ANALYSIS OF SIGNAL PATHWAY PROTEIN-PROTEIN INTERACTIONS DURING BIOTIC AND ABIOTIC STRESS

Submitted by
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Abstract

The overall objective of the work described in this thesis was to characterise the three genes \textit{Hv14.3.3c}, \textit{HvMAPKK1} and \textit{HvFKBP41}, in terms of a role in defence and stress response signalling. These genes had previously been found to be differentially expressed in compatible versus incompatible interactions of barley with the fungus \textit{Rhynchosporium secalis}, suggesting a possible role in the plant defence response, while current literature suggests these genes may also play a role in signal transduction, possibly under a broad range of stresses, including abiotic as well as biotic.

Two main approaches were undertaken to characterise gene function: expression analysis and the identification of protein-protein interactions.

To facilitate expression analysis, full length cDNA fragments of each gene were first obtained using bioinformatics, RACE and genomic walking techniques. Expression was then investigated using quantitative real-time RT-PCR. The results of the expression analysis confirmed that the candidate genes were in fact differentially expressed during infection, suggesting a role in the defence response of barley against \textit{R. secalis}. Analysing their expression in the context of other stresses and treatments, namely frost, drought and ABA, indicated their role may not be limited only to biotic stress, but include abiotic stress as well.

To investigate the possibility that these genes are involved in signalling during the defence response, protein-protein interaction techniques such as yeast two-hybrid and affinity pulldowns were used to identify interacting proteins in an attempt to place the genes within a known signalling network and build and extend on these networks.

Y2H screening was used successfully to identify two putative interactors of \textit{Hv14.3.3c}; an EPSP (5-enolpyruvylshikimate-3-phosphate) synthase and a putative wound-induced protein, and two interactors of \textit{HvFKBP41}; a Rab-type GTPase and the same wound-induced protein. From what is known about the function of these genes in the literature, they fit well with a role in stress response signalling and the potential to be involved in signalling networks with the candidate gene products and also with each other.

Through the trial of many different affinity pulldown techniques, a method for identifying interacting proteins from plant extracts was successfully established, however, issues with protein identification meant that interacting proteins were not identified using this technique.

Steps were then made towards confirming the interactions identified using the Y2H system. Full length cDNA sequences of the identified interactors were obtained and
expression analysis performed, in the aim of investigating co-expression patterns between the genes encoding the interacting proteins and the three candidate genes, to support a potential interaction.

To confirm the Hv14.3.3c-HvEPSP interaction, co-immunoprecipitation and BRET were then used, however confirmation was unsuccessful due to issues with non-specific binding in co-immunoprecipitation and technical issues trying to establish the BRET analysis system in barley.

In summary, the results of this study place the candidate genes *Hv14.3.3c, HvMAPKK1* and *HvFKBP41* as players in signal transduction during the plant defence/stress response. With the identification of previously uncharacterised protein interactions, some progress has also been made towards placing these genes within known signalling networks and identifying potential downstream genes that could possibly play a more specific role in defence response signalling and therefore be potential targets for the generation of resistant or stress tolerant plants.
Statement of Authorship

This work contains no material that has been accepted for the award of any other degree or diploma in any university or other tertiary institute and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference being made in the text.

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I also give permission for the digital version of my thesis to be made available on the web, via the University’s digital research repository, the Library catalogue, the Australasian Digital Theses Program (ADTP) and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

Jenna Malone

July 2009
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- my family and friends for their help and support
### Abbreviations

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACPFG</td>
<td>Australian Centre for Plant Functional Genomics</td>
</tr>
<tr>
<td>AGRF</td>
<td>Australian Genome Research Facility</td>
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<tr>
<td>bp</td>
<td>Base pairs</td>
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<tr>
<td>d</td>
<td>Days</td>
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<tr>
<td>DMSO</td>
<td>Dimethylsulphoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>dNTP</td>
<td>Deoxynucleotide</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra-acetic acid</td>
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<tr>
<td>EST</td>
<td>Expressed sequence tag</td>
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<td>Fig</td>
<td>Figure</td>
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<tr>
<td>g</td>
<td>Grams</td>
</tr>
<tr>
<td>g</td>
<td>Units of relative centrifugal force</td>
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<tr>
<td>Gal4</td>
<td>Yeast transcriptional activator protein</td>
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<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
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<tr>
<td>HEPES</td>
<td>(N-[2-hydroxyethyl] piperazidine-N’-[2-ethanesulphonic acid])</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IMVS</td>
<td>Institute of Medical and Veterinary Science</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropylthiogalactoside</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilo base</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilo Dalton</td>
</tr>
<tr>
<td>kV</td>
<td>Kilo Volts</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
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<tr>
<td>Luc</td>
<td>Luciferase</td>
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<tr>
<td>M</td>
<td>Molar</td>
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<td>Min</td>
<td>Minutes</td>
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<td>ml</td>
<td>Millilitre</td>
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<td>mM</td>
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<td>mg</td>
<td>Milligrams</td>
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<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
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<tr>
<td>ng</td>
<td>Nanograms</td>
</tr>
<tr>
<td>nm</td>
<td>nano metres</td>
</tr>
<tr>
<td>NSW</td>
<td>New South Whales</td>
</tr>
<tr>
<td>o/n</td>
<td>Overnight</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>QLD</td>
<td>Queensland</td>
</tr>
<tr>
<td>Q-PCR</td>
<td>Quantitative real-time PCR</td>
</tr>
<tr>
<td>RACE</td>
<td>Rapidly amplified cDNA ends</td>
</tr>
<tr>
<td>RLuc</td>
<td><em>Renilla reniformis</em> Luciferase</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>SARDI</td>
<td>South Australian Research and Development Institute</td>
</tr>
</tbody>
</table>
SD  Synthetic dropout
SDS  Sodium dodecyl sulphate
SDS-PAGE  SDS-polyacrylamide gel electrophoresis
sec  Seconds
SSH  Suppressive subtractive hybridisation
SA  South Australia
TBS  Tris buffered saline
Tm  Melting temperature
Tris  Tris [hydroxymethyl] amino methane
U  Units
V  Volts
VIC  Victoria
v/v  Volume per volume
w/v  Weight per volume
X-α-Gal  5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside
YFP  Yellow fluorescence protein
YPD  Yeast potato dextrose
Y2H  Yeast two-hybrid
µl  Microlitres
µg  Micrograms
µM  Micromoles
Ω  Ohm
Chapter 1

General Introduction
1.1 Introduction

The main objective of the work described in this thesis was to isolate and characterise three genes; Hv14.3.3c, HvMAPKK1 and HvFKBP41. These genes had previously been found to be differentially expressed in compatible versus incompatible interactions of barley with the fungus Rhynchosporium secalis, suggesting a possible role in the plant defence response. The main aim of this work, therefore, was to investigate this possibility, namely through the use of expression analysis.

Current literature suggests these genes may also play a role in signal transduction, possibly under a broad range of stresses, including abiotic as well as biotic. Thus, secondary aims were to investigate the possibility that the genes may be involved in signal transduction during defence responses, and to identify the signalling networks in which they may be involved. This was namely done through protein-protein interaction studies.

The candidate genes are known to play a role in many diverse plant cellular processes such as development and protein folding. Therefore, if they were found to be involved in plant defence response signalling, manipulation of these genes, while possibly having an effect on resistance or tolerance, may also have an effect on many other cellular processes. Therefore, the identification of genes involved further downstream in the signalling pathway the candidate genes are involved in, may provide more specific targets for providing resistance or tolerance.

To investigate the possibility that these genes are involved in signalling during the defence response, and to identify genes involved downstream in the signalling cascade, techniques such as yeast two-hybrid and affinity chromatography were used to identify interacting proteins in an attempt to place the gene within a known signalling network and build and extend on these networks.

In this chapter the barley/R. secalis interaction is described, highlighting the plant defence response process, problems associated with overcoming R. secalis infection and the need for alternative approaches. Previous work in which the candidate genes were originally identified as possible novel resistance targets, is also described. Following this, the possibility that these genes may not only play a role in providing a broader, more durable defence response to R. secalis than the currently investigated resistance genes, but also the potential to be involved in the defence response to a wider range of stresses, including abiotic, is discussed. A subsequent literature review of the three genes illustrates the current knowledge on the involvement of these genes in the defence response, and a possible role for these genes in signal transduction during the defence response.
1.2 *Rhynchosporium secalis*

1.2.1 Scald disease

Scald disease of barley (*Hordeum vulgare* L.), also known as leaf blotch, leaf spot and leaf blight (Ali, 1974), is caused by the haploid imperfect fungus *Rhynchosporium secalis* (Oudem) J.J. Davis. It is one of the most economically important diseases of barley worldwide, affecting barley in cool, humid areas of temperate zones the most severely (Robbertse et al., 2000; Williams et al., 2003).

After wheat, barley is the second most important cereal crop grown in Australia, with annual production averaging around 6.8 million tonnes (Brown et al., 2009). Thus, diseases that affect barley production are therefore of great importance in Australia. As one of the most damaging foliar and stem pathogens of barley (Jefferies et al., 2000), scald disease is a disease of major interest. Yield losses due to scald disease have been reported to range from 10-40% (Shipton et al., 1974), with losses as high as 48% having been reported in experimental plots in South Australia (Jefferies et al., 2000).

The characteristic symptoms of scald disease initially appear as pale, irregular or diamond shaped lesions on leaves and leaf sheaths (Fig 1.1A). These spots expand and become blue-grey in colour with a water-soaked appearance as infection progresses (Fig 1.1B). Mature lesions become dry and pale brown with a dark purple/brown margin (Fig 1.1C). As the lesions enlarge, they merge, forming large areas of dead tissue (Fig 1.1D). Entire leaves can be destroyed if infection is severe (Ayesu-Offei and Clare, 1971).

The main detrimental effects attributed to scald infection of barley include a decreased kernel weight and percentage of plump grain (Khan and Crosbie, 1988; Schaller, 1951) resulting in a downgrading from malting to feed quality barley (Basson et al., 1990; Garvin et al., 1997).

1.2.2 Infection process

*R. secalis* infection of barley is mainly spread from host to host via splash-dispersion of fungal conidia (Shipton et al., 1974), but can also be transmitted from one generation to the next through fungal mycelial inoculum in seeds (Habgood, 1971). The main source of inoculum is usually infected plant debris on or in soil, where the fungus can continue to sporulate for up to a year (Shipton et al., 1974).

Once fungal conidia contact the host leaf surface, germination takes around one day (Roulin et al., 1997) and involves the production of one or two, or in a few cases three, germ tubes from any surface of the two cells of the conidium (Ayesu-Offei and Clare,
Fig 1.1: Characteristic symptoms of scald infection. A) Initial pale, irregular/diamond shaped lesions B) Expanded, darker water-soaked lesions C) Mature lesions displaying characteristic pale brown colouring with dark margins. D) Lesions that have spread together causing large areas of dead tissue.
1970), that penetrate the leaf cuticle, usually within 24 hours of inoculation. The hyphae grow as mycelium beneath the leaf cuticle, initially above the anticlinal wall of two adjacent host epidermal cells (Ayesu-Offei and Clare, 1970; Lyngs Jorgensen et al., 1993). This initial growth causes swelling of epidermal cells, loss of cell wall rigidity and often separation of the plasma membrane from the cell wall (Lehnackers and Knogge, 1990). It is thought that the fungal infection causes a change in the permeability of the host epidermal cells, altering the turgor balance between these cells and the stomatal guard cells. The change in turgor results in abnormal swelling of guard cells, causing increased opening of stomatal cells in the light and failure to close in the dark. This would lead to water evaporation and dehydration which can result in localised desiccation of infected tissue and wilting of whole leaves (Davis et al., 1994; Ayres, 1972).

As infection proceeds and fungal hyphae grow between epidermal cells, the outer layer of the cell wall breaks down and the inner layer is attacked. It is thought that, since the fungus is capable of producing cellulolytic enzymes in culture (Olutiola and Ayres, 1973), similar enzymes may be involved in the in vivo degradation of the host cell wall during infection (Ryan and Grivell, 1974). This degradation of the epidermal cell wall, as well as the permeability changes, allow nutrients to leak out of the cells and become available to the fungus. As there is no evidence that R. secalis forms haustoria, the leaked nutrients are thought to be sufficient to support hyphal growth (Davis et al., 1994; Jones and Ayres, 1972).

All of these factors ultimately lead to the collapse of epidermal cells after a few days of fungal infection. In the later stages of infection, around 10-14 days, branches of the subcuticular fungal mycelium grow between the collapsed epidermal cells and enter the mesophyll cell layer, causing the mesophyll cells underlying affected epidermal cells to collapse. At this stage the characteristic disease symptoms begin to appear (Lyngs Jorgensen et al., 1993; Ali, 1974).

Fungal mycelium continues to grow beneath the leaf cuticle, forming a closely packed hyphal mat called the subcuticular stroma. Conidia are produced in the stroma before forcing their way up through the cuticle, ready for dispersal, germination and repetition of the cycle (Ayesu-Offei and Clare, 1970).

1.3 Barley - R. secalis interaction

1.3.1 Resistance of barley to scald

There is general consensus that resistance of barley to R. secalis is an inherited trait, and
studies into the inheritance of scald resistance, using a variety of barley cultivars and \textit{R. secalis} races, led to the conclusion that resistance to this pathogen is monogenically inherited (Lehnackers and Knogge, 1990). Resistance is thought to result from early and strong induction of plant defence responses after perception of the fungus (Lauge and De Wit, 1998). This resistance is assumed to be controlled by a gene-for-gene interaction (Flor, 1955), with a gene for resistance in the plant corresponding to a gene for avirulence in the pathogen (Hahn et al., 1993; Lauge and De Wit, 1998). Studies into inheritance of resistance to \textit{R. secalis} in barley have led to the definition of several major resistance genes, however, the designation of resistance genes in barley is complex and inconsistent (Robinson et al., 1996). To date a total of 15 genes for resistance to scald have been identified and named \textit{Rrs1} to \textit{Rrs15}, with \textit{Rrs} short for \textit{Resistance to Rhynchosporium secalis} (Genger et al., 2005; Chelkowski et al., 2003). However, there is confusion as to how many loci exist. For example, the \textit{Rrs1}, 3 and 4 complex on chromosome 3H is regarded by some as a single gene with several alleles (Habgood, 1971) while others maintain that these genes are separate but closely linked (Dyck and Schaller, 1961).

The most effective genes for resistance in the field are \textit{Rrs5} and alleles at the \textit{Rrs1} locus, and the least effective are \textit{Rrs2}, 6, 8, 9 and 11 (Ali, 1976). Genetic studies have shown that some of the resistance genes show incomplete dominance, some complete dominance, and others are recessive genes (Habgood and Hayes, 1971).

\subsection*{1.3.2 The NIP1/Rrs1 gene-for-gene based resistance}

An example of the barley/scald gene-for-gene resistance is based on the corresponding genes \textit{Rrs1} in barley and \textit{NIP1} in \textit{R. secalis} (Hahn et al., 1993).

A class of necrosis-inducing peptides (NIPs) from \textit{R. secalis}, NIP1, NIP2 and NIP3, were first identified by Wevelsiep et al (1991) from fungal culture filtrates. These small peptides of around 10 kDa have been reported to act as toxins on barley as well as on other cereal and bean plants. When injected into leaves they cause scald-like lesions in a genotype-unspecific manner (van't Slot et al., 2007).

NIP1 is an 82-amino acid product of the \textit{NIP1} gene, processed to yield a 60-amino acid secreted mature protein (Rohe et al., 1995). As well as acting as a toxin, NIP1 has also been found to be a race-specific elicitor of defence responses in barley cultivars with the \textit{Rrs1} gene, triggering the expression of plant defence response genes in a cultivar-specific manner (Lauge and De Wit, 1998). Evidence for this includes the co-inoculation of spores from a virulent fungal race together with the NIP1 protein causing conversion of the
interaction from compatible to incompatible, but only on *Rrs1* plants. Transformation of virulent fungal races with the *NIP1* gene giving avirulent transformants is also further evidence to demonstrate that NIP1 is the product of a fungal avirulence gene (Rohe et al., 1995).

All investigated fungal races, that are avirulent on barley cultivars of the *Rrs1* resistance genotypes, carry and express the *NIP1* gene and secrete the elicitor-active NIP1 protein, while races lacking this gene are virulent on *Rrs1* genotypes (Lauge and De Wit, 1998; Hahn et al., 1993). Thus, in compatible interactions, the susceptible host cultivar lacks the resistance gene *Rrs1*, or the fungus does not carry the elicitor-active *NIP1*, leading to no fungal recognition by the host and therefore no expression of the defence response genes. In incompatible interactions, the *Rrs1* gene is present in the resistance cultivar and the fungus carries the elicitor-active NIP1, enabling fungal detection and activation of host defence response genes, resulting in fungal development arrest and ultimately resistance (Rohe et al., 1995).

### 1.3.3 The defence response in compatible and incompatible interactions

A number of reports have investigated the response of barley to *R. secalis*, with the aim of identifying the stage or stages at which fungal growth is inhibited in resistant interactions, and the possible mechanisms of this inhibition.

Lyngs Jorgensen et al (1993) investigated resistance mechanisms by comparing the infection process of *R. secalis* in compatible and incompatible interactions to determine at which stage or stages of infection growth was inhibited in incompatible interactions. They found no differences in the first stages of the infection process, appressoria formation and cuticular penetration, between the compatible and incompatible interactions. However, at the next stage of infection, papillae formation, a difference was visible. Papilla formation occurred at a higher frequency and with significantly increased size in resistant interactions. A strong correlation between larger papilla size and inhibition of fungal penetration was found when comparing the later stages of infection, where only a few hyphae were established subcuticularly and no cell collapse occurred in the resistant interaction, suggesting a possible role for papilla formation in resistance.

A similar study by Xi et al (2000) investigated the differences in the penetration process of *R. secalis* on resistant and susceptible cultivars. A significantly higher amount of host cell wall alterations in resistant cultivars was observed in the form of apposition, the localised thickening of the inner cell wall that appears as a ‘halo’ around the point of attempted penetration by *R. secalis*. These cell wall alterations were thought to be triggered in
response to cuticle penetration by *R. secalis*, and successful penetrations in susceptible cultivars was correlated with no halo formation occurring. Cell wall appositions might not be a general mechanism of resistance against *R. secalis* as they seem to occur in some susceptible interactions as well as resistant (Lehnackers and Knogge, 1990). It is not unlikely that resistance to *R. secalis* is based on a number of different mechanisms, considering the large (15) number of scald resistance genes described.

In a study by Lehnackers and Knogge (1990), where the infection process of two near isogenic barley cultivars, ‘Atlas’, a susceptible cultivar containing the *Rrs2* gene, and ‘Atlas46’ a resistant cultivar carrying *Rrs1* in addition to *Rrs2* (Riddle and Briggs, 1950) was compared, two possible resistance mechanisms emerged. One suggested mechanism was the inhibition of spore germination and germ tube growth. However, other reports from similar studies that used different cultivars have found no evidence of such inhibition playing a role in resistance interactions (Xi et al., 2000), with some finding that spore germination is independent of host genotype (Fowler and Owen, 1971). The second mechanism was the prevention of the establishment of subcuticular mycelia, which appeared to be accompanied by the degradation of successfully penetrated hyphae and of fungal structures on the leaf surface.

### 1.4 Control of scald disease

#### 1.4.1 Current treatment methods

Effective control of leaf scald can be achieved using many commercially available fungicides, particularly from the triazole group, applied as foliar spray, seed dressings or with fertiliser (Jefferies et al., 2000). However, factors such as cost, increased concern over fungicide residues in foodstuffs, effectiveness varying from location to location, impact on the environment, and reports of fungal isolates resistant to some fungicides, do not make the use of fungicides ideal (Jefferies et al., 2000; Kendall et al., 1993). Studies by Cromey et al (2000) into fungicide treatments also showed that it was difficult to control scald with fungicides, with a reduction of only 29% of disease severity in highly susceptible cultivars, and moderately susceptible cultivars also not totally protected from disease using fungicides.

The most effective way to overcome the problems associated with fungicides is the development of barley cultivars resistant to the scald fungus (Jefferies et al., 2000) and an approach to develop such cultivars is the utilisation of breeding programmes. Manipulating resistance genes by plant breeding is considered as one of the most efficient and economic
strategies for control. However, while resistance is widely available and cultivars with different genes for resistance have been developed, there are certain factors that pose limitations on breeding cultivars for resistance against scald (Zhang et al., 1991). A major limitation is the pathogenicity of the fungus. *R. secalis* is pathogenically a highly variable fungus, with a large number of different pathotypes having been identified (Fukuyama et al., 1998). In a study by Ali (1976) where the pathogenicity of 203 *R. secalis* isolates (35 pathotypes) was assessed on 21 barley cultivars, only one cultivar genotype, Atlas46 was found to be resistant to all isolates. This pathogen variability often results in a major gene resistance being rendered ineffective and in a breakdown of resistance over a short period of time. For example, the Australian barley varieties Skiff and Franklin carried high levels of resistance when released commercially in 1989, but by 1993 Skiff was highly susceptible in South Australia and susceptibility in Franklin crops began to emerge in 1997 (Jefferies et al., 2000). Thus, a new cultivar or even the development of transgenic barley carrying a major resistance gene, would not present a long lasting resistance.

Approaches to overcome this problem have included the use of gene pyramiding, introduction of two or more pathotype-specific resistance genes into a single cultivar, as well as cultivar mixtures, having crops containing mixtures of barley varieties carrying different pathotype-specific resistance genes (Burdon et al., 1994). However, Abbott et al (2000) presented evidence to suggest that mixtures were not an effective strategy for controlling scald, especially when one of the cultivars is susceptible (Jefferies et al., 2000), while gene pyramiding has been found to be difficult to achieve and can lead to undesirable agronomic traits (Pink, 2006; Zhang et al., 1991).

### 1.4.2 Identifying new resistance targets

Due to the problems associated with the current control mechanisms, the work undertaken by Dr K. Oldach (ACPFG, University of Adelaide, Australia) aimed to identify genes involved in establishing resistance to *R. secalis*. Targets were differentially expressed genes between compatible and incompatible interactions in the *NIP1/Rrs1* system of *R. secalis* and barley. The differentially expressed genes were identified using suppressive subtractive hybridisation to enrich for differential cDNAs, followed by micro-array analysis. From this, 49 genes displaying a differential expression pattern were found and homology searches, used to assign identity, showed that most of these genes encoded stress-related proteins and proteins involved in signal transduction.
1.4.3 Targeting defence signalling

Plants need to be particularly responsive to environmental stimuli in order to survive. This response requires recognition of the stimuli and initiation of the defence response, and signal transduction links both (Sehnke and Ferl, 1996).

One source of severe stress is infection by pathogens, such as fungi, bacteria and viruses. Plants encounter many pathogens, however most interactions between such pathogens and plants are of the ‘non-host’ variety, meaning that there is no pathogenic activity on the plant and therefore no disease occurs, most likely due to the pathogen lacking the basic pathogenicity factors required to cause disease on the plant. In ‘host’ pathogen systems, however, the pathogen is pathogenic on the plant and can cause disease (Lauge and De Wit 1998).

In such ‘host’ systems, when a pathogen penetrates plant tissue, plant cells perceive signals from pathogens. These signals, known as elicitors or PAMPs (Pathogen-Associated Molecular Patterns), are transmitted to the defence genes via intracellular signalling cascades (Suzuki and Shinshi, 1995). Plants then respond to this pathogen attack by activating multi-step defence responses, including rapid production of reactive oxygen species, strengthening of cell walls, induction of the hypersensitive response (HR), synthesis of pathogen-related proteins (PR proteins) and phytoalexins and localised cell death at sites of infection (Nakagami et al., 2005).

The major R. secalis resistance genes, that are the current targets in breeding for resistance, are involved in this fungal recognition and initiation of the defence response. For example, both the non-specific virulent toxic effect and the R-gene specific avirulence determination of NIP1 are thought to be based on the activation of one or more signalling pathways (Fiegen and Knogg, 2002).

However, little is known about the downstream effects of this recognition, what occurs during the defence response, what genes are involved and what ultimately leads to resistance in some barley cultivars. It is likely that the genes transferring this recognition event as part of a signalling pathway to finally induce the expression of the defence response genes might be conserved among different R. secalis resistance reactions. Therefore, their manipulation, either by breeding or genetic engineering, represents an approach to generate a more durable and broader resistance to R. secalis in barley.

Although different stresses generally elicit a specific final response, many of the signalling intermediates, such as plant hormones, reactive oxygen species, calcium etc are common to
multiple pathways (Roberts et al., 2002). Furthermore, some environmental conditions such as drought, osmotic stress and cold have overlapping physiological effects on the plant. The use of overlapping signalling pathways allows for modulation of responses via cross-talk between pathways at points of intersection (Knight and Knight, 2001). This suggests that the genes involved in signalling transduction during pathogen attack, i.e. of *R. secalis*, may also be involved in the transduction of abiotic stress signals. Therefore, these signalling genes may not only provide an alternative means to overcoming the problem of scald, but may be involved in abiotic stress protection as well.

1.5 Candidate genes

For this study, candidate genes were chosen based on their identification as being differentially expressed in compatible and incompatible interactions of barley with *R. secalis* and therefore their possible role in the defence response, as well as their possible role in stress signal transduction based on their putative identity according to database homology and current literature, as discussed below.

Three candidate barley genes, a putative 14.3.3, MAP kinase kinase and an FKPB-type immunophilin, were chosen for further characterisation in this study.

1.5.1 14.3.3’s

14.3.3 proteins were first described by Moore and Perez (1967) as a family of abundant acidic proteins present in brain extracts, and named due to their migration position on two-dimensional DEAE chromatography and starch gel electrophoresis. A pivotal study by Ichimura et al (1987) found that these 14.3.3 brain proteins were similar in amino acid composition to a protein known to activate tyrosine and tryptophan hydrolases in the presence of calcium and cAMP-dependent kinase. When later studies confirmed that 14.3.3s were in fact activators of tyrosine and tryptophan hydrolases, the now recurrent themes of 14.3.3 protein interaction and the association with protein kinases, were begun, sparking an interest in the 14.3.3 protein family (Ferl, 1996).

Since then 14.3.3s have been found to be ubiquitous in all eukaryotes (Bihn et al., 1997), with an examination of 14.3.3 entries in the public databases revealing over 150 isoforms reported in almost 50 species (Rosenquist et al., 2000). They have a molecular mass of 25 – 32 kDa and occur as both homo- and hetero-dimers of different isoforms (Ferl, 1996). They are highly conserved throughout evolution, with 53% protein sequence conservation across 48 different species, including all amino acid residues in the target binding region (Rosenquist et al., 2000).
A variety of apparently unrelated biological activities, including a role in signal transduction, have been assigned to the 14.3.3 family (Burbelo and Hall, 1995), with the main characteristic shared by 14.3.3s in these processes being interaction with other proteins (Finnie et al., 1999).

1.5.1.1  14.3.3-protein interaction and specificity

The crystal structure has been solved for two mammalian 14.3.3s (Liu et al., 1995; Xiao et al., 1995) and one plant 14.3.3 (Ottmann et al., 2007). The protein sequences of 14.3.3s are highly conserved and the high conservation of the central core region of 14.3.3s makes the animal structure a very likely fit to the general features of all 14.3.3s (Ferl, 1996). The structure shows that 14.3.3 proteins exist as dimers with residues 5 - 21 in one monomer forming contacts with residues Ser58-Glu89 in the opposing monomer (Aitken, 1996). When the L-shaped monomers come together as a U-shaped dimer, they form a broad central groove or channel that contains two binding sites for target proteins (Fig 1.2) (Moorhead et al., 1999). The interior of this large negatively-charged channel contains amino acids that are almost identical throughout the family, meaning this channel would recognise common features of target proteins. Specificity of the interaction of 14.3.3 isoforms with diverse target proteins may therefore involve the outer surfaces of the protein (Aitken, 1996).

Both the N- and C-terminal regions of all 14.3.3 isoforms are highly variable (Obsilova et al., 2008). As the N-terminal end is involved in 14.3.3 dimerisation this may limit the number of possible homo- or heterodimer combinations, a restriction that may confer some specificity on 14.3.3 function (Aitken, 1996), while the variability of the C-terminus may contribute to substrate selectivity.

It is also thought that different isoforms, although they may have many conserved properties, specialise in individual functions on the basis of their isoform diversity, distinct cell-specific and/or developmentally regulated expression and differential response to environmental stimuli (Wu et al., 1997).

Binding of 14.3.3 to other proteins is most commonly through specific phosphorylated target sites, the best characterised of which are the mode 1 (R/KxxpS/TxP) and mode 2 (R/KxxxpS/TxP) sites or close variants of these, where an 'x' can be several, but not all of the 20 amino acids and a lower case 'p' indicates the site of phosphorylation (Yaffe et al., 1997). These are very common motifs, contained in nearly 40% of the Arabidopsis proteome, but 14.3.3 binding will depend on the phosphorylation status, steric availability
Fig 1.2: Structure of 14.3.3. A) Ribbon drawing of 14.3.3 structure, indicating the L-shaped monomers forming a U-shaped dimer and the broad central grooves for target protein binding B) The highly conserved negative channel, the potential region of interaction with signalling proteins (Aitkin 1996). The phosphorylation site, Ser185 is shown.
of binding sites and on sub-cellular localisation of both the 14.3.3 isoform and target protein (Alexander and Morris, 2006). The targets of 14.3.3s are found in all subcellular compartments and have a wide range of functions including transcription factors, biosynthetic enzymes, cytoskeletal proteins, signalling molecules, apoptosis factors and tumour suppressors (Dougherty and Morrison, 2004).

In addition to these motifs, there are also known non-phosphorylated 14.3.3 binding sites described (Petosa et al., 1998). When screening random peptide phage display libraries Wang et al (1998) isolated a number of non-phosphorylated peptides which contained a consensus sequence; WLDL, and bound 14.3.3 with high affinity. A phosphorylation-independent interaction between a 14.3.3 and ExoS, an ADP-ribosyltransferase toxin of Pseudomonas aeruginosa, has also been demonstrated (Masters et al., 1999).

Thus, with both phosphorylation-dependent and independent interactions, the number of proteins that may interact with 14.3.3 is potentially very large, with over 300 different target proteins having been identified to date, namely through the use of affinity purification of interacting proteins from cell lysates by immobilized recombinant 14.3.3 proteins and immunoprecipitation of overexpressed tagged 14.3.3 (Kjarland et al., 2006).

1.5.1.2 Mode of action/function

Although many studies have lead to the association of 14.3.3s with many diverse cellular processes, their function still remains unclear. The main characteristic shared by 14.3.3 proteins in these processes appears to be interaction with other proteins (Finnie et al., 1999) and 14.3.3’s are known to have multiple functions in these interactions. The consequences of 14.3.3 interaction with target proteins can be classified into several generic modes of action (Fig 1.3). Firstly, interaction can directly alter the activity of the target, for example changes in specific activity or half-life of an enzyme. 14.3.3 can also act as an adaptor or scaffold, binding and bringing two proteins together in close proximity, stimulating protein-protein interactions. In addition, the interaction of 14.3.3 and target can cause a conformational change to the target, brought about by interactions at multiple sites on the target. The regulation of subcellular localisation can also be a consequence of 14.3.3 interaction, where 14.3.3s are able to bind cleavable signal peptides, stimulating import into target organelles. Modulation of nuclear import and export is increasingly being revealed as a common mode of action, as well as transport in the endomembrane system (Roberts, 2003; van Hemert et al., 2001).
Fig 1.3: Generic modes of action of 14.3.3. A) Altering target protein activity B) Acting as adaptor, stimulating protein-protein interaction C) Conformational change of target protein D) Regulation of subcellular localisation E) Nuclear import/export F) transport in the endomembrane system (Roberts et al., 2003).
1.5.1.3 Role of 14.3.3s in signal transduction

A wide range of biological functions have been attributed to 14.3.3s, including roles in growth and development regulation, enzyme regulation, response to environmental stress, exocytosis and differentiation. However, it is the likelihood that they play a role in signal transduction that has sparked a large increase into their investigation in recent years (Ferl, 1996).

A key event in signal transduction is the transition of a protein from one activity state to another via phosphorylation. Kinases, phosphatases, transcription factors and enzymes can all be affected by phosphorylation in this way. However, it has become evident that phosphorylation alone does not induce this change in activity state in many cases, and that it is rather the phosphorylation-induced association with 14.3.3s that results in the transition to changes in activity (Sehnke et al., 2002). Most of the more than 60 known 14.3.3 binding proteins in mammalian cells are components of intracellular signalling pathways (Rubio et al., 2004), suggesting they have an important role to play in signalling.

14.3.3s have also been shown to either inhibit (Toker et al., 1990) or activate (Isobe et al., 1992) protein kinase C’s, a family of serine/threonine kinases involved in many signalling processes (van Hemert et al., 2001).

Further evidence that 14.3.3s play a role in signal transduction is their involvement in the mammalian MAP kinase signal transduction cascade. In animal systems, the Ras signal transduction pathway links cell surface receptors to changes in gene expression through a series of protein-protein interactions that mediate the mitogen-activated protein (MAP) kinase cascade. The role of Ras is to activate Raf, a MAPKKK, which phosphorylates MEK, a MAPKK, which in turn phosphorylates MAPK. It has been found that 14.3.3 proteins interact with Raf and it is thought that this interaction is required for Raf activation by Ras, possibly due to 14.3.3 causing a conformational change to Raf (Burbelo and Hall, 1995). An important role for 14.3.3s in Brassinosteroid signal transduction, involved in plant growth and development, has also been shown in Arabidopsis (Gampala et al., 2007) and rice (Bai et al., 2007).

1.5.1.4 Role of 14.3.3s in plant stress response signalling

Due to their role in protein-protein interactions and signalling, the involvement of 14.3.3 proteins in plant stress responses has often been suggested. Such roles have been inferred from the potential of 14.3.3s to regulate both signalling pathways and those proteins involved in the final response.
Abiotic stress
There are numerous lines of evidence to show that 14.3.3s are involved in many metabolic and signalling pathways for plant abiotic stress responses. One of the first plant 14.3.3 genes isolated was identified as a transcript that accumulated in callus and seedlings of rice when exposed to high NaCl concentrations or low temperatures (Kidou et al., 1993). Later studies in rice showed accumulation of almost the entire 14.3.3 gene family under salinity and cold treatment as well as other stress conditions, including heat, oxidation and heavy metals also effecting expression (Yao et al., 2007).

Further expression studies have shown that two 14.3.3 proteins were induced by low temperatures in Arabidopsis (Jarillo et al., 1994), while Chen et al (2006) showed that rice 14.3.3s were differentially expressed under abiotic conditions with different 14.3.3 isoforms induced by salt, PEG6000 (a drought-mimic) and wounding treatment.

Further to this, transformation studies have suggested roles for 14.3.3s in abiotic stress responses with overexpression of an Arabidopsis 14.3.3 in cotton giving a “stay-green” phenotype, improving stress tolerance under moderate drought conditions (Yan et al., 2004).

A 14.3.3 protein from the arbuscular mycorrhizal fungus Glomus intraradices, was found to be up-regulated by drought stress during AM symbiosis, suggesting that the 14.3.3 gene may be involved in the protection that the AM symbiosis confers to the host plant against drought stress (Porcel et al., 2006).

Lipoxygenases are a class of enzymes that play a key role in lipid metabolism and have been implicated in the biosynthesis of stress-responsive signalling molecules. Barley contains two lipoxygenases, Lox1 and Lox2, and it was demonstrated by Holtman et al (2000) that 14.3.3 proteins interact with Lox2 in barley, again suggesting that 14.3.3 binding is involved in stress signalling.

Biotic stress
One of the most well characterised 14.3.3 interactions is that with the plasma membrane H⁺ATPase where 14.3.3 is known to interact with the C-terminal regulatory domain of H⁺ATPase, leading to its activation. Modulation of plasma membrane H⁺ATPase activity has been reported to be involved in defence, wound and general stress signalling in plants (Finnie et al., 2002).

The 14.3.3-H⁺ATPase interaction is also evidence for a role of 14.3.3s in biotic stress signalling. In plants, the receptor for fusicoccin, a wilt-inducing toxin produced by the fungus Fusicoccum amygdalii, has been shown to be a membrane bound-14.3.3 (Korthout
and de Boer, 1994). It has also been found that the binding site for fusicoccin is the 14.3.3-H$^+$ATPase complex and that fusicoccin binding to this complex causes stabilisation of the 14.3.3-H$^+$ATPase interaction, activation of the H$^+$ATPase and stimulation of the hypersensitive response (Baunsgaard et al., 1998). In barley, resistance to avirulent races of powdery mildew is mediated through single-cell hypersensitive cell death (Roberts, 2003). A role of activation of the H$^+$ATPase in the induction of this HR response has also been suggested (Zhou et al., 2000), as well as a role in barley defence to R. secalis where the necrosis inducing peptides NIP1 and NIP3 are known to stimulate H$^+$ATPase activity in plasma membrane vesicles (Wevelsiep et al., 1993).

A cDNA of barley leaf that increased in amount during infection attempts by the powdery mildew fungus (Erysiphe graminis) was found to encode one of the first 14.3.3 proteins identified (Brandt et al., 1992). A 14.3.3 transcript is also induced during the hypersensitive response of soybean to Pseudomonas syringae (Seehaus and Tenhaken, 1998) and rice 14.3.3 expression was found to be induced during early infection with the rice blast fungus Magnaporthe grisea, with the induction appearing earlier and stronger in incompatible interactions (Chen et al., 2006).

Enzymes with potential roles in defence responses have been identified as 14.3.3 binding proteins, including ascorbate peroxidase, thought to be involved in pathogen-induced scavenging of active oxygen species and caffeate $O$-methyltransferase, involved in the production of antimicrobial secondary metabolites (Zhang et al., 1997a; Zhang et al., 1997b). Investigations by Lapointe et al (2001) into the role of the signal transduction pathway in tree-pathogen interactions identified four complete and two partial 14.3.3s. Treatment of trees with chitosan, jasmonates or by wounding increased the mRNA level of these 14.3.3s. Since jasmonates and chitosan are chemical elicitors of pathogen defence reactions in plants, these results suggest a possible role for 14.3.3 proteins in the pathogen defence response of deciduous trees.

1.5.1.5  Hv14.3.3c

To date five barley 14.3.3 homologues have been identified and characterised, and named Hv14.3.3a (Brandt et al., 1992), Hv14.3.3b (genbank accession no. X93170), Hv14.3.3c (genbank accession no. Y14200), Hv14.3.3d and Hv14.3.3e (Schoonheim et al., 2007a), with this study focusing on Hv14.3.3c.
1.5.2 MAP kinases

A specific class of serine/threonine protein kinases, the mitogen-activated protein kinases (MAP kinases) play a central role in the transduction of many extra- and intra-cellular signals in all living organisms (Mishra et al., 2006). The MAP kinase was first discovered by Sturgill and Ray (1986) as a microtubule associated protein, which they named microtubule associated protein-2 kinase or MAP-2 kinase. Later it was found that this kinase was related to a set of proteins that were phosphorylated at tyrosine residues in response to mitogens, and was therefore renamed mitogen-activated protein kinase or MAP kinase (Mishra et al., 2006). As serine/threonine kinases, they belong to a multigene family and are now known to be a family of 40-45 kDa proteins found in all eukaryotes (Hirt, 1997; Mizoguchi et al., 1994).

1.5.2.1 MAP kinase families and MAPKK’s

In yeast and animals, the MAP kinases comprise of three major groups or subfamilies; the Extracellular signal-Regulated Kinases (ERKs), the c-Jun amino(NH₂)-terminal kinases or stress-activated protein kinases (JNKs/SAPKs) and high osmolarity glycerol responsive or p38 kinases (Hog/p38). The ERK kinases are mainly responsible for the transduction of mitogen signals and are involved in differentiation and cell cycle regulation, while the JNK and p38 groups transduce stress and apoptotic signals. All the plant MAP kinase genes reported so far belong to the ERK subfamily, with no plant homologues of the mammalian JNK or p38 groups having been identified (Mishra et al., 2006). Plants contain a large number of MAP kinases with analysis of the Arabidopsis genome by Ichimura et al (2002) identifying 20 MAPKs, 10 MAPKKs and at least 60 MAPKKKs. Using sequence comparison the 20 Arabidopsis MAPKs as well as other plant MAPKs appear to group into at least four distinct families, with members of each group serving similar functions in different species. The first family includes MAPKs that are mostly involved in environmental and hormonal responses and the second involved in environmental stress response and cell cycle regulation while little is known about the function of the third and forth families. The known plant MAPKKs were also found to group into four families, with members again thought to group based on function. The MAPKKK family is the largest and has the greatest structural and compositional variety and was shown to be divided into two main subfamilies based on structural type and function.
It has been shown that MAP kinase pathway components can function in different combinations and have distinct functions in different biological contexts. The specificity of different MAP kinase cascades functioning within the same cell is generated through the presence of docking domains found in various components of MAP kinase molecules and scaffold proteins (Ichimura et al., 2002). It is also thought that, as the MAPKKK family is the largest, each MAPKKK confers responsiveness to distinct stimuli, providing specificity to the response (Chang and Karin, 2001).

1.5.2.2 MAP kinase signalling cascades

MAP kinases play key roles in integrating multiple intracellular signals transmitted by various second messengers. MAP kinase cascades are composed of three protein kinases, MAP kinases (MAPKs), MAP kinase kinases (MAPKKs) and MAP kinase kinase kinases (MAPKKKs). MAPKs are the last component of the cascade and are activated when both tyrosine and threonine residues in the TXY motif are phosphorylated by MAPKKs (Mizoguchi et al., 1997). These dual-specific MAPKKs can only catalyse the activation of specific MAPKs and cannot substitute for each other (Hirt, 1997). MAPKKs themselves are activated when their serine and serine/threonine resides in the S/TXXXS/T motif, or S/TXXXXXS/T in plants, are phosphorylated by MAPKKKs (Fig 1.4) (Mizoguchi et al., 1997; Zhang et al., 2006) or sometimes by other proteins such as raf and mos proteins (Marshall, 1994).

Upstream, MAP kinase pathways may integrate a variety of receptor signals via activation through physical interaction and/or phosphorylation by receptors themselves, intermediate bridging factors or other protein kinases. Examples of these are protein kinase C and GTPase proteins, both of which couple plasma membrane receptors, involved in sensing extracellular stimuli, to distinct intracellular signalling cascades (Hirt, 1997; Nakagami et al., 2005).

Downstream, at the other end of the pathway, activated MAPKs phosphorylate substrates containing the consensus motif ΨXS/TP, where Ψ is a proline or an aliphatic amino acid (Lalle et al., 2005). They may translocate and act in the nucleus to activate certain sets of genes through phosphorylation of specific transcription factors, or stay in the cytoplasm and act on enzymes (Hirt, 1997; Jonak et al., 1999).

1.5.2.3 Role of MAP kinases in plant stress response signalling

In all eukaryotes, MAP kinase pathways serve as highly conserved central regulators of growth, death, differentiation, proliferation and stress responses (Nakagami et al., 2005).
Fig 1.4: Generic MAP kinase pathway. A stimulus is perceived at the plasma membrane and the signal is transferred by phosphorylation through the cascade. Upon phosphorylation, MAPK is translocated to the nucleus where it phosphorylates a transcription factor which may be part of a complex (TFC or transcriptor factor complex), or repressor molecule, altering expression of the target gene (TG) (Roman et al., 2007).
In plants particularly, the induction of sets of MAP kinase cascades is a common response to abiotic and biotic stimuli (Fig 1.5) (Daxberger et al., 2007).

**Abiotic stress**

In a study by Jonak et al (1996), exclusive activation of a MAP kinase pathway in alfalfa by cold and drought was shown. With use of specific antibodies, differentiating between members of the alfalfa MAP kinase family, it was found that only one specific MAPK was activated. This activation was not a general stress response as no activation occurred under salinity, heat shock or ABA treatment, suggesting specificity of MAP kinases for certain stress signalling. At the same time, investigation of the expression of an Arabidopsis MAPKKK and MAPK showed simultaneous induction in response to touch, cold and salinity (Mizoguchi et al., 1996). Teige et al (2004) also showed cold as well as salt stress activation of a MAPKK from Arabidopsis. Yeast two-hybrid screening showed that this MAPKK directly targeted and activated two MAPKs, different to the MAPK found by Mizoguchi et al (1996), and overexpression of this MAPKK lead to constitutive activation of the two MAPK’s, causing upregulation of stress-induced marker genes and increasing salt and freezing tolerance.

Both a MAPKK and MAPK in rice were also induced by cold stress. These two kinases were found to interact via yeast two-hybrid screening, suggesting they probably function in the same signalling pathway and that this pathway is involved in low temperature stress signalling (Wen et al., 2002).

**Biotic stress**

Plant cells have an acute sense for pathogen-derived chemical stimuli, so-called elicitors or PAMPs, which induce the plants defence response. It was found that drastic changes in protein phosphorylation occurred upon yeast derived elicitor treatment in tomato cells and that the addition of kinase inhibitors completely prevented these elicitor induced changes in phosphorylation (Felix et al., 1991). Ligterink et al (1997) identified an elicitor-responsive MAP kinase in parsley that was activated upon binding of a *Phytophthora* derived elicitor peptide to its receptor, followed by its translocation to the nucleus where it was thought to interact with transcription factors that induce expression of defence genes. In Arabidopsis it was also found that the MAP kinase cascade participates in the defence response to the bacterial elicitor flagellin. A MAPKK was activated in cells treated with the elicitor, and in turn phosphorylated and activated a MAPK (Meszaros et al., 2006). Also in Arabidopsis, a MAPKK is responsible for the negative regulation of two resistance
Fig 1.5: MAP kinase pathway involvement in various stress response pathways in plants. MAP kinase cascades are implicated in the signalling defence responses of many biotic and abiotic stresses. The stress is perceived by receptors, MAPKKKs are activated and in turn activate the MAP kinase cascade. Activated MAPK’s go on to induce defence responses (Zhang and Klessig, 2001).

Abbreviations: CWD, cell wall-derived; HR, hypersensitive response.
genes, RPW8.1 and RPW8.2, that stimulate a conserved basal defence pathway in response to powdery mildew infection (Xiao et al., 2005).

A MAPKK was also shown to be biotic stress responsive by You et al (2007), with the identification of a blast (Magnaporthe grisea) and insect (Nilaparvata lugens) responsive putative MAPKK, named OmMKK1, from wild rice.

Song and Goodman (2002) found the expression of a MAPK in rice to be rapidly induced upon various treatments including Magnaporthe grisea itself, as well as jasmonic acid, P. syringae and wounding, all of which induce the same M. grisea disease resistance response. The expression of a barley MAPK was also identified to be rapidly induced by powdery mildew infection (Eckey et al., 2004). In Nicotiana benthamiana, Takahashi et al (2007) demonstrated that MAP kinase cascades, and namely a MAPKK, control non-host resistance including HR cell death to Pseudomonas cichorii. Work by Romeis et al (1999) showed MAP kinases to be involved in gene-for-gene resistance interactions, where two MAP kinases became rapidly activated in a strictly gene-for-gene manner during the Cf-9/Avr9 interaction that confers resistance to the fungal pathogen Cladosporium fulvum in tomato.

WRKY proteins are a family of plant specific transcription factors that bind to the promoters of various wound and pathogen responsive genes, and this binding is thought to be involved in pathogen inducibility of these genes. Work by Young Kim and Zhang (2004) suggests that a MAP kinase cascade in tobacco is responsible for the activation of WRKY transcription factors which in turn induce plant defence response genes.

In transformation studies, silencing of either a gene encoding a MAPK or a MAPKK in tobacco, attenuated resistance gene mediated defence to the tobacco mosaic virus (Liu et al., 2004). In the interaction between tomato and the bacterial pathogen Pseudomonas syringae, a MAPKKK was found to be involved in regulation of host cell death during infection through its overexpression activating pathogen-independent cell death. Two distinct MAP kinase cascades were also shown to be initiated by activation of this MAPKKK (del Pozo et al., 2004). In the same interaction it was also found that resistance to P. syringae, mediated by the Pto resistance gene, was compromised by the individual silencing of either two MAPKKs, or two MAPKs (Ekengren et al., 2003).

1.5.2.4 HvMAPKK1

Based on the rice and Arabidopsis genome sequences, it is expected that barley contains around 10 MAPKKs. The barley MAPKK in this study was named HvMAPKK1, based on its highest sequence homology to the previously named and characterised OsMAPKK1.
1.5.3 Immunophilins and FKBP’s

Immunophilins are a family of highly conserved proteins, all of which have peptidyl-prolyl cis-trans isomerase (PPIase) activity, regulate folding, assembly and trafficking of substrate proteins and act as molecular chaperones (Park et al., 2007). Based of their affinities for specific immunosuppressive drugs of fungal origin, immunophilins are divided into two families, cyclophilins and FKBPs, with little sequence similarity between the members of the two families. Cyclophilins bind to the immunosuppressive drug Cyclosporine A (CsA), while FK506-binding proteins (FKBPs) bind both FK506 and Rapamycin (Barik, 2006). A third family of immunophilins has also recently been identified that bind both classes of immunosuppressive drugs and were named FK506- and CsA-binding proteins or FCBPs (Adams et al., 2005). Both FKBPs and cyclophilins can be classified into single domain and multiple domain members. The single domain members contain a basic PPIase catalytic domain and some of them have signal sequences for targeting to specific organelles (He et al., 2004). The multiple domain members contain not only the catalytic domain but also defined modules that are involved in protein-protein interactions or other functions, such as tetratricopeptide repeats (TPR), WW (small signal transduction molecules) domains, calmodulin-, DNA- and RNA-binding sites and dimerisation modules (Breiman and Camus, 2002; He et al., 2004).

FKBPs comprise a growing family of highly conserved proteins that are widely distributed from E. coli to mammals, with at least 15 FKBPs of varying molecular weights and intracellular locations, having been identified in humans. FKBPs have also been found in plants such as broad bean, maize, wheat and Arabidopsis. Although FKBP genes have been described in plants, FKBP cDNA clones and proteins have been isolated only recently. Though FKBPs may play a general and important role, the exact biological importance of FKBPs in plants is poorly understood (Barik, 2006; Park et al., 2007).

1.5.3.1 Function of immunophilins

Mammalian immune response

During the mammalian immune response immunosuppressive drugs bind to the catalytic pocket of the PPIase domain and inhibit PPIase activity. Additionally, the cyclophilin-CsA and FKBP-FK506 complexes target a common protein, calcineurin, a Ca$^{2+}$-calmodulin-dependent protein phosphatase, involved in numerous signalling processes, inhibiting its phosphatase activity which results in elevated phosphorylation of a number of its substrates. One substrate is the nuclear factor of activated T-cells (NF-AT).
Phosphorylated NF-AT fails to translocate to the nucleus and as a consequence immunologically important genes in the T-cell remain silent, suppressing the immune response.

These drug interaction functions however, only have a clinical relevance and not a physiological one, as healthy mammalian cells never naturally encounter these immunosuppressive drugs, hence the exact biological function of immunophilins remains unclear (Barik, 2006).

**PPIase activity**

To become fully functional and active, newly synthesised proteins must be converted from their primary linear sequence into their well-defined native tertiary structure (Romano et al., 2005). The planar peptide bond in proteins is predominantly in the *trans* conformation. Proline (Pro) residues, however, are unique in that they have a relatively high intrinsic probability of being in the *cis* conformation. The *trans-cis* isomerisation of the peptide bonds on the N-terminal side of Pro is thus essential for proper protein folding. This isomerisation is a rate limiting process that is accelerated by enzymes with PPIase activity, such as the immunophilins. Thus, cyclophilins and FKBP\(\)s have been shown to influence the folding of a number of proteins including collagen, chymotrypsin inhibitor, carbonic anhydrase and ribonuclease (Barik, 2006).

**1.5.3.2 Plant FKBP\(\)s**

The discovery of the first immunophilins in plants did not occur until the early 1990’s, when the first cyclophilin-like cDNA was identified in tomato and maize (Gasser et al., 1990). Since then numerous immunophilins have been identified in plants, with sequencing of the Arabidopsis genome identifying one of the largest families to date, consisting of 29 cyclophilin and 23 FKBP isoforms. The FKBP\(\)s have been found to be localised in the cytosol, nucleus, ER and mitochondria, as well as the chloroplast where the majority are found (Romano et al., 2005). The large number of genes and diversity of structure domains and cellular localisation make immunophilins a versatile superfamily of proteins that clearly function in many cellular processes in plants (He et al., 2004).

In plants, FKBP\(\)s are known to play a role in development. The disruption of an Arabidopsis FKBP leads to pronounced alterations in development, with a defect in one FKBP gene causing the ‘twisted dwarf’ syndrome where all organs are dramatically shorter and the plant displays twisted growth (Kamphausen et al., 2002). However, roles of FKBP\(\)s
in many other cellular processes such as protein folding, trafficking and transcription as well as signal transduction have also been suggested (Harrar et al., 2001).

1.5.3.3 **Role of FKBP type immunophilins in signalling**

The ubiquitous protein chaperone Hsp90 has been shown to regulate more than 100 proteins involved in cellular signalling. In the mammalian system, it is known that FKBP5s bind to the Hsp90 heterocomplex via their conserved TPR domain (Cheung-Flynn et al., 2003). This interaction has been found to also occur in plants, and it has been suggested that the Hsp90-FKBP machinery might be used to target the trafficking of signalling proteins in plants (Owens-Grillo et al., 1996; Pratt et al., 2001).

As well as the cyclophilin-CsA and FKBP-FK506 complexes targeting calcineurin, a Ca^{2+}-calmodulin-dependent protein phosphatase, during the suppression of the immune response in mammals, has also been found to block the Ca^{2+}-dependent signalling pathway in plants. Ca^{2+} is an important second messenger in response to many extracellular stimuli, however the molecular mechanism for its function is poorly understood. This finding suggests a possible role for immunophilins in this signalling pathway (Luan, 1998).

1.5.3.4 **Role of FKBP type immunophilins in plant stress response signalling**

The discovery that binding of the immunosuppressive drugs Cyclosporine and FK-506 to immunophilins selectively blocks transcriptional activation of defence-related genes early in the mammalian T-cell signal transduction pathway (Schreiber and Crabtree, 1992), has placed them as players in defence signalling in animal systems. However their potential role in plant stress response signalling is only beginning to come to light.

It has been suggested that the transcriptional response, or rather expression of immunophilins, may be part of a general requirement for protein folding following synthesis of stress-responsive proteins (Romano 2005).

**Abiotic**

*Shewanella* is a type of bacterium that can grow at very low temperatures, but requires systems to facilitate protein folding, as folding reactions are generally slow at low temperatures. A protein that increased in amount when the bacterium was grown at a low temperature was found to be an FKBP type immunophilin. FKBP may be involved in acceleration of the peptidyl-prolyl isomerisation of proteins at these low temperatures, suggesting a possible role in cold-adaptation (Suzuki et al., 2004).
Wheat root tips express a heat-shock induced FKBP that is part of a chaperone complex with Hsp90. This FKBP is initially localised in the chloroplast but upon heat-shock it is transported to the nucleus where it accumulates, suggesting a role in heat-shock defence (Dwivedi et al., 2003; Kurek et al., 1999). A similar result was found for a broad bean (Vicia faba) FKBP, where the mRNA level of the FKBP was also found to be regulated by heat shock (Luan et al., 1996) as well as for rice, where three large FKBPs were found to be heat stress responsive (Magiri et al., 2006).

Yeast two-hybrid analysis showed that OsSce1 (SUMO-conjugating enzyme 1) protein, involved in temperature stress, physically interacts with the OsFKBP20 protein. OsFKBP20 was identified as stress-inducible EST clone and was found to be heat inducible and lead to high temperature tolerance when overexpressed in yeast cells. It is proposed by Nigam et al (2008) that OsSce1 and OsFKBP20 proteins in concert mediate the stress response of rice plants.

**Biotic**

An antifungal protein isolated from Chinese cabbage and assayed using inhibition tests against B. cinerea and R. solani, was found to be an FKBP protein (Park et al., 2007). There is also evidence for the cyclophilin class of immunophilins being involved in biotic stress responses, with a potato cyclophilin accumulating in the tubers after wounding and fungal infection by Fusarium solani (Godoy et al., 2000).

A bean cyclophilin was also shown to accumulate due to infection by the alfalfa mosaic virus (Marivet et al., 1994), while another cyclophilin was drastically induced by infection of a pepper plant by the bacteria Xanthomonas campestris and Colletotrichum gloeosporioides in incompatible interactions (Kong et al., 2001).

Transformation of carrot plants with microbial factor 3 (MF3), an FKBP from the rhizobacteria Pseudomonas fluorescence, resulted in increased resistance to three fungal pathogens Alternaria dauci, Alternaria radicina and Botrytis cinerea (Baranski et al., 2007).

1.5.3.5 **HvFKBP41**

FKBP genes have been designated a standard nomenclature based on their species of origin and translated protein size. The barley FKBP under investigation has a predicted molecular weight of 41,000 or protein size of 41 kDa and has therefore been named HvFKBP41.
1.5.4 Interaction of candidate genes

In addition to the above evidence for a possible role of the candidate genes *Hv14.3.3c*, *HvMAPKK1* an *HvFKBP41* in plant defence signalling, there is evidence to suggest that the candidate genes themselves may interact with each other or play roles in the same signalling pathways.

For example, 14.3.3s are known to be involved with kinases and MAP kinase signalling cascades, with the first evidence of the interaction of 14.3.3s and plant MAP kinases found by Lalle et al (2005), showing that a MAPK in maize interacts with a 14.3.3 protein. Fanger et al (1998) demonstrated that two MEKKs interact with 14.3.3 proteins, implicating 14.3.3 proteins in the control of MEKK proteins through acting as “scaffold-like” proteins, as the association does not directly influence kinase activity. Furthermore, MEKK3, involved in regulation of transcription factors that mediate pro-inflammatory signals involved in innate immunity, interacts with a 14.3.3 and this interaction is potentially an important regulatory step in MEKK3 signalling (Matituau and Scheid, 2008). An FKBP-rapamycin complex is a high affinity inhibitor of a protein termed FKBP12-rapamycin-associated protein or FRAP, a kinase, though to be involved in the translational control of a growth factor-regulated signalling pathway. A study by Mori et al (2000) showed that a 14.3.3 interacts with FRAP in T-cells, suggesting 14.3.3s may also play a role in the immunophilin-linked immune response. Finally, a study by Ekey et al (2004) to identify genes differentially regulated after infection of barley by powdery mildew discovered that both a MAPK and an FKBP-type immunophilin were differentially expressed, suggesting not only a co-ordinated and therefore possibly interacting role of the two genes, but also a potential role in the pathogen defence response.

This evidence suggest that the candidate genes may all play a role in the same or similar defence signalling pathways. As there is evidence for a role of these genes in response to a range of different stresses both biotic and abiotic, it suggests that plant stress signalling pathways may be highly common and overlapping, supporting the hypothesis that investigation of these genes and the signalling networks they are involved in may lead to the identification of genes that can be manipulated to provide a more durable and broader resistance to stresses, such as *R. secalis* infection, and possibly other biotic and abiotic stresses.
1.6 Project aims

In this project the function of three genes, which have been identified as being differentially expressed in compatible versus incompatible interactions of barley with *R. secalis*, will be investigated, with the aim of determining their role in the *R. secalis*/barley pathogen/host interaction, and possible involvement in signalling pathways. Protein-protein interactions studies, namely yeast two-hybrid screening and affinity pulldowns, will be used to identify and expand on the signalling networks they may be involved in. The specificity of the gene function for this biotic stress will be tested by analysing gene expression in comparison to abiotic stresses, including salinity, drought, frost and ABA treatment.
Chapter 2

Isolation of Full Length cDNAs and Expression Analysis
2.1 Introduction

The main focus of this study was to characterise and assign possible functions in defence response signalling to three candidate barley genes, encoding a putative 14.3.3c, a MAPKK and a FKBP-type peptidyl prolyl cis-trans isomerase. This required the use of full length gene sequences to facilitate the sequence analysis and quantitative real-time PCR analysis of transcript levels described in this chapter, as well as the protein expression and interactions studies described in later chapters. However, as discussed in Chapter 1, the candidate genes were first identified using suppressive subtractive hybridisation, which generates only short, mostly incomplete, cDNA fragments. Therefore, to obtain full length sequences for each gene, RACE and genomic walking techniques were employed.

Rapid Amplification of cDNA Ends (RACE) and genomic walking are polymerase chain reaction-based techniques, which facilitate the cloning of full-length DNA sequences when only a partial sequence is available. Starting points are cDNA libraries made from mRNA in the case of RACE, or digested genomic DNA fragments in genomic walking, to which adaptor tags are ligated to the ends. Priming from the known adaptor sequence can then be achieved, allowing for the regions between the known parts of the sequence and the ends to be amplified (Frohman et al., 1988; Rosenthal et al., 1990).

One way in which the sequence data obtained from this identification of full length gene sequences was then used was in sequence analysis, to compare the barley genes with characterised homologues from other plant species to confirm their identity. Homology searching can be carried out in several ways, with pairwise or multiple sequence alignments, being one of the most common. The most widely used pairwise sequence alignment tool is the Basic Local Alignment Search Tool or BLAST. It is one of the fastest tools available and gives an expectation value for an alignment, which estimates how many times one expects to see such an alignment occur by chance (Altschul et al., 1990).

Sequence similarity is widely used as a means of assigning identity, and often function, to a gene. For example, around 70% of the Arabidopsis genes have been annotated based on sequence similarity to those with known functions in other organisms, while only 9% of genes have been characterised experimentally (Donson et al., 2002). Sequence similarity, however, does not always indicate a biological relationship (homology), or conservation of gene function (orthology) (Xu et al., 2000).

Other methods for assigning identity and/or function are based on the properties of the amino acids in the query sequence. The properties used include hydrophobicity profile,
prediction of transmembrane regions, active sites, and signalling sites, as well as prediction of secondary structure and solvent accessibility (Xu et al., 2000).

To confirm and expand on the differential expression first seen in the microarray analysis described in Chapter 1, indicating a potential role in the defence response, the sequence data was used to design primers, which allowed for the transcriptional activities of the candidate genes to be investigated through analysis of their mRNA abundance and its possible changes in response to stress, via quantitative real-time PCR.

Information on both the physical and functional annotation of a gene can be gained through gene expression analysis (Donson et al., 2002). Under standard or non-stressed conditions, genes are expressed at a basal level. However, under stressed conditions, the expression of genes involved in cellular processes or pathways affected by a stress, such as genes involved in stress response signalling, is altered in response to that stress. Therefore, changes in mRNA levels of genes in response to different stimuli can be used to gain insight into a possible function or role of a gene.

A major drawback of mRNA expression analysis is that mRNAs are not functional entities themselves, but rather are transmitters of information from the genome to the proteome where their function is enacted (Steinmetz and Deutschbauer, 2002). The final activity of the gene product depends on a number of other factors, such as the rate of mRNA translation, mRNA degradation, protein folding and post-translational modifications, protein targeting to the correct cellular compartment, and protein activation. Functional or genetic redundancy can also occur, as well as involvement in multiple processes, other than just stress tolerance or resistance for example (Donson et al., 2002; Steinmetz and Deutschbauer, 2002).

Nevertheless, it is generally accepted, that while post transcriptional events play a role in modulating gene expression, the primary level of control is at transcription (Donson et al., 2002), and therefore transcript profiling is widely used as a first step to investigate the potential role of genes in cellular processes.

Northern blots, in situ hybridisations, reverse transcription polymerase chain reaction (RT-PCR), oligo-arrays, and quantitative real-time RT-PCR (Q-PCR), are all commonly used transcript analysis methods (Alwine et al., 1977; Jin and Lloyd, 1997; Mocharla et al., 1990; Heid et al., 1996).

Disadvantages of northern blot analysis and in situ hybridisation, however, include low sensitivity, labour intensiveness and low-throughput capacity (Bustin, 2000). Oligo-arrays are a more high-throughput method, but are expensive, requiring sophisticated instruments
to manufacture the oligo-array and specialised software for interpretation (Ding and Cantor, 2003). RT-PCR is a relatively quick and sensitive technique, however it is, at best, only semi-quantitative with the amount of product not always related to the amount of input DNA (Freeman et al., 1999).

Q-PCR is currently the method of choice for accurate gene expression analysis, due to its speed, sensitivity, specificity, and most importantly, quantification ability (Nolan et al., 2006). Q-PCR differs from classical PCR by the measurement of the amplified PCR product at each cycle throughout the PCR reaction, in real time, allowing for the quantification of transcript level (Gachon et al., 2004).

Several approaches have been employed to detect the amplified products during Q-PCR. Probe-based Q-PCR, also known as TaqMan (Holland et al., 1991), requires a pair of PCR primers like those used in regular PCR, as well as an additional fluorogenic probe which is an oligonucleotide with both a reporter fluorescent dye and a quencher dye attached. A second method, and the method used in this study, is an intercalator-based method, also known as the SYBR Green method (Bioprobes, 1993), which uses double-stranded DNA dye in the PCR reaction which binds to newly synthesised double-stranded DNA and gives fluorescence.

The SYBR Green method has advantages including being inexpensive, easy to use and sensitive. A major disadvantage, however, is that SYBR Green will bind to any double-stranded DNA in the reaction, including primer-dimers and other non-specific reaction products, which can result in an overestimation of the target concentration (Kim, 2001; Matsenko et al., 2008; Asselbergs and Widmer, 2003).

As Q-PCR relies on measuring the relative mRNA abundance of genes of interest, a key requirement for the accuracy of expression profiling via Q-PCR is normalisation (Vandesompele et al., 2002). Normalisation is achieved by measuring the mRNA levels of ‘standard’ or ‘housekeeping’ genes, genes for which the transcriptional activity does not change substantially under the conditions being investigated.

The mRNA levels of these genes can then be used to calculate reliable normalisation factors, to ensure that any changes in expression levels seen in the genes of interest are not due to factors such as differences in amounts of cDNA, and therefore starting amount of the gene of interest, in each sample (Burton et al., 2004).

In this chapter, the use of RACE and genomic walking techniques to determine the full length coding sequences of Hv14.3.3c, HvMAPKK1 and HvFKBP41, as well as transcript profiling of the genes using Q-PCR, is described.
2.2 Materials and methods

2.2.1 Materials

All chemicals used were analytical or molecular biology grade and solutions were prepared with nanopure water and autoclaved where appropriate.

SuperScript III RT kit, Big-Dye version 3-1 reagents, Elongase and HiFi reagents were purchased from Invitrogen (Waverley, VIC, Australia). Sodium acetate, ethanol, KCl, MgCl₂, and MgSO₄ were from Crown Scientific (Wingfield, SA, Australia). Formaldehyde and NaOH were from Genesearch (Arundel, QLD, Australia). Custom oligonucleotides, ampicillin, ethidium bromide, ficoll, bromophenol blue, glycerol, DTT, EDTA, Trizma-base, MOPS, NaCl, ammonium thiocyanate, guanidine thiocyanate, DMSO, phenol, PIPES, MnCl₂, CaCl₂ and glucose were from Sigma-Aldrich (including Sigma-Proligo, Castle Hill, NSW, Australia). Restriction enzymes, T4 DNA ligase, shrimp alkaline phosphatase and dephosphorylation buffer were purchased from Roche (Castle Hill, NSW, Australia). Taq polymerase reagents, QIAquick Gel extraction Kit, QIAquick PCR Purification Kit and QIAquick Spin Miniprep Kit were from QIAGEN (Doncaster, VIC, Australia). Agar, agarose, bacto-tryptone and bacto-yeast extract were from Becton-Dickinson (North Ryde, NSW, Australia). The SMART PCR RACE kit and Universal Genomic Walker Kit were purchased from Scientifix (Clayton, VIC, Australia). The First Choice RLM-RACE kit, RNAzap, deionised formamide and DNA-free kit were from Applied Biosystems (Scoresby, VIC, Australia). The dNTPS and pGEM T-Easy Kits were from Promega (Annandale, NSW, Australia) and molecular weight markers from Bioline (Alexandria, NSW, Australia). Gene Pulser cuvettes were from BioRad (Regent Park, NSW, Australia) and acetic acid from Merck (Granville, NSW, Australia)

2.2.2 Plant material

*Hordeum vulgare* plant material was obtained from a seed collection maintained at the Waite campus, University of Adelaide, Australia. Primary leaves (from 7-9 day old seedlings) of the near-isogenic cultivars Atlas and Atlas46 (Riddle and Briggs, 1950) were used for inoculation.

2.2.3 Fungal material

Spore suspensions of various *R. secalis* races were a kind gift from Dr Hugh Wallwork (SARDI, SA, Australia). Fungal material was cultured on lima bean agar plates [20% (v/v) lima bean solution, 2% (w/v) Bacto Agar]. Lima bean solution was prepared by
autoclaving 25 g lima beans in 300 ml H$_2$O. Directly after autoclaving the mixture was shaken, left to settle and the resultant supernatant filtered through muslin cloth before use.

2.2.4 Inoculation

2.2.4.1 *R. secalis*
Plants were inoculated as described by Steiner-Lange et al (2003) except that 5 x 10$^5$ instead of 1 x 10$^6$ spores/ml inoculum were used. Inoculation took place in a growth chamber under controlled conditions of 18°C with a 16 h day length. Immediately after inoculation, plants were kept in the dark for 24 h with humidity of 100%. After this, plants were maintained at standard growth conditions of 14 h day length at 18°C with a humidity of 70-80%.

2.2.4.2 *NIP1*
NIP1 inoculation was conducted similarly to that of fungal infection, as described by Steiner-Lange et al (2003). Briefly, the abaxial side of the leaf was treated with 1 droplet of recombinant NIP1 protein, 20 ng/µl, containing 0.5% tween-20. Growth conditions were the same except that treated plants were kept in a sealed container for 24 h after treatment.

2.2.5 Oligodeoxyribonucleotides
Primers were designed with the aid of Primer3 (Rozen and Skaletsky, 2000) in the BioManager suite of programmes at the Australian National Genetic Information Service (ANGIS), or VectorNTI (Invitrogen) software and further analysed using Oligocalculator (http://www.pitt.edu/~rsup/OligoCalc.html).
Primers were obtained from Sigma-Proligo. See Appendix 1 for a list of primers used.

2.2.6 Polymerase chain reaction (PCR)

2.2.6.1 Standard – *Taq* polymerase
Polymerase chain reaction (PCR) was performed in 15 µl, 25 µl or 50 µl reaction volumes containing ~50 ng cDNA, 1 x PCR Reaction Buffer [10 mM Tris-HCl buffer, containing 50 mM KCl, 1.5 mM MgCl$_2$, pH 8.7], 1 x Q-solution, 200 µM each dNTP, 200 nM each primer and 1U *Taq* polymerase.
The standard PCR thermal cycling program was: denaturing at 94°C for 2 min, 30-40 cycles of denaturing at 94°C for 30 sec, annealing at primer Tm (typically 55-62°C) for 30 sec and extension at 72°C (30 sec per 500 bp), followed by a final extension at 72°C for 5 min and holding at 15°C.
2.2.6.2 **Elongase PCR**

When PCR products larger than 2000 bp were to be amplified, the Elongase enzyme mix was used. Reactions contained ~50 ng cDNA, 1 x Elongase buffer A [30 mM Tris-SO₄ buffer (pH 9.1), containing 9 mM (NH₄)₂SO₄, 0.5 mM MgSO₄], 1 x Elongase buffer B [30 mM Tris-SO₄ buffer (pH 9.1), containing 9 mM (NH₄)₂SO₄, 1 mM MgSO₄], 200 µM each dNTP, 200 nM each primer and 1U Elongase enzyme mix. Thermocycling parameters were the same as that described above in section 2.2.6.1, except that extension was performed at 68°C.

2.2.6.3 **HiFi PCR**

For increased accuracy of the PCR, in terms of mis-incorporation, High Fidelity enzyme mix with proofreading ability was used. Reactions contained ~50 ng cDNA, 1 x High Fidelity buffer [60 mM Tris-SO₄ buffer (pH 8.9), containing 18 mM (NH₄)₂SO₄], 2 mM MgSO₄, 200 µM each dNTP, 200 nM each primer and 1U Platinum Taq High Fidelity enzyme mix. The thermal cycling program was the same as that used for Elongase.

2.2.6.4 **Nested PCR**

To increase specificity and the amount of amplified product obtained, nested PCR was employed. Nested PCR reactions, of any of the types of PCR above, were performed as described above, except that two rounds of PCR were performed. After the first PCR reaction (Round 1), the reaction was diluted 1:30 with sterile H₂O and used as template for the second reaction (Round 2) using a second nested pair of primers positioned internally to the first set of primers. Conditions for this reaction were otherwise the same as that used for Round 1.

2.2.6.5 **Semi-nested PCR**

Semi-Nested PCR was performed the same way as nested PCR, except that in Round 2 only one of the primers was nested while the other was the same as that used for Round 1 PCR.

2.2.6.6 **Colony PCR**

Colony PCR was performed as in section 2.2.6.1 except that a match head amount of bacterial cells from a solid colony growing on an agar plate were added to the PCR reaction in place of template DNA, and the initial denaturing step at 94°C was extended to 10 min to facilitate release of the DNA from the bacterial cells.
2.2.7 Agarose gel electrophoresis

PCR products and other nucleic acid fragments were visualised on ethidium bromide-stained agarose gels of 0.8 – 3% (w/v), prepared with 1 x TAE buffer [40 mM Tris-HCl buffer, containing 1 mM EDTA, pH 8 with acetic acid] and the addition of 3 µg/ml ethidium bromide. Samples were prepared with 1 x Ficoll loading dye [15% (w/v) Ficoll 4000, 0.25% (w/v) bromophenol blue, 0.25% w/v xylene cyanol FF] and electrophoresed at approximately 100 volts for 1 h in 1 x TAE buffer. Gels were visualised and photographed using a short wavelength UV transilluminator. DNA fragment sizes were estimated by comparison to fragments present in a molecular weight marker mix (Hyperladder I and II, Bioline, Australia).

2.2.8 DNA and RNA quantification

The concentration of DNA and RNA was determined spectrophotometrically (NanoDrop ND-1000 spectrophotometer) at 260 nm.

2.2.9 Cloning of PCR Products

2.2.9.1 DNA purification - Gel extraction and PCR purification

The QIAquick Gel extraction Kit was used to purify fragments from agarose gel slices, according to the manufacturer’s protocol. The QIAquick PCR Purification Kit was used to purify PCR products from the PCR reaction mix, according to the manufacturer’s instructions.

2.2.9.2 Restriction endonuclease digestion of DNA

DNA was digested in 20 µl or 50 µl reaction volumes containing 1 – 10 ug DNA, 1U each restriction enzyme and 1 x reaction buffer (supplied by the manufacturer as 10 x). Reactions were incubated for 1 – 3 h at 37°C, unless otherwise specified.

2.2.9.3 A-overhang addition

PCR products produced from Elongase or High Fidelity reactions can be not properly adenylated and therefore can not be ligated straight into cloning vectors containing a 3’ T overhang such as pGEM T-easy (section 2.2.9.4), without the addition of an A-overhang first. To do this, 200 µM dATP, 1 U standard Taq polymerase and 1 x polymerase buffer were added to the purified PCR product and the reaction was incubated at 72°C for 20 min before ligation.
2.2.9.4 Ligation

PCR products were routinely ligated into the cloning vector pGEM T-easy using the pGEM-T Easy kit. Reactions of 5 µl contained 0.5 µl pGEM T-easy vector, 2.5 µl 10x rapid ligation buffer, 1 U T4 DNA ligase and 1 – 3.5 µl PCR product (to give an approximate vector:insert ratio of 1:3. Reactions were incubated for 1h at room temperature or 4°C overnight.

2.2.9.5 Transformation of E. coli by heat shock

To prepare competent heat shock cells, the E. coli strain DH5α was grown at room temperature for 16 h with shaking at ~260 rpm in two flasks, each containing 250 ml SOB media [2% (w/v) Bacto-tryptone, 0.5% (w/v) Bacto-yeast extract, 0.1 M NaCl, 2.5 M KCl, pH 7.0]. A further 250 ml SOB containing additional ingredients [10 mM MgCl2, 10 mM MgSO4, 0.035% (v/v) glucose] was added to each flask and the solution divided in two (giving 4 flasks of 250 ml) before growing for a further 2 h. Cells were then chilled on ice for 10 min before centrifugation at 4000 g for 10 min at 4°C. The pelleted cells were resuspended in 2-3 ml ice cold filter sterilised TB buffer [0.15% (w/v) PIPES, 1.08% (w/v) MnCl2, 0.22% (w/v) CaCl2, 1.86% (w/v) KCl, pH 7.4], pooled together and a further 80 ml TB buffer added. Centrifugation was repeated and the cells resuspended in a final volume of 20 ml TB buffer containing 7% DMSO (adding the DMSO slowly with stirring for even dispersion). The cells were incubated on ice for 10 min and aliquots of 100 µl were snap frozen in liquid nitrogen and stored at -80°C until required.

For transformation, the chemically competent cells were thawed on ice for 10 min before the ligation reaction (section 2.2.9.4) or 1 µl of 1:30 dilution of plasmid DNA preparation (section 2.2.9.8) to be transformed, was added to 100 µl of cells. The mixture was left on ice for 30 min before heat shock treatment for 30 sec at 42°C. After heat shock the cells were chilled on ice for 1 min, 600 µl SOB added, and the mixture incubated for 1 h with shaking at ~260 rpm at 37°C. Cells were then plated on LB agar plates [1% (w/v) NaCl, 1% (w/v) tryptone, 0.5% w/v yeast extract, pH 7.0 with 1.5% (w/v) agar] containing the appropriate selective media, routinely 100 µg/ml ampicillin, and grown at 37°C for 16 h. Single colonies were picked at random, replica plated and grown in 5 ml LB media, supplemented with the appropriate selection, overnight at 37°C.

2.2.9.6 Transformation of E. coli by electroporation

To prepare electrocompetent cells, the E. coli strain DH5α was grown in 500 ml LB media (1% w/v NaCl, 1% w/v tryptone, 0.5% w/v yeast extract, pH 7.0) at 37°C with shaking at
~260 rpm until the OD$_{600}$ reached 0.5 – 0.6. The cells were then chilled in an ice bath for 10 min before being pelleted by centrifugation at 3000 g for 20 min at 2°C. Cells were washed by resuspending in 500 ml ice-cold Milli-Q water, pelleted as before, and the wash and centrifugation steps repeated. The cells were resuspended in 10 ml ice cold Milli-Q water, centrifugation repeated and the cells resuspended in 20 ml ice cold 10% (v/v) glycerol. Centrifugation was repeated and the cells were resuspended in a final volume of 4 ml ice cold 10% (v/v) glycerol. Aliquots of 100 µl were snap-frozen in liquid nitrogen and stored at -80°C until required.

Prior to transformation, Gene-Pulser electroporation cuvettes (1.0 mm) were placed on ice and the electrocompetent cells thawed on ice for 10 min. An aliquot of 0.5 – 2 µl of the ligation reaction (section 2.2.9.4) or 1 µl of 1/30 dilution of plasmid DNA preparation (section 2.2.9.8) was added to 40 – 100 µl electrocompetent cells and the mix immediately transferred to the chilled cuvette and placed in the Gene Pulser. Electroporation parameters were 1.8 kV, 25 µF and 200Ω. After transformation, 1 ml SOB was immediately added to the cells, the mixture transferred to an Eppendorf tube and incubated at 37°C for 1 h. Cells were plated on LB agar plates with selection as above.

2.2.9.7 Glycerol stocks

For long term storage of specific clones, 600 µl of cultured cells, grown o/n at 37°C in LB media containing appropriate selection was mixed with 400 µl 50% (v/v) glycerol and the mixture snap frozen in liquid nitrogen and stored at -80°C.

2.2.9.8 Plasmid DNA mini-preparation

Plasmid DNA was prepared from cultures of transformed colonies using the QIAquick Spin Miniprep Kit according to manufacturer’s instructions.

2.2.10 DNA sequencing

Sequencing reactions were performed using Big-Dye version 3.1 reagents. The 10 µl reaction volumes contained 1x Big-Dye buffer, 1 µl Big-Dye, 1 µM primer and 50 – 100 ng plasmid DNA. PCR thermocycling conditions were as follows; 96°C for 30 sec, 25 cycles of 96°C for 10 sec, 50°C for 5 sec and 60°C for 2 min and holding at 15°C.

When the nucleotide sequence analysis was being performed by the Institute of Veterinary Sciences (IMVS) Molecular Pathology, South Australia, DNA was precipitated by the addition of 80 µl 75% isopropanol to the PCR reaction, and incubating for 20 min at RT. The DNA was then pelleted by centrifugation at 16,000 g for 15 min, washed once with
250 µl 75% ethanol, dried in a 37°C heating block, and submitted for sequencing via capillary separation. When analysis was performed by the Australian Genome Research Facility (AGRF), Queensland, DNA was precipitated by combining with 75 µl of freshly prepared ethanol/MgSO$_4$ solution \([70\% \text{ (v/v)} \text{ ethanol}, 0.2 \text{ mM MgSO}_4]\), and incubating for 15 min at RT. DNA was pelleted by centrifugation at 16,000 g for 15 min, dried and submitted for analysis via capillary separation.

### 2.2.11 Bioinformatics

Database searches were performed using the BLAST program (Altschul et al., 1990) of the National Centre for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST). Nucleotide sequences were analysed using the ContigExpress and VectorNTi software programmes (Invitrogen). Protein size predictions were made using the ExPASy ProtParam tool (http://us.expasy.org/tools/protparam.html).

### 2.2.12 3’ RACE

Amplification of the 3’ end regions was achieved using the SMART PCR RACE kit. A cDNA population containing Poly A tails was prepared according to manufacturer’s protocol by Dr K. Oldach (ACPFG, University of Adelaide, Australia), from NIP1 treated Atlas46 epidermal Poly A+ enriched RNA. Semi-nested PCR (section 2.2.6.5) was performed using 0.5 µl of the SMART RACE cDNA, a gene specific forward primer and the 3’ RACE Oligo dT reverse primer (Appendix 1) in the Round 1 PCR. A 1:30 dilution of the Round 1 PCR reaction was used as a template in the Round 2 PCR, along with a second nested gene specific forward primer and the same 3’ RACE reverse primer.

### 2.2.13 5’ RACE

#### 2.2.13.1 SMART RACE

The SMART PCR RACE kit was also used for 5’ RACE. A cDNA population containing the 5’ BD SMART II oligonucleotide binding site was prepared according to manufacturer’s instructions by Dr K. Oldach (ACPFG, University of Adelaide, Australia), from the same RNA as used in 3’ RACE. Nested Elongase PCR reactions were performed using 0.5 µl of the SMART RACE cDNA, two nested gene specific reverse primers, and the UPL+UPS and NUP adaptor primers (Appendix 1).
2.2.13.2 RLM-RACE

An alternative 5' RACE technique using the First Choice RLM-RACE kit was also employed. cDNA was produced by Dr K. Oldach (ACPFG, University of Adelaide, Australia) according to manufacturer’s instruction, from *R. secalis* infected Atlas46 leaf RNA. The cDNA, 0.5 µl, was used in conjunction with gene specific primers and RLM-5’outer and RLM-5’inner adaptor primers (Appendix 1) in a nested Elongase PCR reaction.

2.2.14 Genomic walking

Genomic walking was performed using the Universal Genomic Walker Kit according to manufacturer’s instruction. Briefly, four genomic walking libraries were produced by Dr K. Oldach (ACPFG, University of Adelaide, Australia). Atlas46 genomic DNA was digested with the restriction enzymes *EcoRV* (Library 1), *DraI* (Library 2), *PvuII* (Library 3 and *SspI* (Library 4) and the genomic walking adaptors were then ligated on to the ends of the digested DNA fragments according to manufactures protocol. Adaptor nested primers AP1 and AP2 (Appendix 1) were then used in conjunction with two nested gene specific primers in PCR reactions as previously described.

2.2.15 RNA extraction

With the exception of purchased reagents or unless otherwise indicated, all solutions used for RNA extractions were prepared using autoclaved nanopure H₂O. All non-plastic material required for RNA preparation (mortars, pestle, glassware, spatulas etc) were wrapped in aluminium foil and sterilised by baking at 180°C overnight. Autoclavable plasticware was sterilised by autoclaving while other plastic material (eg electrophoresis gel tanks) was soaked in 0.4 M NaOH for 15 min or sprayed with RNAzap, then rinsed 3 times with autoclaved nanopure H₂O.

Frozen plant tissue samples were ground to a powder in liquid nitrogen using either a sterile mortar and pestle or by using ball-bearings (one of 8 mm diameter and three of 4 mm diameter) and vortexing or shaking the sample in a 10 ml tube. Ground tissue (approx. 100 mg) was mixed with 1 ml Trizol-like reagent [38% (v/v) phenol (pH 4.5), containing 1 M guanidine thiocyanate, 1 M ammonium thiocynate, 5% glycerol, 0.1 M sodium acetate, pH 5] and homogenised by vigorous shaking. The solution was then incubated on a slow rotary wheel at room temperature for 5-8 min. Tissue debris was pelleted by centrifugation at 12,000 g for 10 min a 4°C and the supernatant transferred to a new tube. Chloroform, 200 µl, was then added to the supernatant and the solution mixed by inversion for 30 sec
and incubated at room temperature for 3 min. The solution was centrifuged at 12,000 g for 15 min at 4°C and the upper aqueous phase transferred to a new tube. To this, 500 µl isopropanol was added, the solution mixed by gentle inversion and incubated at room temperature for 10 min. The RNA was then pelleted by centrifugation at 12,000 g for 10 min at 4°C and the supernatant discarded. The RNA pellet was washed once with 1 ml 75% ethanol, leaving the ethanol for 5 min at room temperature before aspirating and leaving the pellet to air dry. The RNA was resuspended in 20-50 µl sterile H$_2$O, and stored at -80°C.

The quality of the RNA isolated, in terms of amount and whether it was degraded, was assessed using agarose gel electrophoresis. To do this, 3 µg RNA (quantified as in section 2.2.8) was added to 15 µl RNA loading buffer containing 12% (v/v) 10 x MOPS [200 mM MOPS, 80 mM sodium acetate, 10 mM EDTA, pH 7.2], 21% (v/v) formaldehyde (37%), 60% (v/v) deionised formamide, 100 µg/ml ethidium bromide and 0.04% (w/v) bromophenol blue. The mixture was heat denatured at 65°C for 10 min and snap cooled on ice for 2 min. Samples were loaded onto agarose gels comprising 75% (v/v) sterile H$_2$O, 10% (v/v) 10 x MOPS, 15% (v/v) formaldehyde (37%) and 1.4% (w/v) agarose. Samples were run at 80 V for ~2 h in 1 x MOPS in electrophoresis tanks which had been pre-treated with RNaseZap.

2.2.16 cDNA synthesis

2.2.16.1 DNase treatment

RNA was DNase treated to remove any DNA contamination using the DNA-free kit. In a 50µl reaction, 1 x DNase I buffer and 2U rDNase was added to the RNA and the reaction incubated at 37°C for 30 min. After incubation, 5µl of DNase Inactivation Reagent and the reaction incubated for a further 2 min at room temperature with occasional mixing. The sample was centrifuged at 10,000 g for 1.5 min at 4°C and the supernatant transferred to a fresh tube and stored at -80°C.

2.2.16.2 First-Strand cDNA synthesis

cDNA was synthesised using the SuperScript III RT kit. In a 20 µl reaction, 5 µg of the DNase treated RNA was combined with 2.5 µM oligo(dT)$_{17}$ adaptor and 0.5 mM dNTP mix, and the reaction incubated at 65°C for 5 min. After incubation the reaction was chilled on ice for 1 min before the addition of 4 µl 5 x First-Strand buffer, 1 µl 0.1M DTT, 40U RNase out Ribonuclease inhibitor and 200U SuperScript III RT. The reaction was then incubated at 50°C for 1 h followed by 70°C for 15 min.
2.2.17 Quantitative (real time) PCR

Quantitative real time PCR (Q-PCR) experiments were conducted by Dr. Neil Shirley (ACPFG, University of Adelaide, Australia) as described in Burton et al (2004), using QuantiTect SYBR Green PCR reagents (Qiagen) in an RG 2000 Rotor-Gene Real-Time Thermal Cycler (Corbett Research, Sydney, Australia). Analysis of the data was carried out using DNA sample analysis system V4.6 software (Corbett Research).

Q-PCR primers were designed as described in section 2.2.5, and were made to amplify fragments from the 3’ end of the transcript incorporating the stop codon. The primers used were 14.3.3c qF and 14.3.3c full R for Hv14.3.3c, MAPKK qF and MAPKK qR for HvMAPKK1 and FKBP qF and FKBP qR for HvFKBP (Appendix 1).

Concentration standards were prepared by amplification from cDNA using the Q-PCR primers and standard PCR conditions (section 2.2.6.1). The PCR products were then purified using HPLC, and sequenced as described in section 2.2.10 to confirm their identities. A dilution series of the HPLC purified PCR products, ranging from 1 x 10^1 to 1 x 10^7 copies per µl, was prepared and used to generate standard curves to relate fluorescence levels to concentration of the PCR product.

Expression levels were measured in cDNA produced as described in section 2.2.16. The cDNA was normalised by the method of Vandesoemple et al (2002), using the control genes HvHSP70, HvTubulin, HvCyclophilin and Hv18sRibosomal RNA. Q-PCR was performed using the following programme: 50°C for 30 min, 95°C for 15 min, followed by 55 cycles of 94°C for 15 sec, 55°C for 30 sec and 72°C for 20 sec. A melt curve was obtained from the products at the end of amplification by heating from 70°C to 95°C to confirm amplification specificity. Four technical replicates of each cDNA were run and used to calculate the mean expression level and standard deviation.
2.3 Results and discussion

2.3.1 Cloning of a Hv14.3.3c cDNA fragment

2.3.1.1 Determining unknown sequence

A 444 bp fragment, encoding for part of a gene that was found to be differentially expressed in the original microarray analysis, was obtained from the SSH mRNA library as described in Chapter 1. When blasted against the NCBI protein database using a blastx search, it was found to have 100% identity to a region of the full length mRNA sequence of Hv14.3.3c, (Accession no. Y14200) (Fig 2.1A).

2.3.1.2 Amplification and cloning of full length Hv14.3.3c

A cloned full length cDNA fragment of Hv14.3.3c was kindly provided by Dr. K. Oldach (ACPFG, University of Adelaide, Australia). This cDNA was obtained as follows: Gene-specific primers 14.3.3c full F and 14.3.3c full R (Appendix 1) flanking the coding region in the Hv14.3.3c mRNA sequence (Fig 2.1B) were used to amplify from cDNA of barley Atlas46 leaves, infected with R. secalis, in a standard PCR reaction. This tissue was selected because it was expected to be enriched for Hv14.3.3c, as Hv14.3.3c expression was shown to be upregulated in the original microarray analysis. The PCR produced a fragment of the expected size (1 kb; data not shown) and sequencing confirmed that it was the full length Hv14.3.3c, with the fragment showing 100% homology to the known Hv14.3.3c gene.

The 1 kb cDNA fragment encodes a predicted 263 aa protein (Fig 2.2) of molecular weight 30 kDa, which was consistent with the 25 – 32 kDa size range of known 14.3.3 family members (Ferl, 1996).

2.3.2 Cloning of a HvMAPKK1 cDNA fragment

2.3.2.1 Determining unknown sequence

A second fragment of 630 bp, for which the gene product was found to be differentially expressed, was also obtained via SSH. When blasted against the NCBI protein database it was found that this gene had not been previously sequenced from barley, with no full-length cDNA or genomic sequences available. The partial sequence showed the highest sequence similarity to a MAPKK from rice (OsMAPKK1; AF216314) (Fig 2.3A). Alignment of the partial HvMAPKK sequence with the full length OsMAPKK1 cDNA sequence indicated that both 5’ and 3’ sequence was required to complete the barley ORF
Fig 2.1: Alignment of partial 14.3.3c sequence from SSH with closest database match Hv14.3.3c. A) Output of Blastn database search with partial 14.3.3c sequence from SSH showing 100% sequence identity to Hv14.3.3c mRNA Y14200. B) Graphical representation of alignment of partial 14.3.3c sequence from SSH with full length coding region and mRNA of Hv14.3.3c, showing the position and orientation of the primers used to amplify Hv14.3.3c from At46 cDNA.
Fig 2.2: Full length Hv14.3.3c cDNA sequence. Deduced ORF and amino acid sequence are indicated. Common restriction sites are also shown.
Fig 2.3: Alignment of partial barley MAPKK sequence from SSH with closest database match OsMAPKK1. A) Output of Blastn database search with partial MAPKK sequence from SSH showing highest sequence similarity to OsMAPKK1 mRNA Accession Number AF216314. B) Graphical representation of alignment of partial MAPKK sequence from SSH with full length coding region and mRNA of OsMAPKK1, showing the position and orientation of the primers used in 5’ and 3’ RACE.
sequence (Fig 2.3B). No ESTs covering the complete unknown regions were available, so 5’ and 3’ RACE and genomic walking techniques were employed to obtain the unknown sequence.

2.3.2.2 3’ RACE

To extend on the existing 630 bp cDNA towards the 3’ end, 3’RACE was used. For the 3’ RACE reaction, cDNA made from NIP1 treated Atlas46 epidermal Poly A+ enriched RNA was used as it should be enriched for \(Hv\text{MAPKK}\) as it was shown to be upregulated at this point in the original microarray analysis.

The SMART PCR cDNA Synthesis Kit produces Poly A tailed cDNA which allows for amplification of the 3’ end using a gene specific forward primer and an oligo-dT reverse primer. Semi-nested Elongase PCR was used to amplify the 3’ region of the \(Hv\text{MAPKK}\). The primers MAPKK 3’F (Fig 2.3B) and Oligo(dT) (Appendix 1) were used in the Round 1 PCR reaction. Separate reactions were run at annealing temperatures of 58, 60, 62 and 64°C to determine the annealing temperature giving the highest specificity. A very light band of around 800 bp was visible in the reactions with annealing at 60 and 62°C (Fig 2.4A). This size is slightly greater than the 600 bp expected from the sequence comparison to \(Os\text{MAPKK1}\). It is possible, however, that the \(Hv\text{MAPKK}\) may differ enough in its 3’ sequence to that of \(Os\text{MAPKK1}\), to result in the 3’ end fragment of \(Hv\text{MAPKK}\) being ~200 bp larger than that of \(Os\text{MAPKK1}\).

The reaction with a 62°C annealing temperature appeared the most specific, so was used as the template for Round 2. Being a semi-nested reaction, only one primer was altered: MAPKK 3’F2 (Appendix 1) was used in place of MAPKK 3’F1 (Fig 2.3B), and the Oligo(dT) primer was used again. Three distinct bands were observed (Fig 2.4B). The fragment of around 800 bp present in Round 1 appeared to have been re amplified in Round 2, suggesting that this band was a true product of \(Hv\text{MAPKK}\), and not an artefact of non-specific priming.

This 800 bp fragment was excised from the gel, purified, ligated into the pGEM-T Easy vector and transformed into \(E.\ coli\) DH5\(\alpha\) via heat shock. Plasmid DNA mini preparations from resultant colonies were digested with \(EcoR I\) restriction endonuclease and the digestion products run on an agarose gel to determine the insert sizes (Fig 2.4C). The fragment was then sequenced using the T7 and SP6 primers (Appendix 1) from DNA preparations of positive clones, determined via the presence of the correct insert size. Sequence analysis showed that the fragment was 100% identical in its overlapping region.
Fig 2.4: Agarose gel analysis of barley MAPKK 3’ RACE fragments. A) Round 1 PCR at different annealing temperatures 1: 58°C, 2: 60°C, 3: 62°C, 4: 64°C. B) Round 2 PCR 1: three amplified fragments, C) EcoR1 digested plasmid DNA 1 – 10: Different colony plasmid DNA with 5 showing presence of correct insert. Bands of interest indicated with arrows.
with the original 630 bp sequence (Fig 2.5A) and showed homology to other MAPKK’s in BLAST searches (Fig 2.5B), indicating that it represented the 3’ end of the barley MAPKK gene.

2.3.2.3 5’ RACE – SMART RACE
The first method undertaken to obtain the sequence located 5’ of the known 630 bp, was SMART RACE. As well as producing poly-A tailed cDNA, the SMART PCR cDNA Synthesis Kit also produces cDNA populations with anchor sequences ligated onto the 5’ ends. This allows for amplification of the 5’ region of a gene using a gene specific reverse primer and a forward primer designed against the anchor sequence. Nested PCR was performed using the primers MAPKK 5’ out and anchor primers UPL + UPS (which bind to each other forming one primer) in Round 1 and MAPKK 5’ and anchor NUP in Round 2 (Appendix 1, Fig 2.3C). Use of this technique, however, failed to produce any amplification products (data not shown).

2.3.2.4 5’ RACE – RLM-RACE
A second type of 5’ RACE, RNA Ligase Mediated Rapid Amplification of cDNA Ends (RLM-RACE) was then attempted. In RLM-RACE, the cDNA is made in such a way as to produce full-length cDNA only, from full-length, capped mRNA, and usually produces a single band after PCR. Nested PCR was again used, using the same gene specific primers as in SMART RACE, and the anchor primers RLM-5’outer for Round 1 and RLM-5’inner for Round 2. The Round 1 PCR produced several fragments (Fig 2.6A), and therefore, to increase the specificity, a second, nested PCR was performed. Round 2, however, produced a similar range of fragments, with at least four fragments being dominant (Fig 2.6B). These fragments were excised from the gel in two gel-slices (Fig 2.6B), purified and cloned into pGEM T-easy. Plasmid DNA of eight resulting clones were extracted and digested with EcoRI revealing at least five different sized insert fragments (Fig 2.6C). One insert of each size was sequenced, however, sequence analysis revealed no overlap between any of the fragments and the known sequence of HvMAPKK, indicating that these fragments were artefacts, and likely the products of non-specific amplification.

2.3.2.5 Genomic walking
As the 5’ RACE techniques were unsuccessful in providing the 5’ region of the barley MAPKK, genomic walking was attempted. A semi-nested PCR was performed, using the same MAPKK 5’ primers as in RACE in both PCR rounds, in conjunction with the genomic walking adaptor primer Ap1 in Round 1, and Ap2 in Round 2 (Appendix 1).
Fig 2.5: Confiming 3’ RACE fragment is the 3’ end of barley MAPKK. A) Graphical representation of alignment of partial MAPKK sequence and 3’ RACE fragment shows 100% sequence similarity in overlapping region. B) Blastn search with 3’ RACE fragment shows sequence homology to other MAPKKs.
Fig 2.6: Agarose gel analysis of barley MAPKK 5' fragments. A) Round 1 PCR products. B) Round 2 PCR products, showing sections excised from gel. C) EcoRI digest of plasmid DNA from 8 colonies resulting from transformation, to check for the presence of correct inserts. 1 – 4: colonies resulting from transformation of upper section fragments, and 5 – 8: lower section fragments.
Separate reactions were performed using different genomic walking library templates. When a portion of Round 1 was run on a 1.4% agarose gel, products were observed from each of the different genomic libraries (Fig 2.7A). Round 2 was then conducted to test the specificity of these products by seeing if they were re-amplified with the semi-nested primer pair. When visualised as in Round 1, it was found that a single band in Library 1 had been re-amplified (Fig 2.7B), suggesting that this fragment, was in fact a specific product of these primers and therefore possibly the 5’ region of the \( \text{HvMAPKK} \). This fragment was excised from the gel and purified and sequenced directly. The fragment showed 100% match to an overlap with the known \( \text{HvMAPKK} \) sequence (Fig 2.8A) and also showed sequence identity to MAPKK orthologues in the database (Fig 2.8B). However, the walk had not reached the 5’ end of the ORF of \( \text{HvMAPKK} \), as no start methionine was present when the sequence was analysed. Therefore, a second genomic walk was performed, walking out from the new 5’ sequence.

Two new nested gene specific primers, MAPKK GW2_Out and MAPKK GW2_In (Appendix 1, Fig 2.8A) were designed to the new 5’ sequence of \( \text{HvMAPKK1} \) and used in a nested PCR reaction with the AP1 and AP2 genomic walking primers. Round 1 gave several fragments of different sizes (Fig 2.9A). Round 2 produced fragments of around 700 bp, 500 bp and 200 bp (Fig 2.9B). The 200 bp fragment was also present in reactions using the products from Library 2 and 4. A fragment of this size was also obtained in every library when trying to amplify the 5’ region of \( \text{HvFKBP41} \) (see section 2.3.3.2), suggesting that this product may be non-specific and likely the product of primer mis-annealing or contamination. The 200 bp product was therefore disregarded, and the Library 1 700 bp and 500 bp fragments excised, purified and sequenced directly using the Round 2 primers. The 700 bp fragment overlapped with the 5’ end of the sequence obtained from the first genomic walk (Fig 2.10A) and matched database MAPKK’s when BLASTed (Fig 2.10B), indicating that this fragment contained more or the rest of the 5’ region of \( \text{HvMAPKK} \). The 500 bp fragment was a truncated version of the 700 bp fragment and was therefore disregarded.

2.3.2.6 Amplification and cloning of full length \( \text{HvMAPKK} \)

To amplify the complete ORF of the barley MAPKK gene, two gene specific primers, MAPKK1 full F and MAPKK full R were designed to the 5’ and 3’ sequences, respectively (Appendix 1, Fig 2.10A). Hi-Fidelity PCR was used to reduce the frequency of mis-incorporation during amplification. A 50 µl reaction, using \( R. \text{secalis} \) infected Atlas46 leaf cDNA as the template, was conducted and produced a single product of
Fig 2.7: Agarose gel analysis of barley MAPKK 5’ genomic walking fragments. A) Round 1 PCR products. 1: Library 1, 2: Library 2, 3: Library 3, 4: Library 4. B) Round 2 PCR products, 1: 1:30 Round 1 Library 1, 2: 1:30 Round 1 Library 2, 3: 1:30 Round 1 library 3, 4: 1:30 Round 1 Library 4.
Fig 2.8: Confirming 5' genomic walking fragment is part of the 5' end of barley MAPKK. A) Graphical representation of alignment of partial MAPKK sequence and 5' genomic walking fragment shows 99% sequence similarity in overlapping region. Site and orientation of second genomic walking primers are shown B) Blastn search with 5' genomic walking fragment shows sequence homology to other MAPKKs.
Fig 2.9: Agarose gel analysis of barley MAPKK 5' genomic walking 2. A) Round 1 PCR products. 1: Library 1, 2: Library 2, 3: Library 3, 4: Library 4. B) Round 2 PCR products. 1: 1:30 Round 1 Library 1, 2: 1:30 Round 1 Library 2, 3: 1:30 Round 1 library 3, 4: 1:30 Round 1 Library 4.
Fig 2.10: Confirming 5' genomic walking 2 fragment is 5' end of barley MAPKK. A) Graphical representation of alignment of genomic walking 1 5' MAPKK sequence and genomic walking 2 fragment shows 99% sequence similarity in overlapping region. B) Blastn search with 5' genomic walking 2 fragment shows sequence homology to other MAPKKs, showing position and orientation of primers used to amplify full length.
around 1 kb (Fig 2.11).

The full length barley MAPKK cDNA fragment was found to contain a single open reading frame encoding a predicted protein of 355 aa (Fig 2.12A) and molecular weight of 40 kDa. When compared to the NCBI protein database, the full length sequence of the barley MAPKK still showed highest sequence similarity to that of the rice MAPKK OsMAPKK1, and was therefore named HvMAPKK1, to continue with the nomenclature theme.

Comparison of the original SSH sequence, the sequence obtained from genomic walking and 3’ RACE, and the full length ORF cDNA sequence revealed the presence of three introns (Fig 2.12B), one in the genomic walking sequence, and two in the original SSH sequence. The latter was unexpected, as the SSH fragment was obtained from cDNA. The presence of introns in the SSH sequence but not in the newly obtained full length cDNA suggests the possibility of different splicing variants of HvMAPKK1 or the presence of a pseudo gene. However, as the OsMAPKK1 cDNA sequence lacked these same introns, and no ESTs indicating splice variants could be found in the database, it was assumed that this was in fact the correct HvMAPKK1 full length cDNA sequence. Further to this, as the full length sequence was obtained from infected Atlas46 tissue, tissue that should be enriched for the presence of the HvMAPKK1 gene of interest, it was decided that, even if other splice variants existed, this version of HvMAPKK1 was the version of interest.

2.3.3 Cloning of a HvFKBP cDNA fragment

A final differentially expressed SSH clone of 1463 bp was also chosen. When blasted against the NCBI database, it was found that this gene has not been previously identified in barley, with the highest similarity being to a full length rice cDNA (Accession No. AK065114), coding for a FKBP-type peptidyl-prolyl cis-trans isomerise (FKBP) (Fig 2.13A). Alignment of the HvFKBP SSH sequence with the full length rice FKBP cDNA (Fig 2.13B) suggested that the barley coding sequence was complete at the 3’ end, but that a small stretch of sequence was required to complete the 5’ end. Therefore, 5’ RACE was undertaken in an attempt to obtain this additional 5’ sequence.

2.3.3.1 5’ RACE

Similarly to HvMAPKK in section 2.3.2.3 and 2.3.2.4 SMART RACE and RLM-RACE techniques were used unsuccessfully in an attempt to obtain the 5’ unknown region of HvFKBP (data not shown).
Fig 2.11: Agarose gel analysis of full length \textit{HvMAPKK1} fragment. Showing 1 kb full length \textit{HvMAPKK1} cDNA fragment.
Fig 2.12: Analysis of full length *HvMAPKK1* cDNA sequence A) Full length cDNA sequence and deduced ORF and amino acid sequence. Common restriction sites are also shown. B) Presence and position of coding region (blue) and introns (black v lines) in amplified sequences.
Fig 2.13: Alignment of partial barley FKBP sequence from SSH with closest database match. A) Blastn database search with partial FKBP sequence from SSH showing highest sequence identity to a rice cDNA, Accession No. AK065114 encoding an FKBP-type peptidyl-prolyl cis-trans isomerase. B) Graphical representation of alignment of partial FKBP sequence from SSH with AK065114 and its corresponding coding region, showing the position and orientation of the primers used in 5’ genomic walking.
2.3.3.2 Genomic walking

Due to the unsuccessful 5’ RACE, genomic walking was performed to obtain the unknown 5’ region of HvFKBP. Nested gene specific 5’ primers FKBP 5’_Out and FKBP 5’_In (Appendix 1, Fig 2.13B) were used with the genomic walking adaptor primers Ap1 and Ap2 respectively, using the general nested PCR strategy described in 1.3.2.4. Round 1 produced several products (Fig 2.14A) while in Round 2, only one fragment of around 1.1 kb from Library 1 was re-amplified (Fig 2.14B). A smear of products were produced from Library 2, and a 200 bp fragment was amplified from all libraries, which may have been the same artefact referred to in section 2.3.2.5. The 1 kb fragment was purified and sequenced, revealing an overlapping match with the original FKBP partial sequence (Fig 2.15). In a BLAST search, the fragment did not show sequence similarity to any other FKBP genes, probably because it contained only a small amount of coding sequence from the extreme 5’ end of the ORF, with the rest of the genomic walking 5’ sequence contained the 5’ untranslated region and promoter region of HvFKBP.

2.3.3.3 Amplification and cloning of full length HvFKBP

To amplify the full length ORF of HvFKBP, two gene specific primers, FKBP full F and FKBP full R (Appendix 1) were designed to the 5’ and 3’ sequences respectively (Fig 2.15). The primers were used in a 50 µl Hi-Fidelity PCR reaction with Atlas46 cDNA, used as the template. The reaction produced two fragments, one of the expected size, 1.4 kb, and another of slightly smaller size (Fig 2.16). Cloning and sequencing of the 1.4 kb fragment confirmed that it contained the full-length HvFKBP ORF. Therefore, no attempt was made to identify the smaller fragment.

The fragment was found to contain an ORF encoding a predicted 362 aa protein (Fig 2.17) of molecular weight 41 kDa. As FKBP gene names are based on their species of origin and translated protein size, and as this was the first time this gene had been identified in barley, it was named HvFKBP41.

2.3.4 Expression analysis

To confirm and expand on the expression analysis of the candidate genes in the SSH and microarray experiments (Chapter 1), the transcriptional activity of the genes was investigated under biotic stress, namely R. secalis infection of barley leaves. To investigate the hypothesis that these genes may also play a role in broader stress signalling, their transcriptional activity under abscisic acid treatment, frost, high salt and drought conditions was analysed.
Fig 2.14: Agarose gel analysis of barley FKBP 5’ genomic walking fragments. A) Round 1 PCR products. 1: Library 1, 2: Library 2, 3: Library 3, 4: Library 4. B) Round 2 PCR products. 1: 1:30 dilution of Round 1 Library 1, 2: 1:30 dilution of Round 1 Library 2, 3: 1:30 dilution of Round 1 Library 3, 4: 1:30 dilution of Round 1 Library 4.
Fig 2.15: Confiming 5' genomic walking fragment is 5' end of barley FKBP. Graphical representation of alignment of genomic walking fragment with original partial FKBP from SSH shows sequence similarity in overlapping region. Position and orientation of primers used to amplify barley FKBP full length cDNA are shown.
Fig 2.16: Agarose gel analysis of full length HvFKBP41 fragment. Showing 1.4 kb HvFKBP41 full length fragment indicated with arrow.
Fig 2.17: Analysis of full length HvFKBP41 sequence

A) Full length sequence and deduced ORF and amino acid sequence. Common restriction sites are also shown. B) Presence and position of promoter, 5' UTR and coding region.
2.3.4.1 *R. secalis* infection

Q-PCR was used to study transcription levels of the genes under uninfected and *R. secalis* infected conditions. As it is known that the fungus infects the epidermal cells (Ayesu-Offei and Clare 1970), it was thought that any initial responses by the plant, in terms of the expression of defence genes, would occur in these cells. Therefore it was decided to investigate expression in epidermal tissue.

Atlas and Atlas46 9 day old seedlings were infected with *R. secalis* strain SA385 and epidermal tissue harvested from each cultivar at 0 h, 3 h, 6 h and 24 h after infection. These timepoints were chosen because previous experiments demonstrated that in incompatible interactions the mRNA of defence-related genes accumulated to maximal levels 24 h post inoculation (Hahn et al., 1993; Steiner-Lange et al., 2003, K. Oldach unpublished). Samples were also taken from uninfected (control) plants at 24 h. Epidermal tissue was collected via epidermal abaxial peels, with epidermal material from ~ 10 leaves collected and pooled into one sample for each timepoint. A ‘rest of leaf’ sample, comprising a single leaf with the epidermal cell layer removed on the abaxial side, was also collected at each timepoint, to compare expression in the epidermal layer versus the rest of the leaf. After around two weeks, the characteristic scald symptoms formed on the leaves of inoculated Atlas control plants, indicating that the infection had been successful.

RNA was then extracted from the tissue, and cDNA produced. Q-PCR analysis was performed on this cDNA for each of the candidate genes *Hv14.3.3c*, *HvMAPKK1* and *HvFKBP41* (Fig 2.18). It was found that all of the genes responded to infection, as indicated by an increase in transcript abundance in the epidermal tissue. *HvMAPKK1* and *HvFKBP* showed the highest expression at 6 h after infection in the resistant cultivar Atlas46, while *Hv14.3.3c* expression was highest at 24 h after infection in the susceptible cultivar Atlas. The expression of all genes was generally higher at 24 h after inoculation than at the comparable time in uninoculated control plants, suggesting differential expression upon *R. secalis* infection and confirming the original microarray results. There was however, some increase in expression after 24 h in the control plants, which suggests that other factors, such as temperature or developmental cycle may also have had an effect on gene expression. Light and/or humidity may have also been a factor at this time, as at the 24 h time point, plants were taken from the 24 hours of dark, 100% humidity treatment and placed under standard daylight conditions. All the genes also showed an increase in expression in both cultivars, when it could be expected that, if these genes play a role in transducing the *Rrs1* mediated defence response signal, differential expression would only be seen in Atlas46 where the *Rrs1* mediate response is occurring.
Fig 2.18: Expression of genes under *R. secalis* infection. Expression level, expressed as copy number and normalised to four housekeeping genes encoding HSP70, Tubulin, Cyclophilin and 18s Ribosomal protein, graphed against each timepoint, hours after infection or 24h water control, for A) *Hv14.3.3c*, B) *HvMAPKK1* and C) *HvFKBP41*. Expression in four different tissue types; Atlas and Atlas46 epidermal (At Ep and At46 Ep respectively) and Atlas and Atlas46 rest of leaf (At Rol and Atlas46 Rol respectively), was analysed. Error bars = standard deviation for each mRNA.
However, all genes responded earlier and, except for Hv14.3.3c, to a greater level, in Atlas46 than Atlas, with expression not increasing until 24 h after infection in Atlas while increasing 6 h after infection in Atlas46. This could be consistent with a role in the defence response, as the faster and possibly stronger response of the genes in the resistant cultivar Atlas46 may be part of a resistance process that was more intense and therefore responsible for the elevated resistance in this cultivar.

Comparison of expression in the epidermal cell layer versus the rest of the leaf indicated that the response was principally in the epidermis, with very low expression levels being observed in the rest of leaf samples. Although these genes were identified as epidermal-expressed genes (K. Oldach, unpublished), they are not epidermal specific, having been found to be expressed in many kinds of plant tissue and at many stages of development (NCBI; Wise et al., 2007). This therefore suggests that the differential expression seen is possibly due to a response to R. secalis infection alone. At these early time points, all fungal activity would be occurring in the epidermal layer, thus if the expression response seen was due to other factors such as a response to light, temperature or physiological state life cycle etc, it could be expected that this would be a whole of plant response and therefore also present in the rest of leaf sample.

While the response of these genes to R. secalis infection in barley has not previously been reported, these genes and their homologues have been investigated in other systems, and the findings support the results of this analysis. For example, one of the first plant 14.3.3s was identified as a barley leaf mRNA that increased in abundance upon inoculation by the powdery mildew fungus (Brandt et al., 1992). In rice, 14.3.3 expression was found to be induced shortly after inoculation with the rice blast fungus Magnaporthe grisea, with the induction appearing earlier and stronger in incompatible interactions (Chen et al., 2006), similar to that of the result found here. Although not a MAPKK, the expression of a barley MAPK was found to be rapidly induced by powdery mildew infection (Eckey et al., 2004) indicating that increased expression of a MAPKK, like the increase seen here, is also credible. A MAPKK was also shown to be biotic stress responsive by You et al (2007), with the identification of a blast (Magnaporthe grisea) and insect (Nilaparvata lugens) responsive putative MAPKK, named OmMKK1, from wild rice.

Similarly to the results observed, HvFKBP41 expression also increases during powdery mildew infection in barley, as does HvMAPKK1 (Wise et al., 2007).
ABA-mediated signalling plays an important role in plant stress responses, causing changes in stomatal aperture, accompanied by gene expression changes.

During *R. secalis* infection, the turgor balance between epidermal cells and guard cells is altered, allowing abnormal swelling of the guard cells, which increases the aperture of stomatal pores in the light meaning that stomata remain open in the dark (Ayres 1972). External application of ABA also causes these changes in stomatal aperture and gene expression, and can be used to identify ABA responsive genes. Therefore, expression of the candidate genes in plants treated with the hormone abscisic acid (ABA) was also investigated.

An ABA cDNA treatment series was made by Alex Smart (ACPFG, SA, Australia). Briefly, Golden Promise seedlings were grown in supported hydroponics in ACPFG growth solution \[5 \text{ mM NH}_4\text{NO}_3, 5 \text{ mM KNO}_3, 2 \text{ mM Ca(NO}_3)_2\cdot4\text{H}_2\text{O}, 2 \text{ mM MgSO}_4\cdot7\text{H}_2\text{O}, 0.1 \text{ mM KH}_2\text{PO}_4, 0.5 \text{ mM Na}_2\text{SiO}_3, 0.05 \text{ mM NaFe(III)EDTA}, 10 \text{ mM H}_3\text{BO}_3, 5 \text{ mM MnCl}_2\cdot4\text{H}_2\text{O}, 5 \text{ mM ZnSO}_4\cdot7\text{H}_2\text{O}, 0.1 \text{ mM CuSO}_4\cdot5\text{H}_2\text{O}, 0.05 \text{ mM Na}_2\text{MoO}_4\] under natural glasshouse light. When seedlings were 10 days old, ABA was added to the hydroponics solution at a final concentration of 10 µM, and shoot samples taken at 0 h, 1 h, 2 h, 4 h, 8 h, and 12 h after the treatment. Shoot samples were also taken from control plants grown without the addition of ABA.

*Hv14.3.3c, HvMAPKK1* and *HvFKBP* expression was compared between the treated and control plants and to the control gene *HvRD17*. *RD17* is short for responsive to dehydration 17, and is a dehydrin, a family of proteins which accumulate in response to dehydrative stress, such as drought, salinity, cold, and frost (Beck et al., 2007). Dehydrins are also called RAB proteins, short for responsive to ABA, as they are also known to accumulate upon ABA treatment (Nylander et al., 2001; Zhu et al., 2000). The orthologue of *HvRD17* in Arabidopsis, known as *COR47*, has been shown to be responsive to ABA (Welin et al., 1994). Therefore barley *RD17* expression was used as a control for successful ABA treatment and also provided a response standard to which the tested genes expression could be compared.

Q-PCR showed that all three genes responded to ABA treatment (Fig 2.19). *HvMAPKK1* and *HvFKBP* both showed a peak in expression 8 h after treatment, similar to *HvRD17*. At this time, *HvRD17* expression had increased 2.5-fold, whereas *HvMAPKK* and *HvFKBP* expression had increased only slightly. *Hv14.3.3c* showed an even greater response than *HvRD17*, with a 3-fold increase in expression. This increase, however, occurred earlier than in the other genes, peaking at 4 h after treatment. At 12 h, expression of the three
Fig 2.19: Expression of genes under 10 µM ABA treatment in leaf. Expression level, expressed as a percentage compared to expression in control plants and normalised to four housekeeping genes encoding HSP70, Tubulin, Cyclophilin and 18s Ribosomal protein, is graphed against each timepoint for each gene. Error bars = standard deviation for each mRNA.
candidates returned to control levels, while expression of HvRD17 remained slightly elevated. The three candidate genes also showed an earlier transient rise in expression at 1 h after treatment, a response that was not evident in the expression profile of HvRD17. This suggests that the candidate genes may play a role in transducing early ABA signals, similar to the role that was suggested they may play during R. secalis infection.

The expression results for Hv14.3.3c are consistent with the findings of Schoonheim et al (2007a) who observed strong ABA induced increases in transcript and protein for all 5 barley 14.3.3 isoforms, including Hv14.3.3c, in germinating seeds. They also showed that silencing of the 14.3.3s negatively affected the progression of the ABA signalling towards activation of target gene expression, suggesting a role for 14.3.3s in the ABA signalling pathway, while interaction studies showing that the 14.3.3s interact with intermediates of the ABA signalling pathway (Schoonheim et al., 2007a).

The HvMAPKK results were also consistent with findings by You et al (2007), showing the wild rice MAPKK orthologue OmMKK1 to be responsive to biotic stress and ABA. They suggested that the induction of OmMKK1 by biotic stress may somehow be linked to ABA-related signalling pathways. ABA was also found to rapidly induce MAP kinase expression in barley aleurone protoplasts. Tyrosine phosphorylation of MAP kinase was found to occur during this activation, leading to the conclusion that ABA activates MAP kinase via a tyrosine phosphatase and that these steps are prerequisites for ABA induced gene expression (Knetsch et al., 1996). Similarly, there is some evidence to support the HvFKBP results, with characterisation of a wheat FKBP promoter revealing the presence of three ABA-responsive elements and induction upon ABA treatment (Kurek et al., 2000).

As ABA is a hormone involved in many abiotic stresses, and the expression results suggested that the candidate genes were ABA responsive, the possibility that they would also be responsive to ABA-mediated abiotic stresses was investigated.

### Frost

A frost cDNA stress series was a kind gift from Jafar Jabbarri (ACPFG, SA, Australia). Briefly, plants were grown at 20°C in a growth chamber for 6 days, before being moved to a frost simulation chamber, where they were exposed to an overnight frost treatment. During the dark cycle, the temperature was cooled from 20°C to 4°C at a rate of 5°C/h. The temperature was then further decreased to -5°C at a rate of 1°C/h, and held at -5°C for 2 h, followed by an increase in temperature to 4°C at 2°C/h and then to 20°C at 5°C/h. Leaf samples were taken directly before the frost at 20°C, and when the temperature reached 4°C, -5°C and 4°C again. The final sample was taken 48 h after the temperature
was returned to 20°C. At the same times, leaf samples were also taken from non-frosted control plants that were being maintained under the pre-frosting growth conditions. Three different cultivars were used to cover a range of possible responses to frost: the cultivar Amagi Nijo, the breeding line WI2585 and the cultivar Golden Promise, which had been categorized as frost tolerant, susceptible, and moderately tolerant, respectively, by (Reinheimer et al., 2004).

Expression of Hv14.3.3c, HvMAPKK1 and HvFKBP was compared between the treated and control plants, and to the expression of the control gene HvDHN8 (Fig 2.20), another barley dehydrin, like HvRD17. Expression of an Arabidopsis orthologue ERD10, short for early responsive to dehydration 10, was shown by Kiyosue et al (1994) to be induced under cold stress, while COR47, the orthologue of dehydrin HvRD17 has also been shown to be cold regulated (Gilmour et al., 1992). Choi et al (1999) showed HvDHN8 to be upregulated during cold treatment.

The expression results showed that Hv14.3.3c and HvDHN8 responded to frost in a similar way, exhibiting very large expression responses by the 4°C time-point following the frost event. At this point, Hv14.3.3c showed a 6-fold increase in expression over control levels, while HvDHN8 showed a massive 400-fold increase. This co-expression with HvDHN8 supports the possibility that Hv14.3.3c may be involved in the cold or frost stress response. HvMAPKK1 and HvFKBP expression was found to peak during the frost event at the -5°C point, with both exhibiting around a 3-fold increase in expression compared to the control.

The later peak in expression of Hv14.3.3c may suggest a role in frost stress recovery whereas the peak in expression of HvMAPKK1 and HvFKBP during the frost event may suggest a role in minimising initial damage or protection. Expression of all genes was highest in the cultivar WI12585, which was expected, as this is the most frost intolerant cultivar, likely to be more affected by the frost and therefore show a greater expression response of genes involved in frost stress.

A role for 14.3.3 involvement in frost tolerance or recovery is also supported by a report that two 14.3.3 proteins were induced by low temperatures in Arabidopsis by Jarillo et al (1994). In sugar beet, low temperature increased the amount of ATPase/14.3.3 complexes and enhanced ATPase activity, resulting in a several-fold rise in the H+ efflux from protoplasts and intact cells (Chelysheva et al., 1999).

Published data also support a role of the MAP kinase signalling cascade in low temperature defence, with a MAP kinase in cotton found to be upregulated by low temperatures by Wang et al (2007). Teige et al (2004) also demonstrated cold stress activation of Arabidopsis MAPKK2, with plants over-expressing MAPKK2 showing increased freezing
Fig 2.20: Expression of genes under frost treatment. Expression level, expressed as a percentage compared to the control, and normalised to four housekeeping genes encoding HSP70, Tubulin, Cyclophilin and 18s Ribosomal protein is graphed against each timepoint for A) *HvDHN8*, B) *Hv14.3.3c*, C) *HvMAPKK1* and D) *HvFKBP41*, with expression in different cultivars shown in different colours. Error bars = standard deviation for each mRNA. 

*Inset: Graphical representation of temperature during the frost treatment and time of sample taking.*
tolerance, while Jonak et al (1996) showed that an alfalfa MAPKK expression and activity was activated by cold stress.

The *HvFKBP* expression results provide the first evidence for a plant FKBP being responsive to cold stress. However, in Shewanella, a bacterium that can grow at very low temperatures but which requires systems to facilitate protein folding, an FKBP type immunophilin accumulated under low temperatures. It was proposed that FKBP may help contribute to cold-adaptation by accelerating peptidyl-prolyl isomerisation of proteins at these low temperatures (Suzuki et al., 2004).

2.3.4.4 Drought

A drought cDNA stress series was a kind gift from Alex Smart (ACPFG). Eight week old plants of the cultivar Sloop were subjected to a cyclical drying treatment that was intended to imitate the effect of intermittent rainfall typically experienced by cereals in Southern Australia during fertilisation and early grain filling. The plants were well watered until the emergence of the first flag leaf, after which the amount of watering was reduced each day until the plants displayed wilting symptoms. Plants were then re-watered to recovery, before again being left to dry to wilting point, this time without any daily watering, before being re-watered a final time. Wilting symptoms took around 2 weeks to appear during the first drought cycle, and leaf and floral samples were taken at 7, 11 and 14 days after the first cycle began. The second drought cycle induced wilting after around 1 week and samples were taken at 6 days after watering ceased (20 days from beginning). Final samples were then taken 2 days after the last re-watering (22 days from beginning). At the same time, control samples were taken from plants that were well watered for the duration of the experiment. *HvDHN8* and *HvRD17* were analysed as controls because the Arabidopsis orthologues, *Cor47* and *ERD10* respectively, had been shown to be responsive to drought and dehydration (Kiyosue et al., 1994; Welin et al., 1994).

The expression of these controls genes was compared to the expression of the candidate genes *Hv14.3.3c*, *HvMAPKK1* and *HvFKBP* (Fig 2.21).

Q-PCR showed that the expression response of the candidate genes in the leaf differed from that of the control genes. All candidate genes showed an increase in expression upon re-watering after the final second drought cycle, while the control genes showed the most response during the middle of the first drought cycle. *HvMAKK1* showed the largest response, with a 3.5-fold increase over the untreated control, while both *Hv14.3.3c* and *HvFKBP* showed a 2-fold increase (Fig 2.21A). Response of the control genes was greater in the floral tissue, with *HvRD17* showing over a 5-fold increase in expression during the
Chapter 2 – Isolation of full length cDNAs and expression analysis

**Fig 2.21: Expression of genes under drought treatment.** Expression level, expressed as a percentage compared to expression in control plants, and normalised to four housekeeping genes encoding HSP70, Tubulin, Cyclophilin and 18s Ribosomal protein is graphed against each timepoint for each gene. **A)** shows expression in leaf tissue and **B)** in floral tissue. Error bars = standard deviation for each mRNA.

*Inset: Graphical representation of cyclic drought treatment and time of sample taking.*
middle of the first drought cycle, and *HvDHN8* showing a 3.5-fold increase slightly later, while the candidate genes all showed no transcriptional response in floral tissue (Fig 2.21B). The increase in expression in leaves after re-watering may suggest a role in drought recovery rather than drought resistance, a role similar to that suggested in relation to frost in section 2.3.4.3. A similar peak in expression was not seen after the first watering, but this may have been due to the sample not being taken at the appropriate time, with a sample taken only 2 days after the second watering, but much later after the first watering.

A role for these genes in the drought stress response is consistent with the literature, over-expression of an Arabidopsis 14.3.3 in cotton lead to increased stress tolerance under moderate drought conditions (Yan et al., 2004), and at least 4 rice 14.3.3s were found to be regulated by drought (Chen et al., 2006). Jonak et al (1996) showed that expression and activity of an alfalfa MAPKK was activated by drought stress, while the protein level of an FKBP was found to increase during water deficiency in lupin (Pinheiro et al., 2005).

### 2.3.4.5 Salt

The transcriptional response of the genes under salt stress was also investigated. The salt stress cDNA series was a kind gift from Jafar Jabbari (ACPFG). Plants were grown in supported hydroponics in ACPFG growth solution under natural glasshouse light. After 7 days, plants were treated with 150 mM NaCl, added in 3 x 50 mM treatments every 12 hours, and supplemental calcium [Ca(NO$_3$)$_2$] was added to achieve a Na$^+$:Ca$^{2+}$ ratio of 15:1. Calcium was added as drastically increasing the concentration of a particular salt such as NaCl increases the ionic interactions in a solution which subsequently decreases the free concentration, or activity, of other ions, namely Ca$^{2+}$. Thus calcium was added in order to counteract this effect.

Leaf samples were taken at 1, 3 and 5 days after 150 mM salt concentration had been reached. Leaf samples were taken at the same time from plants grown in hydroponics to which no additional salt had been added. Cultivars differing in their tolerance to salt, based on levels of sodium accumulation in the leaves, were used: lines Yu6472 and Prior-A are low sodium accumulators (tolerant), WI-2291 and CM 72 are high sodium accumulators (intolerant) and Golden Promise is moderately tolerant (Dr Yuri Shavrukov, ACPFG, personal communication).

DHN8 was run as a pseudo control as it was thought that it may respond to salt stress due to the fact that it is known to be dehydration responsive and high levels of salt cause dehydration.
In the Q-PCR analysis, none of the genes, including *DHN8*, showed expression level differences of more than 0.5-fold relative to controls, a level at which the difference was determined to be statistically insignificant (Fig 2.22). It was also expected that the greatest response in expression might have been seen in the intolerant or high sodium accumulating lines WI-2291 and CM 72 because they would be more affected by the salt treatment. However, this correlation did not occur, with some genes showing tendencies towards greater expression differences in the moderate or tolerant cultivars.

Failure to detect a difference in the expression of the control gene or any of the candidate genes during salt stress, may indicate that the salt treatment was not sufficient to induce a salt stress in the plants. Sodium accumulation in the leaves was measured, however, and it was found that there was a high level of sodium present (Jafar Jabbari, data not shown), suggesting that the plants had in fact taken up the sodium and therefore should have been sufficiently treated as to induce salt stress.

The lack of expression responses in the tested genes may, therefore, suggest that these genes are not responsive to salt stress. Previous findings, however, may contradict these results, with a plant 14.3.3 gene transcript accumulated in callus and seedlings of rice when exposed to high salt (Kidou et al., 1993), while Teige et al (2004) showed salt stress activation of Arabidopsis *MAPKK2*, also induced by cold, with over-expression of this MAPKK conferring increased salt tolerance (Mizoguchi et al., 1996).

It may be possible that the genes responded to the stress earlier or later than the timepoints examined, and therefore a response was not observed. If the genes play a role in recovery from salt stress, as suggested from the frost and drought results, this response would also not have been seen due to samples not being taken after salt treatment had ceased. However, it may also still be possible that these genes do not respond to salt stress, as previous evidence of salt stress response has only been shown for orthologues and different isoforms of these genes in different plant species.
**Fig 2.22: Expression of genes under 150 mM salt treatment.** Expression level, expressed as a percentage compared to expression in the control, and normalised to four housekeeping genes encoding HSP70, Tubulin, Cyclophilin and 18s Ribosomal protein, graphed against each timepoint for A) *HvDHN8*, B) *Hv14.3.3c*, C) *HvMAPKK1* and D) *HvFKBP41*, with expression in different cultivars shown in different colours. Error bars = standard deviation for each mRNA.
2.4 Summary and conclusions

The full length sequences of three partial cDNAs, obtained from suppressive subtractive hybridisation, were successfully obtained. The full length of the first cDNA fragment was obtained by Dr K. Oldach (ACPFG, University of Adelaide, Australia), from a single PCR reaction using primers designed to a previously determined database sequence, with sequence analysis confirming its identities as a barley 14.3.3c, Hv14.3.3c. The second full length fragment was determined from four overlapping cDNA and genomic DNA fragments; the original SSH fragment, a 3’ end 3’ RACE fragment and two 5’ end genomic walking fragments. Subsequent sequence analysis confirmed its identity as a previously unidentified barley MAPKK, with closest homology to rice MAPKK1 leading to it being named HvMAPKK1. Full length of the third fragment was completed with a single 5’ genomic walk, and was determined to be a previously uncharacterised FKBP-type petidyl prolyl isomerise of barley, named HvFKBP41.

Differential expression of these genes during the Rrs1/NIP1 interaction, seen during the original microarray analysis, was then investigated using Q-PCR to analyse the transcriptional activity of the genes during R. secalis infection, with R. secalis infection induced responses confirming the occurrence of differential expression.

To investigate the possibility of these genes being responsive to other stresses, not limited to biotic, their transcriptional activity was then investigated under ABA, frost, drought, and salinity conditions, using stress series provided by Alex Smart and Jafar Jabbari (ACPFG, University of Adelaide, Australia). All genes were found to be responsive to ABA, with HvFKBP41 and HvMAPKK showing a similar response pattern to that of known ABA responsive gene RD17. Hv14.3.3c showed the strongest ABA response, larger and earlier than that of the other two genes and the control. Similarly, all genes were found to be responsive to frost, with Hv14.3.3c showing a similar timed response to that of control gene DHN8, peaking during re-acclimation after the frost event, suggesting a possible role in recovery. HvFKBP and HvMAPKK also responded, however their response peaked earlier than Hv14.3.3c, during the frost event, suggesting a possible role in protection or tolerance. The genes also responded to drought stress, with all genes responding similarly, increasing in expression upon re-watering after drought, again suggesting a possible role in recovery.

Interestingly, the genes were found to be unresponsive to salt treatment. As salinity, frost and drought are all dehydrative stresses, plants often show a similar response to these environmental stresses as they involve triggering of similar or common downstream signal transduction pathways (Beck et al., 2007). Through expression analysis studies in
Arabidopsis, over 300 stress-inducible genes have been identified. Of these genes, more than half of the drought-inducible genes are also induced by high salinity, indicating the existence of significant cross talk between the drought and high-salinity responses (Yamaguchi-Shinozaki and Shinozaki, 2006). Therefore, because the genes were responsive to frost and drought, they were also expected to be responsive to salt stress. However, it is thought that different isoforms of 14.3.3s specialise in individual functions (Wu et al., 1997). Therefore it may be possible that Hv14.3.3c is not involved in the salt stress response, and instead one of the other four isoforms is. This may also be the case for HvFKBP41 and HvMAPKK1, which are both members of a large family of proteins.

ABA is produced under dehydrative conditions and plays an important role in the tolerance response of plants. Exogenous application of ABA induces a number of genes that also respond to dehydration and cold stress (Yamaguchi-Shinozaki and Shinozaki, 2006). Thus, it would be expected that genes that respond to exogenous ABA treatment, are also responsive to dehydrative and cold stresses, supporting the results observed in this study under ABA, drought and frost treatment.

However, it is known that signal transduction cascades, between the initial stress signal and the expression of specific genes, can involve ABA, ie are ABA-dependent, as in the case above, or can also be ABA-independent (Yamaguchi-Shinozaki and Shinozaki, 2006; Zhu, 2002). There are also cases of genes whose expression is governed by both ABA-dependent and independent regulation (Yamaguchi-Shinozaki and Shinozaki, 1992). Therefore it may be possible that the candidate genes respond to drought and frost in an ABA-dependant manner, but to salt in an ABA-independent manner, and this is why different expression results are seen under salt stress.

In general, the candidate genes were found to be responsive to all stresses investigated except salt stress. As discussed in Chapter 1, these genes play a role in a diverse range of cellular processes and are thought to be involved in signalling pathways during stress and defence. Therefore, they are likely to play a role in the stress response to *R. secalis*, frost and drought, and thus a change in their expression during such stress conditions would be expected.

The results suggest that these genes do play a role in the plant defence response, with the next chapter aiming to define this role as a signal transduction role, by placing them as players in defence signal transduction pathways.
Chapter 3

Identifying Interacting Proteins using Yeast Two-Hybrid Screening and Affinity-Pulldowns
3.1 Introduction

The three candidate genes Hv14.3.3c, HvMAPKK1 and HvFKBP41 were found to be differentially expressed under a range of different stresses, both biotic and abiotic (Chapter 2). This differential expression supports a role in the barley defence response.

Based on the gene’s similarity to characterised genes, it was hypothesised that they may play a role in signal transduction during the defence response. Thus, the investigation to identify the signalling networks in which they may be involved is described in this chapter. Being able to associate a protein of unknown function with partners belonging to a known or specific process provides strong evidence of its biological function. In cells, many proteins display their biological function through association within large protein complexes. Thus, understanding protein function as well as unravelling molecular mechanisms within the cell is dependent on the identification of interacting partners (Monti et al., 2005). Therefore, the identification of interacting proteins was attempted, in the aim of gaining insight into the possible biological function of the candidate genes in the defence response.

As the candidate genes are known to play a role in many cellular processes, manipulation of these genes, for example overexpression, while possibly having an effect on resistance or tolerance, is likely to also affect many other processes. Therefore, the identification of genes involved further downstream in the signalling pathway may also provide more specific candidate targets.

Both yeast two-hybrid screening and affinity chromatography pulldowns were used to identify interacting proteins.

The yeast two-hybrid (Y2H) system, first described by Fields and Song (1989) is a common technique used to discover protein-protein interactions, and allows for the identification of physical interactions, such as binding, between two proteins. The system is based on the fact that eukaryotic transcriptional activators consist of two individual domains, a DNA binding domain (BD) and an activation domain (AD), which separately lack function, while together activate transcription (Verschure et al., 2006). In the Y2H system, this is exploited through a protein of interest, or bait protein, being expressed as a fusion protein to the BD, and a second protein, known as a prey protein, fused to the AD. If the proteins interact, the two domains of the transcription factor are brought spatially together, leading to it regaining function and activating transcription of reporter genes, which are used to detect the occurrence of interaction (Young 1998; Fields and Sternglanz 1994).
There are several variations on the Y2H system, with one of the most commonly used systems utilising the GAL4 transcription factor and the reporter genes HIS3, URA3, ADE2 and lacZ. Induction of the HIS3, ADE2 or URA3 reporter genes allows monitoring of transcription activation by growth on plates lacking histidine, adenine or uracil, while induction of the downstream lacZ gene can be used to assay for interaction via ß-galactosidase activity in the presence of X-α-Gal (Fields and Song 1989; Young 1998).

While the Y2H system provides a relatively straightforward and high-throughput means of identifying protein-protein interactions, it also has a number of pitfalls. Proteins are often overexpressed in the Y2H system, which can lead to a modification of the relative concentrations of potential interactors that would occur in the natural environment of the proteins, leading to identification of interactions that may not normally occur. Similarly the system may introduce novel interactors, for example proteins normally residing in a different cellular compartment, or eliminate competing interacting partners, again leading to false interactions. Fusion of proteins to the AD or BD moiety can also inhibit the normal site of interaction, or can impair the proper folding of the protein, thus interfering with the ability of the proteins to interact. Similarly, conditions in the yeast cell may not allow the proper folding or post-translational modifications required for interaction of some proteins (Fields and Sternglanz 1994; Lalonde et al., 2008).

With these factors in mind, alternative techniques were also used as a means of identifying interactions, with the aim of possibly identifying interactions that the yeast two-hybrid system could not, or confirming potential yeast two-hybrid interactions by replicating them using an alternative approach.

Before development of the Y2H system, protein-protein interactions were generally studied using biochemical techniques such as crosslinking, co-immunoprecipitation and co-fractionation by chromatography (Fields and Sternglanz 1994). Affinity chromatography is still a widely used tool today, and is the method of separating biochemical mixtures based on a highly specific biological interaction such as that between antigen and antibody, enzyme and substrate, or receptor and ligand. Immunoaffinity chromatography exploits the highly specific interaction between an antibody and a protein of interest. In principle, a protein (antigen X), bound to an interacting protein, but contained in a mixture of many other proteins, can bind selectively to immobilised anti-X immunoglobulin (IgG), allowing other proteins for which the antibody has no affinity, to be washed away and discarded, isolating protein X and its interactor (Yaciuk 2007; Livingston 1974). Affinity chromatography can also be performed similarly, but without the use of antibodies, by binding the bait protein to an immobilised affinity matrix, such as
sepharose or paramagnetic beads with an affinity for binding amine groups. Unbound sites on the matrix can then be blocked, and the protein-matrix complex used to isolate interacting proteins from a protein complex (Miernyk and Thelen 2008).

Similarly to Y2H, affinity chromatography is not without its downfalls, such as relying on protein-matrix or protein-antibody interaction to not interfere with the proteins ability to interact with target proteins, and the requirement of isolated interacting proteins to be identified via proteomic analysis, using most commonly mass spectrometry, in which concentration and contamination can be an issue. A benefit of affinity chromatography is that the interactions can be allowed to occur in more of a natural environment, such as in a leaf protein extract, rather than the more forced environment of the Y2H system. An advantage of the Y2H system, however, is that low-abundance transcripts isolated from plant tissue stand a good chance of being co-expressed with the bait, due to being under control of the constitutive promoter ADH1 and therefore being highly expressed in the yeast (Schoonheim et al., 2007b).

Another downfall of affinity chromatography is the need for purified or recombinant bait protein. Heterologous expression of recombinant proteins is a commonly used method for obtaining a protein. A variety of biological systems are available for heterologous expression, including bacterial, fungal, viral, mammalian and insect systems (Kost 1997), as well as recently developed cell-free expression systems (Sawasaki et al., 2002). Bacterial systems, most commonly E. coli systems, are generally the most widely used due to their low cost, speed and simplicity as well as an increasing number of available cloning vectors and mutant host strains. They are also highly productive, allowing for large quantities of protein expression to be achieved (Terpe 2006). Heterologous expression systems also allow for proteins to be expressed as a fusion with an affinity tag, providing an easy means of protein purification after expression. One such commonly used tag is the polyhistidine tag, comprising of six histidine residues, which allows for purification of the recombinant protein via immobilized metal affinity chromatography (Janknecht and Nordheim 1991).

Due to the complimentary advantages and disadvantages, both the yeast two-hybrid system and affinity chromatography were used to identify interacting proteins of Hv14.3.3c, HvMAPKK1 and HvFKBP41. The expression and purification of recombinant Hv14.3.3c and antibody development, for use in affinity chromatography, and the development and comparison of different affinity chromatography approaches is also described in this chapter.
3.2 Materials and methods

3.2.1 Materials

Matchmaker Yeast Two-Hybrid Kit including the *Saccharomyces cerevisiae* yeast strains AH109 [MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1UAS-GAL1TATA-HIS3, GAL1UAS-GAL2TATA-ADE2, URA3::MEL1UAS-MEL1TATA-LacZ] and Y187 [MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, met-, gal80Δ, URA3:: GAL1UAS-GAL1TATA-LacZ], Herring testes DNA, pGBKT7, pGADT7, X-α-Gal and synthetic dropout media (-2 [-leu, -trp], and -4 [-ade, -his, -leu, -trp]) were from Scientifix (Clayton, VIC, Australia). Kanamycin, zymolase enzyme, LiAc, PEG 3500, HEPES MgATP, BSA, NaPi, DTT, adenine hemi-sulphate, SDS, imidazole, glass beads (212-300 micron), NBT-BCIP, PVP, coomassie brilliant blue R, PMSF, IPTG, NaF, lysozyme, phenol, chloroform, isoamyl alcohol (IAA), protease inhibitor cocktail and mouse anti-His-alkaline phosphatase conjugated monoclonal antibody were from Sigma-Aldrich (Castle Hill, NSW, Australia). Glycine, silver nitrate, sodium carbonate, sodium thiosulphate, mouse anti-Myc monoclonal antibody, KH₂PO₄ and NaH₂PO₄ were from Genesearch (Arundel, QLD, Australia). Hypond-P PVDF, Immobiline™ DryStrip gels, HiTrap NHS-activated HP 1 ml columns and Sepharose 4B and CL-6B were from GE Healthcare (Rydalmere, NSW, Australia). NuPage 4-12% Bis-Tris pre-cast gels, NuPage MES SDS Running Buffer and Dynabeads M-208 Totosylactivated were from Invitrogen (Waverley, VIC, Australia). Protein A agarose was from Roche (Castle Hill, NSW, Australia). Centricon centrifugal filters were from Millipore (North Ryde, NSW, Australia) and QIAexpress heterologous expression system, including Ni-NTA affinity resin and pQE30 vector, from Qiagen (Doncaster, VIC, Australia). Methanol was from Merck (Granville, NSW, Australia).

3.2.2 Polymerase chain reaction (PCR)

PCR was performed as described in section 2.2.6 except that ~20 ng plasmid DNA was used in place of cDNA template.

3.2.3 Cloning of PCR products

Cloning was performed as described in section 2.2.9 except for the following alterations.

3.2.3.1 Restriction endonuclease digestion of DNA

When required, phosphatase treatment was carried out after restriction enzyme digestion.
described in section 2.2.9.2. The digestion reaction was stopped by heat deactivation at 65°C for 15 min and 1U Shrimp Alkaline Phosphatase enzyme and 1x Dephosphorylation Buffer added. The reaction was incubated at 37°C for 30 min before being either heat deactivated again or purified using the QIAquick PCR purification kit.

3.2.3.2 Ligation

When ligating into vectors other than pGEM T-easy, a similar reaction was used to that described in section 2.2.9.4, except that the pGEM T-easy vector was replaced with the vector of choice and 1U T4 DNA ligase and 1x ligation buffer used.

3.2.4 Generation of Y2H constructs

The three candidate genes Hv14.3.3c, HvMAPKK1 and HvFKBP41 were cloned into the pGBK T7 Gal4 binding-domain (BD) bait vector (Fig 3.1) using standard cloning techniques. Briefly, full length candidate genes were amplified from plasmids containing full length cDNA fragments of each (described in chapter 2) using high fidelity PCR. The primers used for each gene were designed to incorporate restriction sites on the ends of the full length fragments, to create compatible restriction sites for cloning into the pGBK T7 bait vector. The primers 14.3.3c Y2H-F and 14.3.3c Y2H-R (Appendix 1) were used to amplify a cDNA fragment corresponding to the full length coding region of Hv14.3.3c from plasmid DNA preparation (section 2.3.1.2) and incorporated a BamHI site at the 5’ end and a NotI site at the 3’ end. Similarly the primers MAPKK Y2H-F and MAPKK Y2H-R (Appendix) were used to amplify the complete coding region of HvMAPKK1 from plasmid DNA preparation (section 2.3.2.6) and incorporated a NdeI site at the 5’ end and an EcoRI site at the 3’ end. Finally, FKPB Y2H-F and FKBP Y2H-R (Appendix) primers were used to amplify the HvFKBP41 ORF from plasmid DNA preparation (section 2.3.3.3) and incorporated an NdeI site at the 5’ end and an EcoRI site at the 3’ end.

Full length HvWound and HvEPSP determined in chapter 4, were cloned into both the pGADT7 Gal4 activation-domain (AD) prey vector and pGBK T7 bait vector. The primers Wound Y2H F and R (Appendix 1) were used to amplify a cDNA fragment corresponding to the full length coding region of HvWound from plasmid DNA preparation (section 4.3.2.2), and incorporated a NdeI site at the 5’ end and a BamHI site at the 3’ end. The primers EPSP Y2H F and R (Appendix 1) were used to amplify a cDNA fragment corresponding to the full length mature peptide of HvEPSP from plasmid DNA preparation (section 4.3.1.3), and incorporated a NdeI site at the 5’ end and a EcoRI site at the 3’ end.
Fig 3.1: Gal4 binding domain (BD) plasmid, pGBK7. The pGBK7 plasmid expresses proteins fused to the Gal4 DNA binding domain (BD) and Myc epitope tag. The candidate genes were inserted into pGBK7 using the multiple cloning site (MCS). Hv14.3.3c was cloned into pGBK7 at the BamHI and NotI sites, HvMapKK1 cloned in at NdeI and EcoRI sites and HvFKBP41 cloned in at NdeI and EcoRI sites.
PCR products were run on agarose gels and the wanted fragments excised from the gel and purified. The fragments and vectors were then digested with the corresponding restriction endonucleases and the fragments ligated into the vectors (Fig 3.1). The constructs were then transformed into *E. coli* DH5α cells via heat shock transformation as described in section 2.2.9.5, except that 50 µg/ml kanamycin was used for selection in place of ampicillin for pGBK7 constructs. Plasmid DNA was extracted and DNA sequencing of the constructs conducted using the sequencing primers MML 3’ BD for pGBK7, MML 3’ AD for pGADT7 and T7 for both (Appendix 1), to check that the inserts were in the correct frame for expression.

### 3.2.5 Generation of a Y2H prey library

The Y2H prey library was produced by Dr K. Oldach (ACPFG, University of Adelaide, Australia). Briefly, a library of A46 infected and NIP1 treated whole leaf and epidermis cDNAs was produced using the Matchmaker Yeast two-hybrid kit according to manufacturer’s instruction. The cDNAs were then cloned into the pGADT7 Gal4 activation-domain (AD) prey vector using the *SmaI* site in the multiple cloning site (Fig 3.2).

### 3.2.6 Bait/prey vector swap

To swap the orientation from prey vector to bait, both Int36 and Int114 were released from pGADT7 with *NdeI* and *BamHI*, and ligated into pGBK7 digested with the same enzymes. For Hv14.3.3c, no restriction sites were common to both vectors that could be used to directly release from pGBK7 and re-ligate into pGADT7, and so Hv14.3.3c had to be re-amplified and cloned into the prey vector. The forward Y2H primer, 14.3.3c Y2H-F, which incorporated a *BamHI* site, was used in conjunction with a second reverse primer 14.3.3c Y2H Sac, which incorporated a *SacI* site, to amplify Hv14.3.3c from plasmid DNA preparation (section 2.3.1.2). This fragment was digested with *BamHI* and *SacI* and ligated into pGADT7, digested with the same enzymes.

### 3.2.7 Yeast Transformation

Yeast transformation *Saccharomyces cerevisiae* strains Y187 and AH109 were streaked from frozen glycerol stocks and grown on YPDA agar plates [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose, 0.01% (w/v) adenine hemi-sulphate with 2% (w/v) agar] at 30°C for 2 days.

To make competent cells for transformation, single colonies were used to inoculate 1 ml YPDA media [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose, 0.01% (w/v) agar]
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Fig 3.2: Gal4 activation domain (AD) plasmid, pGADT7. The pGADT7 plasmid expresses proteins fused to the Gal4 DNA activation domain (AD) and HA epitope tag and also contains a nuclear localisation signal. Prey cDNA’s were cloned into pGADT7 at the SmaI site in the multiple cloning site (MCS).
adenine hemi-sulphate] and cell clumps dispersed by vortexing. The mix was then transferred into a flask containing 50 ml YPDA and incubated at 30°C with shaking at ~200 rpm for 16 h or until an OD<sub>600</sub> greater than 1.5 was reached. 30 ml of the culture was then added to 300 ml YPDA and grown for a further 2 – 3 h until OD<sub>600</sub> of 0.4 – 0.6 was reached. Cells were pelleted by centrifugation at 1000 g for 5 min at room temperature and washed by resuspension in 50 ml sterile Milli-Q water. Centrifugation was repeated and the cells resuspended in a final volume of 1.5 ml fresh TE/LiAc solution [10 mM Tris-HCl buffer (pH 7.5), containing 1 mM EDTA, 100 mM LiAc].

For transformation, 100 µl of the competent cells was added to 1-2 µg of construct plasmid DNA, along with 0.1 mg Herring testes carrier DNA, and the mixture vortexed vigorously. 600 µl fresh PEG/LiAc solution [10 mM Tris-HCl buffer (pH 7.5), containing 40% (w/v) PEG 4000, 1 mM EDTA, 100 mM LiAc] was added and the mix vortexed vigorously before incubation at 30°C for 1 h with shaking at ~200 rpm. After incubation, 70 µl DMSO was added, the solution gently mixed by inversion, and heat shock performed at 42°C for 15 min.

The mixture was chilled on ice for 1 min and cells collected by centrifugation for 30 sec at 16,000 g. Cells were resuspended in 0.1 ml sterile TE buffer [10 mM Tris-HCl buffer (pH 7.5), containing 1 mM EDTA] and plated onto SD agar plates [0.67% (w/v) yeast nitrogen base, 2% (w/v) glucose, 0.07% (w/v) drop out media, with 2% (w/v) agar] containing the appropriate drop out growth selection.

For transformation into the mating strain Y187, the bait construct alone was used and the growth selection was done using SD plates lacking tryptophane (SD<sup>−1</sup>). For co-transformations into AH109 to test self activation, the bait construct and empty pGADT7 activation domain (AD) prey vector (or vice versa) were used in the same amounts (1-2 µg of each) and growth selection was performed using SD plates lacking tryptophan and leucine (SD<sup>−2</sup>). For co-transformation to test interaction, the bait and prey constructs were used in the same amount and the growth selection done using SD lacking tryptophan, leucine, histidine and adenine (SD<sup>−4</sup>). For detection of β-galactosidase activity, and therefore the occurrence of interaction, 20 µg/ml X-α-Gal [5-bromo-4-chloro-3-indolyl-D-galactopyranoside] was added to the appropriate SD plates.

### 3.2.8 Screening for putative protein-protein interactions

To screen for interactors, a protocol modified from the Matchmaker Yeast two-hybrid kit was used as follows. Bait constructs were transformed into the mating strain Y187 as above. After 3 days growth at 30°C on SD<sup>−1</sup> plates an individual colony was transferred to
50 ml SD\(^1\) media [0.67\% (w/v) yeast nitrogen base, 2\% (w/v) glucose, 0.07\% (w/v) drop out media] and grown at 30°C with shaking at ~ 200 rpm for 16 h or until OD\(_{600}\) reached 1.5. The cells were pelleted by centrifugation for 5 min at 1000 g, resuspended in 50 ml YPDA media containing 25 µg/ml kanamycin and transferred to a 2 L culture flask. An aliquot (1.5 ml) of the prey library (\(R.\) secalis infected barley cDNA library:Gal4 (AD) prey construct transformed into AH109) was thawed in warm water and added. The strains were left to mate for 21-24 hours at 30°C with slow shaking of 30-50 rpm. After mating, cells were pelleted by centrifugation at 1000 g for 5 min and washed twice with 50 ml YPDA media containing 25 µg/ml kanamycin, centrifuging as before between each wash. Cells were resuspended in a final volume of 7.5 ml YPDA + 25 µg/ml kanamycin and 200 µl aliquots plated on to 150 mm diameter SD\(^4\) plates. The plates were sealed and incubated upside down for 5-10 days at 30°C. Resultant colonies were then transferred to SD\(^4\) plates containing 20 µg/ml X-α-Gal and incubated for 1-2 days at 30°C to screen for putative protein-protein interactions. Positive (blue) colonies were transferred to SD\(^2\) plates for regeneration and stored at 4°C. Redundant clones were then identified, and prey inserts sequenced as described below.

### 3.2.9 Yeast DNA extraction

#### 3.2.9.1 Zymolase treatment

A match-head amount of cells from positive colonies growing on SD\(^2\) plates from above were transferred to 500 µl H\(_2\)O. The cells were pelleted by centrifugation at 16,000 g for 1 min, and resuspended in 100 µl solution containing 15 U/ml Zymolase enzyme. 25 µl of glass beads (212-300 micron) were added and the sample incubated at 37°C for 30 min. The sample was then mixed via vortex for 1 min and incubated for a further 10 min at 95°C. The sample was chilled on ice for 5 min before centrifugation for 1 min at 16,000 g to pellet cellular debris. The sample was stored at -20°C until required.

#### 3.2.9.2 Phenol/chloroform extraction

An overnight culture of each positive clone was grown by inoculating ~4 ml YPDA media with a single colony and growing overnight at 30°C with shaking at ~200 rpm. The cells were then pelleted via centrifugation at 16,000 g and 200 µl ice cold extraction buffer [50 mM Tris-HCl buffer (pH 7.4), containing 130 mM NaCl, 5 mM EDTA, 5% (w/v) SDS] added. Glass beads, 20 µl, were then added, followed by 200 µl phenol/chloroform/IAA (24:25:1). The solution was then vortexed 3 x 2 min on a platform vortex, incubating on ice for 2 min between each vortex. Cell debris was pelleted via
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centrifugation for 15 min at 16,000 g, and the supernatant transferred to a fresh tube. DNA was then precipitated via the addition of 500 µl 100% ethanol and 20 µl 3 M sodium acetate (pH 5), followed by incubation at -20°C overnight. DNA was pelleted via centrifugation as before, and the pellet washed 2 x in 300 µl 70% (v/v) ethanol. The pellet was air dried for ~ 10 min and resuspended in 20 µl sterile water.

3.2.10 PCR amplification of interacting prey and identity determination

Inserts from putative interacting prey constructs were amplified by PCR to eliminate redundant clones and to facilitate identification of the insert cDNA.

A standard PCR reaction of 15 µl and 50 cycles, using 1µl of yeast DNA, extracted as above, as template and the primers T7 and MML 3’ AD (Appendix 1) was performed. To check for redundancy, 5 µl of the PCR reaction was then digested with HaeIII, and the digested products run on a 3% agarose gel. Clones with the same banding pattern were considered the same and duplicates eliminated. To determine the identity of the remaining putative interactors, the prey construct was transformed into E. coli as described below, plasmid DNA extracted and the insert sequenced using the T7 primer.

Alternatively, a nested PCR using a standard Taq polymerase PCR was used. The Round 1 PCR reaction was performed as described above except that the primers PGA F and PGA R (Appendix 1) were used. For Round 2, a 25 µl reaction using a 1:30 dilution of Round 1 as template and the nested primers T7 and MML 3’ AD was performed. The Round 2 reaction was then used in a digestion as described above, to check for redundancy. To determine the identity of the remaining putative interactors, the Round 2 fragments could be directly sequenced (after PCR purification as described below), without needing to be cloned and transformed into E. coli.

3.2.11 Large scale PCR purification

For large scale, high throughput PCR purification a ‘homemade’ PCR purification system was used in place of the QIAquick PCR purification kit. A 96 well PCR plate was used in place of a column. A small hole was made in the bottom of each tube of the PCR plate, 20 µl glass beads were added to each tube and then 40 µl of Sepharose CL-6B added on top. The plate was spun at 1000 g for 1 min to remove the storage buffer. Subsequently, 40 µl of the PCR reaction from above was added gently to the surface of the sepharose, the plate spun as before, and the purified PCR fragments collected in a fresh PCR plate placed underneath the purification plate.
3.2.12 Transformation into *E. coli*.

Prey construct containing putative interactors were transformed into *E. coli* for preparation of plasmid DNA, sequencing and storage. Extracted prey constructs from section 3.2.9 or purified PCR fragments from section 3.2.11 were used to transform DH5α cells via electroporation (section 2.2.9.6), followed by plasmid DNA preparation (section 2.2.9.8) and sequencing (section 2.2.10).

3.2.13 Self activation and co-transformation to confirm interactions

Putative interactors were tested for self-activation by transformation into AH109 cells with empty bait construct as described above in section 3.2.4. Any self-activating clones were considered to be false interactors and discarded. To confirm interaction with the bait genes, the bait and prey construct were co-transformed into AH109 together, and any prey construct that no longer appeared to interact were considered false positives and discarded.

3.2.14 Generation of Hv14.3.3c *E. coli* expression construct

The Hv14.3.3c/pQE30 expression construct was a kind gift from Dr K. Oldach (ACPFG, University of Adelaide, Australia). Briefly, the full length coding region of Hv14.3.3c (Fig 2.2) was amplified from plasmid DNA (section 2.3.1.2) using a Hi Fidelity PCR. The primers used were designed to incorporate restriction sites on the ends of the full length fragment, to create compatible restriction sites for cloning into the pQE30 expression vector. The primers 14.3.3c pQE-F and 14.3.3c pQE-R (Appendix 1) were used and incorporated a BamHI site at the 5′ end and a NotI site at the 3′ end. The PCR product was electrophoresed on an agarose gel, and the wanted fragment excised from the gel and purified. The fragment and pQE30 vector were then digested with the corresponding restriction endonucleases and the fragment ligated into the vector. The construct was then transformed into *E. coli* M15 chemically competent cells via standard heat shock transformation (section 2.2.9.5), except that 50 µg/ml kanamycin was used for selection as well as 100 µg/ml ampicillin. Plasmid DNA was extracted and DNA sequencing of the construct conducted using the pQE30 sequencing primers pQE_F and pQE_R (Appendix 1), to check that the insert was in the correct frame for expression. A glycerol stock of a positive colony was made for storage (section 2.2.9.7).

3.2.15 Expression induction in *E. coli* using IPTG

The glycerol stock from above was used to inoculate 10 ml LB media containing 100 µg/ml ampicillin and 25 µg/ml kanamycin and grown at 37°C for 16 h with shaking at
~260 rpm. The culture was then added to 90 ml LB media containing 100 µg/ml ampicillin and 25 µg/ml kanamycin and grown for a further 2 hours or until the OD$_{600}$ reached ~0.6. To this, 1 mM IPTG was added and the culture grown for a further 4 h. Cells were harvested by centrifugation at 4500 x g for 15 min and stored at -80°C until required.

### 3.2.16 Purification of His-tagged protein

Native purification of His-tagged proteins was performed using protocols modified from the Ni-NTA affinity purification kit (Qiagen). Induced cells from above were thawed on ice for 15 min and resuspended in 5 ml lysis buffer [50 mM NaH$_2$PO$_4$ buffer (pH 8), containing 300 mM NaCl, 10 mM imidazole]. Lysozyme was added to a concentration of 1 mg/ml and the solution incubated on ice for 30 min. Cells were then sonicated on ice, for 6 x 30 sec or until the lysis solution turned transparent. The solution was centrifuged at 4500 g for 20 min at 4°C to pellet cellular debris and the supernatant was added to 0.5 ml Ni-NTA agarose equilibrated in lysis buffer. The solution was then incubated for 1 h at room temperature or 4°C overnight with gentle mixing on a rotary wheel. The agarose was settled by centrifugation at 300 g and washed 4 times with wash buffer [50 mM NaH$_2$PO$_4$ buffer (pH 8), containing 300 mM NaCl, 20 mM imidazole], settling the resin and removing the supernatant between washes. For more stringent washes if required, a wash buffer containing 40 mM or 60 mM imidazole was used. Bound His-tagged proteins were eluted with 3 x 500 µl aliquots of elution buffer [50 mM NaH$_2$PO$_4$ buffer (pH 8), containing 300 mM NaCl, 250 mM imidazole], settling the agarose via centrifugation as before between each elution and retaining the supernatant, containing the eluted protein, each time. Eluted proteins were concentrated and buffer exchanged into 1 x PBS buffer [1.5 mM KH$_2$PO$_4$ buffer (pH 7.4), containing 5 mM KCl, 150 mM NaCl, 10 mM Na$_2$HPO$_4$] using Centricon centrifugal filters according to manufacturer’s instruction.

### 3.2.17 SDS-polyacrylamide gel electrophoresis (PAGE)

Proteins were analysed using SDS-polyacrylamide gel electrophoresis (Laemmli, 1970). Gels with a resolving layer of 8 – 14% (w/v) and staking layer of 5% (w/v) were routinely used. Protein samples were prepared by addition of 1 x loading dye [45 mM Tris-HCl buffer (pH 6.8), containing 10% (v/v) glycerol, 1% SDS, 0.01% bromophenol blue, 50 mM DTT] followed by boiling for 10 min and chilling on ice for 2 min before loading. Gels were run at 100 V for ~ 2 h in 1 x running buffer [25 mM Tris, 150 mM glycine]
Gels were routinely stained with coomassie stain [40% (v/v) ethanol, 12% (v/v) acetic acid, 0.1% (w/v) coomassie brilliant blue R] for ~1-2 h followed by destaining [40% (v/v) ethanol, 12% (v/v) acetic acid] overnight. For higher resolution, NuPage 4-12% Bis-Tris pre-cast gels were used and run in 1 x NuPage MES SDS Running Buffer.

### 3.2.18 2 dimensional PAGE (2D PAGE)

2D PAGE was performed by the proteomic group, ACPFG, VIC, Australia. Briefly, 250 µg protein sample was loaded on a pH 4-7 IPG strip (Immobiline™ DryStrip gels) and a 10% polyacrylamide gel used for the second dimension.

### 3.2.19 Silver staining of proteins

When a more sensitive detection method was required, silver staining was used. After SDS-PAGE, the gel was incubated in a solution of 50% (v/v) methanol, 12% (v/v) acetic acid o/n at room temperature with gentle shaking. The gel was then washed 3 x 20 min in 95% (v/v) ethanol and fixed in Formaldehyde Fixing Solution [40% (v/v) methanol, 0.05% (v/v) formaldehyde (37%)] for 30 min. After fixing, the gel was washed 3 x 5 min in RO H₂O, incubated for 1 min in 0.02% (w/v) sodium thiosulphate and washed for a further 3 x 1 min in RO H₂O. The gel was then incubated for 10 min in 0.1% (w/v) silver nitrate, washed 3 x 1 min in RO H₂O, and was ready for colour development. Thiosulphate developing solution [3% (w/v) sodium carbonate, 0.1% formaldehyde (37%), 0.04% (w/v) sodium thiosulphate] was added to the gel and incubated with gentle shaking until the bands reached the desired intensity. Developing was stopped by washing in 50% (v/v) methanol, 12% (v/v) acetic acid for 10 min and acetic acid removed by a further wash in 50% methanol for 30 min (Echan and Speicher, 2002).

### 3.2.20 Western blots

Proteins were transferred from polyacrylamide gels to Hypond-P PVDF membranes, using the BioRad electroblotting system, under a constant voltage of 100 V for 1 h. After transfer, membranes were blocked with skim milk powder dissolved in 1 x TBS [0.05 M Tric-HCl buffer (pH 7.4), containing 0.15 M NaCl] at room temperature overnight.

After blocking, membranes were washed 3 x 10 min in 1 x TBS. For detection of His-tagged protein, mouse anti-His-Alkaline Phosphatase conjugated monoclonal antibody was routinely used. The antibody was diluted 1:5000 with 1 x TBS, and incubated on the membrane for ~2 h at room temperature with gentle rocking. The membrane was washed 3 x 10 min in 1 x TBS before the addition of ~2ml NBT/BCIP detection solution. The solution
was incubated on the membrane until the desired colour intensity was reached, and the reaction stopped by rinsing in RO water.

For detection with anti-Hv14.3.3 polyclonal antibody, the polyclonal serum was diluted 1:1000 with 1 x TBS, and incubated with the membrane as above. After washing, an anti-rabbit-AP conjugated monoclonal secondary antibody was added to the membrane and, incubation, washing and colour detection performed as above.

3.2.21 Antibody production

A polyclonal rabbit anti-Hv14.3.3c antibody was produced by the IMVS. The antibody was raised against a synthetic peptide synthesised by Auspep, (Parkville, VIC, Australia), designed to a specific, non-conserved region of Hv14.3.3c. Peptides were designed with the aid of a Protein Hydrophobicity Plot [http://www.vivo.colostate.edu/molkit/hydropathy/index.html] and antigen profiler (http://www.openbiosystems.com/Blog.aspx?ArticleId=81).

3.2.22 Antibody purification

The HiTrap NHS-activated HP affinity column was used for purification of anti-Hv14.3.3c antibody for polypeptide serum. Briefly, the column was prepared by binding of the peptide to which the antibody was raised, according to manufacturer’s instruction. Polyclonal serum was then passed through the column to allow binding of the anti-Hv14.3.3c antibody to the peptide. Unbound components of the serum were then washed away and the anti-Hv14.3.3c antibody eluted from the column using low pH [0.1 M glycine, pH 2.5].

The antibody was then exchanged into 1 x PBS buffer and concentrated using Centricon centrifugal filters according to manufacturer’s instruction.

3.2.23 Affinity Pull-downs using recombinant Hv14.3.3c

3.2.23.1 Dynabeads

Dynabeads M-208 tosylactivated were prepared by washing 3 x 5 min in 1 X PBS on a rotary wheel. Purified Hv14.3.3c protein, prepared as described in sections 3.2.13-15 was then added to the Dynabeads at 3 µg protein per 10^7 beads and incubated o/n at 4°C with gentle mixing on a rotary wheel. Unbound protein was removed by washing 3 x 5 min with 1 x PBS containing 0.1 – 1% BSA.

Plant protein extract was prepared by grinding ~100 mg (either Atlas or Atlas46 9 day old seedlings, control or R. secalis 24 h infected) leaf tissue in a mortar and pestle under liquid
nitrogen. Ground tissue was then added to ~ 5 ml Extract Buffer [50 mM HEPES-OH buffer (pH 7.5) containing 50 mM NaF, 1 mM PMSF, 1 mM benzamidine, 5 mM NaPPi, 1 mM DTT and 1% PVP] containing protease inhibitor cocktail, incubated for ~1 h at 4°C with gentle mixing and then centrifuged at 13,000 g for 5 min. The supernatant was then added to the Hv14.3.3c coupled Dynabeads and incubated for ~ 2 h at 4°C on a rotary wheel. The beads were again washed 4 x 5 min with 1 x PBS or until all unbound proteins removed. Bound proteins were eluted with Elution Buffer [0.1 M glycine, pH 2.5] and the pH of the elution neutralised using 1 M NaH$_2$PO$_4$. For control samples, BSA-bound Dynabeads were used in place of Hv14.3.3c linked Dynabeads.

3.2.23.2 Sepharose column

Affinity-pulldown experiments were also performed using HiTrap NHS-activated HP 1 ml columns according to manufacturer’s instruction. Briefly, columns were prepared by washing with 3 x 2 ml ice cold 1 mM HCl. Purified Hv14.3.3c, 5 mg, was buffer exchanged into Coupling Buffer [0.2 M NaHCO$_3$ buffer (pH 8.3), containing 0.5 M NaCl] using Centricon centrifugal filters, added to the column and incubated for 30 min at room temperature. The column was then washed and deactivated by 6 x 2 ml washes, alternating between Wash Buffer A [0.5 M ethanolamine, 0.5 M NaCl, pH 8.3] and Wash Buffer B [0.1 M acetate, 0.5 M NaCl, pH 8.3], incubating the column in Wash Buffer A for 30 min at room temperature at wash 3.

The column was then prepared for affinity-pulldown by washing with 3 ml Extraction Buffer [50 mM HEPES-OH buffer (pH 7.5), containing 50 mM NaF, 1 mM PMSF, 1 mM benzamidine, 5 mM NaPPi, 1 mM DTT and 1% PVP], 3 ml Elution Buffer 2 [100 mM glycine, pH 2.5] and 10 ml Extract Buffer, before the plant extract was added. Plant extract was prepared as described in section 3.2.22.1, added to the column and incubated for ~ 1 h at room temperature. Unbound proteins were washed from the column using 5 x 1 ml Extract Buffer. Bound proteins were eluted from the column twice, first using 1 ml Elution Buffer 1 [1 mM Hv14.3.3c Raf-1 phosphopeptide in 1 x PBS] and then 1 ml Elution Buffer 2, washing with 5 x 1ml Extract Buffer in between. After elution the column was re-equilibrated in Extract Buffer by washing with 1 x 3 ml and re-used for the subsequent samples.

For a control, a column that had been washed and deactivated without prior binding of Hv14.3.3c protein was used.
3.2.23.3 Sepharose resin

A modified protocol to that used by Moorhead et al. (1999) was also attempted. CH-Sepharose 4B was prepared according to manufacturer’s instruction, similarly to that done for the Sepharose columns, and 5 mg purified Hv14.3.3c protein bound to 5 ml swollen sepharose. After ligand binding the resin was blocked and deactivated by incubation in either 1 M ethanolamine (pH 8) or 0.1 M Tris-HCl buffer (pH 8) for 1 h.

Plant protein extract was prepared as in section 3.2.23.1, except that 250 g of tissue, a mixture of Atlas and Atlas46, infected and non-infected, was prepared in 100 ml Extract Buffer containing 500 μM MgATP and the extract buffer exchanged into Coupling Buffer [50 mM Hepes-OH buffer (pH 7.5), containing 1 mM DTT] using dialysis. Hv14.3.3c-sepharose and plant extract were incubated at 4°C o/n with gentle mixing. Unbound proteins were washed from the resin with 5 x 50 ml Buffer C containing 500 mM NaCl.

Bound proteins were eluted once with 1 ml 100 mM glycine (pH 2.5). Sepharose bound with BSA in place of Hv14.3.3c was used as a control.

3.2.24 Affinity-pulldowns using anti-Hv14.3.3c antibody

Plant protein extract was prepared as in section 3.2.23.1 using ~ 500 mg leaf material (a mixture and Atlas and Atlas46, infected and non infected tissue) in 10 ml Extract Buffer. The plant extract supernatant was split in half (~ 4 ml) and 2.5 μg anti-Hv14.3.3c antibody (purified as described in section 3.2.21) added to one sample, and 2 μg commercial mouse anti-myc antibody added to the other as a control. Plant extract and antibody were incubated at 4°C for ~3 h with gentle mixing. Protein A agarose was prepared by equilibrating in Extract Buffer before 50 μl was added to each sample. The mixture was then incubated o/n as before. Unbound proteins were removed by washing the Protein A agarose with 5 x 2 ml Wash Buffer [20 mM NaH₂PO₄ buffer, pH 8.3]. Bound proteins were eluted from the agarose with 500 μl 100 mM glycine (pH 2.5).

3.2.25 Mass-spectrometry

Mass-spectrometry was performed by Dr J. Patterson (ACPFG, Melbourne, Australia). Briefly, MS and MS/MS data was acquired using a nanospray source on a QStar XL hybrid quadrupole-TOF LC-MS/MS (Applied Biosystems/MDS Sciex, U.S.A.) using the AnalystQS software (Applied Biosystems/MDS Sciex) operating in a data-dependent acquisition mode.
3.3 Results and discussion

3.3.1 Y2H screens

The first technique employed to identify proteins that interacted with Hv14.3.3c, HvMAPKK1 or HvFKBP41 was yeast two-hybrid screening.

3.3.2 Hv14.3.3c Y2H screen

3.3.2.1 Identifying interactors

The Hv14.3.3c/pGBKT7 construct was first tested for self activation before being used as bait in a screen and was found to be negative for self activation (Fig 3.5). The bait construct was then screened against a \textit{R. secalis} infected barley cDNA prey library. Around 20\% of the selected colonies resulting from the mating showed \(\beta\)-galactosidase activity, suggesting interaction was occurring (Fig 3.3). DNA was extracted from the selected positive clones (27) and PCR amplification followed by digestion of the inserts performed to determine the number of unique clones and to eliminate redundancy, based on the insert size and digestion pattern. Only three clones were eliminated based on clear redundancy (Fig 3.4). The remaining clones were transformed into \textit{E. coli}, with 19 clones successfully transformed, and the inserts sequenced (Table 3.1). The sequences were first analysed for the presence of an in-frame protein, that is, the sequence can only be translated in one frame in the yeast, so the sequence was analysed to determine whether translation in this frame produced a putative protein. Often the sequence produced a non-sense protein or contained many stop codons when translated in this frame and was therefore disregarded. The putative identity of inserts in the remaining clones that produced in-frame proteins (7) was then determined via protein blast analysis using NCBI. The final elimination step was to test for self activation, where a further 4 clones were disregarded as they were self activating (data not shown) and therefore produced a false positive interaction with Hv14.3.3c, leaving 3 potential interactors, Int36, Int96 and Int114 (Table 3.1).

A common type of false positive in Y2H screens are so called ‘sticky proteins’ that result from AD-proteins which seem to have intrinsic properties that make them more likely to bind to multiple partners. They are often only identified when carrying out multiple screens with different baits in the same AD-prey library (Vidalain et al., 2004). After multiple isolations, irrespective of bait, during many screens conducted with this same prey library (data not shown), it was decided that Int92, identified to encode part of
Fig 3.3: Selection of putative interacting proteins from mating with bait Hv14.3.3c/pGBKT7. Colonies showing β-galactosidase activity (blue) on SD-4 x-α-Gal plates. 27 were selected for further analysis and named based on their grid position.
Fig 3.4: Agarose gel analysis of HaeIII digested yeast DNA. 1 – 27 Digested DNA from 27 positive colonies from Fig 3.3. Clones in lanes 8 and 14 were eliminated based on redundancy to clone in lane 6 (red arrows) and clone 23 eliminated based on redundancy to clone in lane 5 (blue arrows).
Lane 1: colony 2, Lane 2: colony 6, Lane 3: colony 12, Lane 4: colony 13,
Lane 5: colony 16, Lane 6: colony 17, Lane 7: colony 18, Lane 8: colony 19,
Lane 9: colony 22, Lane 10: colony 36, Lane 11: colony 38, Lane 12: colony 58,
Lane 13: colony 64, Lane 14: colony 67, Lane 15: colony 73, Lane 16: colony 92,
Lane 17: colony 114, Lane 18: colony 120, Lane 19: colony 123, Lane 20: colony 127,
Lane 21: colony 129, Lane 22: colony 133, Lane 23: colony 137, Lane 24: colony 138, Lane 25: colony 139, Lane 26: colony 140, Lane 27: colony 141, based on grid position from Fig 3.3.
Fig 3.5: Confirming Hv14.3.3c interactions using Y2H. A) Hv14.3.3c interaction with original Int36 (HvEPSP) and Int114 (HvWound) prey, and self activation test. B) Reverse orientation interaction with Hv14.3.3c in pGADT7 and Int36 and Int114 in pGBK7, and self activation test. C) Hv14.3.3c interaction with full length mature HvEPSP and full length HvWound, and self activation test.
Table 3.1: Correct reading frame, putative identity and self activation of putative Hv14.3.3c interactors. Red crosses represent an insert in the incorrect frame or non-self activating, and green tick the opposite. Encircled clones are those determined to be of most interest.

<table>
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<th>Clone</th>
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<th>Putative Identity</th>
<th>Self activation</th>
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</tr>
<tr>
<td>6</td>
<td>✗</td>
<td></td>
<td></td>
</tr>
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<td>12</td>
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</tr>
<tr>
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<td></td>
<td></td>
</tr>
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<td></td>
</tr>
<tr>
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<td>Glucan endo-1,3-beta-glucosidase</td>
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<td>✗</td>
<td></td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>✓</td>
<td>5-enolpyruvylshikimate-3-phosphate synthase</td>
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</tr>
<tr>
<td>38</td>
<td>✓</td>
<td>40S ribosomal</td>
<td>✓</td>
</tr>
<tr>
<td>58</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>64</td>
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<td>73</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>92</td>
<td>✓</td>
<td>Alpha-tubulin</td>
<td>✗</td>
</tr>
<tr>
<td>114</td>
<td>✓</td>
<td>Putative wound induced gene</td>
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</tr>
<tr>
<td>120</td>
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<td></td>
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an alpha-tubulin protein, was a ‘sticky’ proteins, and was therefore disregarded.

Based on sequence homology, Int36 was identified to encode a partial fragment of 5-enolpyruvylshikimate-3-phosphate synthase, also known as EPSP synthase, the penultimate enzyme of the shikimate pathway, catalysing the reversible addition of the enolpyruvyl moiety of phosphoenolpyruvate to shikimate 3-phosphate and is also the target of the broad spectrum herbicide glyphosate (Della-Cioppa et al., 1986). There have been no previous reports of a 14.3.3 interacting with an EPSP synthase or being involved with the shikimate pathway. There are, however numerous lines of evidence to suggest that EPSP synthase and the shikimate pathway are involved in the plant defence response, supporting the possibility for a role of a 14.3.3/EPSP synthase interaction during the defence response. Firstly, a well established defence mechanism of plants to pathogenic and other stress is the synthesis of secondary metabolites. EPSP synthase is a critical enzyme in the production of chorismate, the end product of the shikimate pathway and the precursor of the three aromatic amino acids, as well as other essential aromatic compounds, including secondary metabolites (Herrmann 1999). One secondary metabolite derived from the three aromatic amino acids is lignin, the second most abundant biopolymer that not only imparts mechanical strength to all plants but also plays vital roles in plant defence against microorganisms, in wound healing, in maintenance of structural integrity, and in water transport capacity (Herrmann 1995). Other secondary metabolites include the phytoalexins, low-molecular-weight antimicrobial compounds that accumulate in plants as a result of infection or stress and are associated with resistance in plants to diseases caused by fungi and bacteria (Kuc 1995). Therefore, it may be possible that the interaction of EPSP synthase with Hv14.3.3.c may be linked in some way to the production of secondary metabolites during the defence response.

Secondly, as well as being involved in the production of secondary metabolites, there is also further evidence of EPSP synthase and shikimate pathway involvement in the defence response. For example, the activity levels of two isoforms of EPSP synthase were found to increase in cultured maize cells upon fungal elicitor treatment (Forlani 2002), while an EPSP synthase, MAP kinase and an immunophilin were all found to be differentially expressed upon infection of barley with powdery mildew (Eckey et al., 2004), suggesting not only a role in the defence response, but possibly a link between the candidate genes Hv14.3.3c, HvMAPKK1 and HvFKBP41 and EPSP synthase.

Finally, EPSP synthase, and in fact the entire shikimate pathway, is localised in the chloroplast (Herrmann 1999), therefore, it may be unlikely that EPSP synthase could interact with Hv14.3.3c. However, 14.3.3s have been found to be present in chloroplasts
Chapter 3 – Identifying interacting proteins using yeast two-hybrid screening and affinity-pulldowns

(Sehnke et al., 2000), which would make this interaction possible. Similarly, it is known that EPSP synthase is synthesised as a precursor protein in the cytosol, before being translocated into the chloroplast (Della-Cioppa et al., 1986; Hollander-Czytok and Amrhein 1987). Therefore it may be possible that EPSP synthase and Hv14.3.3c interact during this time, or even that Hv14.3.3c, with 14.3.3s known to play a role in protein localisation (Chapter 1), is involved in EPSP synthase translocation.

Int114 showed highest sequence similarity to that of a rice gene, named a putative wound responsive gene. Little is known about this uncharacterised gene, however it was first identified as an EST showing differential expression during the rice/rice blast fungus interaction (Jantasuriyarat et al., 2005).

Wound and defence responses are intimately connected, since many pathogens create, or enter through wounds in the epidermis (Dyer et al. 1989), therefore, although this gene has been named wound responsive, it may in fact also play a role in other defence responses.

A study by Keith et al (1991) showed that levels of DAHP synthase, the first enzyme in the shikimate pathway, increased in Arabidopsis leaves subjected to either wounding or infiltration with pathogenic Pseudomonas syringea strains. A similar study showed the same response to wounding in potato and tomato tissue (Dyer et al., 1989). It may be possible therefore, that Hv14.3.3c, the shikimate pathway, EPSP synthase and a wound responsive gene are all involved in the plants response to fungal infection.

With over 300 different 14.3.3 target proteins having been identified to date (Kjarland et al, 2006), it may have been expected that the Y2H screen would identify more putative interactors, including some of the interactors previously reported. However, the interaction studies reported so far have mainly been based on two different techniques, namely affinity purification of interacting proteins from cell lysates by immobilized recombinant 14.3.3 proteins, such as that also attempted in this study (section 3.3.7), and immunoprecipitation of overexpressed tagged 14.3.3 from cells (Kjarland et al, 2006), with very little work using the Y2H system having been reported, especially in plant systems. Most work utilising the Y2H system has only been used to confirm and analyse previously identified interactions (Bornke 2005), and very few large-scale Y2H screens using 14.3.3s as baits, have been undertaken (Schoonheim et al., 2007b; Zhang et al., 1997a). In a study by Zhang et al (1997a) where an Arabidopsis 14.3.3 was used as a bait in a Y2H screen, only ‘several’ interactors were identified, with only one, a 5-hydroxyferulic acid O-methyltransferase, named. In the Schoonheim et al (2007b) study, while over 130 interactors of barley 14.3.3s were identified, all five 14.3.3s, A through E, were used as baits and the interactors of 14.3.3c alone were not specified.
Similarly to that discussed for HvMAPKK1 (section 3.3.3), phosphorylation state may have played a role in failure to identify more interactors. Phosphorylation state is important for 14.3.3 interactions, as binding of 14.3.3 to target proteins is most commonly through specific phosphorylated target sites. Therefore, if the phosphorylation state in the yeast cell nucleus is incorrect, interaction between Hv14.3.3c and some targets may not occur.

14.3.3 proteins also exist as dimers, where the monomers come together to form a broad central groove or channel that contains binding sites for target proteins. As it is known that the addition of the AD or BD fusion moiety can inhibit the normal site of interaction, or may impair the proper folding of the protein (Fields and Sternglanz 1994), it may also be possible that the 14.3.3c protein or its interactor was sterically inhibited to properly interact.

Although over 300 14.3.3 target proteins have been identified, this is the first report of interaction with an EPSP synthase. The main reason for this is likely to be that a majority of 14.3.3 interaction studies, especially the earlier studies, were done in animal systems where a shikimate pathway does not exist. The fact that the fungal infected prey library used in this study is different to that used in other studies, and therefore the composition of prey cDNAs is different, may also be a potential reason.

It is also thought that, although there are many different isoforms of 14.3.3s that have many conserved properties, there is specificity in the interaction with different isoforms (Aitken 1996; Wu et al., 1997). Therefore the interaction with EPSP synthase may be 14.3.3c specific, and, as only one study to date, by Schoonheim et al (2007b), has investigated 14.3.3c interactions in barley, not all possible interactions may have been found.

The artificial nature of the Y2H system may be another reason why this interaction has not been identified in other interaction studies. The overexpression of proteins in the Y2H system can lead to identification of interactions that may normally only occur in very low abundance, which may be too low to detect in other systems such as affinity-pulldowns. Similarly, certain interactions may only occur very briefly, or under certain conditions or at certain times in the natural environment. For example, in the case of HvEPSP, interaction with Hv14.3.3c may only occur for a brief time while EPSP is in the cytosol (Della-Cioppa et al., 1986; Hollander-Czytok and Amrhein 1987). In affinity-pulldown systems the tissue being used would have to have been taken from a plant at the right time, or under the correct conditions, when this interaction occurs, or it would not be detected, while in the more forced nature of the Y2H system, this interaction is more likely to occur and be detected.
3.3.2.2 Confirming the interaction

To confirm the original interaction with the partial clones, Int36 and Int114 were checked again for interaction with Hv14.3.3c via co-transformation using the Y2H system, and the interaction was confirmed (Fig 3.5A). They were also re-tested for self-activation via co-transformation with empty pGBK7 vector and found not to be self-activating (Fig 3.5A).

To further investigate the interaction, the orientation of the interacting bait and prey vectors was swapped, that is, \( \text{Hv14.3.3c} \) was cloned into the prey vector pGADT7 and the two putative interactors were cloned into pGBK7. Upon co-transformation, no activity was found (Fig 3.5B), suggesting that the proteins no longer interacted in this orientation. However, it is known that fusing proteins to the activation or binding domain can disrupt structure or function, and it is not uncommon that interactions seen in one orientation (i.e. A as bait, B as prey) are not always seen in the other (i.e. B as bait, A as prey) (Aloy and Russell 2002). Therefore, disruption of one or both of the proteins in the interaction pair may occur in this orientation and hence an interaction was not seen.

Next, the putative interaction between Hv14.3.3c and the mature protein encoded by Int36 and Int114 was assessed. The full length coding sequence of Int114 (named \( \text{HvWound} \)) and mature peptide sequence of Int36 (named \( \text{HvEPSP} \)) was determined as described in Chapter 4, and prey constructs containing these produced. It was found that the complete HvWound protein still interacted with Hv14.3.3c, however the HvEPSP mature peptide did not (Fig 3.5C). It was thought that this may have been a result of using a truncated form of the protein. As discussed in Chapter 4, the full length EPSP synthase gene contains an N-terminal chloroplastic signal peptide, which could have interfered with the Y2H system, and was therefore not included in the full length sequence. This may have led to improper folding of the HvEPSP protein, inhibiting the interaction. It has also been shown that 14.3.3s can interact with chloroplastic signal peptides (Sehnke et al., 2000), and therefore omission of this signal peptide in HvEPSP may also have effected the ability to interact.

Interaction of Hv14.3.3c with the whole proteins of the interactors was not tested in the reverse orientation, as it was thought that if the partial proteins did not interact in this configuration, possibly due to interference, then it may be likely that the full proteins may not be able to either.
3.3.3 HvMAPKK1 Y2H screen

3.3.3.1 Identifying interactors

HvMAPKK bait was found to be negative for self activation (Fig 3.8) before used to screen the prey library, and 48 positive colonies resulting from the mating were selected (Fig 3.6). Of these, 17 clones were eliminated based on redundancy (Fig 3.7), and the remaining 31 clones were selected for transformation into *E. coli* for identification. All transformations were unsuccessful, possibly due to the zymolase DNA extraction producing a very low amount of DNA (data not shown) and also because the DNA produced from the zymolase extraction is not very pure, which can affect the efficiency of the transformation. Because of this, yeast DNA extraction for the 31 clones was repeated, this time using the phenol/chloroform method as this method is thought to recover more DNA that is a lot more pure than that of the zymolase method. This time only 6 clone transformations were unsuccessful, possibly due to the extracted DNA again, however it is also possible that the clones produced proteins that were toxic to *E. coli* and therefore no transformed *E. coli* colonies were obtained. As in the Hv14.3.3c screen the inserts of the putative interactors were sequenced, production of an in-frame protein analysed, putative identity determined and self-activation tested (Table 3.2). This left only two putative interactors, Int8 and Int59.

3.3.3.2 Confirming the interaction

To confirm the original interaction, Int8 and Int59 were checked again for interaction with HvMAPKK1. However, it was found that the two putative interactors no longer interacted with HvMAPKK1 (Fig 3.8). A reason for this may be that Y2H interactors can be wrongly identified due to the presence of two or more different plasmids in the cells of a single yeast colony, of which only one encodes a genuine interactor (Vidalain et al., 2004). *E. coli* cells mostly only accept one plasmid during transformation (Weston et al., 1979), therefore it may be possible at this step that the plasmid containing the interacting prey was lost. It may also be that colonies were mixed up during the procedure, or that insufficient selection was not used at a particular point, leading to escape of false positives.

3.3.3.3 Repeat screen

The HvMAPKK1 screen was repeated twice, however was again unsuccessful at identifying any interactors, with self-activators very prominent. It was thought that the lack of interaction being detected may be due to the phosphorylation state of the MAP kinases being expressed in yeast, as MAP kinases only become active once specific tyrosine,
Fig 3.6: Selection of putative interacting proteins from mating with bait HvMAPKK1/pGBKT7. Colonies showing β-galactosidase activity (blue) onto SD-4 x-α-Gal plates, 48 were selected for further analysis and named based on their grid position.
Fig 3.7: Agarose gel analysis of *HaeIII* digested yeast DNA. 1 – 48

Digested DNA from 48 positive colonies from Fig 3.6. 17 clones were eliminated based on redundancy, with redundant clones indicated with coloured arrows.

Lane 1: colony 4, Lane 2: colony 8, Lane 3: colony 10, Lane 4: colony 17, Lane 5: colony 19, Lane 6: colony 25, Lane 7: colony 30, Lane 8: colony 31, Lane 9: colony 33, Lane 10: colony 35, Lane 11: colony 37, Lane 12: colony 39, Lane 13: colony 43, Lane 14: colony 46, Lane 15: colony 48, Lane 16: colony 56, Lane 17: colony 59, Lane 18: colony 61, Lane 19: colony 70, Lane 20: colony 71, Lane 21: colony 74, Lane 22: colony 83, Lane 23: colony 94, Lane 24: colony 101, Lane 25: colony 108, Lane 26: colony 110, Lane 27: colony 111, Lane 28: colony 114, Lane 29: colony 116, Lane 30: colony 120, Lane 31: colony 135, Lane 32: colony 143, Lane 33: colony 144, Lane 34: colony 148, Lane 35: colony 155, Lane 36: colony 156, Lane 37: colony 157, Lane 38: colony 160, Lane 39: colony 161, Lane 40: colony 162, Lane 41: colony 163, Lane 42: colony 166, Lane 43: colony 173, Lane 44: colony 176, Lane 45: colony 180, Lane 46: colony 183, Lane 47: colony 186, Lane 48: colony 194, based on grid position from Fig 3.6.
**Fig 3.8: Confirming HvMapKK1 interactions.** HvMapKK1 interaction with original Int59 and Int8 prey, and self activation test.
Table 3.2: Correct reading frame, putative identity and self activation of putative HvMAPKK1 interactors. Red crosses represent an insert in the incorrect frame or non-self activating, and green tick the opposite. Encircled clones are those determined to be of most interest.

<table>
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<td>Immunophilin:peptidyl-prolyl cis-trans isomerase</td>
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<tr>
<td>194</td>
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</table>
threonine or serine residues are phosphorylated (Mizoguchi et al., 1997; Zhang et al., 2006). If the phosphorylation state was not correct, activation and therefore the ability to bind to target proteins could be compromised. *S. cerevisiae* is thought to possess endogenous serine/threonine kinases (Lim et al., 1993), which are present in the nucleus, but no genes with homology to known tyrosine kinases have been identified in yeast to date. However, work by Schieven et al (1986) described tyrosine kinase activity in yeast, while Zhu et al (2000) found that several Ser/Thr kinases of yeast could phosphorylate tyrosine. Therefore, if yeast does contain tyrosine kinases or if tyrosine is phosphorylated by other means, HvMAPKK1 phosphorylation should not have been affected.

It may also be that, while upstream in the MAP kinase pathway, MAPKKKs may integrate a variety of receptor signals via activation through physical interaction with other proteins such as receptors (Hirt 1997; Nakagami et al., 2005), and downstream MAPKs may interact with many different proteins to effect gene expression (Lalle et al., 2005; Hirt 1997), in the middle of the pathway, MAPKK may not interact with many, if any, proteins other than pathway MAP kinases. As a Y2H screen is not comprehensive, nor are MAPKs and MAPKKKs abundant transcripts, the chance of identifying a real interaction might be very low and only numerous additional screens might have produced such an interactor to be identified.

### 3.3.4 HvFKBP Y2H screen

#### 3.3.4.1 Identifying interactors

To try to overcome some of the problems that occurred in the Hv14.3.3c and HvMAPKK1 Y2H screens, the method was changed slightly. To address the problem of unsuccessful transformation into *E. coli* for identification, a nested PCR amplification step was introduced as described in section 3.2.9. This allowed for amplification, dilution and re-amplification of the prey inserts, which meant that they could then be directly sequenced, without cloning into *E. coli* first. Analysis of yeast plasmid DNA can often be difficult, thought to be due to the poor quality and low purity of the purified yeast plasmid DNA, with preparations often containing significant amounts of contaminants, such as residual phenol, cellular proteins and polysaccharides (Caldwell and Becker, 1993), and requires cloning into *E. coli* first. The nested PCR step overcomes the problem of not being able to directly sequence from the yeast DNA. It also allows for insert identification before the transformation step, meaning uninteresting clones, such as known self-activators, interactors or sticky proteins, can be quickly eliminated. This change to the method also improved elimination of redundancy. The Round 2 PCR reaction could be used for *HaeIII*
digestion, and, as it contained a greater amount of amplified product, and often a more specific product, digested fragments were a lot more visible and gave a clear banding pattern, making it easier to compare, identify and eliminate redundant clones.

HvFKBP/pGBKT7, found to not be self-activating (Fig 3.11), was used to screen the *R. secalis* infected barley cDNA prey library, using the modified method. After the mating, 73 resultant positive clones were selected (Fig 3.9), and 20 redundant clones eliminated (Fig 3.10). The remaining clones were then identified by direct sequencing of the Round 2 PCR fragment. For some clones, the sequence quality obtained was poor and they could not be identified. Re-sequencing of the fragments was unsuccessful, and it was thought that the poor quality was possibly due to the PCR fragment being too small to directly sequence, which suggests the clone contained little or no prey insert. It may also have been due to the presence of multiple fragments in the reaction, the product of non-specific amplification during the PCR reaction, or possibly the presence of multiple prey constructs in the one yeast cell, both of which are unable to be directly sequenced. Translation of in-frame proteins was analysed, eliminating 9 clones, while self-activation eliminated a further 3, leaving 6 different non-self-activating putative interactors (Table 3.3).

Transcribed in the correct frame, Int7 and Int23 encoded proteins of 160 and 150 aa. However, when trying to assign identity, they were found to show no homology to known database sequences, neither plant nor fungal, and it was therefore decided that they were non-sense proteins.

After having been isolated in screens with different baits (own screens and screens of Dr K. Oldach, personal communication), it was decided that Int6, encoding part of a protease inhibitor and Int125 and Int128, found to be identical and to encode a beta-expansin, were ‘sticky’ proteins, and therefore were disregarded.

This left two putative interactors, Int63 and Int156. Interestingly, Int156 was the same as Int114, identified as a putative Hv14.3.3c interactor, encoding a putative wound responsive gene. It could be possible that this protein is sticky, occurring multiple times with different baits, and therefore not a true interactor. However, it was not isolated in the HvMAPKK1 screen, nor during any other screen with this library, suggesting it may be a true interactor and therefore a possible association between Hv14.3.3c and HvFKBP41, possibly linking them to the same signalling pathway.

Int63 was identified as a Rab type GTP-binding protein. GTP-binding proteins, also known as G proteins or small GTPases, are involved in guanine nucleotide binding and hydrolysis and constitute a superfamily consisting of more than 100 members, classified into at least five sub families: the Ras, Rho, Rab, Sar1/Arf, and Ran families. They are important signal
Fig 3.9: Selection of putative interacting proteins from mating with bait HvFKBP41/pGBKT7. Colonies showing β-galactosidase activity (blue) on SD-4 x-α-Gal plates were considered positive, with 73 selected for further analysis and named based on their grid position.
Fig 3.10: Agarose gel analysis of HaeIII digested yeast DNA. 1 – 73 Digested DNA from 73 positive colonies selected from Fig 3.9. 20 clones eliminated based on redundancy with redundant clones indicated with coloured arrows.
Lane 1: colony 2, Lane 2: colony 5, Lane 3: colony 6, Lane 4: colony 7, Lane 5: colony 8, Lane 6: colony 11, Lane 7: colony 14, Lane 8: colony 17, Lane 9: colony 21, Lane 10: colony 23, Lane 11: colony 24, Lane 12: colony 26, Lane 13: colony 27, Lane 14: colony 28, Lane 15: colony 29, Lane 16: colony 30, Lane 17: colony 33, Lane 18: colony 36, Lane 19: colony 37, Lane 20: colony 39, Lane 21: colony 43, Lane 22: colony 47, Lane 23: colony 53, Lane 24: colony 55, Lane 25: colony 58, Lane 26: colony 60, Lane 27: colony 63, Lane 28: colony 70, Lane 29: colony 72, Lane 30: colony 74, Lane 31: colony 77, Lane 32: colony 79, Lane 33: colony 80, Lane 34: colony 84, Lane 35: colony 86, Lane 36: colony 97, Lane 37: colony 99, Lane 38: colony 100, Lane 39: colony 101, Lane 40: colony 102, Lane 41: colony 103, Lane 42: colony 109, Lane 43: colony 110, Lane 44: colony 113, Lane 45: colony 117, Lane 46: colony 118, Lane 47: colony 119, Lane 48: colony 120, Lane 49: colony 125, Lane 50: colony 126, Lane 51: colony 127, Lane 52: colony 128, Lane 53: colony 129, Lane 54: colony 130, Lane 55: colony 133, Lane 56: colony 134, Lane 57: colony 136, Lane 58: colony 137, Lane 59: colony 138, Lane 60: colony 140, Lane 61: colony 144, Lane 62: colony 145, Lane 63: colony 146, Lane 64: colony 148, Lane 65: colony 150, Lane 66: colony 153, Lane 67: colony 154, Lane 68: colony 155, Lane 69: colony 156, Lane 70: colony 157, Lane 71: colony 159, Lane 72: colony 160, Lane 73: colony 163, based on grid position from Fig 3.9.
**Fig 3.11: Confirming HvFKBP41 interactions.** HvFKBP41 interaction with original Int63 (HvGTPase) and Int156/114 (HvWound), and self activation test.
Table 3.3: Correct reading frame, putative identity and self activation of putative HvFKBP41 interactors. Red crosses represent an insert in the incorrect frame or non-self activating, and green tick the opposite. Encircled clones are those determined to be of most interest. The question mark represents clones that had an unclear frame.

<table>
<thead>
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</tr>
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<td>Putative protease inhibitor</td>
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</tr>
<tr>
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<tr>
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<td>×</td>
</tr>
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<td>×</td>
<td></td>
<td></td>
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<tr>
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</tr>
<tr>
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<td></td>
<td></td>
</tr>
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<td><strong>FusA conserved domain containing</strong></td>
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</tr>
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</tr>
<tr>
<td>145</td>
<td>×</td>
<td></td>
<td></td>
</tr>
<tr>
<td>148</td>
<td>×</td>
<td></td>
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</tr>
<tr>
<td>156</td>
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<td><strong>Putative wound induced protein</strong></td>
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transduction molecules, involved in secondary messenger cascades and regulate a wide variety of cell functions. The Rab family is the largest family, with 57 Rabs identified in Arabidopsis (Pereira-Leal and Seabra 2001) and is also known to regulate intracellular vesicle trafficking (Takai et al., 2001; Dombrowskia et al., 2007).

Following perception of elicitor signals or PAMPs, plant receptors are activated, which then in turn activate their effectors. Effectors transfer the elicitor signals to second messengers which further amplify the elicitor signal to other downstream reactions. GTP binding proteins are one known type of such effector (Zhaoa et al., 2005), supporting a potential role in defence response signalling and linking HvFKBP to this role through this putative interaction. Elicitor signal transduction pathways also lead to production of plant secondary metabolites, which may also link the HvFKBP/GTP binding protein interaction, with the Hv14.3.3c/EPSP synthase interaction, thought to have a possible role in secondary metabolite synthesis as discussed earlier.

Further lines of evidence that support a role for a GTP-binding protein in defence response signalling include a study by Nahm et al (2003), which showed a Rab type GTP-binding protein from rice was differentially regulated by cold, salt, dehydration, and ABA, while a rice homologue with 93% sequence similarity to that of Int63 was identified as a cDNA induced by Magnaporthe grisea infection (Meng et al unpublished). Y2H screening of a tomato cDNA library for interactors with Pseudomonas syringae avirulence factors identified two small GTPases in tomato (Bogdanove and Martin, 2002) and one in Arabidopsis (Speth et al., 2009). Constitutive expression of this GTPase in Arabidopsis enhanced plant defences, conferring resistance to P. syringae infection (Speth et al., 2009). This is the first report of a plant FKBP/GTPase interaction, however Bai et al (2007) reported the direct interaction of a mammalian GTPase with an FKBP. A 14.3.3 was also found to interact with a GTPase in humans (Jin and Lloyd 2004), as well as 14.3.3s being known to interact with GTPase regulators (Benzing et al., 2000; Ishii and Kurachi 2003), while GTP-binding proteins are known to be associated with MAP kinase signalling cascades, with MAP kinase pathways integrating a variety of upstream signals through interaction with G proteins, for example the G protein Ras (Jonak et al., 1999), possibly also linking Hv14.3.3c and HvMAPKKK1 into this signalling network.

3.3.4.2 Confirming the interaction

To confirm the original interaction, Int63 and Int156/114 were checked again for interaction with HvFKBP41 via co-transformation, and the interaction was confirmed.
They were also re-tested for self activation via co-transformation with empty pGBK7 vector and found not to be self-activating (Fig 3.11).

Due to time constraints, the effect of swapping vector orientations was not tested. The full length gene sequences were also not tested as, as described in Chapter 4, the full length sequence of Int63 was obtained from database EST sequences, allowing for primers for qPCR to be designed for expression analysis, without the full length open reading frame needing to be cloned. Similarly, the full length Int156/114 sequence, HvWound, had already been obtained through the Hv14.3.3c work, and could therefore be used in expression analysis, so no further interaction analysis was conducted until the results of the expression analysis were known.

### 3.3.5 Affinity-pulldowns

As an alternative to Y2H, and to try to overcome some of the associated problems described above, or to confirm the results of the Y2H screens, affinity-pulldown chromatography was employed. As the methods had yet to be established, it was decided to first focus on identifying interactors of one of the candidate genes, Hv14.3.3c, for which more background information and similar studies had already been conducted.

### 3.3.6 Heterologous expression of Hv14.3.3c protein

Recombinant Hv14.3.3c protein was successfully expressed and purified using the QIAexpress system. When products of the purification were viewed on an SDS-PAGE gel, a single strong band of approximately 30 kDa could be seen, suggesting successful expression of tagged Hv14.3.3c protein (Fig 3.12A). The absence of any other bands in the elution also confirmed the success of the purification, with the washing steps in the purification being stringent enough as to remove any non-specific contaminating proteins. Detection of the purified protein with an Anti-His tag monoclonal antibody via western blot analysis provided further evidence that the 30 kDa protein was in fact 6xHis-tagged Hv14.3.3.c (Fig 3.12B).

### 3.3.7 Affinity-pulldowns using recombinant Hv14.3.3c

#### 3.3.7.1 Dynabeads

The first method employed to identify proteins that interacted with Hv14.3.3c involved the use of Dynabeads. The beads are magnetic polystyrene beads coated with a polyurethane layer that have activated hydroxy groups that can covalently bind any ligand containing amino or sulfhydryl groups, with high efficiency.
Fig 3.12: SDS-PAGE analysis of Hv14.3.3c expression and purification. A) Purification of His-tagged Hv14.3.3c protein 1 E. Coli lysate from induced cells 2 Ni-NTA resin flow through 3 – 5 Wash 1, 2, 3 6 – 9 Elution 1, 2, 3, 4. B) SDS-PAGE gel of purified Hv14.3.3c protein and C) duplicate western blot showing anti His-tag antibody recognition of putative Hv14.3.3c protein band.
Interacting proteins were compared in extracts from Atlas and Atlas46, control and 24 h *R. secalis* infected, 9 day old seedlings. Initial experiments showed positive results with putative interacting proteins present in the elution, and different proteins present when different tissue sample was used (Fig 3.13A). Mass-spectrometry was then performed to identify the proteins, using a sample of the elute (Fig 3.13B). The results showed that the bands represented mainly Rubisco protein, which is a common contaminant in plant extract samples due to its high abundance, as well as an ATPase subunit and a 14.3.3 protein. As 14.3.3s are known to interact with H+ATPases (Finnie et al, 2002) and form dimers with other 14.3.3 monomers, pulldown of a 14.3.3 and ATPase subunit were expected.

The experiment was repeated but scaled up, using twice as much leaf tissue and dynabeads, in an attempt to pulldown more interactors and a larger quantity of each for identification. A fifth sample of unlinked Dynabeads incubated with plant extract (a mixture of all four extracts) was used as a control. The results showed that more putative interactors had been pulled-down, however, the proteins appeared the same in each sample and were also present in the control (Fig 3.14).

This suggested that the proteins were binding non-specifically to the Dynabeads, possibly due to insufficient blocking during 14.3.3c binding. Proteins covalently bind to the beads, and it is though that during this binding step, all places on the beads available from binding will be bound with protein, blocking the ability for proteins from the plant extract to bind non-specifically later.

The method was repeated numerous times using different blocking conditions, such as altering the amount of protein, both 14.3.3c and BSA, added, however the problem could not be overcome.

3.3.7.2 Sepharose column

To try to overcome the problem of non-specific binding to the dynabeads, a different affinity matrix, sepharose, was used. The sepharose column works in a similar manner to that of the Dynabeads, covalently binding of ligands containing primary amine groups.

According to the manufacturer, non-specific adsorption of proteins to the columns is negligible due to the hydrophilic properties of the base matrix.

As a means of further increasing specificity, the Raf-1 phosphopeptide (Muslin et al., 1996) was also used to elute the protein. This peptide has a high affinity for binding to 14.3.3s in the active site, so should compete with any interactors for binding, causing them to become unbound and come out in the elution. This should achieve elution of only proteins bound to 14.3.3c, overcoming the problem of non-specific binding to the resin.
Fig 3.14: Silver stained SDS-PAGE gel of repeat Dynabead affinity pulldown. A) Proteins eluted from the beads when using 1 Atlas control 2 Atlas 24 h infected 3 Atlas46 control 4 Atlas46 24 h infected tissue and 5 non-Hv14.3.3c bound dynabeads.
Similarly to that performed in the Dynabead experiments, pulldowns with protein extracts from Atlas and Atlas46, control and 24 h *R. secalis* infected 9 day old seedlings, were compared. A control column with no recombinant Hv14.3.3c bound to it was also used to test for non-specific binding. The results, however, showed that non-specific binding had again occurred, despite deactivation of the resin after 14.3.3c binding, which should prevent further protein binding. The Raf-1 phosphopeptide elution from the control column appeared to contain the same proteins as that eluted from the test columns, although there was less protein in terms of amount (Fig 3.15A). The presence of protein in the control elution did not appear to be due to insufficient washing, as all unbound proteins appeared to have been washed from the column before elution (Fig 3.15B), and was therefore likely to be due to non-specific binding to the resin again.

It may be expected that proteins, and most likely different proteins, be present in both the Raf-1 peptide and the low pH elution due to the fact that 14.3.3s interact with target proteins, not only at their active site, but on other sites of the 14.3.3 protein as well (Petosa et al, 1998), with the peptide only eluting active site bound proteins and the low pH disrupting all interactions, however, this would not explain proteins in the control.

### 3.3.7.3 Sepharose resin

A similar approach was then attempted, following a modified protocol from Moorhead et al (1999) who successfully identified interacting proteins of 14.3.3s using cauliflower extracts and sepharose resin, as opposed to columns as above. Using this method, few non-specific proteins were present in the control elution, suggesting successful blocking of the sepharose resin (Fig 3.16A). However, there were also very few proteins present in the 14.3.3c pulldown elution (Fig 3.16B). It was thought that maybe the wash conditions were too stringent and lead to disassociation of interactions, however the same solutions were used successfully in the study by Moorhead et al (1999). No protein binding could also be the result of lack of phosphorylation, as 14.3.3 is known to bind to phosphorylated target sites. However, plant extracts were prepared in the presence of protein phosphatase inhibitors to maintain the phosphorylation state of the proteins, so this should not have been the problem. There are also non-phosphorylated 14.3.3 binding sites (Petosa et al, 1998), therefore these protein interactions should still have occurred in the event of a phosphorylation problem, and some proteins in the elution would be expected.

Due to the problems associated with using protein-affinity matrices, the large amounts of material and cost associated, and the unsuccessful identification of interactors, a different approach was employed.
Fig 3.15: Silver stained SDS-PAGE gel of Sepharose Column affinity pulldown. A) Proteins eluted from the beads in peptide elution then low pH elution when using 1 Atlas control 2 Atlas 24 h infected 3 Atlas46 control and 4 Atlas46 24 h infected tissue and 5 Non-Hv14.3.3c bound column. B) All samples from Atlas 24 h pulldown showing 1 protein extract flow through 2 – 4 wash 1, 2, 3 5 peptide elution 6 – 8 second wash 1, 2, 3 9 low pH elution.
Fig 3.16: Coomassie stained SDS-PAGE gel of Sepharose resin affinity pulldown. A) Control pulldown with BSA 
1 Plant extract preparation 
2 – 6 Binding of BSA and blocking of resin 
7 Plant extract flow through 
8 – 9 Washing off unbound proteins 
10 Elution. B) Pulldown with Hv14.3.3c 
1 – 5 binding of Hv14.3.3c and blocking of resin 
6 Plant extract flow through 
7 – 8 Washing off unbound proteins 
9 Elution.
3.3.8 Anti-Hv14.3.3c antibody

A polyclonal anti-Hv14.3.3c antibody was produced by IMVS and was raised against a Hv14.3.3c peptide. The peptide was a 13 aa synthetic peptide designed to the last amino acid residues at the N-terminal end of 14.3.3c (Fig 2.2), as this area is the most variable between the 14.3.3s and therefore the most 14.3.3c specific (Aitken 1996). This peptide was also chosen as it covered a region of the Hv14.3.3c protein predicted to have high hydrophilicity and therefore most likely to be on the surface of the protein and accessible to the antibody for binding.

To test whether the polyclonal antibody raised against this peptide was able to recognise recombinant Hv14.3.3c, a western blot was used. To test the specificity of the antibody for Hv14.3.3c, its ability to recognise recombinant Hv14.3.3a and Hv14.3.3b (as Hv14.3.3d and e had not been identified at this time) was also tested. Heterologous expression constructs of Hv14.3.3a and b were a kind gift from Dr. A. Comis (ACPFG, University of Adelaide, Australia) and were expressed and purified in the same way as Hv14.3.3c (section 3.2.14 and 3.2.15) (Fig 3.17). Results of the western blot showed that the polyclonal antibody was specific for Hv14.3.3c (Fig 3.18).

3.3.9 Affinity-pulldowns using anti-Hv14.3.3c antibody

Having a Hv14.3.3c specific antibody allowed for affinity-pulldowns to be conducted from a different approach. The antibody could be added to plant protein extract where it would bind native plant Hv14.3.3c. Under the correct buffer condition, Hv14.3.3c should stay bound to any interacting protein. Therefore if a resin such as Protein A agarose, which has an affinity for binding IgGs, was used to bind and pull out the anti-Hv14.3.3c antibody from the plant extract, it is possible that it could bring with it native Hv14.3.3c and any bound interactors. This approach could also overcome the previous problem of proteins binding non-specifically to the resin, as this resin does not have an affinity for proteins.

The pulldown was conducted and the results indicated that putative interacting proteins had been obtained when using the anti-Hv14.3.3c antibody, with no proteins present in the control where an anti-Myc antibody had been used in place of anti-Hv14.3.3c (Fig 3.19A), as expected if no non-specific binding is occurring, as Myc is an animal specific transcription factor, meaning there would be nothing for the anti-Myc antibody to bind to (Gaubatz et al., 1995).

Mass-spectrometry was used to try to determine the identity of the putative interacting proteins. The protein bands were excised from the gel in sections and analysed by mass-
Fig 3.17: Coomassie stained SDS-PAGE gels of Hv14.3.3a and b expression. A) Hv14.3.3b expression showing 1 induced *E. coli* cell lysate 2 Flow through 3 wash 1 4 wash 4 5 Elution B) Hv14.3.3c expression showing 1 induced *E. coli* cell lysate 2 Flow through 3 wash 1 4 wash 4 5 elution 6 purified Hv14.3.3c.
Fig 3.18: Confirming Anti-Hv14.3.3c antibody specificity. SDS-PAGE gel and duplicate western blot of 1 Hv14.3.3a 2 Hv14.3.3b and 3 Hv14.3.3c, showing anti Hv14.3.3c antibody recognises only Hv14.3.3c protein band.
Fig 3.19: Coomassie stained SDS-PAGE gel of anti-Hv14.3.3c antibody pulldown. A) Pulldown with anti-Hv14.3.3c antibody showing 1 Plant extract flow through 2 – 4 washing and 5 Elution, followed by control pulldown with anti-Myc antibody showing 6 Plant extract flow through 7 – 9 washing and 10 Elution. B) Replica gel showing both 1 low pH and 2 peptide elution and bands cut out for mass spectrometry analysis.
spectrometry (Fig 3.19B). However it was found that there was too much Rubisco contamination present in many of the samples to get an accurate identification of other protein bands. Some proteins were identified, including a heat shock protein, ribosomal subunits, 14.3.3s, and other components of the Calvin cycle, such as phosphoribulokinase, already known to interact with 14.3.3s or not of interest.

To try to overcome the Rubisco contamination, it was decided to run a 2D gel in an attempt to get better separation of the proteins and a more localised spot of Rubisco. It was found, however, that there was not enough protein present in the sample for a 2D gel, with only a few faint protein spots visible on the gel, that were unable to be identified through mass-spectrometry (Fig 3.20).

The fact that the antibody pulldown was successful, while all other attempts using recombinant Hv14.3.3c failed, could suggest that there may have been a problem with the recombinant Hv14.3.3c protein. The antibody pulldown allows for native 14.3.3c protein present in the leaf extract to be utilised whereas the recombinant protein pulldowns do not. Therefore, it may be possible that the recombinant Hv14.3.3c was mis-folded or modified in such a way as to inhibit protein interactions, a common disadvantage associated with heterologous expression of plant protein in E. coli (Kost 1997), while the antibody could pull out native 14.3.3c that was not modified and therefore able to bind its targets.
Fig 3.20: Coomassie stained 2D gel of anti-Hv14.3.3c antibody pulldown. Circle spots were excised from the gel for mass-spec analysis.
3.4 Summary and conclusions

Using the Y2H system two previously uncharacterised interactors of Hv14.3.3c and HvFKBP41 were identified. The two putative Hv14.3.3c interactors were found to be an EPSP synthase and a putative wound-induced gene, and the two HvFKBP41 interactors, a small GTPase and the same wound induced gene. Both the GTPase and EPSP synthase have potential or established roles in defence responses and signalling, supporting the possible role of Hv14.3.3c and HvFKBP41 in defence response signalling through interacting with these proteins. The finding that both Hv14.3.3c and HvFKBP41 interact with the same protein, the wound induced gene product, potentially links these genes to the same signalling pathway or network.

An improved, more high-throughput and cost-effective Y2H screening method was also successfully applied through the introduction of a nested PCR step allowing for prey sequencing and identification without cloning into \textit{E. coli}.

Issues with non-specific binding in the recombinant protein affinity-pulldowns meant that interacting proteins were not identified using these techniques.

As negative results such as the occurrence of this non-specific binding are rarely published it is hard to determine whether this is a common problem associated with affinity-pulldown techniques. Some studies, however, have reported some non-specific binding, but have claimed this non-specific binding to be insignificant and not to have affected the results.

A study by von Rechenberg et al (2005) compared different amino-reactive resins for the identification of ampicillin- and penicillin-binding proteins using affinity-pulldown assays. While Dynabeads were found to be better than other resins tested, they still showed some non-specific binding.

Moorehead et al (2006), from which the sepharose pulldown protocol used in this study was adapted, also had a few micrograms of proteins in their BSA-sepharose control, however the proportion of protein in the control compared to the 14.3.3-bound sample was deemed insignificant. It may be possible that the amount of protein extract used could have affected this. They used protein extract from 1 kg of starting material, but still the same amount, 5 ml, of sepharose used in this study. It may be possible that there is a certain amount of non-specific binding that can occur on 5 ml of sepharose and, as this study used less protein extract, less protein bound to the 14.3.3-bound sepharose sample, making the ratio of specific to non-specific binding look more significant in comparison to the
Moorehead et al (2006) study where more proteins were available to bind to the 14.3.3-sepharose.

Other studies, such as that of Rubio et al (2004) who identified over 200 human 14.3.3 interacting proteins, have not reported the use of a control.

Through the trial of many different affinity-pulldown techniques, a method for identifying interacting proteins from plant extracts using an antibody in place of recombinant protein, was finally identified, although this technique still requires some improvement in the area of protein identification. Had identification not been a problem and with time permitting, to extend on this method the aim was to compare the different proteins pulled down when using infected or stressed tissue versus not infected or stress, allowing for the identification of differential proteins and therefore interacting proteins most likely to be involved in the defence response.

The problems associated with identification also meant that further Hv14.3.3c interactors were not identified nor the putative interactors from the Y2H screen confirmed. As there are problems associated with the reliability of Y2H interactions, such as the artificial nature of the interaction, the results generally have to be confirmed *in vitro* or by some other means (Zhang et al 1997b). The next chapter deals with confirmation of these interactions.
Chapter 4

Isolation of Interactor Full Length cDNAs, Expression Analysis and Confirming the Hv14.3.3c-HvEPSP interaction
4.1 Introduction

In the previous chapter, putative Hv14.3.3c and HvFKBP41 interactors were identified. To facilitate Y2H interaction studies with the full length proteins (previously described in Chapter 3), as well as the expression analysis and protein expression and purification described in this chapter, the full length sequences of the interactors needed to be obtained. In this chapter, isolation of full length gene sequences of the putative interactors, using the same techniques as that described in Chapter 2, is presented.

Expression analysis of the genes under the same stress conditions as in Chapter 2 was also investigated. To be able to interact, it is expected that two proteins need to be present at the same time and in the same location. Although protein level is not reliant on transcription only, it is thought that comparing the transcriptional activity of two genes may give an indication as to their potential to interact and it has been shown in a number of studies that coexpression is correlated with functional relationships, such as physical interaction between the encoded proteins (Lee et al., 2004).

In a study by Jansen et al (Jansen et al., 2002) the relationship between mRNA expression and protein-protein interactions was investigated by comparing the expression pattern of genes coding proteins involved in known protein complexes. It was found that proteins of the same complex showed significant coexpression, both in terms of similarities of absolute mRNA levels, as well as expression profiles.

Therefore, in addition to providing insight into the potential role of these genes in defence response signalling, as discussed in Chapter 2, expression analysis was also performed in an attempt to provide support for the putative interaction with the candidate proteins, by investigation of co-expression patterns of their corresponding genes.

The identification of potential interactors with Hv14.3.3c and HvFKBP41 presented in Chapter 3 was achieved using the Y2H system. There are, however, problems associated with the reliability of Y2H interactions, such as the artificial nature of the interaction and the fact that it can yield high levels of false-positive results. Due to this, Y2H interactions are generally only considered putative and have to be confirmed by other means (Zhang et al., 1997b; Miernyk and Thelen 2008).

As well as being used to identify interacting proteins, such as that discussed in Chapter 3, co-immunoprecipitation (co-IP) is also a commonly used technique for the analysis of putative protein-protein interactions, with the co-IP of proteins the most convincing evidence that two or more proteins physically interact in vivo (Miernyk and Thelen 2008).
There are different approaches commonly being used for protein production: chemical synthesis, *in vivo* expression and cell-free protein synthesis. Chemical synthesis cannot be used to produce long peptides, while *in vivo* expression can only produce proteins that are not fatally toxic to the host cell (Endo and Sawasaki, 2006). Recombinant proteins expressed in the cytoplasm of bacteria can be insoluble and therefore inactive (Geisse et al., 1996). Cell-free translation systems, however, can synthesise proteins with high accuracy and at speeds approaching *in vivo* rates and can also be used to express proteins that seriously interfere with cell physiology (Endo and Sawasaki, 2006). Therefore, as well as the use of an *in vivo* expression system, the *E. coli* QIAexpressionist system discussed previously (Chapter 3), a cell-free system was also used to produce proteins for co-IP.

The most commonly used cell-free systems are based on extracts from *E. coli*, rabbit reticulocytes or wheat germ extracts (Endo and Sawasaki, 2006). One such system is the TNT Coupled Wheat Germ Extract System®, which allows for the cell-free transcription and translation of DNA into protein. Proteins produced from such systems have been used in many functional studies including protein-protein interaction studies, and recently, proteins expressed using this systems have also been used in assays to confirm yeast two-hybrid interactions (Roberts and Paterson, 1973; Rodriguez Millaa et al., 2006; Hino et al., 2008; Xu and Stern, 2004).

In addition to co-IP, bioluminescent resonance energy transfer (BRET) was also used in this study as an alternative means of confirming interactions. There are a number of techniques available to study protein-protein interactions in living cells, with fluorescence resonance energy transfer (FRET) being one of the most common. In this technique, two candidate proteins to be tested for interaction are labelled with a donor fluorophore and a compatible acceptor fluorophore, whose excitation spectrum overlaps with the emission spectrum of the donor. If donor and acceptor fluorophore come into close proximity, via interaction of the candidate proteins, radiationless transfer of energy from the excited donor to the acceptor occurs. This can then be detected by virtue of a secondary peak or shoulder in the emission spectrum upon excitation of the energy donor (Gordon et al., 1998).

Techniques such as FRET have the added advantage over co-IP of allowing for the confirmation that the interaction occurs in the natural environment, and also the ability to monitor the dynamics of specific interactions in a living cell in real time (Mendelsohn and Brent, 1999). However, in plant systems, it can become difficult to distinguish the GFP interaction signal from autofluorescence. The bioluminescence resonance energy transfer
(BRET) approach resembles FRET in every way except that a bioluminescent energy donor, ie luciferase, replaces the fluorescent donor, overcoming the problem of distinguishing a signal from autofluorescence (Xu et al, 1999; Subramanine et al., 2006).

A versatile BRET system employing the blue-light-emitting luciferase from the marine anthozoan *Renilla reniformis* (RLuc) and YFP was first developed in bacteria by Xu et al 1999. In this study, an improved method by Subramanine et al 2006, for use in plant systems and including a set of recombination cloning vectors, was employed.

The expression analysis described in this chapter suggests a potential role for all three of the identified interactors, *HvEPSP*, *HvWound* and *HvGTPase* in the defence response, and their co-expression with their putative interactors, *Hv14.3.3c* or *HvFKBP*, also provides evidence for a potential interaction. However, due to time constraints and the amount of work involved in trying to confirm these interactions, it was decided to focus on only one putative interaction: that of *Hv14.3.3c* and *HvEPSP*. Although this interaction pair had not shown co-expression patterns more similar than the other pairs of interactors it was decided that this was the most interesting candidate based on its cellular functions, and the fact that a role for EPSP synthase in defence response signalling had not been previously investigated. The finding that *HvEPSP* contains a known 14.3.3 binding motif also gives more credibility to the potential interaction, and therefore, this chapter describes attempts to confirm this interaction.
4.2 Materials and methods

4.2.1 Materials

BRET vectors were a kind gift from Ass. Prof. A. Von Arnim (University of Tennessee, Knoxville, USA). The TNT Coupled Wheat Germ Extract System was from Promega (Annandale, NSW, Australia). Urea, 5052, anti-mouse-AP conjugated monoclonal secondary antibody and NPS were from Sigma-Aldrich (Castle Hill, NSW, Australia). LR Clonase II kit, pCR8-GW-TOPO TA cloning kit and pDEST17 vector were from Invitrogen (Waverley, VIC, Australia). Mouse monoclonal anti-HA tag antibody was from Genesearch (Arundel, QLD, Australia).

4.2.2 Quantitative (real time) PCR

Q-PCR was performed as described in section 2.2.17, except that the primers used were EPSP qF and qR for HvEPSP, Wound qF and qR for HvWound and GTPase qF and qR for HvGTPase (Appendix 1).

4.2.3 In Vitro Transcription/Translation using wheat germ extract

4.2.3.1 In Vitro transcription/translation

1 µg of Int36/pGADT7 plasmid DNA from section 3.3.2 was digested with NotI to linearise the plasmid DNA, followed by subsequent purification using the QIAquick PCR Purification Kit. In vitro transcription/translation was then performed using the TNT Coupled Wheat Germ Extract System according to manufacturer’s instruction.

4.2.3.2 Co-immunoprecipitation from wheat germ extract

Protein A agarose was prepared for antibody binding by washing in 100 mM Tris-HCl buffer (pH 8) to remove storage buffer. The antibody (anti-His for Hv14.3.3c pulldown or anti-HA tag for Int36) was bound to the resin by adding 2 µg of antibody in 20 µl 100 mM Tris-HCl buffer (pH8) to 20 µl Protein A agarose and incubating at 4°C o/n with gentle mixing. Co-immunoprecipitation samples were prepared by adding ~50 µg recombinant Hv14.3.3c to 20 µl of the wheat germ reaction and incubating for ~1h at room temperature with gentle mixing. The sample was then added to either anti-His (to bind Hv14.3.3c in an attempt to pulldown Int36 with it) or anti-HA (to bind Int36 in an attempt to pulldown Hv14.3.3c with it) protein A agarose and incubated o/n at 4°C with gentle mixing on a rotary wheel. Unbound proteins were washed from the resin with 5 x 500 µl PBS and
bound proteins eluted by adding 40 µl SDS loading buffer and boiling for 10 min. Protein A agarose with no antibody bound and wheat germ reaction sample with no Hv14.3.3c added were used as controls.

The co-IP products were run on SDS-PAGE gels and probed with either anti-His or anti-HA antibody, to detect the presence of Hv14.3.3c or Int36, using western blot analysis.

4.2.4 Western blot analysis

Western blot analysis was performed as described in section 3.2.19 for detection with anti-Hv14.3.3c and anti-His antibodies. For detection with mouse anti-Myc or anti-HA monoclonal antibody, the antibody was used at a dilution of 1:1000, in conjunction with an anti-mouse-AP conjugated monoclonal secondary antibody.

4.2.5 Generation of HvEPSP E. coli expression construct

HvEPSP was cloned into the expression vector pDEST17 using Gateway recombination as follows. Firstly, the full length mature peptide of HvEPSP with NH₂-terminal Myc tag, was amplified from HvEPSP/pGBKT7 plasmid DNA (section 3.2.6), using a standard PCR reaction and primers EPSP TopoF and EPSP TopoR (Appendix 1). The amplified product was run on an agarose gel, purified and cloned into the pCR8-GW-TOPO vector according to manufacturer’s instructions.

Ligation products were transformed into E. coli DH5α cells via heat shock transformation. Positive colonies were identified via restriction mapping with EcoRI to look for the presence of correct inserts and sequencing to check for correct orientation using the GW1 and GW2 primers (Appendix 1). Plasmid DNA was prepared from a positive clone using the QIAquick Spin Miniprep Kit. The Myc-tagged HvEPSP fragment was transferred from the pCR8-GW-TOPO entry vector into the E. coli expression destination vector pDEST17 via a Gateway cloning LR reaction. Briefly, ~100 ng of the entry clone was mixed with 100 ng of destination vector. TE buffer, 4 µl, and 2 µl LR Clonase II enzyme mix were then added and the reaction incubated at 25°C o/n. After incubation, 2 µl of Proteinase K solution was added to the reaction and incubated for a further 37°C for 20 min. Plasmids were then transformed into E. coli BL21* cells via heat shock transformation, and positive clones selected via restriction mapping with EcoRI. A glycerol stock of a positive colony was prepared for storage.
4.2.6 Expression induction in *E. coli* using auto-induction media

The glycerol stock from above was used to inoculate 100 ml LB media containing 2% (v/v) 50 x 5052 [25% (w/v) glycerol, 2.5% (w/v) glucose, 10% (w/v) lactose], 5% (v/v) 20 x NPS [0.5 M NH$_4$SO$_4$ buffer (pH 6.8) containing 1 M KH$_2$PO$_4$, 1M Na$_2$PO$_4$, 1 mM MgSO$_4$, 100 µg/ml ampicillin and 25 µg/ml kanamycin, and grown at 37°C for 48 h with shaking. Cells were harvested by centrifugation at 4500 g for 15 min and stored at -80°C until required.

4.2.7 Purification of His-tagged HvEPSP protein

Purification of His-tagged proteins was performed similarly to that described in section 3.2.15, however denaturing purification was used instead of native. Cells were prepared as in the native purification, but were resuspended in urea lysis buffer [8M urea, 0.1 M NaH$_2$PO$_4$, 10 mM Tris-Cl, pH 8.0]. No lysozyme was added and the solution was incubated for ~1 h on ice with gentle shaking and only sonicated if lysis was not complete (solution did not look transparent). The Ni-NTA agarose was equilibrated using urea lysis buffer, and washed with urea wash buffer [8M urea, 0.1 M NaH$_2$PO$_4$, 10 mM Tris-Cl, pH 6.3]. Bound his-tagged proteins were eluted in the same manner but using urea elution buffer [8 M urea, 0.1M NaH$_2$PO$_4$, 10 mM Tris-Cl, pH 4.5]. Eluted proteins were concentrated and buffer exchanged into 1 x PBS buffer using Centricon centrifugal filters according to manufacturer’s instruction.

4.2.8 Co-immunoprecipitation of recombinant Hv14.3.3c and HvEPSP

Co-immunoprecipitation samples were prepared by mixing 50 µg each protein in 100 µl PBS and incubating at 4°C for ~ 2 h with gentle shaking. After incubation ~2 µg of antibody (anti-Hv14.3.3c for pulldown of Hv14.3.3c or anti-Myc tag for Myc-HvEPSP) was added to the sample, and the sample incubated for a further ~2 h at 4°C. Protein A agarose was prepared by washing with 1 x PBS to remove storage buffer, and 30 µl added to each sample. The samples were then incubated o/n at 4°C with gentle mixing on a rotary wheel. Unbound proteins were washed from the resin with 5 x 500 µl TBS containing 50 - 150 nM NaCl. Co-immunoprecipitated proteins were eluted by adding 40 µl SDS loading buffer and boiling for 10 min. Hv14.3.3c or Myc-HvEPSP proteins were used separately as positive controls, and samples with no antibody added used as negative controls.
4.2.9 Generation of BRET expression constructs

BRET constructs were made using Gateway technology, similarly to that described in section 4.2.5. Briefly, Hv14.3.3c was amplified from Hv14.3.3c/pGBKT7 plasmid DNA (section 3.2.4) using the primers 14.3.3c YFP F and either 14.3.3c YFP NR (including the Hv14.3.3c stop codon for NH₂-terminus YFP tag fusion) or 14.3.3c YFP CR (excluding the Hv14.3.3c stop codon for COOH-terminus YFP tag fusion). HvEPSP was amplified from HvEPSP/pGADT7 plasmid DNA (section 4.3.1.3) using primers EPSP Rluc F and either EPSP Rluc NR (including the HvEPSP stop codon for NH₂-terminus Rluc tag fusion) or EPSP Rluc CR (excluding the HvEPSP stop codon for COOH-terminus Rluc tag fusion). The fragments were amplified using standard PCR reactions, cloned into the entry vector pCR8-GW-TOPO and transferred from the entry vector to the destination vectors YFP-attR, attR-YFP, hRluc-attR or attR-hRluc using a Gateway cloning LR reaction as previously described.
4.3 Results and discussion

4.3.1 Cloning of a HvEPSP synthase cDNA fragment

4.3.1.1 Determining unknown sequence

A 1.1 kb cDNA fragment, named Int36, was obtained from the Y2H library as described in section 3.3.2 and extended to the 3’ end of the transcript sequence, as indicated by the presence of the polyA tail. A full length barley EPSP synthase had not been previously identified. Alignment with a homologue with the highest sequence similarity, a rice EPSP synthase OsEPSP (accession no. AF413082) indicated that 600 bp were required to complete the 5’ region of the barley ORF (Fig 4.1A). No barley ESTs that covered the unknown 5’ were available in the database (Fig 4.1B), therefore, techniques to extend the gene in the 5’ direction were employed.

4.3.1.2 Genomic walking and 5’ RACE

RACE techniques are not always successful in providing the 5’ regions of genes, e.g., HvMAPKK1 and HvFKBP in Chapter 2. However, tomato and petunia EPSP synthase genes of 9 kb, containing 7 very large introns, have been described (Gasser et al., 1988), indicating that genomic sequencing may be a difficult approach for obtaining the full-length barley EPSP synthase ORF sequence. Therefore, both 5’ RACE and genomic walking were undertaken concurrently to recover further cDNA/genomic sequence of the barley EPSP synthase gene.

The nested primers EPSP 5’out and EPSP 5’In (Appendix 1) were designed to the 5’ region of the known 1.1 kb sequence (Fig 4.1A). 5’ SMART RACE and genomic walking were performed as described in sections 2.2.13 and 2.2.14, respectively.

In Round 1, the SMART RACE template produced no product, while one fragment of similar size was amplified from each of the genomic walking libraries (Fig 4.2A), which may have been the artefact referred to in section 2.3.2.5. In Round 2, the only SMART RACE products amplified were too small to contain any further 5’ sequence of EPSP synthase (Fig 4.2B). Round 2 genomic walking amplifications produced the same non-specific fragment in all libraries, one small fragment in library 1 and one fragment larger than 1 kb in library 3. When cloned and sequenced, the ~1 kb fragment showed 100% identity to the original sequence in the overlapping region (Fig 4.3A) and showed homology to database EPSP synthase gene sequences from other species (Fig 4.3B).
Fig 4.1: Alignment of Int36 sequence from Y2H with closest database match *OsEPSP*. A) Graphical representation of alignment of partial Int36 sequence from Y2H with homologue of highest sequence similarity, *OsEPSP*, Accession Number AF413082. B) Graphical representation of alignment of *OsEPSP* with barley EST’s showing no EST covering unknown 5’ region available.
Fig 4.3: Confirming 5’ genomic walking fragment is 5’ end of barley EPSP. A) Graphical representation of alignment of putative EPSP synthase Int36 sequence and 5’ genomic walking fragment shows sequence similarity in overlapping region. Site and orientation of second 5’ extension primers are also shown B) Output of Blastx search with 5’ genomic walking fragment shows sequence homology to other EPSP synthase genes.
However, although the additional genomic walking fragment extended past the 5’ end of the rice EPSP synthase gene, suggesting the complete 5’ region had been obtained, a complete ORF or start methionine could not be found. Alignment with OsEPSP also showed the presence of a 92 bp section of sequence towards the end of the genomic walking fragment not present in the rice sequence. As this fragment was obtained from genomic DNA it was likely that this was an intron, and would therefore interrupt a complete ORF.

The first 700bp of the genomic walking fragment showed little similarity to that of OsEPSP cDNA, so it was also thought that this end may contain intron sequence. It was therefore decided to try to obtain further 5’ sequence using 5’ RACE from the position at 700 bp into the genomic walking sequence to try to avoid amplification of intron sequence and obtain more 5’ sequence.

5’ RACE was therefore attempted as before, but using new gene specific primers EPSP 5’2 out and in (Fig 4.3A, Appendix 1) and SMART RACE Atlas46 cDNA library. Reactions were run at different annealing temperatures to try to improve the specificity of amplification. Round 1 produced very faint smeared products in the reactions run at 58°C and 60°C (Fig 4.4A), while Round 2, run at an annealing temperature 2°C higher than that of Round 1, produced multiple products in the reactions run at 60°C and 62°C, while only one product at 64°C (Fig 4.4B). A representative of each sized fragment was excised from the gel, cloned and sequenced. It was found that the single specific fragment of ~ 200 bp from the 64°C reaction was correct, containing additional 5’ sequence (Fig 4.5). As the fragment was so small, and did not extend past the 5’ end of OsEPSP, it was not expected to contain the complete 5’ region of the barley EPSP synthase gene and sequence analysis confirmed this, with the absence of a start methionine.

Previous characterisation of EPSP synthase genes from petunia, tomato and maize (Gasser et al., 1988; Forlani, 2002), has shown the presence of an N-terminal chloroplastic signal peptide which is cleaved off to produce a mature peptide upon transport into the chloroplast. When compared the petunia and tomato protein sequences (Fig 4.6) it was found that the start of the mature peptide had been reached, and that the 5’ region that was still unknown would therefore contain the chloroplastic signal peptide. As this signal peptide would be a hindrance for Y2H, where a chloroplastic signal peptide could compete with the nuclear localisation signal peptide required for localisation in the yeast nucleus, no further 5’ RACE/walking was undertaken. The start methionine of the EPSP synthase gene
Fig 4.4: Agarose gel analysis of second putative barley EPSP 5’ RACE. **A)** Round 1 SMART RACE using Atlas46 cDNA, run at 1: 58°C, 2: 60°C, 3: 62°C. **B)** Round 2 using 1:30 dilution of 1: Round 1 58°C, run at 60°C, 2: Round 1 60°C, run at 62°C and 3: Round 3 62°C run at 64°C. Excised regions are highlighted in red.
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Fig 4.5: Confirming 5’ RACE fragment is 5’ end of barley EPSP. A) Graphical representation of alignment of RACE 5’ fragment with 5’ genomic walking fragment, and OsEPSP shows sequence similarity in overlapping region. B) Output of Blastx search with 5’ RACE fragment shows sequence homology to other EPSP synthase genes.
Fig 4.6: Barley EPSP mature peptide. Protein alignment of petunia and tomato EPSP synthase with barley EPSP protein contig from 5’ RACE + genomic walking + Int36, showing position of start of mature peptide (red arrow)
was therefore not present, however, the internal start methionine in the Y2H expression vector or other protein expression vectors would overcome the problem of the gene not including a start methionine.

4.3.1.3 Amplification and cloning of full length mature \textit{HvEPSP}

To amplify cDNA sequence encoding the mature peptide of the barley EPSP synthase, two gene specific primers, EPSP-mat F and EPSP-mat R (Appendix 1) were designed to the second 5’ RACE and original 3’ sequences. A Hi-Fidelity PCR reaction using these primers and Atlas46 template cDNA produced an expected 1.3 kb fragment (Fig 4.7) which was subsequently excised, purified, cloned and sequenced.

The mature peptide was found to encode a 445 aa protein (Fig 4.8), with 75% sequence homology to both petunia and tomato EPSP synthase mature peptide cDNA sequence and 83% and 81% similarity to the petunia and tomato mature peptide protein sequence respectively. The mature peptide also had a predicted molecular weight of 48kDa, the same as that of all previously characterised EPSP synthases (Herrmann, 1999). This suggested that a barley EPSP synthase gene had been obtained, and was subsequently named \textit{HvEPSP} synthase (\textit{HvEPSP}).

Binding of 14.3.3 to other proteins is most commonly through specific phosphorylated target sites (Yaffe et al., 1997). The mode 1 target site R/K\textit{XXpS/T\textit{XX}} was found to be present in HvEPSP, in the original Y2H fragment, corresponding to amino acids 204 – 209, KLISVP (Fig 4.8A), supporting the potential for Hv14.3.3c and HvEPSP to interact as found in the Y2H screen in Chapter 3.

4.3.2 Cloning of a \textit{HvWound} cDNA fragment

4.3.2.1 Determining unknown sequence

A 527 bp cDNA fragment, named Int114, was obtained from the Y2H library as described in section 3.3.2. Database searches revealed the full length sequence had not been previously identified in barley, and that the partial sequence showed highest sequence similarity to a gene from rice, Accession No. AK112035 annotated as a putative wound induced gene. Alignment with this rice putative wound induced gene indicated further 5’ sequence was required to complete the full length sequence from barley (Fig 4.9A). An EST search identified barley ESTs covering the complete full length sequence. A contig was made from the ESTs and used for primer design to amplify the cDNA sequence containing the ORF from barley, as described below (Fig 4.9B).
Fig 4.7: Agarose gel analysis of a DNA fragment encoding the full length *HvEPSP synthase* mature peptide. Showing 1.3 kb *HvEPSP synthase* mature peptide fragment.
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Fig 4.8: Analysis of full length *HvEPSP* cDNA sequence. A) Full length mature peptide cDNA sequence and deduced amino acid sequence of *HvEPSP*, with original Y2H prey sequence in grey and additional 5’ sequence in blue and 14.3.3 binding motif circled. Common restriction sites are also shown. B) Presence and position of coding region (blue) and introns (black v line) in amplified sequences.
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Fig 4.9: Alignment of Int114 sequence from Y2H with closest database match OsEPSP. A) Graphical representation of aligment of partial Int114 sequence from Y2H with homologue with highest sequence similarity, a rice putative wound induced gene, Accession Number AK112035. B) Graphical representation of alignment of Int114 sequence with barley EST’s showing ESTs covering complete unknown regions are available.
4.3.2.2 Amplification and cloning of full length HvWound

To amplify the complete ORF of the wound induced gene from barley, two gene specific primers, Wound full F and Wound full R (Appendix 1) were designed to the known 3’ sequences and EST 5’ contig sequence (Fig 4.9B). A Hi-Fidelity PCR reaction using these primers and Atlas46 cDNA produced an expected 1 kb full length fragment (Fig 4.10), which was subsequently cloned and sequenced.

The amplified full length fragment encoded a predicted 325 aa, 36 kDa protein (Fig 4.11). As this was only a putative, uncharacterised gene, its size could not be compared to published data as confirmation of its identity, however, the full length sequence still showed highest sequence homology to the rice putative wound induced gene, suggesting that the correct full length fragment had been obtained.

A common phosphorylated 14.3.3 binding motif was not found in the HvWound sequence. There are, however, known non-phosphorylated 14.3.3 binding sites (Petosa et al., 1998) and therefore the interaction between Hv14.3.3c and HvWound, identified in the Y2H screen in Chapter 3 may still be possible. A literature review by Hallberg (2002) listed published non-phosphorlated 14.3.3 binding motifs as being DALDL, WLDLE, DLLST, RSESEE and LRELQL. None of these sites could be found in HvWound, however, as the motif sequences are quite variable, it may be possible that a modified version is present.

4.3.3 Cloning of a HvGTPase cDNA fragment

Int63 was a 125 bp fragment identified in the Y2H screen as encoding an HvFKBP interactor. When compared to database sequences it was found to show highest sequence homology to an Arabidopsis Rab-type GTPase protein (NP 187601). Alignment with this gene at the DNA level indicated both 5’ and 3’ sequences were required to complete the full length ORF from barley (Fig 4.12A).

A BLAST search revealed barley ESTs covering the complete gene sequence (Fig 4.12B). A contig was made from the ESTs to determine the full length ORF sequence of Int63. However, due to time constraints, the full length ORF was not cloned, and the deduced barley sequence was instead used only to design Q-PCR primers. Expression analysis of the gene would first be conducted, with the aim of determining whether this putative interactor appeared of interest.

4.3.3.1 Sequence analysis

The deduced barley full length sequence of Int63 encoded a 215 aa 23 kDa protein (Fig
Fig 4.10: Agarose gel analysis of full length HvWound fragment. Showing expected 1 kb HvWound fragment.
**Fig 4.11: Analysis of full length *HvWound* cDNA sequence.** Full length cDNA sequence and deduced ORF and amino acid sequence of *HvWound*, with original Y2H prey sequence in grey and additional 5’ sequence identified in blue. Common restriction sites are also shown.
Fig 4.12: Alignment of Int63 sequence from Y2H with closest database match *AtGTPase*. A) Graphical representation of alignment of partial Int63 sequence from Y2H with homologue with highest sequence similarity, an Arabidopsis GTPase, Accession Number NP 187601. B) Graphical representation of alignment of Int63 sequence with barley EST’s showing ESTs covering unknown 5’ and 3’ region are available.
Fig 4.13: Analysis of full length *HvGTPase* cDNA sequence. Deduced full length, ORF and amino acid sequence of HvGTPase from barley EST sequences, with original Y2H prey sequence in grey and additional EST sequence in blue. Common restriction sites are also shown.
4.13) and is in agreement with the known molecular mass of 21 to 30 kDa for small GTPases (Yang, 2002). The full length sequence was most similar to the gene sequence of a GTPase named Rab8 from Arabidopsis and tobacco. In Arabidopsis, the Rab GTPases have been classed into eight subfamilies, designated AtRabA–AtRabH, with Rab8 falling into the E subfamily. However, very few GTPases or GTP-binding proteins appear to have been identified in barley. There seems to exist no consistent nomenclature across genes from different species. Therefore the full length Int63 sequence was named HvRab-type GTP-binding protein or \textit{HvGTPase} for short.

4.3.4 Expression analysis

As putative interactors with Hv14.3.3c and HvFKBP, it was presumed that HvEPSP, HvWound, and HvGTPase may also play a role in the plant stress response. To be true interactors, these genes may share similar expression patterns, as they would be required to be expressed in the same tissues at the same time to be available for interaction. If they are part of the same signal transduction pathways, they are also likely to respond similarly, in terms of timing and possibly magnitude of expression response.

Therefore, to investigate a possible role for these interactor genes in stress response, and to investigate whether there was any co-expression of the interactors with their corresponding bait gene, the transcriptional activity of these genes under the same stress conditions in which Hv14.3.3c and HvFKBP showed a possible response (Chapter 2), was investigated by Q-PCR.

4.3.4.1 \textit{R. secalis} infection

The expression patterns of the putative interactors were found to be similar to that of the candidate genes, with expression increasing in the susceptible interaction 24 h after infection and earlier, at 6 h after infection, in the resistant interaction (Fig 4.14). This supports the potential for these genes to be true interactors and again also suggesting the possibility that an earlier response in Atlas46 may be linked to resistance, as discussed in Chapter 2. This increase in expression of the interactors during infection, however, was not as strong as the candidate genes, when comparing to the water mock-inoculations.

Unlike the candidate genes, the interactors showed significant levels of expression in the ‘rest of leaf’ sample, suggesting that this response may not be localised to the epidermis. It may also be that the level of basal expression of these genes is generally higher in the whole plant, and in the epidermal samples this basal expression level is seen in addition to
Fig 4:14: Expression of interactors under *R. secalis* infection. Expression level, expressed as copy number and normalised to four housekeeping genes encoding HSP70, Tubulin, Cyclophilin and 18s Ribosomal protein, graphed against each timepoint, hours after infection or 24h water control, for A) *HvEPSP*, B) *HvWound* and C) *HvGTPase*. Expression in four different tissue types; Atlas and Atlas46 epidermal (At Ep and At46 Ep respectively) and Atlas and Atlas46 rest of leaf (At Rol and Atlas46 Rol respectively), was analysed. Error bars = standard deviation for each mRNA.
the response to infection, which may mask the effects of infection alone on the genes’ expression response.

Although interacting with different candidate genes, *HvEPSP* and *HvGTPase* showed very similar expression patterns and this expression pattern matched more closely to that of *HvFKBP41* than *Hv14.3.3c*. This may suggest that the interactor genes are also be linked in some way, for example, involvement in the same signalling network.

The fact that EPSP synthase was found to be differentially expressed upon infection of barley with powdery mildew (Eckey et al., 2004) supports the expression results observed. Similarly, the rice homologue of *HvWound* was found to show differential expression during the rice/rice blast fungus interaction (Jantasuriyarat et al., 2005).

Constitutive expression of a GTPase in Arabidopsis conferred resistance to *P. syringae* infection (Speth et al., 2009; Bogdanove and Martin, 2002) suggesting GTPase expression is linked with resistance, supporting the results.

### 4.3.4.2 ABA

Interestingly, under ABA treatment, *HvGTPase* expression pattern was most similar to that of *Hv14.3.3c*, rather than putative interactor *HvFKBP41*, with an early peak in expression at 4 h in response to ABA (Fig 4.15). *HvWound’s* expression matched more closely to *HvFKBP41*’s expression than *Hv14.3.3c*’s.

*HvEPSP* showed expression patterns similar to that of *HvWound*, with a very differently timed peak in expression to that of *Hv14.3.3c*, possibly not supporting an interaction occurring. At the point of *HvEPSP* expression peak, 8 h, however, *HvEPSP* and *Hv14.3.3c* expression levels are very similar, as, although *Hv14.3.3c* peaks in expression earlier than *HvEPSP*, it is still being expressed at the same level as *HvEPSP* at its peak, supporting a potential interaction.

GTPases are known to be involved in ABA-mediated signalling, with expression of a GTPase from both Arabidopsis and rice shown to be induced upon ABA treatment (Jayasekaran et al., 2006; Nahm et al., 2003), supporting the expression results.

ABA has also been implicated as a signalling molecule in wounding, and is known to elicit wound responses in plants (Lerner, 1999), supporting the *HvWound* results.

### 4.3.4.3 Frost

In the frost intolerant cultivar WI12, in which the candidate genes had shown highest expression (Fig 2.21), all genes except *Hv14.3.3c* showed a very similar expression pattern, slightly increasing in expression during the frost event at -5°C (Fig 4.16C).
Fig 4.15: Expression of interactors under 10 µM ABA treatment in leaf. Expression level, expressed as a percentage compared to expression in control plants and normalised to four housekeeping genes encoding HSP70, Tubulin, Cyclophilin and 18s Ribosomal protein, graphed against each time point for each gene. Error bars = standard deviation for each mRNA.
Fig 4.16: Expression of interactors under frost treatment. Expression level of each gene, expressed as a percentage compared to the control and normalised to four housekeeping genes encoding HSP70, Tubulin, Cyclophilin and 18s Ribosomal protein, graphed against each timepoint in cultivar A) Amaji nijo B) Golden promise and C) WI12. Error bars = standard deviation for each mRNA.
Hv14.3.3c did not show a similar expression pattern to either putative interactor in any of the cultivars, while HvFKBP41 showed slight similarity to HvWound expression, but not to the extent seen in the other treatments. The most closely matched expression patterns were that of HvEPSP and HvGTPase, with almost identical expression in Golden promise (Fig 4.16B) and very similar in Amagi nijo (Fig 4.16A), again suggesting a possible link between the two. HvWound showed an expression response greater than all other genes in the tolerant cultivar Amaji nigo, while it showed little to no response in intolerant cultivar WI12, suggesting possible down-regulation in response to cold temperatures.

Expression of the rice GTPase homologue, responsive to ABA, is also differentially regulated by cold temperatures (Nahm et al., 2003), supporting the HvGTPase expression results.

4.3.4.4 Drought

In leaf tissue under drought stress, putative interactors HvGTPase and HvWound showed similar expression patterns to that of Hv14.3.3c and HvFKBP41, increasing upon re-watering after the final drought cycle and therefore suggesting a potential role in recovery. HvEPSP showed expression patterns different to that of all other genes, decreasing after rewatering at this same time point (Fig 4.17).

The rice GTPase, differentially expressed during ABA and cold treatment, is also known to be differentially regulated by dehydration (Nahm et al., 2003), supporting the results. Similarly, a Rab-type GTPase from Pennisetum glaucum, a food grain crop of India, was also found to be upregulated during dehydration, with the study suggesting the GTPase to be a potential candidate gene for developing dehydration tolerance in planta (Agarwal et al., 2008). Further to this, a GTPase from wild barley was identified as the potential cause of tolerance to dehydration (Suprunova et al., 2007).

4.3.5 Co-immunoprecipitation from wheat germ transcription/translation

The first method used in an attempt to confirm the Hv14.3.3c-HvEPSP interaction was in vitro transcription/translation of Int36, followed by co-immunoprecipitation with recombinant Hv14.3.3c. Confirmation of the original interaction between Hv14.3.3c and the partial HvEPSP, Int36, was first attempted. In vitro transcription/translation was the first method used as it allowed producing a workable and detectable amount of Int36 protein, without having to generate expression constructs, express and purify the protein via other means such as heterologous expression in E. coli. Linearised Int36 in Y2H plasmid pGADT7 was used as the template as it contained a start and stop codon for
Fig 4.17: Expression of interactors under drought treatment. Expression level, expressed as a percentage compared to expression in control plants and normalised to four housekeeping genes encoding HSP70, Tubulin, Cyclophilin and 18s Ribosomal protein, graphed against each timepoint for each gene. Error bars = standard deviation for each mRNA.
translation and also allowed for the HA-epitope tag (present at the NH$_2$-terminus of Int36) to be expressed as a fusion to Int36, facilitating later co-IP. *In vitro* transcription/translation of Int36 was performed and co-IP with Hv14.3.3c attempted. However, upon western blot analysis to detect the presence of the proteins, it was found that neither Hv14.3.3c nor Int36 could be detected (results not shown).

For co-immunoprecipitation using anti-His protein A agarose to bind Hv14.3.3c, even if interaction did not occur, it was expected that Hv14.3.3c still be present in the pulldown product. A potential explanation for the missing protein detection could have been problems with the co-IP conditions itself, such as too stringent washes, not enough antibody to bind Hv14.3.3c, or enough Hv14.3.3c present to be bound and detected. The co-IP was repeated a number of times, using less stringent washing and slightly more Hv14.3.3c and antibody (although, the small volumes used in the wheat germ system restricted the amounts that could be increased), however, no improvements were achieved.

It was also thought that transcription/translation of HvEPSP may not have been successful, as no Int36 was detected when anti-HA protein A agarose was used to pulldown. To test this, a small amount of the wheat germ extract (after the transcription/translation reaction had been performed) was run on an SDS-PAGE gel and probed with anti-HA antibody via western blot analysis. It was found that no HvEPSP could be detected, suggesting transcription/translation had been unsuccessful or that Int36 protein levels were too low to detect. Transcription/translation was repeated three times with identical results.

As the wheat germ system was not successful in confirming the Hv14.3.3c-Int36 interaction, interaction confirmation of Hv14.3.3c with the mature HvEPSP protein was not attempted using this approach.

### 4.3.6 Heterologous expression of HvEPSP

Due to unsuccessful transcription/translation of Int36 using the wheat germ extract method, it was decided to use heterologous expression in *E. coli* to obtain larger amount of protein for co-IP. As this involved the construction of an expression vector and protein purification, expression of the mature HvEPSP, rather than testing the partial Int36 protein first, was attempted.

Construction of a HvEPSP/pQE30 construct was first attempted so that expression in *E. coli* and purification using Ni-NTA affinity purification could be performed as described for Hv14.3.3c (Chapter 3). However, after many attempts, HvEPSP was not successfully cloned into the pQE30, possibly due to the quality of the pQE30 vector stock used, and a different technique was employed.
HvEPSP was instead cloned into the expression vector pDEST17 using Gateway recombination, which still allows for \textit{E. coli} expression and Ni-NTA purification, but using a slightly different expression induction process and different \textit{E. coli} cell line. In addition to the 6×His tag provided by pDEST17, HvEPSP was also cloned from the Y2H construct HvEPSP/pGBK7 with the NH$_2$-terminal Myc tag, allowing for expression of HvEPSP with an additional Myc tag to facilitate co-IP as described below.

After expression induction, HvEPSP protein was purified in the same way as that described in Chapter 3 for Hv14.3.3c, however, no HvEPSP protein was obtained (Fig 4.18A). Overexpression of foreign genes in prokaryotes such as \textit{E. coli} can commonly lead to the production of intracellular aggregates of the recombinant protein defined as inclusion bodies (Luo et al, 2006) These inclusion bodies are insoluble and therefore require a denaturing method of purification, to resolubilise the protein. A denaturing purification, using urea instead of imidazole, was employed in an attempt to purify HvEPSP, with the thought that expression may have been successful, but inclusion bodies may have been formed. Urea purification results in the protein being denatured, therefore re-naturing the protein was required for correct folding into an active state for possible interaction with Hv14.3.3c. The eluted protein was run through a Centricon filter, exchanging the denaturing urea buffer for PBS, provided re-naturing conditions.

After SDS-PAGE analysis of the elution from the Centricon filter it was found that HvEPSP expression and purification had been successful, determined by the presence of a protein band at around the expected size of 50 kDa, which was 48 kDa for the HvEPSP mature peptide as determined in chapter 3, but slightly larger due to the presence of the two tags, 6×His and Myc. (Fig 4.18A).

To further confirm that HvEPSP had been successfully expressed and purified, a western blot was used. A small amount of the putative HvEPSP protein was probed with anti-His tag monoclonal antibody via western blot analysis. The same 50 kDa band was detected, confirming that this protein was HvEPSP (Fig 4.18B).

\textbf{4.3.7 Co-immunoprecipitation of recombinant Hv14.3.3c and HvEPSP}

Co-IP was first attempted by mixing the two proteins, Hv14.3.3c and HvEPSP together and using anti-Myc protein A agarose to pulldown HvEPSP (Fig 4.19). At first the co-IP appeared to have been successful, with both HvEPSP and Hv14.3.3c protein being detected (via probing with anti-His antibody) in the pulldown products, meaning that Hv14.3.3c had been bound to HvEPSP. However, it was also found that Hv14.3.3c protein was detected in the control sample where only Hv14.3.3c was added. For Hv14.3.3c to be present, there
Fig 4.18: SDS-PAGE analysis of HvEPSP mature peptide expression and purification. A) Purification of His-tagged HvEPSP peptide showing native purification 1 E. Coli lysate from induced cells 2 Ni-NTA resin flow through 3 Wash 1 4 Wash 4 5 – 7 Elution 1, 2, 3, and denaturing purification 8 E. coli lysate from induced cells 9 Ni-NTA resin flow through 10 Wash 1 11 Wash 4 12 – 14 Elution 1, 2, 3. B) SDS-PAGE gel and duplicate western blot showing anti His-tag antibody recognition of putative HvEPSP peptide band.
Fig 4.19: SDS-PAGE analysis of co-immunoprecipitation. SDS-PAGE gel and duplicate western blot showing anti-His antibody immunodetection of co-immunoprecipitated proteins from anti-myc pulldown of 1 Hv14.3.3c + HvEPSP 2 Hv14.3.3c alone 3 HvEPSP alone.
must have been non-specific binding of Hv14.3.3c to the Protein A agarose or Myc antibody, as no HvEPSP was present for it to bind to. While it appeared that more Hv14.3.3c was present in the test sample than the control, and that some Hv14.3.3c-HvEPSP binding may have occurred, the results were not conclusive.

The co-IP was repeated using pulldown in both directions, that is, using anti-Myc protein A agarose to pulldown His-Myc-HvEPSP as before, but also using anti-Hv14.3.3c protein A agarose to pulldown His-Hv14.3.3c. The stringency of the wash conditions was increased and more controls included for comparison. However, similar results to the first co-IP were obtained (Fig 4.20). Both HvEPSP and Hv14.3.3c were present in the anti-Myc and anti-14.3.3c pulldown of Hv14.3.3c + HvEPSP, suggesting interaction had occurred. However, Hv14.3.3c was again present in the anti-Myc pulldown when no HvEPSP was added, and similarly, HvEPSP was present in the anti-14.3.3c pulldown when no Hv14.3.3c was added, suggesting non-specific binding. Both HvEPSP and Hv14.3.3c protein was detected in the control sample of protein A agarose alone, without any antibody present, confirming that non-specific binding to the agarose was occurring, despite the stringent washes.

It was also found that a HvEPSP protein band was present in a pulldown sample where no HvEPSP protein had been added (lane 2 and 5, Fig 4.20A), suggesting HvEPSP contamination. However, this band was not detected by the anti-His antibody (lane 2 and 5, Fig 4.20B), suggesting that it was not HvEPSP. In the control samples run as size references, it was found that the Myc antibody contained a band the same size as HvEPSP. Antibodies consist of a heavy and light chain, which are 50 kDa and 25 kDa in size respectively. Therefore it was thought that the contaminating band may be antibody heavy chain, occurring due to denaturation upon elution and SDS-PAGE.

Due to the non-specific binding of the proteins to the protein A agarose resin and the possible presence of antibody bands masking the results, confirmation of the interaction was inconclusive.

One reason the co-IP may not have been successful could be that the proteins were not active or in their active conformation. Recombinant HvEPSP protein formed inclusion bodies that needed to be resolubilised via denaturation. Possibly, after denaturation, even with a renaturation step as used here, it is seldom possible to recover full activity of the protein (Kost, 1997). Therefore it is likely that the HvEPSP protein did not properly renature or refold into its native form, which may have inhibited its ability to interact with Hv14.3.3c.
Fig 4.20: SDS-PAGE analysis of repeat co-immunoprecipitation. A) SDS-PAGE gel of co-immunoprecipitated proteins from: anti-myc pulldown of 1 Hv14.3.3c + HvEPSP 2 Hv14.3.3c alone 3 HvEPSP alone, and anti-Hv14.3.3c pulldown of 4 Hv14.3.3c + HvEPSP 5 Hv14.3.3c alone 6 HvEPSP alone. Controls include 7 resin only pulldown of Hv14.3.3c + HvEPSP 8 Hv14.3.3c protein 9 HvEPSP protein 10 anti-Hv14.3.3c antibody and 11 anti-myc antibody for size comparison. B) Duplicate western blot analysis of 1 – 7 detected with anti His-tag antibody.
Similarly to the discussion in Chapter 3, other shortcomings of the heterologous expression system, such as the inability of *E. coli* to perform post-translational modifications of proteins (Dubessay et al., 2004), or the common mis-folding of proteins (Kost, 1997), may also have resulted in reduced or no activity of either or both HvEPSP and Hv14.3.3c, inhibiting the interaction.

### 4.3.8 Bioluminescence resonance energy transfer (BRET)

Bioluminescence resonance energy transfer (BRET) was used as an alternative means of confirming the Hv14.3.3c-HvEPSP interaction.

Efficient resonance energy transfer depends on the close juxtaposition of the energy donor and acceptor moieties (Subramanine et al., 2006) and, similarly to the heterologous expression systems previously discussed, expression of the proteins as fusions to the donor and acceptor moieties, can interfere with the ability of the proteins to interact. Therefore, the proteins of interest were expressed as both N- and C-terminal fusions. HvEPSP mature protein was cloned into the RLuc expression vector in both an N- and C-terminal orientation, and Hv14.3.3c was cloned into the YFP expression vector in a similar fashion. To first establish the method, a control vector, expressing both RLuc and YFP was transformed into barley leaf tissue (Dr A. Jacobs, ACPFG, University of Adelaide, Australia). Upon bombardment it was found that YFP was being expressed (Fig 4.21), detected by visualisation under a fluorescence stereo microscope (Leica FLIII) using a 500/20 nm excitation filter, suggesting successful transformation and expression of the construct. However, in subsequent luminescence measurement, luciferase expression could not be detected. It was thought that this may have been due to the level of expression (due to the number of cells transformed via bombardment) being too low to detect. However, there were also problems with preparation of the sample to assay, using the Dual-Glo™ Luciferase Assay System (Promega), which may have also affected detection. A step of the assay is to homogenise the leaf sample in a perspired quantity of assay solution, and the lysate from this step is used to measure luciferase activity. This process, however, did not produce much lysate, possibly due to the difference of using barley leaf tissue as opposed to tobacco or Arabidopsis in which this protocol had been used before (Subramanine et al., 2006).

Therefore, due to not being able to establish a working method, the Hv14.3.3c and HvEPSP BRET constructs were not tested for interaction.
Fig 4.21: YFP expression of BRET control vector. Single cell of barley leaf showing YFP expression after transformation with BRET RLuc:YFP expression vector via bombardment. Image courtesy of Dr A. Jacobs, ACPFG, University of Adelaide, Australia.
4.4 Summary and conclusions

The full length sequences of the three partial cDNAs, obtained from Y2H screening and identified as putative interactors with Hv14.3.3c and HvFKBP41, were successfully obtained. The full length of the first interactor, Int36, was obtained from 3 overlapping cDNA and genomic DNA fragments; the original Y2H fragment, and two 5’ end fragments, one from a genomic walk and the second from 5’ RACE. Subsequent sequence analysis confirmed its identity as a previously unidentified barley EPSP synthase, and the presence of a known 14.3.3 binding motif supported the finding of an interaction between Hv14.3.3c and HvEPSP.

Full length sequence of Int114, found to interact with both Hv14.3.3c and HvFKBP41, and Int63 was identified from database ESTs. Sequence analysis confirmed the identity of Int114 as a putative wound induced gene, while Int63 was found to be a Rab-type GTPase.

Expression analysis to investigate stress responsiveness and co-expression between candidate gene and interactor was performed. During _R. secalis_ infection, differential expression similar to that of the candidate genes (Chapter 2) was observed, supporting a potential role of the interacting genes in the defence response. The interactor genes showed expression patterns similar to that of the candidate genes, supporting their putative interaction, as well as similar patterns to each other, indicating the possibility that they may be linked to each other, e.g. belonging to the same signalling pathway.

The three interactor genes also showed very similar expression patterns during powdery mildew infection in barley, with all decreasing in expression in the resistant interaction (Wise et al., 2007), again supporting a potential link between them.

During ABA treatment, close expression pattern matches were seen between non-interacting pairs, suggesting a potential link between the other candidate genes and the interactors, for which interactions were not identified in the Y2H screen, as well as between the interactors themselves.

_HvGTPase_ and _HvEPSP_ showed almost identical expression patterns during _R. secalis_ infection and frost. EPSP synthase catalyses the rate limiting step for the production of precursors of secondary metabolites (Herrmann 1999), while GTPases are effectors involved in the transduction of elicitor signals to secondary messengers that lead to downstream reactions such as the production of secondary metabolites (Zhaoa et al., 2005). This, and the co-expression results obtained, suggest that _HvGTPase_ and _HvEPSP_ may be linked during stress response signalling, possibly via GTPase acting on EPSP, effecting its expression and therefore secondary metabolite production.
However, *HvGTPase* and *HvEPSP* showed very different expression patterns during ABA and drought, with *HvGTPase* being expressed similarly to *Hv14.3.3c* and different to all other genes during ABA treatment, and *HvEPSP* showing a decrease in expression after rewatering after drought, where all other genes showed an increase. This may therefore indicate a specificity for a role of HvGTPase and HvEPSP during infection and frost.

The expression results from this chapter and that in Chapter 2 show that in general, the candidate genes and putative interactors show similar expression patterns, supporting the potential for interaction. However, the genes showing the most similar expression patterns seem to differ under different treatments or stresses, which may suggest some specificity.

Unfortunately, the Hv14.3.3c-HvEPSP interaction was not conclusively confirmed using any of the techniques employed. Non-specific binding to resins was again an issue as discussed in Chapter 3. Due to the size of the proteins being investigated, antibody contamination was also an issue, as antibody bands on SDS-PAGE gels masked and confused the results. Other co-IP methods exist whereby the antibodies can be covalently linked to the resin matrix, which allows for elution of co-immunoprecipitated products from the resin, without elution of the antibodies. Time permitting, use of such a method may have overcome the problem of antibody band interference but would not help overcome non-specific binding to the resin.

BRET appears to be a good approach to overcome the problems with co-IP, however, more time is required to establish the technique for a barley plant interaction system.
Chapter 5

Conclusions and Future Work
This study consisted of two main parts, expression analysis and the identification of protein-protein interaction.

Expression analysis was successful, confirming that the candidate genes under investigation were in fact differentially expressed during infection, suggesting a role in the defence response of barley against \textit{R. secalis}. They were also found to be responsive to other stresses and treatments, namely frost, drought and ABA, indicating their role in defence may not be limited only to biotic stress, but include abiotic as well.

The results of co-expression pattern analysis between the candidate genes being investigated and their putative interactors identified in this study, further supported their potential to be true interactors. The results also indicated some possible specificity for certain gene responses and interactions to occur under certain stresses. For example, \textit{HvGTPase} and \textit{HvEPSP}, which showed almost identical expression patterns during \textit{R. secalis} infection and frost, but very different patterns during ABA treatment.

To expand on this expression analysis, future work could be to investigate expression of these genes at the protein level. As transcript level, measured in this study, does not always represent the actual level of protein translated, this may provide a more accurate indication of the activity of these gene products and their potential to interact. Western blot analysis using whole leaf protein extracts from \textit{R. secalis} infection series and the Hv14.3.3c antibody were attempted in this study (data not shown), however it was found that the 14.3.3c protein level was too low to be detected successfully. Optimisation of the protein extraction method or addition of a protein enrichment step would be required, as well as specific antibodies made for the other five proteins.

The first stage of protein-protein interaction analysis involved the identification of putative interactors. Two different approaches were taken. The first approach, Y2H screening, was used successfully to identify two putative interactors of Hv14.3.3c; an EPSP synthase and a putative wound induced protein, and two interactors of HvFKBP41; a Rab-type GTPase and the same wound induced protein. From what is known about these genes in the literature, they fit well with a role in stress response signalling and the potential to be involved in signalling networks with the candidate gene products and with each other.

It may be possible that HvMAPKK1 acts earlier in the signalling pathway, possibly being involved in transducing pathogen or stress perception. HvGTPase could also be involved in this initial perception, acting as an intermediate and passing the signal to secondary messengers. 14.3.3s have many diverse functions and it is possible that Hv14.3.3c may be involved in the phosphorylation-induced activation of HvMAPKK1, or may also play a
role in the import of HvEPSP from the cytosol to the chloroplast. HvEPSP might be involved in the synthesis of secondary metabolites that are involved in the plant’s defence mechanism and this synthesis may be affected or regulated by HvGTPase. HvFKBP41 could play a role in assembly and folding of downstream defence response genes, with the expression of immunophillins thought to be a general requirement for protein folding following synthesis of stress-responsive proteins.

The second approach employed to identify interacting proteins was affinity pulldowns. Through the trial of many different affinity pulldown techniques, a method for identifying interacting proteins from plant extracts was successfully established, although this technique still requires some improvement in the area of protein identification. Problems with Rubisco contamination prevented the identification of putative interactors via mass-spectrometry. If this work were to be continued in the future, employing techniques to remove or reduce Rubisco in the tissue extract before performing the pulldown, for example by using an anti-Rubisco antibody to bind and remove the Rubisco, could solve the contamination problem and lead to the identification of more interactors. With this technique established and working, it would then be very possible to compare the interactors pulled down from different tissue types, such as stressed and non-stressed tissue and identify differential interactions specific to certain stresses or treatments. In the case of *R. secalis* infection, it may also be possible to compare interactions at different stages of infection, with the ultimate aim of identifying highly specific proteins involved in the downstream defence response that could be targets of manipulation to engineer resistance to the fungus.

It may be possible that one or more of the interacting genes identified in this study are involved further downstream than the candidate genes in the defence response, and may be specific enough to be manipulated, ie overexpressed or expressed in certain tissues or under certain conditions, to confer resistance or tolerance to stress. However, similarly to the candidate genes, the interactors are involved in both numerous and important cellular processes and their manipulation may therefore have many other, possibly detrimental, effects than just an effect on resistance or stress tolerance. A way around this could be to use mutants, such as those identified through Targeting Induced Local Lesions IN Genomes (TILLING) (McCallum et al., 2000). Mutant lines can give access to different versions of the gene of interest that have only subtle modifications in the gene product’s function. Assessing TILLING lines with
mutations in the candidate genes and correlating the modified phenotype under stress with potentially modified protein-protein interactions could further elucidate the interactions observed in this study.

In addition to this, further work that could be undertaken to extend on this study, could also be to try to extend on the signalling networks and pathways that these genes may be involved in by repeating this work with the three identified interactors, possibly identify protein that they interact with which may be more specific downstream defence/stress response genes, with the potential to be manipulated.

The second stage of the protein-protein interaction work undertaken in this study involved attempts to confirm the Hv14.3.3c-HvEPSP interaction. However, after attempting many different methods, the interaction could not be conclusively confirmed. Co-immunoprecipitation of recombinant proteins may provide a viable means of confirming the interaction if alterations, such as the use of covalently-linking resin, are employed.

Similarly, BRET may also provide a means of confirming the interaction if the method can be adjusted to suit its use in a barley leaf system. There is a commercially available RLuc cDNA that has been adapted for human codon usage without altering the amino acid sequence. Subramanine et al (2006) found that in transiently transformed Arabidopsis seedlings, the enzyme activities for both unfused luciferase and several protein fusions were consistently higher with hRLuc than with the original RLuc cDNA. Therefore, use of this modified RLuc may overcome the problem of low luciferase levels if this was in fact the problem.

In general, the results of this study place the candidate genes Hv14.3.3c, HvMAPKK1 and HvFKBP41 as players in signal transduction during the plant defence/stress response. Some progress has also been made towards the identification of potential downstream genes that could possibly play a more specific role in defence response signalling and therefore be potential targets for the generation of resistant or stress tolerant plants.
Appendix
## Appendix 1 – Primers

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Sequence 5’ → 3’</th>
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<tbody>
<tr>
<td>14.3.3c full F</td>
<td>CGCAAAACGCAGATTTGGCAATTCAAG</td>
</tr>
<tr>
<td>14.3.3c full R</td>
<td>ACCACAACCTTCCCCGGAACATCA</td>
</tr>
<tr>
<td>14.3.3c qF</td>
<td>TGGCAGCCTCCCAGCTACTAGCT</td>
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<tr>
<td>14.3.3c Y2H F</td>
<td>TGGGATCCCATGTCGGCAACGAGGAGTTT</td>
</tr>
<tr>
<td>14.3.3c Y2H R</td>
<td>TACGCGGCACACACACACCTCAGCAGCC</td>
</tr>
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<td>14.3.3c pQE F</td>
<td>TGGGATCCCATGTCGGCAACGAGGAGTTT</td>
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<td>14.3.3c YFP F</td>
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<td>14.3.3c YFP NR</td>
<td>TTACTGCCCCTCAGCTGAGTCTAGTTT</td>
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<td>14.3.3c YFP CR</td>
<td>TTCTTTATGTCCCTGTGAATCACATGCC</td>
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<td>MAPKK 3’ F</td>
<td>TTCTTTCTTTCTTTCTTTGTCAGGCTGAGCC</td>
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<td>MAPKK 5’ out</td>
<td>GGCTTTATGTCCCTGTGAATCACATGCC</td>
</tr>
<tr>
<td>MAPKK 5’ in</td>
<td>CGGATGCAGAAGTCAGGCGAGGAAAC</td>
</tr>
<tr>
<td>MAPKK GW2_out</td>
<td>ACCTGATGTATTCTCATATGTGGCAGTCAGTTT</td>
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<td>MAPKK GW2_in</td>
<td>GCAGGCAAAAGATCAGGCGAGGAAAC</td>
</tr>
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<td>MAPKKqR</td>
<td>GGGTGGGCAATCAGTAGTGGAGG</td>
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<tr>
<td>FKBP 5’_out</td>
<td>GGATGCTGTATTCTTGAGTATCATCCAA</td>
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<tr>
<td>FKBP 5’_in</td>
<td>CTGGGGGAGTTGGAGTATCAGT</td>
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<td>ATCTGTACCCGAGATGCGGCAATAAGT</td>
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<tr>
<td>FKBP full R</td>
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<tr>
<td>FKBP qF</td>
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<tr>
<td>FKBP qR</td>
<td>CCTACTGTAGATCTGCCCTGAGGAAACCTGAGT</td>
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<tr>
<td>FKBP Y2H F</td>
<td>ATCCATATGATGCTGATTTTATCTTGAGTAAATTTTAG</td>
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<tr>
<td>FKBP Y2H R</td>
<td>GAATTCCGATACTTGAACTGAGGAAACCTGAGT</td>
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<td>EPSP 5’ out</td>
<td>CAGCCATTACGCAAGGAGGCTGATCCATTAT</td>
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<td>GAGAGCTTAACCTTGCCAGCGG</td>
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<tr>
<td>EPSP 5’2 out</td>
<td>GCCTCGAGCATGTCAGGCGAGGAGGAGG</td>
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<td>EPSP 5’2 in</td>
<td>TGTTCAACAGGTTATCCACGACCAGG</td>
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<tr>
<td>EPSP TopoF</td>
<td>GAGGAGCAAGCTGATCTCAGAGGAAGGAG</td>
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<td>EPSP TopoR</td>
<td>CGTACCAGCGCAATTCCGCGTACCTT</td>
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<tr>
<td>EPSP qF</td>
<td>CACGAGGATGCGGAGGAGGAG</td>
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EPSP qR
CAAGGCCAACGCACTACAAATAATTACA

EPSP mat F
CCGCGGGGCAGGCCAGGGGT

EPSP mat R
GGCTACAGGATACAGCGCAATTGC

EPSP Y2H F
ATTGATATGGCGGGCCAGGGGT

EPSP Y2H R
ATAGAATTCTCAAGCGCAATTATGCGTAG

EPSP Rluc F
ATGGCGGGCCAGGGGT

EPSP Rluc NR
CTAGTTTCTTGACAAAGGTGCTTAGGACGTC

EPSP Rluc CR
GGCTACAGGATACAGCGCAATTGC

Wound full F
CGACAGACTTACCTTCTGATATGATC

Wound full R
TGCGAACGGAGGAATATGATACAA

Wound qF
GCTAAGAGTTTGTATTGTGGAGGCAATAG

Wound qR
CAATTTTGCAGAGGCAATATGATACAC

Wound Y2H F
TAGCATATGATGGCAATGGAGGGAACA

Wound Y2H R
ATAGGATCCGAGGAAATATGATACAAATAGA

GTPase qF
GAGTGCAAAGACAAACATG

GTPase qR
TCGCAACTTCTTCTTCTCCGTGG

RLM-5' outer
GCTGATGGCGATGAATGAACACTG

RLM-5' inner
CGCGGATCCGAACACTGCGTTTGCTTTGATG

T7
TAATACGACTCTATAG

SP6
ATTTAGGTGACACTATAG

Oligo(dT)17
TTTTTTTTTTTTTTTTTT

UPL
CTAATACGACTCTATAGGCAAGCAGTGGTAGTTTCAACCGAGAGT

UPS
CTAATACGACTCTATAGGC

NUP
AAGCAGTGATATCAACCGAGAGT

AP1
GTAATACGACTCTATAGGC

AP2
ACTATAGGGCAGCGGTGG

MML 3' BD
AGATGGTGCAGAGAGGATG

MML 3' AD
TTTCGTTTAAAAACCTAAGAGTC

pGA F
CTATTCGATGATGAAGATACCCCAC

pGA R
TGGCGAAGAAGTCCAAAGCTT

pQE F
CGGAACAAATTTCACAC

pQE R
GTTCTGAGGTCATTACTGG

GW1
GTTGCAACAAATTGATGAGCAATGC

GW2
GTTGCAACAAATTGATGAGCAATTA
References


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