Molecular characterisation of differentially expressed genes in the interaction of barley and *Rhynchosporium secalis*

Submitted by

Jafar Sheikh Jabbari, B.Sc., M.Sc.

A thesis submitted in fulfillment of the requirements for the degree of Doctor of Philosophy

Discipline of Plant and Food Science

School of Agriculture, Food and Wine

Faculty of Sciences

University of Adelaide, Waite Campus

Glen Osmond, SA, 5064

Australia

Chapter 1: General Introduction

1.1 Introduction

The work in this thesis describes the functional analysis of three defence candidate genes, which were selected from a library, enriched for differentially expressed genes in the barley-*Rhynchosporium secalis* interaction. The overall aim of the work was to isolate full-length clones of the genes and investigate the potential biological role of corresponding proteins in barley disease resistance and analyse their putative function. The study included expression analysis during development of barley as well as under different biotic and abiotic stresses, mutant complementation, heterologous protein expression, protein subcellular localisation and producing overexpression and knock down transgenic barley lines. Since the common theme among these genes assumed a role in plant defence response, this chapter deals mainly with plant defence against pathogens. In the following sections, plant defence responses, the barley-*Rhynchosporium* interaction and some techniques that were used to address the functional analysis of candidate genes are introduced. Gene specific reviews will be presented in detail for each gene in later chapters where experimental data are presented.

1.2 Plant defence against pathogens

Constant exposure of plants to potential pathogens has resulted in evolution of a number of strategies to resist invasion. Plants possess an effective basal resistance against potential pathogens. To allow successful infection and tissue colonisation pathogens avoid, tolerate, manipulate, or suppress defence responses (Cui *et al.*, 2005; Nomura *et al.*, 2005; Schulze-Lefert, 2004; Zimmerli *et al.*, 2004). Basal resistance include inducible responses as well as pre-formed physical and chemical barriers.

Inducible defences are triggered by microbe-associated molecular patterns (MAMPs/PAMPs) (Bent and Mackey, 2007; Nurnberger and Lipka, 2005), and elicitors. MAMPs often constitute indispensable structural components of the pathogen (Nurnberger *et al.*, 2004) such as lipopolysaccharide and lipooligosacharides (Gerber and Dubery, 2004; Gross *et al.*, 2005; Keshavarzi *et al.*, 2004; Newman *et al.*, 2002; Silipo *et al.*, 2005; Zeidler and Castranova, 2004), peptidoglycan, bacterial flagellin (Felix *et al.*, 1999; Gomez-Gomez and Boller, 2002), bacterial elongation factor Tu (Kunze *et al.*, 2004),

fungal chitin or peptides such as Pep-134 in oomycetes (Jones and Takemoto, 2004; Zipfel and Felix, 2005) and cold shock proteins (Felix and Boller, 2003). Perception of MAMPs or MIMPs (see below) initiates MAMP/MIMP-triggered immunity (MTI) which is sufficient to halt microbial infection (Jones and Dangl, 2006). It also reduces the severity of symptoms in compatible interactions as seen in Arabidopsis enhanced disease susceptibility (*eds*) mutants (Glazebrook *et al.*, 1996; Parker *et al.*, 1996). It has been shown that a threshold level of MAMP must be present before the response is activated (Melotto *et al.*, 2006). The direct contribution of MAMP perception to whole-plant disease resistance was shown in Arabidopsis plants lacking the flagellin receptor Fls2. These plants showed increased susceptibility to infection by *Pseudomonas syringae* pv. *tomato* strain DC3000 (Zipfel *et al.*, 2004).

Elicitors are molecules composed of both pathogen and plant origins that are recognised by plant surveillance system. Release of effector proteins as well as chitin and glucan oligomers from pathogen cell walls can act as elicitors (van Loon *et al.*, 2006). Fragments of both the cuticle and the plant cell walls which are released by pathogen-secreted, plant cell wall-degrading enzymes such as xylanases, pectate lyases and polygalacturonases can also elicit defence responses (Bent and Mackey, 2007; Huckelhoven, 2007). It has been proposed to call these plant derived compounds MIMPs (microbe-induced molecular patterns) (Ignatius *et al.*, 1994; Mackey and McFall, 2006). MIMPs compose a very significant class of elicitors and were shown in many studies to induce defence-related responses (Bent and Mackey, 2007; Davis *et al.*, 1984; Hahn *et al.*, 1981; Schweizer *et al.*, 1998). To overcome basal defences, microbes have evolved strategies to avoid recognition or to suppress plant defences. Effectors are pathogen proteins and toxins that interact with the host which interfere with recognitions of MAMPs and MIMPs or alter resistance signalling and responses or promote pathogenesis. When an effector is recognized by a host defence receptor (resistance protein), such effectors are also named avirulence genes. Effector-triggered immunity (ETI) is referred to recognition of effectors which are used by microbes to suppress MTI (Chisholm *et al.*, 2006). The ability of effectors to suppress basal defences is host specific, which likely contributes to the ability of a microorganism to be a "pathogen" only on a subset of hosts (Bent and Mackey, 2007).

Resistance to a pathogen can be achieved at both the species (non-host) and cultivar level. Non-host resistance can result from successful preformed barriers or toxic chemicals as

well as from active defences induced upon pathogen recognition (Mysore and Ryu, 2004). Cultivar level resistance is induced by direct or indirect gene-for-gene recognition of pathogen avirulence and a host-encoded resistance (R) protein (Cunha *et al.*, 2006). The induced responses will be covered later (Section 1.2.2).

1.2.1 Preformed defence mechanisms

Preformed antimicrobial secondary metabolites and physical barriers constitute a plant's first line of defence against invading organisms (Mysore and Ryu, 2004; Thordal-Christensen, 2003). These passive defences include the presence of a waxy exterior surface, cell walls and constitutively produced secondary metabolites and enzymes with antimicrobial properties (Osbourn, 1996). Chemical and structural signals from plant surfaces induce pathogen cell differentiation and expression of pathogenicity genes. For example, hyphal differentiation of rust fungi is induced by topography of the plant (Hoch and Staples, 1987) and surface wax composition of barley is important in differentiating appressoria of the powdery mildew fungus (Tsuba *et al.*, 2002). After breaching the cuticle by pathogens, cell walls present another obstacle for successful tissue colonisation. Although physical barriers can prevent microorganism entry into tissues, pathogens have evolved strategies to overcome these. For instance, pathogens employ a cell wall–degrading secretome including cellulases, polygalacturonases, xylanases, and proteinases to degrade cell walls (reviewed in Annis and Goodwin, 1997). Motile foliar bacteria enter the leaf apoplast through wounds and stomata or the penetration pegs of fungal appressoria generate considerable force sufficient to puncture plant barriers (reviewed in Knogge, 1996).

Plants produce a diverse array of constitutive antimicrobial secondary metabolites which are termed “phytoanticipins” (Vanetten *et al.*, 1995). Some of these compounds are constitutive and exist in biologically active forms in healthy plants (reviewed in Ingham, 1973). Others such as saponins (Osbourn, 1996), cyanogenic glycosides and glucosinolates (Giamoustaris and Mithen, 1995) occur as inactive precursors which are activated often by plant enzymes. The enzymes are released as a result of breakdown in cell integrity caused by tissue damage or pathogen attack. Compounds belonging to the latter category are still regarded as constitutive because their synthesis is not transcriptionally activated. Because in healthy plants these compounds are commonly

sequestered in vacuoles or organelles, the extent of tissue damage will determine the concentrations encountered by an invading pathogen. This implies that necrotrophs are likely to cause substantial release of these compounds while biotrophs may avoid the release by minimizing damage to the host. The exposure level of a potential pathogen to these compounds also depends on host genotype, age, and environmental conditions (Price *et al.*, 1987) and de novo synthesis of enzymes (Osbourn, 1996). Some pathogens can overcome toxic secondary metabolites by producing detoxifying enzymes, for instance *Gaeumannomyces graminis* var. *avenae* can detoxify avenacinA-1 antimicrobial saponin of oat roots (Papadopoulou *et al.*, 1999) and *Botrytis cinerea* can detoxify the tomato saponin α-tomatine or the phytoalexin resveratrol and this ability has been correlated with the aggressiveness of this fungus (Quidde *et al.*, 1998; Schoonbeek *et al.*, 2001). When passive defences are breached, plants rely on their innate immune system to prevent disease with active responses.

1.2.2 Inducible defence responses

Pathogens overcome preformed obstacles, and access the plant interior. Recognition of pathogen presence by plant receptors initiates inducible responses that trigger a series of signalling cascades and activate numerous defence pathways. Early transcription-independent defence induces rapid biochemical changes such as altered ion fluxes across the plant plasma membrane (Felle *et al.*, 2004), changes in the phosphorylation state of regulatory proteins (Chandra and Low, 1995), transient increase in cytosolic Ca^{2+} (Stab and Ebel, 1987), activation of Ca^{2+} dependent protein kinases (Garcia-Brugger *et al.*, 2006), increase in nitric oxide (Wendehenne *et al.*, 2004) and cyclic nucleotides (Durner *et al.*, 1998), generation of active oxygen species (Duan and Schuler, 2005; Jabs *et al.*, 1997) and more.

The successive activation of components of signal transduction cascades eventually leads to the expression of plant defence. Such defence comprises the deposition of callose and lignin for cell wall fortification (papilla) (Kauss, 1987; Zeyen *et al.*, 2002), hypersensitive response (HR) (Maleck and Dietrich, 1999), the oxidative burst, accumulation of phytoalexins (Dixon, 1986) and synthesis of pathogenesis-related (PR) proteins (Bol *et al.*, 1990; Bowles, 1990; Linthorst, 1991). Other responses include enhanced transcription of genes encoding enzymes involved in the flow of carbon from the primary to the secondary

metabolism of plants, such as peroxidases, lipooxygenases, superoxide dismutases and phenylalanine ammonia lyase (PAL), a key enzyme in the biosynthesis of phenolic compounds with antimicrobial activity (Montesinos, 2000) and the induction of systemic resistance in dista1 plant organs.

Accumulation of reactive oxygen species (ROS) in plant cells is implicated in mediating cell wall cross-linking, the induction of defence gene expression, and the induction of the hypersensitive response (Torres and Dangl, 2005). Nitric oxide (NO) also is an important signalling molecule in plant defence. NO functions in combination with ROS to potentiate the hypersensitive cell death against *P. syringae* in both soybean cells and *Arabidopsis* leaves (Delledonne, 2005).

Induced biochemical defences include the production of phytoalexins and antimicrobial proteins, which include PR proteins such as chitinases and glucanases. PR proteins will be discussed in detail in Chapter 3. Phytoalexins are non-proteinaceous low-molecular-mass secondary metabolites, which display an enormous chemical diversity and exhibit antimicrobial and antifungal activities. They include phenolics, terpenoids, polyacetylenes, and fatty acid derivatives and are produced by plants in response to diverse stresses, but predominantly against fungal infection (Grayer and Kokubun, 2001; Panikulangara *et al.*, 2004; Pedras *et al.*, 2002). Increased phytoalexin biosynthesis often correlates with enhanced resistance to several pathogens including pathogenic fungi (Thomma *et al.*, 1999; Yang *et al.*, 2004).

The defence responses not only occur locally at the site of attempted ingress but also in distal tissues and result in enhanced systemic resistance to subsequent pathogen encounter. These systemic resistance responses include systemic acquired resistance (SAR) and induced systemic resistance (ISR). The resistance conferred by SAR is long-lasting systemic resistance that is often effective against a broad spectrum of pathogens. SAR is characterized by the spectrum of both disease resistance and expression of a particular set of genes in resistant tissue. For example, in tobacco, SAR provides a significant leve1 of protection against tobacco mosaic virus, *Pseudomonas syringae* pv *tabaci, Cercospora nicotianae, Phytophthora parasitica, Peronospora tabacina,* and *Erwinia carotovora* (Friedrich *et al.*, 1996; Hunt and Ryals, 1996; Vernooij *et al.*, 1995). In Arabidopsis, the SAR response involves the induction of a subset of the genes expressed in tobacco (Uknes

et al., 1992) suggesting that the types of SAR genes expressed may be unique among species. It was believed that SAR develops in response to pathogen-induced tissue necrosis (Durrant and Dong, 2004). However, recently it was shown (Mishina and Zeier, 2007) that MAMPs recognition, not tissue necrosis, contributes to bacterial induction of SAR in Arabidopsis. Characterization of several SAR-deficient Arabidopsis mutants has revealed that establishment of SAR is dependent on systemic accumulation of salicylic acid (SA), existence of a functional SA signalling pathway and systemic expression of a set of PR and other defence genes (Durrant and Dong, 2004; Grant and Lamb, 2006; Mishina and Zeier, 2006). SA levels are elevated at the onset of SAR in cucumber (Metraux *et al.*, 1990), tobacco (Malamy *et al.*, 1990), and Arabidopsis (Uknes *et al.*, 1993). The nature of the mobile signal in SAR was remained elusive for many years. An Arabidopsis mutant (*dir1*) impaired specifically in the systemic character of SAR, implicated involvement of a lipid transfer protein (Maldonado *et al.*, 2002), suggesting that the mobile signal may contain a lipid moiety. However, very recently Park and colleagues (2007) showed that the methyl salicylate is the mobile SAR signal in tobacco plants.

In addition to pathogens it has been found that treatment of plants with the synthetic chemical 2, 6-dichloroisonicotinic acid (INA) can induce SAR (Metraux *et al.*, 1990). In both tobacco and Arabidopsis, INA induces the same spectrum of pathogen resistance and gene expression as does pathogen infection (Lawton *et al.*, 1995; Uknes *et al.*, 1992; Vernooij *et al.*, 1995; Ward *et al.*, 1991). Chemically induced resistance has been described also in barley by INA (Kogel *et al.*, 1994; Wasternack *et al.*, 1994) and by benzo (l,2,3) thiadiazole-7-carbothioic acid S-methyl ester (BTH) in wheat (Gorlach *et al.*, 1996).

ISR develops in response to colonization of plant roots by non-pathogenic, root-colonizing bacteria and depends on jasmonic acid (JA) and ethylene signalling (Pieterse *et al.*, 1998; Ton *et al.*, 2002). ISR is independent of the production of salicylic acid (SA) by the plant and is not associated with the accumulation of PRs (Pieterse *et al.*, 1996; van Loon *et al.*, 1998; Verhagen *et al.*, 2004). Recent studies have shown the involvement of a secretory hydrophobin-like elicitor Sm1 from *Trichoderma virens* in the induction of ISR in cotton (Djonovic *et al.*, 2006) and maize (Djonovic *et al.*, 2007). Similarly, lipopeptides surfactins and fengycins from *Bacillus spp* have been indicated in the induction of ISR in

beans and tomato (Ongena *et al.*, 2007). The induction correlated with stimulation of enzymatic activities of lipoxygenases and hydroperoxide lyase which are key enzymes in the lipoxygenase pathway, leading to synthesis of antifungal phytooxylipins (Ongena *et al.*, 2004). Many of the pathogens investigated could be restricted by both SAR and ISR and induction of both SAR and ISR in the same plant leads to additively increased protection (van Wees *et al.*, 2003). These observations indicate that SAR and ISR are complementary types of induced resistance with partly overlapping, partly specific actions against different types of pathogens.

1.2.3 Difference in compatible and incompatible interactions

Plants with mutation in defence pathways can show enhanced susceptibility or reduced resistance which supports the idea that there is a continuum of possible interactions ranging from extreme susceptibility to complete resistance (Glazebrook, 2001). The success of a plant resistance depends on the type and magnitude of activated signalling pathways, their effectiveness against individual pathogens and pathogen ability to suppress those responses. As outlined above, MTI is effective in defence against non-host pathogens and restricting growth of pathogens and reduction of symptoms in compatible interactions. However, ETI is faster and stronger than MTI (Tao *et al.*, 2003; Thilmony *et al.*, 2006; Truman *et al.*, 2006) and often culminates in HR (Greenberg and Yao, 2004). Microarray data shows that many of the rapid gene expression changes that occur during gene-for-gene responses (ETI) also occur during susceptible interactions, but with slower kinetics and at reduced magnitude (Tao *et al.*, 2003; Zierold *et al.*, 2005a). This pattern can result from the suppressive action of microbial virulence factors and reprogramming the host to support pathogenesis (Chisholm *et al.*, 2006; Hauck *et al.*, 2003; Ridout *et al.*, 2006). The defence responses described so far are a collective overview of many pathosystems. In the next section, barley interaction with *R. secalis* will be reviewed.

1.3 Barley–*Rhynchosporium* pathosystem

Scald disease of barley (*Hordeum vulgare* L.) caused by *Rhynchosporium secalis* (Oudem) J. J. Davis, is a serious disease in all of the major barley growing regions of the world (Graner and Tekauz, 1996; Shipton *et al.*, 1974; Xue and Hall, 1992) and it is reported to

be most severe on barley in cool, humid areas of temperate zones (Robbertse *et al.*, 2000; Xue and Hall, 1992).

In field trials yield losses caused by *R. secalis* have been measured at 10-70% (Anon, 1974; Anon, 1976; Shipton *et al.*, 1974; Zhang *et al.*, 1992), in Western Australia over 45% (Khan, 1986) and in South Australia 48% (Jefferies *et al.*, 2000). Crop surveys have indicated average annual yield loss of 10-40% throughout southern Australia (Abbott *et al.*, 1991; Khan, 1986) and 15% in Victoria (Brown, 1985). Scald damage is mainly attributed to a reduction in 1000-kernel weight (Khan and Crosbie, 1988; Scott *et al.*, 1992), detrimental effects on quality of malt and feed barley grain (Basson *et al.*, 1990; Edney *et al.*, 1998) and plumpness (James *et al.*, 1968; Schaller, 1951).

In Australia, in addition to barley, scald commonly infects volunteer barley grass (*Hordeum leporium* Link) which is abundant around farms (Ayesu-Offei, 1971; Khan *et al.*, 1968; Smith and Murray, 1971). *R. secalis* is pathogenically a very variable fungus and a large number of different pathotypes have been identified (Fukuyama *et al.*, 1998; Goodwin *et al.*, 1992; Salamati and Tronsmo, 1997). Due to the economic importance of scald disease there are numerous references in the literature on different aspects of the barley-*Rhynchosporium* pathosystem, which covers the pathogen, histopathology, host and pathogen interaction, genetics, biochemistry and molecular biology.

1.3.1 *Rhynchosporium secalis*

In addition to barley, *R. secalis* infects rye (*Secale cereale* L.), couch grass (*Elymus reperis* L.) and over 20 other grass species from ten genera (Kilby and Robinson, 2001; Sprague, 1950). Numerous studies on host specialisation of *R. secalis* have shown contrasting results.

On one hand some studies indicate that *R. secalis* has a preference for a particular species, for instance, isolates from rye, couch grass and barley did not show cross infection (Robinson *et al.*, 1996). Similarly samples collected from rye and couch grass were pathogenic only on the original host (Cromey and Mulholand, 1987). Others also found strict host specialisation (Blum *et al.*, 1987; Caldwell, 1931; Dyck and Schaller, 1961a;

Kajiwara, 1968; Mazars *et al.*, 1983; Rai and Strobel, 1969; Shipton *et al.*, 1974; Spencer and Gorin, 1961).

In contrast, other investigations indicate that there is not a strict host specialization among isolates from different species. Isolates from rye caused minor lesions on barley (Kilby and Robinson, 2001; Lyngs Jorgensen and Smedegaard-Petersen, 1995) and isolates collected from couch grass attacked barley (Jackson and Webster, 1976c). Other investigators also observed less host specialization (Caldwell, 1937; Kay and Owen, 1973). In addition, isozyme analyses showed no genetic evidence for distinct *R. secalis* populations on various host species (Goodwin *et al.*, 1993).

In laboratory experiments the possibility of adaptation from other hosts to barley and *vice versa* has been shown (Kay and Owen, 1973). Under optimal conditions, it was shown that isolates from rye, couch grass and wall barley (*Hordeum murinum* L. ssp. Murinum) increased their pathogenicity to barley after serial passages over barley leaves. Similarly it has been shown in Australia that isolates from barley and barley grass may infect the alternate host (Ali, 1981; Ali *et al.*, 1976).

Numerous reports indicate high variability in the pathogenicity spectrum of *R. secalis* worldwide (Ali *et al.*, 1976; Ceoloni, 1980; Cromey, 1987; Cromey and Mulholand, 1987; Houston and Ashworth, 1957; McDonald *et al.*, 1999; Williams and Owen, 1973b; Xu *et al.*, 1997). Physiological races of *R. secalis* specialized to particular barley cultivars have been demonstrated (Obendorf, 1997; Owen, 1958; Riddle and Briggs, 1950; Riederer and Schreiber, 2001; Schein, 1957). Existence of *formae speciales* among *R. secalis* have also been shown (Caldwell, 1937; Owen, 1958).

Isolates collected from the same lesion were found not only to differ in pathotypes but also in sporulation rate and isozymes (Brown, 1990; Habgood, 1973; Hansen and Magnus, 1973; Newman and Owen, 1985; Robbertse *et al.*, 2000; Zhang *et al.*, 1992). Even variation in pathogenicity between single spore cultures of *R. secalis* derived from the same parental isolates has been reported (Hansen and Magnus, 1973; Tekauz, 1991). It was shown that 14 new pathotypes were recovered from plants inoculated with a mixture of five pathotypes (Jackson and Webster, 1976a). The possibility that individual isolates, even when single spore derived, subsequently evolve into mixtures (Hansen and Magnus,

1973) suggests that the virulence profile of single *R. secalis* isolates may change over time. In a study between 1973 and 1983 it was found that there was a considerable change in the pathogenicity pattern of *R. secalis* in central Norway, expressed by the increasing number of virulence genes in the local isolates (Elen, 1987). These changes make test results equivocal and comparison with previous and other independent studies difficult. Spontaneous mutation (Goodwin *et al.*, 1994), parasexual cycles (Burdon *et al.*, 1994) and an unknown sexual stage (Salamati *et al.*, 2000) are believed to be possible sources of variation. Two greenhouse studies involving pathogenicity markers (Jackson and Webster, 1976a; Starling *et al.*, 1971) and isozyme markers (Newman and Owen, 1985) have demonstrated the generation of recombinant genotypes.

Many researchers have reported that *R. secalis* isolates are characterised by unnecessary virulence. In other words they are virulent on host barley containing resistance genes to which they have not been exposed (Ali and Boyd, 1973; Goodwin *et al.*, 1990; Jackson and Webster, 1976b; Salamati and Tronsmo, 1997; Tekauz, 1991; Xue and Hall, 1991). However, it should be noted that the diversity of races distinguished in a trial depends on the number of barley cultivars and their scald resistance genes (Ceoloni, 1980; Jackson and Webster, 1976a; Tekauz, 1991). In addition comparisons among studies of pathogenic variability are complicated by other factors, including the influence of environmental conditions on scald symptom development (Skoropad, 1960; Williams and Owen, 1973b), the genetic variability among differentials even within individually named cultivars (Ceoloni, 1980), the effect of inoculum concentration on host reaction (Jackson and Webster, 1976a), and the scoring scale used to assess the disease (Lyngs Jorgensen *et al.*, 1993). For these reasons, critical comparisons between experiments are not possible. This problem is further complicated by the lack of agreement about the identity of resistance genes in barley cultivars (Habgood and Hayes, 1971).

1.3.2 Infection process and changes in host tissue

Infection is initiated by splash-dispersed spores (Shipton *et al.*, 1974) that require 4 to 12 hours of free moisture to germinate (Jarosz and Burdon, 1988). In addition to spores from previous crop residues and wild hosts (Ali and Boyd, 1973; Owen, 1958), the disease is also transmitted from one generation of host to the next one by mycelial inoculum in seeds (Habgood, 1971; Skoropad, 1959). Generally, both conidial cells germinate with up to

three germ tubes, forming an appressorium prior to penetration attempts (Lyngs Jorgensen *et al.*, 1993). Appressorium formation can also occur directly on the conidium (Ryan and Grivell, 1974). Below each appressorium an infection hyphae forms and penetrates the cuticle within 24 hours of inoculation (Ayesu-Offei and Clare, 1970; Caldwell, 1937; Xi *et al.*, 2000a). In a few cases penetration initiates from the tips of germ tubes without discernible appressoria (Ayesu-Offei and Clare, 1970). Following penetration of wax and cuticle layers the fungus initially grows above the anticlinal walls of two adjacent host epidermal cells (Ayesu-Offei and Clare, 1970), which has been reported to be rich in pectic substances (Martin and Junpier, 1970). It is at this stage that swelling of epidermal cells and loss of the cell wall rigidity occurs. This is often accompanied by a separation of the plasmalemma from the cell wall (Ayesu-Offei and Clare, 1970; Lehnackers and Knogge, 1990). These changes have been speculated to be the cause of permeability change in host cells (Jones and Ayres, 1972; Wevelsiep *et al.*, 1993).

The increased permeability causes solute loss from epidermal cells and subsequently turgor pressure between stomatal guard cells and their surrounding epidermal cells is altered, which results in increased opening of stomatal cells and finally failure of stomata to close in the dark (Ayres, 1972; Branchard and Laffray, 1987). Beside changes in turgor pressure, kinetin has also been shown to enhance stomatal opening in barley (Digby and Cooper, 1972) and it has been reported (Evans and Banerjee, 1973) that *R. secalis* produces cytokinins in culture and that abnormally high levels of cytokinins have been isolated from leaves infected by *R. secalis.*

As infection progresses the outer layer of the cell wall erodes and the inner layer is attacked. At this stage the cuticle still remains largely intact (Ryan and Grivell, 1974). Since the fungus is capable of producing cellulytic enzymes in culture, it is believed that similar enzymes may be involved in the *in vivo* degradation of host cell walls (Ryan and Grivell, 1974). Subsequently epidermal cells collapse. After about 10 days of the disease and profuse branching of fungal mycelium, the mesophyll cells underlying affected epidermal cells also collapse and finally necrotic lesions form (Lehnackers and Knogge, 1990). Evidence for the infection of mesophyll cells only after collapse of epidermal cells is supported by measuring CO_2 fixation by the host, which was not reduced by infection until the collapse of epidermal cells (Jones and Ayres, 1974). Concomitantly, the thick hyphae develop into a loosely packed subcuticular prosenchyma, later developing into a

stroma and subsequent sporulation (Lyngs Jorgensen *et al.*, 1993). Penetration of the cytoplasm has never been reported.

After collapse of epidermal cells, macroscopic symptoms appear on the leaf as grey, water-soaked patches 8-12 days post inoculation. The lesions are associated with large masses of mycelium. These lesions usually develop into oval-shaped, pale-brown or white centres surrounded by a dark-brown margin (Ayesu-Offei and Clare, 1971; Fowler and Owen, 1971).

1.3.3 Histopathology of infection in susceptible and resistant barley cultivars

Responses of barley cultivars to *R. secalis* in compatible and incompatible interactions have been investigated to identify the inhibition stage of fungal growth and possible mechanism. Localized thickening of the inner host cell wall called apposition is the visible response of barley cells to an infection at the point of attempted penetration (Jones and Ayres, 1974; Xi *et al.*, 2000a). Apposition is seen as a circular or semicircular ridge. In the centre of an apposition, a circular area appears which is called a "halo" and is believed to be the result of ridges around the point of attempted penetration (Ayesu-Offei and Clare, 1970; Xi *et al.*, 2000a).

The role of the apposition in relation to resistance of barley to *R. secalis* is not clear. Some studies show that appositions occur at higher frequencies following penetration attempts in resistant cultivars than susceptible ones and they seem to be larger in resistant cultivars (Ayesu-Offei and Clare, 1970). There are also observations that race-specific resistance enhances the formation of appositions (Lyngs Jorgensen *et al.*, 1993). Similarly Xi *et al.* (2000a) correlated the successful penetration of susceptible cultivars with no halo being formed around the point of penetration. The failure of penetration with different germ tubes, but the same conidia, was found to be associated with the formation of halo, occasionally, successful penetration also occurred despite halo. However, despite these reports it is believed that the role of appositions in the resistance to *R. secalis* is insignificant as they are found on both resistant and susceptible cultivars (Lehnackers and Knogge, 1990). Nevertheless appositions could be of importance if their chemical

composition varies between susceptible and resistant cultivars (Lyngs Jorgensen *et al.*, 1993).

In general, the cutin and wax layers of the leaf cuticle is believed not to contribute to protection of barley against *R. secalis* (Ayres and Owen, 1971; Martin, 1965). Abrasion of leaves in resistant cultivars had no effect on the resistance of the host (Ali, 1974). However, it should be noted that abrasion does not result in complete loss of cuticle.

The inhibition of conidial germination has been suggested to be a mechanism of resistance (Lehnackers and Knogge, 1990; Shipton *et al.*, 1974). However other reports have found no evidence of a role for inhibition of spore germination in resistant cultivars (Fowler and Owen, 1971; Xi *et al.*, 2000a). In addition, germination and formation of appressoria by *R. secalis* has been found to be independent of host genotype. The percentage of spore germination, the number of germ tubes per germinated spore and the number of appressoria per hundred germ tubes was found to be the same for susceptible and resistant cultivars (Fowler and Owen, 1971).

Subcuticular mycelium of *R. secalis* has been reported in both susceptible and resistant barley cultivars (Fowler and Owen, 1971), but in resistant cultivars the frequency and extension rate of the hyphae were limited and superficial and accompanied with the formation of abnormal conidia (Ali, 1974). It is believed that a critical step in barley defence is the result of subcuticular mycelium growth prevention, degradation of fungal mycelium (Lehnackers and Knogge, 1990) and inhibition of sporulation, which could explain the differences between resistant and susceptible cultivars in race-specific interactions (Habgood, 1977; Kari and Griffiths, 1993; Xue and Hall, 1991). Reduced sporulation and increased latency period (Fowler and Owen, 1971; Habgood, 1977) seem to act in race non-specific resistance.

1.3.4 Factors affecting disease symptoms

The expression of disease symptoms is determined by the combined influence of host genotype, pathogen genotype and environmental conditions (Boyd *et al.*, 1987). The effect of environmental factors on the infection of barley by *R. secalis* has been the subject of investigations by many researchers (Ayesu-Offei and Clare, 1970; Fowler and Owen,

1971; Polley, 1971; Skoropad, 1960). There are indications that the germination of conidia and growth of germ tubes proceeds at relative humidity greater than 95%, and light slows these processes. Both temperature and leaf surface wetness affect the period from germination to penetration and the maximum infection occurs when the leaf surface remains wet for more than 14 hours and the temperature is 20 to 25°C. Temperature can also affect both the resistance of the host and the pathogenicity of the pathogen (Ali, 1974; Yarwood, 1959).

1.3.5 Resistance genes

Resistance of barley to *R. secalis* is an inherited character. Resistance of barley to the scald disease often follows the gene-for-gene model (Flor, 1971) and is based on a gene for avirulence in the pathogen and a corresponding resistance gene in the plant (Hahn *et al.*, 1993; McDonald *et al.*, 1989). Existence of such a model in the barley-*Rhynchosporium* interaction was shown (Hahn *et al.*, 1993) at the molecular level by isolation of the *Nip1* avirulence gene in specific fungal pathotypes. The product of *Nip1* is recognised by resistance gene *Rh3* (*Rrs1*) in barley.

Genetic interpretation of scald resistance can vary, depending on the pathotypes being used, the genetic background in which a resistance gene resides, environmental conditions in which the screening is conducted and the scoring system used (Ali *et al.*, 1976; Garvin *et al.*, 1997; Tekauz, 1991; Xue *et al.*, 1991). Ceoloni (1980) believes that the genetics of some resistances is more complicated due to additional factors or different alleles at the same locus. Closely linked genes could not have been detected with the races used in the genetic analysis so far reported. The detection of certain resistance genes and their distinction to previously reported ones is always dependent on the ability of the virulence genes employed to differentiate between host genotypes.

1.3.5.1 Major resistance genes

The designation of scald resistance genes in barley is complex and inconsistent (Robinson *et al.*, 1996). At least 15 different resistance genes from cultivated and wild barley have been identified (Genger *et al.*, 2005) but, there is confusion as to the number of loci. On the one hand, the *Rh*, *Rh3*, and *Rh4* locus on chromosome 3H (Habgood and Hayes, 1971;

Wells and Skoropad, 1963) is thought to be at least two different but closely linked genes (Dyck and Schaller, 1961b), however others regard it as a single gene with several alleles (Habgood and Hayes, 1971).

Genetic studies of resistance have shown that some of the resistance genes show incomplete dominance, some complete dominance, some complementary effects and others are recessive genes (Ali *et al.*, 1976; Habgood and Hayes, 1971). Experiments have indicated that certain designated genes alone or in combination, confer effective resistance against a majority of *R. secalis* pathotypes, whereas other genes and gene combinations appear to be less effective (Ali *et al.*, 1976; Hansen and Magnus, 1973; Kajiwara and Iwata, 1963; Williams and Owen, 1973b).

1.3.5.2 Minor resistance genes

Studies on the inheritance of scald resistance genes have resulted in the identification of a range of resistance genes with different levels of partial resistance to scald (Habgood, 1977). Partial resistance is considered to be race non-specific because of its expression and because it appears to be polygenic in several cultivars (Habgood, 1974). However, it was found (Kari and Griffiths, 1997) that partial resistance does not operate equally against all races. Quantitative resistance is described by increased incubation period (Boyd *et al.*, 1987; Williams and Owen, 1973a; Xue and Hall, 1991), reduced infection frequency (Habgood, 1977), a reduced diseased leaf area (Boyd *et al.*, 1987; Robinson *et al.*, 1996) and reduced sporulation rate of the pathogen (Habgood, 1977; Kari and Griffiths, 1993; Xue and Hall, 1991). As a result of the combined effects of these quantitative trait loci (QTLs), disease development rate slows down and minimum damage to the crop occurs (Xi *et al.*, 2000b).

QTLs have shown additive effects and no significant interaction has been found (Habgood, 1974; Jensen *et al.*, 2002). A number of QTLs have been mapped on chromosomes 3H, 4H and 6H (Gronnerod *et al.*, 2002; Jensen *et al.*, 2002; Spancer *et al.*, 1998) and on chromosomes 2H, 3H, 4H and 7H (Abbott *et al.*, 1992; Backes *et al.*, 1995; Becker and Heun, 1995; Gronnerod *et al.*, 2002). Many of the QTLs on chromosomes 7H and 3H, seem to map in the region of major resistance genes (Bjornstad *et al.*, 2002; Genger *et al.*, 2005).

1.3.6 Biochemical and molecular studies

1.3.6.1 Fungal toxins

R. secalis produces toxins and application of those compounds to plants produce visible or physiological symptoms in barley and seem not to be host-specific, since treatment of non-host plants results in visible symptoms of scald (Ayesu-Offei and Clare, 1971). Toxins produced by fungal hyphae are responsible for the collapse of host cells. When cut stems of barley were immersed in culture filtrates of *R. secalis*, the grey water-soaked patches developed on leaves within 1 hour of treatment. Microscopic examination of these treated leaves showed that the mesophyll cells and the anticlinal walls of epidermis had both collapsed which resembles the early symptoms observed in infected leaves (Ayesu-Offei and Clare, 1970).

Different groups of toxic compounds have been identified by employing analytical techniques. One group is Rhynchosporosides, a group of low molecular mass α-1-O-propanediol glucosides, which in bioassays of detached leaves caused necrosis at leaf tips and margins and subsequent chlorosis (Auriol *et al.*, 1978; Mazars *et al.*, 1983; Rafenomananjara *et al.*, 1983). Another phytotoxin isolated from culture filtrates of the fungus is a glycoprotein. Microscopic studies of host tissue treated with the glycoprotein toxin revealed cellular disturbances accompanied by a progressive plugging of xylem vessels with polysaccharide materials (Mazars *et al.*, 1989a) which is most likely the result of toxin-induced host responses (Mazars *et al.*, 1984; Mazars *et al.*, 1989b). This glycoprotein was also found to elicit the formation of plant cell wall materials (pectin and hemicellulose) in barley and lignin biosynthesis in non-host plants (Mazars *et al.*, 1990).

Necrosis inducing proteins (Nips) Nip1, Nip2 and Nip3 are low molecular weight toxic proteins isolated from culture filtrates of the fungus, which induce tissue necrosis in leaves of barley and non-host wheat (Wevelsiep *et al.*, 1991). Further characterisation of the Nip1 in different races of *R. secalis* identified 4 isoforms (Rohe *et al.*, 1995). They also showed that Nip1 type I and II are the elicitor of the resistance response in cultivars containing resistance gene *Rh3* (*Rrs1*) and that it might diffuse through the stomata into the leaf and across the cell wall to reach its plant cell targets (Hahn *et al.*, 1993; Wevelsiep *et al.*, 1991). In further studies on Nips, it was concluded that the peptides Nip1 and Nip3 but not

Nip2 stimulate an H^+-ATPase in the plasma membrane and thereby induce necrosis formation. The necrosis-inducing activity and toxicity of Nip3 was shown in beans as well (Wevelsiep *et al.*, 1993). It was shown (Fiegen and Knogge, 2002) that race specificity, H^+-ATPase stimulation and necrosis-inducing activity of Nip1 isoforms were affected in a similar way. They concluded that all three activities are mediated through a single plant receptor. This finding further was confirmed by binding studies using a Nip1 type which revealed a single class of binding sites with identical binding characteristics in microsomes from near-isogenic resistant and susceptible barley (van't Slot *et al.*, 2007).

1.3.6.2 Barley defence-related gene expression in response to *R. secalis*

Molecular investigations into the response of barley to inoculations with *R. secalis* have resulted in the identification of some genes, which are up regulated upon inoculation with spores. It was found (Roulin *et al.*, 1997) that in leaves of a susceptible cultivar, the activity of the enzyme β-1, 3-glucanase (PR-3) increased only slightly, up to two-fold, over a period of 10 days while in resistant backcross lines its activity increased earlier and to higher levels after inoculation with fungal spores. Another study (Hahn *et al.*, 1993) also showed that transcripts of a *PR-9* and *PrHv-1* (PR-5) were expressed earlier and at a higher level in resistant cultivars in response to inoculation. In that study a rapid transient expression of acidic *PR-5* and *PR-9* transcripts upon treatment with Nip1 phytotoxin was also observed. Later investigations (Steiner-Lange *et al.*, 2003) showed that *PR-1*, *PR-5* and *PR-9* were expressed in leaf mesophyll tissue of resistant plants, while a germin-like protein (PR-16) was synthesized in epidermal tissue of both resistant and susceptible cultivars upon inoculations. The same study also resulted in isolation of epidermally expressed *PR-10*, *LoxA* (lipoxygenase) and *pI2-4* (unknown function) genes. A putative protease inhibitor (SD10) was also found to be preferentially expressed in epidermis (Hahn *et al.*, 1993).

Upon inoculation of resistant barley with spores of *R. secalis* levels of *Ltp4* and *Ltp2* (Lipid transfer protein) transcripts increased above basal levels, concomitantly with the increase in *PrHv-1* mRNA (García-Olmedo *et al.*, 1995). These LTPs are present in crude cell wall preparations and are potent inhibitors of bacterial and fungal plant pathogens (Molina *et al.*, 1993). Further studies comparing the expression of these genes in near-isogenic cultivars Atlas (*Rh2*) and Atlas 46 (*Rh2, Rh3*) led to the assumption that these

genes are under the control of the *Rh3* gene and that the proteins encoded might be responsible for the resistance of the Atlas 46 cultivar to the fungus (García-Olmedo *et al.*, 1995). Other LTPs isolated from germinating barley seeds also have been shown to have antifungal properties (Gorjanovic *et al.*, 2005). In another study analysis of intercellular washing fluids of a resistant barley cultivar resulted in isolation of proteins with antifungal activities against spores of *R. secalis* (Zareie *et al.*, 2002). These proteins included a ß-1, 3-glucanase, a chitinase and three thaumatin-like proteins.

1.3.7 Problems controlling scald

Currently scald disease is controlled by deployment of resistance genes, fungicide application and crop rotation. Although various sources of resistance to scald are available in both cultivated barley (Goodwin *et al.*, 1990) and wild barley (Abbott *et al.*, 1992), the variability of the pathogen complicates breeding. Pathogen variability greatly increases the likelihood that a specific pathogenicity may already exist in the *R. secalis* population even before the corresponding resistance genes are deployed in barley cultivars (Jarosz and Burdon, 1996). The highly variable nature of the pathogen has led to breakdown of single major resistance genes within short periods (Houston and Ashworth, 1957; Zhang *et al.*, 1987). For example, the Australian cultivars Skiff and Franklin carried high levels of resistance to scald when they were released in 1989. By 1993, Skiff was highly susceptible in South Australia and susceptible Franklin crops were first observed in 1997 (Cselenyi *et al.*, 1998). Another example is cultivar Atlas 46 which was introduced in California in 1947, and considered resistant. In 1953, it was infected in several locations, and by 1956 it was extremely susceptible in all parts of the state (Houston and Ashworth, 1957). Comparisons of tests on six cultivars in 1973 and 1995 (Salamati and Tronsmo, 1997) showed an increase in susceptibility in these cultivars to isolates studied in 1973 (Hansen and Magnus, 1973). It is believed that race non-specific resistance (Fowler and Owen, 1971; Habgood, 1971; Habgood, 1974) may offer a lasting, but not completely effective protection against scald disease. On the other hand, pathotypes of *R. secalis* that are resistant to commonly used fungicides have been identified in field populations, reducing fungicide effectiveness in some instances (Kendall *et al.*, 1993; Locke and Philips, 1995; Taggart *et al.*, 1998).

These problems necessitate searching for alternative control means. Candidate gene approaches by isolation and characterisation of differentially expressed genes in compatible and incompatible interactions using functional genomics studies may allow to provide molecular tools to engineer longer lasting or broader resistance.

1.4 Candidate gene identification and characterisation

Since completion of the genome sequences of several species, biologists have developed numerous techniques to understand the function of many genes. One of the central goals of functional genomics is to describe the biological function of every gene product in a particular species. Approaches have been devised to understand how these gene products are expressed and interact during growth and development. These approaches include transcript analysis, mutant characterisation (Ross-Macdonald *et al.*, 1999), identifying protein– protein interactions (Uetz *et al.*, 2000), determining subcellular localisation of proteins (Huh *et al.*, 2003; Morin *et al.*, 2001; Simpson *et al.*, 2000), production and analysis of transgenic lines and more. In the following section, functional analysis techniques used in this project are discussed.

1.4.1 Candidate gene identification

In most pathosystems, susceptible or resistant genotypes commonly differ quantitatively for gene expression. Differentially expressed genes between genotypes contrasting for response to infection by pathogens are considered candidates for having a role in resistance. In one method, global expression analysis was used to isolate such genes. Zierold and colleagues (2005b) used microarray based transcriptome analysis of barley epidermis in *Mlo* and *mlo5* cultivars and identified candidate genes that may play a role in papilla-based defence or, conversely, in supporting fungal growth by those cells in which a haustorium was formed. Similarly, the Affymetrix barley GeneChip hybridised to RNA from barley leaves in different interactions identified 22 differentially expressed genes in susceptible and resistant interactions of barley with *Blumeria graminis* f. *hordeum* (*Bgh)* (Caldo *et al.*, 2004). Their findings provided a link between the recognition of general and specific pathogen-associated molecules in gene-for-gene specified resistance. Their data also supported the hypothesis that host-specific resistance has evolved from the

recognition and prevention of the pathogen's suppression of plant basal defence. In further analysis they hypothesized (Caldo *et al.*, 2006) that the regulation of basal defence influences host-cell accessibility to the fungal pathogen and drives allelic diversification of gene-specific resistance phenotypes.

Another approach for identifying differentially expressed genes is Suppression Subtractive Hybridisation (SSH). The SSH is a powerful technique (Diatchenko *et al.*, 1996) to obtain a library enriched for differentially expressed genes in compatible and incompatible interactions of plants with a pathogen or any other process in living organisms. This technique has been used to identify differentially expressed genes of *Gossypium barbadense* infected with *Verticillium dahlia* (Zuo *et al.*, 2005), in a moderately resistant potato cultivar and *Phytophthora infestans* interaction (Ros *et al.*, 2004), in *Medicago truncatula* genes differentially expressed at different stages of the symbiotic interaction with *Sinorhizobium meliloti* (Godiard *et al.*, 2007) and to obtain novel *in planta*-expressed genes from *P. parasitica* (Bittner-Eddy *et al.*, 2003).

Homology based cloning is another frequently used technique to isolate candidate genes. In this method the sequences of functionally characterised genes is used to isolate orthologs or paralogues. To identify the homologous genes EST mining of publically available sequences or a cDNA library screening with a heterologous probe under low stringency condition is employed. Once candidate genes have been isolated, various analyses could provide evidence for their biological activity or their role in a biological process.

1.4.2 Expression analysis for gene characterisation

Profiling temporal and spatial expression patterns of genes provides an important basis for functional analysis of unknown genes by correlating those patterns with biological processes of interest (Ruan *et al.*, 1998). In addition by studying global gene expression, co-ordinately expressed genes can be identified which can help to discover the networks involved in the process. Comprehensive spatial and temporal analysis of transcription patterns of a gene coupled with comparisons to transcript profiles of other genes of known biological function, may also provide informative clues as to the function of the target genes. For instance, Eulgem and colleagues (2004) were able to compare the responses

controlled by three genetically distinct resistance gene-mediated signalling pathways in Arabidopsis interaction with *P. parasitica*. They found that all three pathways could converge, leading to up-regulation of common sets of target genes. Similarly using gene expression profiling in Arabidopsis, it was found that in addition to controlling the expression of PR genes, NPR1 also directly controls the expression of genes involved in the secretory pathway (Wang *et al.*, 2005).

Global expression datasets for barley under biotic and abiotic stresses are publically available from BarleyBase (Shen *et al.*, 2005) available from http://www.plexdb.org/ and Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/query/blast.html). Analysis of those data alone or in combination with data from custom designed experiments can provide insights to the function of gene(s) of interest in stress tolerance.

1.4.3 Gain- and loss-of-function

Gain-of-function is achieved by ectopic, temporal or spatial over-expression of a gene or can also be used to complement a mutant phenotype by expression of a gene suspected to be responsible for the mutant phenotype. Gain-of-function has been used in analysis of genes involved in plant defence. For instance, Kristensen and colleagues (2001) used transient expression of peroxidases to investigate their role in barley defence against powdery mildew. Transient over-expression of wild-type *Mlo* in mutant *mlo* barley confirmed the role of this gene in barley susceptibility to powdery mildew (Shirasu *et al.*, 1999). By expression in a single-cell system it was demonstrated (Zhou *et al.*, 2001) that Mla1 triggers full resistance in the presence of the severely defective *rar1-2* mutant allele, a gene which is required for the function of other *Mla* alleles.

Loss-of-function analysis requires silencing of a gene in a plant and studying the produced phenotype or analysis of transgenic plant under stress conditions to identify gene function and its role in stress tolerance. If a putative resistance gene is silenced, its function could be confirmed if the plants lose their resistance. For instance, transient silencing of *Mlo* in barley has shown its role in barley susceptibility to powdery mildew (Schweizer *et al.*, 2000). However, inactivating a gene that is a member of a multi-gene family may not produce noticeable phenotype due to functional redundancy. The most common method of gene silencing is through antisense and RNA interference (Finnegan *et al.*, 2001) both of

which operate post-transcriptionally. Antisense can target members of a multi-gene family simultaneously if the DNA sequence similarity is high among members. On the other hand, RNAi is efficient in targeting a single gene or multiple genes simultaneously depending on the sequences used for silencing.

Transient and stable expression of gene fusions between reporter genes (*uidA*, *gfp*) and the gene of interest are other common approach to elucidate gene function via transgenesis (Shen *et al.*, 2007).

1.4.4 Protein subcellular localisation

Most cellular processes are spatially restricted to defined regions of the cell. Therefore, subcellular location is a key characteristic of many proteins that ultimately limits the range of possible functions and thus 'compartmentation' is seen as one of their key attributes. Protein subcellular localisation can be achieved by biochemistry, immunocytochemistry and fluorescent analog cytochemistry (i.e. microinjection of fluorescently tagged proteins). These methods are time consuming and laborious. Discovery of the green fluorescent protein (GFP) from jelly fish has proved invaluable as a non-destructive *in vivo* marker for subcellular localization (Chalfie *et al.*, 1994) and discovery of its derivatives and orthologs from other species, have been very useful in co-localisation studies of proteins fused to different fluorescent proteins (Dixit *et al.*, 2006).

In addition to *in vivo* functional genomics technologies, *in vitro* techniques such as bioassay have been used in functional analysis of gene products.

1.4.5 Heterologous protein expression

Bioassay is a useful *in vitro* method to test putative biochemical function or antifungal properties of a defence-related protein. However, isolation of intact proteins from plant tissues in a biochemically active form is a difficult task. Often proteins are present at low abundance or are associated with other proteins or in complexes. Heterologous systems such as *E. coli*, *Pichia pastoris* and insect cells can be used to produce recombinant proteins in large amounts. In these systems the gene of interest is cloned into a special vector in frame with N- or C-terminal affinity tags. The plasmid is transferred to a cell in

which the expression of the protein can be induced. The expressed protein then is purified by affinity chromatography (Hochuli *et al.*, 1987) and purified protein is used for functional analysis.

1.5 Objectives

Our understanding of plant defence mechanisms has progressed considerably through molecular approaches in recent years. However, most of the studies have been conducted on the model plant Arabidopsis due to availability of the powerful genetic capabilities. Comparatively molecular processes during interaction of crop plants with pathogens and especially necrotrophic fungi is less well understood.

Several studies have reported isolation of barley genes whose expression is induced by *Rhynchosporium* (Section 1.3.6.2). However, the function of the genes or their contribution to scald resistance has remained unknown. Since the first site of contact between a pathogen and a host is the epidermis and *R. secalis* primarily infects epidermal tissue, isolating genes upregulated in epidermis of resistant cultivars may allow identification of genes that are involved in mediating resistance. To identify such genes a SSH approach was taken to create a cDNA library enriched for genes upregulated in epidermis tissue of a resistant cultivar. This was achieved by subtraction of expressed genes in the epidermis of a resistant cultivar (Atlas 46) with the near-isogenic susceptible cultivar (Atlas) 24 hours after inoculation (Dr Klaus Oldach, ACPFG, University of Adelaide). The investigated pathosystem is a well defined interaction in which the resistance is based on the expression of the *Rrs1* resistance gene in Atlas 46 and its corresponding avirulence gene *Nip1* in *R. secalis* (Rohe *et al.*, 1995). Using near-isogenic lines should help to eliminate the vast number of genes that were not specific to the defence response. Thus, the SSH library is enriched for genes encoding proteins specifically involved in *Rrs1*-based scald resistance. Functional analysis of such genes in this pathosystem can provide evidence for their role and the mechanisms involved in successful defence against necrotrophic pathogens such as *R. secalis*.

Most approaches employing differential gene expression techniques identify a large number of candidate genes that cannot all be analysed unless *via* a high throughput

system. Such a system has not been developed for necrotrophic pathosystems. Thus, for functional characterisation, clones were selected based on annotation, similarity to genes encoding proteins with known or suggested activity and their possible involvement in biotic and/or abiotic stress tolerance in barley. The functional analysis aimed to use a broad variety of tools (*in vitro* and *in vivo*) to validate the homology-based annotation and their role in plant protection. Broadly, the results will present experimental evidence to evaluate expression based candidate gene approaches. Moreover, investigating hypothesised roles in crop plants based on annotation and function shown in model plants serves as a case study on the value of applying knowledge gained from model to crop plants.

In this project the function of clones from the SSH library and their potential contribution to scald resistance and abiotic stress tolerance was investigated. Table 1.1 shows characteristics of three clones selected for analysis which provide an opportunity to look at three different pathways and processes. The putative encoded proteins would have direct antimicrobial activity (s134), involve in biochemical pathways affecting pathogen nutrient acquisition from host cells (n194), and provide a physical or chemical barrier to pathogen ingress (d1057). Within the overall objective of this project the more specific aims were:

- To obtain full length cDNA and genomic sequence of the clones and their family members
- To characterise isolated genes by a variety of *in silico* analysis
- To investigate spatial and temporal expression in barley leaves in response to scald and abiotic stresses including frost, drought and salinity. In addition, to use publically available datasets to analyse expression of the genes in other barley pathosystems and during barley development
- To localise the cellular targeting of the gene products in barley cells by GFP fusion
- To analyse anti-fungal activity of the gene encoding a PR-17 protein (s134 clone) by using recombinant protein
- To verify complementation of an Arabidopsis *fiddlehead* mutant by its potential homolog (d1057 clone) in barley
- To produce transgenic lines in which those genes are over-expressed or silenced
- To characterise transgenic lines as much as time allows.

The isolation and characterisation of *HvPR-17* (s134), *HvGolS* (n194) and *HvFdh* (d1057) are described in Chapters 3, 4 and 5, respectively.

Table 1.1 SSH clones selected for characterisation. SSH library was derived from epidermis tissue of barley plants challenged with *R. secalis* conidiospores. The library was enriched for genes preferentially expressed in incompatible interaction.

cDNA clone	Gene	Size (bp)	e value	Database hit and putitative function
s134	*HvPR-17c*	473	0	Barley fungal-elicitor inducible protein. Small multi-gene family.
n194	*HvGols1*	297	e^{-177}	Galactinol synthases in Arabidopsis. Two members in barley.
d1057	*HvFdh*	347	0	*Fiddlehead* gene in Arabidopsis. Single gene in barley.

Chapter 2: General Materials and Methods

2.1 Introduction

This section outlines materials and methods commonly used throughout this project. Specific protocols will be presented in detail in later chapters where experimental data are presented.

2.2 Materials

2.2.1 Plant materials

Seeds of barley cultivars Atlas and Atlas 46 were kindly provided by Dr Wolfgang Knogge from University of Adelaide and seeds of other cultivars were obtained from Dr Ursula Reimold-Langridge (ACPFG, Waite Campus) unless otherwise stated. Golden Promise was included in most experiments because stable transformations were done in this background. The reasons for selection of other cultivars are given where the experiments using those cultivars are described.

Arabidopsis *fiddlehead* mutant seeds were kindly provided by Dr Alexander Yephremov from Max-Planck Institute for Plant Breeding (Cologne, Germany).

2.2.2 Fungus isolates

Rhynchosporium secalis isolates SA385 and SA6 grown from single spores were obtained from Dr Hugh Wallwork (South Australian Research and Development Institute). The isolate differentiate the known scald resistance gene (*Rh3*) in the cultivars used for biotic stress. *Pyrenophora teres* f. sp. *teres* culture was kindly provided by Andrew Craig (University of Adelaide).

2.2.3 Interaction of barley cultivars and *R. secalis* isolates

Golden Promise is a susceptible cultivar with no known resistance gene to scald (Harlan and Martini, 1936). Resistance of Atlas depends on locus *Rh2* (Dyck and Schaller, 1961a), while its near-isogenic line Atlas 46 in addition to *Rh2* locus contains the *Rh3* (Rrs1)

resistance locus which has been introgressed from cultivar Turk. The relationship between the resistance locus present in the barley cultivars and fungal isolates have been summarised in Table 2.1.

2.3 Plant growth condition

Generally, barley seeds (unless otherwise advised) were planted in Coco fibre mix (180 l of each Coco peat and Waikerie sand were mixed and steamed for one hour and then the following supplements were added: dolomite lime 540 g, agricultural lime 1800 g, hydrated lime 720 g, gypsum 540 g, superphosphate 450 g, iron sulphate 1350 g, iron chelate 90 g, Micromax 540 g, calcium nitrate 1350 g and after cooling 1800 g of Osmocote mini 3-4 mm [16-3-9+te]) in plastic pots (10x8.5x9 cm) and were grown in a controlled environment growth cabinet programmed at 17°C with a daily 16 h photoperiod at 300 µEinsteins m^{-2} s^{-1} and watered as needed. A mixture of fluorescent and incandescent bulbs supplied light.

Arabidopsis seeds were planted in plastic pots (6.5x8x5.5 cm) containing ACPFG soil mix (1 part sand, 1 part peat, 1 part perlite supplemented with 1 g/l $FeSO_4$, 3 g/l Osmocote and 2 g/l pH amendment [2 g dolomite, 1 g gypsum, 1 g lime]) and were grown in a growth room set at 21ºC day and 18ºC night temperature. Day length was 18 h and light levels were on average 75 µEinsteins m^{-2} s^{-1}. Humidity was not controlled, but relative humidity ranged between 50% and 80%.

2.4 Initiation of fungal cultures and preparation of spores

Lima bean agar (LiBA) prepared as described by Williams *et al.* (2003). Potato dextrose agar (Sigma-Aldrich, Castle Hill, NSW, Australia) was prepared by reconstituting 39 g of the medium in 1 litre of Mill-Q water and then autoclaving. *P. teres* was cultured on this medium.

R. secalis cultures were initiated on LiBA from silica gel stored spores. To maintain the virulence of the fungus, these cultures were used in two subsequent subcultures and then

discarded. *P. teres* cultures were initiated by cutting a 5 mm disc from an old culture and placing it in the middle of a fresh potato dextrose agar plate.

R. secalis conidia were harvested as described previously (Dyck and Schaller, 1961a). The resulting suspension of mycelium, spore and agar was filtered through Miracloth (Calbiochem, Darmstadt, Germany) and the number of spores in suspension was determined with a haemocytometer and adjusted to $1x10^6$ spores/ml. For subculturing 1 ml of scraping was spread onto fresh medium plates.

2.5 Inoculations and disease symptom assessment

Barley seedling primary leaves (7-8 days after sowing, Fig. 2.1A) were sprayed with 200 µl of inoculum/leaf (Fig. 2.1B) or Milli-Q water (control plant) in 0.05% Tween-20 (Steiner-Lange et al., 2003) and transferred into an enclosed plastic container filled with 1 cm water to maintain high humidity and kept in dark for 24 hours. At each time point for whole leaf samples three primary leaves from each treatment were harvested. For epidermis samples 20 leaves were peeled and pooled (Fig. 2.1C).The harvested material was immediately frozen in liquid nitrogen and stored at −80°C until extraction of RNA. Samples of five inoculated plants were evaluated for symptoms 2-3 weeks after inoculation to confirm if inoculation was successful. The experiment was repeated at least two times and samples were processed separately.

Scald symptoms on secondary leaves from transgenic plants was rated on a five-point scale 21 days post-inoculation according to Ali (1974) as shown in Table 2.2.

2.6 Drought experiment

Pots (8″) were filled with equal amounts of Coco fibre potting mix, watered to saturation and left for three weeks to lose moisture. Six seeds of cultivars Haruna Nijo, Barque 73 and Golden Promise were planted using forceps in separate pots and grown in a growth chamber (Section 2.3). Two pots of each cultivar were watered regularly as control plants and the other pots were only watered 200 ml/pot after planting. The first sampling was done as the plants started to wilt (wilt1) followed by a second sampling

Table 2.1 Barley-*Rhynchosporium* interaction. Differentiation of scald resistance locus in barley cultivars by fungal isolates.

Cultivar	R locus	Fungal isolate	
		SA385	SA6
Atlas 46	Rh2, Rh3	Resistant	Resistant
Atlas	Rh2	Susceptible	Resistant
Golden Promise	NA	Susceptible	Susceptible

Table 2.2 Scald symptom scoring. Criteria used for scoring severity of disease symptoms in transgenic barley lines.

Scale	Symptom description
0	no visible symptoms
1	small lesions confined to leaf tips and margins
2	larger lesions at leaf margins or in central portion of the leaf blade
3	large lesions, covering large areas of the leaves
4	wilted leaves with no discrete lesions within wilted area

24 h later (wilt2, Fig. 2.2A). Then pots were watered to saturation and third sampling was done 24 h after watering (rewatered). At each sampling third leaves from four plants were harvested and pooled (Fig. 2.2B). The longitudinal half of each sample was frozen in liquid nitrogen for RNA extraction and the other half used for measuring leaf water content by dividing fresh weight to dry weight after drying to a constant weight at 60ºC (Appendix E). Two biological samples were taken and two cDNA was synthesised from each and pooled for Q-PCR analysis. These cultivars were selected because of their difference in drought tolerance.

2.7 Salt experiment

Seeds were germinated in petri dishes and were grown in a hydroponic solution as described by Genc *et al.* (2007), except that the final concentration of NH_4NO_3 was 5 mM. The system consisted of two sets of supported hydroponics (regularly bottom flooded) and plants were grown under natural light in a glasshouse (Fig. 2.3A). After one week, the nutrient solution was replaced and for one set salt treatment commenced. The NaCl concentration of the hydroponic solution was increased by 50 mM every 12 hours to reach a final concentration of 150 mM. Supplemental calcium ($CaCl_2$) was added to achieve a Na^+/Ca^{2+} ratio of 15:1. At the start of treatment, the second leaf was emerging. At different times after the last addition of NaCl, second leaves (third leaf for Golden Promise) were harvested from six plants. The longitudinal halves of the samples were used for RNA extraction and the other half for Na^+ and K^+ ion measurement. Two biological samples were taken and two cDNA was synthesised from each and pooled for Q-PCR analysis. Five cultivars or breeding lines, Yu-6472, CM72, WI2291, Prior(A) (seeds kindly provided by Dr Yuri Shavrukov, ACPFG) and Golden Promise were selected because of their differences in Na^+ accumulation in leaves (Dr Yuri Shavrukov, personal communication).

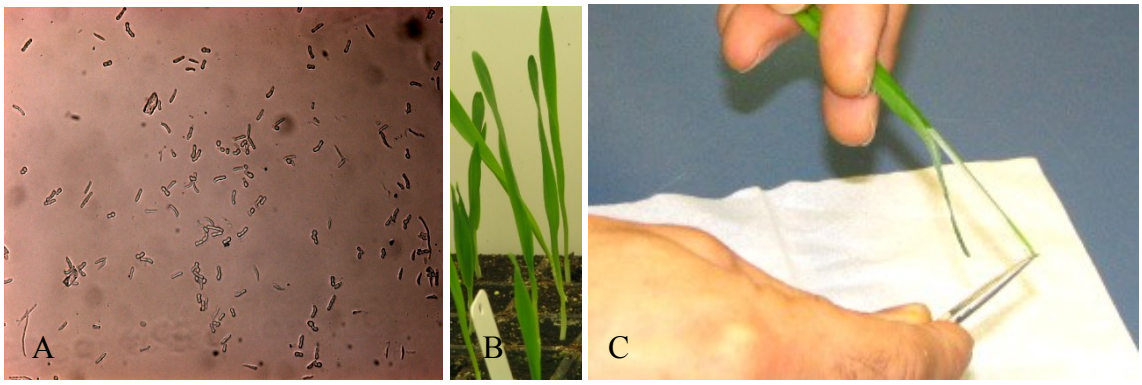


Figure 2.1 Inoculation and peeling of barley leaves. (A) Spores isolated from *R. secalis* culture used for spray inoculation. (B) 7 days old barley seedlings used for inoculation. (C) Peeling epidermal tissues from young barley seedling leaf.

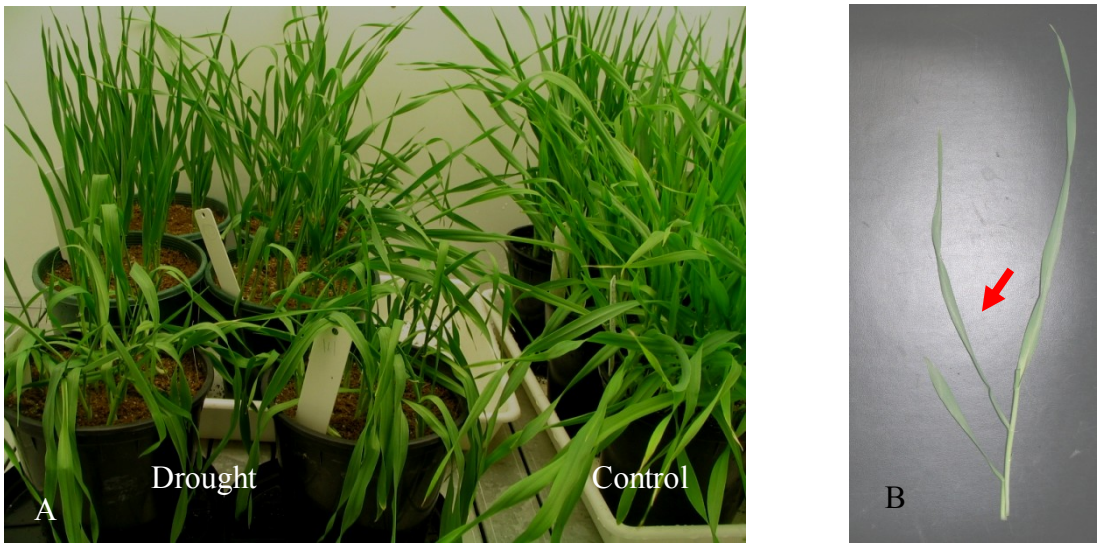


Figure 2.2 Drought treated and control plants. (A) The plants at front row (left side) are at wilt2 sampling stage. Control plants at right show more growth. (B) A drought treated plant at harvest. Red arrow points to third leaf harvested for analysis.

2.8 Frost experiment

Plants were grown in a growth cabinet (Section 2.3) for 6 days and one set of plants transferred to a frost chamber (Fig. 2.4). The treatment began at 20ºC during dark cycle and temperature was reduced by 5ºC/h to 3.5ºC and then it was reduced by 1ºC/h until the temperature reached -5.5ºC and was held at -5.5ºC for 2 hours. Temperature was then increased by 2ºC/h until the chamber reached 3.5ºC. Then it was increased by 5ºC/h until 10ºC and the plants were returned to the growth room. Samples of four leaves from each cultivar were taken and frozen in liquid nitrogen at the beginning of the experiment (20 ºC), as the temperature dropped at 4^{o}C (pre-frost 4), once the temperature reached −5.5^{o}C (frost -5.5), as the temperature increased to 4^{o}C (post-frost 4) and during recovery 48 h after the plants were returned to the growth room (48 h 20ºC) as shown in Figure 2.5. The control plants during treatment time were kept in the dark as the treated plants. Two biological samples were taken and two cDNA was synthesised from each and pooled for Q-PCR analysis. Cultivars: Haruna Nijo, Golden Promise, Galleon, Amagi Nijo, WI2585 were used based on differences in their cold tolerance (Reinheimer *et al.*, 2004).

2.9 Measurement of ion concentration

The leaf samples were extracted with nitric acid (0.5 M) and Na^+ and K^+ concentration was measured using a Flame-photometer Model 860 (Sherwood Scientific, Cambridge, UK). The data is presented in Appendix E.

2.10 Extraction and purification of nucleic acids

Genomic DNA was extracted from 7-10 day old seedling leaves stored at −80ºC according to Pallota *et* al.(2000). Total RNA was extracted from 150 mg of tissue using Tri-Reagent solution (Molecular Research Centre, INC, Cincinnati, Ohio) according to manufacturer's instructions. High salt solution was used for precipitation step and the RNA was redissolved in 80 µl of double-autoclaved Milli-Q water and spectrophotometrically quantified, then stored at -80°C. Poly A^+ RNA was extracted from 50 mg of leaf tissue 24

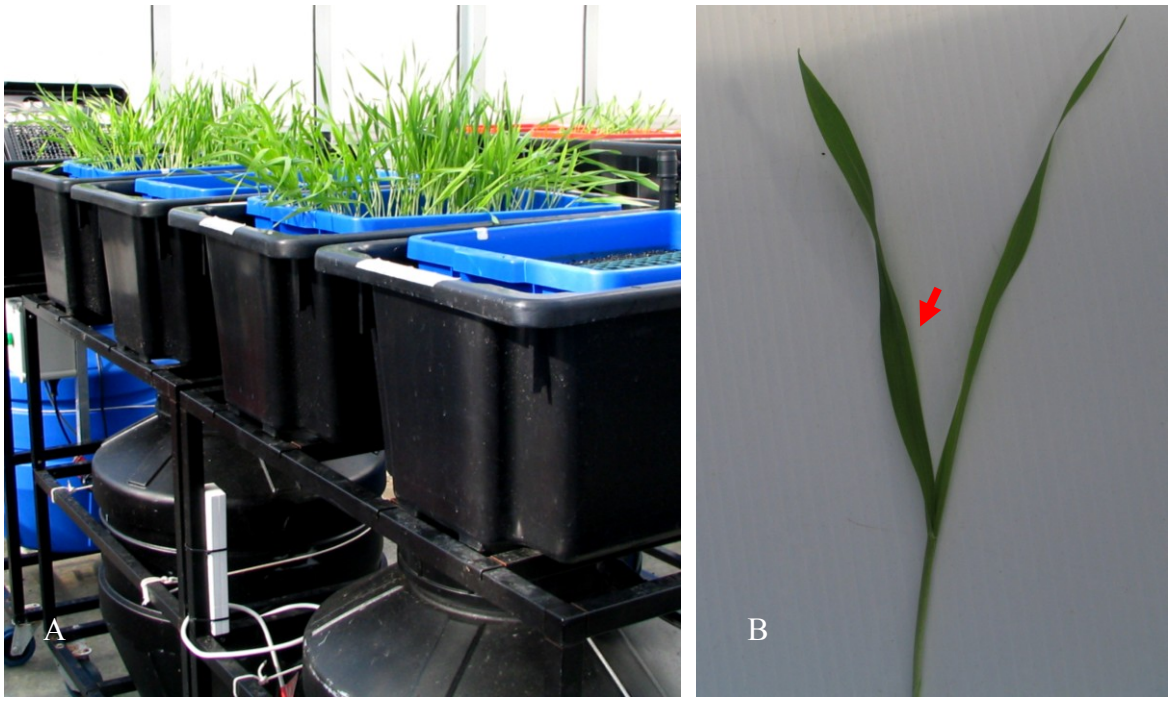


Figure 2.3 Salt experiment set up. (A) Supported hydroponic system used for growing barley plants. (B) A plant at harvest time after salt treatment. Red arrow points to second leaf at harvest.

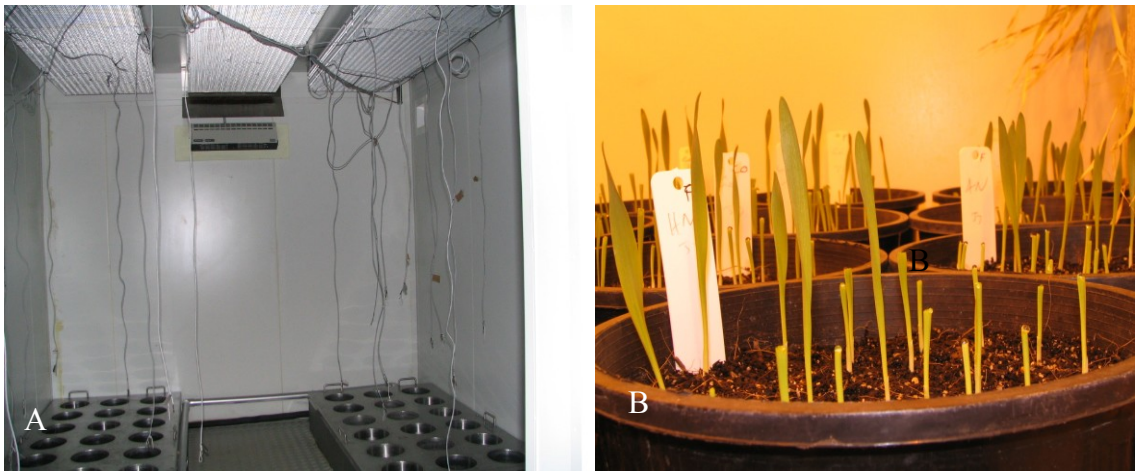


Figure 2.4 Frost experiment. (A) Frost chamber used to treat plants. (B) Plants after treatment did not show any frost injury.

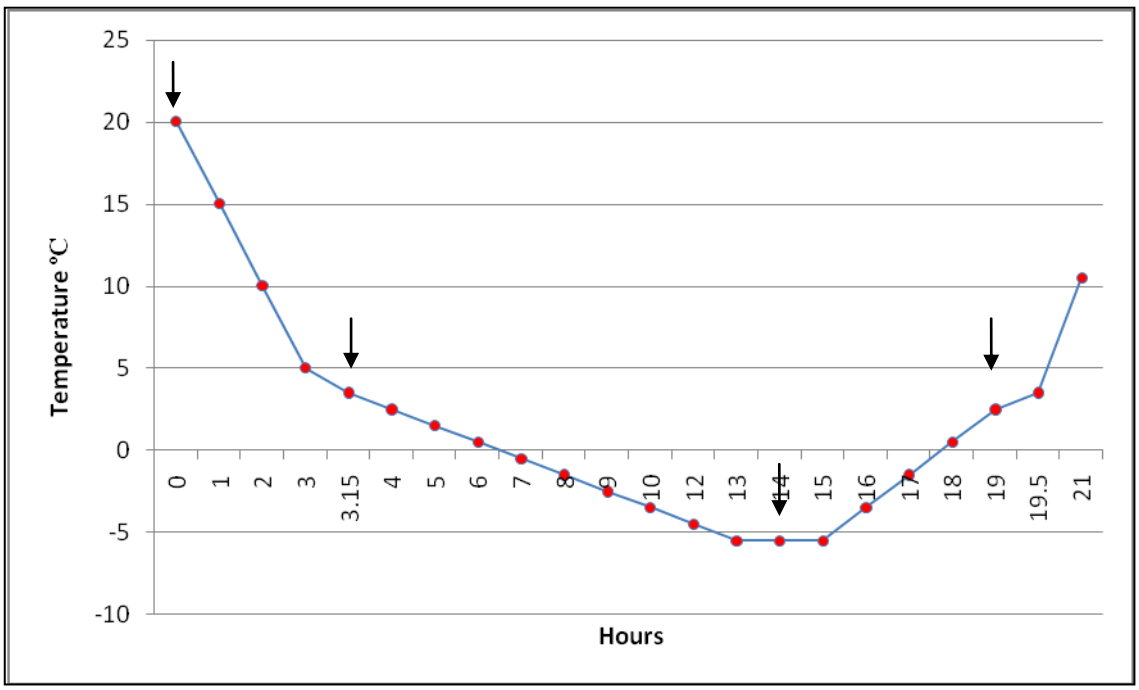


Figure 2.5 Frost chamber temperatures during treatment. The arrows indicate sampling times.

hours post inoculation with isolate SA385 using Genoprep^{tm} mRNA kit (GenoVision, West Chester, PA, USA) as recommended by the manufacturer.

To purify vectors, DNA fragments from PCR or enzymatic reactions, a QIAquick PCR purification or QIAquick Gel Extraction kit (QIAGEN) was used according to manufacturer's instructions.

The quality of RNA and DNA was assessed visually by gel fractionation. A denaturing agarose gel was used for RNA and TAE agarose gel for DNA. Concentration of RNA and DNA was measured by a Nano-Drop 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE USA). To concentrate dilute DNA or RNA solutions a SAVANT Speed Vac Concentrator (SAVANT Instruments INC, NY, USA) SVC100H Model was used.

2.11 PCR amplification

Four different DNA polymerases including *Taq* DNA polymerase, ProofStart DNA polymerase (QIAGEN, Doncaster, Vic, Australia), *PfuUltraHF* DNA polymerase (Stratagene, La Jolla, Ca, USA) and Immolase DNA polymerase (Bioline, Australia) were used in PCR reactions. The reaction set up for each polymerase was according to manufacturer's recommendations. For amplification of fragments from genomic DNA, a solution containing 50 ng/µl DNA, from plasmid 1-5 ng/µl and for nested or semi-nested PCR 1:50 dilution of first PCR product was used as template. The total volume of reactions was made up to 25 µl, unless stated otherwise. Thermocyclers DNAEngine TETRAD 2 and PTC-100^{TM} Programmable Thermal Controller (MJ Research, Reno, MV, USA) were used for cycling.

2.12 Synthesis of cDNAs and RACE

BD SMART^{TM} RACE cDNA Amplification kit (BD Biosciences Clontech, CA, USA) was used for Rapid Amplification of cDNA Ends (RACE). To synthesise 5'- and 3'-RACE ready cDNAs, 2 µl poly A^+ RNA (Section 2.10) extracted from *R. secalis* inoculated barley leaves was used as manufacturer's instructions. The first-strand reaction

product was diluted with 250 µl Tricine-EDTA buffer, and cDNA was stored at -20°C and directly used as template in RACE reactions.

BD SMART^{TM} PCR cDNA Synthesis kit (BD Biosciences Clontech, CA, USA) was used to synthesise dscDNA following instructions for SMART cDNA Synthesis for Library Construction. Total RNA (1 µl) extracted from Atlas 46 leaf material 24 hours post *R. secalis* inoculation was used as template.

First PCR for 3'-RACE was performed using ProofStart DNA polymerase with CDSIIA primer as reverse and a gene specific forward primer (GSPF5) with dscDNA as template. A semi-nested reaction was performed for n194 and d1057 by using 1 µl of 1:50 dilution of the first PCR product as template and replacing GSPF5 with GSPF6 (nested). The PCR products were gel-purified, subcloned into pGEM-T Easy (Section 2.14) and at least 10 positive clones were sequenced for each gene by using SP6 and T7 primers. Cycling programs (CP) 1 and 2 (Appendix A) were used in first and semi-nested PCR reactions, respectively.

To amplify 5' end of clones, 1.25 µl of 5'-RACE-Ready cDNA was used as template in a PCR reaction with ProofStart DNA polymerase. Reverse gene specific primer (s134R, n194R, d1057R3, Appendix C) and a 1:4 mix of Smart Universal Primer A long (SmUPAL) and short (SmUPAS) as forward primer were used for priming first PCR. Nested PCR was performed by using nested gene-specific reverse primer (s134 R2, n194R3, d1057R4) and Smart Nested Universal PrimerA (SmNUPA). CPs 3 and 4 were used in first and nested PCR reactions, respectively. Nested PCR products were cloned and sequenced as described for 3'-RACE products.

2.13 Real-time reverse transcript PCR (Q-PCR)

Total RNA was extracted from harvested samples. The RNA was treated with DNase using a DNA-free kit (Ambion, Austin, Texas) according to manufacturer's instructions to remove any contaminating DNA. First strand cDNA primed with oligo dT was synthesised from 2 µg of treated RNA using the Thermoscript III RT-PCR kit (Invitrogen, Mount Waverley, VIC, Australia) as recommended by the manufacturer.

Gene-specific Q-PCR primer pairs were designed for each gene to amplify a fragment between 150-300 bp and were named s134F16/R16, n194 F16/R16 and d1057F16/R16 (Appendix C). These primers were used for expression analysis of genes in biotic and abiotic stress series. Other set of primers were designed to measure expression level of total transcripts of GOI in transgenic lines. Primer pairs q134F1/R1, q194F1/R1 and q1057F1/R1 were used in transcript analysis of transgenic plants. The BLASTn algorithm was used to search EST databases and assess the uniqueness of these primers in amplifying the target sequence. PCR product size and specificity was confirmed by agarose gel electrophoresis and sequencing.

The transcript levels were quantified as outlined in Burton *et al.* (2004). Dr Neil Shirley (University of Adelaide) is acknowledged for conducting Q-PCR of stress experiments and Dr Paul Bogacki (South Australian Research and Development Institute) for analysing expression of transgenic lines.

The normalisation provides an opportunity to either eliminate or reduce sampling errors (such as quality and quantity of RNA). Normalisation factors for each cDNA were calculated based on Vandesompele *et al*. (2002) which uses internal control genes that have a relatively stable expression. Data obtained from Q-PCR were normalised with the best of three control genes of barley stably expressed genes run simultaneously with samples, including *Glyceraldehydes-3-phosphate* (*GAPDH*), *α-Tubulin* (*Tubulin*), *Heat shock protein 70* (*HSP70*)and *Cyclophilin* (Burton *et al.*, 2004) and *SF400* and *SF427* encoding proteasome subunits (Bogacki *et al.*, 2008). The control genes data are given in Appendix F.

2.14 Ligation of DNA fragments into plasmid vectors

For cloning into plasmids with T overhang (pGEM Teasy), purified PCR fragments first were A-tailed and then ligated overnight according to manufacturer's recommendations (Promega, Madison, USA).

Restriction enzyme digested vectors and DNA fragments to be ligated were purified (Section 2.10). Double digestion of fragments or vectors was done by sequential digestion and purification. Restricted vectors producing compatible ends were dephosphorylated

(see below) before setting up the ligation reaction. The ligation reaction of fragment into appropriate vector was set up as described above without A-tailing.

To dephosphorylate restricted vectors a reaction containing 1 µl calf intestine alkaline phosphatase (Roche), 5.5 µl of 10x buffer (supplied), and 48.5 µl of purified digested vector in 55 µl volume was set up. The reaction was incubated at 37°C for 1 hr and the enzyme inactivated by heating at 65°C for 15 min followed by column purification (Section 2.10).

2.15 Preparation of competent cells and transformation of *E. coli*

The *E. coli* strain DH5α was used throughout experiments unless stated otherwise. For positive selection of transformed cells one or combination of ampicillin (50 µg/ml), kanamycin (25 µg/ml), and spectinomycin (25 µg/ml) was added to growth medium depending on the antibiotic resistance gene encoded by the vector.

E. coli strain DH5α chemically and electro-competent cells were prepared using the protocols described by Inoue *et al.* (1990) and Rakes *et al.* (Rakesh and Robert 1996), respectively. Competent cells were transformed according to instructions in pGEM Teasy manual (Promega) and cells were plated out onto pre-warmed LB plates supplemented with appropriate antibiotic and incubated at 37°C for 16 hr. Gene Pulser (Bio-Rad, Herculus, CA, USA) set to 1.8 KV, 25 µFD and 200 Ω was used for electro transformation. For white/blue selection of colonies, X-Gal (80 µg/ml) and IPTG at 0.1 mM final concentration were added to the medium.

2.16 Extraction of plasmid DNA

A colony of recombinant bacteria was inoculated into 3 ml LB medium (0.5% w/v NaCl, 1% w/v tryptone and 0.5% w/v yeast extract, pH 7.5) supplemented with antibiotic for selection and were grown over night at 37°C with vigorous shaking. Plasmid was extracted using the "QIAprep Spin Miniprep" kit (QIAGEN) following the manufacturer's instructions.

2.17 DNA sequencing

Nucleotide sequence analysis of DNA was performed at the Institute of Medical and Veterinary Science (IMVS, Adelaide, SA, Australia) or at the Australian Genome Research Facility (AGRF, Brisbane, Australia) on an ABI 3700 capillary sequencer.

2.18 Preparation of [α-^{32}P]-radio labelled DNA probes

Two fragments were amplified for each gene by using a plasmid containing full length of the genes. Template for the 3' end of genes was amplified by s134F5/R3, n194F5/R and d1057F5(3')/R and for full length by s134F3/R3, n194F3/R and d1057F6/R primer pairs using ProofStart and CP 5 (Appendix A). The amplified fragments were gel-purified and sequenced (Appendix B) and used for probe preparation according to Sutton *et al.* (2003).

2.19 Southern blot analysis

Southern blot analysis was performed according to Pallota *et al.* (2000). After hybridization membranes were washed sequentially for 20 min in solution 1 (2x SSC, 0.1% w/v SDS), solution 2 (1x SSC, 0.1% w/v SDS), solution 3 (0.5x SSC, 0.1% w/v SDS), solution 4 (0.2x SSC, 0.1% w/v SDS) and solution 5 (0.1x SSC, 0.1% w/v SDS) at 65°C. The signal intensity of membranes was checked with a Geiger-Muller counter between each transfer from one solution to the next and washing was stopped when low background signal was detected. Hybridisation signals were detected by exposing the membrane to X-ray film (Fuji, Super HRG 30) using an intensifying screen at -80°C for varying lengths of time depending on the bound radioactivity estimated by a Geiger counter. Films were developed in an automatic AGFA CP1000 developer (Wayville, SA, Australia).

2.20 Northern blot analysis

Total RNA (15-20 µg) was fractioned in a denaturing agarose gel (1.2% agarose, 1x MOPS solution pH 7.0, 2.2 M formaldehyde). To the samples (12 µl) 3 µl of 5x loading buffer (0.16% saturated bromophenol blue aqueous solution, 2 mM EDTA pH 8.0, 7.2%

of 12.3 M formaldehyde, 20% glycerol, 30.8% deionised formamide, 20 mM MOPS, 0.02% ethidium bromide) was added and incubated at 65°C for 15 min, then cooled on ice. Samples were loaded into the gel and run in 1x MOPS solution (20 mM MOPS, 5 mM sodium acetate, 1 mM Na_2EDTA, pH 7.0).

Gels were blotted onto Hybond-N^+ membrane by capillary transfer using 10x SSC as the transfer buffer for 12 h. The membrane was rinsed in 2x SSC for 1 min and then cross-linked as Southern membranes. Sealed membranes were stored at -20°C. Hybridisation, washing and signal detection was performed as described in Section 2.19.

2.21 Construction of over-expression and antisense vectors

A plasmid containing the full-length cDNA of each gene was used as template to amplify the coding region of the genes. BamHI restriction site and four extra nucleotides were added to the 5' end of both forward and reverse primers. Forward primer F7 and reverse primer R7 of genes was used in these reactions. ProofStart enzyme was used to amplify coding sequence of *HvPR-17c* and *HvGolS1* by using CP 10 and *PfuUltraHF* was used to amplify *HvFdh* following CP 11 (Appendix A).

Binary vector pPZPUbi.cas (derived from pPZP, Hajdukiewicz *et al.*, 1994) carries spectinomycin resistance gene for selection of transformed *E. coli* and *Agrobacterium* cells. It also carries a copy of hygromycin phosphotransferase (HPT) under the control of the CaMV35S promoter. The expression of a gene of interest (GOI) is driven by maize ubiquitin1 promoter (Christensen *et al.*, 1992).

The vector pPZPUbi.cas and amplified coding fragments of genes were digested with BamHI, purified (Section 2.10), ligated (Section 2.14) and transformed into *E. coli* (Section 2.15). The plasmid minipreps from positive colonies were sequenced with PZPR and PZPF primers (Appendix D) to verify the insert sequence as well as orientation. Perfect match colonies with sense and antisense orientation were selected for stable transformation into barley. The resultant vectors were named pHvGOISE for over-expression and pHvGOIAS for antisense constructs, respectively.

2.22 Construction of dsRNAi vectors

A three step cloning was used for constructing a dsRNAi binary vector for stable transformation. Two primer sets, incorporating restriction recognition site at 5' terminal (Table 2.3), one for sense and other for antisense fragment were designed for each gene: ProofStart enzyme was used to amplify all fragments following CP 6 and amplicons were gel purified. Primer pairs (F10/R10) and (F11/R11) of each gene were used to amplify sense and antisense fragments, respectively.

First, antisense fragments digested with BglII and XbaI were ligated into vector pHannibal (Wesley *et al.*, 2001) digested with the same enzymes and transformed into *E. coli*. Positive transformants were selected on ampicillin supplemented LB plates. Ligating compatible ends of BamHI and BglII eliminates the recognition site for BamHI, which was used for excising the fragment to be subcloned into the binary vector. In the second step, sense fragments were digested with XhoI and KpnI and ligated into the previous vector containing the antisense fragment digested with the same enzymes and positive clones were selected and sequenced.

Finally, a fragment containing sense and antisense region of each gene was released from pHannibal by BamHI digestion and the released fragment was ligated into the single BamHI site of vector pPZPUbi.cas. The resulting vectors were transformed into *E. coli* and positively selected clones were used for DNA extraction and sequenced with vector primers to identify the vectors with correct orientation. The resultant vectors were named pHvGOIRNAi and stably transformed into barley.

The following online prediction programs were used to predict the most effective region for gene silencing using dsRNAi:

1. https://rnaidesigner.invitrogen.com/rnaiexpress/, BLOCK-iT RNAi Designer from Invitrogen
2. www.idtrna.om/scitools/applications, siRNA Designer from Integrated DNA Technologies
3. www.ambion.com/techlib/misc/siRNA-finder.html, siRNA target Finder from Ambion

The overlapping fragment suggested by all programs was selected as RNAi region. The selected region for each gene is shown in related chapter.

2.23 Construction of GFP fusion vectors

The vectors were constructed in two stages. In the first step, GFP coding region without start codon was amplified using ProofStart, with plasmid pHvOXO (kindly provided by Dr Klaus Oldach, University of Adelaide) as GFP template using primer pair GFPfuF3/R3 incorporating extra nucleotides and restriction sites at the 5' end of primers (Appendix C) resulting in a BamHI-BlnI-A-GFP codon-BglII fragment for further cloning. CP 7 was used for PCR. The amplified fragment was sequentially digested with BamHI and BglII, and ligated into pPZPUbi.cas vector restricted with BamHI to obtain vector pGFPfu. Positive clones were selected on spectinomycin supplemented LB agar plates and sequenced to verify the accuracy and correct orientation of the insert.

In the second step, fragments for coding region of genes without stop codon (*HvGOIORFfu*) were PCR-amplified using *PfuUltraHF* incorporating additional nucleotides and restriction sites as BamHI-coding sequence-GGAG-BlnI fragment. Primer pairs F7/R12 of each gene were added to the reaction and CP 8, 8 and 9 (Appendix A) were used to amplify fragments for *HvPR-17c*, *HvGolS1* and *HvFdh*, respectively. The additional nucleotides added to the GFP fragment and coding region of genes including the BlnI restriction site encodes the amino acids "Gly-Gly-Leu-Gly-Gly" as spacer residues between fusion proteins. Vector pGFPfu and gel-purified PCR fragments were digested with BamHI followed by BlnI and ligated. After transformation, minipreps from positive colonies were sequenced to identify colonies carrying an accurate copy of the gene. The resultant vectors were named pHvGOI:GFP.

2.24 Transient expression by microprojectile bombardment

Barley or Arabidopsis leaves were harvested and cut into 15 mm length pieces and incubated on Petri dishes containing 1% water agar for 3-4 h prior to DNA delivery. The protocol of Gordon-Kamm *et al.* (1990) was used to precipitate 5 µg of vector on 2 µg

Table 2.3 Design of primers used for constructing RNAi vectors. Introduced restriction recognition sites into gene specific primers for amplifying sense and antisense fragments of RNAi. The restriction sites were designed to enable cloning the fragments into restriction sites of pHannibal and subsequent release of the cloned region in a single fragment for ligation into pPZP.Ubi.

Primer	Sense fragment (5'→3')	Antisense fragment (5'→3')
Forward	XhoI, BamHI	BglII
Reverse	KpnI	XbaI, BamHI

gold particles (1 µm in diameter, Bio-Rad, Munich, Germany), then resuspended in 100 µl ethanol. To each Macrocarrier 5 µl of the suspension was added and allowed to dry before bombardment using a PSD-1000/He Particle Delivery System (Bio-Rad). A 900 psi rupture disc pressurised with Helium was used to shoot the DNA-coated gold particles into leaves at 27 Hg column vacuum. Bombarded segments were kept on the same plate for 24 h in the dark and then examined under a microscope.

2.25 Fluorescence and confocal microscopy

Bombarded leaf segments were mounted on glass slides and examined using a Zeiss Axioskop 20 microscope for transmitted light and incident-light fluorescence (Carl Zeiss, Oberkochen, Germany) equipped with a HBO 50W high-pressure mercury lamp. GFP fluorescence of transformed cells was detected using filter set 487 (excitation 450-490 nm, beam splitter FT 510 and barrier filter LP 520). Leica MZ FLIII stereo-fluorescence microscope with GFP1 filter (excitation 425/60 nm, barrier filter 480 nm) also was used. Images were captured with a DC 300F digital camera (Leica Microsystems GmbH, Wetzlar, Germany) and processed with IM1000 Image Manager V1.10 (Leica Microsystems) software.

For cell wall staining, bombarded leaf segments were stained in propidium iodide (10 µg/ml) for 1 h, followed by 3 rinses in water and mounted on a glass slide under a cover slip. A Leica TCS-SP1 laser scanning confocal microscope (LSCM) equipped with an argon laser was used for imaging at an excitation wavelength of 488 nm. The GFP emission was collected in 500-550 nm channel and propidium iodide emission at 600-700 nm. Images from both channels were overlaid to produce composite pictures. To produce three-dimensional clips, Z stacks taken from a cell were combined. Drs Alexander Johnson and Stuart Roy (ACPFG, Adelaide University) are acknowledged for help with confocal microscopy.

2.26 Subcellular localisation of GFP-fusion protein

Vectors pHvGOI:GFP were transiently expressed in barley leaves (Section 2.24) and location of HvGOI:GFP fusion protein was studied under fluorescence and LSCM (Section 2.25).

2.27 Stable transformation of barley

Stable barley transformation was performed in the Barley Transformation Group (University of Adelaide) by Dr Rohan Singh and Ms Konstanze Beck-Oldach based on the protocol by Tingay and colleagues (1997) with modifications according to Matthews *et al.* (2001).

2.28 Genetic analysis of transgenic lines

To confirm stable barley transformation, DNA was extracted from individual T_0 and T_1 plants and PCR reactions with primers HygF/R using Immolase polymerase was employed to amplify the hygromycin resistance gene fragment (*hyg*) using CP 19. For some lines PCRs were also carried out specifically to the transgene using a vector and a gene-specific primer to amplify a fragment with known length. Primer pairs s134F16/PZPR CP 25 (209 bp), s134F16/PZPF CP 27 (182 bp) and d1057F8/PZPF CP 26 (710 bp) were used to validate the *PR-17c* over-expression, *PR-17c* antisense and *HvFdh* over-expression lines, respectively.

2.29 Phenotyping transgenic plants

To identify developmental phenotypes, T_1 plants were allowed to grow in a glasshouse to maturity. The observed phenotypes were photographed and noted.

For disease assay, plants were grown in a completely randomised design and inoculated with strain SA6. The resistance of transgenic lines to scald was assessed by scoring symptoms as described (Section 2.5). The disease assay was repeated twice and TTEST function of Microsoft Excel was used to calculate significance of differences in

comparison to non-transgenic progeny. Lines showing significant differences in both experiments were reported as being significantly different.

Drought tolerance of transgenic lines was examined by random planting of four uniformed size seedlings (germinated in a petri dish) in a 6" pot filled with 1400 g Coco mix. The *HvGolS1* transgenic plants were grown in a growth chamber under the condition as described at Section 2.3 and *HvFdh* lines were grown in a glasshouse with supplementary light. The pots were watered with 200 ml water in regular intervals and watering was stopped after three weeks. The time taken for plants to wilt was noted by regular checks. The pots were watered two weeks after onset of wilting and the recovery rate of individual plants was recorded.

2.30 Sequence analysis

Data from sequencing were viewed by the Vector NTI V.9 (VNTI) software (Invitrogen, Mount Waverley, VIC, Australia). Sequence alignments were carried out using AlignX application of VNTI or ClustalX. ContigExpress program in the VNTI V.9 suite was used for editing raw sequences and assembly of contiguous sequences (contig).

2.31 Homology searches and extension of sequences

Genbank (http://www.ncbi.nlm.nih.gov) non-redundant barley EST database was screened for homologous ESTs by BLAST (Altschul *et al.*, 1998) searches using the gene of interest cDNA as query. ESTs that had overlapping 3' or 5' end with a query were assembled into a contig using the VNTI ContigExpress program (Section 2.30). Extensions of the original fragments were further blasted to retrieve more ESTs and the assembly procedure was performed progressively until no ESTs extending beyond the previous contig was found. Also TIGR Plant Transcript Assemblies at http://plantta.tigr.org/cgi-bin/plantta_release.pl were searched for query matches.

2.32 Online analysis tools

Tools and software offered by the EXPASY proteomics server (http://kr.expasy.org/) were used (last revision on December 2007) for various analyses:

- Physical and chemical properties of proteins were predicted using the ProtParam program (Gasteiger *et al.*, 2005) at http://kr.expasy.org/tools/protparam.html
- Secondary structure of proteins using PSIpred program (Jones, 1999) at http://bioinf.cs.ucl.ac.uk/psipred/. The predicted structure was edited by GSviwe software.
- Phosphorylation sites using Netphos and Netphosk programs (Blom *et al.*, 1999) at http://www.cbs.dtu.dk/services/NetPhosK/
  and http://www.cbs.dtu.dk/services/NetPhos/, respectively
- Molecular weight and pI in different phosphorylation states were predicted by Scansite Molecular Weight & Isoelectric Point Calculator at http://scansite.mit.edu/calc_mw_pi.html
- O-Glycosylation of amino acid residues at http://ogpet.utep.edu/ogpet_result.php
- Conserved domains within protein sequences by Conserved Domain Database (CDD) at http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi (Marchler-Bauer and Bryant, 2004)
- Signal peptide cleavage sites using SignalP (Emanuelsson *et al.*, 2007) server at http://www.cbs.dtu.dk/services/SignalP/
- Mitochondrial targeting by MITOPROT (Claros and Vincens, 1996) at http://ihg.gsf.de/ihg/mitoprot.html
- Chloroplast localisation using ChloroP 1.1 (Emanuelsson *et al.*, 1999) server at http://www.cbs.dtu.dk/services/ChloroP/
- Identifying putative mitochondrial, plastid and ER targeting sequences using Predator (Small *et al.* 2004) at http://urgi.versailles.inra.fr/predotar/predotar.html
- Subcellular location of eukaryotic proteins using TargetP (Emanuelsson *et al.*, 2007) at http://www.cbs.dtu.dk/services/TargetP/
- Subcellular localisation by PSORT at http://psort.ims.u-tokyo.ac.jp/form.html (Nakai and Kanehisa, 1991)

- Subcellular localisation by CELLO V.2.5 at http://cello.life.nctu.edu.tw/ (Yu *et al.*, 2004)
- Protein localisation by SherLoc (Hoglund *et al.*, 2006) at http://www-bs.informatik.uni-tuebingen.de/Services/SherLoc/
- Prediction of transmembrane region of proteins at http://www.predictprotein.org (Rost *et al.*, 1996)
- Intron junctions in genomic DNA using NetPlantGene (Hebsgaard *et al.*, 1996) server at http://www.cbs.dtu.dk/services/NetPGene/

2.33 Transcript profiling using Barley1 22k GeneChip

Data from hybridisation of RNA from different experiments to barley Affymetrix chip deposited in BarleyBase (Shen *et al.*, 2005) and available from http://www.plexdb.org/ and data at http://www.ncbi.nlm.nih.gov/geo/query/blast.html represented by Gene Expression Omnibus (GEO) were used to investigate the expression pattern of the genes of interest (GsOI). cDNA sequences of GsOI were blasted against barley GenChip exemplars to retrieve a representing contig and its probe set sequences. The retrieved and query sequences were aligned and locations of probe sets were checked to ensure perfect match in the region between the contig and query sequence. The data normalised using the Robust Multi-array Analysis (RMA) were collected from each hybridisation and the average values of replica hybridisations extracted from the database. Data were retrieved from datasets BB3, BB4, BB7 and BB9 hybridisations which represent barley transcriptome analysis during developmental stages (Druka *et al.*, 2006) , interaction with powdery mildew fungus (Caldo *et al.*, 2004), mlo5-mediated response to *Bgh* (Fischer, unpublished) and barley-*Fusarium graminearum* interaction (Boddu *et al.* 2006), respectively. Also data series GSE6325 (Walia *et al.*, 2007b), GSE3097 (Walia *et al.*, 2006) and GSE5605 (Walia *et al.*, 2007a) deposited at GEO representing the barley transcriptome under salt stress and jasmonic acid treatment were used. From GEO series, only good quality data as indicated in the database were collected and the average of biological replicates was used for analysis.

2.34 Phylogenetic analysis

Predicted protein sequences of GsOI were used as queries in the BLASTp program (http://www.ncbi.nlm.nih.gov/BLAST/) to identify non-redundant protein homologues with an initial e-value cut off of e^{-10}. Similarly, a tBLASTn search was carried out to retrieve ESTs and presence of ORF was detected by VNTI program. To retrieve all possible plant homologues also a tBLASTx search was done against TIGR Plant Transcript Assemblies (TA) at (http://www.plantta.tigr.org/search.html) database using the protein of interest as query sequence. In addition, the gene index assemblies at the Computational Biology and Functional Genomics Laboratory were screened for non-plant homologous sequences (http://compbio.dfci.harvard.edu/tgi/tgipage.html). Retrieved EST and TA sequences containing an ORF were translated into a protein sequence. Then, protein sequences from each species were separately aligned to remove duplicates and produce a non-redundant list of protein sequences. Remaining proteins within the list were blasted against CCD to ensure that they possess the domain of interest.

To examine the phylogenetic relationships among different members of a protein from various species, protein sequences were edited (signal peptide was removed) and aligned using ClustalX (Thompson *et al.*, 1997). The sequence relationships were determined using a distance based algorithm, the neighbour joining, (Saitou and Nei, 1987). This method makes pair wise comparisons of whole sequences, thus evolutionary divergence is determined based on a single coefficient of sequence similarity or difference. Bootstrap scores for all nodes in the tree were obtained from 1000 runs. The alignment file was displayed using the GeneDoc program (Nicholas *et al.*, 1997) and the tree was visualised using the TreeView software (Page, 1996).

Chapter 3: Characterisation of barley PR-17 family

3.1 Introduction

One of the plants' induced defence responses is expression of proteins referred to as "pathogenesis-related proteins" (PR). PR proteins were defined as host encoded proteins, which are induced specifically in pathological or related situations (Antoniw and Pierpoint, 1978; Bohlmann *et al.*, 1998) and by application of chemicals that mimic the effect of pathogen infection or induce similar responses (Bol *et al.*, 1990). However, some PRs are also expressed in healthy plants and their expression is regulated during infections. To avoid confusion, the term "inducible defence-related proteins" has been suggested. Recently, van Loon *et al.* (2006) used the term for all microbe-induced proteins and their homologues that include constitutively present proteins whose expression increases during most infections.

PR proteins in most plants usually occur as multigene families. At present PR proteins are divided into 17 different families and show diverse properties (Table 3.1). In addition to known families, a putative novel family (PR-18) comprises fungus- and SA-inducible carbohydrate oxidases such as a hydrogen peroxide-generating protein with antimicrobial properties from sunflower (van Loon *et al.*, 2006). Most of the existing families include members that are secreted to the extracellular space, where they are believed to have a role in resistance.

Generally, PR proteins exhibit hydrolase or inhibitory activities which are consistent with a role in defending the plant against pathogen infection either by active hydrolysis of fungal cellular components or by a toxic effect on their growth environment (Muthukrishnan *et al.*, 2001). Several PR proteins show antifungal activity *in viv*o, *in vitro*, or both (Kitajima and Sato, 1999) indicating a simple form of defence against the invading pathogen. On the other hand, evidence suggests that extracellular PR proteins may also have an indirect role in resistance. For example, peroxidases (PR-9) induced during barley–powdery mildew interaction, may have a role in oxidative cross-linking of plant cell wall components and preventing the pathogen penetration (Thordalchristensen *et al.*, 1992). Also, an oxalate oxidase involvement in signal transduction has been suggested (Zhou *et al.*, 2000). Moreover, constitutively expressed or developmentally regulated and vacuole localised PR proteins (Kitajima and Sato, 1999) could act as an effective second line of defence when the pathogen causes tissue damage. Induction and accumulation of

PR proteins concomitant with SAR also suggest a causal relationship in resistance. Although PR proteins have been characterised as proteins induced by pathogen attack, evidence suggests that they may have important functions in plant development and abiotic stress responses.

Members of homologous PR proteins in different species are developmentally regulated. For instance, PR-1, PR-4 and PR-5 type proteins (Casacuberta *et al.*, 1991; Hejgaard *et al.*, 1991; Hejgaard *et al.*, 1992) as well as PR-3 (Jacobsen *et al.*, 1990; Kragh *et al.*, 1990; Leah *et al.*, 1991), PR-2 (Hoj *et al.*, 1989; Leah *et al.*, 1991) and a ribosome-inactivating protein (Leah *et al.,* 1991) are synthesized in cereal seeds. It has been shown that in tobacco, tomato, and pea seeds pathogen-inducible basic PR-2 and PR-3 have a roll in cell wall degradation and emergence of radical as well as protection of the exposed inner tissues of the seed against microbial entry (Leubner-Metzger, 2005; Morohashi and Matsushima, 2000; Wu *et al.*, 2001). Chitinases homologous to PR-3 and PR-4 are required as morphogenetic factors in carrot somatic embryogenesis (Kragh *et al.*, 1996). In banana fruits it has been shown that a PR-8 protein constitute a storage form of nitrogen (Peumans *et al.*, 2002). Many other PR proteins such as PR-1 to PR-6 also have been shown to be induced during senescence in various species (summarised in Quirino *et al.*, 2000). Transgenic approaches also have suggested developmental roles of PR. In a transgene study, a PR-2 protein was shown to be necessary for normal pollen development in tobacco (Worrall *et al.*, 1992). By introducing a chimeric gene fusion of the osmotin promoter and beta-glucuronidase into tobacco it was shown that osmotin promoter had a very high natural level of activity in mature pollen grains during anther dehiscence and in pericarp tissue at the final, desiccating stages of fruit development and in corolla tissue at the onset of senescence (Kononowicz *et al.*, 1992).

Abiotic stresses such as osmotic stress, low temperature, salinity and wounding can also elicit PR proteins induction. Over-expression of osmotin in transgenic tobacco resulted in improved salt and drought tolerance (Barthakur *et al.*, 2001). PR proteins with antifreeze activity have been isolated from winter rye (Hon *et al.*, 1995), bittersweet nightshade (*Solanum dulcamara*) (Huang and Duman, 2002) and include β-1,3-glucanases, chitinases and thaumatin-like proteins. In the apoplast of winter rye these proteins accumulate during cold acclimation, and low-temperature-induced extracts exhibit both enzyme and

Table 3.1 Pathogenesis-related protein families. Current families of pathogenesis-related proteins adapted from van Loon *et al.* 2006.

NOTE:
This table is included on page 56 of the print copy of the thesis held in the University of Adelaide Library.

antifreeze activities (Hiilovaara-Teijo *et al.*, 1999). Similar proteins are induced under the same conditions in freezing-tolerant wheat and barley, not in freezing-sensitive maize (Antikainen and Griffith, 1997). It has been shown that PR-3, PR-4 and PR-5 affect seed germination in Arabidopsis in the presence of high salt (Seo *et al.*, 2008).

Many PR protein family members have been cloned and characterised from different species. Although PR-17 family members have been cloned from tobacco (Okushima *et al.*, 2000), wheat (Gorlach *et al.*, 1996) and barley (Christensen *et al.*, 2002), the function of the family and their role in plant defence have remained uncharacterized. In this chapter, cloning new members, functional analysis, antimicrobial activity and the role of the PR-17 family in barley defence is reported.

3.2 Materials and methods

General materials and methods were presented in chapter 2. This section describes materials and methods specifically used for characterisation of barley PR-17 members, which are covered in this chapter.

3.2.1 Isolation of full length cDNA and genomic sequences of barley PR-17 members

The clone s134 (*HvPR-17c*) sequence was used to design primers for 3'- and 5'-RACE (Section 2.12) and the products of RACE were sequenced and assembled into contigs. The full length of *HvPR-17c* and *HvPR-17d* cDNA were amplified from 5'-RACE ready cDNA by using ProofStart and s134F3/R3 and s134F17/R17 primer pairs and subsequently was subcloned into pGEM-T Easy vector. Corresponding genomic DNA region was amplified similarly using genomic DNA from Atlas, Atlas 46, and Turk cultivars by CP 13 and 19 for *HvPR-17c* and *HvPR-17d*, respectively. PCR products were cloned and ten colonies for each cultivar were sequenced.

3.2.2 Heterologous expression, purification and desalting

To express and purify recombinant HvPR-17c protein, the QIAexpress (QIAGEN) system was employed. Two PCR fragments were generated using *PfuUltraHF* polymerase and CP 12. Primer pairs s134F13/R13 and s134F14/R14 were used for amplifying fragments to be ligated into pQE30 (N-terminal His-tag) and pQE70 (C-terminally tagged, Appendix G) vectors, respectively. BamHI, HindIII, SphI and BglII restriction sites were introduced into 5' end of s134F13, R13, F14 and R14 primer, respectively. Amino acid residues 1-22 were not included for expression, since they constitute a signal peptide and predicted to be cleaved off the mature protein. The insert of vectors were sequenced to verify accuracy and were named pQE30HvPR-17c and pQE70HvPR-17c.

The vectors were transformed into competent *E. coli* strain M15 cells (QIAGEN) and were subjected to colony-blot procedures using primary mouse anti-his antibody and alkaline phosphatase conjugated anti-mouse secondary antibody (Sigma). The colonies with the strongest signal in the colony assay were picked from the plate and used for inoculation of

large-scale culture for preparative purification. HIS-Select® HF Nickel Affinity Gel (Sigma) was used to purify recombinant protein under native conditions. The procedures were carried out according to manufacturers' instructions.

To change buffer and concentrate the protein a Centricon centrifugal device fitted with Ultracel ^{R} YM10 (10 KDa cut off, Millipore, USA) was used according to manufacturer's instructions. The exchange buffer consisted of 10 mM sodium acetate at pH 4.5, pH 5.8, pH 6.5, pH 7.5 and pH 8. Protein concentration was measured at A_{280} nm in spectrophotometer (Nano-Drop) and the concentration was adjusted by adding required amount of the same buffer. The success of concentration was checked by running equal amount of sample before and after ultra filtration on SDS-PAGE. In addition, an aliquot of each concentrated protein solution was picked and dithiothreitol (DDT) to a final concentration of 1 mM was added to each sample.

3.2.3 Polyacrylamide gel electrophoresis

To monitor the expression level and purification efficiency SDS-PAGE (resolving gel 14% and stacking gel 4.0%) (Laemmli, 1970) was used to fractionate proteins in an XCell Surelock Mini-Cell system (Invirogen) run at 100 V. The gel was stained with staining solution (0.5% comassie brilliant blue R, 10% v/v glacial acetic acid, 40% v/v ethanol, 50% water) for 1 h and de-stained overnight in de-staining solution (staining solution without comassie). A broad range SDS-PAGE prestained standard (Bio-Rad) was used to estimate protein molecular weights.

3.2.4 Western blot

The protein samples were run in duplicate gels in the same tank. One gel was used for staining and visualisation of bands and the other gel for electro blotting onto a Hybond-P membrane using a wet transfer system (Mini-Blot Electrophoretic Transfer Cell, BioRad) under a constant 400 mA for 1 h. The presence of proteins on the membrane was detected by incubating with mouse Anti-His antibody (Sigma) followed by incubation with a secondary antibody (Anti-mouse alkaline phosphatase conjugate). The blot was developed in NBT/BCIP (Sigma) by covering the membrane with the solution until desired staining

intensity was achieved and reaction was stopped by rinsing in water for 5 min. Manufacturers' instructions were used in all of the procedures used for Western blot.

3.2.5 Bioassay

Two methods were used to investigate the anti-fungal activity of purified recombinant proteins. In the disc method, fresh cultures of *Pyrenophora teres* were initiated on plates containing potato dextrose agar. A disc (1 cm in diameter) from a culture was cut and placed in the centre of a plate and incubated at 18ºC. After the fresh growth started from the disc into new medium, Wattman papers (5 mm diameter) were placed around growing fungal hyphae and 10 µl of various protein solutions were placed on separate discs. Buffer alone was used as negative control and Mancozeb fungicide (0.01% w/v) as positive control. The plates were incubated and antifungal activity was assessed daily for one week. In the second method, conidia of *R. secalis* isolate SA385 were harvested from a LiBA plate (Section 2.4.). These were suspended in sterilised Mili-Q water and spore number was adjusted to $1x10^7$/ml. 2 µl of the spore suspension was mixed with 8 µl of each protein solution and incubated at 20ºC in the dark. The bioassay mix was examined under the microscope every day for one week. Protein solutions in both methods included 3.5 mg/ml and 2 mg/ml concentrations at the pH5.8, pH 6.5, pH 7.5 and pH 8 with and without addition of DDT to a final concentration of 1 mM.

3.3 Results

3.3.1 Isolation of three genes encoding barley PR-17 family members

The first step in the characterisation of clone s134 was the isolation of the gene sequence using RACE PCR. Sequencing of 5'-RACE products (Fig. 3.1) led to the identification of two fragments with similar size named *HvPR-17a5'* and *HvPR-17c5'*. The result indicated existence of other homologues. Although the clone contained a poly A^+ tail, a primer was designed from the 5' region to amplify the remaining sequence of the genes by 3'-RACE. The cloned products of 3'-RACE PCR (Fig. 3.2) was sequenced which resulted in three homologous fragments of similar length. They were named *HvPR-17a3'*, *HvPR-17c3'* and *HvPR-17d3'*. Joining of 3'- and 5'-RACE sequences produced two contiguous sequences (Fig. 3.3 A and B) and one 3' fragment. One contig was formed by overlapping sequences of *HvPR-17a3'* and *a5'* (*HvPR-17a1*) and the other by *HvPR-17c3'* and *c5'* (*HvPR-17c*). Northern analysis with gene specific 3' probe (Section 3.3.5) indicated that *HvPR-17c* is a full-length transcript.

The gene-specific primer set covering the full open-reading frame of the *HvPR-17c* was used to amplify the full-length of the gene from genomic DNA and cDNA of different cultivars (Fig. 3.4). The amplified fragments' sequences were identical which indicated lack of an intron in that region of *HvPR-17c*.

The sequence of *HvPR-17d3*' was used to search the TIGR barley gene index and a tentative contig *TC139571* showed perfect match at its 3' end with query sequence. However, *TC139571* did not contain a poly A^+ tail and its 3' end stretched beyond the poly A^+ tail of *HvPR-17d*3'. A forward primer was designed from the contig *TC139571* 5' end and a reverse primer based on *HvPR-17d3'* and the full length of the gene was amplified by RT-PCR from *R. secalis* inoculated Atlas 46 leaf cDNA and genomic DNA of three cultivars (Fig. 3.5). Sequencing of the PCR products revealed an 86 bp intron in genomic region. Alignment of *HvPR-17d* cDNA and genomic DNA sequences are shown in Figure 3.6.

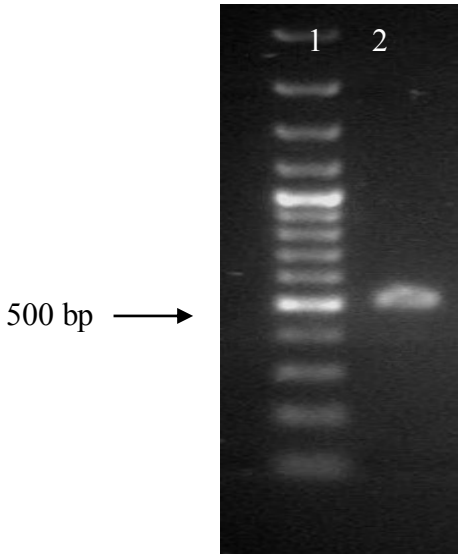


Figure 3.1 s134 5'-RACE. Nested 5'-RACE product amplified by primer pair s134R2/SmNUPA was fractionated on a 2% agarose gel. Lane 1: DNA size marker, Lane 2: 5'-RACE product.

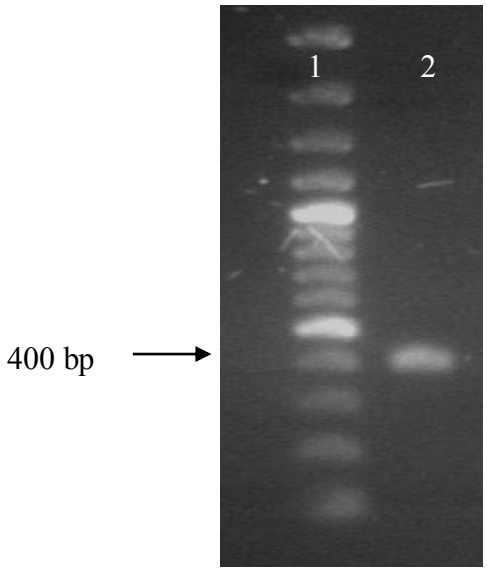


Figure 3.2 s134 3'-RACE. The 3'-RACE product using primer pair s134F5/CDSIIA was fractionated on 2% agarose gel and purified. Lane 1: DNA size marker, Lane 2: 3'-RACE product.

(A)

```
                 1                                                  50
   pBH6-12     (1) ACGAGATGAAGATATCCATCGCAGCAGCCGCTGTCCTCCTCCTGGCCTTG
HvPR-17a3'     (1) --------------------------------------------------
HvPR-17a5'     (1) ACGAGATGAAGATATCCACCGCAGCAGCCGCTGTCCTCCTCCTGGCCTTG
                 51                                                 100
   pBH6-12    (51) GCAGCCACGGCTAGCGCGGTCACGTTCGACGTGACGAACGAGGCGTCGAG
HvPR-17a3'     (1) --------------------------------------------------
HvPR-17a5'    (51) GCAGCCACGGCTAGCGCGGTCACGTTCGACGTGACGAACGAGGCGTCGAG
                101                                                 150
   pBH6-12   (101) CACAGCCGGCGGCCAGCGATTCGACAGGGAGTACGGCGCCGCCTACGCCA
HvPR-17a3'     (1) --------------------------------------------------
HvPR-17a5'   (101) CACAGCCGGCGGCCAGCGATTCGACAGGGAGTACGGCGCCGCCTACGCCA
                151                                                 200
   pBH6-12   (151) AGCAAGTGCTCTCGGATGCTTCCTCCTTCACCTGGGGCATCTTCAACCAG
HvPR-17a3'     (1) --------------------------------------------------
HvPR-17a5'   (151) AGCAAGTGCTCTCGGATGCTTCCTCCTTCACCTGGGGCATCTTCAACCAG
                201                                                 250
   pBH6-12   (201) CCGGACCCCTCAGACCGCAGGCCCGCCGATGGCGACACCGTCACCCTCGC
HvPR-17a3'     (1) --------------------------------------------------
HvPR-17a5'   (201) CCGGACCCCTCAGACCGCAGGCCCGCCGATGGCGACACCGTCACCCTCGC
                251                                                 300
   pBH6-12   (251) CGTCCGCGACACGAACGGCATCGCCTCCACCAGCGGCAGCACCATCGAGC
HvPR-17a3'     (1) --------------------------------------------------
HvPR-17a5'   (251) CGTCCGCGACACTAACGGCATCGCCTCCACCAGCGGCAGCACCATCGAGC
                301                                                 350
   pBH6-12   (301) TCAGCGCCCGCTCCGTCGGCGGTATTACCGGCGACAACCTCAAGGAGCAG
HvPR-17a3'     (1) --------------------------------------------------
HvPR-17a5'   (301) TCAGCGCCCGCTCCGTCGGCGGTATTACCGGCGACAACCTCAAGGAGCAG
                351                                                 400
   pBH6-12   (351) GTGGACGGGGTGCTGTACCACGAGGTGGTGCACGTGTGGCAGTGGGGGCT
HvPR-17a3'     (1) --------------------------------------------------
HvPR-17a5'   (351) GTGGACGGGGTGCTGTACCACGAGGTGGTGCACGTGTGGCAGTGGGGGCT
                401                                                 450
   pBH6-12   (401) GCAGGACTACCACGAGCACCACCGGGATCTTCGAGGGGATCGCGGACTACG
HvPR-17a3'     (1) -----------------ACCACGGGATCTTCGAGGGGATCGCGGACTACG
HvPR-17a5'   (401) GCAGGACTACCACGAGCACCACG----------------------------
                451                                                 500
   pBH6-12   (451) TGCGGCTCAAGGCCGGGTACGTAGCGGCGAACTGGGTGAAGGAGGGCGGC
HvPR-17a3'    (34) TGCGGCTCAAGGCCGGGTACGTAGCGGCGAACTGGGTGAAGGAGGGCGGC
HvPR-17a5'   (424) --------------------------------------------------
                501                                                 550
   pBH6-12   (501) GGCAGCCGGTGGGACGAGGGATACGACGTGACGGCCAGGTTCCTGGACTA
HvPR-17a3'    (84) GGCAGCCGGTGGGATCAGGGGTACGACGTGACGGCCAGGTTCCTGGACTA
HvPR-17a5'   (424) --------------------------------------------------
                551                                                 600
   pBH6-12   (551) CTGCGACTCGCGCAAGCCCGGGTTCGTGGCGGAGATGAACGGCAAGCTCA
HvPR-17a3'   (134) CTGCGACTCGCGCAAGCCCGGGTTCGTGGCGGAGATGAACGGCAAGCTCA
HvPR-17a5'   (424) --------------------------------------------------
                601                                                 650
   pBH6-12   (601) AGGACGGCTACAACGACGACTACTTCGTGCAGATCCTCGGGACGAGCGCG
HvPR-17a3'   (184) AGGACGGCTACAACGACGACTACTTCGTGCAGATCCTCGGGACGAGCGCG
HvPR-17a5'   (424) --------------------------------------------------
                651                                                 700
   pBH6-12   (651) GACCAGCTGTGGAACGACTACAAGGCCAAGTACTCCCAGGGCTGATCCGT
HvPR-17a3'   (234) GACCAGCTGTGGAACGACTACAAGGCCAAGTACTCCCAGGGCTGATCCGT
HvPR-17a5'   (424) --------------------------------------------------
                701                                                 750
   pBH6-12   (701) TTCATGTTGCATCGCATGCATGTGTACCGGTCTACGTCTATGTACTGATA
HvPR-17a3'   (284) TTCATGTTGCATCGCATGCGTGTGTACCGGTCTACGTCTATGTACTGATA
HvPR-17a5'   (424) --------------------------------------------------
```

```
                751                                                 800
   pBH6-12  (751) TACTAGTAGTATACTTGTATTACTACACGGTTGATGTACTGCCTGGAATG
HvPR-17a3'  (334) TACTAGTAGTATACTTGTATTACTACACGGTTGATGTACTGCCTGGAATG
HvPR-17a5'  (424) ------------------------------------------------
                801                                         847
   pBH6-12  (801) GAATAAATCAACGTTCGCAAGGTTGAGCA-----------------
HvPR-17a3'  (384) GAATAAATCAACGTTCGCAAGGTTGAAAAAAAAAAAAAAAAAAAAA
HvPR-17a5'  (424) ------------------------------------------
```

(B)

```
                1                                                   50
HvPR-17c3'    (1) ------------------------------------------------
HvPR-17c5'    (1) AAGCAGGGGTATCAACGCAGAGTACGCGGGGATCTACCACTAGCTCAGCG
                51                                                  100
HvPR-17c3'    (1) ------------------------------------------------
HvPR-17c5'   (51) ACACAGGCACGTAGTAGCAACACCCACTACAAAATGAAGCTTCAGGTAGC
                101                                                 150
HvPR-17c3'    (1) ------------------------------------------------
HvPR-17c5'  (101) CACGGTCGCCTCCTTCCTCCTGGTGGCCTTGGCCGCGACGGCCCAGGCAG
                151                                                 200
HvPR-17c3'    (1) ------------------------------------------------
HvPR-17c5'  (151) TGACGTTCGACGCGTCGAACAAGGCGTCGGGCACCTCCGGCGGCCGGCGG
                201                                                 250
HvPR-17c3'    (1) ------------------------------------------------
HvPR-17c5'  (201) TTCGAGCAGGCCGTCGGCCTCCCATACTCCAAGAAGGTCCTCTCCGAGGC
                251                                                 300
HvPR-17c3'    (1) ------------------------------------------------
HvPR-17c5'  (251) CTCCGCCTTCATCTGGAAAACCTTCAACCAGCGTGCCGTCGGCGACCGCA
                301                                                 350
HvPR-17c3'    (1) ------------------------------------------------
HvPR-17c5'  (301) AGCCTGTCAACGCAGTCACCCTCGTCGTCGAGGACATCAGCGGCGTCGCC
                351                                                 400
HvPR-17c3'    (1) ------------------------------------------------
HvPR-17c5'  (351) TTCACCAGCGCCAACGGCATCCACCTCAGCGCCCAGTACGTCGCCAGCAT
                401                                                 450
HvPR-17c3'    (1) ------------------------------------------------
HvPR-17c5'  (401) CTCCGGCGACGTCAAGAAGGAGGTGACCGGCGTGCTGTACCACGAGGCGA
                451                                                 500
HvPR-17c3'    (1) ------------------------------------------------
HvPR-17c5'  (451) CGCACGTGTGGCAGTGGAACGGGCAGGGCAAGGCGAACGGCGGGCTCATC
                501                                                 550
HvPR-17c3'    (1) --------CGCCGACTACGTGCGGCTCAAGGCCGGGTTCGCGCCGGGGCA
HvPR-17c5'  (501) GAGGGGATCGCCGACTACGTGCGGCTCAAGGCCGGGTTCGCGCCGGGGCA
                551                                                 600
HvPR-17c3'   (43) CTGGGTGAAGCCGGGGCAGGGCGACCGGTGGGATCAGGGGTACGACGTCA
HvPR-17c5'  (551) CTGGGTGAAGCCGGGGCAGGGCGACCGGTGGGATCAGGGGTAC-------
                601                                                 650
HvPR-17c3'   (93) CGGCGAGGTTCCTCGACTACTGCGACTCACTGAAGCCCGGGTTCGTCGCG
HvPR-17c5'  (594) ------------------------------------------------
                651                                                 700
HvPR-17c3'  (143) CAGCTCAACGCCAAGATGAAGAGTGGGTACACCGACGACTTCTTCGCGCA
HvPR-17c5'  (594) ------------------------------------------------
                701                                                 750
HvPR-17c3'  (193) GATTCTCGGCAAGAACGTGCAGCAGCTGTGGCGGGACTACAAATCCAAGT
HvPR-17c5'  (594) ------------------------------------------------
                751                                                 800
HvPR-17c3'  (243) TTGGAGCCTGAATACACGATTAGCCTACTTGACGGTGTGATGGCATAGCG
HvPR-17c5'  (594) ------------------------------------------------
                801                                                 850
HvPR-17c3'  (293) CACTATATATATCGAAATGATCAACAAAACAAGTGCTTTATGCCCTGTAT
```

```
HvPR-17c5'  (594) ---------------------------------------------------
                  851                                             900
HvPR-17c3'  (343) AATAAATAAAAGATAAATAAAACGACTTTGGCAAAAAAAAAAAAAAAAAA
HvPR-17c5'  (594) ---------------------------------------------------
                  901       913
HvPR-17c3'  (393) AAAAAAAAAAAAA
HvPR-17c5'  (594) -------------
```

Figure 3.3 *HvPR-17* contigs. Contigs formed by joining of 3'- and 5'-RACE products. (A) Contig formed by overlapping sequences of *HvPR-17a3'* and *a5'* was named *HvPR-17a1*. Alignment shows that the contig is similar to *pBH6-12* (Section 3.3.3) with six mismatched nucleotides shown in bold font. (B) Contig formed by overlapping sequences of *HvPR-17c3'* and *c5'* was named *HvPR-17c*. Overlapping regions are in bold characters. Nucleotide sequences were aligned using the Vector NTI V.9 AlignX program.

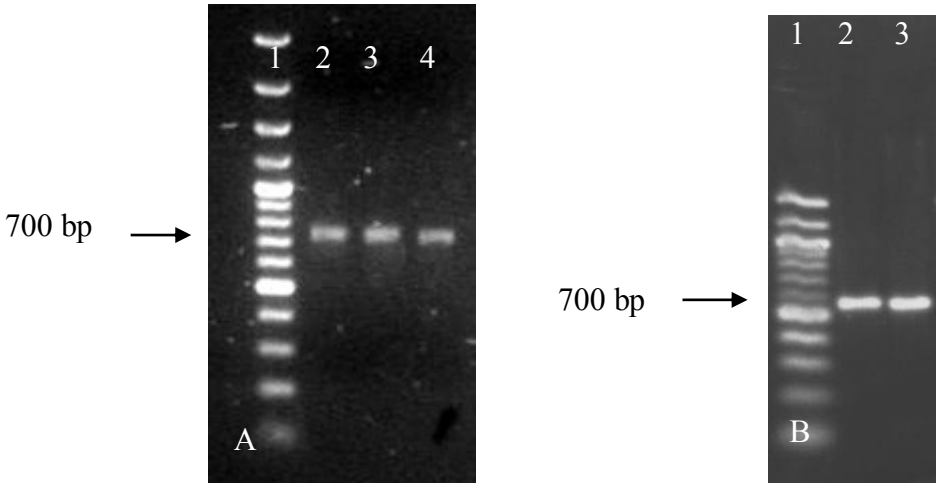


Figure 3.4 Amplified fragments of *HvPR-17c*. (A) Amplified genomic region of *HvPR-17c* fragments from Atlas (Lane 2), Atlas 46 (Lane 3) and Turk (Lane 4) cultivars using primer s134F3/R3. (B) Lane 2: cDNA from Atlas, Lane 3: genomic DNA from Atlas. Lane 1 in both pictures is marker DNA.

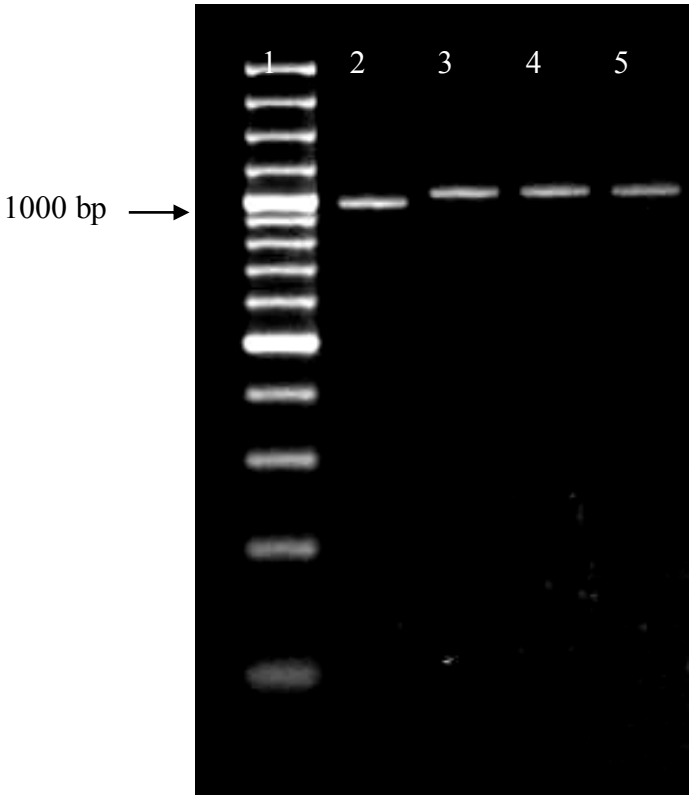


Figure 3.5 Fragments of *HvPR-17d*. cDNA and genomic DNA amplified by s134F17/R17 primer set targeting *HvPR-17d*. Lane1: marker, Lane 2: Atlas 46 cDNA, Lane 3: Atlas genomic DNA, Lane 4: Atlas 46 genomic DNA, Lane 5: Golden Promise genomic DNA.

```
                        1                                                  50

HvPR-17d cDNA     (1) ATAAATTTGGCCCTCCACCGCCAGTCTGCCACTCACAGAAGCACTTAGCA
HvPR-17d gDNA     (1) -------------------------------------------CTTAGCA
                      51                                                  100
HvPR-17d cDNA    (51) CGATGAAGATTGCCATTGCAGCAGCAGCAGCCGCTCCCCTCCTCCTGCTC
HvPR-17d gDNA     (8) CGATGAAGATTGCCATTGCAGCAGCAGCAGCCGCTCCCCTCCTCCTGCTC
                     101                                                  150
HvPR-17d cDNA   (101) CTGGCCCTGGCCGGCACGGCCCGCGCCGTGACTTTCGACGCGACGAACAC
HvPR-17d gDNA    (58) CTGGCCCTGGCCGGCACGGCCCGCGCCGTGACTTTCGACGCGACGAACAC
                     151                                                  200
HvPR-17d cDNA   (151) GGTTCCGGACTCGGCCGGCGGCCAGAGATTCAACCAGGACGTTGGCGTCG
HvPR-17d gDNA   (108) GGTTCCGGACTCGGCCGGCGGCCAGAGATTCAACCAGGACGTTGGCGTCG
                     201                                                  250
HvPR-17d cDNA   (201) ACTACGCCAAGCAGGTGCTCTCCGATGCCTCGTCCTTCATCTGGACCACC
HvPR-17d gDNA   (158) ACTACGCCAAGCAGGTGCTCTCCGATGCCTCGTCCTTCATCTGGACCACC
                     251                                                  300
HvPR-17d cDNA   (251) TTCAACCAGCCCAACCCGGGAGACCGTAGGGACTACGACTCGGTCACCCT
HvPR-17d gDNA   (208) TTCAACCAGCCCAACCCGGGAGACCGTAGGGACTACGACTCGGTCACCCT
                     301                                                  350
HvPR-17d cDNA   (301) CGCTGTCGTCGACAACATCGAGCCTGTGGCCCAGACCGTCGGCAACGCTA
HvPR-17d gDNA   (258) CGCTGTCGTCGACAACATCGAGCCTGTGGCCCAGACCGTCGGCAACGCTA
                     351                                                  400
HvPR-17d cDNA   (351) TCCAACTCCGAGCCCAATACGTCGCCGGCTTCGACGGCGACGTCAAGCAA
HvPR-17d gDNA   (308) TCCAACTCCGAGCCCAATACGTCGCCGGCTTCGACGGCGACGTCAAGCAA
                     401                                                  450
HvPR-17d cDNA   (401) GAGGT----------------------------------------------
HvPR-17d gDNA   (358) GAGGTAATAACCCCGGCGGCACGGCGCGACGTCGCATGCTTATGTATACG
                     451                                                  500
HvPR-17d cDNA   (406) -------------------------------------------GAAAGGCG
HvPR-17d gDNA   (408) TATAGCTTCTTAGGTTTCAACACAAGATTGATGTGCGCAGGTGAAAGGCG
                     501                                                  550
HvPR-17d cDNA   (414) TACTGTACCACGAGGCGACGCACGTGTGGCAGTGGATAGACCACTACGGC
HvPR-17d gDNA   (458) TACTGTACCACGAGGCGACGCACGTGTGGCAGTGGATAGACCACTACGGC
                     551                                                  600
HvPR-17d cDNA   (464) GAAAAACCGGGGCTCTTCGAGGGGATCGCCGACTACGTGCGGCTCAAGGC
HvPR-17d gDNA   (508) GAAAAACCGGGGCTCTTCGAGGGGATCGCCGACTACGTGCGGCTCAAGGC
                     601                                                  650
HvPR-17d cDNA   (514) CGACCTCGCGCCGGGGCACTGGGTGAAGGACGGGGGCGGCGACCGGTGGG
HvPR-17d gDNA   (558) CGACCTCGCGCCGGGGCACTGGGTGAAGGACGGGGGCGGCGACCGGTGGG
                     651                                                  700
HvPR-17d cDNA   (564) ATCAGGGGTACGACGTGACGGCCAGGTTCCTGGACTACTGCGACTCGCTC
HvPR-17d gDNA   (608) ATCAGGGGTACGACGTGACGGCCAGGTTCCTGGACTACTGCGACTCGCTC
                     701                                                  750
HvPR-17d cDNA   (614) AAGCCAGGGTTCGTGGCGGAGATGAACGGCAAGCTCAAGGACGGCTACAG
HvPR-17d gDNA   (658) AAGCCAGGGTTCGTGGCGGAGATGAACGGCAAGCTCAAGGACGGCTACAG
                     751                                                  800
HvPR-17d cDNA   (664) CGACGACTACTTCGTGCAGATCCTGGGGAAGAGCGTGGACGAGCTGTGGA
HvPR-17d gDNA   (708) CGACGACTACTTCGTGCAGATCCTGGGGAAGAGCGTGGACGAGCTGTGGA
                     801                                                  850
HvPR-17d cDNA   (714) GCGACTACAAGGCCAAGTACCCCCAGCCCCAGAGCTGATGGTCGACGTAC
HvPR-17d gDNA   (758) GCGACTACAAGGCCAAGTACCCCCAGCCCCAGAGCTGATGGTCGACGTAC
                     851                                                  900
HvPR-17d cDNA   (764) GATGCATGCAGTTTATTGTTGTATGTGTACCGGTATACATCGACGTTCGA
HvPR-17d gDNA   (808) GATGCATGCAGTTTATTGTTGTATGTGTACCGGTATACATCGACGTTCGA
                     901                                                  950
HvPR-17d cDNA   (814) ACTGATCCAGTATACTTGTGTTAGAACACAGTTGCTGTATTGACTGGAGC
HvPR-17d gDNA   (858) ACTGATCCAGTATACTTGTGTTAGAACACAGTTGCTGTATTGACTGGAGC
                     951                                                 1000
HvPR-17d cDNA   (864) GGAATAAATTGACGTTGGCACGGTGTGTGTTAACGAACTGAGGAAAAAAA
```

```
HvPR-17d gDNA   (908) --------------------------------------------------
                      1001
HvPR-17d cDNA   (914) AAAAAAAAAA
HvPR-17d gDNA   (908) ----------
```

Figure 3.6 Alignment of cDNA and genomic DNA of *HvPR-17d* sequences. The alignment reveals an 87 bp intron in the genomic copy. Alignment of nucleotide sequences were conducted using the Vector NTI V.9 suite AlignX program. The intron sequence is in bold font.

3.3.2 Sequence analysis of the barley *HvPR-17*

After cloning, the nucleotide sequences of the *HvPR-17* family members were analysed for the presence of open reading frames (ORFs). Encoded proteins were predicted to contain either a single ORF (*PR-17c* and *b*) or a long ORF (*PR-17a* and *d*) with one short ORF within the main ORF. Downstream of the initiation codon two in frame stop codons are found in *PR-17a*, four in *PR-17b* and three in *PR-17c* and *PR-17d*. The longest ORF in double ORF genes were selected for translation. All four proteins were predicted to have a signal peptide of different length. Isoelectric points also varied from 4.7 to 9.3. Table 3.2 shows analysis of the deduced proteins and alignment of the amino acid sequences is given in Figure 3.7. The predicted mature protein sequence for HvPR-17c shares 58%, 84% and 66.3% amino acid identity, respectively, to HvPR-17a, HvPR-17b and HvPR-17d. Sequence homology is restricted to conserved blocks separated by minor insertions, deletions and areas of multiple amino acid substitutions. A similarity tree (Fig. 3.8) constructed from the deduced amino acid sequences using ClustalX, represents the sequence similarities among the barley PR-17 proteins.

3.3.3 Similarity search and phylogenetic analysis

Next, public databases were searched for identifying similar sequences and functional annotation. BLASTn searches against NCBI barley nucleotide database showed that *HvPR-17a1* represents *pBH6-12* (Accession No. Y14201) with six mismatch nucleotides (Fig. 3.3A). The mismatches cause an I_5 to T and E_{171} to Q conversion in pBH6-12 in comparison to our sequence. Another homologue found was *pBH6-17* (Accession No. Y14202). These two previously isolated genes together with three newly isolated genes in this study form a small family in barley. *pBH6-12* and *pBH6-17* have been named *HvPR-17a* and *HvPR-17b*, respectively (Christensen *et al.* 2002). To follow the suggested nomenclature, the three isolated genes in this study were named *HvPR-17a1, HvPR-17c* and *HvPR-17d*. The remaining work conducted in the project was mainly focused on *HvPR-17c* as a representative of the family as it was found in the SSH library.

Database searches using BLASTp (Altschul *et al.* 1997) revealed several HvPR-17-like sequences. Proteins identified in the database having highest sequence similarity to HvPR-17c were WAS-2 from wheat (Accession No. AD46133) and a barley hypothetical protein

(Accession No. CAA74594) with 93% and 84% identity respectively. HvPR-17d was found to be similar to WCI-5 (Accession No. AAC49288) from wheat with 87% identity. However, the function of the proteins is unknown. Therefore, the Conserved Domain Database was searched which identified a domain named plant basic secretory protein (BSP). These proteins (pfam 04450) are believed to be part of the plants' defence mechanism against pathogens.

As there was no experimental data on the function of BSP domain, the occurrence of proteins with a BSP domain across all species was investigated by screening entries in the Genbank non-redundant protein and EST database and the TIGR Transcript Assemblies (Section 2.34). Homologous proteins were identified from plants, bacteria and fungi but no homologue was found from animal sequences. Non-redundant PR17–like proteins found from dicotyledonous species were 19, from monocotyledonous species 18, from gymnosperms 6, from fungi 16 and from bacteria 9 protein sequences. All of these proteins contained a BSP domain. Most of plant and bacterial PR-17 were found to possess a signal peptide, whereas none of fungal proteins was predicted to have a signal peptide. Multiplicity of PR-17 genes was found to be a common feature in plant genomes whereas in all fungal and most bacterial genome this gene exists as a single copy. Isoelectric point of fungal proteins was 5.5-7 with most being in a range of 5-6, bacterial at 4.9-9 and plants 4.5-9.6.

Phylogenetic analysis was conducted to reveal relationship of the proteins among kingdoms and families. All the identified proteins were aligned using the ClustalX program. Phylogenetic analysis was performed on aligned amino acid sequences using the neighbour joining algorithm and bacterial sequences were used to define an out-group. Sequences were found to form six distinct groups (Fig. 3.9) and were named subfamilies A-F. The phylogenetic tree tentatively categorises the plant members into three subfamilies (D-F).

Subfamily A is comprised of bacterial proteins. Subfamily B includes one protein from each of rice, Arabidopsis, *Medicago*, grapevine and *Aspergillus*. All members of this subfamily lack a signal peptide. Subcellular localisation programs predicted plastid localisation for the rice member. Other members were not predicted to localise in any subcellular structures, suggesting that they are most likely cytoplasmic proteins. This

Table 3.2 Analysis of HvPR-17 family members. Signal peptides were predicted using SignalP program. Other data were calculated by Vector NTI V9 program.

Gene	Molecular weight	Isoelectric point	Signal peptide	Number of amino acid residues
HvPR-17a	24909.15	4.8	1-21	229
HvPR-17b	24230.35	8.58	1-22	225
HvPR-17c	24386.83	9.32	1-25	225
HvPR-17d	25487.44	4.7	1-25	232

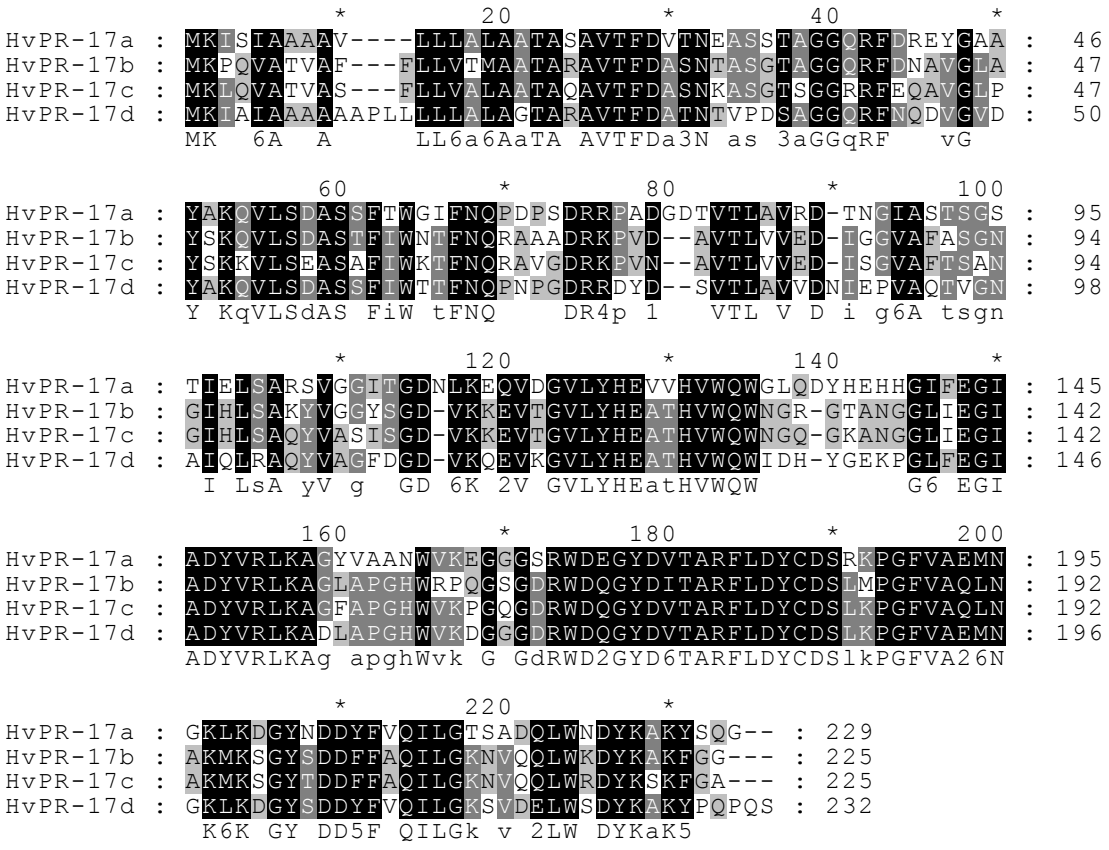


Figure 3.7 Sequence alignment of deduced amino acid sequence of PR-17 family members in barley. Dashes indicate the places in which a sequence has been expanded to allow for optimal sequence alignment. The level of shade indicates the degree of conservation of the residue among sequences. Alignments of the deduced amino acid sequences were conducted using the ClustalX program and displayed by the GeneDoc program.

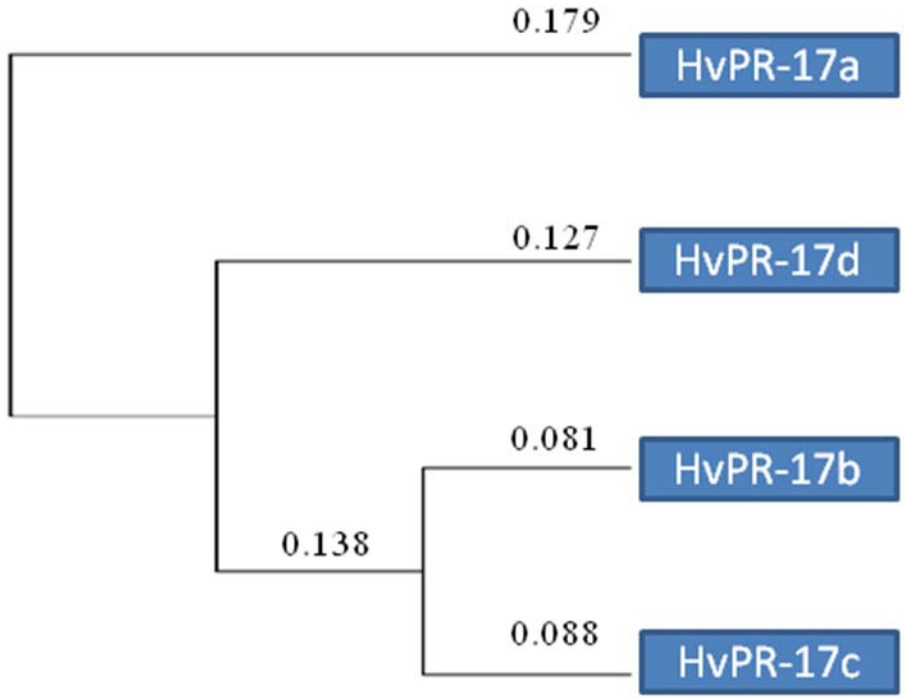


Figure 3.8 Similarity tree of HvPR-17s. The Alignment of sequences conducted using the ClustalX program and the tree displayed by the TreeView program. The numbers on nodes represents branch lengths.

subfamily is composed of sequences representing plants and fungi indicating that this class evolved prior to the divergence of these kingdoms.

Subfamily C all belongs to fungal species of basidiomycetes and ascomycetes and they exist as a single copy gene. N-terminal signal peptides were not predicted for any of them and they appear to lack nuclear localisation signals or transit peptides. They all are predicted from genome sequences.

Subfamily D was found to be composed of sequences belonging to pine, a gymnosperm, with all but one predicted to have a signal peptide.

Subfamily E is from monocotyledonous plants with the majority being secretory. Barley PR-17 protein sequences are related to this subfamily which includes genes from wheat, rice and maize. HvPR-17b and HvPR-17c grouped together based on amino acid sequence similarity, whereas the other two barley members are more similar to wheat proteins.

Subfamily F belongs to dicotyledonous plants and comprises two subgroups. Subgroup FI is composed of four Arabidopsis secretory proteins whereas subgroup FII includes secretory and non-secretory proteins from different species.

3.3.4 Prediction of PR-17c secondary structure and post-translational modifications

The structure and post-translational modification of proteins affects their activity. Various programs at the EXPASY web site (Section 2.32) were used to predict possible post-translational modification of HvPR-17c and the secondary structure of the encoded protein. Among different possible modifications only putative phosphorylation sites for serine 28, 32, 35 and tyrosine 170, 179, 199 of pre-protein and also kinase specific phosphorylation for serine 28 were identified. No phosphorylation site was predicted for threonine residues. The possible secondary structure of the HvPR-17c (Fig. 3.10) was determined using PSIPRED. It shows that the protein mostly will form helices and coil structures. The instability index was computed to be 22.30 that suggest a stable protein.

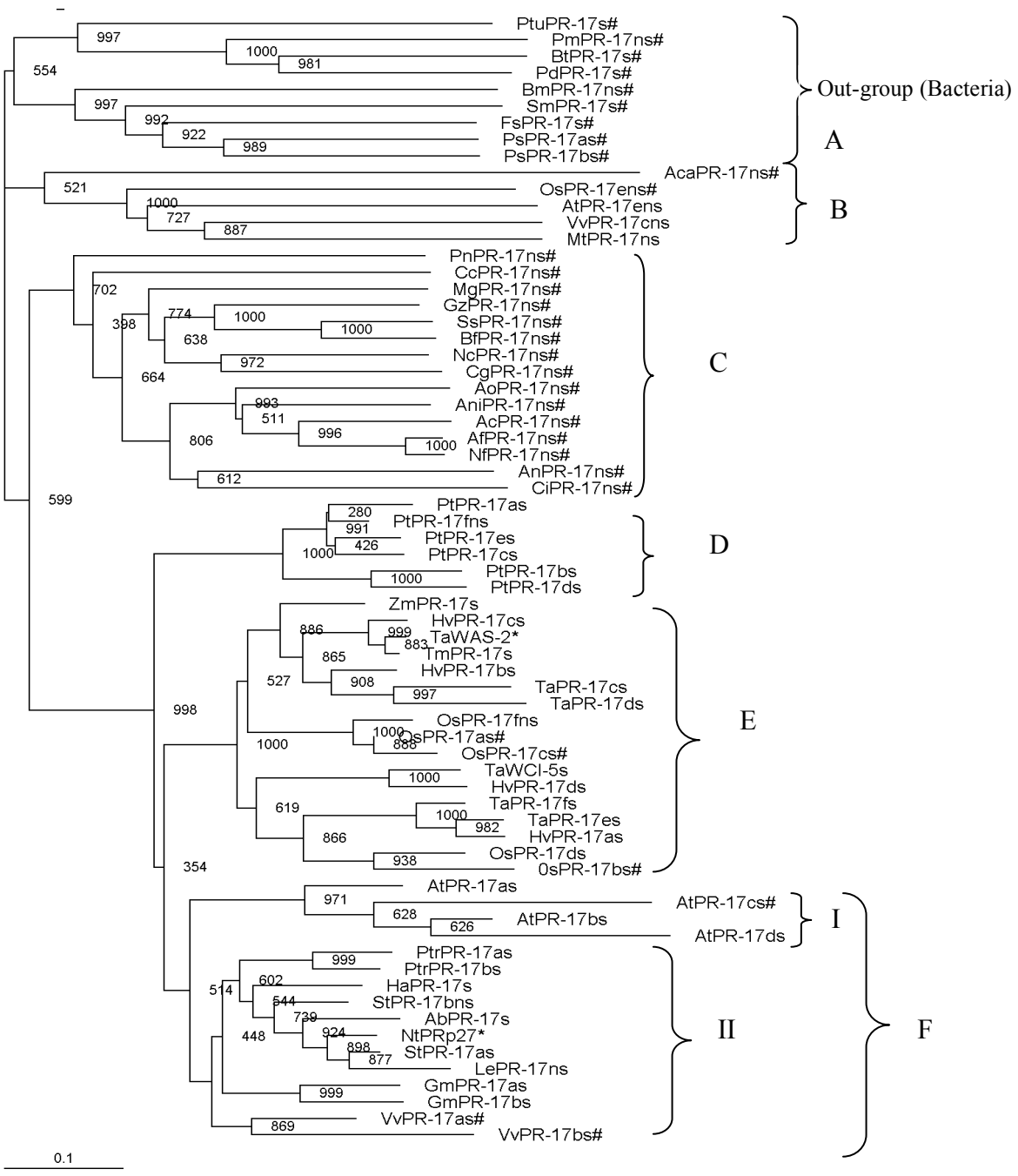


Figure 3.9 Phylogenetic relationships of PR-17 polypeptides from diverse organisms. Other species proteins similar to HvPR-17 were retrieved as described in Section 2.34 and used in a multiple sequence alignment together with barley homologues isolated in this work. The ClustalX program was used to align the retrieved sequences and a phylogenetic tree was created using the neighbour joining method. The TreeView program was used to display the tree. The "s" and "ns" after PR-17 indicates secretory and non-secretory proteins, respectively. Names ending with # and * refers to hypothetical and proteins described in the literature, respectively. Branch length reflects the extent of sequence

divergence. The reliability of the cluster analyses was tested by bootstrap confidence limits and indicated as success per 1000 bootstrap trials as presented on nodes. The abbreviations are followed by the accession number of the sequence or TA number for TIGR database entries: Ab: *Atropa belladonna* (AbPR-17: CAC40754), Ac: *Aspergillus clavatus* (AcPR-17: XP_001268987), Aca: *Ajellomyces capsulatus* (AcaPR-17: XP_001543729), Af: *Aspergillus fumigatus* (AfPR-17: XP_752642), Ani: *Aspergillus niger* (AniPR-17: XP_001392666), An: *Aspergillus nidulans* (AnPR-17: XP_658600), Ao: *Aspergillus oryze* (AoPR-17: BAE60785), At: *Arabidopsis thaliana* (AtPR-17a: NP_565369, AtPR-17b:, NP_179117 AtPR-17c: AAD25574, AtPR-17d: NP_179120, AtPR-17e: NP_181818), Bf: *Botryotinia fuckeliana* (BfPR-17: XP_001551481), Bm: *Blastopirellula marina* (BmPR-17: ZP_01089104), Bt: *Bacteroides thetaiotaomicron* (BtPR-17: NP_812875), Cc: *Coprinopsis cinerea* okayama (CcPR-17: EAU92318), Ci: *Coccidioides immitis* (CiPR-17: XP_001242950), Cg: *Chaetomium globosum* (CgPR-17: XP_001223830), Ha: *Helianthus annuus* (HaPR-17: DY917817), Hv: *Hordeum vulgare* (HvPR-17a: CAA74593, HvPR-17b: CAA74594, HvPR-17c: this study, HvPR-17d: this study), Gm: *Glycine max* (GmPR-17a: TA58392_3847, GmPR-17b: EV267727), Gz: *Gibberella zeae* (GzPR-17: XP_386366), Le: *Solanum lycopersicum* (LePR-17: AAO22065), Mg: *Magnoporta grisa* (MgPR-17: XP_361710), Mt: *Medicago truncatula* (MtPR-17: ABO82201), Nc: *Neurospora crassa* (NcPR-17: XP_962280), Nf: *Neosartorya fischeri* (NfPR-17: XP_001264492), Nt: *Nicotiana tubacum* (NtPRp27: BAA81904), Os: *Oryza sativa* (OsPR-17a: EAY79024, OsPR-17b: EAY79025, OsPR-17c: EAZ16514, OsPR-17d:, AAG13532 OsPR-17e: EAY99186, OsPR-17f: NP_001064925), Pd: *Parabacteroides distasonis* (PdPR-17: YP_001304711), Pm: *Parabacteroides merdae* (PmPR-17: ZP_02033826), Pn: *Phaeosphaeria nodorum* (PnPR-17: EAT86970), Ps: *Pedobacter spp* (PsPR-17a: ZP_01883454, PsPR-17b: ZP_01886653), Pt: *Pinus taeda* (PtPR-17a: CO164243, PtPR-17b: CF390465, PtPR-17c: DR093655, PtPR-17d: CO159008, PtPR-17e: DR163772, PtPR-17f: CO158229), Ptr: *Populus trichocarpa* (PtrPR-17a: TA1427_153471, PtrPR-17b: CK113943), Ptu: *Pseudoalteromonas tunicate* (PtuPR-17: ZP_01131968), Sm: *Stenotrophomonas maltophilia* (SmPR-17: ZP_01644420), Ss: *Sclerotinia sclerotiorum* (SsPR-17: EDN96401), St: *Solanum tuberosum* (StPR-17a: AAO22065, StPR-17b: CK265676), Ta: *Triticum aestivum* (TaWAS-2: AAD46133, TaWCI-5: AAC49288, TaPR-17c: CK214216, TaPR-17d: CV760462, TaPR-17e: CV769412, TaPR-17f: TA65180_4565), Tm: *Triticum monococcum* (TmPR-17: TA2167-4568), Vv: *Vitis vinifera* (VvPR-17a: CAN65930, VvPR-17b: CAN65929, VvPR-17c: EC996050), Zm: *Zea mays* (ZmPR-17:TA174521_4577).

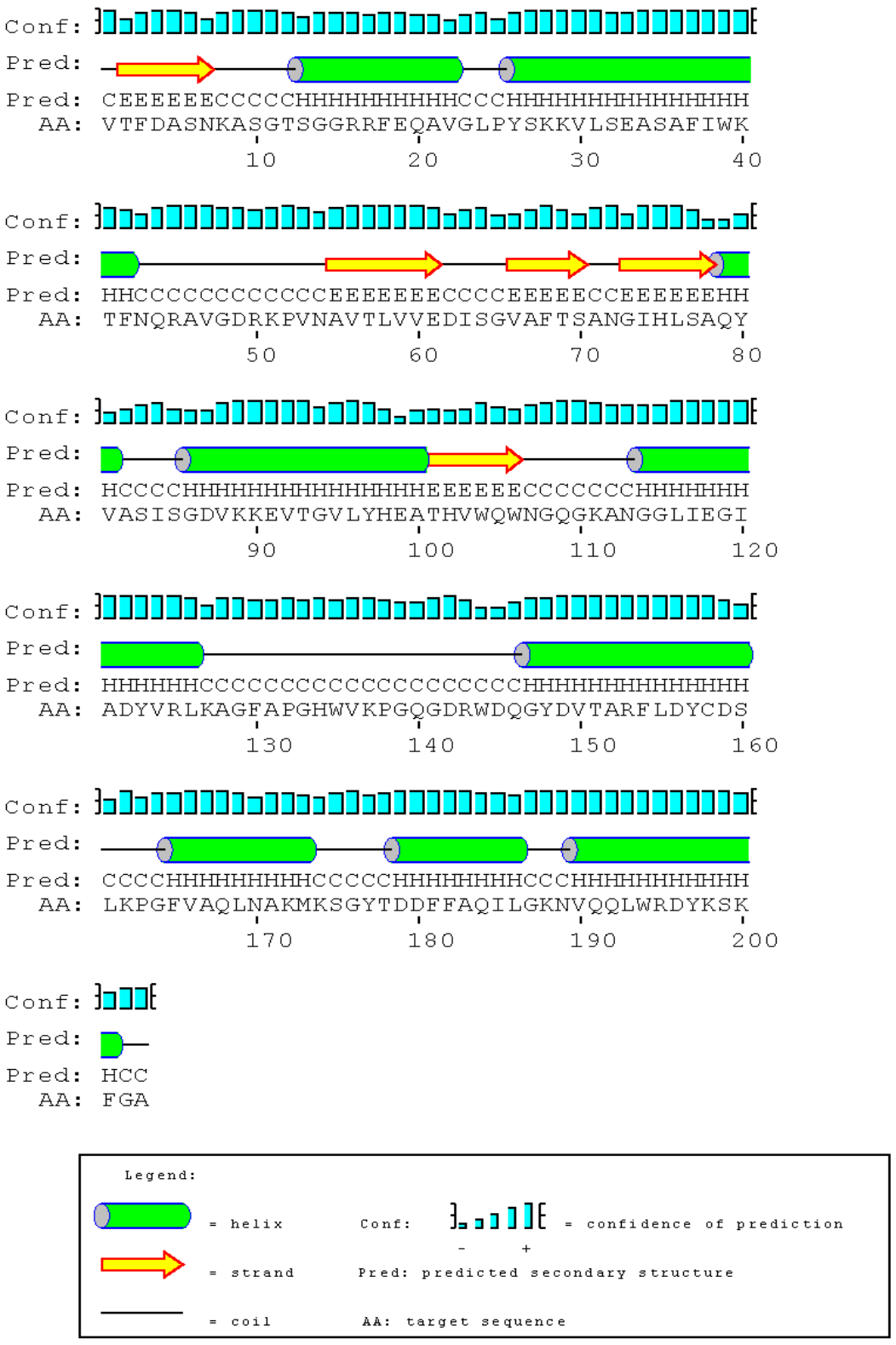


Figure 3.10 Secondary structure of HvPR-17c predicted by PSIPRED. The prediction schematically depicts the helix, β strand and coil structures. The numbers indicate the amino acids in the primary structure of the protein.

3.3.5 DNA gel blot analysis

To investigate the genomic organisation of *HvPR-17* family genes a probe template from the 3' region (3' probe) and a template from full-length sequence was used for labelling. Visualisation of the PCR product in an agarose gel revealed bands of expected sizes of 292 bp and 754 bp (Fig. 3.11). The locations of primers have been shown on *HvPR-17c* sequence in Figure 3.12. DNA gel blot analysis using the 3' probe (Fig. 3.13B) indicated hybridisation to one band whereas the full-length probe (Fig. 3.13C) hybridised to up to five bands. Also shown here is the hybridisation pattern with the 3' probe for EcoRV digested DNA gel blot (Fig 3.13D). This hybridisation pattern indicates the specificity of the 3' probe for *PR-17c* and confirms the existence of up to five family members.

3.3.6 Analysis of *PR-17* expression under biotic stresses

Generally, expression of genes is assumed to be associated with a function under stress condition. Northern blot hybridisations, Q-PCR and publically available microarray data were used to study the spatial and temporal expression of *HvPR-17* transcripts in leaf tissues under biotic stresses. Temporal expression patterns of *PR-17c* after mock and necrotrophic pathogen (*R. secalis*) inoculation in leaves of near-isogenic barley cultivars Atlas and Atlas 46 was studied by Northern blot analysis with the gene specific 3' probe. The RNA gel blot analysis showed that the gene is induced transiently at 6-24 hours post-inoculation in both susceptible (Atlas) and resistant (Atlas 46) cultivars (Fig. 3.14). To investigate the transcript level in more detail and past 24 hours another experiment was carried out with more time points (Fig. 3.15). These two hybridisations collectively showed that induction of mRNA is temporally regulated between 6-24 h after inoculation and reached undetectable levels after 48 h. In this experiment, RNA was also extracted from leaf 24 hours after wounding. The blot detected slight induction by wounding in Atlas 46. To get an insight of the transcript level of *PR-17c* in epidermal tissue of barley leaf, the abundance of barley *PR-17c* transcript in response to inoculation with *R. secalis* was examined via Q-PCR (Fig. 3.16). A low level of transcript was detected before inoculation. In both cultivars, the gene was induced by pathogen presence but it accumulated differentially in resistant and susceptible cultivars. The transcript accumulated sharply and reached its peak in Atlas 46 earlier than Atlas at 6 h post-

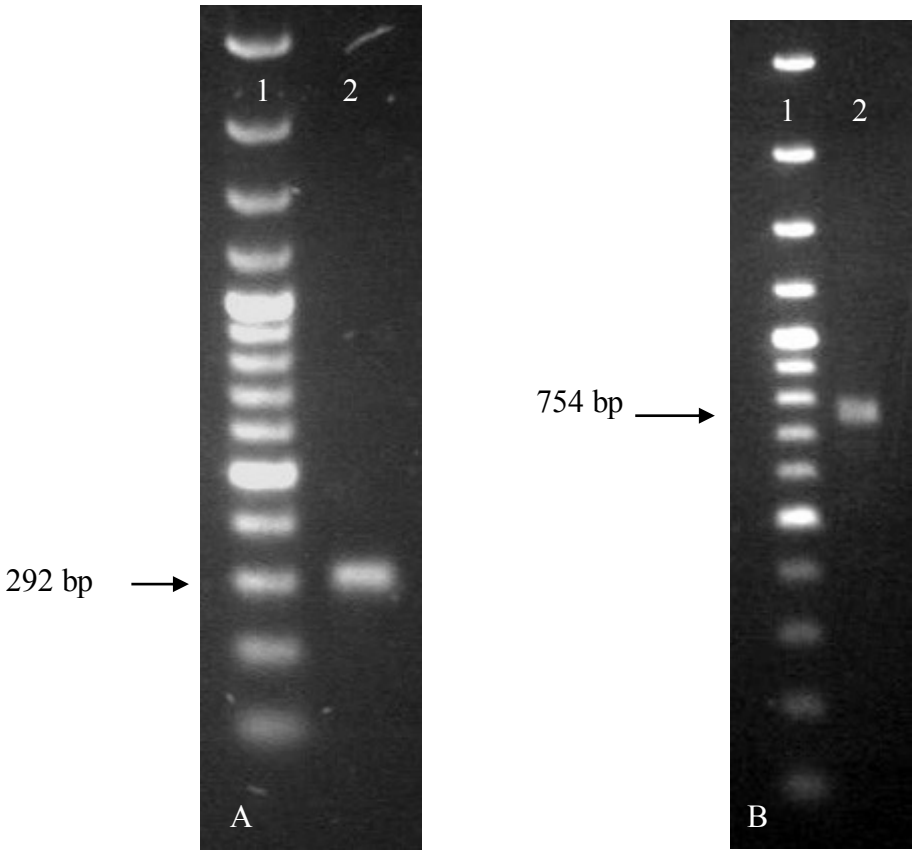


Figure 3.11 *HvPR-17c* amplified probe templates. (A) 3' probe amplified by primer pairs s134F5/R3, and (B) full-length probe amplified by primers s134F3/R3. Lane 1: DNA marker, Lane 2: PCR product.

Figure 3.12 Full-length sequence of *HvPR-17c* cDNA. The primers that were used for amplification of probes' template are shown in bold and arrows indicate their orientation. The sequence region used for RNAi construct is underlined.

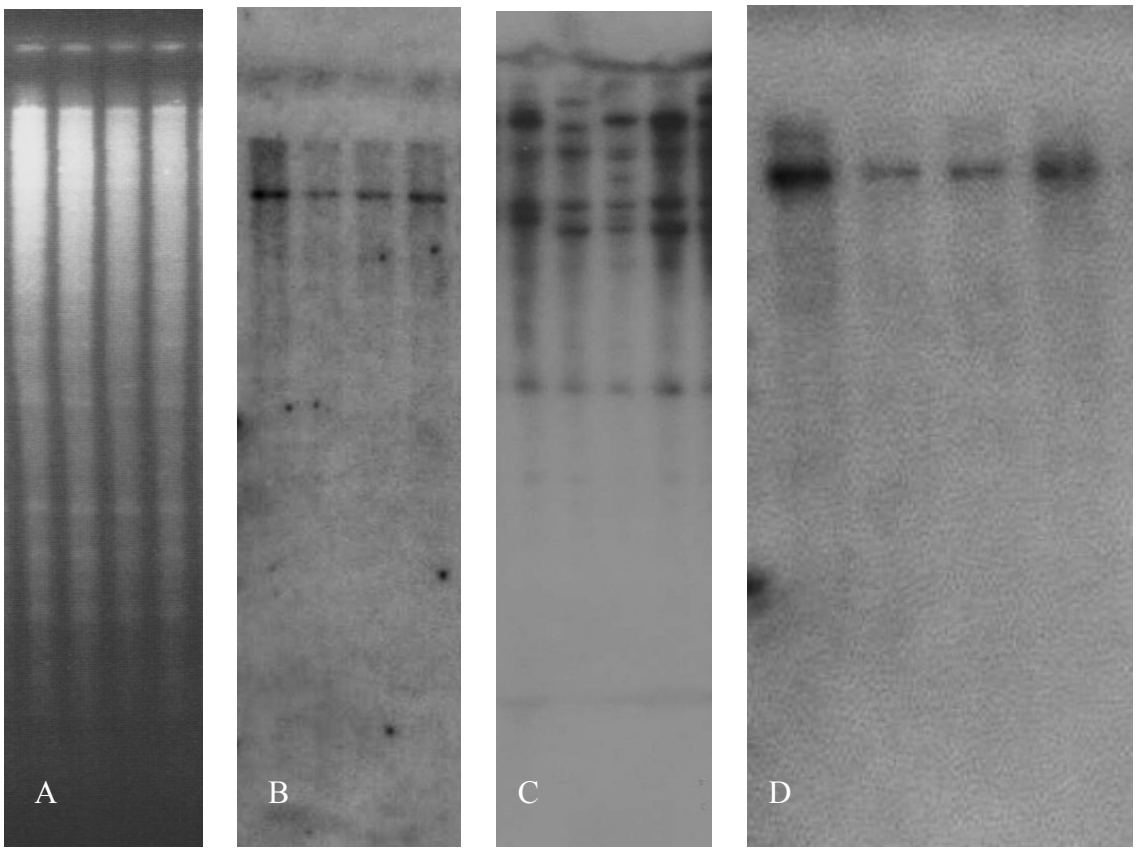


Figure 3.13 Southern blot analysis of barley cultivars with *HvPR-17c* probes. (A) Gel fractionated genomic DNA digested with HindIII restriction enzyme stained with etidium bromide. (B) Filter probed with the 3' probe and (C) full-length probe. (D) 3' probe hybridised to EcoRV digested DNA. Cultivars from left to right: Golden Promise, Atlas, Sahara and Atlas 46.

inoculation. In Atlas after initial induction, there was a lag phase before an increase in mRNA levels occurred. The mock-inoculated plants showed low level of transcript in comparison with inoculated plants at 24 h post-treatment.

To investigate expression of other members and wether *HvPR-17c* is induced by other pathogens, publicly available data from hybridisations of the 22K barley1 GeneChip (Section 2.33) under other pathological conditions were interrogated for expression levels of *PR-17* family members. Barley *PR-17a*, *PR-17b*, *PR-17c* and *PR-17d* are represented by probe sets 634_at, 590_s_at, 358_at, and HW03022_u_at on barley1 chip, respectively. Two data sets represent gene expression levels in barley interacting with the powdery mildew fungus (*Blumeria graminis* f. sp *hordei, Bgh*). Probe set intensities representing barley PR-17 family members in the interaction of barley line CI16151 with *Bgh* 5874 (incompatible) and *Bgh* k1 (compatible) are shown in Figure 3.17. Expression data in *Mlo* (compatible) and *mlo5* (incompatible) interactions are presented in Figure 3.18. Among *PR-17* transcripts in both experiments, the *PR-17a* has the highest level of expression, in contrast *PR-17d* with the lowest level. The expression levels in the compatible interaction follow a similar trend as in the incompatible interaction, but with a lower intensity during the compatible interaction. Examination of the pathogen and mock (water) inoculated spikes of the barley cultivar Morex from 0 to 144 hours after inoculation with *Fusarium graminearum* Schwabe a semi-necrotrophic pathogen exhibited an increase in transcript number in response to pathogen with *PR-17a* having highest and *PR-17c* lowest probe intensities, respectively (Fig. 3.19).

3.3.7 Expression analysis under abiotic stresses and development

Induction of some PR proteins under abiotic stresses and during development has been previously shown. To investigate whether the induction of PR-17 is specific to defence responses, the kinetics of *HvPR-17c* expression during frost, salinity and drought was studied. Before cold treatment, varying levels of transcript was measured in different cultivars (data not shown). When the expression level of the gene in frost-treated plants were compared to controls at least in one time point there was higher level of transcript in cold treated plants. The most pronounced response was detected in cultivar Galleon at frost -5.5ºC showing 6.5 fold increase in transcript (Fig. 3.20).

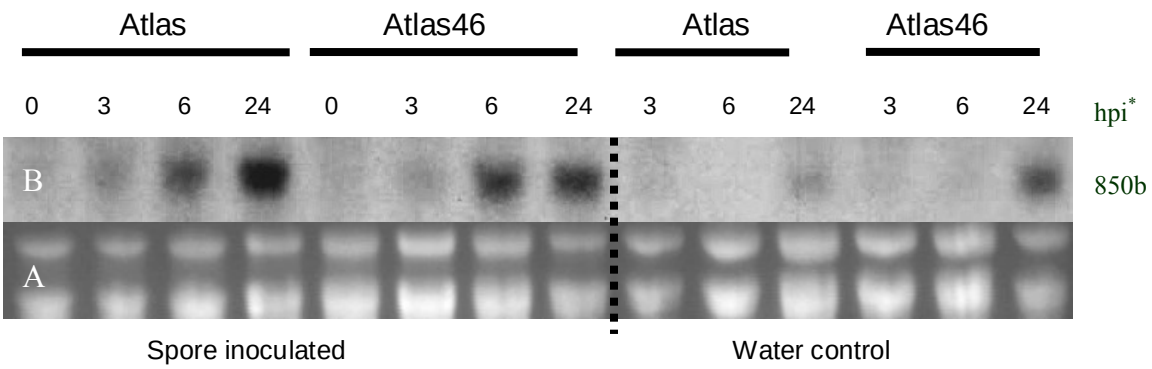


Figure 3.14 Temporal expression levels of *HvPR-17c* in barley plants inoculated with *R. secalis*. (A) Denaturing agarose gel of total RNA (20 µg/lane) isolated from the leaves of barley (susceptible cultivar Atlas and resistant cultivar Atlas 46) at different times after spore and mock inoculations. (B) Northern blot with *HvPR-17c* 3' probe. Ribosomal 18S and 28S RNA were used as loading control in the gel. * Hours post-inoculation.

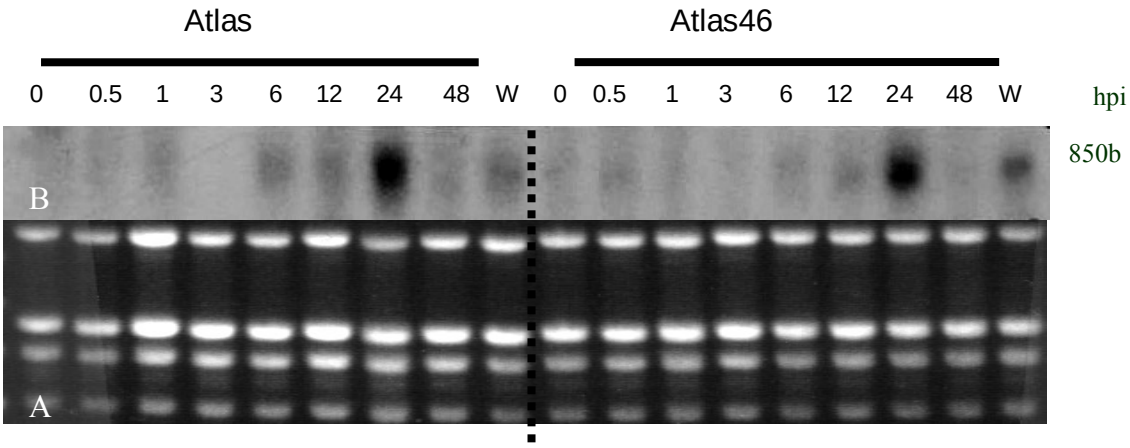


Figure 3.15 Temporal expression levels of *HvPR-17c* in *R. secalis* inoculated and wounded barley leaf was studied by hybridizing the 3' probe to total RNA extracted from susceptible cultivar Atlas and resistant cultivar Atlas 46 tissue at different times after spore inoculations and wounding. (A) Denaturing agarose gel of total RNA (15 µg/lane). (B) Northern blot with *HvPR-17c* 3' probe. W: wounded leaf sample. The sample for wounding was taking 24 hours after wounding. Ribosomal 18S and 28S RNA were used as loading control in the gel.

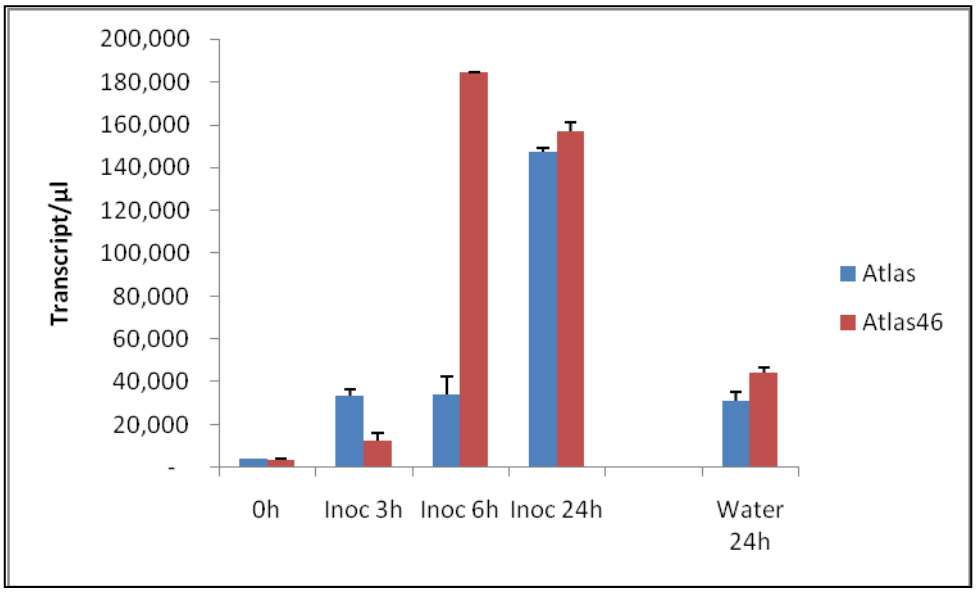


Figure 3.16 Normalised expression levels of *HvPR-17c* in barley leaf epidermis. Levels of mRNA are presented as the number of copies per micro litre of cDNA after normalisation. Error bars show standard deviations of four replicates for each mRNA. 0h: before inoculation, Inoc 3h, Inoc 6h, Inoc 24h, represents expression level 3, 6 and 24 hours post inoculation respectively, Water 24h: mock-inoculated plants 24 hours later. Data were normalised against *Cyclophilin*, *α-Tubulin* and *Heat Shock Protein 70* mRNA levels.

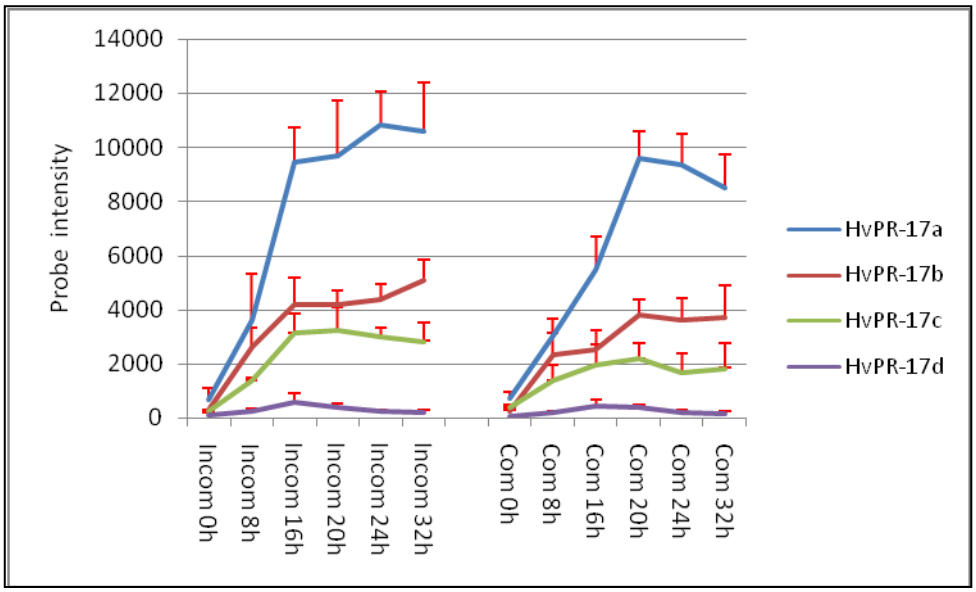


Figure 3.17 Expression of *HvPR-17* members in response to *Blumeria graminis* f. sp *hordei* (*Bgh*). Normalised average probe set intensity levels of *HvPR-17* family members in the incompatible interaction (Incom) between cultivar CI16151 (*Mla6*) and *Bgh* 5874 (*AvrMla6*) and compatible interaction (Com) of the same barley cultivar with *Bgh* K1 (*AvrMla13*). Probe intensities were RMA normalised averages of three biological replicates. Error bars represent standard deviations. Data were extracted from BB4 database available in BarleyBase web site.

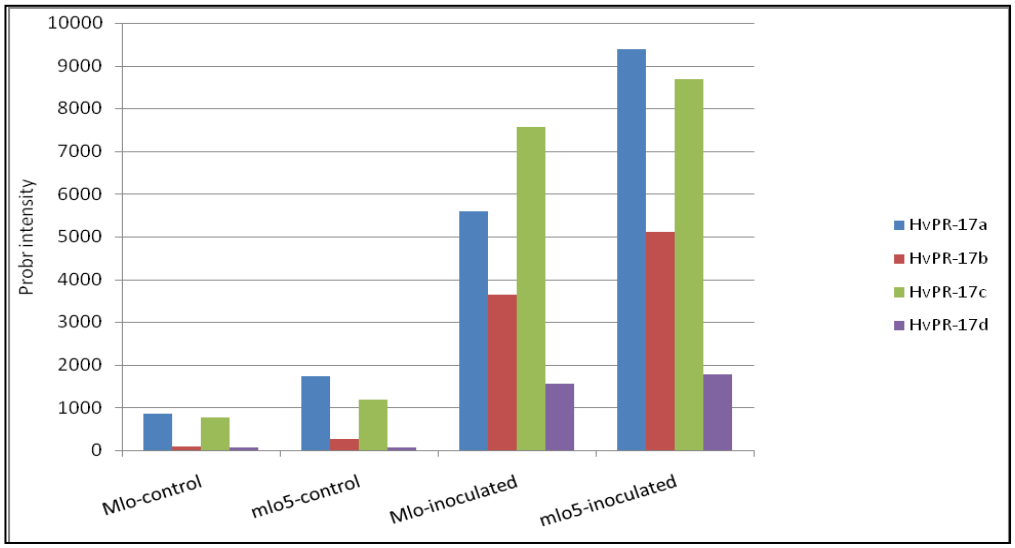


Figure 3.18 Expression of *HvPR-17* members in cultivars containing different *Mlo* alleles in response to *Blumeria graminis* f. sp *hordei* (*Bgh*). Normalised probe set intensity levels of *HvPR-17* family members in the interaction between cultivars with *Mlo* (susceptible) and *mlo5* (resistant) alleles inoculated with *Bgh*. Probe intensities were RMA normalised. The control plants were inoculated with water. Data were extracted from BB7 database available in BarleyBase web site.

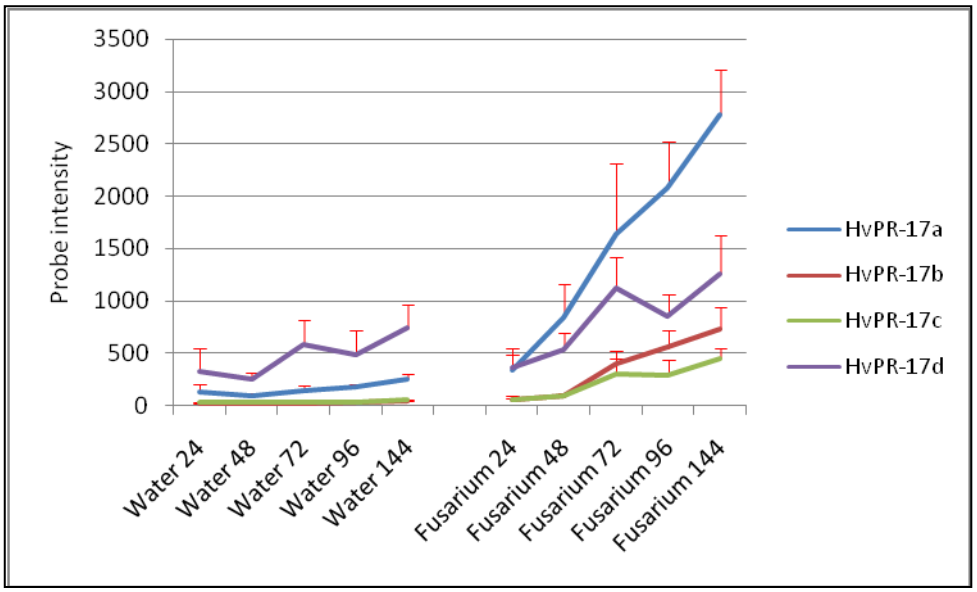


Figure 3.19 Expression of *HvPR-17* members in response to *Fusarium graminearum*. Normalised average probe set intensity levels of *HvPR-17* family members in the interaction between cultivar Morex and *F. graminearum*. Probe set intensities were RMA normalised averages of four independent replications of the experiment and a fifth replication at 24 and 72 h after inoculation. Error bars represent standard deviations. Data were extracted from BB9 database available in BarleyBase web site.

Salt treatment effect on expression of the gene was dependent on cultivars. The overall transcript level was very low in all cultivars. The level of *PR-17c* in salt-treated Yu-6472 and CM72 was slightly higher than control plants. But WI2291 responded to salt treatment by down-regulating the expression level (Fig. 3.21).

Plants exposed to drought showed different levels of tolerance. At the time of harvest, drought-treated plants were at three-leaf stage and no tillering was observed, whereas the control plans were at more advanced stage of growth with numerous tillers. Haruna Nijo was the first cultivar to wilt at 20 days followed by Barque73 at 22 days and Golden Promise 29 days after planting. The response of *HvPR-17c* to drought was a reduction in transcript levels. Although mRNA levels were different among cultivars, the level of transcript for each cultivar was higher in control plants in comparison with drought-treated plants at any time point. Interestingly, 24 h after rewatering treated plants the transcript levels did not show a large change (Fig. 3.22).

Expression of the genes during barley development showed that *PR-17* genes were upregulated during seed germination in embryo with *PR-17a* having the highest expression. A secondary peak of smaller magnitude was also observed in seedling crowns for three of the genes (Fig. 3.23). In some stages tissue specific expression of members were observed. For instance, *PR-17d* was upregulated in bracts and caryopsis 12-16 days post pollination in comparison to other members.

3.3.8 Bioassay

Antifungal activity of PR proteins can be demonstrated *in vitro* by bioassay using heterologously expressed protein. The barley *PR-17c* cDNA (Fig 3.12) fragments were amplified (Fig. 3.24) to express N- and C-terminally tagged proteins in *E. coli*. HvPR-17c protein containing C-terminal His-tag was expressed with pQE70. The solubility test showed that the majority of the protein was present in the soluble fraction. Therefore, expressed protein was purified under native conditions. SDS-PAGE of various fractions of the protein extract showed a single band at a position consistent with the predicted mass of 23.5 KDa (Fig. 3.25). The presence of the recombinant protein was also verified by immunodetection in a Western blot using an anti-his antibody (Fig. 3.26).

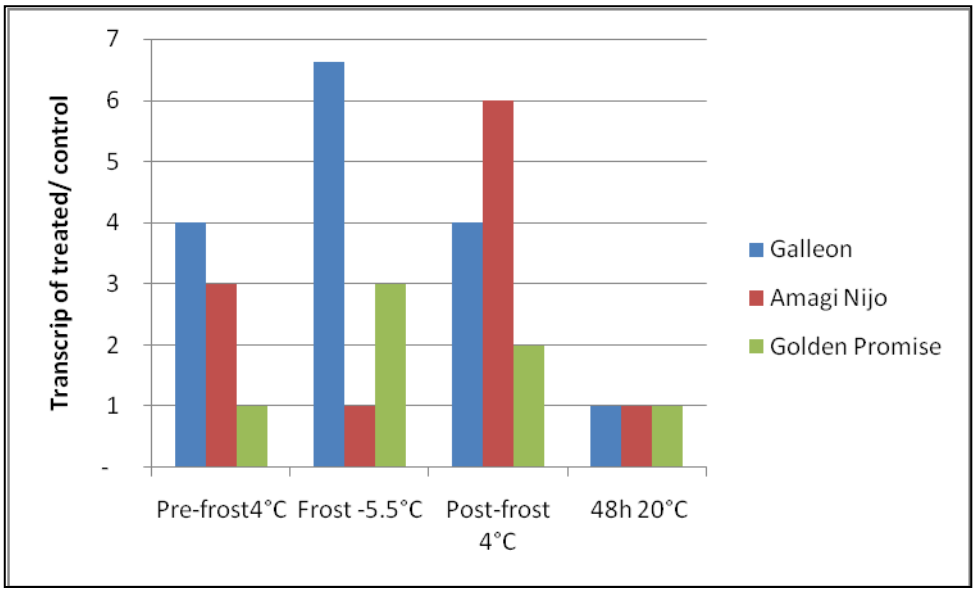


Figure 3.20 Q-PCR analysis of the effect of frost treatment on *HvPR-17c* expression. The transcript levels at leaves of different barley cultivars at different times during frost treatment and control plants kept at 17ºC. Expression levels were calculated for copy number/µl of cDNA. Fold change in expression levels of each cultivar in treated plants in comparison to control is shown. Data were normalised against *GAPDH*, *Cyclophilin* and *α-Tubulin* levels and are mean values of triplicates in the PCR.

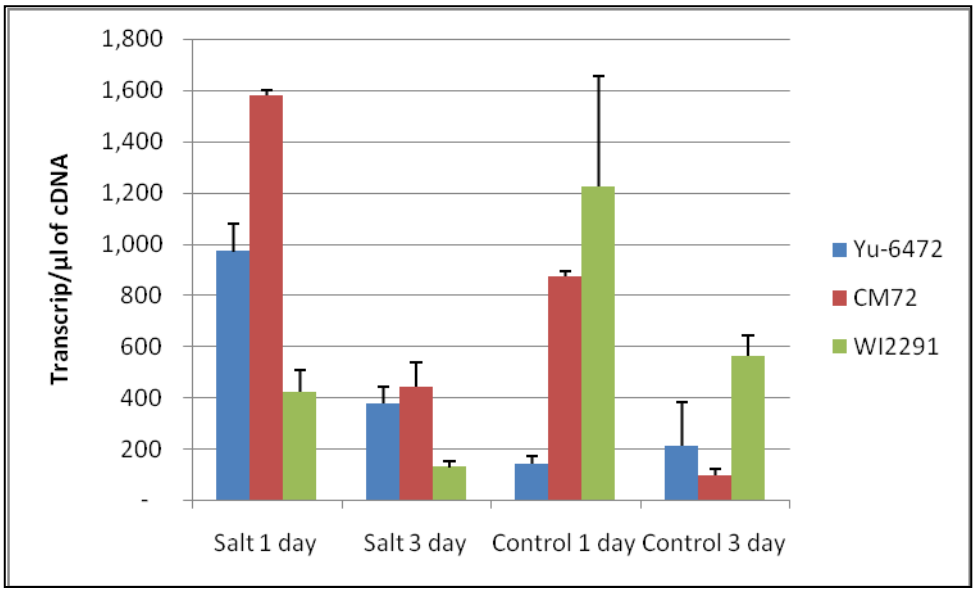


Figure 3.21 Quantitative RT-PCR analysis of the *HvPR-17c* in salt-treated barley cultivars. Two sets of plants were grown under the same condition in a hydroponic system until emergence of the second leaf. For one set salt was added to a final concentration of 150 mM, 50 mM at 12 hours interval. Leaf samples were harvested one and three days after last salt addition. Data were normalised against *Cyclophilin*, *α-Tubulin* and *GAPDH* mRNA levels. Data are mean values of triplicates in the PCR including standard deviations.

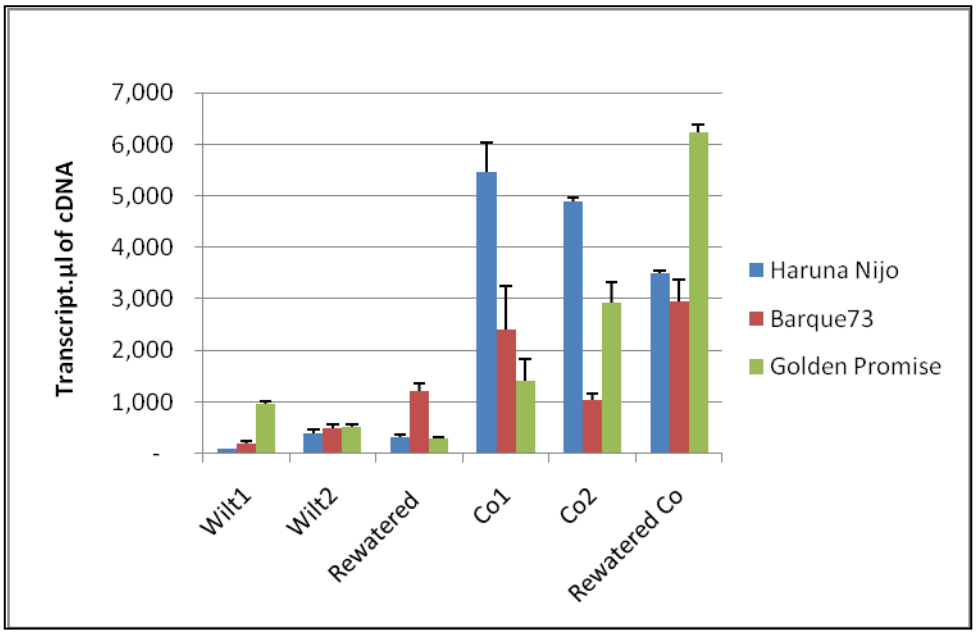


Figure 3.22 Q-PCR analysis of response of barley *PR-17c* to drought in different barley cultivars. Two sets of plants were grown under the same condition in a growth cabinet. Wilt1: wilting of leaves was observed, Wilt2: sampling 24 hours after Wilt1, Rewatered: plants were watered to saturation and sample taken 24 hours later, Co1, Co2 and Rewatered Co represent non-treated controls from corresponding stages of treated-plants. Data were normalised against *Cyclophilin*, *GAPDH* and *Heat Shock Protein 70* mRNA levels. Data are mean values of triplicates in the PCR including standard deviations.

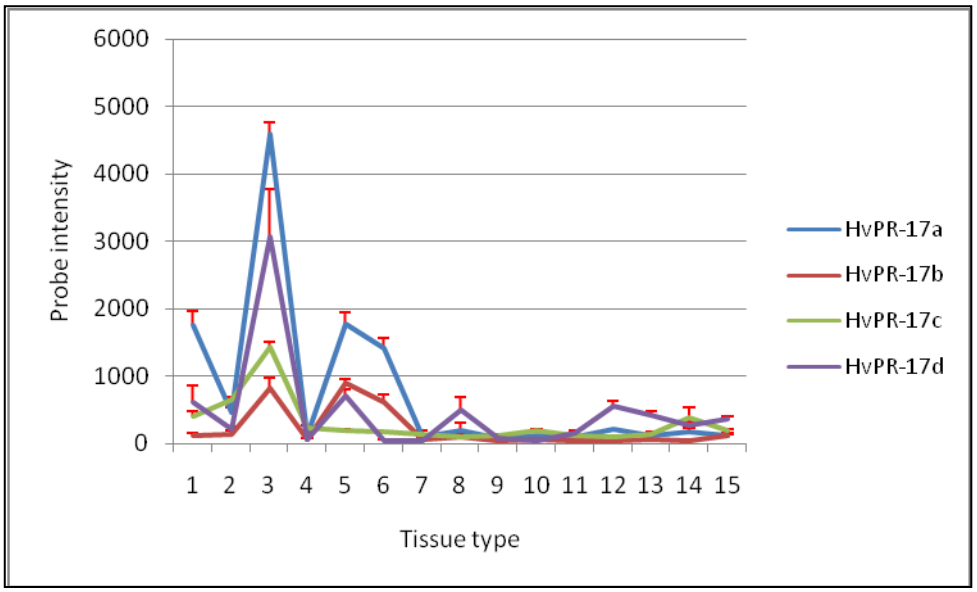


Figure 3.23 Expression of *HvPR-17* members during barley development. Normalised average probe set intensity levels of *HvPR-17* family members during development in Morex. Probe set intensities were RMA normalised average of three biological replicates. The numbers in X-axis represent following tissue types: 1: coleoptyle, 2: radical, 3: embryo during germination, 4: seedling root, 5: seedling crown, 6: leaf, 7: immature inflorescence, 8: bracts, 9: pistil, 10: anthers, 11: caryopsis 5 day after pollination (DAP), 12: caryopsis 10 DAP, 13: caryopsis 16 DAP, 14: embryo 22 DAP, 15: endosperm 22 DAP. Error bars represent standard deviations. Data were extracted from BB3 database available in BarleyBase web site.

To conduct the bioassay the purified protein was concentrated and desalted using ultra filtration (Fig. 3.27). The protein denatured at pH 4.5. Other protein solutions at different pH and with or without addition of DDT were used to study their effect on *R. secalis* spores. Microscopic examination of bioassay mix was performed daily up to one week and no morphological difference was observed between treated and control samples (Data not shown). The disc assay was used to study a potential anti-fungal effect of the protein on *P. teres* culture. Similarly, no inhibition zone appeared around discs treated with protein after one week of incubation at any of the pH and concentrations examined, but disc treated with Mancozeb (a fungicide) as a positive control clearly inhibited growth of the fungus (Fig. 3.28).

3.3.9 Subcellular localisation

To confirm *in silico* apoplastic predictions for the localisation of PR-17 family proteins, GFP fusion was employed. The *HvPR-17cORFfu* fragment was amplified (Fig. 3.29) to construct the pHvPR-17c:GFP vector. Targeting of the PR-17c:GFP fusion protein was observed in multiple independent bombardment experiments. When optical sections taken from a cell expressing HvPR-17c:GFP were combined, secretion of this extracellular peptide via the endoplasmic reticulum secretory pathway was observed. GFP fluorescence was visible in the endoplasmic reticulum, in the Golgi apparatus, and within secretory vesicles (Fig 3.30A). Moreover, analysis of single optical sections and pictures taken with UV microscope demonstrated that HvPR-17c:GFP was primarily localised to the cell periphery and individual secretory vesicles were visible (Fig. 3.30B and C).

3.3.10 Generation of transgenic lines

The bioassay did not reveal antifungal properties toward tested pathogens. Many factors can affect the activity of heterologously expressed proteins *in vitro*. Therefore, the possible *in vivo* role of PR-17 protein in defence was examined by producing and characterising over-expression and knockdown transgenic lines. The coding region of *HvPR-17c* was amplified (Fig. 3.31) for constructing over-expression (pHvPR-17cSE) and antisense (pHVPR-17cAS) vectors. Antisense and RNAi approach was used to generate knockdown transgenic lines. To construct the RNAi vector (pHvPR-17cRNAi) a sense

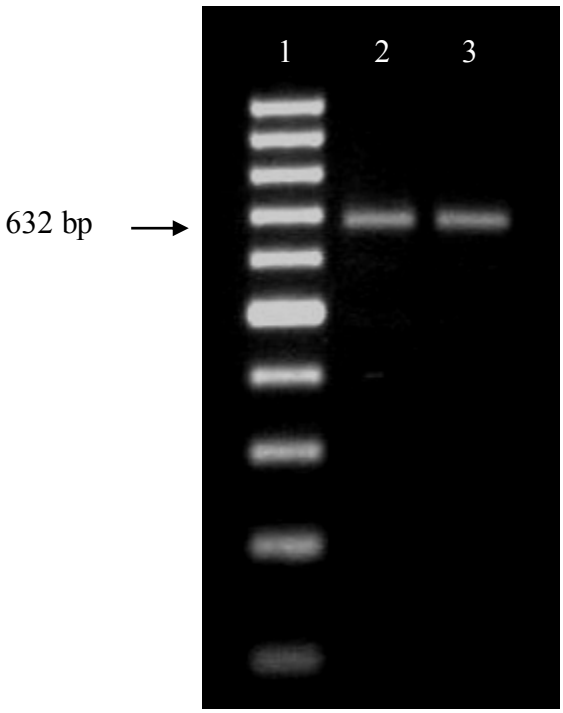


Figure 3.24 Amplification of *PR-17c* fragments for protein expression. The barley *PR-17c* cDNA fragments were amplified and gel-purified for sub-cloning into expression vectors to express N- and C-terminal tagged proteins. Lane1: 100 bp DNA ladder, Lane 2: fragment for sub-cloning into pQE30 using primer set s134F13/R13, Lane 3: fragment for sub-cloning into pQE70 using primer set s134F14/R14.

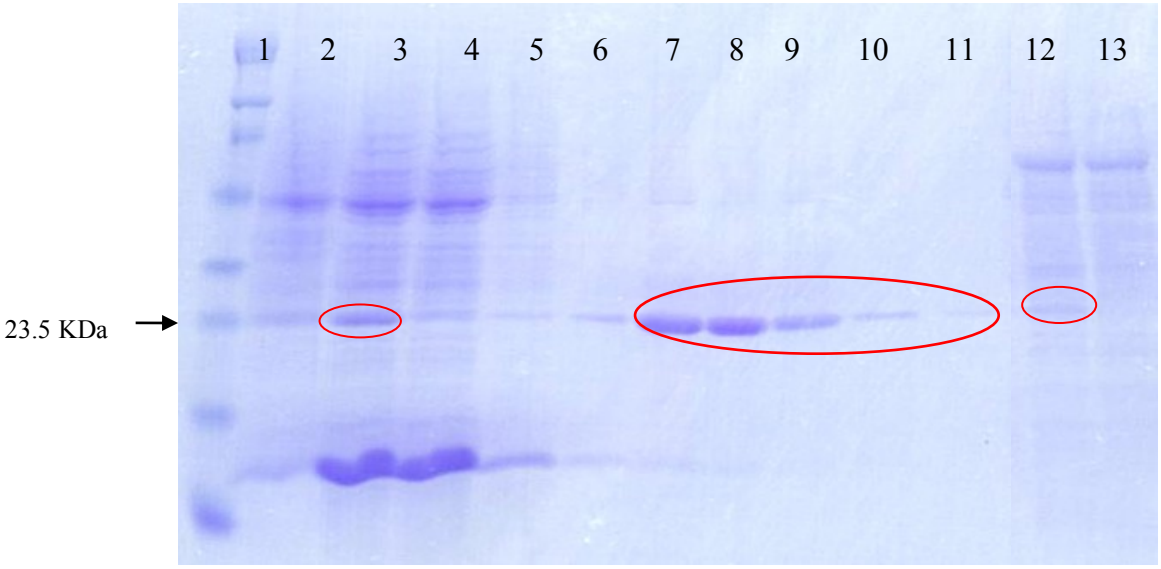


Figure 3.25 SDS-PAGE analyses of proteins at different stages of purification of HvPR-17c. The protein was expressed in the *E. coli* M15 strain using the QIAexpress system. Proteins were separated on a 14% SDS–PAGE and stained with comassie blue R-250. Lane 1: molecular weight protein marker, Lane 2: insoluble fraction of cell lysate, Lane 3: soluble fraction of cell lysate, Lane 4: flow through, Lanes 5-6: first and second wash, Lanes 7-11: HvPR-17c eluted fractions from His-select agarose solution, Lane 12: induced culture cell lysate, Lane13: un-induced culture cell lysate. The recombinant protein band is marked by red eclipses.

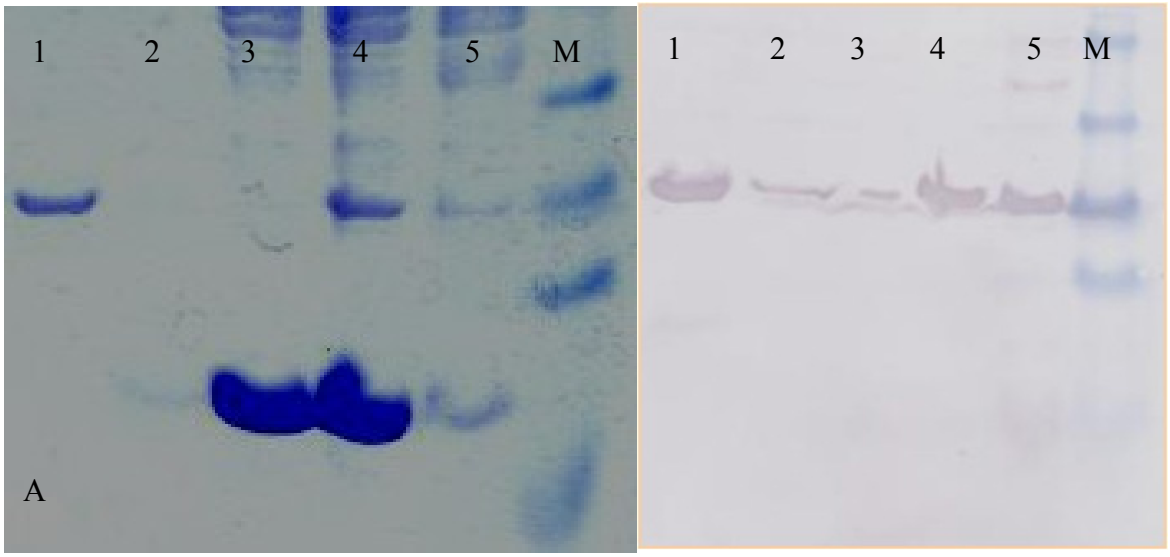


Figure 3.26 Western blot analyses of different protein fractions and purified HvPR-17c protein with anti-his antibody. (A) Duplicate gel stained with comassie blue. (B) Western blot. Lane 1: purified protein, Lane 2: first wash, Lane 3: flow through, Lane 4: soluble cell lysate, Lane 5: non-soluble cell lysate, M: molecular weight marker.

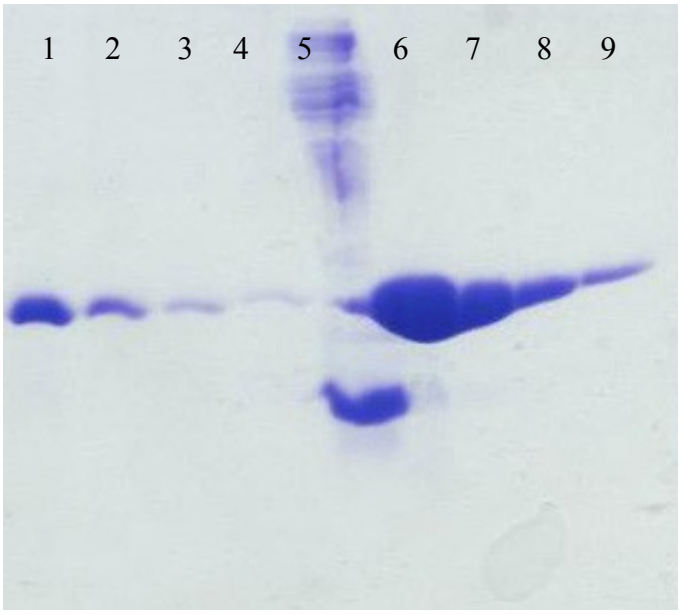


Figure 3.27 SDS-PAGE gel of the purified and concentrated HvPR-17c protein after buffer change. Lanes 1-4: elution 1-4 before concentration, Lane 5: molecular mass marker, Lanes 6-9: eluted samples of 1-4 after concentration.

Figure 3.28 Disk assay to investigate recombinant HvPR-17c antifungal properties. *Pyrenophora teres* was grown on a potato dextrose agar by cutting a small disc from a culture and locating it in the centre of a fresh plate. After 2 days, discs were placed around the parameter of the growth front and were soaked with 10 µl of protein solution. The growth was assessed daily up to one week. Disc 1: positive control (Mancozeb 0.01%), Disc 2-5: protein in pH 5.8, 6.5, 7.5 and 8 solution (2 mg/ml), respectively.

and an antisense fragment of a 3' region (Fig 3.12) was amplified from *HvPR-17c* using two separate primer combinations (Fig. 3.32). The constructs were stably transformed into barley. A total of six over-expression, eight antisense and seven RNAi lines were regenerated. The presence of the transgene was verified by PCR amplifying the hygromycin resistance gene in all lines (Fig. 3.33). In over-expression and antisense lines transgene presence was verified, in addition, by a primer pair consisting of a gene specific and a vector-anchored primer (Fig. 3.34).

3.3.11 Expression and phenotypic analysis of transgenic lines

Analysis of the *PR-17c* transcripts in transgenic lines using Q-PCR showed various levels of transcripts in comparison with the wild type plant (Fig. 3.35).

For disease resistance analysis eight plants from each line were evaluated for scald symptoms (Fig. 3.36). The t-test revealed significant reduction in symptoms in comparison to non-transgenic progeny in antisense line 57-5. The symptom score average was 1 for this line while the non-transgenic progeny score was two. The following developmental phenotypes were also observed:

1. Variegated leaves (Fig. 3.37) were observed in the progeny of over-expression lines 56-3 in a ratio of 2:9 and 56-4 in 2:5 ratios. These plants stayed variegated and produced some albino leaves. No seed set was seen in these plants.
2. Albino plants (Fig. 3.38) grew from RNAi line 57-4 progeny in a 1:2 ratio.

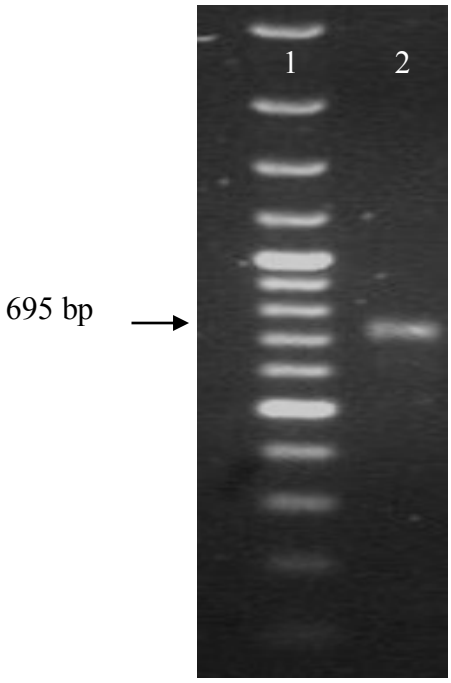


Figure 3.29 Amplification of the barley *PR-17cORFFu* fragment. PCR fragment was amplified using primer set s134F7/R12. Lane 1: 100 bp DNA ladder, Lane 2: PCR product.

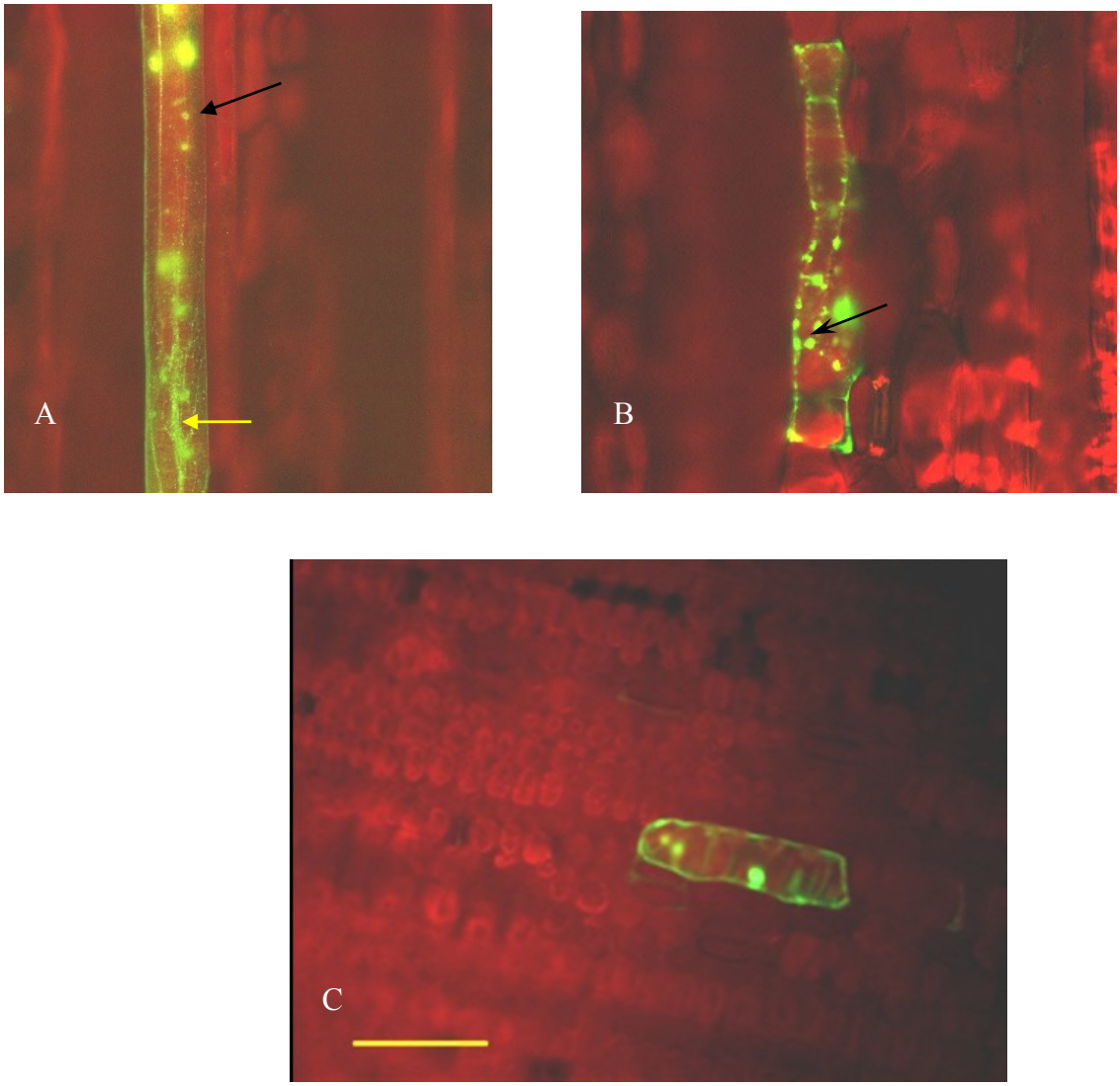
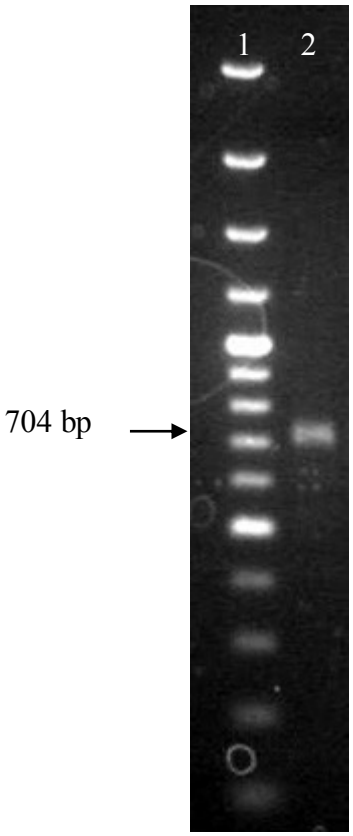


Figure 3.30 Barley epidermal cells bombarded with pHvPR-17c:GFP construct. (A) Combined images of a cell expressing the fusion protein taken by confocal laser scanning microscope show extracellular and ER localisation of the protein, (B) an image of another cell under CLSM shows individual secretory vesicles. (C) A cell expressing fusion protein under fluorescence microscope. Yellow arrow points toward endoplasmic reticulum in A and black arrow to secretory vesicles in A and B. Bar= 100 µm.

(A)



(B)

AATTAT**GGATCC**ACCATGAAGCTTCAGGTAGCCACGGTCGCCTCCTTCCTCCTGGTGGCCTTGGCCGCGACG
GCCCAGGCAGTGACGTTCGACGCGTCGAACAAGGCGTCGGGCACCTCCGGCGGCCGGCGGTTCGAGCAGGCC
GTCGGCCTCCCATACTCCAAGAAGGTCCTCTCCGAGGCCTCCGCCTTCATCTGGAAAACCTTCAACCAGCGT
GCCGTCGGCGACCGCAAGCCTGTCAACGCAGTCACCCTCGTCGTCGAGGACATCAGCGGCGTCGCCTTCACC
AGCGCCAACGGCATCCACCTCAGCGCCCAGTACGTCGCCAGCATCTCCGGCGACGTCAAGAAGGAGGTGACC
GGCGTGCTGTACCACGAGGCGACGCACGTGTGGCAGTGGAACGGGCAGGGCAAGGCGAACGGCGGGCTCATC
GAGGGGATCGCCGACTACGTGCGGCTCAAGGCCGGGTTCGCGCCGGGGCACTGGGTGAAGCCGGGGCAGGGC
GACCGGTGGGATCAGGGGTACGACGTCACGGCGAGGTTCCTCGACTACTGCGACTCACTGAAGCCCGGGTTC
GTCGCGCAGCTCAACGCCAAGATGAAGAGTGGGTACACCGACGACTTCTTCGCGCAGATTCTCGGCAAGAAC
GTGCAGCAGCTGTGGCGGGACTACAAATCCAAGTTTGGAGCCTGAATACA**GGATCC**AATTAT

Figure 3.31 PCR amplification of *PR-17c* ORF. (A) Coding fragment of *PR-17c* incorporating BamHI restriction site at both ends was amplified by primer set s134F7/R7. (B) The sequence of the amplified fragment. The underlined sequence indicates extra nucleotides incorporated. Nucleotides in bold are BamHI recognition site.

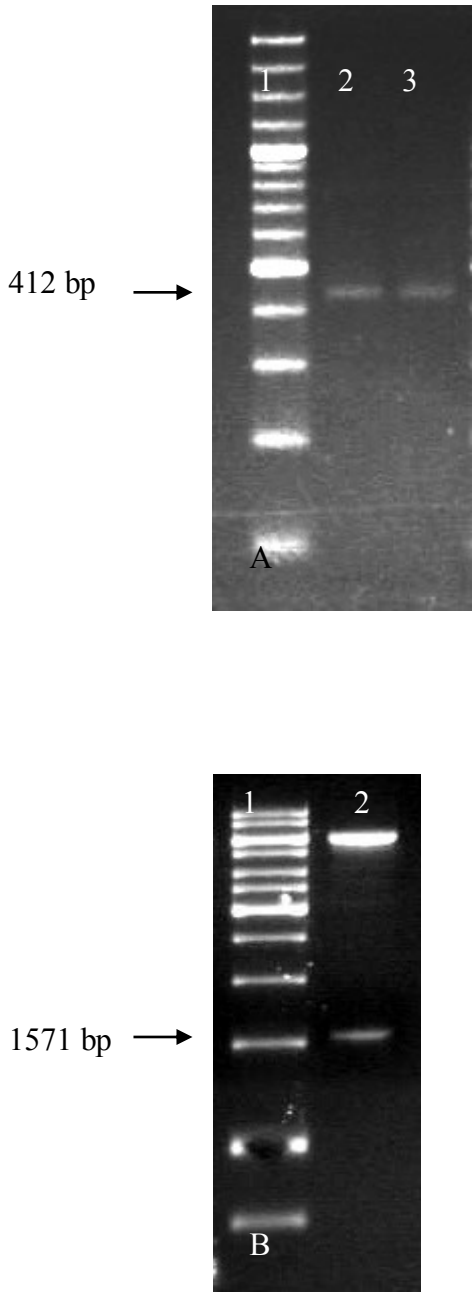


Figure 3.32 PCR amplification of RNAi fragments. (A) Sense and antisense fragments amplified by primers s134F10/R10 (sense) and s134F11/R11 (antisense) for cloning into pHannibal vector. Lane 1: DNA marker, Lanes 2 and 3: sense and antisense fragment, respectively. (B) Excising *HvPR-17* RNAi sequence from pHannibal vector. Fragment containing sense and antisense region of the gene was cut from pHannibal by BamHI restriction and purified for cloning into pPZPUbi. Lanes 1: marker DNA, Lane 2: BamHI digested pHannibal vector containing sense and antisense fragments (lower band) and vector backbone (upper band).

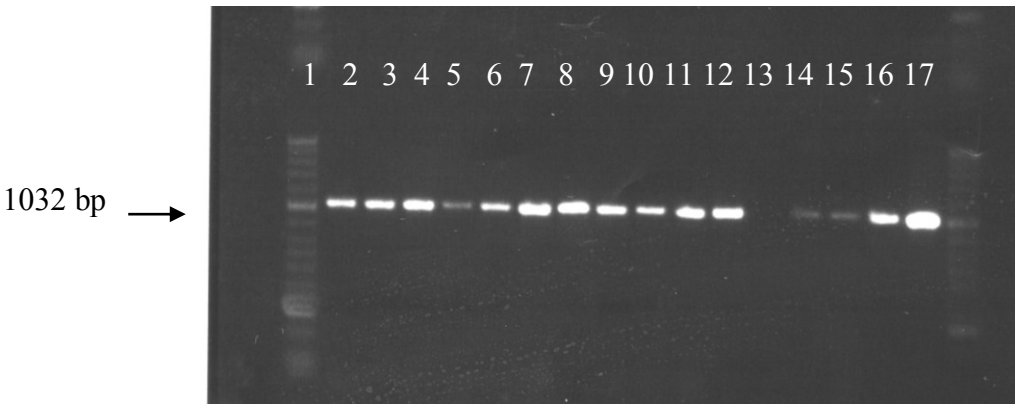


Figure 3.33 Validating *PR-17c* transgenic lines. Presence of transgene was verified by amplifying hygromycin resistance gene by primer pair HygF/R.. Lane 1: DNA size marker, Lanes 2-7: over-expression lines, Lanes 8-12: antisense lines, Lane 13: wild type, Lanes 14-17: RNAi lines.

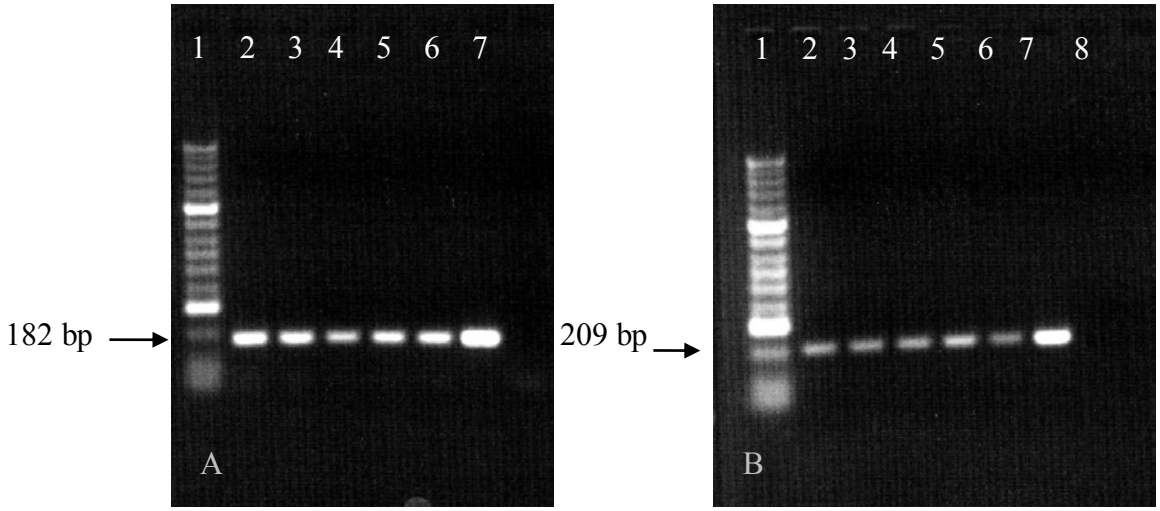


Figure 3.34 Validating presence of over-expression and antisense *PR-17c* construct in transgene. (A) The presence of pHvPR-17cAS in transgenic plants was verified by amplifying a 182 bp fragment by s134F16 gene specific primer and vector derived PZPF. Lane 1: marker, Lanes 2-6: transgenic antisense lines, Lane 7: pHvPR-17cAS vector as positive control. (B) The presence of pHvPR-17cSE in transgenic plants was verified by amplifying a 209 bp fragment by s134F16 gene specific primer and vector derived PZPR. Lanes 2-6: transgenic lines, Lane 7: pHvPR-17cSE vector as positive control, Lane 8: wild type.

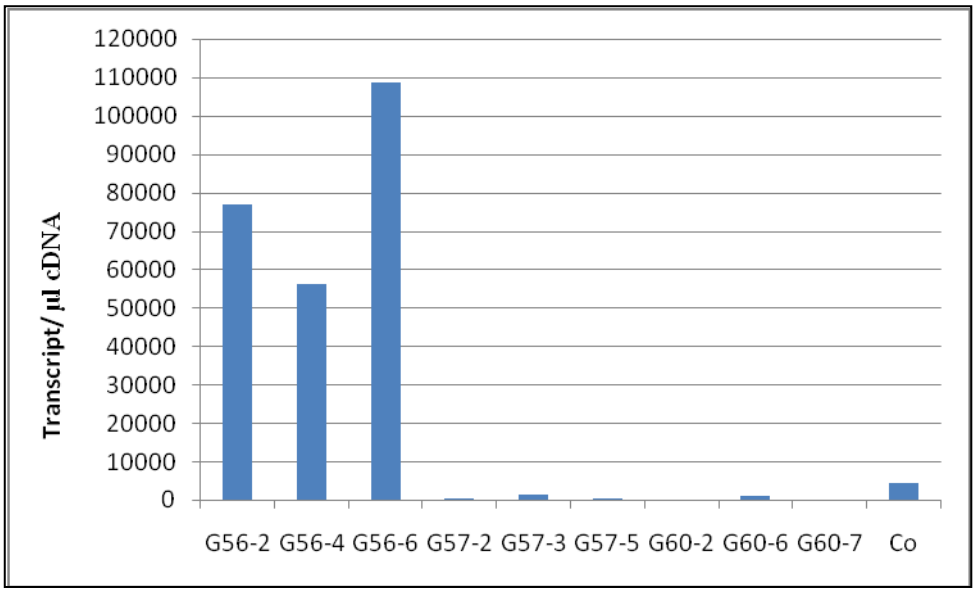


Figure 3.35 *HvPR-17c* transcript analyses in T_1 transgenic lines. Primer set qs134F1/R1 was used to measure transcripts in transgenic lines. The data were normalised against *SF400* and *SF427* mRNA levels. G56: over-expression, G57: RNAi, G60: antisense lines. Control is a wild type progeny of the G57 line.

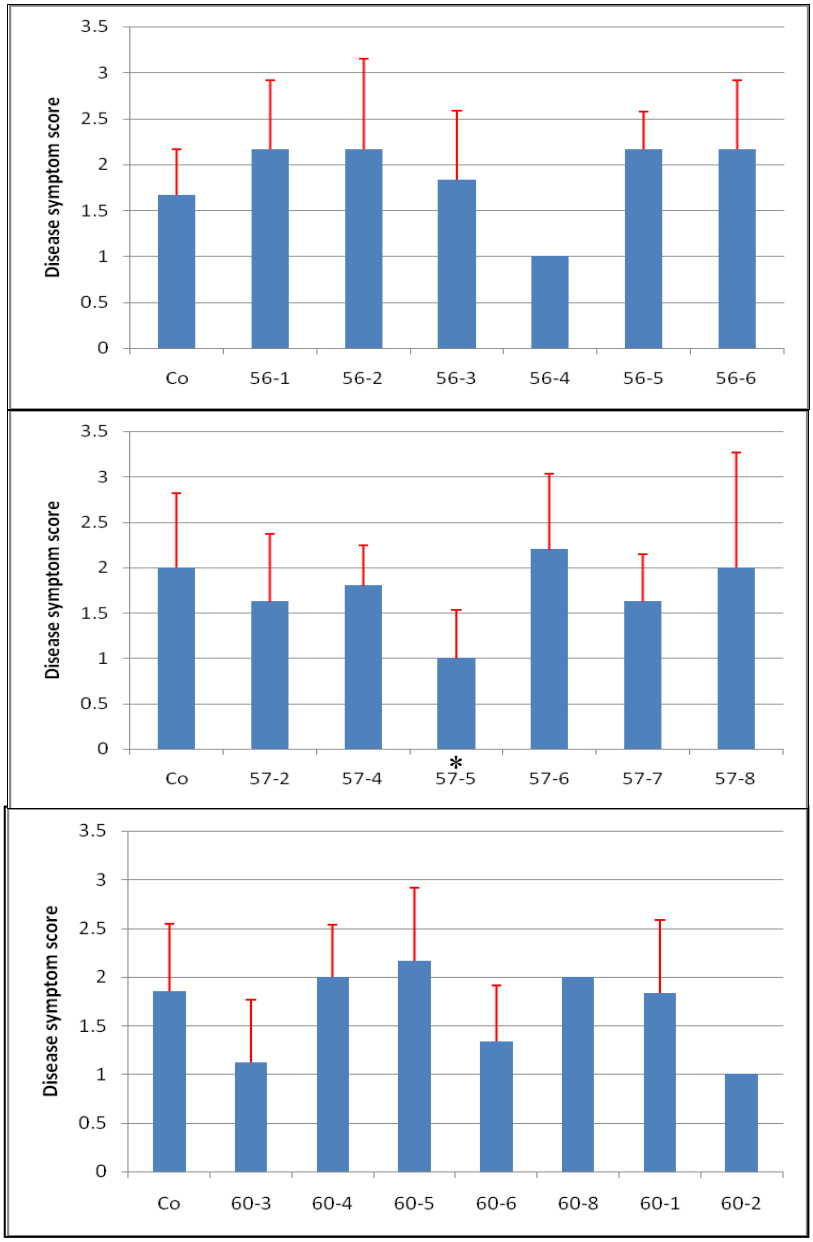


Figure 3.36 Scoring *Hv-PR-17c* transgenic lines for scald symptoms. Eight T_1 plants from each line was inoculated with *R. secalis* strain 6 and scored for disease symptoms 14 days post-inoculation. The experiment was repeated twice. Controls are non-transgenic progeny of the lines. Lines 56, 57 and 60 represent over-expression, antisense and RNAi lines, respectively. Students' t-test was used to determine significance of difference. Significant differences occurred in both experiments for the same line are reported.* indicates significant difference to control plants at 95% confidence.

Figure 3.37 A variegated T_1 plant from progeny of 56-4 over-expresser transgenic line. Different types of variegated leaves such as green centre region with pale margins or pale centre with green margins were observed. These plants flowered but failed to produce any seeds.

Figure 3.38 An albino plant grown from RNAi (G57-4) transgenic line.

3.4 Discussion

Expression of PR proteins is one of the induced responses of plants in response to pathogen attack. The function or biochemical activities of some PR family members are known. However, the function of the majority of PR such as PR-17, the most recent family of PR, remains unknown. In the study reported here, the isolation and molecular characterisation of barley PR-17 family members were undertaken, and their expression in response to biotic and abiotic stresses were examined by conducting experiments and interrogating publically available data sets. Protein subcellular localisation and antifungal activity of the heterologously expressed protein were investigated by GFP fusion and *in vitro* bioassays, respectively. Transgenic barley plants with *PR-17c* being over-expressed or suppressed were regenerated for *in planta* functional analysis and disease resistance.

3.4.1 Cloning and analysis of *HvPR-17* genes

In this study, three genes homologous to clone s134 were isolated from barley cDNA and genomic DNA. An additional homologue (pBH6-17, Accession No. Y14202) was identified in the Genbank protein database. The cloned genes were named *HvPR-17*, as the classification of PR proteins is based on similarities in molecular weight, amino acid sequences and enzymatic or biological activities of the proteins and gene sequences are annotated on the basis of homology to a family member (van Loon *et al.*, 2006). Two of the genes isolated in this study were new members and named *HvPR-17c* and *HvPR-17d*. Other member was named *HvPR-17a1* because of its high homology to *HvPR-17a* (pBH6-12, Accession No. Y14201). Comparison of the family member sequences showed them to be highly similar at both DNA and deduced protein amino acid sequences (58%-84% identity). Cloning the genomic regions showed that *PR-17c* lacks any intron in its coding region whereas, *HvPR-17d* has a 86 bp intron which contains the normal plant intron/exon junction signals of 'GT' at the 5' boundary and 'AG' at the 3' boundary (Breathnach and Chambon, 1981).

Two *HvPR-17* homologues have been described from wheat (Gorlach *et al.*, 1996; Kuwabara *et al.*, 1996) and one from tobacco (Okushima *et al.*, 2000). One of the wheat homologues (WCI-5) which shares 87% identity to HvPR-17d was induced by application of Benzothiadiazole (BTH), a chemical activator of resistance. In addition, BTH also

enhanced the accumulation of cell-wall bound phenolics, increased resistance to powdery mildew (Hukkanen *et al.*, 2007) and induced oxidative burst and a hypersensitive response in rice (Chen *et al.*, 2007). Recent studies have shown that BTH acts as a functional analog of SA and activates SA signalling pathway. It also recently was shown that WRKY45 transcription factor was up-regulated by BTH treatment 3 hour after application and knockdown of the gene abolished BTH effects (Shimono *et al.*, 2007). The finding suggests that WRKY45 may have a role in regulation of this family. WCI-5 was expressed co-ordinately with a lipoxygenase, a cystein proteinase, and two other proteins with unknown function, and the expression pattern of these genes was similar to those of chemically induced SAR genes in dicotyledonous species. Importantly, the WCI-5 transcript was also found to accumulate in wheat leaves infected by the wheat powdery mildew fungus (Gorlach *et al.*, 1996) along with PR-1. The other wheat homologue (WAS-2) which shares 92% identity to HvPR-17c is a secreted protein induced by abscisic acid (Kuwabara *et al.* 1999). The transcript of another member of the family, NtPRp27 from tobacco, was accumulated in response to TMV infection, mechanical wounding as well as drought and ABA treatments (Okushima *et al.,* 2000). In addition, NtPRp27 clearly responded to jasmonic acid and ethylene, which are thought to mediate wound and pathogenic signals (Creelman and Mullet, 1997; Xu *et al.*, 1994).

The genomic organisation of barley *PR-17* genes was determined by using two probes hybridised to Southern blots. It was evident that the *PR-17c* 3' probe bound to one major DNA fragment whereas the full-length probe hybridised to up to five DNA fragments indicating existence of at least five homologues. DNA fragments may be produced by internal restriction enzyme cleavage sites within the genes, producing two or more DNA fragments. However, since the sequences does not contain internal recognition sites for the restriction enzyme used, multiple bands indicate the presence of multiple genes with similar sequences which cross-hybridise. Direct evidence for presence of a gene family in barley was provided by cloning multiple family members. Similarly, multiple bands in Southern blots with a *HvPR-17b* full-length probe has been reported (Christensen *et al.*, 2002).

3.4.2 Phylogenetic analysis

Sequence assembly and phylogenetic analysis of the genes provided the first detailed profile of the copy number complexity and evolutionary dynamics of the PR-17s in different organisms. The collection and phylogenetic analysis of PR-17s from different species raises questions about naming PR-17 proteins' conserved domain as "Plant Basic Secretory" proteins. In this study, it was found that PR-17-type proteins are not exclusive to plants and exist in fungi and bacteria. In addition, not only are some of them predicted to be localised in the cytoplasm and plastids but also their pH among different kingdoms ranges from 4.5 to 9.8. A clade was found to include non-secretory sequences from rice, Arabidopsis, *Medicago*, grapevine and *Aspergillus*, which indicates they are ancestral sequences common to organisms before the divergence of monocotyledons, dicotyledonous and fungi. Most of bacterial sequences were found to be secreted proteins. Plant species contain a small family of these proteins whereas bacteria and fungi have them as single copy genes. Multigene families could arise from single or multiple genes by DNA duplication over a long time period (Ohta, 2000). The functional and structural diversification of the newly formed genes would arise through the occurrence of mutation and recombination (Wagner, 2001).

The four barley PR-17 sequences analysed in this study grouped into the same subfamily with other Poaceae, demonstrating that barley PR-17s share a common evolutionary origin based on conserved sequence and structural characteristics such as amino acid homology and conserved motifs with PR-17s from other grasses. The other PR-17s appear also to be grouped according to their taxonomy, forming clusters of fungi, bacteria, gymnosperms, Solanaceae, Brassicaceae , Fabaceae, Vitaceae, Pinaceae and Salicaceae. In rice and Arabidopsis PR-17 proteins, a higher level of conserved amino acid identity exists among subfamily members than between PR-17s belonging to different subfamilies. The extremely limited functional data for proteins in these subfamilies renders it difficult to speculate on proposed activity of barley PR-17s.

3.4.3 Expression profiling under biotic and abiotic stresses

Understanding the temporal and spatial expression patterns of genes in response to a stress provides an important basis for functional analysis of unknown genes. It should be noted that transcript levels are not always representative of protein levels or enzyme activity

(Donson *et al.*, 2002), because post-translational modifications are often a requirement for activity (Gygi *et al.*, 1999). Nevertheless, transcript profiling experiments have been informative in elucidating plant basal defence, and are proving useful in revealing mechanisms of induced defence responses. As outlined in the Introduction, pathogenesis-related proteins have been shown to be regulated by both biotic and abiotic stresses. PR proteins are also regulated during normal growth and development in a tissue-specific manner and in response to developmental cues (van Loon *et al.*, 2006) suggesting that they also play a role in certain developmental process.

The transcripts of *PR-17c* were detected in both compatible and incompatible interactions from 6 to 24 hours post inoculation with *R. secalis* in epidermal and whole leaf tissues. Nonetheless, the Q-PCR results showed that in epidermis tissue in the incompatible interaction, the transcripts were accumulated earlier and with greater magnitude in comparison with the compatible interaction. Affymetrix GeneChip hybridisation data also showed a similar pattern of accumulation of transcripts in barley-*Bgh* interactions in *Mla6* and *mlo5* mediated resistance for all four *PR-17* family members. Similarly, Zierold and colleagues (2005a) found that in resistant plants carrying the *mlo5* gene, transcript abundance of up-regulated genes was higher compared with the near-isogenic, susceptible line, such as seen here in response of resistant and susceptible cultivars. The data are in agreement with the expression of several other genes that have been shown to be responsive in compatible interactions and being more strongly activated in incompatible interactions (Bell *et al.*, 1986; Constabel and Brisson, 1992; Lamb *et al.*, 1992).

In response to *F. graminearum* up-regulation of mRNA levels was observed 24 hours post inoculation. Most of *R. secalis* (Ayesu-Offei and Clare, 1970) and *Bgh* (Clark et al., 1993) conidia germinate within 12 hours and *F. graminearum* spores germinate 24 hours after inoculation (Boddu *et al.*, 2006). One can speculate that the genes were up-regulated concomitant with germination of spores. This observation suggests that expression of *PR-17s* in barley interaction with the pathogens is triggered by non-specific elicitors during spore germination and penetration. This finding is consistent with the observations that fungal attachment and germination are accompanied by the release of proteins, carbohydrates, lipids, glycoproteins, and peptides from the spores (Tucker and Talbot, 2001) and many of these molecules can trigger basal host defence responses (Kiba *et al.*, 1999). The result also shows that *PR-17* family members responded to attack by

necrotrophic, biotrophic and semi-necrotrophic pathogens, although the level of accumulation was different for individual members. *PR-17a* showed the highest level of induction in all interactions whereas, other members accumulated to different levels depending on the pathosystem.

The exposure of barley plants to abiotic stresses revealed that the transcript levels of *PR-17c* were influenced by at least one form of stress. Frost treatment increased expression of *PR-17c* in comparison to control plants and the increase in transcript accumulation was dependent on the cultivar. Similarly, salt treatment caused a slight increase in mRNA levels in two of the cultivars examined. Conversely, drought caused a decrease in transcript levels. However, in all three treatments the highest level of expression was much lower than the levels observed by *R. secalis* inoculation. Drought-stressed plants 24 hours after rewatering still had low level of *PR-17c* transcripts, which indicates repression of PR protein signalling pathway in drought-exposed plants requires more time to return to normal levels.

HvPR-17 genes are activated by not only pathogen invasion, but also show developmental stage- and organ-specific expression even in healthy plants as has been reported for some other PR (van Loon *et al.*, 2006). Endogenous expression levels of *PR-17c* were only determined for leaf tissue in this study. *HvPR-17c* was found to be expressed in all cultivars at a low level. Tissue series data from cultivar Morex (Druka *et al.* 2006) were used to determine a temporal expression pattern of the barley *PR-17s* transcript accumulation during developmental stages. All family members were expressed at higher levels in geminating embryos and there was an up-regulation of three members in seedling crown except *PR-17c*. In addition, *PR-17d* was up regulated at anthesis and caryopses at 10 DAP. This observation could suggest an important protective role for these peptides against an eventual pathogen attack during germination. Differential expression of the members at different tissues indicates tissue specific regulation of the family members.

The results presented in this chapter illustrate that the barley *PR-17s* are diverse in their expression patterns, indicating these proteins have roles both in normal cellular development and in response to a range of biotic and possibly abiotic factors. The large and rapid alteration of barley *HvPR-17* family transcript levels in plants exposed to biotic

stress implies that they are an integral component of the defence-response mechanism of barley. To examine such role in plant defence additional approaches were required.

3.4.4 Subcellular localisation

As many cellular processes are spatially constrained to distinct cellular compartments, protein subcellular localisation often provides clues as to the functional role of a protein (Nair and Rost, 2002). *In silico* analysis showed that barley *PR-17* encoded proteins contain putative typical N-terminal hydrophobic signal peptides (Emanuelsson *et al.*, 2007) of 21-25 amino acids which could direct their translocation into the endoplasmic reticulum. Signal peptides target proteins to the ER, and it is known that proteins containing a C-terminal KDEL or HDEL sequence may be retained in the ER, whereas proteins without these sequences may continue to the Golgi complex (Denecke *et al.*, 1992). A retention signal was not detected in the amino acid sequences. These predictions suggest that barley PR-17 proteins are exported to the extracellular space.

In this study, the localisation of *PR-17c* gene product was determined by using a GFP fusion construct transiently expressed in barley epidermal cells. GFP itself is not able to cross most membranes, with the exception of the nuclear membrane through the nuclear pores (Grebenok *et al.*, 1997). The result obtained suggests that indeed PR-17c is a secreted protein. Although most of protein localised in the periphery of cells, some protein resided in the cytoplasm and structures resembling an ER network and vesicles. The pattern of fluorescence is quite similar to that with the secretory ZmES4-GFP gene (Dresselhaus *et al.*, 2005). The extracellular localisation was also supported by an immuno blot of PR-17a and PR-17b (Christensen *et al.* 2002) with intercellular washing fluids in *Bgh*-inoculated barley. Previous investigations of the subcellular localisation of various PR proteins, have also demonstrated that a proportion of each of the PR proteins investigated has been localised to the extracellular space.

The extracellular location is indicative of a protective role, since it is the first contact area with an invader pathogen. Extracellular proteins are thought to play a central role in plant defence responses against pathogens (Lee *et al.*, 2004) and changes in abundance of plant-secreted proteins have been identified in response to fungal pathogens (Rep *et al.*, 2002; Zareie *et al.*, 2002). In addition, *in silico* analysis indicated that HvPR-17s are very stable

proteins. Perhaps because of the need to function in a hostile environment, these PR proteins have pH and thermal stabilities and are quite resistant to proteolysis. Extracellular localisation and expression profile suggested that this family may have a direct role in resistance, so additional experiments were carried out.

3.4.5 Do HvPR-17s have a role in resistance

Evidence for the causal role of PR-17 proteins in plant defence is lacking. Two approaches (*in vitro* and *in planta*) were undertaken to examine such a role. Bioassays are a simple way of determining a protein's anti-microbial activity but require sufficient quantity of protein in solution. To obtain such quantity of a protein, heterologous expression of genes in a bacterial host system is the simplest and cheapest available approach. The HvPR-17c protein expressed in *E. coli* was used in different concentrations and in a range of pH (10 mM sodium acetate, pH 5.5-8) for this purpose. This condition is most likely to retain the natural structure of protein. Since protein activity is influenced by pH, selecting a broad range could increase the chance of obtaining biologically active protein.

Spore bioassay (Ayres and Owen, 1970; Zareie *et al.*, 2002) was chosen to study the effect of recombinant protein on *R. secalis* spores, as growth characteristics of *R. secalis* were not found to be suitable for a disc bioassay. The antifungal activity of the protein was also tested by radial growth inhibition method as described by Schlumbaum *et al.* (1986) against *Pyrenophora teres*. No activity against any of these pathogens was observed. It was hypothesised that lack of anti-fungal activity could be due to oxidation of the protein. For instance, it has been shown that the oxidised from of thaumatin lacks activity. Addition of 1.25 mM DTT rendered thaumatin with strong proteolytic activity (van der Wel and Bel, 1980). In this study, DDT at a 1 mM concentration was used in bioassay mixtures in separate experiments but still no anti-fungal activity was observed. The lack of anti-fungal activity could be due to several reasons. Firstly, the inherent lack of antifungal properties of the protein or towards the pathogens tested here. Not all PR proteins inhibit growth of all pathogens (Chadha and Das, 2006). Secondly, the lack of activity could also be attributed either to the involvement of the protein in an enzyme complex (Dhugga, 2005). Thirdly, incorrect folding of the protein in a heterologous system (Baneyx and Mujacic, 2004) could be another reason. Finally, the possibility of lack of a range of substrate molecules in the bioassay mix (Perrin *et al.*, 2001) or lack of post-translational

modification of the protein. This modification could include phosphorylation as predicted by Netphos program. The result prompted us to investigate resistance of transgenic lines.

Taking a reverse genetics approach to alter expression levels of a gene and subsequently study the phenotypic effects may provide hints about the relatedness of the protein to defence against pathogens. In this study, both RNAi and antisense silencing methods were used. RNAi interference was expected to target the *PR-17c* whereas antisense expression is expected to silence all family members because of cross hybridisation of their highly similar sequences. The expression of *PR-17c* was measured in transgenic lines and effective over-expression or suppression of the gene was observed.

Transgenic approaches have been used to demonstrate PR proteins direct role in plant defence. In several instances partial resistance against pathogens has been shown to be associated with constitutively expressed PR proteins (Liu *et al.*, 2004; Oldach *et al.*, 2001; Pflieger *et al.*, 2001; Vleeshouwers *et al.*, 2000). Typically, disease development is slowed, or pathogen proliferation and symptom severity are reduced. Inoculation and scoring disease symptoms revealed no difference between over-expressing transgenic lines and wild type plants. Many of the PR proteins are encoded by more than one gene and different PR protein types often act co-ordinately. Several studies have shown the combined and synergistic activity of different PR proteins in transgenic plants (Grover and Gowthaman, 2003; Melchers and Stuiver, 2000). Enhanced protection achieved by combined over-expression of PR proteins suggests that they are part of a far larger array of defence systems. In this system, each component could contribute more or less to basal resistance against an attacker, as well as to the enhanced resistance in plants with induced resistance. No studies have shown definitively that inactivation of a specific PR protein results in enhanced susceptibility to a pathogen. Nevertheless, transgenic studies corroborate the conclusion that resistance can be enhanced against some pathogens in some plant species.

One of antisense lines produced in the project showed significant reduction (2 fold) on scald symptom. In addition, among both over-expressed and suppressed lines developmental phenotypes were observed. However, whether those phenotypes are due to the function of PR-17 will require further study such as Western blot to examine the protein level and its correlation to the phenotype. Alternatively, the phenotype could be

caused by disruption of other genes in the integration site that is required for pathogenesis or normal development.

In this study, five barley PR-17 family members were identified and their characteristics and expression profile indicated a role in barley defence. However, a direct role for a member (PR-17c) in resistance against *R. secalis* was not found. The expression level of *HvPR-17a* was found to be the highest among the members of this family. Future transgenic studies by over expressing this gene and assaying resistance against a wide range of barley pathogens may demonstrate a direct role in defence. On the other hand, the identification of tissue and developmental specific expression of members provides an excellent opportunity to clone their promoter region for further analysis or to use them for tissue specific targeting of defence related genes. The cloned promoters can also be used in a yeast-one-hybrid screen of cDNA libraries from infected barley to identify transcription factors switching on the expression of the genes and signalling pathways involved.

Chapter 4: Characterisation of n194

4.1. Introduction

Galactinol synthase (GolS, EC 2.4.1.123) catalyses the synthesis of galactinol (O-α-D-galactopyranosyl-[1→1]-L-myo-inositol) from *myo*-inositol and UDP-D-Galactose (Liu *et al.*, 1998), which is required for the synthesis of raffinose family oligosaccharides (RFO). RFO (α-1,6-galactosyl$_n$ –Suc $1 \leq n \leq 15$) are found in most higher plants (French, 1954). These water-soluble, non-reducing carbohydrates are synthesised by sequential addition of galactosyl (Gal) units to sucrose linked via α-(1,6) glycosidic linkages (Panikulangara *et al.*, 2004). Synthesis of raffinose and stachyose by addition of Gal units from galactinol to sucrose and raffinose are catalysed by raffinose synthase (RS, EC 2.4.1.67) (Lehle and Tanner, 1973) and stachyose synthase (STS, EC 2.4.1.67) (Peterbauer *et al.*, 1999), respectively. Oligosaccharides with higher degree of polymerisation than four are synthesised by transfer of Gal residues from galactinol (Tanner *et al.*, 1967) or non-galactinol dependent pathways (Bachmann *et al.*, 1994; Tapernoux-Luthi *et al.*, 2004).

The only known function of galactinol is its role in the biosynthesis of RFO and since GolS potentially catalyses a metabolic key step in RFO synthesis, it has been proposed to have a main regulatory role in the carbon partitioning between sucrose and RFO (Peterbauer *et al.*, 2002; Saravitz *et al.*, 1987). Experimental support for such a proposal has been provided by showing strong correlation of GolS activity and RFO levels during soybean seed development in a temporal manner (Kuo *et al.*, 1997), the occurrence of the *GolS1* transcript and raffinose in radicle tips of tomato embryo (Gurusinghe and Bradford, 2001), and also in *Ajuga reptans* leaves between *GolS1* expression levels and GolS activities with RFO accumulation (Sprenger and Keller, 2000). In addition over-expression of a *GolS* isoform in Arabidopsis caused an increase in galactinol and raffinose content (Taji *et al.*, 2002). In other studies differences in the total amount of RFO deposited during seed development have been also related to variation in the level of galactinol synthase activity (Handley *et al.*, 1983; Lowell and Kuo, 1989; Saravitz *et al.*, 1987). However, such a direct correlation has not been found in all cases. In some plants such as potato, pea seeds and low phytic acid barley mutants RFO accumulation was correlated with *myo*-inositol levels, the galactinol precursor (Karner *et al.*, 2004; Keller *et al.*, 1998). These studies suggest that RFO accumulation in seeds of some species is modulated not only by GolS but also by the levels of the initial substrates.

GolS accumulation is correlated with exposure to environmental water deficit stresses caused by cold, heat, drought and during the developmentally induced desiccation in the late maturation stage of seed. For instance, in germinating tomato seeds *GolS* mRNA accumulation was induced by dehydration but not by cold, whereas both stresses induced mRNA accumulation in seedling leaves (Downie *et al.*, 2003). In Arabidopsis at normal temperature *GolS1* expression is restricted to meristematic and vascular tissue but after heat shock its expression was induced in all cells and tissues. (Panikulangara *et al.*, 2004). The induction was dependent on *Heat Shock Factor 3*. They also demonstrated a correlation between the induction of *GolS1* mRNA and increase in raffinose-galactinol levels after heat shock.

RFO have been shown to have multiple roles in plants. In seeds they accumulate during the late stages of maturation coinciding with acquisition of desiccation tolerance (Brenac *et al.*, 1997b; Kuo *et al.*, 1997). They are used for storage, translocation, utilization of carbon (Keller and Matile, 1985; Sprenger and Keller, 2000) and against different abiotic stresses such as those caused by heat, frost (Bachmann *et al.*, 1994; Hinesley *et al.*, 1992; Santarius, 1973), drought (Downie *et al.*, 2003) and salt (Gilbert *et al.*, 1997). Functional evidence for a direct role of RFO in plants' abiotic stress tolerance have been shown in transgenic Arabidopsis over-expressing *AtGolS2* and transgenic petunia with reduced α-galactosidase activity (Pennycooke *et al.*, 2003) which showed increased tolerance to drought and freezing, respectively. In addition, rice UDP-glucose 4-epimerase (*UGE*), which interconverts UDP-D-glucose and UDP-D-galactose is known to be induced by various abiotic stresses. In a study over-expression of *OsUGE-1* in Arabidopsis conferred tolerance to salt, drought and freezing stress in transgenic plants. Those transgenic plants showed a higher level of raffinose than the wild-type plants. This observation suggests that an elevated level of raffinose caused by over-expression of *OsUGE-1* may have resulted in enhanced tolerance to abiotic stress (Liu *et al.*, 2007).

Vitrification of cytoplasm during seed maturation, which is accompanied by water loss has been considered to be a critical point enabling seeds to tolerate desiccation (Buitink *et al.*, 2000; Hoekstra *et al.*, 2001; Obendorf, 1997; Williams and Leopold, 1989). Raffinose and sucrose have been proposed to be involved in this process in drying seeds by stabilizing sensitive macromolecular structures (Crowe *et al.*, 1987). Hincha and colleagues (2003) showed that RFO with increasing degree of polymerization are better able to reduce

soluble marker leakage from liposome during air-drying and after rehydration by preventing membrane fusion. RFO are also involved in the stabilization of membranes under water stress by replacing dissipating water between polar head groups of membranes (Brenac *et al.*, 1997a; Crowe *et al.*, 1992). This function of RFO could help cellular stress tolerance under freezing and desiccation which targets membranes (Crowe *et al.*, 1992; Oliver *et al.*, 2002; Steponkus, 1984). The interaction of RFO with membrane lipids in dry state also reduces the gel to liquid crystalline lipid phase transition temperature (T_m) (Hincha *et al.*, 2003). T_m reduction has been suggested to be the result of direct hydrogen bonding interaction between the sugars and the phospholipids' head groups (Oliver *et al.*, 2002; Oliver *et al.*, 1998). Raffinose has been shown to stabilize membranes during freeze-drying of lobster muscle microsomes (Crowe *et al.*, 1984), desiccation-tolerant pea seed embryo protoplasts (Xiao and Koster, 2001) and along with stachyose during freezing of spinach thylakoids (Hincha, 1990).

In addition to RFO direct interaction with membrane components, it has been hypothesised that these compounds act as osmolytes, which helps cells to adjust osmotic pressure that is altered by drought, salinity and cold. There is also evidence that suggests they might increase stress tolerance by radical scavenging, protection from photo inhibition or detoxification (Bohnert and Jensen, 1996; Bohnert *et al.*, 1995; Nishizawa *et al.*, 2008; Pharr *et al.*, 1995).

Important roles of RFO in abiotic stress tolerance of dicotyledonous plants were discussed above but the role of GolS in monocotyledonous plants has not been studied in detail. No report was found either on the role of GolS or RFO in plant-pathogen interaction. Isolation of the full-length of clone n194 cDNA revealed similarity to genes encoding GolS in plants. In this chapter, isolation and characterisation of barley *GolS* genes and its role in biotic and abiotic stress tolerance of barley have been presented.

4.2 Materials and Methods

In this section material and methods specifically used for the characterisation of barley *GolS* members is given. The general material and methods were described in Chapter 2.

4.2.1 Isolation of full-length cDNA and genomic DNA of *HvGolS1* and *HvGolS2*

The clone n194 (*HvGolS1*) sequence was used to search EST databases and assemble a contig (Section 2.31). Based on the contig sequence gene specific primers were designed for 3'- and 5'-RACE (Section 2.12). The sequences obtained from RACEs were used to amend the contig. For cloning *HvGolS2*, Genbank barley EST databases were searched using *HvGolS1* as query and a contig was assembled. The full-length sequences of the genes was amplified from 5'-RACE ready cDNA by ProofStart and n194F3/R3 and GolS2F/R primer pairs following CP 13 and CP 21 for *GolS1* and *GolS2*, respectively. Genomic sequence of the genes was amplified from Atlas46, Atlas and Turk genomic DNA by using *PfuUltraHF* polymerase and primer pair n194R/F3 (*HvGolS1*) following CP 15 and primers GolS2F/R (*HvGolS2*) following CP 21. After ligating gel-purified PCR products to pGEM-T Easy vector, 10 clones were sequenced for each amplicons.

4.2.2 Heterologous expression and purification of recombinant HvGolS1

To express and purify recombinant HvGolS1 proteins a similar strategy was used as Section 3.2.2. Two PCR fragments were amplified using *PfuUltraHF* polymerase and primer pairs n194F13/R13 and n194F14/R14 by CPs 12 and 22, respectively. The plasmids resulting from ligation of fragments to vectors were named pQE30HvGolS1 and pQE70HvGolS1.To check expression of the 6xHis-tagged proteins, minipreps under denaturing condition following Ni-NTA Spin Handbook (QIAGEN) instructions was prepared.

4.3 Results

4.3.1 Isolation of two barley *GolS* family members

To characterise the clone n194 its full-length sequence and one homologue's was cloned. Nested 3'-RACE (Fig. 4.1) with n194 clone specific primer resulted in amplification of homologous fragments. These fragments vary in the position of poly A^+ tails. Some representative sequences are shown in Figure 4.2. 5'-RACE PCR was not successful, but full-length of n194 (*HvGolS1*) was amplified from cDNA and genomic DNA (Fig. 4.3) by designing primers from a contig assembled by joining clone n194, 3'-RACE and overlapping EST sequences extended toward 5' end of the gene. Sequencing of genomic region showed the existence of two introns (Fig. 4.4).

Homology search of databases indicated the existence of a *GolS1* homologue in barley. A contig was assembled including ploy A^+ tail and was named *HvGolS2*. The sequence of the contig was amplified by PCR (Fig. 4.5). Sequencing of the PCR products revealed existence of three introns. Alignment of *HvGolS2* cDNA and genomic DNA sequences are shown in Figure 4.6.

To investigate the number of *GolS*-like genes in barley, Southern hybridisation was performed using a 3' and a full-length probe spanning 3' end and full length of the *GolS1* gene (Fig. 4.7), respectively. DNA gel blot analysis with the 3' probe (Fig. 4.8A) hybridised to one band, but full-length probe (Fig. 4.8B) hybridised to two strong bands. This hybridisation pattern indicates the specificity of the 3' probe for detection of *HvGolS1* and confirms the existence of at least two *GolS* genes in the barley genome.

4.3.2 Sequence analysis

After cloning, the nucleotide sequences of *HvGolS* cDNAs were analysed for the presence of ORFs. They both contain a single ORF and downstream of the first stop codon four more in frame stop codons were found. *GolS1* encodes a protein with 323 amino acid residues, molecular weight of 36948 Da, and pI of 5.06. Similarly, *GolS2* encodes a putative protein of 329 amino acid residues with a molecular weight of 38211 Da and pI of

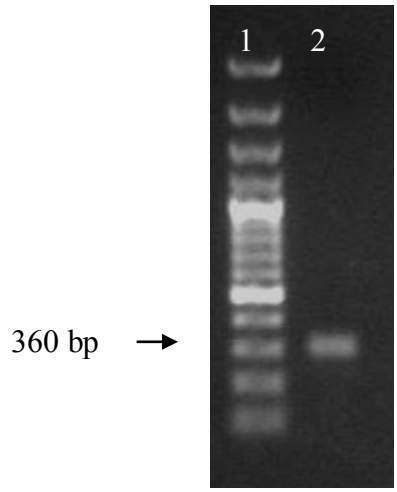


Figure 4.1 *HvGolS1* 3'-RACE. The *GolS1* nested 3'-RACE product using primer pairs n194F6/CDSIIA was fractionated in a 2% agarose gel and purified. Lane 1: DNA marker, Lane 2: PCR product.

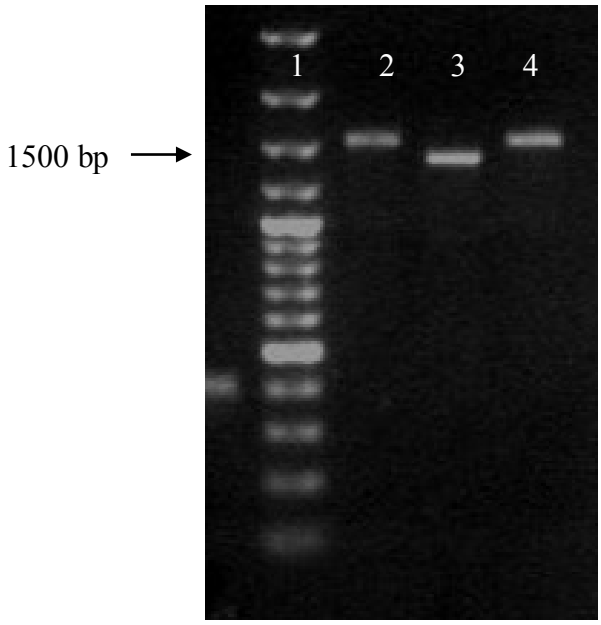


Figure 4.2 Amplification of *HvGolS1* full-length. Lane 2: Atlas 46 genomic DNA, Lane 3: Atlas 46 cDNA fragment, Lane 4: Atlas genomic DNA. The fragments were amplified by using the primer pair n194R/F3 and indicate existence of introns in genomic DNA.

```
                1                                                    50
GolS1A3'    (1) CTGCCTTCCAGTTAGGCGTATGTATTCTACTAATAAATACTAGTAATACG
GolS1B3'    (1) CTGCCTTCCAGTTAGGCGTATGTATTCTACTAATAAATACTAGTAATACG
GolS1C3'    (1) -TGCCTTCCAGTTAGGCGTATGTATTCTACTAATAAATACTAGTAATACG
GolS1D3'    (1) CTGCCTTCCAGTTAGGCGTATGTATTCTACTAATAAATACTAGTAATACG
GolS1E3'    (1) CTGCCTTCCAGTTAGGCGTATGTATTCTACTAATAAATACTAGTAATACG
GolS1F3'    (1) CTGCCTTCCAGTTAGGCGTATGTATTCTACTAATAAATACTAGTAATACG
                51                                                  100
GolS1A3'   (51) TAGGAAAGATTTCGTCAGTACTACGTAATATCGTCGTCGATTTAGCATCT
GolS1B3'   (51) TAGGAAAGATTTCGTCAGTACTACGTAATATCGTCGTCGATTTAGCATCT
GolS1C3'   (50) TAGGAAAGATTTCGTCAGTACTACGTAATATCGTCGTCGATTTAGCATCT
GolS1D3'   (51) TAGGAAAGATTTCGTCAGTACTACGTAATATCGTCGTCGATTTAGCATCT
GolS1E3'   (51) TAGGAAAGATTTCGTCAGTACTACGTAATATCGTCGTCGATTTAGCATCT
GolS1F3'   (51) TAGGAAAGATTTCGTCAGTACTACGTAATATCGTCGTCGATTTAGCATCT
                101                                                 150
GolS1A3'  (101) ACACGTTTTGGTATTGTAGATCATCATCAATCGATCATATACAGTGTTCT
GolS1B3'  (101) ACACGTTTTGGTATTGTAGATCATCATCAATCGATCATATACAGTGTTCT
GolS1C3'  (100) ACACGTTTTGGTATTGTAGATCATCATCAATCGATCATATACAGTGTTCT
GolS1D3'  (101) ACACGTTTTGGTATTGTAGATCATCATCAATCGATCATATACAGTGTTCT
GolS1E3'  (101) ACACGTTTTGGTATTGTAGATCATCATCAATCGATCATATACAGTGTTCT
GolS1F3'  (101) ACACGTTTTGGTATTGTAGATCATCATCAATCGATCATATACAGTGTTCT
                151                                                 200
GolS1A3'  (151) TTTTCCGATTGAGGTACGTACGGCCACCATAGTGTTTTTCGGTTTGTATA
GolS1B3'  (151) TTTTCCGATTGAGGTACGTACGGCCACCATAGTGTTTTTCGGTTTGTATA
GolS1C3'  (150) TTTTCCGATTGAGGTACGTACGGCCACCATAGTGTTTTTCGGTTTGTATA
GolS1D3'  (151) TTTTCCGATTGAGGTACGTACGGCCACCATAGTGTTTTTCGGTTTGTATA
GolS1E3'  (151) TTTTCCGATTGAGGTACGTACGGCCACCATAGTGTTTTTCGGTTTGTATA
GolS1F3'  (151) TTTTCCGATTGAGGTACGTACGGCCACCATAGTGTTTTTCGGTTTGTATA
                201                                                 250
GolS1A3'  (201) AGGAAGGAGTATATTGTAAACACGTACGTGCAAAAAAAAAAAAAAAAAAAA
GolS1B3'  (201) AGGAAGGAGTATATTGTAAACACGTACGTGCAAGCACACGGTTGGTCCGA
GolS1C3'  (200) AGGAAGGAGTATATTGTAAACACGTACGTGCAAGCACACGGTTGGTCCAA
GolS1D3'  (201) AGGAAGGAGTATATTGTAAACACGTACGTGCAAGCACACGGTTGGTCCAA
GolS1E3'  (201) AGGAAGGAGTATATTGTAAACACGTACGTGCAAGCACACGGTTGGTCCAA
GolS1F3'  (201) AGGAAGGAGTATATTGTAAACACGTACGTGCAAGCACACGGTTGGTCCAA
                251                                                 300
GolS1A3'  (251) AAAAAA------------------------------------------
GolS1B3'  (251) AAAAAAAAAAAAAAAAAAAAAAA-------------------------
GolS1C3'  (250) GTATGATTATAGATCGACCTGCTACGTACACGTATGCATGGCTGAAACGT
GolS1D3'  (251) GTATGATTATAGATCGACCTGCTACGTACACGTATGCATGGCTGAAACGT
GolS1E3'  (251) GTATGATTATAGATCGACCTGCTACGTACACGTATGCATGGCTGAAACGT
GolS1F3'  (251) GTATGATTATAGATCGACCTGCTACGTACACGTATGCATGGCTGAAACGT
                301                                                 350
GolS1A3'  (257) ------------------------------------------------
GolS1B3'  (274) ------------------------------------------------
GolS1C3'  (300) TTTGGAAGCAAAAAAAAAAAAAAAAAAAAAAAA----------------
GolS1D3'  (301) TTTGGAAGCTATGAAGAAAAAAAAAAAAAAAAAAAAAAAA---------
GolS1E3'  (301) TTTGGAAGCTATGAAGATGAAAAAAAAAAAAAAAAAAAAAA--------
GolS1F3'  (301) TTTGGAAGCTATGAAGATATATATTTTCTCTGAAAAAAAAAAAAAAAAA
                351
GolS1A3'  (257) -------
GolS1B3'  (274) -------
GolS1C3'  (334) -------
GolS1D3'  (342) -------
GolS1E3'  (343) -------
GolS1F3'  (351) AAAAAAA
```

Figure 4.3 Poly A^+ variation of *HvGolS1*. Poly A^+ tail (bold sequences) position of *HvGolS1* revealed by sequencing 3'-RACE fragments cloned in pGEM-T Easy vector.

```
                    1                                                  50
HvGolS1gDNA     (1) --------------------CGCACAAGTCCACAGCACACAGCAGACCCA
                                        n194F3 ------------>
HvGolS1cDNA     (1) CTCGTGCCGAATTCGGCACG**CGCACAAGTCCACAGCACACAGCAG**ACCCA
                    51                                                 100
HvGolS1gDNA    (31) TCCCATCCCATCCATCCTTTGATTTGAAGCAAGACAAGAGGGACCGACCG
HvGolS1cDNA    (51) TCCCATCCCATCCATCCTTTGATTTGAAGCAAGACAAGAGGGACCGACCG
                    101                                                150
HvGolS1gDNA    (81) AGCAAGCAATGGCTCCCATGCTCAAGCGGATCGTGGAGGACGAGCCCAAG
HvGolS1cDNA   (101) AGCAAGCAATGGCTCCCATGCTCAAGCGGATCGTGGAGGACGAGCCCAAG
                    151                                                200
HvGolS1gDNA   (131) AAGGCGGCGTACGTGACCTTCCTCGCCGGCTCCGGCGACTACTGGAAGGG
HvGolS1cDNA   (151) AAGGCGGCGTACGTGACCTTCCTCGCCGGCTCCGGCGACTACTGGAAGGG
                    201                                                250
HvGolS1gDNA   (181) CGTGGTCGGCCTTGCCAAGGGCCTCCGCGCCGTCAACTCCGCCTACCCGC
HvGolS1cDNA   (201) CGTGGTCGGCCTTGCCAAGGGCCTCCGCGCCGTCAACTCCGCCTACCCGC
                    251                                                300
HvGolS1gDNA   (231) TCGTGGTGGCCGTGCTCCCCGACGTCCCCGAGGAGCACCGCCAGGAGCTG
HvGolS1cDNA   (251) TCGTGGTGGCCGTGCTCCCCGACGTCCCCGAGGAGCACCGCCAGGAGCTG
                    301                                                350
HvGolS1gDNA   (281) CTCAAGCAGGGCTGCGTCGTCCGGGAGATCGTGCCCGTCTACCCGCCGGA
HvGolS1cDNA   (301) CTCAAGCAGGGCTGCGTCGTCCGGGAGATCGTGCCCGTCTACCCGCCGGA
                    351                                                400
HvGolS1gDNA   (331) GAGCCAGACCCAGTTCGCCATGGCCTACTACGTCATCAACTACTCGAAGC
HvGolS1cDNA   (351) GAGCCAGACCCAGTTCGCCATGGCCTACTACGTCATCAACTACTCGAAGC
                    401                                                450
HvGolS1gDNA   (381) TCCGCATCTGGGAGGTACCTTACTCACTGACCTCTAGCTCTCTCGCCATC
HvGolS1cDNA   (401) TCCGCATCTGGGAG-----------------------------------
                    451                                                500
HvGolS1gDNA   (431) **TCTCTTCTTCTTCCTCCATATTTGATGATCCATGGATGTTGATTGATTCT**
HvGolS1cDNA   (415) --------------------------------------------------
                    501                                                550
HvGolS1gDNA   (481) **TGTGTACGTACGTACGTGTAG**TTCGTGGAGTACGAGAGGATGGTGTACCT
HvGolS1cDNA   (415) ----------------------TTCGTGGAGTACGAGAGGATGGTGTACCT
                    551                                                600
HvGolS1gDNA   (531) GGACGCGGACATCCAGGTGTACGACAACATCGACCACCTCTTCGACCTCG
HvGolS1cDNA   (444) GGACGCGGACATCCAGGTGTACGACAACATCGACCACCTCTTCGACCTCG
                    601                                                650
HvGolS1gDNA   (581) AGATGGGCAGCTTCTACGCCGTCAAGGACTGCTTCTGCGAGAAGACGTGG
HvGolS1cDNA   (494) AGATGGGCAGCTTCTACGCCGTCAAGGACTGCTTCTGCGAGAAGACGTGG
                    651                                                700
HvGolS1gDNA   (631) AGCCACACCCGGCAGTACGAGATCGGCTACTGCCAGCAGTGCCCCGACAG
HvGolS1cDNA   (544) AGCCACACCCGGCAGTACGAGATCGGCTACTGCCAGCAGTGCCCCGACAG
                    701                                                750
HvGolS1gDNA   (681) GGTGGCGTGGCCGGAGCGCGAGCTGGGCGTGCCCCCGCCGCCGCTCTACT
HvGolS1cDNA   (594) GGTGGCGTGGCCGGAGCGCGAGCTGGGCGTGCCCCCGCCGCCGCTCTACT
                    751                                                800
HvGolS1gDNA   (731) TCAACGCCGGCATGTTCGTGCACGAGCCCAGCATGGCCACCGCCAAGGCC
HvGolS1cDNA   (644) TCAACGCCGGCATGTTCGTGCACGAGCCCAGCATGGCCACCGCCAAGGCC
                    801                                                850
HvGolS1gDNA   (781) CTCCTCGACAGGCTTGTCGTCACCGACCCCACCCCGTTCGCCGAGCAGGA
HvGolS1cDNA   (694) CTCCTCGACAGGCTTGTCGTCACCGACCCCACCCCGTTCGCCGAGCAGGA
                    851                                                900
HvGolS1gDNA   (831) CTTCCTCAACATGTTCTTCAGGGACGTGTACAAGCCCATCCCGCCGGTGT
HvGolS1cDNA   (744) CTTCCTCAACATGTTCTTCAGGGACGTGTACAAGCCCATCCCGCCGGTGT
                    901                                                950
HvGolS1gDNA   (881) ACAACCTCGTGCTCGCCATGCTCTGGAGGCACCCGGAGAACATCCAGCTC
HvGolS1cDNA   (794) ACAACCTCGTGCTCGCCATGCTCTGGAGGCACCCGGAGAACATCCAGCTC
                    951                                               1000
HvGolS1gDNA   (931) GGCGAGGTCAAGGTCGTCCACTACTGCGCCGCGG**TACGTCTTTCATATCT**
HvGolS1cDNA   (844) GGCGAGGTCAAGGTCGTCCACTACTGCGCCGCGG----------------
```

Figure 4.4 Alignment of cDNA and genomic DNA of *HvGolS1* sequences revealed location of two introns. Alignment of nucleotide sequences were conducted using the AlignX program of Vector NTI V.9 suite. The intron sequences are shown in bold font. The sequence of region used for the RNAi construct (Section 4.3.7) is underlined. The sequences in bold italic mark the primers and the direction of arrows indicates orientation of primer

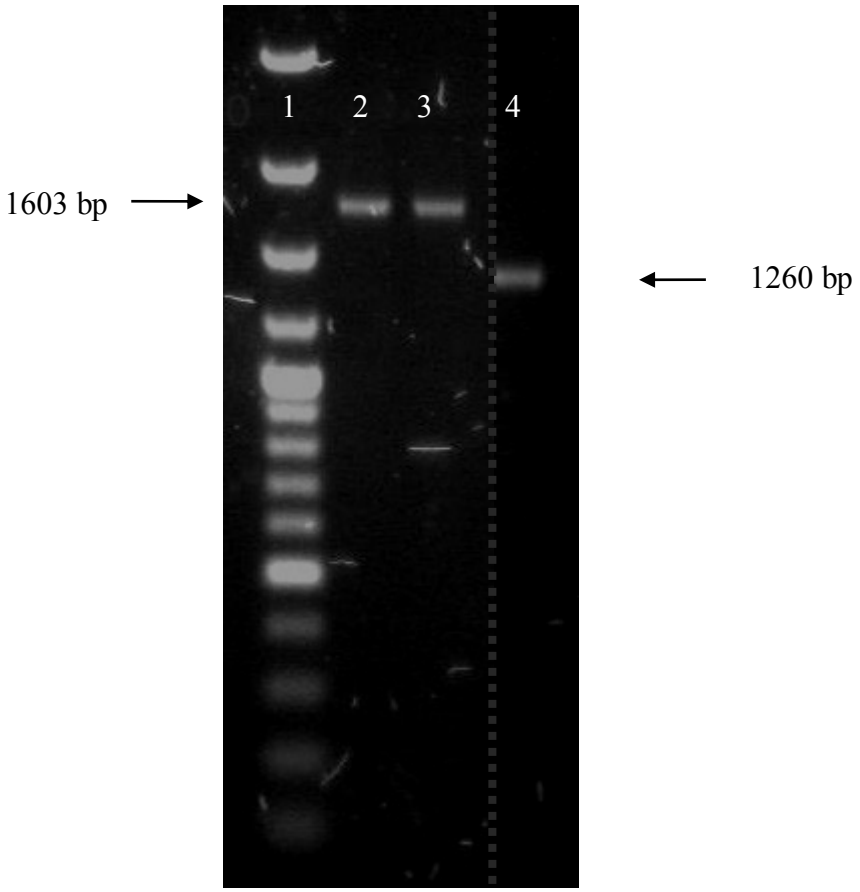


Figure 4.5 PCR amplification of *HvGolS2*. PCR products amplified from genomic DNA of Atlas and Atlas 46 (Lanes 2 and 3) and cDNA of Atlas 46 (Lane 4) by using the primer pairs GolS2R/F indicates existence of introns in genomic DNA. The dashed vertical line indicates that pictures have been cut.

```
                    1                                                  50

HvGolS2cDNA       (1) CTCGTGCCGCTCGTGCCGAATTCGGCACGAGCTCTCTAAATCCGACACAA
HvGolS2gDNA       (1) ----------------------GCACGAGCTCTCTAAATCCGACACAA
                    51                                                 100
HvGolS2cDNA      (51) AAACTCAAGCCCGAGCCCAAAGCTTCCTCCCTCCCGGAGACGAGATGGCT
HvGolS2gDNA      (27) AAACTCAAGCCCGAGCCCAAAGCTTCCTCCCTCCCGGAGACGAGATGGCT
                   101                                                 150
HvGolS2cDNA     (101) CCCGAGCTGGCCGGCAAGATGACCGCCAAGGCAGCCGTGGCGGCGGCCAA
HvGolS2gDNA      (77) CCCGAGCTGGCCGGCAAGATGACCGCCAAGGCAGCCGTGGCGGCGGCCAA
                   151                                                 200
HvGolS2cDNA     (151) GCCCGCGACGAGGGCGTACGTGACGTTCCTGGCGGGGTCTGGGGACTACT
HvGolS2gDNA     (127) GCCCGCGACGAGGGCGTACGTGACGTTCCTGGCGGGGTCTGGGGACTACT
                   201                                                 250
HvGolS2cDNA     (201) GGATGGGCGTGGTTGGGCTCGCCAAGGGCTTGCGCAAGGTTGGCTCGGCC
HvGolS2gDNA     (177) GGATGGGCGTGGTTGGGCTCGCCAAGGGCTTGCGCAAGGTTGGCTCGGCC
                   251                                                 300
HvGolS2cDNA     (251) TACCCGCTGGTGGTGGCCGTGCTGCCCGACGTGCCCGAGCTCCACCGCAA
HvGolS2gDNA     (227) TACCCGCTGGTGGTGGCCGTGCTGCCCGACGTGCCCGAGCTCCACCGCAA
                   301                                                 350
HvGolS2cDNA     (301) GATCCTCGTCTCCCAGGGCTGCATCGTCCGCGAGATCGCCCCCGTGTACC
HvGolS2gDNA     (277) GATCCTCGTCTCCCAGGGCTGCATCGTCCGCGAGATCGCCCCCGTGTACC
                   351                                                 400
HvGolS2cDNA     (351) CGCCCGAGAACCAGACCCAGTTTGCGATGGCCTACTACGTCATCAACTAC
HvGolS2gDNA     (327) CGCCCGAGAACCAGACCCAGTTTGCGATGGCCTACTACGTCATCAACTAC
                   401                                                 450
HvGolS2cDNA     (401) TCCAAGCTCCGCATCTGGGAG----------------------------
HvGolS2gDNA     (377) TCCAAGCTCCGCATCTGGGAGGTAAATTGCTACACCGCCCGCATGTCTCG
                   451                                                 500
HvGolS2cDNA     (422) --------------------------------------------------
HvGolS2gDNA     (427) CTCACGTGTTTTTTTTCTCAAACGGAGCCAAAAAGCTTTGCCTCATCAAA
                   501                                                 550
HvGolS2cDNA     (422) --------------------------------------------------
HvGolS2gDNA     (477) TTTAATAAGAACTTTTTTTAGTGTGTATGGCTAGTAATGGTATGCTGATC
                   551                                                 600
HvGolS2cDNA     (422) -------------------------TTTGTGGAGTACGAGAGGATGGTG
HvGolS2gDNA     (527) TGTTCTTGGTTTTGTTTTGAGCGCAGTTTGTGGAGTACGAGAGGATGGTG
                   601                                                 650
HvGolS2cDNA     (446) TACCTTGACGCCGACATCCAGGTGTTCGACAACATCGACGAGCTGTTTGA
HvGolS2gDNA     (577) TACCTTGACGCCGACATCCAGGTGTTCGACAACATCGACGAGCTGTTTGA
                   651                                                 700
HvGolS2cDNA     (496) TCTGCCCAAGGGGCGCTTCTACGCTGTGATGGACTGCTTCTGCGAGAAGA
HvGolS2gDNA     (627) TCTGCCCAAGGGGCGCTTCTACGCTGTGATGGACTGCTTCTGCGAGAAGA
                   701                                                 750
HvGolS2cDNA     (546) CGTGGAGTCACACCCGGCAGTACCAGATCGGCTACTGCCAGCAGTGTCCC
HvGolS2gDNA     (677) CGTGGAGTCACACCCGGCAGTACCAGATCGGCTACTGCCAGCAGTGTCCC
                   751                                                 800
HvGolS2cDNA     (596) GACAGGGTGACGTGGCCGGCCGCCGAGATGGGCCCGCCGCCGGCGCTTTA
HvGolS2gDNA     (727) GACAGGGTGACGTGGCCGGCCGCCGAGATGGGCCCGCCGCCGGCGCTTTA
                   801                                                 850
HvGolS2cDNA     (646) CTTCAACGCCGGCATGTTCGTGCACGAGCCCAGCATGGCCACCGCCAAGG
HvGolS2gDNA     (777) CTTCAACGCCGGCATGTTCGTGCACGAGCCCAGCATGGCCACCGCCAAGG
                   851                                                 900
HvGolS2cDNA     (696) CGCTCCTGGAAACCCTCCGCGTGACGCCGACCACCCCATTCGCGGAGCAG
HvGolS2gDNA     (827) CGCTCCTGGAAACCCTCCGCGTGACGCCGACCACCCCATTCGCGGAGCAG
                   901                                                 950
HvGolS2cDNA     (746) G--------------------------------------------------
HvGolS2gDNA     (877) GCAAGCAGCTAGTGCTTTTGTTCCGAGAGTCCAGATTTAGCGATGATGGC
                   951                                                1000
HvGolS2cDNA     (747) ---------------------------------------ATTTCTTGAAC
```

```
HvGolS2gDNA   (927) GTTCGTGCTCACCTTGTTCTTGCTCCCTTCATCTGCAGGATTTCTTGAAC
                   1001                                              1050
HvGolS2cDNA   (758) ATGTTCTTCAGGGAGCAGTACAAGCCGATCCCGCTGGTCTACAACCTTGT
HvGolS2gDNA   (977) ATGTTCTTCAGGGAGCAGTACAAGCCGATCCCGCTGGTCTACAACCTTGT
                   1051                                              1100
HvGolS2cDNA   (808) GCTGGCAATGCTCTGGAGGCACCCGGAGAACGTCCAGCTGGAGAAGGTCA
HvGolS2gDNA  (1027) GCTGGCAATGCTCTGGAGGCACCCGGAGAACGTCCAGCTGGAGAAGGTCA
                   1101                                              1150
HvGolS2cDNA   (858) AGGTGGTGCACTACTGCGCTGCGG--------------------------
HvGolS2gDNA  (1077) AGGTGGTGCACTACTGCGCTGCGGTAAGTGGGCGTGACCTCTTGGCTGGT
                   1151                                              1200
HvGolS2cDNA   (882) --------------------------------------------------
HvGolS2gDNA  (1127) GGTATGAATTTCGTTCGGTTTTTTGATTACTTTCTTGCATTCTTACGGAT
                   1201                                              1250
HvGolS2cDNA   (882) ----------------------GATCGAAGCCATGGAGGTTCACGGGA
HvGolS2gDNA  (1177) GGATTGGTTACACCTGGTCGCAGGGATCGAAGCCATGGAGGTTCACGGGA
                   1251                                              1300
HvGolS2cDNA   (908) AAAGAGGACAACATGGACAGGGAGGACATAAAGATCCTCGTTAGGAACTG
HvGolS2gDNA  (1227) AAAGAGGACAACATGGACAGGGAGGACATAAAGATCCTCGTTAGGAACTG
                   1301                                              1350
HvGolS2cDNA   (958) GTGGGATATCTACAACGACGAGAGCCTCGATTTCAAGGGCCTGCCCGCCC
HvGolS2gDNA  (1277) GTGGGATATCTACAACGACGAGAGCCTCGATTTCAAGGGCCTGCCCGCCC
                   1351                                              1400
HvGolS2cDNA  (1008) TGGCCGCGGACGCCGACGAGCTCGAGGCGGCCGCGACGAAGCCGCTCCGC
HvGolS2gDNA  (1327) TGGCCGCGGACGCCGACGAGCTCGAGGCGGCCGCGACGAAGCCGCTCCGC
                   1401                                              1450
HvGolS2cDNA  (1058) GCGGCCCTTGCGGAAGCTGGCACTGTCAAATACGTCACCGCGCCCTCGGC
HvGolS2gDNA  (1377) GCGGCCCTTGCGGAAGCTGGCACTGTCAAATACGTCACCGCGCCCTCGGC
                   1451                                              1500
HvGolS2cDNA  (1108) TGCGTAATCCCCGGTCGCCTAGCTCCGGCAGCTGCGCGCGCCAGCAGGCC
HvGolS2gDNA  (1427) TGCGTAATCCCCGGTCGCCTAGCTCCGGCAGCTGCGCGCGCCAGCAGGCC
                   1501                                              1550
HvGolS2cDNA  (1158) CTCGTGGAGTGCCCGTGCCACGTATAAGCATTTGCATTTTAGTATTTTGC
HvGolS2gDNA  (1477) CTCGTGGAGTGCCCGTGCCACGTATAAGCATTTGCATTTTAGTATTTTGC
                   1551                                              1600
HvGolS2cDNA  (1208) GTCTGTTCGGTTCCAAGTTAGCAGTTAGCAGTATCATTTCAAGTTTAAGA
HvGolS2gDNA  (1527) GTCTGTTCGGTTCCAAGTTAGCAGTTAGCAGTATCATTTCAAGTTTAAGA
                   1601                                              1650
HvGolS2cDNA  (1258) TAGGAGGTTTGATCGAAACAGTCCACCTATGTGAATACCCTTACACCCTT
HvGolS2gDNA  (1577) TAGGAGGTTTGATCGAAACAGTCCACC----------------------
                   1651                                              1700
HvGolS2cDNA  (1308) CGTGAAGTAGAGTGCTGACGTCCTGTATGAAAGAATTATGTGTTGAATCA
HvGolS2gDNA  (1604) --------------------------------------------------
                   1701                                              1750
HvGolS2cDNA  (1358) ATAGGAAGAAGCAAACATTATGGCGAAAAAAATAAAAAATAAAAAAAAAA
HvGolS2gDNA  (1604) --------------------------------------------------
                   1751
HvGolS2cDNA  (1408) AAAAA
HvGolS2gDNA  (1604) -----
```

Figure 4.6 Alignment of cDNA and genomic DNA of *HvGolS2*. The alignment of sequences revealed existence of three introns. Alignment of nucleotide sequences were conducted using the AlignX program of Vector NTI V.9 suite. The intron sequences are shown in bold font.

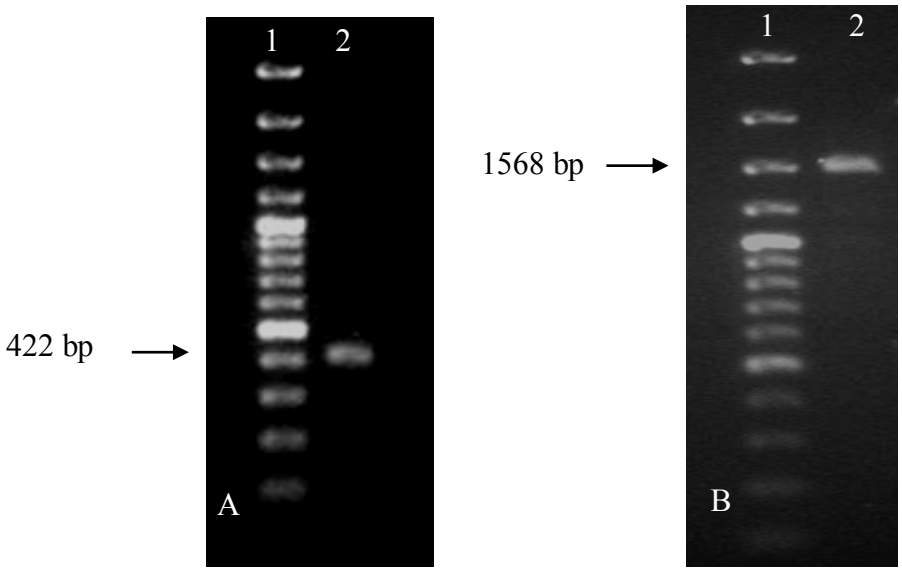


Figure 4.7 *HvGolS1* amplified probe templates. (A) The 3' probe amplified by primer pair n194F5/R from cDNA, and (B) full-length probe amplified with primers n194F3/R from genomic DNA. Lane 1: DNA marker, Lane 2: PCR product. The location of primers is shown in Figure 4.4.

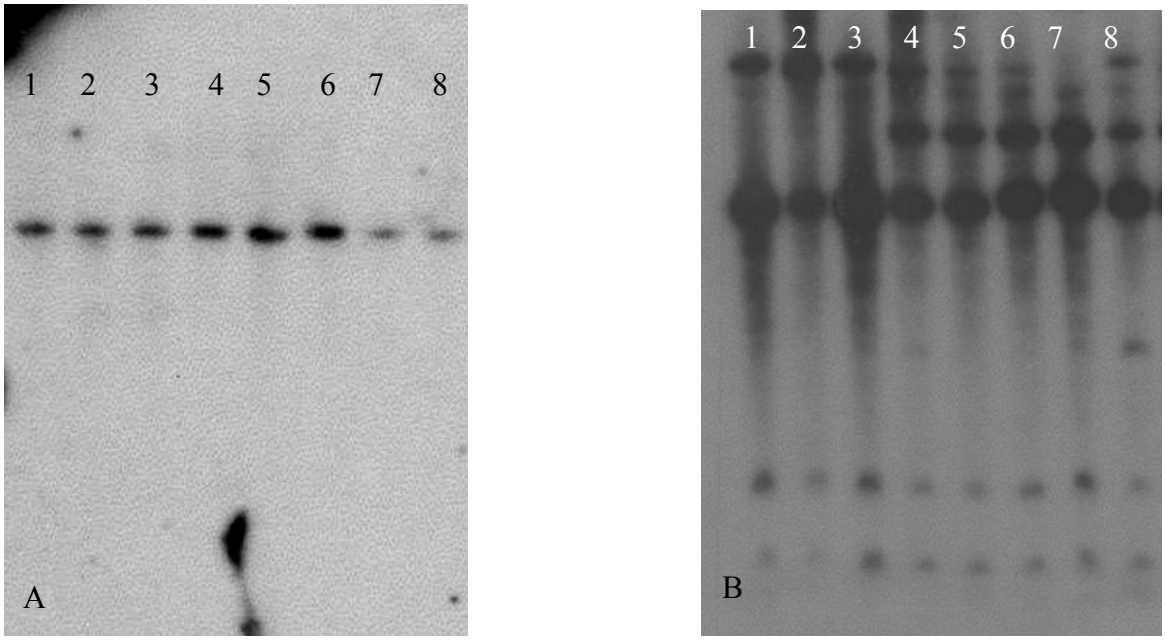


Figure 4.8 Southern blot analyses of different barley cultivars with *HvGolS1* probes. Genomic DNA digested with HindIII restriction enzyme and probed with the 3' probe (A) and full-length probe (B). Cultivars from left to right: Atlas, Atlas 46, Atlas 68, Turk, Clipper, Galleon, Golden Promise and Haruna Nijo.

5.67. The predicted protein sequences of HvGolSs share 74% identity and 80% similarity to each other. *GolS1* has a typical poly A^+ signal (AATAAA) starting at nucleotide position 1123, whereas *GolS2* lacks this signal.

Post-translational modification of proteins affects their activity. Various programs at the EXPASY site were used to predict possible post-translational modifications of HvGolS proteins. Among different possible modifications putative phosphorylation sites for GolS1 threonine 192, 206, 264 and tyrosine 18, 91, 106, 155, 178, 222, 286 and kinase-specific phosphorylation for serine 316 was identified. GolS2 predicted to have a phosphorylation site for serine 73, threonine 10, 198, 205, 212, 270, 329 and tyrosine 98, 113, 228, 292, 332 and also kinase-specific phosphorylation for threonine 329.

4.3.3 Similarity search and phylogenetic analysis

Next, public databases were searched for identifying similar sequences and functional annotation. Search of Conserved Domain Database with the *GolS1* and *GolS2* encoded amino acid sequences identified them as Glycosyl transferase family 8. This family includes enzymes that transfer sugar residues to donor molecules (pfam01501). The protein identified with highest sequence similarity to HvGolS1 was an uncharacterised galactinol synthase from wheat (Accession No. BAF51566) showing 95% identity. HvGolS2 was found to be similar to the characterised (Zhao *et al.* 2003) maize galactinol synthase 3 protein (Accession No. AAO48782) with 84% identity.

Multiple sequence alignments and phylogenetic analysis can be used to predict the function of newly isolated genes based on their sequence conservation with sequences of experimentally characterised proteins. Non-redundant full-length GolS proteins used for phylogenetic study included 21 from dicotyledonous and 10 from monocotyledonous species. All of these proteins contained a Glycosyl transferase domain. Identified proteins were aligned by ClustalX program (Fig. 4.9) which showed the sequences of these proteins are much conserved among different species. A common feature of the proteins was the existence of an APSSA motif at the end of the protein except one sequence. An un-rooted phylogenetic analysis was performed on aligned amino acid sequences using neighbour joining algorithm. Sequences were found to form four distinct groups (A-D) with at least one member with experimentally determined galactinol synthase activity in

each group (Fig. 4.10). Grasses sequences formed one clade indicating that these sequences share highest homology amongst each other.

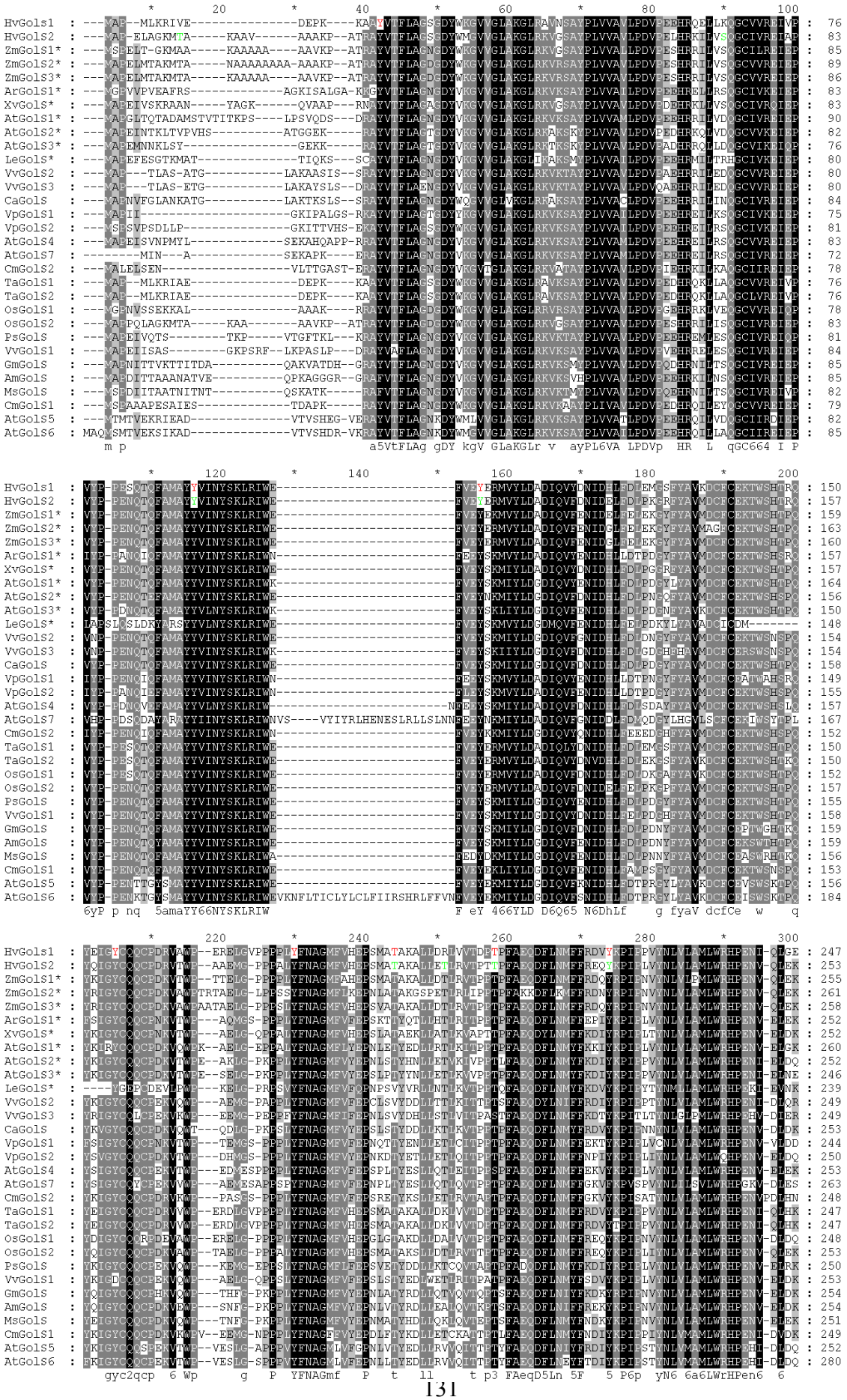
4.3.4 Subcellular localisation

Similarity searches and alignment with experimentally determined GolS indicated GolS activity for the cloned genes. GolS has been shown to be localised in the cytoplasm and both proteins were predicted to be localised in the cytoplasm by CELLO, WolfPSORT and SherLoc programs. To confirm the localisation, GFP fusion was employed. The *HvGols1ORFfu* fragment was amplified (Fig. 4.11) to construct the pHvGolS1:GFP vector for transient expression in barley and Arabidopsis leaves. The targeting of GolS1:GFP was observed in multiple independent bombardment experiments with GFP fluorescence being visible in cytoplasm (Fig 4.12).

4.3.5 Expression analysis

Generally, expression of genes is assumed to be associated with a biological function in expressed tissue. Northern hybridisation, Q-PCR and barley GeneChip data were used to study the spatial and temporal expression of *HvGolS* transcript under biotic and abiotic stresses as well as development. Barley *GolS1* and *GolS2* genes are represented by probe sets 3810_at and 3811_at on the Barley1 chip, respectively.

Northern blot analysis with the gene specific 3' probe showed that *GolS1* gene is transiently up-regulated at 12-24 hours post-inoculation with *R. secalis* only in resistant (Atlas 46) cultivar in comparison to control (Fig. 4.13). Transcript levels in epidermal tissue of barley leaves in response to inoculation with *R. secalis* was examined via Q-PCR (Figure 4.14) and showed a low level of transcript before inoculation. In both cultivars, the gene was upregulated by pathogen but the transcripts accumulated sharply 6 h post-inoculation and reached its peak in Atlas 46 (resistant cultivar) at 24 h. In contrast, in Atlas (susceptible cultivar) accumulation of transcripts was slow and started at 24 h after inoculation.



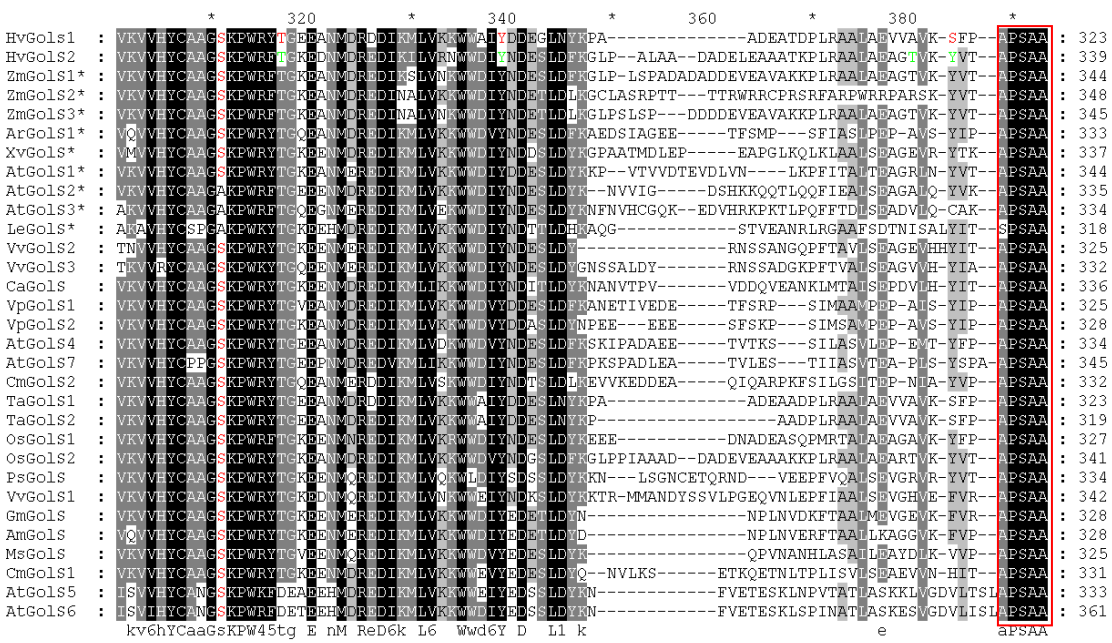


Figure 4.9 Multiple sequence alignment of plant's galactinol synthase proteins. Alignment was based on pairwise similarity using ClustalX program. A pentapeptide motif at the C-terminal end is boxed. Predicted phosphorylation residues in this study are shown in red and green for barley GolS1 and GolS2 proteins, respetively. A putative serine phosphorylation site in position 311 sggested by Sprenger and Keller (2000) is marked in red. Dashes indicate introduced gaps. Shading represents the degree of sequence identity at each residue position. Sequence names followed by an * indicates that GolS activity of the protein has been shown with heterologously expressed protein in *E. coli*. The abbreviations and accession numbers are as following: Am: *Ammopiptanthus mongolicus* (AmGolS: ABF66656), Ar: *Ajuga reptans* (ArGolS1: CAB51534), At: *Arabidopsis thaliana* (AtGols1: AAB63818, AtGols2: AAG09103, AtGols3: AAC33195, AtGolS4: AAB71970, AtGolS5: BAB10052, AtGolS6: CAB79480, AtGolS7: AAC24075), Ca: *Capsicum annuum* (CmGolS: ABQ44212), Cm: *Cucumis melo* (CmGolS1: AAL78687, CmGolS2: AAL78686), Gm: *Glycine max* (GmGolS: AAM96867), Hv: *Hordeum vulgare*, Le: *Lycopersicnm esculentum* (LeGolS: AAL26804), Ms: *Medicago sativa* (MsGols: AAM97493), Os: *Oryza sativa* (OsGolS1: NP_001060697, OsGolS2: EAY89768), Ps: *Pisum sativum* (PsGolS: CAB51130), Ta: *Triticum aestivum* (TaGolS1: BAF51566, TaGolS2: BAF51565), Vp: *Verbascum phoeniceum* (VpGolS1: ABQ12640, VpGolS2: ABQ12641), Vv: *Vitis vinifera* (VvGolS1: CAO40163, VvGolS2: CAN66209, VvGolS3: CAO17390), Xv: *Xerophyta viscosa* (XvGolS: ABK27907), Zm: *Zea mays* (ZmGolS1: AAQ07248, ZmGolS2: AAQ07249, ZmGolS3: AAQ07250).

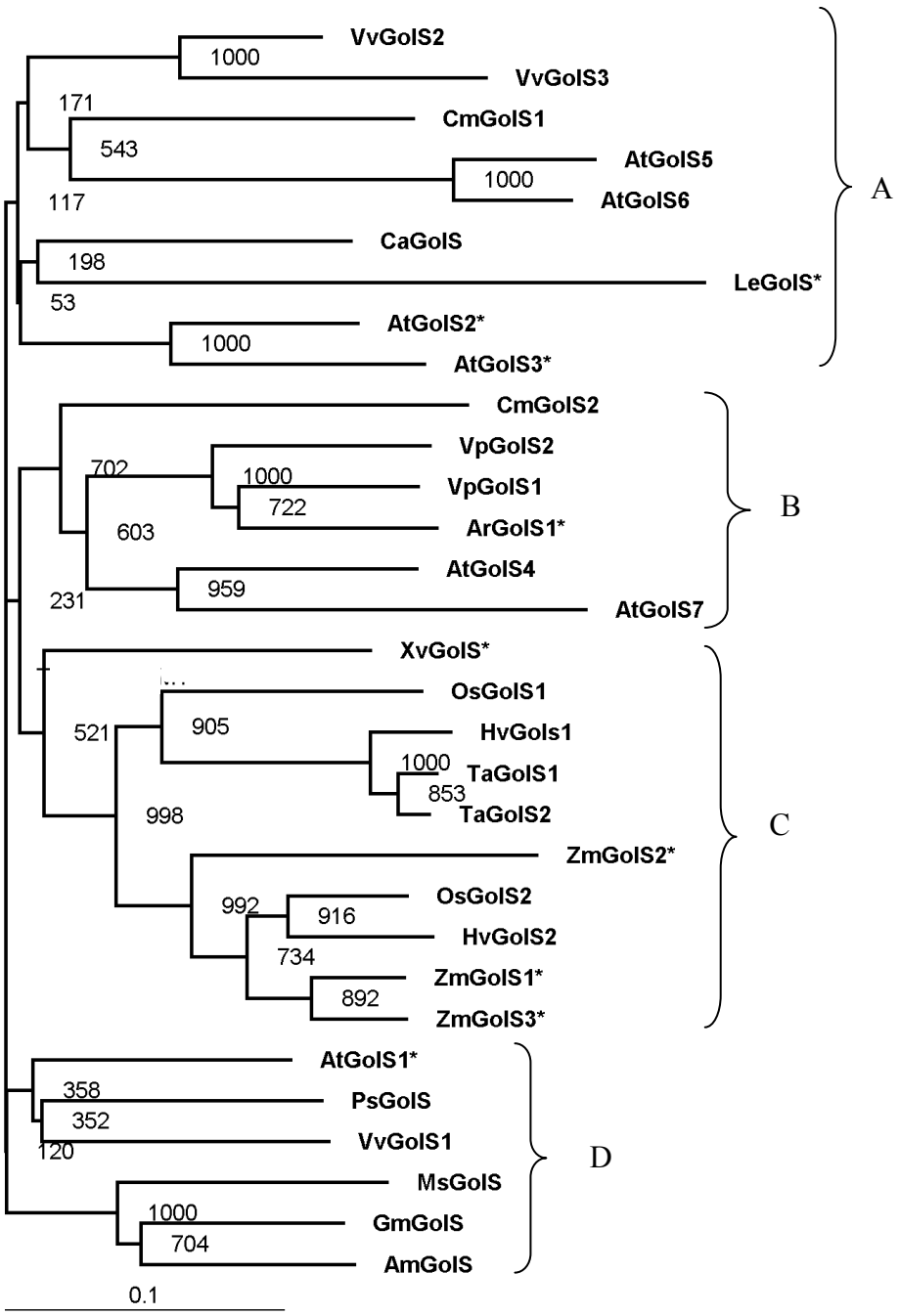


Figure 4.10 Phylogenetic relationships of galactinol synthase proteins from diverse plants. Other species proteins similar to HvGolS were retrieved as described in Section 2.34 and used in a multiple sequence alignment together with barley homologues isolated in this work (Fig. 4.9). The ClustalX program was used to align the retrieved sequences and a phylogenetic tree was created using the neighbour-joining method. The TreeView program was used to display the tree. Branch length reflects the extent of sequence divergence. The reliability of the cluster analyses was tested by bootstrap confidence limits and indicated as success per 1000 bootstrap trials presented on nodes. The proteins accession numbers are as in Figure 4.9.

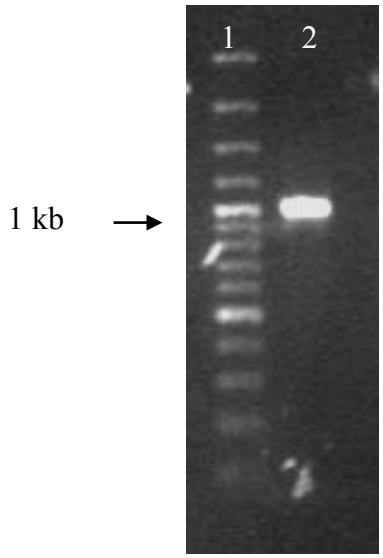


Figure 4.11 Amplification of the *HvGolS1ORFFu* fragment. PCR fragment was amplified using the n194F7/R12 primer set. Lane1: 100 bp DNA ladder, Lane 2: PCR product.

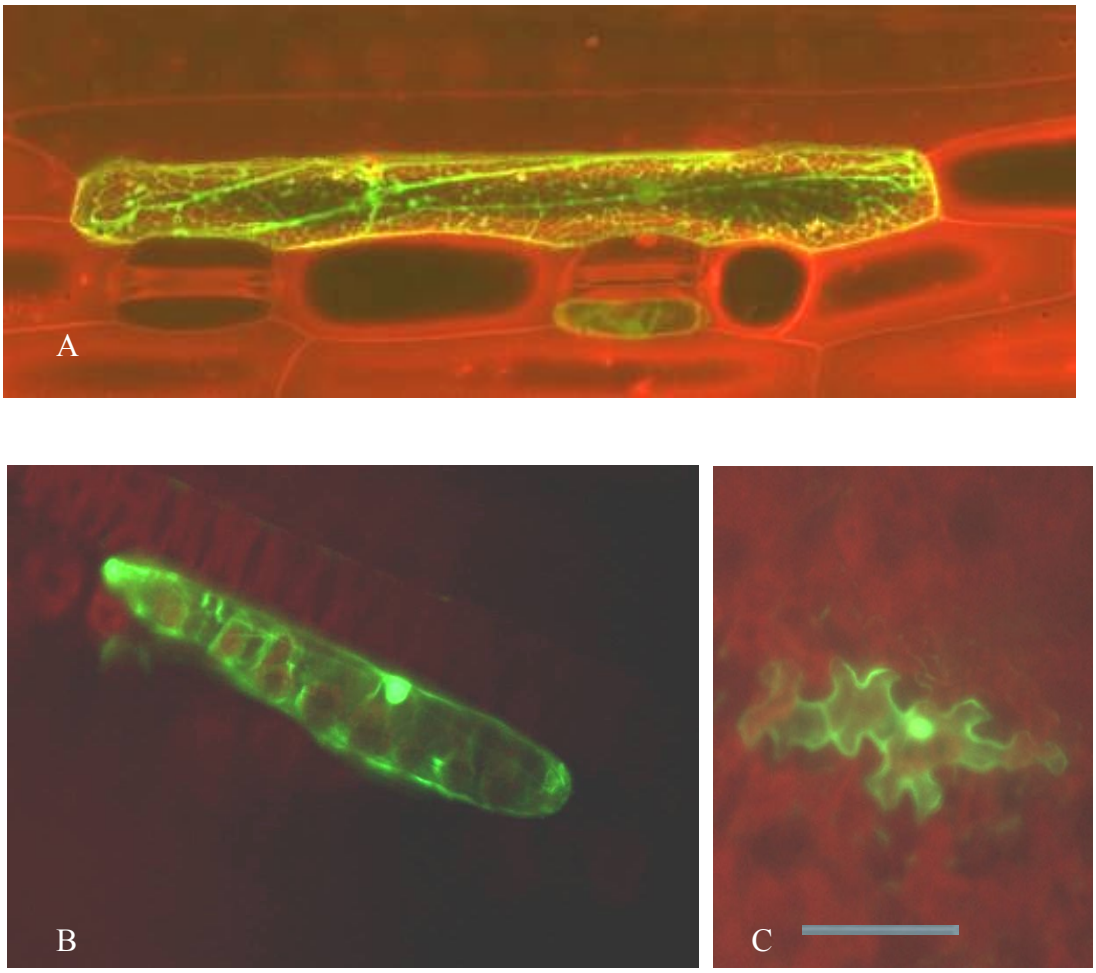


Figure 4.12 Barley and Arabidopsis epidermal cells bombarded with pHvGolS1:GFP construct showed cytoplasmic localisation. (A) Combined shots of a cell expressing fusion protein taken by confocal laser scanning microscope. (B) A barley cell expressing fusion protein under fluorescence microscope. (C) An Arabidopsis cell expressing the same construct under fluorescence microscope (C). Bar= 100 µm.

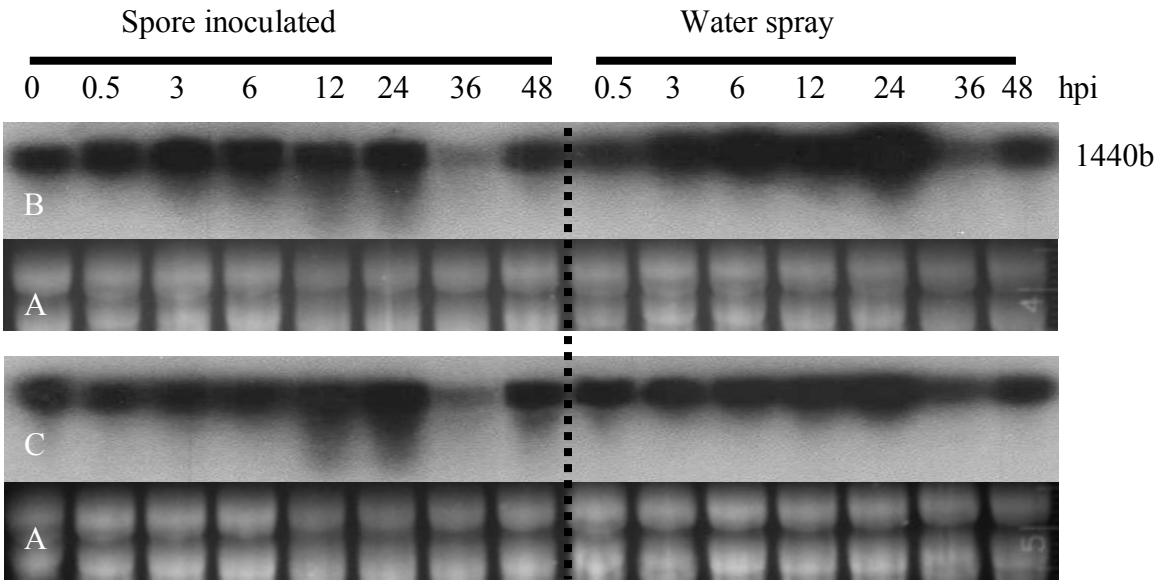


Figure 4.13 Temporal expression levels of *HvGolS1* in leaves of barley plants inoculated with *R. secalis*. (A) Denaturing agarose gel of total RNA (20 µg/lane) isolated from barley leaves. The tissue collection time after inoculation are indicted on top of lanes. Northern blot hybridised with *HvGolS1* 3' probe for susceptible cultivar Atlas RNA (B) and resistant cultivar Atlas 46 RNA (C). Ribosomal 18S and 28S RNA were used as loading control.

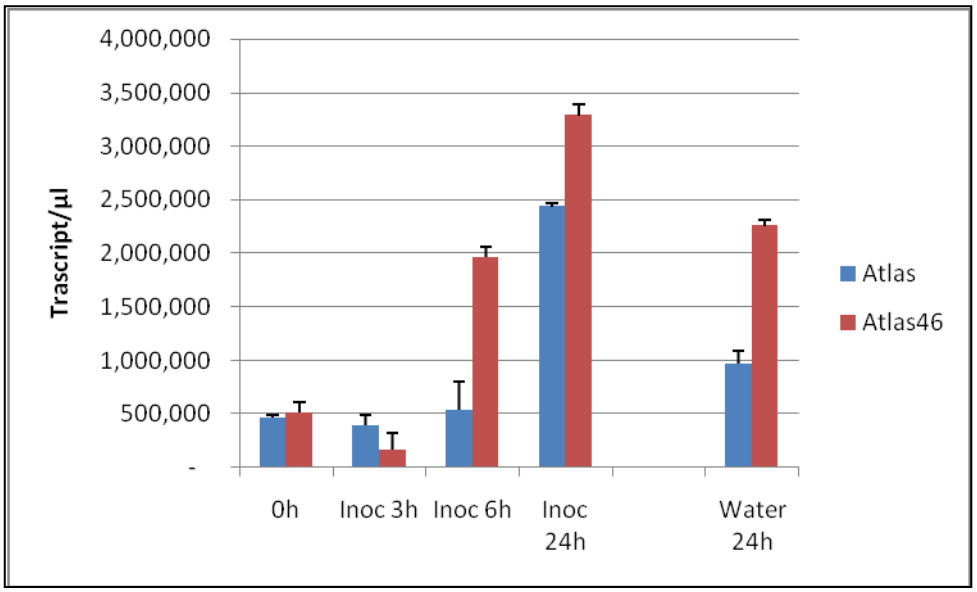


Figure 4.14 Normalised expression levels of *HvGolS1* in barley leaf epidermis. Levels of mRNA are presented as number of copies per micro litre of cDNA after normalisation. Error bars show standard deviations of four replicates for each mRNA. 0h: before inoculation, Inoc 3h, Inoc 6h, Inoc 24h, represents 3, 6 and 24 hours post inoculation respectively, Water 24h: mock-inoculated plants 24 hours after treatment. Data were normalised against *Cyclophilin*, *α-Tubulin* and *Heat Shock Protein 70* mRNA levels.

Expression level of both barley *GolS* genes did not show a strong response to the *Blumeria graminis* f sp *hordei* and *Fusarium gramineaeum* (Figs. 4.15, 4.16, 4.17). However, both genes were expressed at a higher level in seedling roots, leaves, pistil, anthers and bracts in comparison with other tissues examined. During seed maturation, tissue specific expression was observed. *GolS1* transcript was increased in embryos 22 day after pollination (DAP), whereas *GolS2* mRNA level was increased in endosperm at the same time (Fig. 4.18).

Induction of *GolS* genes under abiotic stresses have been shown in dicotyledonous plants. Therefore, for comparison the kinetics of *HvGolS1* expression during frost, drought and salinity was studied. Figure 4.19 shows transcript levels in frost-treated and control plants. Before cold treatment, varying levels of transcript were measured in different cultivars. Cold exposure generally caused a five-fold increase in transcript levels. Interestingly, two cold tolerant cultivars, Haruna Nijo and Amagi Nijo showed the highest expression peak.

The response of *HvGolS1* to drought was a reduction in transcript levels. Although mRNA levels were different among cultivars, at any time point the level of transcript for each cultivar was higher in control plants than in drought-treated plants. Interestingly, rewatered plants showed a differential response. Barque73, a drought tolerant barley cultivar showed fast recovery and its galactinol synthase level reached that of control plants (Fig. 4.20).

Salt treatment did not have a consistent effect on *GolS1* transcript level and was dependent on the cultivars and the time after treatment (Fig. 4.21). The expression levels did not show a correlation with tissue Na^+ concentration (Appendix E). Publicly available barley GeneChip data from various salinity experiments deposited in GEO (Section 2.33) were interrogated for expression of barley *GolS* genes under salinity stress. As the quality of data was not of a good standard at different data points or replications, only averages of biological replicates with good quality data were used in this analysis. Expression levels in shoot tissue of cultivar Golden Promise shows that both genes transcript level slightly increased in response to salinity, but application of jasmonic acid (JA) upregulated only the transcript of *GolS2* (Fig. 4.22). Pre-treatment of plants with JA followed by salinity treatment had less influence on the accumulation of mRNA than by salinity alone.

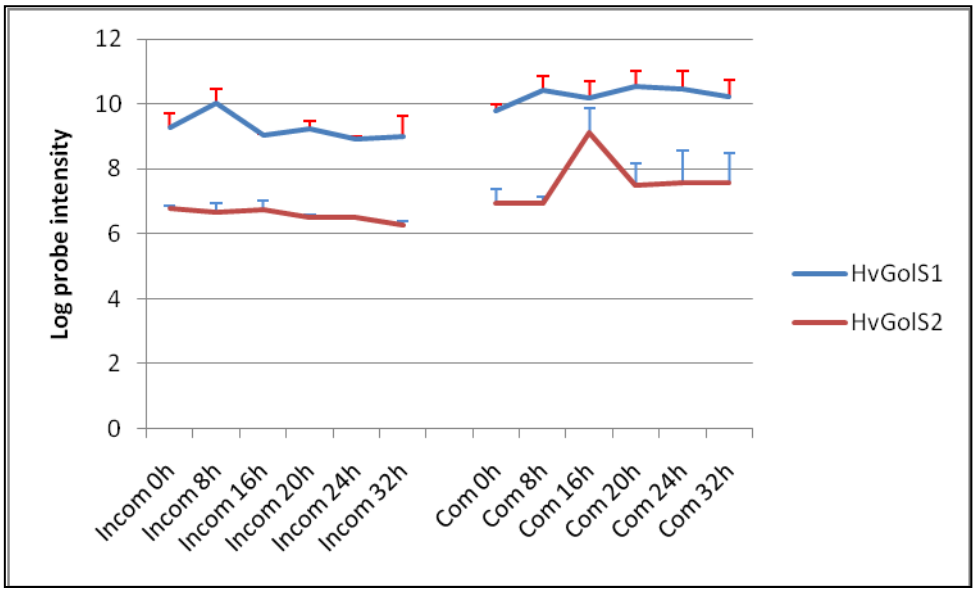


Figure 4.15 Expression of *GolS* genes in response to *Blumeria graminis*. Normalised average probe set intensity levels of barley *GolS* genes in incompatible interaction (Incom) between barley cultivar CI16151 (*Mla6*) and *Blumeria graminis* f. sp *hordei* 5874 (*AvrMla6*) and compatible interaction (Com) of the same barley cultivar with *Bgh* K1 (*AvrMla13*). Probe intensities were the RMA normalised average of three biological replicates. The error bars represent standard deviations.

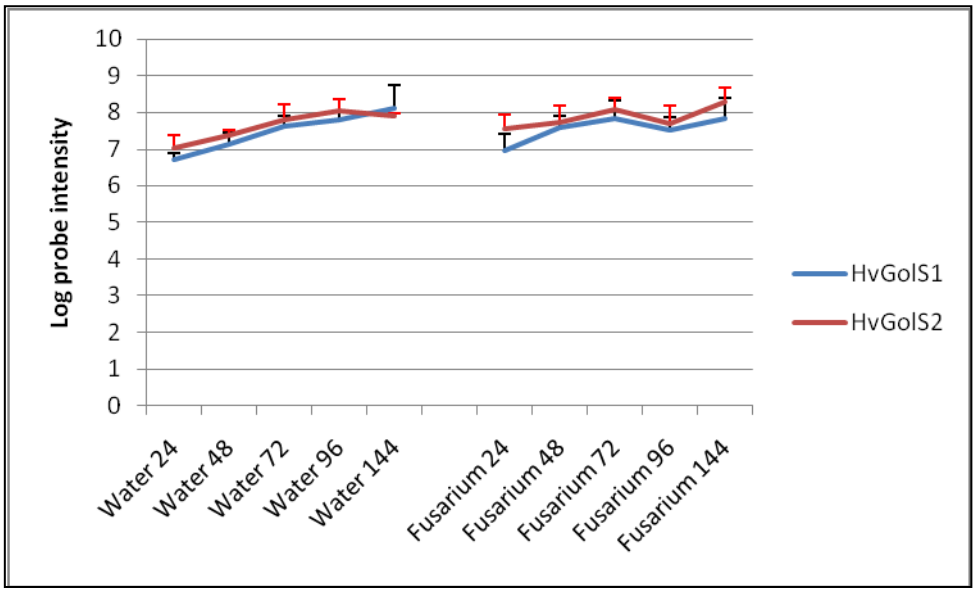


Figure 4.16 Expression of *GolS* genes in response to *Fusarim graminearum*. Normalised average probes set intensity levels of barley *GolS* genes in interaction between barley cultivar Morex and *Fusarim graminearum*. Probe intensities were the RMA-normalised average of four independent replications of the experiment and an extra fifth replication at 24 and 72 h after inoculation. The plants were spore or water inoculated and leaves were harvested at different times after inoculations. The error bars represent standard deviations.

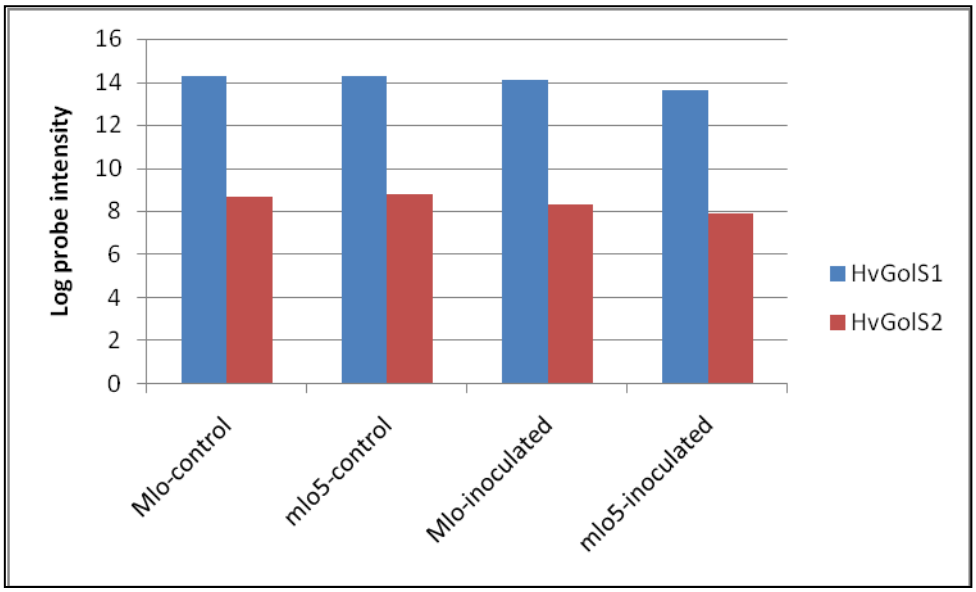


Figure 4.17 Expression of *GolS* genes in response to *Blumeria graminis* in *mlo* resistance. Normalised probe set intensity levels of barley *GolS* genes in interaction between barley cultivars with *Mlo* (compatible) and *mlo5* (incompatible) alleles inoculated with *Blumeria graminis* f. sp *hordei*. Probe intensities were RMA-normalised. The control plants were inoculated with water.

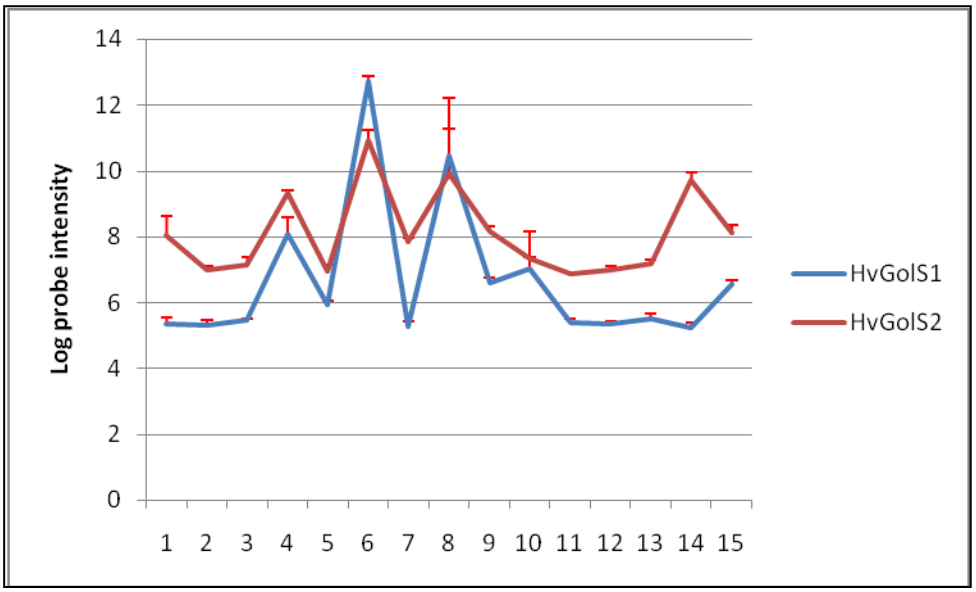


Figure 4.18 Normalised average probe intensity levels of *HvGolS1* and *HvGolS2* during development in cultivar Morex. Probe intensities were the RMA-normalised average of three biological replicates. The error bars represent standard deviations. The numbers on the X-axis represent following tissue types: 1: coleoptyle, 2: radical, 3: embryo during germination, 4: seedling root, 5: seedling crown, 6: leaf, 7: immature inflorescence, 8: bracts, 9: pistil, 10: anthers, 11: caryopsis 5 day after pollination (DAP), 12: caryopsis 10 DAP, 13: caryopsis 16 DAP, 14: embryo 22 DAP, 15: endosperm 22 DAP.

Comparison of shoot and root response to salinity in cultivar Golden Promise after 25 days exposure to salinity shows that expression of *GolS1* is only up-regulated in root tissue (Fig. 4.23). In a time course study in shoots of cultivar Morex slight up-regulation of *GolS1* transcript at 3, 8 and 27 h after salinity treatment in comparison to control treatment was observed, but levels of mRNA were different at each time point (Fig. 4.24).

4.3.6 Recombinant HvGolS1 expression

Biochemical activity of candidate GolS can be demonstrated *in vitro* using heterologously expressed protein. The coding region of *HvGolS1* cDNA was amplified (Fig. 4.25) and cloned into pQEs expression vectors in order to generate recombinant proteins containing terminal histidine tags. Recombinant GolS1 was expressed with both pQE30HvGols1 and pQE70HvGolS1 constructs and SDS-PAGE analysis of the resulting protein appeared as a single band at a position consistent with the predicted molecular weight of 38.7 KDa (Fig. 4.26).

4.3.7 Generation and analysis of transgenic lines

The possible role of HvGolS1 protein activity in drought tolerance and defence was examined by producing and characterising over-expression and knockdown transgenic lines. The coding region was amplified (Fig. 4.27) for constructing over-expression (pHvGolS1SE) and antisense (pHvGols1AS) vectors. To suppress endogenous *HvGolS1* transcript levels in transgenic barley antisense and RNAi approach was used. To construct the RNAi vector a sense and an antisense fragment of a 3' region (Fig. 4.4) was amplified from *HvGolS1* using two separate primer combinations (Fig. 4.28). The cloned fragments were excised from pHannibal (Fig. 4.29) and ligated into pPZPUbi to produce pHvGolS1RNAi construct. The constructs were stably transformed into barley. A total of twelve over-expression, six antisense and four RNAi lines were regenerated. The presence of the transgene was verified by PCR amplifying the hygromycin resistance gene in all lines (Fig. 4.30).

For disease resistance analysis eight plants from each line were evaluated for scald symptoms (Fig. 4.31). The t-test revealed significant reduction in symptoms in line 47-4

(RNAi) and increase in 45-3 (over-expression) and 47-1 (RNAi) in comparison to non-transgenic progeny.

Drought tolerance of selected lines was evaluated as described in Section 2.29. The appearance of wilting among pots varied by twelve days (Fig. 4.32), but no difference was observed among transgenic and non-transgenic progeny in the same pot or the rate of recovery after resuming watering.

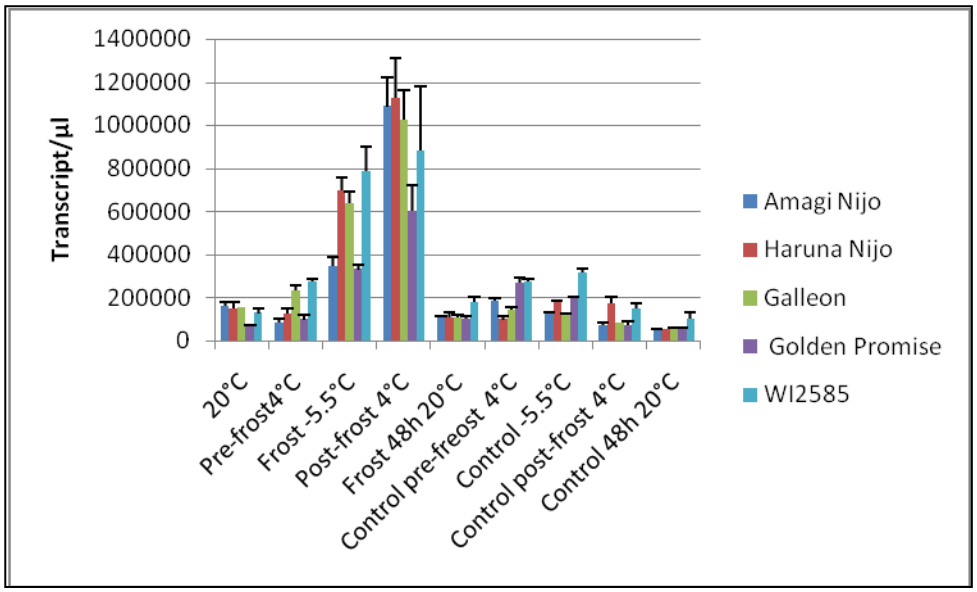


Figure 4.19 Effect of frost treatment on *HvGolS1* expression analysed in leaves at different times during frost treatment. Expression levels were calculated for copy number/µl of cDNA. Data were normalised against *GAPDH*, *Cyclophilin* and *α-Tubulin* mRNA levels. Data were mean values of triplicates in the PCR including standard deviations.

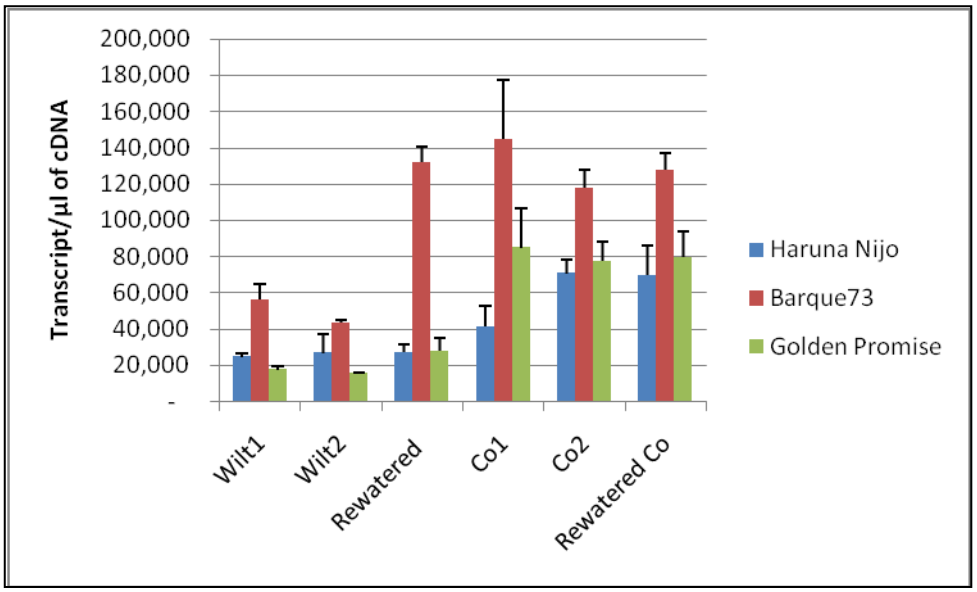


Figure 4.20 Response of barley *GolS1* to drought in different barley cultivars. Two sets of plants were grown under the same condition in a growth cabinet. Wilt1: wilting of leaves was observed, Wilt2: sampling 24 hours after Wilt1, Rewatered: plants were watered and samples taken 24 hours later, Co1, Co2 and Rewatered Co, controls for correspondingly treated plants. Data were normalised against *Cyclophilin*, *GAPDH* and *Heat Shock Protein 70* mRNA levels. Data were mean values of triplicates in the PCR including standard deviations.

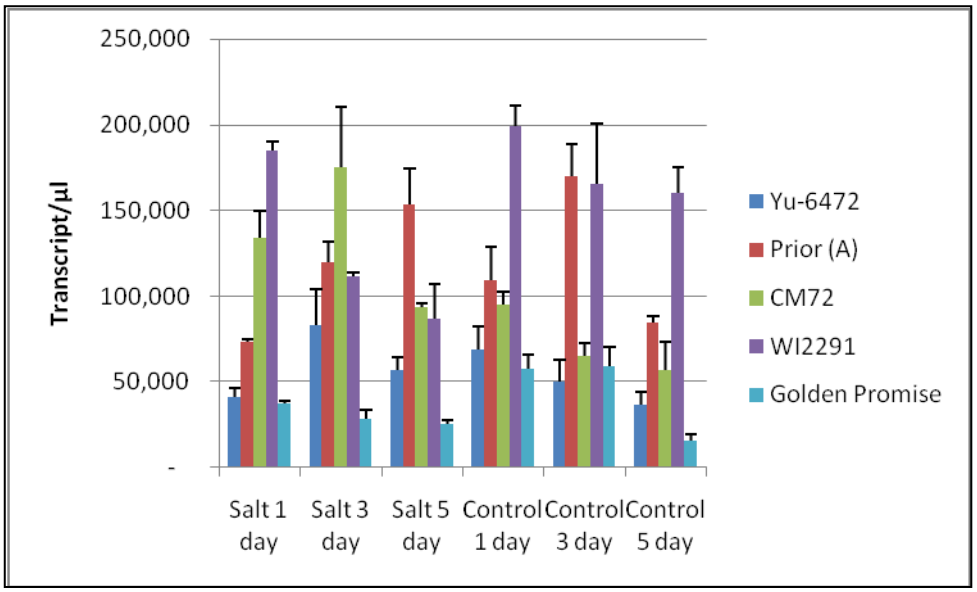


Figure 4.21 Quantitative RT-PCR analysis of the *HvGolS1* in salt treated barley cultivars. Two sets of plants were grown under the same condition in a hydroponic system until emergence of the second leaf. For one set salt was added to a final concentration of 150 mM, with a stepwise increase of 50 mM at 12 hours interval. Leaf samples were harvested one, three and five days after last salt addition. Data are mean values of triplicates in the PCR including standard deviations and were normalised against *Cyclophilin*, *Tubulin* and *GAPDH* mRNA levels.

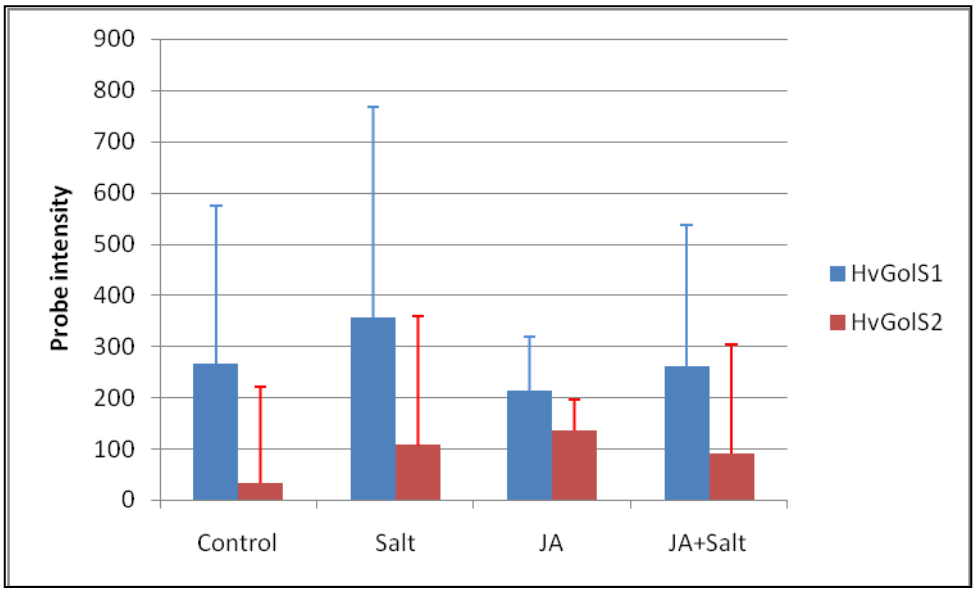


Figure 4.22 Response of *GolS* genes to salinity and JA application in cultivar Golden Promise. Normalised average probe intensity levels of barley *GolS* genes in leaf tissue in response to salinity (Salt), JA application (12 µM) and pretreatment with JA followed by salinity treatment (JA+Salt) in Golden Promise cultivar. Salinity stress of ~ 18 dS m^{-1} was imposed gradually in four equal steps by adding NaCl to growth medium during four days period. Probe intensities were the average of two independent replications per experiment. The error bars represent standard deviations.

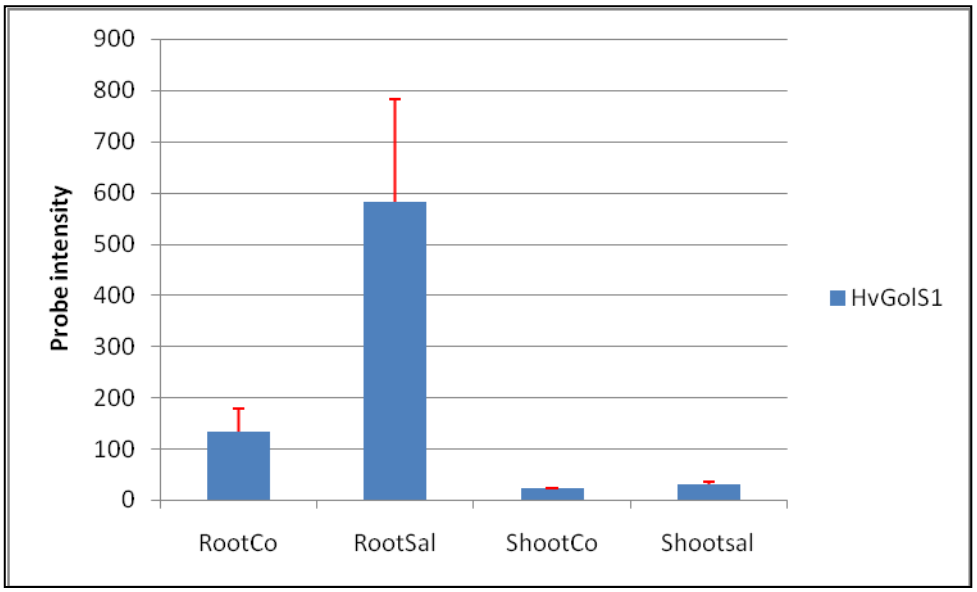


Figure 4.23 *HvGolS1* gene expression in response to salinity in shoot and root tissues in cultivar Golden Promise. Salinity stress was imposed over a period of five days in five equal steps to reach a final concentration of 150 mM NaCl and sampling was performed five days after last NaCl addition. Probe intensities were the normalised average of three independent replications of the experiment. The error bars represent standard deviations.

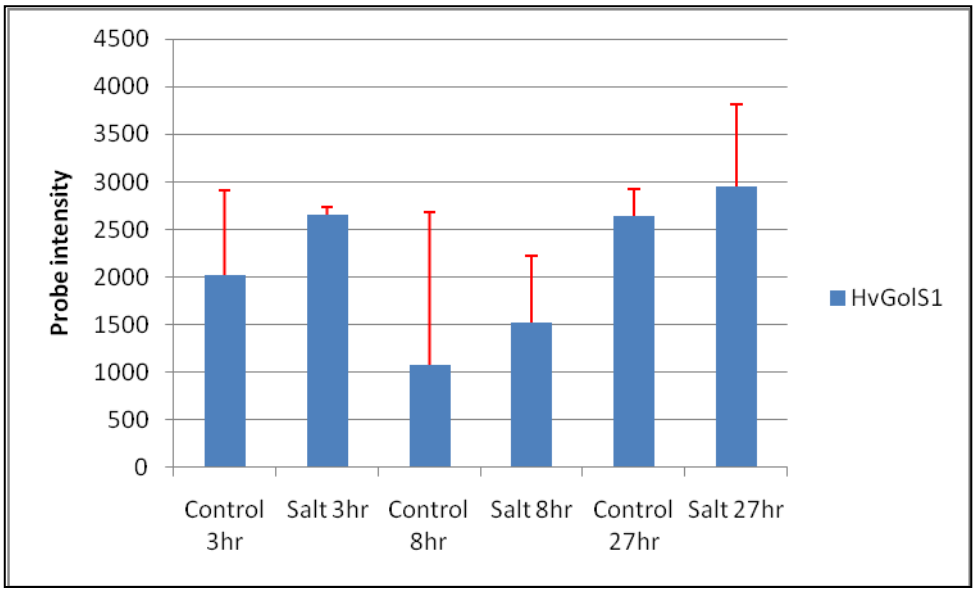


Figure 4.24 Expression of *HvGolS1* gene in response to salinity in Morex cultivar at different time points post addition of 100 mM NaCl. Gradual salt stress was imposed starting on day 14 after transplanting until day 17 with NaCl concentration increments of 25 mM NaCl per day up to 100 mM. Probe intensities were the normalised average of three independent replications of the experiment. The error bars represent standard deviations.

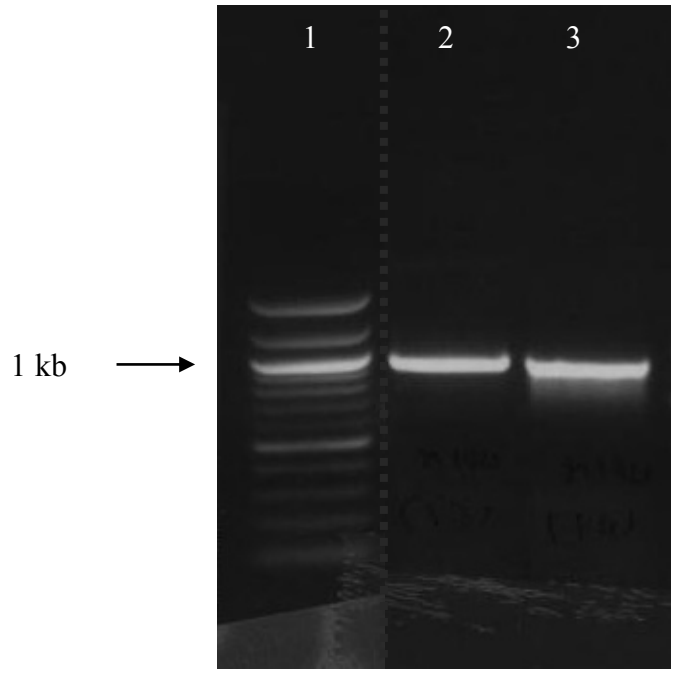


Figure 4.25 Cloning *HvGolS1* coding region for protein expression. The barley *GolS1* cDNA fragments were amplified and gel-purified for sub-cloning into expression vectors to express N- and C-terminal tagged proteins. Lane1: 100 bp DNA ladder, Lane 2: fragment for sub-cloning into pQE30 using primer set n194F13/R13, Lane 3: fragment for sub-cloning into pQE70 using primer set n194F14/R14. The dashed vertical line indicates that pictures has been cut.

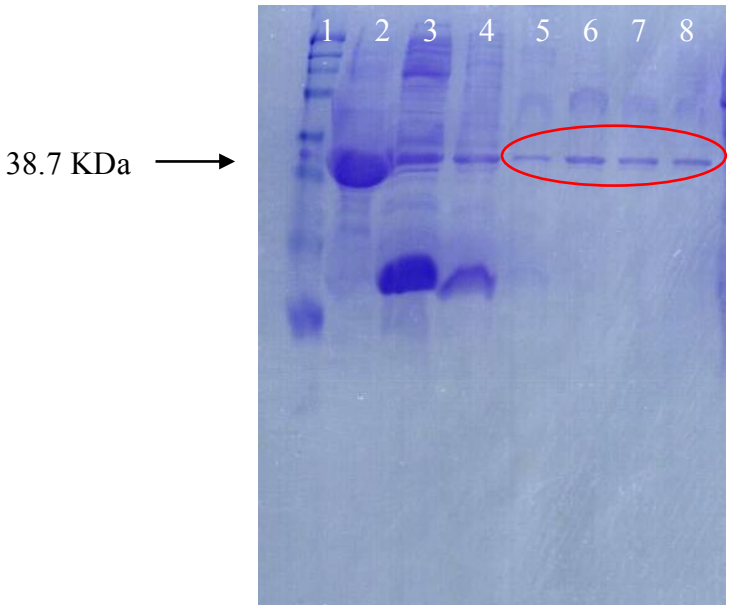
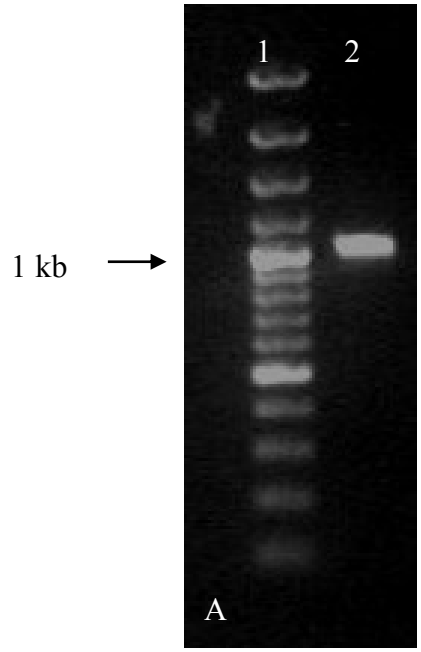


Figure 4.26 SDS-PAGE analysis of HvGolS1 protein. Analysis of protein at different stages of purification, expressed in *E. coli* using QIAexpress system and pQE30HvGolS1. Proteins were separated on a 14% SDS–PAGE and stained with comassie blue R-250. Lane 1: molecular weight protein markers, Lane 2: cell lysate, Lane 3: flow through, Lane 4: first wash, Lanes 5-8: HvGolS1 recombinant protein eluted fractions from the column. The recombinant protein band is marked by red ellipses.



(B)

TATATT**GGATCC**ACCATGGCTCCCATGCTCAAGCGGATCGTGGAGGACGAGCCCAAGAAGGCGGCGTACGTG
ACCTTCCTCGCCGGCTCCGGCGACTACTGGAAGGGCGTGGTCGGCCTTGCCAAGGGCCTCCGCGCCGTCAAC
TCCGCCTACCCGCTCGTGGTGGCCGTGCTCCCCGACGTCCCCGAGGAGCACCGCCAGGAGCTGCTCAAGCAG
GGCTGCGTCGTCCGGGAGATCGTGCCCGTCTACCCGCCGGAGAGCCAGACCCAGTTCGCCATGGCCTACTAC
GTCATCAACTACTCGAAGCTCCGCATCTGGGAGTTCGTGGAGTACGAGAGGATGGTGTACCTGGACGCGGAC
ATCCAGGTGTACGACAACATCGACCACCTCTTCGACCTCGAGATGGGCAGCTTCTACGCCGTCAAGGACTGC
TTCTGCGAGAAGACGTGGAGCCACACCCGGCAGTACGAGATCGGCTACTGCCAGCAGTGCCCCGACAGGGTG
GCGTGGCCGGAGCGCGAGCTGGGCGTGCCCCCGCCGCCGCTCTACTTCAACGCCGGCATGTTCGTGCACGAG
CCCAGCATGGCCACCGCCAAGGCCCTCCTCGACAGGCTTGTCGTCACCGACCCCACCCCGTTCGCCGAGCAG
GACTTCCTCAACATGTTCTTCAGGGACGTGTACAAGCCCATCCCGCCGGTGTACAACCTCGTGCTCGCCATG
CTCTGGAGGCACCCGGAGAACATCCAGCTCGGCGAGGTCAAGGTCGTCCACTACTGCGCCGCGGGTTCGAAG
CCGTGGAGGTACACCGGCGAGGAGGCCAACATGGACAGGGACGACATCAAGATGCTGGTGAAGAAATGGTGG
GCCATCTACGACGACGAGGGCCTCAACTACAAGCCTGCCGCCGACGAGGCCACCGACCCGCTGCGTGCTGCC
CTCGCCGAGGTCGTTGCCGTCAAGTCCTTCCCAGCGCCCTCCGCCGCGTAGTCATCACGCGCCT**GGATCC**AA
TATA

Figure 4.27 Amplifying *HvGolS1* fragment for vector construction. (A) Coding fragment of *HvGolS1* incorporating BamHI restriction site at both ends was amplified by primer set n194F7/R7. Lane 1: DNA marker, Lane 2: PCR product. (B) The sequence of amplified fragment. The underlined sequence indicates extra nucleotide incorporated into primers. Nucleotides in bold are the BamHI recognition site.

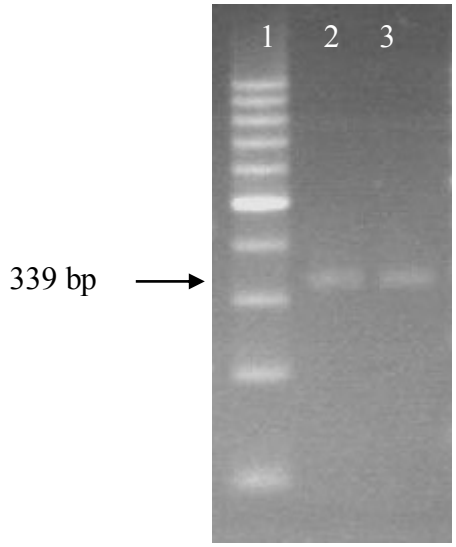


Figure 4.28 Amplifying sense and antisense fragments for RNAi. Sense and antisense fragments amplified by primers n194F10/R10 (sense) and n194F11/R11 (antisense) for cloning into pHannibal. Lane 1: DNA marker, Lane 2: sense fragment, Lane 3: antisense fragment.

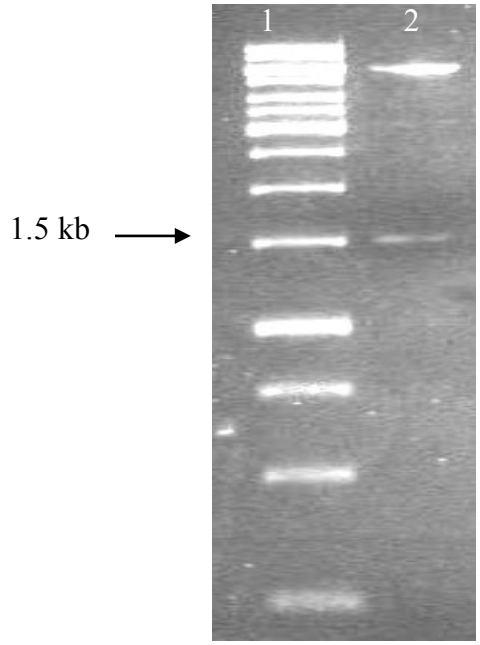


Figure 4.29 Purifying *HvGolS1* containing fragment from pHannibal. Fragment containing sense and anti-sense region of the *HvGolS1* gene was cut from pHannibal by BamHI restriction and purified for cloning into pPZPUbi. Lanes 1: marker, Lane 2: excised fragment containing sense and anti-sense fragment for RNAi (bottom band) and pHannibal vector (top band).

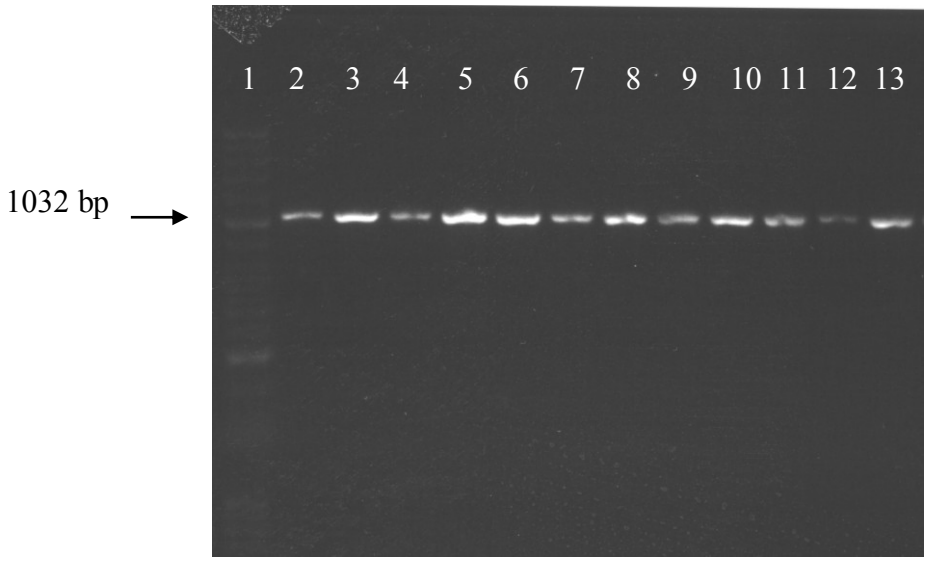


Figure 4.30 PCR verification of *HvGolS1* over-expression lines. Presence of the transgene in transgenic lines transferred with pHvGolS1SE was verified by amplifying the hygromycin resistance gene by primer pair HygF/R. Lane 1: marker, Lanes 2-12: transgenic line, Lane 13: pHvGolS1SE plasmid.

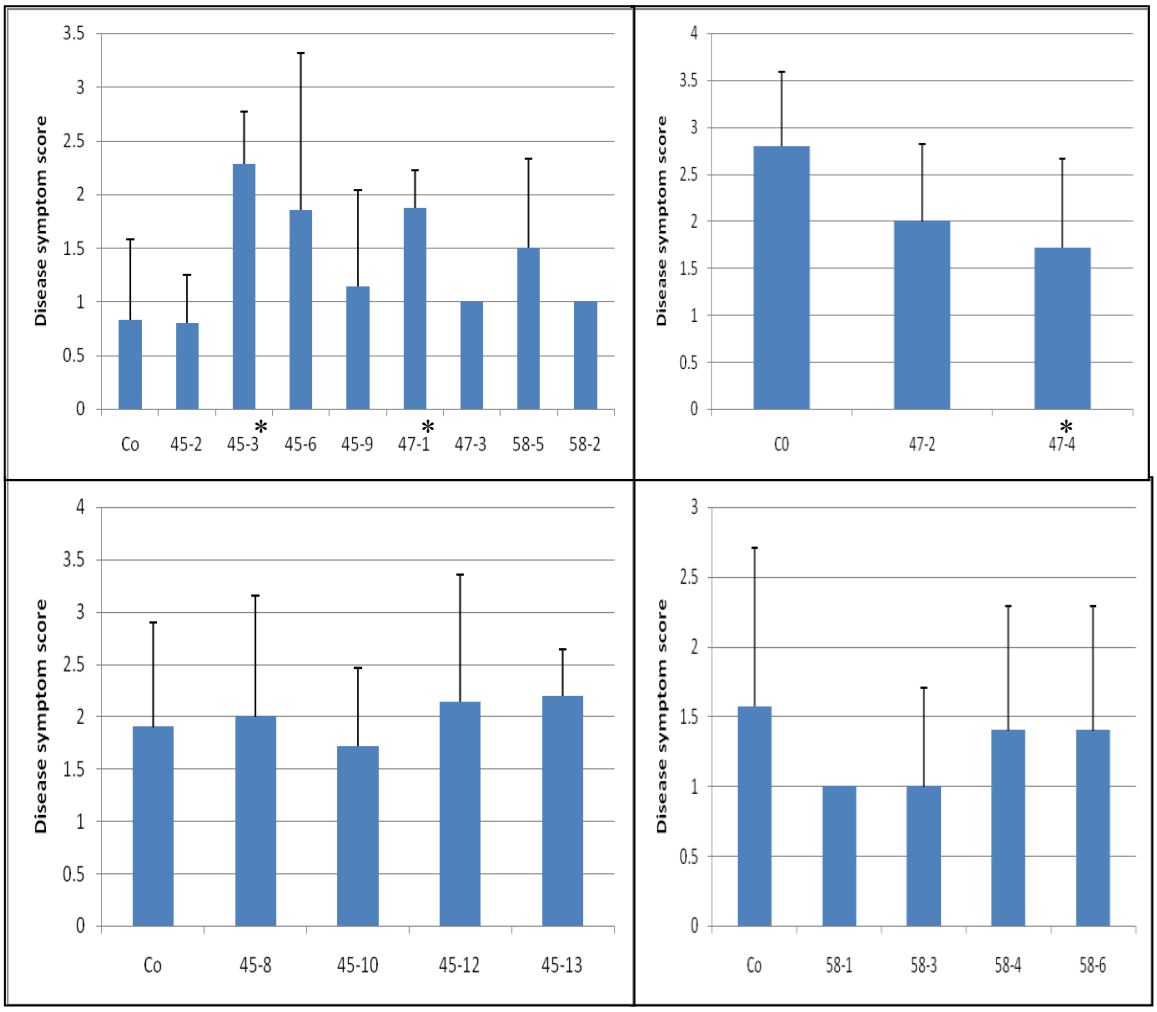


Figure 4.31 Scoring *HvGolS1* transgenic lines for scald symptoms. Eight T_1 plants from each line was inoculated with *R. secalis* strain SA6 and scored for disease symptoms 14 days post-inoculation. The experiment was repeated twice. Controls are non-transgenic progeny of the lines. Lines 45, 47 and 58 represent over-expression, RNAi and antisense lines, respectively. Students' t-test was used to determine significance of difference. Significant differences occurring in both experiments are reported. * indicates significant difference to control plants at 95% confidence.

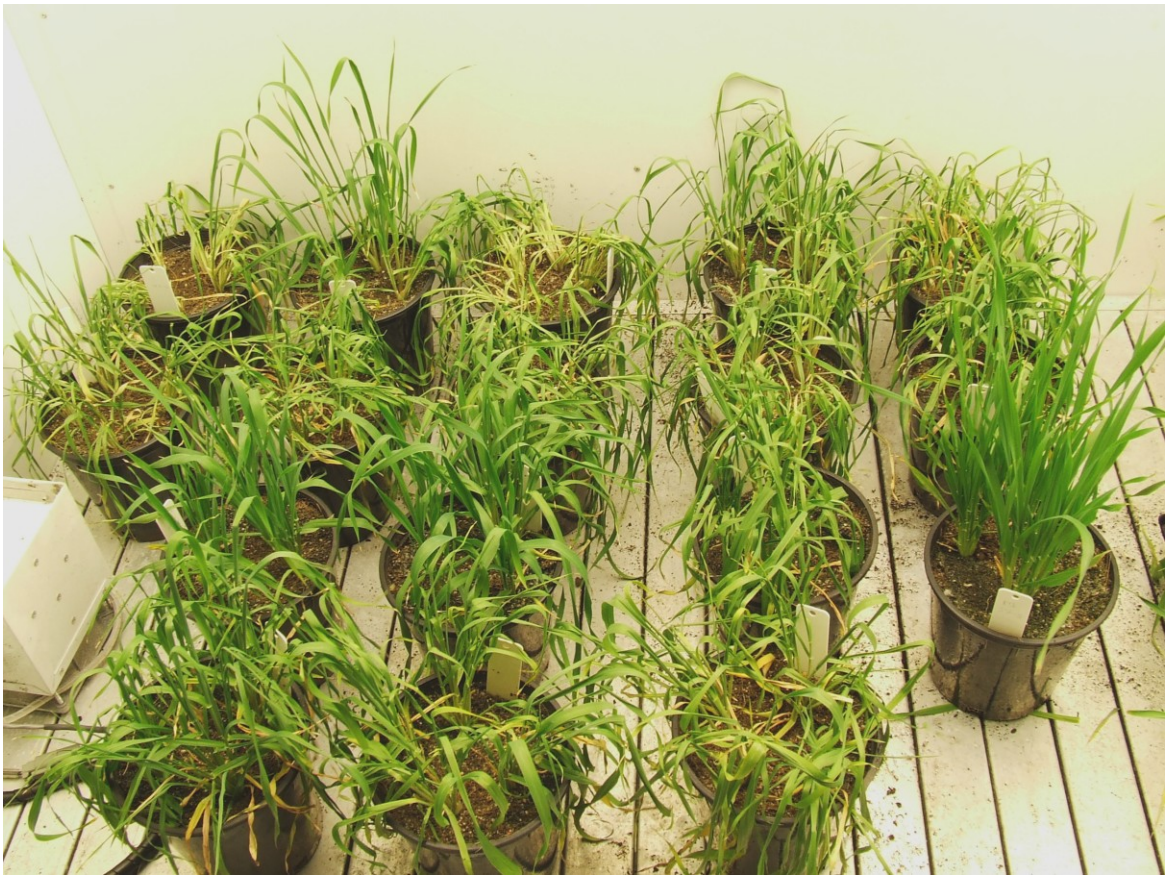


Figure 4.32 Drought tolerance experiment of barley *GolS1* transgenic lines. Eight T_1 plants from each line were grown in two separate pots (4 plants/pot) which were transgene and non-transgene progeny in some pots. Variation in the onset of wilting was observed among different pots was 14 days.

4.4 Discussion

In the study reported in this chapter, the isolation and molecular characterisation of two new barley genes was undertaken and their homology with *GolS* genes from other species was shown. Their expression in response to biotic and abiotic stresses was examined and transgenic plants were regenerated in which the *HvGolS1* transcript levels were modified for *in planta* functional analysis.

4.4.1 Cloning and analysis of *HvGolS* genes

Two members of a gene family were isolated from barley cDNA and genomic DNA by 3'-RACE combined with EST mining. The cloned genes were named *HvGolS1* and *HvGolS2*. The relatively large number of homologous proteins has been shown to have galactinol synthase activity and is the basis to assign GolS activity to the two uncharacterised members isolated here (see also Section 4.4.3). Other evidence also supports the assigned function. *In silico* analysis showed that barley *GolS* genes-encoded proteins do not contain any sequence to direct the protein to a subcellular compartment or the secretory pathway. The prediction was supported by cytoplasmic localisation of the GFP fusion protein. The pattern of fluorescence was quite similar to that of cytoplasmic CFP:TGBp1 (Samuels *et al.*, 2007) in wheat epidermal cells and pUbi:GFP in onion epidermis (Dresselhaus *et al.*, 2005). Previous investigations of the subcellular localisation of various GolS have also indicated co-localisation of GolS with other RFO synthesising enzymes such as raffinose and stachyose synthase in the cytoplasm (Bachmann and Keller, 1995; Sprenger and Keller, 2000; Zhao *et al.*, 2004b).

HvGolS1 and HvGolS2 show highest homology to a wheat (Accession No. BAF51566, 95% identity) and rice protein (Accession No. ABF95621, 90% identity), respectively. Comparison of two sequences revealed high similarity at both DNA and deduced protein amino acid residues. Cloning genomic regions showed that *GolS1* and *GolS2* have two and three introns, respectively. Similarly, most reported *GolS* genes have two introns positioned in two highly conserved regions. In seven Arabidopsis *GolS* genes there is a third intron (Downie *et al.*, 2003). GolS proteins identified to date and in this study contain a conserved hydrophobic pentapeptide (APSSA) carboxy-terminal (Downie *et al.*, 2003; Sprenger and Keller, 2000; Taji *et al.*, 2002). *HvGolS1* 3'-RACE produced homologous fragments with different polyadenylation site. Such occurrence has been

found previously in numerous gene transcripts in plants (Giranton *et al.*, 1995; Golovkin and Reddy, 1996; Hartung and Puchta, 2000; Xiao *et al.*, 2002).

Other *GolS* genes characterised from cereals include one from rice (WSI76, Accession No. BAA05538) which was induced by water stress (Takahashi *et al.*, 1994) and three from maize (*ZmGolS1*, *ZmGolS2*, and *ZmGolS3*; Accession No. AAQ07248, AAQ07249;, AAQ07250, respectively) sharing 84% identity across their entire length at the nucleotide level and at least 92% identity at the protein level (Zhao *et al.*, 2004b). The recombinant proteins from maize cDNAs synthesized galactinol *in vitro*.

The genomic organisation of barley *HvGolS* genes was determined by using two probes. One band was detected when hybridising genomic DNA to the 3' probe whereas the full-length probe hybridised to two DNA fragments. The absence of sites for the restriction enzymes used in either the exons or introns of *HvGolS* was confirmed by sequencing the genomic sequence. Two bands indicate the presence of at least two genes with similar sequences which cross-hybridise due to existence of similar DNA motifs. Direct evidence for existence of at least two *GolS* genes in barley was provided by cloning two family members. The number of *GolS* genes varies in different species, from one in tomato (Downie *et al.*, 2003) up to seven members that have been reported in Arabidopsis (Taji *et al.*, 2002).

4.4.2 Transcript profiling

The role of RFO in plant-pathogen interactions has not been investigated. As a first step to characterise their possible role in plant defence, expression of *HvGolS* in response to various pathogens was determined. The transcripts of *HvGolS1* were found to be upregulated by inoculation with *R. secalis* in both compatible and incompatible interactions in epidermis but with different kinetics. In whole leaf tissues, up-regulation was observed only for resistant genotype in comparison to controls. However, Affymetrix GeneChip hybridisation data indicated that transcripts of *GolS1* and *GolS2* were not altered greatly by *Blumeria graminis* and *Fusarium graminearum* inoculations in comparison to control plants. This finding indicates that expression of the gene could be regulated differently in response to different types of pathogens. One way that GolS can affect plant-pathogen interaction is through reduction of osmotic potential by leading to the synthesis of RFO. A reduction in osmolality could affect fungal uptake of nutrients. which is a prerequisite for successful colonization and infection (Divon and Fluhr, 2007).

Characterisation of a *Stagonospora nodorum* mutant for mannitol 1-phosphate dehydrogenase (*Mpd1*) showed that mutants were unable to sporulate in host wheat plants (Solomon *et al.*, 2005). Since one role of mannitol is the adjustment of osmotic pressure, the inability could be due to disruption of the optimum osmotic balance.

One of plants' responses to water stress caused by frost, salinity or drought is the expression of genes involved in osmolyte synthesis. The accumulation of solutes contributes to the recovery of plants and restoring osmotic potential balance between plant cells and the external water potential. RFO have been proposed to act as an osmoprotectant in dicotelydenous plants, but such a role in monocotelydonus species has not been investigated broadly. RFO biosynthesis requires the presence of galactinol and GolS is believed to be a control point in the biosynthesis of RFO hence affecting osmotic potential of plant cells. The exposure of barley plants to frost had a great influence on transcript abundance of *GolS1* and up to six-fold increase was measured in leaves of treated plants in comparison to control plants. The increase in transcript accumulation was dependent on the cultivar. Interestingly, cultivars Haruna Nijo and Amagi Nijo, which have shown significant frost tolerance (Reinheimer *et al.* 2004), demonstrated the highest level of expression. Similarly, induction of *GolS* genes upon cold exposure has been reported in other species. Studies have demonstrated parallel increase in both GolS gene expression and enzyme activities and RFO accumulation in many species during the process of cold acclimation. Such events have been demonstrated in the crown of winter hardy alfalfa cultivars (Castonguay and Nadeau, 1998; Cunningham *et al.*, 2003), seedling leaves of tomato (Downie *et al.*, 2003), leaves of *Ajuga reptans* (Sprenger and Keller, 2000), pine needles (Hinesley *et al.*, 1992) and Arabidopsis (Taji *et al.*, 2002). Increase in GolS enzyme activity in kidney bean seeds has also been reported upon exposure of plants to cold. This increase was correlated with an increased transcript level in vegetative tissues which disappeared by re-exposure to room temperature (Liu *et al.*, 1998). In our experiment also re-exposure of treated plants to normal growth condition resulted in transcript levels similar to controls. However, such a response is not universal. *GolS* were not up-regulated by cold in germinating tomato and maize seeds but induced in tomato seedling leaves (Downie *et al.*, 2003; Zhao *et al.*, 2004a). Furthermore, *GolS* genes were not up-regulated by cold or dehydration stress in *Cucumis melo* leaves (Volk *et al.*, 2003). It has been suggested that genetic factors controlling seed and vegetative cold tolerance are largely independent (Foolad and Lin, 2001). Comparison of the barley *GolS1*

expression pattern with maize (Zhao *et al.*, 2004b) in which cold stress failed to induce *ZmGolS* transcript accumulation indicates differential regulation of expression in cereals.

Three of the *GolS* genes in Arabidopsis are members of the core binding factor (CBF) regulon. CBF3 is a transcription factor that modulates a complex set of biochemical changes in response of plants to low temperature (Maruyama *et al.*, 2004). Overexpression of *CBF3/DREB1a* in transgenic Arabidopsis plants induced *AtGolS3* transcript and caused increased frost tolerance and the accumulation of osmoprotectants such as proline and raffinose, indicating physiological relevance of GolS induction (Gilmour *et al.*, 2000). Raffinose was capable of reducing the damaging effects of cold on components of the photosynthetic machinery in isolated thylakoid membranes of spinach (Santarius, 1973; Santarius and Heidrun Milde, 1997). Other mechanisms of cell protection by RFO were discussed in the Introduction.

In our study, barley plants were exposed to salinity stress to determine whether plants exposed to salinity will respond to decreases in external water availability by increasing transcription of *GolS* gene, which could result in an increase in RFO levels. The data from exposure of barley plants to salt in this work and microarray data revealed that the transcript levels of *HvGolS* were not greatly influenced by salinity. Salt treatment caused a slight increase or decrease in mRNA levels depending on the cultivars examined and the time after exposure to stress. Since the cultivars displayed different rates of growth, the pattern of transcript accumulation could be due to growth stage rather than the salt treatment. Analysis of data from salt and jasmonic acid treatments of cultivar Golden Promise showed a slight increase in levels of both *GolS1* and *GolS2* transcripts in leaves. However, in roots the expression of *GolS1* was increased by five-fold indicating the tissue specificity of this gene in response to salinity. In a time course study in leaves of Morex, also *GolS1* expression was slightly higher than controls. Similar to our experimental result, salt treatment failed to induce *ZmGolS* transcript accumulation in maize (Zhao *et al.*, 2004a). In contrast, induction of *GolS* has been observed in Coleus plants treated with salinity (Gilbert *et al.*, 1997). The lack of *GolS* response in barley leaves to salt stress could be due to the fact that barley at seedling stage is damaged by ionic effects rather than osmotic effects of salt (Storey and Wyn Jones, 1978), but, during germination osmotic effects of salt are the primary stress component (Mano *et al.*, 1996).

Drought-treated plants had a lower level of *GolS1* than control plants. However, rewatering plants restored transcript levels but not to the level of control plants in two of the cultivars. Interestingly, in Barque73 that is a drought tolerant cultivar the levels after rewatering equalled those of control plants. This pattern could be due to growth arrest in drought-treated plants and the time required to resume their growth after rewatering. Similarly, in perennial ryegrass shoots and roots *GolS* did not accumulate under drought stress and its transcript level was reduced in mature leaves but fructan levels increased (Pavis *et al.*, 2001). In contrast, in germinating seeds and tomato leaves, maize and Arabidopsis *GolS* induction has been reported in response to drought stress (Downie *et al.*, 2003; Zhao *et al.*, 2004a). In another study by Peters and colleagues (2007) it was found that in the resurrection plant, *Xerophyta viscose*, sucrose and RFO particularly raffinose levels increased under water deficit conditions. In this study, the transcripts of *GolS2* were not measured and it is possible that its transcripts will respond to drought. Differential or tissue specific expression of different members of gene families has been reported and was shown in previous and this chapter by analysis of the Barley1 GeneChip data. Differential expression of *GolS* family members have been reported in maize, Arabidopsis and *Ajuga reptans* (Taji *et al.*, 2002; Zhao *et al.*, 2004a; Zhao *et al.*, 2004b). Lack of induction of GolS1 under drought and salinity but its up-regulation in cold treated barley is opposite to expression pattern in rice which showed strong overlap of gene expression in response to drought and high salinity but not to cold (Rabbani *et al.*, 2003).

Developmental regulation of *GolS* gene expression has been shown. Endogenous expression levels of *GolS1* were only determined for leaf tissue in this study. *GolS1* was found to be expressed in all cultivars at low levels. Tissue series data from cultivar Morex (Druka *et al.*, 2006) were used to determine temporal expression pattern of the barley *GolS* genes transcripts during development. An increase in both barley *GolS1* and *GolS2* mRNA levels during developmental stages and organ-specificity was observed. *GolS1* transcript accumulated in anthers, which could be concomitant with maturation drying. Accumulation of RFO such as stachyose and raffinose during seed development has correlated with desiccation tolerance implying a role in tolerance (Blackman *et al.*, 1992; Brenac *et al.*, 1997b). Similarly, maize *GolS3* was predominately responsible for participating in galactinol production during seed development whereas *ZmGolS2* was mainly responsible for galactinol production in germinating seeds exposed to sudden desiccation. Furthermore, tissue-specific differences in expression of *GolS* genes has been

documented also in leaves of the raffinose-translocating species *Ajuga reptans* (Sprenger and Keller, 2000) and in leaves and seeds of melon (Volk *et al.*, 2003).

4.4.3 Phylogenetic analysis

Sequence alignment and phylogenetic analysis of galactinol synthases from different species revealed a high level of identity in amino acid sequences. The sequence similarity among known, full length GolS protein sequences defined four distinct phylogenetic groups. The two barley GolS sequences isolated in this study belong to the same group (C) with other Poaceae, such as maize, rice and wheat, indicating that barley GolS share a common evolutionary origin with other grasses. In this group three maize genes have been shown to have galactinol synthase activity by characterising heterologously expressed proteins in *E. coli* (Zhao *et al.*, 2004b). The other GolS encoded proteins appear not to be grouped according to the botanical classification. The seven members of the AtGolS family had proteins in three groups exhibiting as much variation within as among species. This contrasts with the ten monocot GolS (including the two isolated in this study) that were clustered in a single group. Group A is comprised of three experimentally shown GolS proteins. This group contained two Arabidopsis proteins that were present in mature, dehydrated seeds and up-regulated in drought- and cold-stressed vegetative tissues (Taji *et al.*, 2002) and a tomato protein expressed in both seeds and leaves (Downie *et al.*, 2003). In those three proteins alanine substitutes a serine residue which was identified as a putative phosphorylation site (Sprenger and Keller, 2000). In other GolS proteins there is a conserved serine residue in that position. Although, the predictions made in this study did not identify those residues as a putative phosphorylation site. Group B accommodated the *Ajuga reptans* GolS1 induced by cold in mesophyll cells (Sprenger and Keller, 2000), a *Cucumis melo* protein, two Arabidopsis and two *Verbascum phoeniceum* proteins. Monocots comprised group C containing only proteins from barley, rice, *Xerophyta viscosa* and maize. Group D was a cluster of GolS from Fabaceae, Vitaceae and Brassicaceae including an Arabidopsis protein with corresponding transcripts present in mature, dehydrated seeds and up-regulated by drought and cold stress (Taji *et al.*, 2002).

4.4.4 Transgenic plants

The role of GolS and RFO in plant defence and cereal abiotic stress tolerance has not been investigated before. In this study transgenic barley lines in which *GolS1* over-expressed or suppressed were produced for *in planta* functional analysis. Disease scoring showed significant differences in symptom between three transgenic lines and controls. To determine whether the differences are due to transgene expression and altered level of RFO requires further analysis.

Synthesis of osmolytes and protective compounds is one of the strategies employed by plants to survive drought and generally water deficit stress. These compounds which include the water-soluble carbohydrates, glucose, sucrose, raffinose and fructans may act by stabilizing membranes and proteins or mediating osmotic adjustment (Bohnert *et al.*, 1995; Hare *et al.*, 1998; Hoekstra *et al.*, 2001). One of the aims of this study was to evaluate the putative role of the RFO in drought tolerance of barley by modifying expression of *GolS1*. In drought experiment, variation on the onset of wilting was observed among different pots but no difference was observed among the plants growing together in any pot, which included in some pots non-transgenic progeny. The differences among pots could be attributed to differences in growth rate and possible positional variation in the growth chamber. The protective role of RFO in water deficit tolerance has been shown in transgenic Arabidopsis plants over-expressing *AtGolS2* which caused an increase in endogenous galactinol and raffinose (Taji *et al.*, 2002). As in this study the RFO level in transgenic lines was not determined, the possibility that RFO has a similar role in barley cannot be ruled out. Although, studies have suggested involvement of other compatible osmolytes such as proline, betaine, polyols, fructans and soluble sugars (Kishitani *et al.*, 1994; Koster and Lynch, 1992; Murelli *et al.*, 1995; Nomura *et al.*, 1995; Pavis *et al.*, 2001) in cereals abiotic stress tolerance.

Cloning and characterisation of barley GolS genes suggested a possible role in defence and frost tolerance indicated by up-regulation of transcripts upon treatment. Such a response was not observed in plants subjected to salinity and drought indicating that barley may not genetically programmed to use RFO for protection under those stresses. Transgenic barley lines produced in this study are an important reverse genetics tool to test effect of *GolS* in frost tolerance in barley. Other *in vivo* and *in vitro* experiments also could be used to characterise the transgenic lines produced in this study further. Leaf

samples from abiotic stress treated plants can be used for chlorophyll efflux and ion-leakage analysis and the rate of chlorophyll extraction and electrical conductance due to ion leakage from leaves would indicate protection from damage to cell membranes. Heterologous protein expression in *E. coli* was undertaken as a first step to investigate its putative role as a galactinol synthase. The constructs can be used to express and purify the protein in large scale for *in vitro* galactinol synthase activity. This method has been used in previous studies to confirm the galactinol synthase activity of the putative GolS genes (Downie *et al.*, 2003; Taji *et al.*, 2002; Zhao *et al.*, 2004b). Metabolic profiling comparing substrates and final products of GolS biosynthesis pathway and RFO composition of tissues in transgenic lines with varying level of GolS1 expression will also be helpful in demonstrating their biochemical activity in barley.