and their interacting protein partners are then responsible for triggering the induction of other gene clusters, most notably those involved in the accumulation of the plant hormone ethylene, which may itself be the key signal for the observed induction of genes that regulate defence/stress responses and detoxification pathways within 6 hai. In the compatible interaction, the pathogen may have evaded this early defence response as the early induction of recognition genes was suppressed. Induction of the ethylene-forming enzyme may also have been suppressed as a consequence, thereby depriving the plant of the signal required to switch on the full repertoire of defence components.

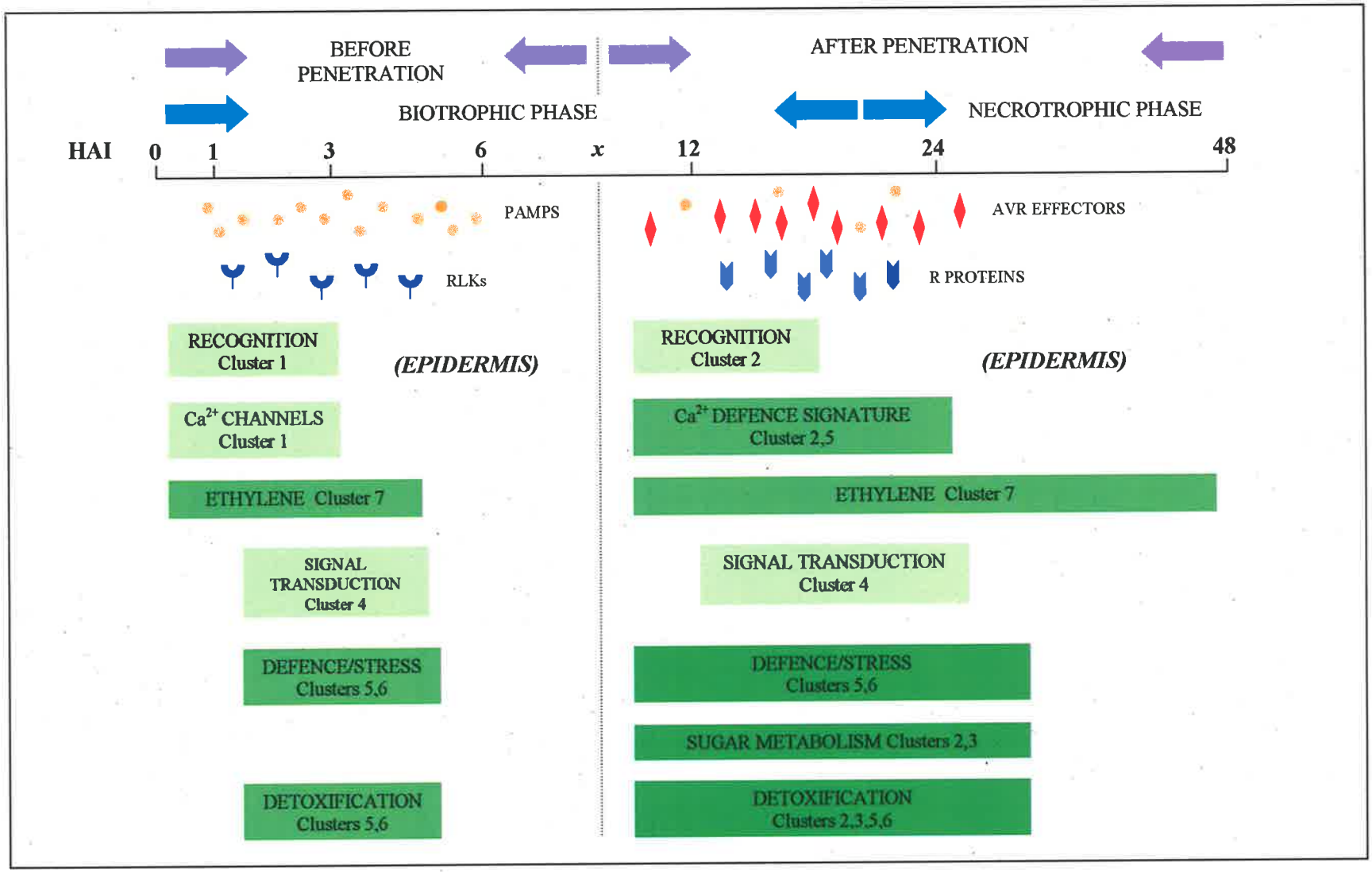


Figure 15: Proposed model of events taking place in the leaf epidermis during the first 24 h of the barley-*P. teres* incompatible interaction (higher shade of green corresponds to greater level of induction).

After fungal penetration of the leaf surface, which is presumed to occur within 12 hai, the primary mode of pathogen recognition and initiation of defence responses is thought to involve the more specific interaction of plant proteins with AVR effectors rather than PAMPs. As a result of this more specific interaction, the defence response is "amplified", leading to increased induction of genes associated with defence/stress responses and detoxification pathways from 12 to 24 hai. It is thought that the same signal transduction pathway controls both the innate (before penetration) and host-specific (after penetration) defence responses. Ethylene could be the signal that triggers common defence components and defence-specific Ca^{2+} signatures activated from 12 to 24 hai may be responsible for the "amplified" effect observed in the latter response. In the compatible interaction, pathogen AVR effectors are not recognised early by the plant and no defence response is initiated as a consequence. In addition, changes in carbohydrate partitioning are also proposed to occur only after pathogen penetration. This may coincide with the formation of metabolic sinks caused by fungal penetration (thought to be absent during the pre-penetration phase), as well as *P. teres* switching to a necrotrophic mode of existence – which will ultimately be of detriment to the fungus if the metabolic sinks are replenished with an energy source to keep the surrounding cells alive.

5.3 Quantitative differences in pathogen-induced gene expression distinguish the barley-*P. teres* incompatible interaction from a compatible one

Unlike other studies in which expression profiles of pathogen-induced genes during compatible and incompatible interactions have been shown to be qualitatively similar but quantitatively different (Tao et al., 2003; Zierold et al., 2005), this was not the case here. Although a quantitative difference was observed in all clusters, expression profiles between barley-*P. teres* compatible and incompatible interactions differed. The incompatible interaction was characterised by genes that were up-regulated while induction of the same genes was suppressed or they were down-regulated in the compatible interaction. Similar studies have also shown that in general, transcript abundance of pathogen induced genes appears to be elevated in incompatible compared to compatible interactions (Tao et al., 2003; Boyd et al., 1994; Bull et al., 1992). Furthermore, these same studies have shown that the amplitude of the responses increases later in the compatible interaction. Although this is not clearly

evident in the work presented here, it is possible that similar expression profiles do exist in the barley-*P. teres* compatible interaction at later time points and that large gaps between sampling times (e.g. from 12 to 24 hai and from 24 to 48 hai) have prevented this observation.

5.4 Future work

5.4.1 Detailed cytological analysis of the barley-*P. teres* interaction within 24 hai

Although previous studies have described how *P. teres* infects barley leaf tissue (Keon and Hargreaves, 1983; Schäfer, 2000), the development of the fungus has not been monitored within 24 hai and therefore it is not yet possible to correlate the expression profiles generated in this study with fungal development. Using confocal microscopy, it would be possible to conduct a detailed cytological analysis of the barley-*P. teres* interaction within 24 hai. This information would enable the gene clusters identified in this study to be grouped according to how they are activated in response to specific stages of fungal development.

5.4.2 Functional characterisation of differentially expressed genes

This study has identified 28 pathogen-induced genes associated with the barley net blotch resistance response that could serve as candidates for genetic manipulation to generate a transgenic breeding line with enhanced resistance against both net blotch-causing pathogens. The next step would be to verify their association with net blotch resistance by subjecting them to functional analysis as their expression profiles alone cannot prove this. Molecular approaches based on either over-expressing or silencing candidate defence genes can be used, however the large number of candidates means that a hierarchical system of gene impact or epistasis must be set in place. In such a system, signal transducing components such as protein kinases or CBLs may have a higher impact on the outcome of the barley-*P. teres* interaction than PR proteins, for instance, which may be part of a more redundant set of terminal effectors.

If any of the candidate genes are to be over-expressed, some important factors need to be considered. Firstly, the fact that (a) these genes were isolated from leaf epidermis,

and (b) their differential expression was only demonstrated in this tissue, means that, ideally, expression of their corresponding transgenes should also be targeted to the epidermis. Secondly, considering the adverse effects many constitutively over-expressed transgenes have had on plant size and/or seed production, limiting their expression only when it is needed at sites of infection would also be desirable. For best effect, promoters that are both epidermis-specific and pathogen-inducible should therefore control the expression of candidate transgenes. At the time of writing, and as a follow-up to the work presented here, transgenic barley lines over-expressing seven of the differentially expressed HvPtr genes have been generated and are awaiting analysis.

5.4.3 Mapping of differentially expressed genes

This study has also paved the way to identify new loci associated with net blotch resistance based on mapping expression QTLs (eQTLs). The potential of using this approach has been highlighted in recent studies in which candidate defence response genes have been mapped relative to phenotypic variation for disease resistance and shown to co-localise with major resistance genes or QTLs (Pflieger et al., 2001; Wang et al., 2001; Liu et al., 2004). In these studies, the researchers were restricted to mapping locations for commonly studied defence genes such as those encoding PR proteins assuming that they were involved in their plant-pathogen interaction of choice. The advantage gained from the present study is that a suitable EST library has already been generated and candidate genes can be selected for mapping based on their differential expression profiles e.g. the HvPtr genes. A similar strategy has already been shown to work effectively. Ren et al. (2004) isolated ESTs differentially regulated by brown planthopper (BPH) feeding using SSH and showed that some of these ESTs mapped to known QTLs and BPH resistance genes. Thus locating eQTLs based on mapping differentially expressed resistance-related ESTs may prove to be beneficial in identifying new genetic sources of net blotch resistance.

To enable the identification of eQTLs controlling the expression of each HvPtr gene, access to an adequate mapping population will be needed. From there, a "summation approach" (Brem et al., 2002; Schadt et al., 2003; Kliebenstein et al., 2006) can be used to identify "broad effect" eQTLs associated with net blotch resistance. This

approach is based on superimposing individual eQTLs and identifying common regions controlling the expression of the majority of HvPtr genes. Alternatively, a recent report has described how it is possible to identify eQTLs controlling defined gene networks (network eQTLs) (Kliebenstein et al., 2006). As seven individual co-regulated gene networks putatively associated with net blotch resistance were identified in this study, identifying the eQTLs that regulate these networks may also prove to be a promising strategy in discovering new genetic loci that confer disease resistance in barley.

5.4.4 Identification of novel variants of candidate defence response genes by mutational screening

The expression profiles of more than 250 SSH clones potentially involved in the net blotch resistant response remain unknown. Based on the ratio of clones that were found to be differentially expressed from the 45 selected, it is anticipated that conducting expression profiling on the remainder of the clones in the library will reveal more candidates for functional analysis. A good high-throughput strategy for functionally characterising a larger number of candidate genes is by a process called "reverse genetics", in which each gene is mutated in turn and the resulting impact on the plant is tested. The recent development of the first structured chemically mutagenised populations in barley (Caldwell et al., 2004) has provided the scientific community with an excellent resource to apply "reverse genetics" to functionally characterise barley genes. This strategy is based on TILLING (for **T**argeting **I**nduced **L**ocal **L**esions **IN** **G**enomes) – a high-throughput, non-transgenic method that incorporates a molecular screen of the mutant population to identify point mutations in genes of interest (Henikoff et al., 2004). TILLING is an attractive strategy to identify novel variants of candidate net blotch resistance-related genes – particularly those for which no putative function was assigned because they had no homology with any previously characterised genes. Their effects on plant phenotype will enable the discovery of novel genes that are involved in conferring resistance to the barley net blotches and perhaps to a wider range of barley leaf diseases.

5.4.5 Biochemical analysis of the barley-*P. teres* interaction

This study would benefit greatly if more was known about certain biochemical aspects of the barley-*P. teres* compatible and incompatible interactions. One of the main findings of this study was the potential involvement of ethylene and Ca^{2+} as signalling molecules for the induction of defence responses against the net blotch fungi. It would be of particular interest to monitor ethylene emission and Ca^{2+} levels after *P. teres* inoculation in both net blotch resistant and susceptible barley lines to determine if indeed a correlation exists between the production of these two molecules and the expression profiles of some of the functional gene clusters identified in this study. By using Ca^{2+} channel blockers and inhibitors of ethylene such as 1-methylcyclopropene and silver thiosulphate, it would be possible to evaluate the functional roles these gene clusters may have in conferring resistance to the barley net blotches. In addition, there is also evidence that carbohydrate metabolism is differentially regulated in the barley-*P. teres* incompatible interaction. The profiling of sugar metabolites by gas chromatography-mass spectrometry may thus reveal important associations that take place between gene clusters controlling sugar transport and cellular responses to *P. teres* infection.

5.5 Conclusions and perspectives

This study has, for the first time, shed light on barley genes that are differentially induced in response to *Ptm* and *Ptt* infection in the epidermis of a resistant compared to susceptible barley cultivar. Detailed expression profiling of resistance-related genes has revealed similarities in the way that net blotch resistant plants respond to infection by both *formae* of *P. teres* at the transcriptional level. In addition, it has enabled the generation of a putative model implicating functional groups of genes that may contribute to net blotch resistance during the early stages of infection. By performing further biochemical, genomic, and functional characterisation of these genes, a more comprehensive understanding of their role in the barley-*P. teres* incompatible interaction can be developed.

Appendix A: List of 73 SSH clones subjected to differential screening by Northern blot analysis and summary of the results obtained.

73 SSH clones were selected for differential screening by Northern blot

47 SSH clones produced a clear hybridisation signal

23 SSH clones were pathogen-induced

12 SSH clones were differentially expressed

1. SF404	27. NF428	51. SF450	62. NF267
2. NF163	28. SF504	52. NF123	63. NF434
3. NF93	29. NF61	53. NF401	64. NF528
4. NF420	30. NF6	54. NF251	65. NF579
5. SF206	31. SF382	55. SF179	66. SF310
6. SF532	32. NF47	56. NF242	67. SF432
7. NF541	33. NF12	57. SF199	68. NF229
8. NF699	34. SF424	58. SF13	69. NF294
9. SF528	35. SF357	59. NF586	70. NF472
10. SF223	36. NF53	60. SF346	71. NF717
11. NF457	37. SF576	61. NF404	72. SF606
12. NF309	38. NF312		73. SF641
13. SF614	39. SF472		
14. NF57	40. NF272		
15. SF394	41. SF269		
16. SF48	42. SF344		
17. NF721	43. SF633		
18. NF250	44. NF408		
19. NF13	45. NF520		
20. NF777	46. NF158		
21. SF216	47. SF79		
22. SF93	48. NF165		
23. SF52	49. NF723		
24. SF599	50. SF182		
25. NF373			
26. SF615			

Appendix B: Functional categorisation and sequence similarities of barley (*Hordeum vulgare* L.) cDNAs isolated from the NF and SF SSH libraries

^Clone identity	Size (bp)	Protein/DNA similarity	Sequence origin	BLASTn		BLASTx		Best barley EST match	
				Acc. No. of similar sequence	Similarity	Acc. No. of similar sequence	Similarity	Acc. No. of similar sequence	Similarity

^Note: Protein/DNA similarities for clones in **bold** type were derived from the corresponding cDNA sequence. Protein/DNA similarities for all other clones were derived from the best barley EST match. The prefix CON is short for contig and was used to name 2 or more clones sharing matching/overlapping sequences.

Stress or defence response

SF581	62	Pathogenesis-related protein bpr1-1 precursor	*H. vulgare*	Z48728	3e-26 (100%, 62bp)	S71554	2e-99 (98%, 174aa)	CA018463	6e-28 (100%, 62bp)
SF577	147	Germin-like protein	*H. vulgare*	X93171	8e-63 (99%, 128bp)	T05956	2e-52 (100%, 99aa)	BM443154	e-65 (100%, 126bp)
SF534	322	Glutathione S-transferase	*T. aestivum*	AF002211	e-44 (92%, 134bp)	O04437	2e-70 (94%, 138aa)	BJ548886	0.0 (100%, 320bp)
NF469	223	Hypothetical protein wali7	*T. aestivum*	L28008	5e-71 (94%, 188bp)	T06984	4e-04 (100%, 16aa)	CB864403	e-116 (99%, 215bp)
NF531	88	Senescence-associated protein	*A. thaliana*	-	-	NP_569030	9e-18 (46%, 113aa)	BQ768730	2e-38 (97%, 88bp)
NF720	225	Putative peroxidase	*O. sativa*	-	-	AAM08517	3e-43 (68%, 121aa)	CD053691	e-115 (99%, 218bp)
NF714	173	Probable peroxidase	*H. vulgare*	-	-	T04454	7e-17 (97%, 43aa)	CB872909	5e-87 (99%, 166bp)
SF297	107	Probable peroxidase	*H. vulgare*	-	-	T04454	4e-74 (100%, 136aa)	CB872909	e-42 (96%, 98bp)
SF602	125	Chitinase	*H. vulgare*	X78671	2e-56 (100%, 113bp)	S48847	2e-24 (100%, 50aa)	BJ461085	5e-58 (100%, 113bp)
NF251	278	Calmodulin-binding protein	*A. thaliana*	-	-	NP_194589	6e-07 (44%, 67aa)	CB872700	3e-56 (100%, 113bp)
SF147	363	Pathogen induced protein 2-4	*H. vulgare*	-	-	AAP04431	2e-59 (99%, 118aa)	CB860123	0.0 (99%, 356bp)

SF209	218	Putative senescence-associated protein	*P. sativum*	-	-	BAB33421	2e-36 (95%, 71aa)	CB883683	e-116 (99%, 218bp)
SF245	122	Chitinase II precursor	*T. aestivum*	-	-	AAD28730	e-65 (65%, 198aa)	BF265478	2e-52 (100%, 106bp)
SF260	106	Wheat aluminium induced protein wali 5	*T. aestivum*	-	-	JQ2361	e-35 (75%, 90aa)	CB874251	2e-48 (100%, 99bp)
SF269	97	Pit2 protein, Pi starvation induced	*N. tabacum*	-	-	T03677	e-26 (71%, 77aa)	BQ460116	2e-42 (100%, 89bp)
NF447	135	Glutathione S-transferase 1 (GST class-phi)	*T. aestivum*	-	-	P30110	4e-20 (87%, 48aa)	BJ461431	3e-63 (99%, 128bp)
NF422	166	Chitinase	*O. sativa*	-	-	JC5844	3e-64 (69%, 172aa)	CB879431	e-81 (100%, 155bp)
NF408	123	Hypersensitive-induced reaction protein 1	*H. vulgare*	-	-	AAN17462	4e-19 (100%, 46aa)	BM374317	2e-52 (99%, 110bp)
SF346	121	Senescence-associated protein-like protein	*O. sativa*	-	-	AAO72638	8e-25 (53%, 97aa)	CB866039	4e-53 (99%, 111bp)
NF430	71	Glutathione transferase	*T. aestivum*	-	-	CAC94004	6e-48 (81%, 115aa)	BF267321	4e-27 (100%, 63bp)
SF372	507	Calcineurin B-like	*P. sativum*	-	-	AAM91028	e-22 (90%, 53aa)	AV921566	0.0 (96%, 476bp)
NF669	173	HS1	*H. vulgare*	-	-	AAW03319	4e-41 (77%, 109aa)	CA009446	4e-81 (100%, 152bp)
SF522	257	Aldehyde dehydrogenase	*O. sativa*	-	-	AAG43027	e-29 (75%, 82aa)	CB865533	e-139 (100%, 250bp)
NF213	191	Aldehyde dehydrogenase	*O. sativa*	-	-	AAG43027	6e-15 (100%, 36aa)	BJ456941	e-102 (100%, 188bp)
NF835	131	Senescence-associated protein-like	*O. sativa*	-	-	BAD28167	6e-24 (62%, 81aa)	BJ458966	2e-11 (100%, 35bp)
CON2	134	Pathogenesis-related protein 1a	*H. vulgare*	X74939	4e-55 (99%, 115bp)	S37166	5e-27 (67%, 89aa)	BG300939	e-62 (100%, 121bp)
CON4	316	Catalase 1	*H. vulgare*	U20777	e-142 (97%, 302bp)	AAC17729	6e-47 (100%, 88aa)	BF260686	e-169 (100%, 301bp)
CON6	290	Mitochondrial aldehyde dehydrogenase, putative (ALDH)	*A. thaliana*	AB055519	e-44 (100%, 94bp)	NP_190383	6e-11 (65%, 55aa)	CB871488	e-108 (100%, 198bp)
CON9	123	Hypothetical protein wali6	*T. aestivum*	-	-	T06986	2e-45 (93%, 88aa)	BM816157	4e-43 (100%, 88bp)

CON13	173	Putative heat shock protein	*O. sativa*	AF358772	e-24 (88%, 112bp)	AAL83988	2e-13 (94%, 38aa)	CD056629	2e-89 (100%, 166bp)
CON27	196	Pathogenesis-related protein 4	*H. vulgare*	Y10814	2e-57 (97%, 127bp)	T06169	e-10 (96%, 30aa)	CB879514	2e-99 (98%, 196bp)
CON36	181	Superoxide dismutase (Cu-Zn) 4A	*Z. mays*	-	-	P23345	3e-74 (89%, 152aa)	BJ486091	5e-93 (100%, 172bp)
CON45	217	Pathogenesis-related protein 1 precursor	*H. vulgare*	-	-	Q05968	2e-17 (100%, 39aa)	BM370330	e-116 (99%, 217bp)
CON50	312	Hypersensitive-induced reaction protein 1	*H. vulgare*	-	-	AAN17462	3e-41 (98%, 87aa)	CB880077	e-172 (99%, 312bp)
Metabolism									
SF413	119	Putative phospho-2-dehydro-3-deoxyheptonate aldolase 1	*O. sativa*	-	-	BAC10194	4e-62 (92%, 120aa)	CB881467	2e-57 (100%, 112bp)
SF625	158	Steroid 5-alpha-reductase	*A. thaliana*	-	-	NP_191096	3e-39 (94%, 77aa)	CD056828	6e-83 (100%, 155bp)
SF545	281	Putative glucosyltransferase	*O. sativa*	-	-	AAP53972	e-08 (58%, 51aa)	BJ454315	e-128 (99%, 240bp)
SF532	237	UDP-glucose 4-epimerase	*O. sativa*	-	-	BAC02925	5e-33 (78%, 88aa)	AV945958	e-129 (99%, 237bp)
SF542	233	ADP-ribosylation factor-like protein	*A. thaliana*	-	-	BAB08314	5e-52 (86%, 112aa)	BQ760852	e-120 (100%, 218bp)
SF551	183	L-allo-threonine aldolase	*A. thaliana*	-	-	NP_566228	7e-06 (44%, 50aa)	CD054074	5e-93 (99%, 176bp)
SF557	94	1-aminocyclopropane-1-carboxylate oxidase	*S. bicolor*	-	-	T14643	7e-60 (84%, 138aa)	BJ552816	2e-44 (98%, 94bp)
SF32	65	Pyruvate, orthophosphate dikinase	*Z. mays*	-	-	AAA33498	e-61 (97%, 118aa)	CB872047	7e-25 (100%, 57bp)
NF478	354	Tryptophan synthase beta chain 1 (Orange pericarp 1)	*Z. mays*	M76685	e-82 (86%, 342bp)	P43283	e-57 (93%, 115aa)	CB881907	0.0 (99%, 342bp)
NF468	237	Probable alpha-galactosidase	*H. vulgare*	Y13848	e-125 (99%, 237bp)	T04423	2e-37 (97%, 78aa)	AV931851	e-129 (99%, 237bp)
NF402	356	GTP cyclohydrolase II/3,4-dihydroxy-2-butanone-4-phosphate synthase-like protein	*O. sativa*	-	-	AAO72560	4e-26 (80%, 66aa)	CA023946	0.0 (99%, 349bp)

NF428	144	Tryptophan synthase beta chain 1 (Orange pericarp 1)	*O. sativa*	-	-	P43283	2e-19 (100%, 45aa)	BQ761805	3e-69 (100%, 132bp)
NF375	202	Peptide methionine sulfoxide reductase	*L. sativa*	-	-	Q9SEC2	6e-58 (80%, 125aa)	BJ461526	e-111 (100%, 202bp)
NF456	277	Putative acyl-CoA synthetase	*O. sativa*	-	-	BAC01252	5e-38 (78%, 90aa)	AV919025	e-138 (97%, 269bp)
NF231b	383	ATP synthase subunit alpha	*T. durum*	X80470	0.0 (98%, 383bp)	CAA56641	e-11 (94%, 36aa)	AJ461865	e-67 (100%, 130bp)
NF213a	258	Putative dihydrolipoamide dehydrogenase precursor	*O. sativa*	-	-	BAB39219	3e-39 (91%, 83aa)	BM378036	e-138 (99%, 252bp)
SF633	447	Adenine phosphoribosyl-transferase	*H. vulgare*	ABO12046	e-163 (98%, 308bp)	BAB08003	3e-60 (100%, 117aa)	AV921087	0.0 (98%, 414bp)
SF344	322	Putative beta-glucosidase homolog	*O. sativa*	-	-	BAB67933	9e-20 (52%, 86aa)	CA011331	e-168 (98%, 314bp)
SF348	242	Putative 33kDa oxygen evolving protein of photosystem II	*O. sativa*	-	-	BAB64069	e-60 (96%, 119aa)	CB882054	e-125 (99%, 230bp)
SF106	154	Serine carboxypeptidase II-3 precursor	*H. vulgare*	-	-	P52711	5e-20 (100%, 46aa)	BJ552462	2e-76 (100%, 144bp)
NF272	210	Photosystem I iron-sulfur protein - barley chloroplast	*H. vulgare*	-	-	A32364	5e-31 (98%, 63aa)	BE216544	e-105 (99%, 201bp)
NF178	65	Ntdin	*N. tabacum*	-	-	BAA88985	8e-30 (65%, 89aa)	CB876541	2e-19 (100%, 50bp)
NF230	236	Copalyldiphosphate synthase No1	*L. sativa*	-	-	BAB12440	2e-08 (37%, 67aa)	AV837091	e-125 (99%, 232bp)
NF313	393	Sterol delta-7-reductase (ST7R)	*A. thaliana*	-	-	NP_175460	2e-53 (74%, 130aa)	BQ765594	0.0 (100%, 393bp)
NF61	240	Acetyl-CoA C-acyltransferase precursor	*B. napus*	-	-	T07989	e-25 (79%, 67aa)	BJ461684	e-118 (98%, 233bp)
NF98	180	Glutamate dehydrogenase A (GDH A)	*N. plumbaginifolia*	-	-	O04937	e-15 (68%, 61aa)	AJ435893	2e-90 (100%, 170bp)
SF21	195	Malate dehydrogenase	*O. sativa*	-	-	P43279	e-61 (85%, 134aa)	CB880534	3e-98 (98%, 195bp)
SF24	313	Methylthioadenosine/S-adenosyl homocysteine nucleosidase	*O. sativa*	-	-	AAL58883	9e-15 (84%, 46aa)	BU967324	e-169 (99%, 306bp)
SF111	424	Putative invertase	*A. thaliana*	-	-	AAM65926	4e-44 (87%, 96aa)	AV918599	0.0 (100%, 424bp)

SF152	300	Putative indole-3-glycerol phosphate lyase	*O. sativa*	-	-	AAP44679	e-29 (73%, 84aa)	CB880438	e-150 (97%, 294bp)
SF179	105	Putative chorismate synthase	*A. thaliana*	-	-	NP_564534	e-56 (74%, 142aa)	BJ548425	4e-28 (87%, 105bp)
SF61	236	Vacuolar invertase	*T. aestivum*	-	-	T06226	e-13 (68%, 57aa)	CB874333	e-125 (99%, 236bp)
SF251	308	Isovaleryl-CoA-dehydrogenase precursor	*A. thaliana*	-	-	AAL32645	8e-24 (70%, 81aa)	CA018558	2e-91 (99%, 176bp)
SF265	178	Oxysterol-binding family protein	*A. thaliana*	-	-	NP_200750	5e-20 (66%, 59aa)	CB876546	6e-93 (99%, 178bp)
SF267	324	ADP-ribosylation factor, putative	*A. thaliana*	-	-	NP_188935	4e-21 (83%, 55aa)	CA017513	e-163 (97%, 317bp)
SF391	161	Photosystem II 10kDa polypeptide, chloroplast precursor	*H. vulgare*	X97771	e-80 (100%, 154bp)	Q40070	e-18 (100%, 43aa)	CB877702	2e-82 (100%, 154bp)
SF424	72	Glyceraldehyde-3-phosphate dehydrogenase	*H. vulgare*	M36650	3e-23 (98%, 65bp)	P08477	3e-52 (100%, 102aa)	CB881308	e-29 (100%, 65bp)
SF472	245	ATP synthase beta subunit	*T. aestivum*	X74545	e-81 (98%, 164bp)	no significant homology		CD057062	e-120 (100%, 218bp)
NF339	179	Putative eceriferum 3 (CER3)	*O. sativa*	-	-	BAB43991	3e-27 (93%, 63aa)	CA015800	2e-89 (99%, 172bp)
NF841	193	Glutamine-dependent asparagine synthetase 1	*H. vulgare*	-	-	AAK49456	3e-17 (95%, 46aa)	CB881043	3e-95 (98%, 186bp)
SF324	124	Putative ubiquinol-cytochrome C reductase complex ubiquinone binding protein	*O. sativa*	-	-	BAA95821	e-33 (91%, 72aa)	CB865695	3e-63 (100%, 124bp)
SF475	278	Putative cytochrome P450	*L. rigidum*	AF321870	2e-74 (89%, 256bp)	AAK38091	e-45 (91%, 92aa)	BM443167	e-151 (99%, 278bp)
SF595	176	Flavin mono-oxygenase (FMO)	*A. thaliana*	-	-	CAD39838	5e-23 (82%, 58aa)	BE215373	8e-92 (99%, 174bp)
NF399	144	(1-3,1-4)-beta-D-glucanase	*H. vulgare*	-	-	CAA36801	7e-16 (94%, 39aa)	CB879831	2e-40 (100%, 86bp)
NF401	182	Thioredoxin-like protein	*A. thaliana*	-	-	AAM63651	9e-40 (69%, 107aa)	BJ458089	e-41 (95%, 114bp)
SF382	126	Glyceraldehyde-3-phosphate dehydrogenase-related	*A. thaliana*	-	-	NP_178071	e-43 (88%, 98aa)	CB865835	2e-60 (100%, 117bp)

NF361	70	Tryptophan decarboxylase	*H. vulgare*	-	-	BAD11769	e-108 (92%, 207aa)	CD054136	3e-20 (95%, 64bp)
SF588	135	Putative 3-isopropylmalate dehydratase large subunit	*O. sativa*	-	-	XP_463952	3e-37 (97%, 73aa)	BJ553059	9e-54 (96%, 122bp)
SF29	135	Putative alkaline/neutral invertase	*O. sativa*	-	-	XP_466154	4e-38 (80%, 91aa)	BQ661324	9e-70 (100%, 135bp)
SF206	333	Delta-24-sterol methyltransferase	*T. aestivum*	-	-	AAB49338	8e-41 (97%, 85aa)	CB867589	9e-69 (100%, 134bp)
CON8	243	Vacuolar ATP synthase subunit G 1	*N. tabacum*	-	-	O82702	e-18 (47%, 110aa)	BU967399	e-130 (99%, 237bp)
CON12	322	Deoxymugineic acid synthase 2	*H. vulgare*	AB063249	e-170 (99%, 316bp)	BAC10595	2e-28 (98%, 64aa)	BJ460219	e-175 (99%, 322bp)
CON14	132	Extracellular invertase	*T. monococcum*	-	-	AAN80141	9e-58 (94%, 117aa)	CB881780	3e-38 (100%, 80bp)
CON16	341	Glutamate dehydrogenase A (GDH A)	*N. plumbaginifolia*	AL606728	4e-51 (88%, 189bp)	O04937	2e-23 (79%, 63aa)	BQ664365	0.0 (99%, 334bp)
CON22	329	ATP synthase beta chain	*H. vulgare*	X00408	e-163 (97%, 318bp)	P00828	2e-53 (100%, 105bp)	CB860582	e-61 (98%, 136bp)
CON23	230	Vacuolar ATP synthase subunit G 1	*N. tabacum*	-	-	O82702	6e-20 (77%, 61aa)	CD057863	e-127 (100%, 230bp)
CON29	217	Probable alpha-galactosidase	*H. vulgare*	Y13848	e-105 (99%, 208bp)	T04423	6e-22 (100%, 48aa)	BQ765587	e-112 (99%, 209bp)
CON32	229	Photosystem II protein D1	*H. vulgare*	-	-	P05337	e-29 (100%, 62aa)	BE437922	e-121 (100%, 219bp)
CON34	287	Putative acyl-ACP thioesterase	*O. sativa*	-	-	BAA83582	4e-06 (51%, 45aa)	AV943449	e-152 (100%, 272bp)
CON53	339	Herbicide binding protein D1	*L. perenne*	-	-	AAK53381	4e-61 (100%, 113aa)	BE438132	e-171 (99%, 309bp)

Protein degradation

SF400	130	20S proteasome alpha subunit F	*O. sativa*	-	-	BAA28276	2e-76 (93%, 148aa)	CB874893	4e-68 (100%, 130bp)
SF399	251	Putative zinc protease	*O. sativa*	-	-	BAB63563	4e-05 (50%, 40aa)	CB881959	e-136 (99%, 249bp)
SF580	95	Putative ubiquitin carboxyl-terminal hydrolase	*O. sativa*	-	-	BAB56080	4e-63 (78%, 147aa)	AJ466635	2e-41 (100%, 85bp)

NF508	379	Polyubiquitin (UBQ3)	*A. thaliana*	AF184280	e-105 (91%, 287bp)	NP_851029	8e-48 (96%, 99aa)	BJ547448	e-136 (96%, 281bp)
SF575	135	26S proteasome regulatory particle triple-A ATPase subunit 6	*O. sativa*	-	-	BAB17626	5e-57 (97%, 90aa)	CB862615	2e-64 (100%, 124bp)
SF387	252	Ubiquitin conjugating enzyme	*H. vulgare*	AY220735	e-113 (95%, 241bp)	AAP04430	6e-82 (97%, 148aa)	BJ454565	e-133 (99%, 244bp)
SF325	130	Cysteine protease XBCP3	*A. thaliana*	-	-	NP_563855	7e-21 (55%, 67aa)	AV919131	4e-62 (100%, 120bp)
NF115	113	ATP-dependent clp protease	*P. sativum*	-	-	P35100	e-41 (83%, 102aa)	CB864170	3e-47 (99%, 101bp)
SF271	243	ATP-dependent clp protease	*P. sativum*	-	-	P35100	7e-16 (84%, 50aa)	CB881041	e-122 (98%, 236bp)
SF427	105	20S proteasome alpha subunit E	*O. sativa*	-	-	Q9LSU1	4e-66 (96%, 130aa)	CB876055	4e-49 (100%, 98bp)
SF78	222	26s proteasome regulatory particle triple-A ATPase subunit 1	*O. sativa*	-	-	XP_468146	e-72 (97%, 139aa)	BJ550115	e-115 (100%, 211bp)
CON7	269	Subtilisin-chymotrypsin inhibitor 2	*H. vulgare*	-	-	T06181	9e-19 (67%, 68aa)	BQ767599	e-139 (100%, 249bp)
CON47	353	Cysteine proteinase	*Z. mays*	-	-	S60456	4e-47 (92%, 91aa)	BM816477	0.0 (100%, 353bp)
CON48	315	Subtilisin-chymotrypsin inhibitor 2	*H. vulgare*	-	-	T06181	2e-32 (98%, 68aa)	CA592288	e-174 (99%, 315bp)
CON52	335	CI2E	*H. vulgare*	-	-	AAM22827	3e-16 (60%, 73aa)	CA018873	0.0 (99%, 335bp)

Cell division/growth

NF616	185	Putative G10 protein	*O. sativa*	-	-	BAB63707	6e-82 (93%, 145aa)	BJ449123	9e-95 (99%, 179bp)
SF605	210	Putative 60S ribosomal protein L36	*O. sativa*	-	-	BAB93221	4e-49 (92%, 105aa)	BJ543946	e-102 (99%, 192bp)
NF449	213	Pin1-type peptidyl-prolyl cis/trans isomerase	*Malus x domestica*	-	-	AAK83088	e-20 (66%, 71aa)	BU973498	e-107 (99%, 200bp)
NF47	198	Cell cycle control protein - related	*A. thaliana*	-	-	NP_173930	e-21 (49%, 112aa)	BU970654	e-103 (100%, 191bp)
SF176	205	60S ribosomal protein L10A	*A. thaliana*	-	-	NP_197636	5e-22 (76%, 67aa)	CB859155	e-109 (100%, 201bp)

SF283	372	Microsomal signal peptidase 25kDa subunit, putative (SPC25)	*A. thaliana*	-	-	NP_181525	4e-49 (70%, 132aa)	BM816840	e-144 (96%, 297bp)
SF301	249	Probable microsomal signal peptidase 25 kDa subunit	*A. thaliana*	-	-	P58684	2e-14 (66%, 57aa)	CB873813	e-130 (98%, 246bp)
SF484	204	60S ribosomal protein L39	*Z. mays*	-	-	P51425	e-23 (96%, 51aa)	CA029660	6e-90 (95%, 204bp)
SF301	249	Microsomal signal peptidase 25kDa subunit (SPC25) - like protein	*O. sativa*	-	-	XP_466287	4e-55 (76%, 132aa)	CB873813	e-130 (98%, 246bp)
SF562	163	Actin-depolymerizing factor 1	*Petunia x hybrida*	-	-	Q9FV12	6e-43 (73%, 111aa)	CA001428	e-78 (98%, 156bp)
SF235	190	Actin	*S. italica*	-	-	AAG10041	4e-47 (100%, 92aa)	BJ545527	2e-83 (95%, 181bp)
CON18	242	Ribosomal protein S7	*Z. mays*	-	-	NP_043077	e-28 (98%, 64aa)	CA027358	e-128 (99%, 235bp)

Transport

NF232	422	MDR-like ABC transporter	*O. sativa*	-	-	CA059586	7e-66 (90%, 140aa)	AJ472818	e-147 (100%, 266bp)
SF619	215	Histidine amino acid transporter	*O. sativa*	AJ557777	3e-17 (86%, 99bp)	CAD89802	e-168 (84%, 343aa)	AU252292	e-114 (99%, 215bp)
NF723	212	Proline transport protein	*O. sativa*	-	-	T50690	e-04 (53%, 39aa)	AJ435173	e-111 (100%, 203bp)
SF613	275	MATE efflux protein family	*A. thaliana*	-	-	NP_188997	7e-25 (41%, 146aa)	BQ767795	e-147 (98%, 275bp)
SF528	327	Adenine nucleotide translocator 2	*T. aestivum*	-	-	Q41630	5e-16 (100%, 41aa)	CB866080	e-173 (99%, 315bp)
SF66	203	Sorbitol transporter	*P. cerasus*	-	-	AAO39267	5e-30 (69%, 88aa)	AV943008	e-108 (99%, 203bp)
SF141	478	Putative potassium transporter	*O. sativa*	-	-	CAD21001	3e-27 (89%, 64aa)	BJ461597	0.0 (99%, 470bp)
SF174	150	Chloride channel	*O. sativa*	-	-	BAB97268	2e-75 (80%, 171aa)	BJ455187	e-78 (100%, 150bp)
SF215	111	Delta-type tonoplast intrinsic protein	*T. aestivum*	-	-	AAD10495	3e-16 (68%, 54aa)	CB881822	5e-40 (98%, 101bp)
SF464	221	Chloride channel	*O. sativa*	-	-	BAB972667	3e-23 (90%, 60aa)	CB871733	e-117 (99%, 221bp)

NF322	319	Putative outward rectifying potassium channel	*O. sativa*	-	-	AAO32309	4e-32 (86%, 76aa)	BU992021	e-124 (100%, 227bp)
NF423	129	Major facilitator superfamily antiporter	*Z. mays*	-	-	AAN33180	7e-08 (61%, 44aa)	BM443568	e-59 (99%, 122bp)
Signalling									
SF623	392	14-3-3-like protein A	*H. vulgare*	X62388	0.0 (99%, 392bp)	P29305	3e-53 (100%, 103aa)	CB883485	0.0 (99%, 392bp)
SF618	469	Receptor-like protein kinase	*A. thaliana*	-	-	NP_200898	9e-17 (49%, 79aa)	BJ483958	0.0 (99%, 354bp)
SF426	431	Nonphototrophic hypocotyl 1b	*O. sativa*	AB018443	9e-56 (84%, 297bp)	BAA84779	8e-47 (84%, 107aa)	BJ552475	0.0 (99%, 428bp)
NF596	160	Plasma membrane H+-ATPase	*H. vulgare*	AJ344078	4e-71 (99%, 140bp)	CAC50884	2e-25 (100%, 59aa)	CB865950	e-71 (99%, 140bp)
NF303	129	GAL83 protein	*S. tuberosum*	-	-	CAB52141	5e-30 (67%, 94aa)	CB873408	2e-63 (100%, 122bp)
NF66	366	Putative receptor-like protein kinase	*O. sativa*	-	-	AAP68881	7e-07 (69%, 42aa)	CA010770	0.0 (99%, 366bp)
NF675	203	Xylanase inhibitor	*T. aestivum*	AJ438880	3e-32 (95%, 89bp)	CAD27730	2e-09 (90%, 31aa)	CA012477	2e-59 (96%, 136bp)
NF294	140	Putative leucine-rich repeat protein	*O. sativa*	-	-	AAO23085	e-22 (94%, 52aa)	CB865384	e-68 (100%, 131bp)
SF45	164	Leucine-rich repeat protein family	*A. thaliana*	-	-	NP_187250	e-24 (52%, 109aa)	BQ660406	8e-83 (100%, 157bp)
SF223	520	Putative protein kinase Xa21, receptor type precursor	*O. sativa*	-	-	BAC10827	e-34 (48%, 175aa)	BI954443	2e-12 (82%, 128bp)
SF232	152	Putative casein kinase I	*O. sativa*	-	-	BAB92346	5e-21 (96%, 50aa)	BJ469951	2e-77 (99%, 152bp)
SF474	118	Serine/threonine protein kinase 1, nonphototropic hypocotyl protein 1-like	*A. sativa*	-	-	T08033	e-28 (85%, 68aa)	CB859159	2e-54 (99%, 111bp)
NF205	159	Protein kinase family	*A. thaliana*	-	-	NP_195303	5e-10 (66%, 45aa)	BQ762202	5e-84 (100%, 159bp)
NF688	132	AKIN gamma	*M. truncatula*	-	-	AAO61674	2e-26 (59%, 100aa)	AV927851	8e-51 (96%, 133bp)
NF829	188	Putative mitogen-activated protein kinase wjumk1	*O. sativa*	-	-	CAD54742	3e-17 (76%, 59aa)	AV934964	6e-96 (100%, 177bp)

SF225	193	Putative SSR alpha subunit	*O. sativa*	-	-	BAD53577	7e-21 (63%, 80aa)	CB858640	e-104 (100%, 193bp)
CON56	193	Putative leucine-rich repeat protein	*O. sativa*	-	-	AAO23085	2e-24 (91%, 58aa)	CB878837	e-102 (99%, 193bp)

Gene expression/regulation

SF453	200	Putative RNA binding protein	*O. sativa*	-	-	AAG59664	e-46 (62%, 145aa)	BU989069	e-106 (100%, 194bp)
NF576	217	Small nuclear ribonucleo protein polypeptide G - related	*A. thaliana*	-	-	NP_187757	3e-26 (84%, 66aa)	CB882517	e-115 (99%, 217bp)
NF579	327	CONSTANS B-box zinc finger family protein	*A. thaliana*	-	-	NP_195607	e-25 (42%, 147aa)	BJ460535	e-178 (99%, 320bp)
NF257b	233	Putative ribosomal protein	*O. sativa*	-	-	AAP21401	7e-31 (73%, 83aa)	AV929320	e-123 (98%, 233bp)
NF733	245	Ring zinc finger protein-like protein	*O. sativa*	-	-	AAO72540	4e-11 (89%, 28aa)	BQ761454	e-130 (100%, 234bp)
NF93	146	Putative cytidine deaminase	*Z. mays*	-	-	AAN40022	3e-75 (80%, 168aa)	CB882097	5e-71 (100%, 135bp)
NF754	186	Histone H3.2	*M. sativa*	-	-	AAB36495	9e-17 (100%, 43aa)	BG418556	e-101 (100%, 186bp)
NF69	246	High mobility group box protein 2	*O. sativa*	-	-	BAB85204	2e-17 (63%, 71aa)	CB875335	e-131 (100%, 238bp)
NF165	170	Probable transcription activator	*O. sativa*	-	-	T03990	e-20 (79%, 64aa)	AV922592	3e-67 (100%, 131bp)
NF256	253	KOW domain-containing transcription factor family protein	*A. thaliana*	-	-	NP_192575	2e-04 (74%, 27aa)	CA016143	e-130 (99%, 240bp)
SF449	172	Putative PHD-type zinc finger protein	*A. thaliana*	-	-	AAM65374	e-24 (84%, 57aa)	CD054311	5e-93 (100%, 172bp)
NF420	198	Putative U2 snRNP auxiliary factor	*O. sativa*	-	-	BAB90507	4e-10 (68%, 47aa)	CA016751	7e-93 (99%, 178bp)
SF342	500	Putative myb family transcription factor	*A. thaliana*	-	-	AAO64031	2e-13 (60%, 61aa)	BJ476673	0.0 (99%, 473bp)
SF313	156	Putative nucleotide-binding protein	*O. sativa*	-	-	CAC09490	2e-13 (89%, 39aa)	CB881616	2e-80 (100%, 153bp)
SF582	82	Putative finger transcription factor	*O. sativa*	-	-	AAU10743	6e-23 (72%, 74aa)	BQ663786	3e-21 (100%, 51bp)

CON3	181	Protein translation factor SUI1 homolog (GOS2 protein)	*O. sativa*	-	-	P33278	e-43 (88%, 96aa)	CB872588	2e-98 (100%, 181bp)
CON10	281	Zinc finger protein	*O. sativa*	AY225189	e-16 (94%, 59bp)	BAA90806	2e-21 (67%, 65aa)	CB864489	e-158 (100%, 281bp)
CON15	172	Translation initiation factor 5A	*O. sativa*	-	-	AAC67555	8e-35 (79%, 74aa)	CB883293	5e-93 (100%, 172bp)
CON19	188	Protein translation factor SU1 homolog	*S. bakko*	-	-	O48650	2e-14 (88%, 42aa)	CB873914	e-102 (100%, 188bp)
CON38	398	Putative translation initiation factor SUI1	*O. sativa*	-	-	BAB89060	e-31 (87%, 72aa)	BM372386	0.0 (99%, 378bp)
CON40	177	Putative SCARECROW gene regulator-like	*O. sativa*	-	-	AAM08829	2e-36 (87%, 63aa)	BJ462110	2e-37 (97%, 92bp)

Unknown function

SF390	183	Ethylene-forming-enzyme-like dioxygenase-like protein	*O. sativa*	-	-	BAA95829	3e-11 (52%, 61aa)	CD058506	e-90 (98%, 176bp)
NF392	193	NTGP1 (isoprenylated plant protein)	*N. tabacum*	-	-	AAD00116	6e-07 (100%, 25aa)	BQ662617	e-96 (99%, 186bp)
NF760	370	Probable inorganic pyrophosphatase-related protein	*A. thaliana*	-	-	NP_190930	2e-46 (86%, 97aa)	CB872224	0.0 (99%, 355bp)
SF404	140	Blue copper-binding protein homolog	*T. aestivum*	-	-	AAD10251	2e-25 (50%, 110aa)	BQ763672	e-25 (100%, 59bp)
NF804	180	Putative ABA-responsive protein	*O. sativa*	-	-	AAL31061	9e-47 (60%, 138aa)	BM370615	9e-98 (100%, 180bp)
NF163	187	Possible apospory-associated protein	*P. ciliare*	-	-	AAA80576	3e-45 (90%, 95aa)	CB882420	4e-94 (99%, 180bp)
SF77	298	Putative integral membrane protein NRAMP	*H. vulgare*	-	-	CAD55951	4e-48 (100%, 99aa)	BU970534	e-166 (100%, 297bp)
SF167	150	Putative nuclear protein	*O. sativa*	-	-	AAL86478	6e-18 (89%, 46aa)	BG416829	2e-74 (100%, 143bp)
SF198	346	OsNAC4 protein	*O. sativa*	-	-	BAA89798	2e-06 (78%, 37aa)	CA005352	e-163 (100%, 293bp)
SF237	117	Brain and reproductive organ-expressed protein - related	*A. thaliana*	-	-	NP_199062	4e-14 (53%, 63aa)	BJ468054	e-46 (98%, 107bp)
SF275	77	Similar to microtubule-associated protein	*O. sativa*	-	-	BAC55760	3e-52 (96%, 106aa)	CA028770	2e-35 (100%, 77bp)

SF468	166	CBS domain containing protein	*A. thaliana*	-	-	NP_190422	e-16 (74%, 54aa)	CA017252	4e-87 (99%, 166bp)
NF390	342	ARP protein NADPH oxidoreductase homolog	*A. thaliana*	-	-	S57614	5e-11 (68%, 45aa)	BJ485736	e-175 (98%, 332bp)
SF593	260	Copine-related	*A. thaliana*	-	-	NP_196946	e-10 (59%, 44aa)	CD054055	e-140 (99%, 260bp)
SF636	225	Cyanate hydratase	*O. sativa*	-	-	Q9FWK4	5e-06 (57%, 47aa)	BE215175	2e-96 (94%, 213bp)
NF9	115	Dormancy-associated protein	*C. lanceolata*	-	-	AAW02792	2e-14 (75%, 48aa)	CB876507	9e-51 (99%, 107bp)
NF379	288	LAs17 binding protein-like	*O. sativa*	-	-	XP_477400	4e-78 (87%, 164aa)	BJ477011	e-103 (100%, 190bp)
SF115	236	mutT domain protein-like	*A. thaliana*	-	-	AAM65320	(51%, 70aa)	CB859295	5e-23 (96%, 63bp)
NF301	139	SNF7 protein-like	*O. sativa*	-	-	BAD35619	7e-17 (54%, 98aa)	BQ665106	e-62 (99%, 127bp)
SF194	113	Ethylene-forming-enzyme-like dioxygenase-like protein	*O. sativa*	-	-	XP_476310	6e-21 (55%, 98aa)	AL501569	e-12 (94%, 51bp)
CON11	237	Similar to nodulin	*O. sativa*	-	-	BAC20893	3e-19 (63%, 71aa)	BI778591	e-112 (96%, 237bp)
CON21	335	Putative histidine decarboxylase	*O. sativa*	-	-	AAG12476	5e-21 (60%, 69aa)	no significant homology	
CON31	171	Glycine rich protein	*H. vulgare*	Z48625	3e-50 (99%, 107bp)	CAA88559	4e-04 (95%, 20aa)	AV909884	4e-78 (97%, 162bp)
CON46	413	Ethylene-forming-enzyme-like dioxygenase-like protein	*O. sativa*	-	-	BAC22234	e-27 (58%, 99aa)	AV931573	0.0 (97%, 410bp)
CON51	214	Auxin-regulated protein	*Z. elegans*	-	-	AAM12952	5e-24 (73%, 69aa)	CB881366	e-115 (100%, 211bp)

Unknown protein

SF52	232	Hypothetical protein 62 - barley chloroplast	*H. vulgare*	-	-	T04410	e-31 (100%, 70aa)	BJ451637	e-122 (100%, 221bp)
NF453	344	Hypothetical protein	*O. sativa*	BA000029	e-177 (97%, 344bp)	BAC20633	e-13 (100%, 37aa)	CA023790	e-179 (97%, 344bp)
NF710	339	Hypothetical protein	*O. sativa*	-	-	NP_039397	e-07 (51%, 66aa)	BE437950	e-155 (95%, 329bp)

NF703	214	Expressed protein	*A. thaliana*	-	-	NP_173069	2e-35 (86%, 79aa)	CB876098	e-109 (100%, 199bp
SF504	164	Expressed protein	*A. thaliana*	-	-	NP_198754	6e-15 (87%, 49aa)	CA005827	6e-83 (100%, 155bp)
SF599	256	Unknown protein	*O. sativa*	-	-	BAC55659	5e-20 (88%, 52aa)	BJ447630	e-133 (99%, 244bp)
SF257	236	Unknown protein	*O. sativa*	-	-	AAG46152	5e-42 (74%, 128aa)	BF065281	e-122 (100%, 221bp)
SF289	146	Hypothetical protein	*O. sativa*	-	-	AAO39879	7e-43 (65%, 117aa)	CA013637	5e-71 (100%, 135bp)
NF538	53	Expressed protein	*A. thaliana*	-	-	NP_565808	5e-10 (43%, 79aa)	BQ656365	e-16 (100%, 43bp)
NF778	264	Hypothetical protein F8K7.5	*A. thaliana*	-	-	D86349	5e-50 (95%, 97aa)	CA005883	e-139 (99%, 254bp)
NF174	128	Expressed protein	*A. thaliana*	-	-	NP_192654	e-14 (45%, 81aa)	AV836765	3e-60 (100%, 119bp)
NF204	261	Hypothetical protein	*A. thaliana*	-	-	C86159	8e-28 (64%, 91aa)	BJ465895	e-134 (100%, 244bp)
NF222	406	Putative protein	*A. thaliana*	-	-	NP_201222	9e-14 (48%, 95aa)	BJ486105	0.0 (100%, 406bp)
NF371	151	Unknown protein similar to WD-repeat protein	*O. sativa*	-	-	BAC57818	3e-14 (78%, 47aa)	BU968627	9e-64 (95%, 144bp)
SF79	415	Unnamed protein product - Similar to *A. thaliana* putative acyl-CoA dehydrogenase	*O. sativa*	-	-	BAA96762	4e-56 (93%, 113aa)	BI780003	0.0 (99%, 407bp)
SF253	355	Putative protein RabGAP/TBC domain-containing protein	*A. thaliana*	-	-	NP_200289	4e-32 (55%, 120aa)	BG343762	0.0 (98%, 348bp)
NF373	433	Expressed protein	*A. thaliana*	-	-	NP_196828	8e-20 (52%, 87aa)	CB881169	0.0 (100%, 433bp)
NF440	263	Hypothetical protein 3 H^+-transporting ATP synthase chain I	*N. tabacum*	-	-	T02946	3e-13 (90%, 40aa)	BM100888	e-141 (100%, 256bp)
SF326	176	Unnamed protein product	*O. sativa*	-	-	BAA90391	5e-08 (65%, 43aa)	BU968638	4e-88 (100%, 166bp)
SF615	318	Unknown protein	*A. thaliana*	-	-	AAM65272	7e-58 (44%, 254aa)	BM816741	2e-84 (100%, 158bp)
SF364	228	Expressed protein	*A. thaliana*	-	-	NP_568167	9e-27 (76%, 75aa)	CA030007	e-121 (99%, 228bp)

SF250	487	Unnamed protein product	*O. sativa*	-	-	BAA87824	3e-04 (82%, 23aa)	AL510734	e-164 (97%, 321bp)
SF119	170	Hypothetical protein	*O. sativa*	-	-	BAD46202	2e-20 (50%, 115aa)	CB882680	6e-50 (100%, 102bp)
CON5	304	Expressed protein Universal stress family protein	*A. thaliana*	-	-	NP_566108	6e-06 (81%, 27aa)	BM369974	e-156 (98%, 294bp)
CON17	555	Unnamed protein product Similar to isocitrate dehydrogenase	*O. sativa*	-	-	BAA88179	2e-60 (91%, 122aa)	BU996054	e-179 (99%, 325bp)
CON20	153	Expressed protein Myosin-related	*A. thaliana*	-	-	NP_172024	e-08 (29%, 135aa)	BU989174	9e-82 (100%, 153bp)
CON24	329	Expressed protein	*A. thaliana*	-	-	NP_194807	3e-42 (73%, 109aa)	BQ467286	2e-23 (100%, 56bp)
CON25	149	Hypothetical protein p85RF	*P. armeniaca*	-	-	T51098	8e-28 (70%, 79aa)	CB880708	3e-73 (98%, 149bp)
CON26	189	Unknown protein	*A. thaliana*	-	-	AAM61190	2e-52 (88%, 105aa)	CB872966	e-101 (99%, 189bp)
CON28	234	Unknown protein	*O. sativa*	-	-	NP_908355	2e-37 (83%, 81aa)	BF619223	8e-74 (100%, 140bp)
CON33	171	Unnamed protein product	*O. sativa*	-	-	BAA96588	3e-17 (73%, 61aa)	CD056932	2e-92 (100%, 171bp)
CON35	304	Hypothetical protein 62	*H. vulgare*	-	-	T04410	9e-32 (100%, 70aa)	CB881687	e-146 (96%, 298bp)
CON42	424	ORF46c	*P. koraiensis*	-	-	NP_817268	e-05 (72%, 33aa)	CB878804	0.0 (99%, 421bp)
CON49	163	Unknown protein Similar to F-box protein FBL2 from *H. sapiens*	*A. thaliana*	-	-	AAK28636	e-27 (70%, 86aa)	BQ766914	e-81 (98%, 163bp)
CON55	478	Hypothetical protein	*D. radiodurans*	-	-	D75542	2e-27 (51%, 135aa)	CB879837	0.0 (99%, 467bp)

Novel

SF416	84	No significant homology	-	-	-	-	-	No significant homology
SF523	163	No significant homology	-	-	-	-	-	No significant homology
SF564	124	No significant homology	-	-	-	-	-	No significant homology
NF457	184	No significant homology	-	-	-	-	-	No significant homology
NF435	186	No significant homology	-	-	-	-	-	No significant homology

NF309	88	No significant homology	-	-	-	-	-	No significant homology
NF132	214	No significant homology	-	-	-	-	-	No significant homology
NF459	134	No significant homology	-	-	-	-	-	No significant homology
SF614	210	No significant homology	-	-	-	-	-	No significant homology
NF738	155	No significant homology	-	-	-	-	-	No significant homology
SF199	142	No significant homology	-	-	-	-	-	No significant homology
NF237	200	No significant homology	-	-	-	-	-	No significant homology
NF241	310	No significant homology	-	-	-	-	-	No significant homology
SF259	206	No significant homology	-	-	-	-	-	No significant homology
SF264	300	No significant homology	-	-	-	-	-	No significant homology
SF298	149	No significant homology	-	-	-	-	-	No significant homology
SF394	111	No significant homology	-	-	-	-	-	No significant homology
SF418	127	No significant homology	-	-	-	-	-	No significant homology
SF466	69	No significant homology	-	-	-	-	-	No significant homology
SF467	84	No significant homology	-	-	-	-	-	No significant homology
SF306	248	No significant homology	-	-	-	-	-	No significant homology
SF327	84	No significant homology	-	-	-	-	-	No significant homology
SF420	98	No significant homology	-	-	-	-	-	No significant homology
SF596	201	No significant homology	-	-	-	-	-	No significant homology
CON41	360	No significant homology	-	-	-	-	-	No significant homology

No/poor homology

SF455	165	No significant homology	-	-	-	-	-	AV931008	3e-69 (97%, 146bp)
SF48	306	No significant homology	-	-	-	-	-	BQ767270	e-128 (96%, 264bp)
SF57	226	No significant homology	-	-	-	-	-	CA000146	e-112 (99%, 208bp)
SF415	140	No significant homology	-	-	-	-	-	BG416301	3e-72 (100%, 137bp)
SF398	286	No significant homology	-	-	-	-	-	BU975076	e-118 (92%, 280bp)
SF514	328	No significant homology	-	-	-	-	-	BQ765322	e-176 (100%, 312bp)
NF721	174	No significant homology	-	-	-	-	-	CB880649	2e-89 (98%, 174bp)

NF441	376	No significant homology	-	-	-	-	-	CB8791437	0.0 (99%, 367bp)
NF304	128	No significant homology	-	-	-	-	-	CB867444	6e-61 (100%, 118bp)
NF118	359	No significant homology	-	-	-	-	-	AL500101	0.0 (98%, 337bp)
NF711	137	No significant homology	-	-	-	-	-	BQ763761	2e-67 (100%, 129bp)
NF727	306	No significant homology	-	-	-	-	-	CA005820	e-172 (100%, 306bp)
NF231a	129	No significant homology	-	-	-	-	-	BE420997	3e-56 (97%, 126bp)
SF635	172	No significant homology	-	-	-	-	-	CB880536	e-68 (94%, 160bp)
NF194	128	No significant homology	-	-	-	-	-	CA001260	4e-56 (99%, 116bp)
NF250	246	No significant homology	-	-	-	-	-	CA005520	e-130 (99%, 240bp)
NF267	146	No significant homology	-	-	-	-	-	CB877902	2e-21 (96%, 62bp)
NF13	202	No significant homology	-	-	-	-	-	AV914866	3e-52 (100%, 106bp)
SF18	86	No significant homology	-	-	-	-	-	BM373244	5e-36 (100%, 78bp)
SF63	215	No significant homology	-	-	-	-	-	CB879658	e-113 (99%, 212bp)
SF67	91	No significant homology	-	-	-	-	-	BJ484246	e-39 (100%, 84bp)
SF109	219	No significant homology	-	-	-	-	-	AJ436284	e-115 (100%, 212bp)
SF462	309	No significant homology	-	-	-	-	-	CB881903	e-144 (95%, 302bp)
NF870	146	No significant homology	-	-	-	-	-	CB878196	4e-72 (100%, 139bp)
NF383	143	No significant homology	-	-	-	-	-	BQ765346	e-65 (100%, 128bp)

SF318	126	No significant homology	-	-	-	-	-	BQ461228	2e-64 (100%, 126bp)
SF329	155	No significant homology	-	-	-	-	-	BF622342	e-81 (100%, 155bp)
SF509	96	No significant homology	-	-	-	-	-	CB878266	3e-40 (98%, 87bp)
NF404	233	No significant homology	-	-	-	-	-	CB879430	e-123 (99%, 229bp
SF457	195	No significant homology	-	-	-	-	-	CD055529	6e-59 (100%, 115bp)
NF391	172	No significant homology	-	-	-	-	-	CA017140	3e-79 (98%, 167bp)
SF357	144	No significant homology	-	-	-	-	-	BI778486	7e-64 (97%, 135bp)
SF617	198	No significant homology	-	-	-	-	-	CA007288	2e-24 (100%, 57bp)
SF535	159	No significant homology	-	-	-	-	-	CB878846	2e-58 (100%, 114bp)
SF553	181	No significant homology	-	-	-	-	-	BM375430	7e-83 (98%, 170bp)
NF123a	117	No significant homology	-	-	-	-	-	CD054869	3e-44 (96%, 113bp)
SF526	202	No significant homology	-	-	-	-	-	AJ463250	2e-99 (99%, 187bp)
SF93	194	No significant homology	-	-	-	-	-	CD056058	4e-32 (100%, 70bp)
NF777b	279	No significant homology	-	-	-	-	-	BF265893	e-131 (97%, 260bp)
NF54	82	No significant homology	-	-	-	-	-	BM101140	2e-10 (100%, 35bp)
SF224	256	No significant homology	-	-	-	-	-	AL499816	2e-44 (98%, 96bp)
SF292	564	No significant homology	-	-	-	-	-	BJ449615	e-96 (89%, 276bp)
CON30	274	No significant homology	-	-	-	-	-	CB879028	e-132 (96%, 263bp)

CON39	266	No significant homology	-	-	-	-	-	CA010175	e-139 (99%, 256bp)

RUBISCO

NF666	207	Ribulose-bisphosphate carboxylase small chain precursor	*T. aestivum*	M37328	3e-63 (92%, 177bp)	RKWTS	7e-80 (95%, 145aa)	CB879668	e-111 (99%, 207bp)
CON1	286	Ribulose-1,5-bisphosphate carboxylase	*W. microscopica*	-	-	AAK72538	e-45 (100%, 86aa)	CB879222	e-138 (99%, 255bp)
CON43	296	Ribulose bisphosphate carboxylase	*L. chinensis*	-	-	CAA90004	3e-52 (98%, 98aa)	CB882113	e-163 (99%, 296bp)
CON44	324	Ribulose bisphosphate carboxylase	*P. sanctum*	-	-	CAA90006	e-60 (99%, 108aa)	CB879343	e-180 (99%, 324bp)
CON54	163	Ribulose-1,5-bisphosphate carboxylase/oxygenase large chain	*T. aestivum*	-	-	NP_114267	6e-21 (95%, 49aa)	CB882113	7e-77 (98%, 155bp)
CON57	239	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	*C. paniculata*	AY137459	e-126 (99%, 239bp)	AAO43794	7e-21 (90%, 51aa)	CD054477	e-128 (99%, 239bp)
CON58	219	Ribulose bisphosphate carboxylase large chain (RuBisCo large subunit)	*A. crassa*	-	-	P25413	4e-38 (100%, 73aa)	CB879222	e-119 (100%, 219bp)

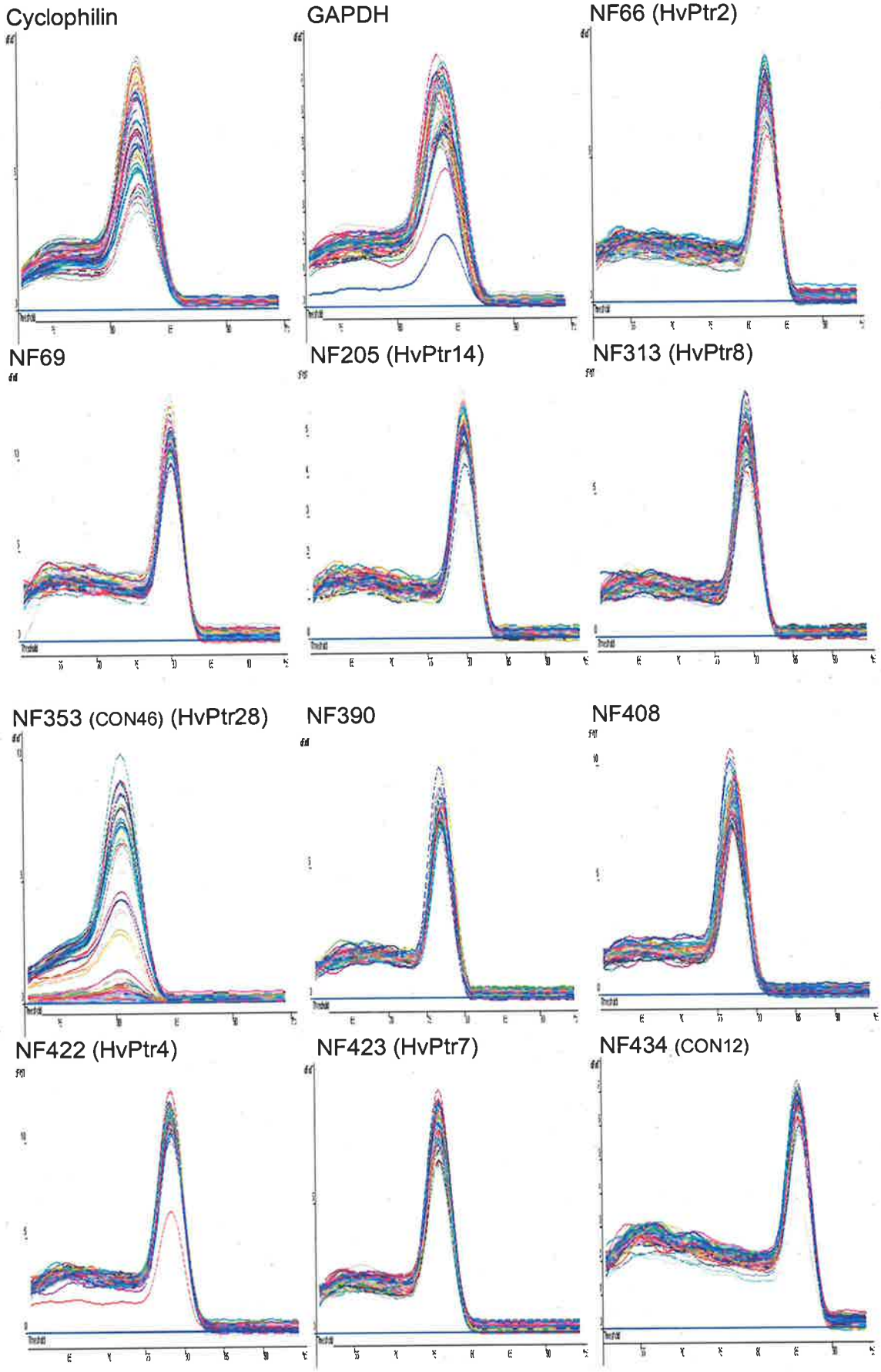
Appendix C: PCR primers and PCR product sizes together with optimal acquisition temperatures for the SSH clones analysed by Q-PCR

Gene	Primers	Product size (bp)	Acquisition temperature (°C)
*GAPDH**	(F) GTGAGGCTGGTGCTGATTACG (R) TGGTGCAGCTAGCATTTGAGAC	198	80
*α Tubulin**	(F) AGTGTCCTGTCCACCCACTC (R) AGCATGAAGTGGATCCTTGG	248	80
*Cyclophilin**	(F) CCTGTCGTGTCGTCGGTCTAAA (R) ACGCAGATCCAGCAGCCTAAAG	122	79
NF353	(F) CGCACAAAATCCTTGGAAATAGGTA (R) GATACATGAAGATCAAGCTCAACGACTAC	203	75
NF804	(F) GTCCGGTTGACCTGGTGTGTAG (R) CTCCTACCATTGTAAGTATTACCGGAGT	106	75
SF310	(F) GGCGTCTTCATCACCTGCAACTAC (R) TTTTATTTACTCGCTCGGTCCCTCT	150	77
SF512	(F) GATGGGCATATACAAAGGTATGAGA (R) ATCAGTGCATATTCTAACTGGAGGA	122	73
SF551	(F) GGACGTCACTGTCTGAAATTTGGTA (R) ATCCACGCATAACACCTGACAAACT	123	77
SF595	(F) GAACTTCTCCCATTCTAGTGCATCC (R) ACCTATGCAAATCTCCACACTTCAG	123	74
NF205	(F) ATGCCTGCTGCAGTGAACTTGT (R) CACGAGCACTCTCTCCCTATGTTAC	139	75
NF472	(F) TGTATCGATCAAGCAAGACTTCTCA (R) ACTACAACATGTCTGACTTGCGAGA	131	75
NF552	(F) ACTATAGATTCAGGGGAAGGGGTTT (R) TCACGGCACTTTACATACAGCTACA	127	75
NF66	(F) ACACAACAGCTATGTCTTCCAGCTC (R) GGGAAAGCACTCAGTTATAGGCAGT	133	78
SF475	(F) AACATACAAGGCAATGGAACTTGGT (R) TCTCTGGATTGAATTCTCTTGCATC	128	74
NF408	(F) AGTTGTGCTATCACTGCGCTTG (R) AGGAAGAACTAGTAGCAGCATGGAAA	90	72
SF372	(F) GAGAGGAGAAAATGGGAATTTCAGA (R) GCTCTGAAGGATGTTGAATTTGAGA	136	72
SF45	(F) ACTCGACTGGTTACAAAATCTTGGTC (R) AATTTTGTCCGGGCAATCAGTTCT	125	74
SF474	(F) ACTCGTAGTTAGGACGGTAGGAGATG (R) TATGATCCCGATACAAGATTACTCCAC	80	73
SF528	(F) CGTTGGAAGGCAATAATTTCTGTAG (R) CTCGAACTCAAACTCAACCCTATGA	142	76
SF593	(F) AGGGCTTTAAATCACCAGCTAATTG (R) ATACTGAAGATGCTCGGTGGAAAAT	120	73
SF77	(F) CACTAATTCCTCTCATCACCCTTGTT (R) AGTATGAGGTATCCGTTGATGAGCA	130	75
SF387	(F) CTTGCATGCAGAAGATTGTGTTG (R) GGCAATGATAGGTTGATGTTTTGAC	138	75
SF623	(F) GCTGCGTAAATGGACTCAGAAATAAAC (R) GAATCCTACAAGGACAGCACCTTGAT	185	79
SF324	(F) GAGAAGCTGTCCCATAGATACTGAATG (R) CACTCTTGGTGGATTATAGGACATGA	90	73

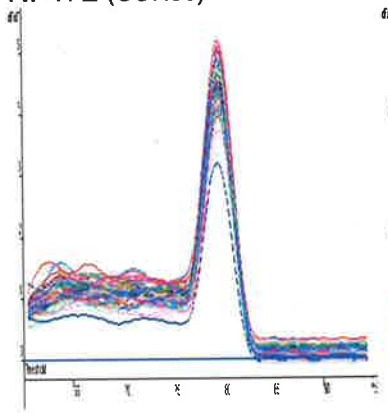
SF400	(F) AAGCACGGGTGTTTCTATCAAAGTA	108	75
	(R) TGAGTTGAGGAAGGACTAAGCTTGC		
SF427	(F) ATTCCAGCCAGCGCGTAGGTAT	89	75
	(R) CCATCACCAAGTCGCCTTTAGTAGT		
SF449	(F) TTGCTGTGTTGTTATTCGGTGTATG	129	75
	(R) CTAGGACACCAAGAGTCCAGAACAG		
SF464	(F) CTGATTTTGCTTCTCAGTGTGACTT	102	77
	(R) GATTTCCTATCGTTGGGATTCTC		
SF575	(F) GTGGTTTAAGCTTATAGCGGCACAT	90	76
	(R) AGCTGCACGGCACTGCAAGTTAC		
SF265	(F) CCCTTGAGAAGGGCGACAT	150	77
	(R) ACGGGGTAGGGGAGATGTCTT		
SF260	(F) CATATTGTTGGCCGTATAGCAC	92	76
	(R) AGGTGTGCCGCCTAGAATAAAG		
NF423	(F) GCTGCTACCTAACTAATGGCAAAC	77	72
	(R) TGCATATATATACATGCTGTCTGGT		
NF313	(F) AGGATCTCTGGGGCATAGTGAA	117	74
	(R) CCCGTCAAAGATTATTGCTTCC		
SF113	(F) CTAGGCTCCATCGTCGCTTATC	118	77
	(R) GCGTGTGGCAAACATCACAT		
NF390	(F) AATGAGCAGAGACTGCAACTGG	115	72
	(R) GCATGGACAGAGGGAGAAGTTT		
NF579	(F) ACACACTCTAAGGTCCGTGCAA	112	75
	(R) TGTAAATTGCTTCGGTTGCTGT		
SF618	(F) GATGAAGAGCAGGCGATACTGA	102	78
	(R) GATGTTGAAGTTTGCCTCATGG		
SF532	(F) GGATGATGGTGGTGTATGCTCTA	119	80
	(R) GATGTGCAGAGACCTGTGGAACT		
NF434	(F) AGGGACCTTGCTTACAAGTTCGT	110	80
	(R) ACTGTTGGGATTCACCCCTCT		
SF522	(F) GGAATCACTTCCTGCTTCTCGT	127	75
	(R) GATTGGGCCTCATGGTAGTGAT		
SF468	(F) CGTCGAAGAAATCCCCAAAGTA	130	79
	(R) GACTCGACCAAGATCCACAGG		
SF232	(F) TTGCCGATCAGATGATAAATCG	115	74
	(R) TAGACCTGATTAGCCCGCCTAC		
NF69	(F) TTGGAAGTAGATGTGCCTTTGTG	115	75
	(R) CTAGTGGCATTCTCGGCTCTCT		
SF111	(F) AAAACTGGCTTCATTGCCTTGT	123	76
	(R) GCAGGCAAGGAAATTTCAGACT		
SF613A	(F) GGTCAGATATGCGGTCTCCTTT	112	77
	(R) AGATAAGGTCGCTTGCTTTGCT		
NF422	(F) GTCAGGAGCATGTCCGATTAAC	94	73
	(R) TGCCATCTTATTGTGTTTCTGG		
NF717	(F) GGACATGGATTATTGGATGAGGA	110	78
	(R) CTTAGGATGGGAGCATTCACCTT		
SF66	(F) GCGCATCCATGTATGTCTACAA	106	73
	(R) TACTGTTAGCGGTCGTCCAAAA		

* From Burton et al. (2004).

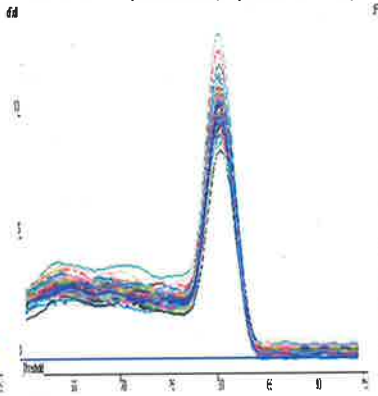
Appendix D: Melt profiles of Q-PCR products obtained using gene specific primers



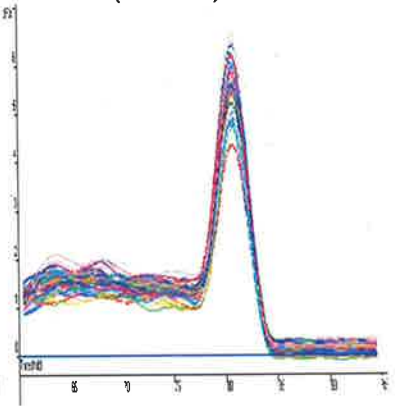
NF472 (CON56)



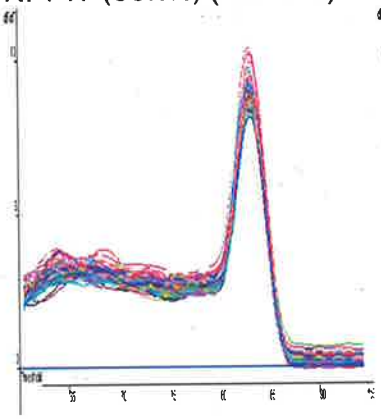
NF552 (CON29) (HvPtr13)



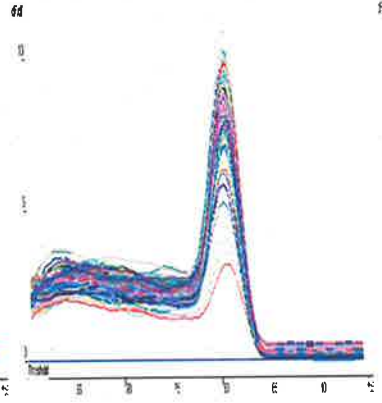
NF579 (HvPtr9)



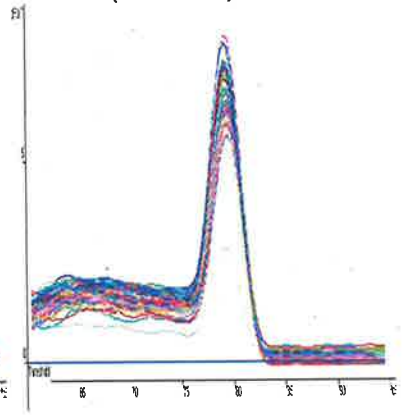
NF717 (CON16) (HvPtr21)



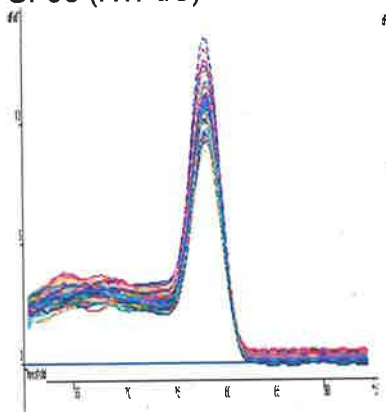
NF804 (HvPtr22)



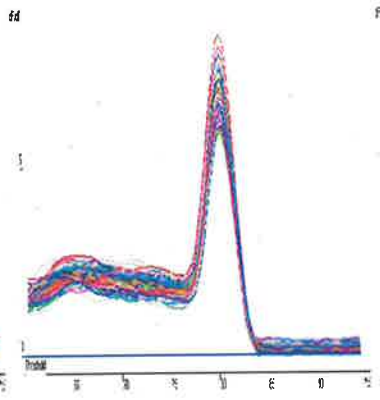
SF45 (HvPtr18)



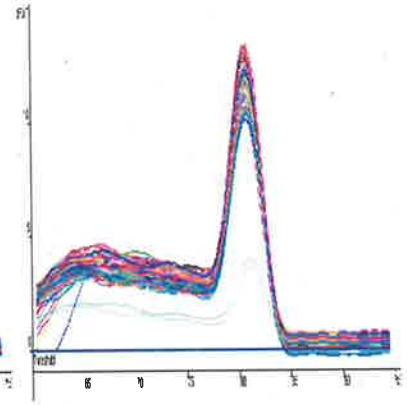
SF66 (HvPtr6)



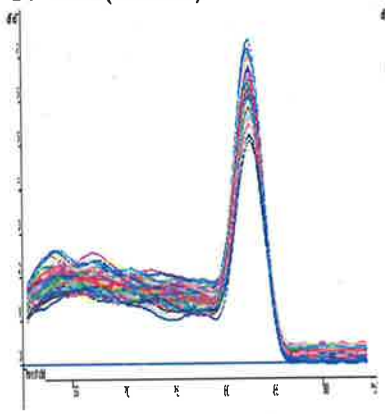
SF77



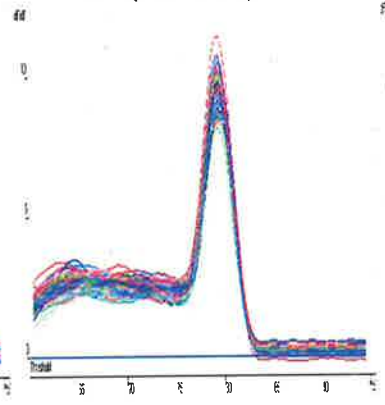
SF111 (HvPtr11)



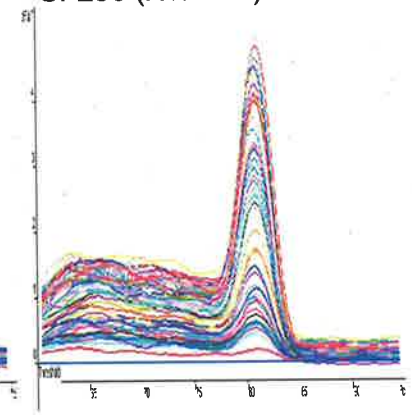
SF113 (CON47)



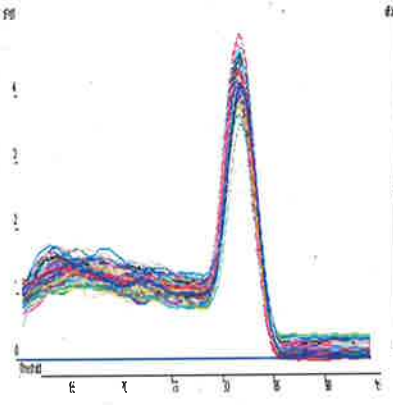
SF232 (HvPtr19)



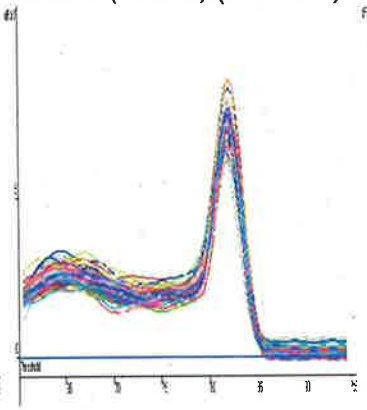
SF260 (HvPtr25)



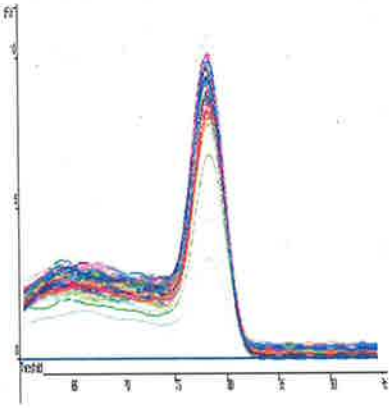
SF265



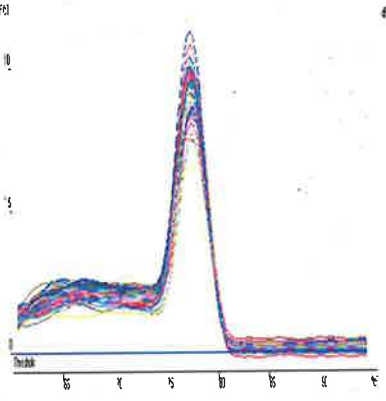
SF310 (CON45) (HvPtr26)



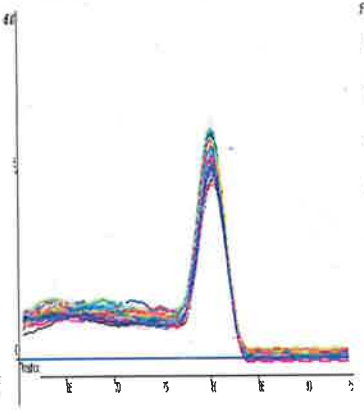
SF324



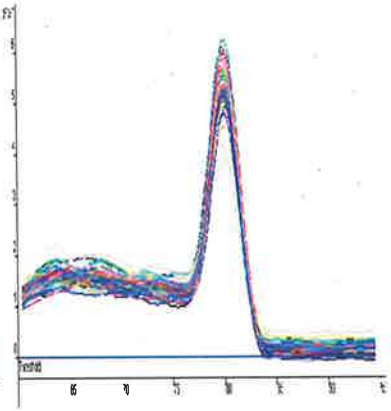
SF372 (HvPtr10)



SF387



SF400



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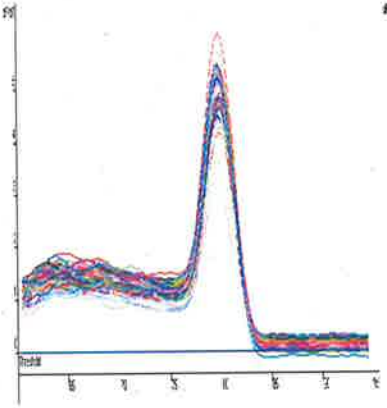

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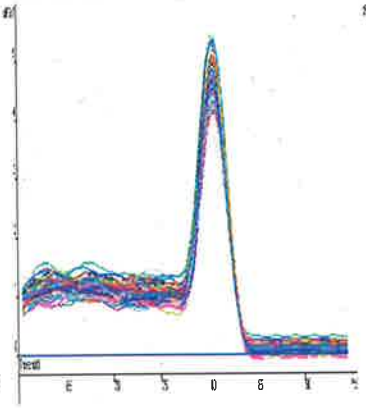
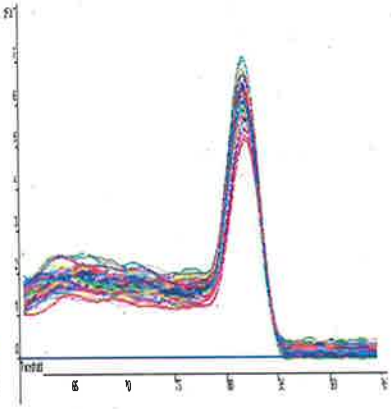
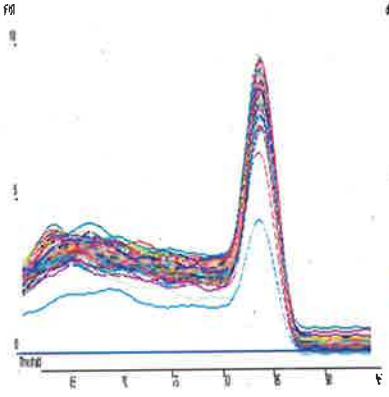


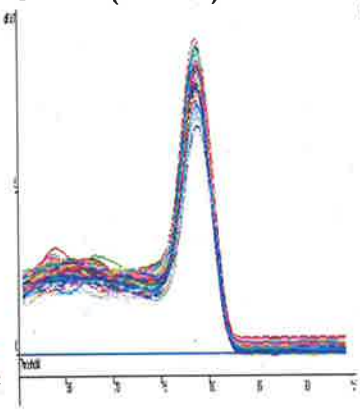
SF464



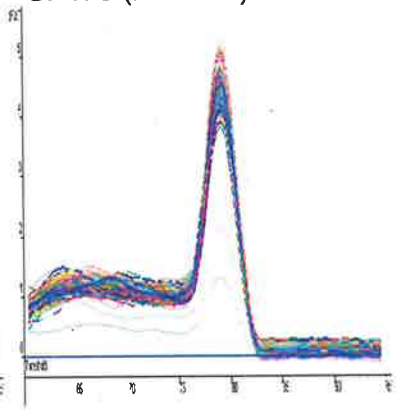
SF468 (HvPtr16)



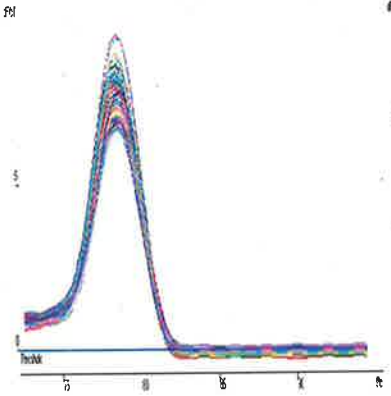
SF474 (HvPtr1)



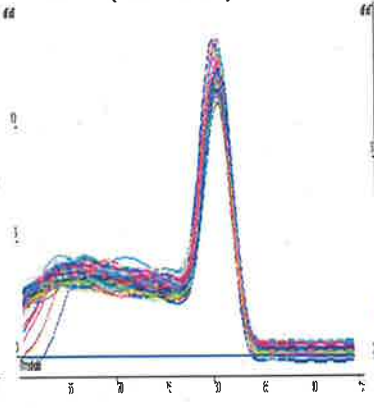
SF475 (HvPtr17)



SF512 (CON6) (HvPtr12)



SF522 (HvPtr15)



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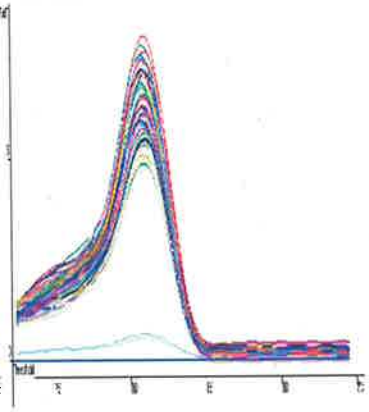
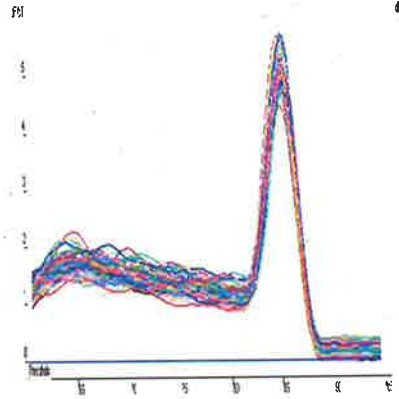
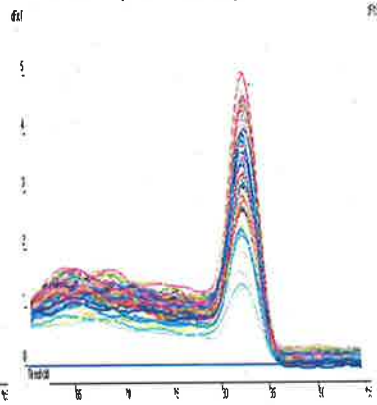


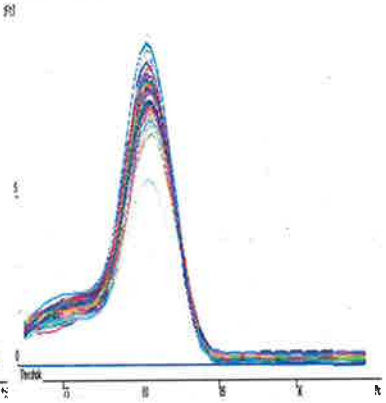
SF532



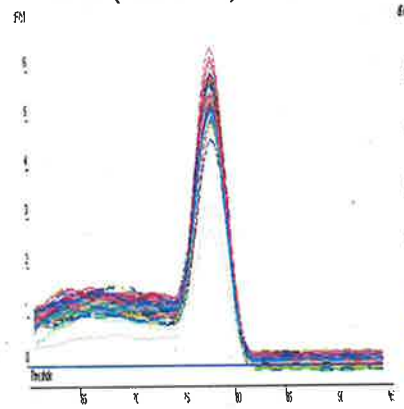
SF551 (HvPtr20)



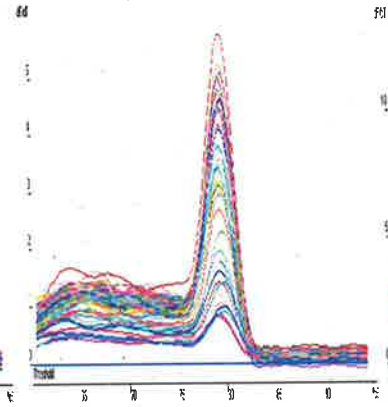
SF575



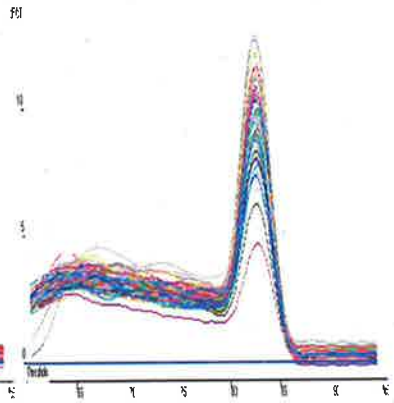
SF593 (HvPtr24)



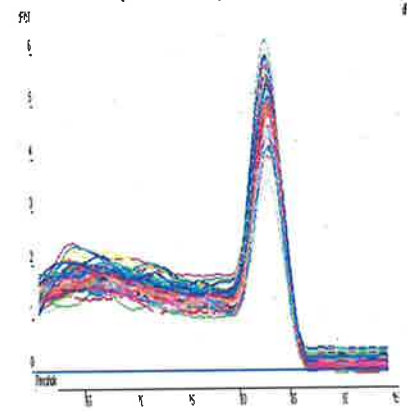
SF595 (HvPtr27)



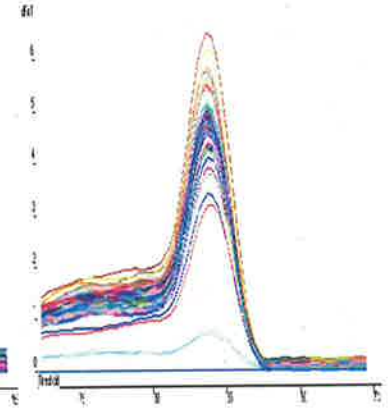
SF613 (HvPtr23)



SF618 (HvPtr5)



SF623 (HvPtr3)



Appendix E: Normalisation factors (NormFs) calculated by geNorm for the tissue series of cDNAs based on the combination of the three best reference genes (SF400, SF427, and SF575).

cDNA SERIES	NormF
BC1	1.6931
BC3	1.5098
BC6	0.8648
BC12	1.0078
BC24	1.0491
BC48	1.9839
BNF1	1.7043
BNF3	1.7049
BNF6	1.2849
BNF12	1.2485
BNF24	2.4976
BNF48	1.6578
BSF1	1.6748
BSF3	1.2102
BSF6	0.9740
BSF12	1.5489
BSF24	1.8779
BSF48	1.9199

cDNA SERIES	NormF
CC1	1.6823
CC3	1.2173
CC6	0.5583
CC12	1.0544
CC24	0.7010
CC48	0.6680
CNF1	0.9754
CNF3	0.6283
CNF6	0.7561
CNF12	0.6054
CNF24	0.9349
CNF48	0.9891
CSF1	0.9933
CSF3	1.0287
CSF6	0.5763
CSF12	0.0204
CSF24	0.6761
CSF48	0.8506

BC: B87/14 mock (water) inoculation; BNF: B87/14 inoculated with the *Ptt* isolate; BSF: B87/14 inoculated with the *Ptm* isolate; CC: CI9214 mock (water) inoculation; CNF: CI9214 inoculated with the *Ptt* isolate; CSF: CI9214 inoculated with the *Ptm* isolate. The numbers in the cDNA series column represent hours after inoculation.

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