THE BARLEY EXPANSIN FAMILY

Submitted by

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ABSTRACT

Expansins are plant proteins that have been shown to induce cell wall extension and stress relaxation under acid pH conditions. The expansin gene family has been investigated in *Arabidopsis*, rice, maize, tomato and wheat. In barley (*Hordeum vulgare*), however, no systemic identification or characterisation of expansin genes has been reported. This study was undertaken to characterise the expansin family in barley and to investigate the mechanism of action of expansins in the cell wall via heterologous expression of barley expansin genes in *Escherichia coli*.

The expansins are usually encoded by a superfamily of genes. On the basis of phylogenetic sequence analysis, four sub-families of expansins are currently recognised in plants and are designated α-expansins (EXPA), β-expansins (EXPB), expansin-like A (EXLA) and expansin-like B (EXLB). In Chapter 2 the analysis of barley EST data deposited in the public databases is described. This resulted in the discovery of 34 partial or complete barley expansin genes (17 EXPB, 14 EXPA and 3 EXLA). Primers for mRNA transcript studies using quantitative PCR (Q-PCR) across a range of tissues were designed for genes for which 3' untranslated region (3'UTR) sequences were available. The Q-PCR results and barley Affymetrix data discussed in Chapter 3 show that the barley expansin genes are transcribed across a wide range of tissues and at various stages of cell wall development. This matches previously published information that expansins participate in a diverse range of developmental processes, including seed germination, fruit softening, root development, leaf growth and stem elongation. Their mechanism of action is yet to be determined unequivocally but is believed to involve the disruption of hydrogen bonds between cellulose microfibrils and "cross-linking" glycans in the cell wall; this in turn is believed to facilitate the wall extension and stress relaxation processes mentioned above.

In order to investigate the mode of action of expansins in the cell wall, an efficient expression system was required to produce biologically active recombinant expansin protein to characterise the function of the expansins. Complementary DNAs were used to build constructs that allowed expression of three full-length expansin genes in *E. coli.* The expression studies in which a number of approaches were used to obtain active protein are presented in Chapter 4.

Finally, the potential roles of expansins amongst a host of other proteins involved in cell wall-modification are discussed, along with functional assay results and proposed commercial applications.

STATEMENT OF AUTHORSHIP

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

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Maria Lombardi

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ABBREVIATIONS

°C	Degrees centigrade
Å	Angstrom
A	Absorbance
AXAH	Arabinoxylan arabinofuranohydrolase
AGPs	Arabinogalactan proteins
ATPase	Adenosine Triphosphatase
BLAST	Basic local alignment search tool
bp	Base pairs
BSA	Bovine serum albumin
CBD	Cellulose Binding Domain
CBH	Cellobiohydrolase
CesA	Cellulose-synthase
cDNA	Complementary DNA
cm	Centimetre
Csl	Cellulose synthase-like
d	Days
Da	Daltons
dap	days after pollination
DEPC	Diethylpyrocarbonate
DMSO	Dimethy sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetra-acetic acid
EST	Expressed sequence tag
EXLA	expansin-like A
EXLB	expansin-like B
EXPA	α-expansin
EXPB	β-expansin
FAE	Ferulic acid esters
fra	kotanin-like protein
g	Gram
GH45	Glycoside Hydrolase family 45
GRP	Glycine-rich protein
h	Hour
H^+	Hydronium
HPLC	High-performance liquid chromatography
HRGP	Hydroxyproline-rich glycoprotein
HvEXP	Barley expansin
Hz	Hertz
IMAC	Immobilised metal affinity chromatography
IPTG	Isopropylthiogalactoside
kb	Kilobase
kDa	Kilo Dalton
LB	Luria-Bertani
M	Molar
MCS	Multiple cloning site
MES	2-(N-morpholino) ethanesulfonic acid
mg	Milligram
min	Minutes
mm	Millimeters
mRNA	Messenger RNA
NCBI	National Centre for Biotechnology Information
NEB	New England Biolabs
NF	Normalisation Factor
ng	Nanogram
Ni-NTA	Nickel-nitrilotriacetic acid
nm	Nanometers
nr	non-redundant
OD	Optical density
OH	Hydroxyl group
•OH	Hydroxyl radical
ORF	Open Reading Frame
PBS	Phosphate buffered saline
PMSF	Phenylmethylsulphonyl fluoride
PCR	Polymerase chain reaction
pH	Potential of Hydrogen
PRP	Proline-rich protein
Q-PCR	Quantitative PCR
RACE	Rapid Amplification of cDNA Ends
RG-I	Rhamnogalacturonan I

RG-II	Rhamnogalacturonan II
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Room temperature (22°C)
SDB	Super Duper Buffer
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
s	Seconds
TA	Transcript Assemblies
TC	Tentative Contig
TEMED	Tetramethylethylene-diamine
TIGR	The Institute of Genomic Research
T_m	Melting temperature
Tris	Tris[hydroxymethyl] amino methane
U	Units
UTR	Untranslated region
UV	Ultraviolet
µ	micro
v/v	Volume for volume
w/v	Weight for volume
x g	Units of centrifugal force
XTH	Xyloglucan endotransglycosylases/ Hydrolases

CHAPTER 1

INTRODUCTION

1 INTRODUCTION

In this thesis, a study of the cell wall modifying protein family of expansins in barley is described. In the Introduction a general overview of plant cell walls with a particular focus on the primary cell walls of monocotyledonous plants will be provided, together with a description of cell wall elongation and the main proteins associated with this process. In this way, I hope to provide an insight into the involvement of expansins in cell wall elongation and their proposed mechanism of action within the wall.

1.1 PLANT CELL WALLS

Plant cells are encased within a complex polysaccharide wall, which is the main source of cellulose, the most abundant and useful biopolymer on Earth. The complex polysaccharide plant cell wall constitutes the raw material that is used to manufacture textiles, paper, lumber, films, thickeners and other products useful to mankind (Himmel *et al.*, 1999). The cell wall not only strengthens the plant body, but also has roles in plant growth, maintaining cell shape, and providing protection against pathogens, dehydration and other environmental factors. Cell walls are categorised according to whether they are deposited during cell growth, when they are referred to as primary cell walls; or after it ceases when they are called secondary cell walls.

1.1.1 Primary cell wall

The primary cell wall is generally a structure of crystalline cellulose microfibrils embedded in a complex polysaccharide matrix, with smaller quantities of structural proteins. The structure of cellulose is generally conserved across land and marine plant cell walls, but the structures and relative amounts and types of other cell-wall components are highly variable (Carpita and McCann, 2000). The primary cell wall is further characterised and categorised, based on the family and order in the Plantae kingdom to which it belongs, and on its composition.

The variable compositions of the primary cell walls of angiosperms (flowering plants) and gymnosperms have been analysed and, based on their major polysaccharides and architecture, have been catalogued into two types of primary cell wall, Type I and Type II (Carpita and McCann, 2000). However, it is now known that wall compositions do not fall clearly into these categories, but rather exhibit a continuum across the plant kingdom (Harris, 2005). The type I/II categories will therefore not be mentioned further here. Nevertheless, walls of the gymnosperms as well as most angiosperms, including the dicots and non-commelinoid monocots (Heslop-Harrison *et al.*, 1984), have walls composed of three major classes of polysaccharides, namely cellulose, xyloglucan and pectin (Carpita and Gibeaut, 1993). In general terms walls of the commelinoid monocots (Poaceae)

have more glucuronoarabinoxylan and less xyloglucan and pectin (Carpita and Gibeaut, 1993). Within the commelinoid monocots are the Poales from the family of Poaceae that includes barley, maize, millet, rice, and wheat. The primary wall of the Poales also contains (1,3;1,4)-β-D-glucans (mixed-linked glucans), which until recently were thought to be specific to grasses and cereals (Carpita and Gibeaut, 1993). However (1,3;1,4)-β-D-glucans have been found in isolated cases in fungi (Fontaine *et al.*, 2000), lichens (Honegger and Haisch, 2001; Olafsdottir and Ingolfsdottir, 2001), in some algae and in bryophytes (Popper and Fry, 2003). Sørensen *et al*. (2008) and Fry *et al*. (2008) have demonstrated that (1,3;1,4)-β-D-glucan is abundant in walls of horsetail (*Equisetum*) species. In the primary walls of the Poales, the (1,3;1,4)-β-D-glucan has been the subject of extensive research because of the socioeconomic importance of cereals and the importance of the non-cellulosic polysaccharides of walls in human health (Braaten *et al.*, 1994).

1.1.2 Structure of the cell wall

As noted above, the basic structure of the primary cell wall consists of crystalline cellulose microfibrils embedded in a gel-like matrix of non-cellulosic polysaccharides, and some protein. Crystalline cellulose is synthesised by a diverse range of organisms (Saxena *et al.*, 1990; Matthysse *et al.*, 1995; Pear *et al.*, 1996; Tsekos *et al.*, 1996) and is composed of unbranched polymers of (1,4)-β-linked glucosyl residues arranged in linear chains. Each glucosyl residue is oriented 180° from the next residue to form cellobiose, which is considered by some groups to be the structural repeating unit of cellulosic chains (Figure 1-1). This chemical structure allows the chains to take on a flat, ribbon-like conformation. Cellulose rarely occurs as a single chain, and cellulose microfibrils therefore form by the spontaneous crystallisation of about 36 (1,4)-β-linked glucan chains via hydrogen bonding and Van der Waals forces (Cousins and Brown, 1995). The multiple hydroxyl groups of the glucosyl residues from one chain form hydrogen bonds with oxygen atoms and OH groups on the same or on a neighbouring chain. These forces hold chains firmly together and provide cellulose with a high tensile strength (Wainwright *et al.*, 1976). The microfibrils are embedded in the non-cellulosic polysaccharide matrix of the wall (Figure 1-2).

Non-cellulosic polysaccharides of the wall are often substituted polysaccharides containing backbones of neutral sugar residues that can form hydrogen bonds with the surface of the cellulose microfibrils. Non-cellulosic wall polysaccharides include mannan, arabinoxylan, glucuronoarabinoxylan, glucomannan, xyloglucan and pectin. Xyloglucan and heteroxylan are

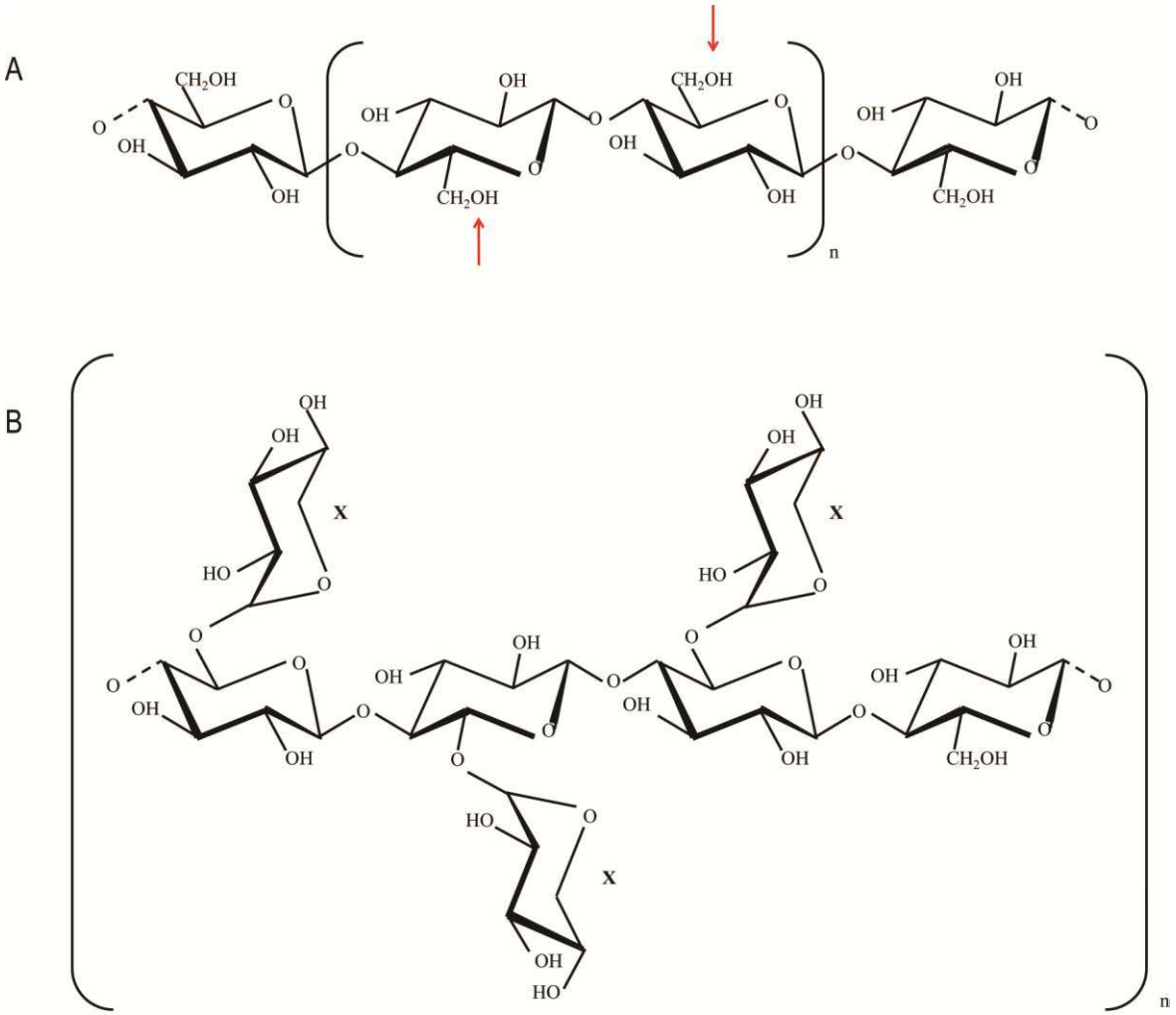

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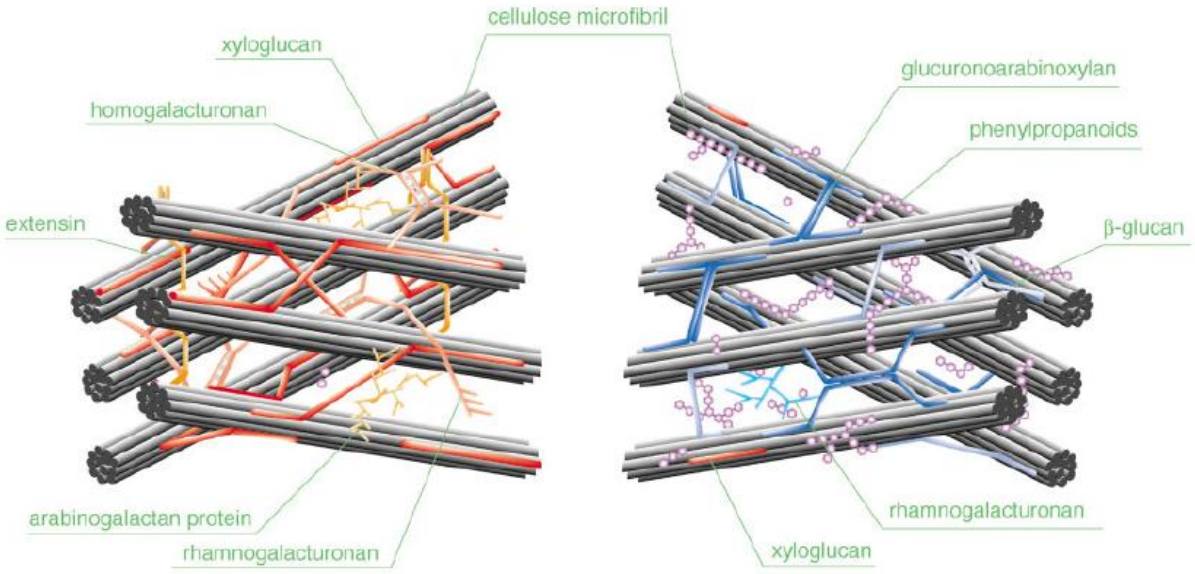
Figure 1-1 Structures of cellulose, xyloglucan and (1,3;1,4)-β-D-glucan.

A. The structural formula of cellulose. The arrows point to the basic repeat unit, which is a cellobiose molecular (Brown, 2004). B. Representative xyloglucan repeat unit. X = xylose. C. A portion of a (1,3;1,4)-β-D-glucan molecule (Burton *et al.*, 2004).



Dicot type wall

(*Arabidopsis thaliana*)

Monocot type wall

(*Oryza sativa*)

Figure 1-2 Schematic structural models of walls as represented by Arabidopsis and rice cell walls, respectively (Yokoyama and Nishitani, 2004).

Note the higher levels of pectin and xyloglucans in the dicot walls, and the relatively high level of glucuronoarabinoxylan in monocot walls.

two of the most abundant non-cellulosic polysaccharides (McNeil *et al.*, 1984) in dicot and monocot walls, respectively, and their structures vary among plant species (Vincken *et al.*, 1997).

The walls characterised of dicots and non-commelinoid monocots have (1,4)-β-glucan- or (1,4)-β-mannan- based polymers as their main non-cellulosic polysaccharide and a considerable amount of pectin (Carpita and Gibeaut, 1993). The principal interlocking polysaccharide is widely believed to be xyloglucan. Xyloglucan consists of a (1,4)-linked β-D-glucan backbone with either xylosyl residues or short side chains of xylosyl, galactosyl, fucosyl and/or arabinosyl side chains attached at regular intervals (Figure 1-1). Three substituted glucosyl residues are generally followed by a single unsubstituted glucosyl residue. Xyloglucan forms an extended molecule that can interact with cellulose fibrils via hydrogen bonding, as shown in Figure 1-2 (Hayashi *et al.*, 1987; Carpita and Gibeaut, 1993). Other non-cellulosic polysaccharides such as glucomannans and glucuronoarabinoxylans are also present in the primary wall of dicots and non-commelinoid monocots but are generally found in lower amounts than xyloglucan (McNeil *et al.*, 1984; Read and Bacic, 1996).

The pectic polysaccharides of the dicots and non-commelinid monocots are highly complex polymers that are usually composed of three domains: homogalacturonan, rhamnogalacturonan I (RG-I) and small proportions of rhamnogalacturonan II (RG-II). The backbone homogalacturonan contains a linear chain of (1,4)-linked α-D-galacturonyl acid residues. Rhamnogalacturonan I has a backbone composed of alternating (1,4)-linked α-D-galacturonyl and (1,2)-linked α-L-rhamnosyl residues. The rhamnosyl residues contain side chains of various neutral sugars, primarily arabinan and galactan. Rhamnogalacturonan II contains at least eight (1,4)-linked α-D-galacturonyl residues to which four structurally distinct side chains are attached (Vidal *et al.*, 2000; Glushka *et al.*, 2003). The wall pectin molecules can be stabilised by calcium ions and borate diesters (Hall and Cannon, 2002). It is widely accepted that pectin plays a key role in determining the plastic extensibility of plant cell walls (Jarvis, 1992) and it has been suggested that the pectin polymers provide charged surfaces that modulate wall pH and ion balance, and define the pore size of the wall (Baron Epel *et al.*, 1988; Read and Bacic, 1996).

Heteroxylans consist of a (1,4)-linked β-D-xylan backbone substituted primarily with arabinosyl residues (McNeil *et al.*, 1984). Other substitutents, such as glucuronyl residues and ferulic acid esters (FAE) may be attached to glucuronoarabinoxylans. Grass cell walls also contain xyloglucans, glucomannans and pectins, but these are typically lower in abundance than in the walls of dicots (McNeil *et al.*, 1984; Shibuya and Nakane, 1984; Carpita and Gibeaut, 1993). The

glucuronoarabinoxylans are bound and interlaced with cellulose microfibrils in much the same way as the xyloglucan-cellulose network is formed in dicot walls, as shown in Figure 1-2 (Carpita, 1983). The glucuronoarabinoxylans, found in monocot walls appear to replace, in part at least, the pectic substances that predominate in dicot walls (Carpita, 1996).

The cell walls of all plants also contain small amounts of structural proteins, which may have a reinforcing role (Lei and Wu, 1991; Kieliszewski *et al.*, 1992). Several classes of structural proteins have been described and these are classified according to their amino acid compositions. The structural proteins include hydroxyproline-rich glycoprotein (HRGP), glycine-rich protein (GRP) and proline-rich protein (PRP). These structural proteins are often located in specific cell or tissue types, and may be more characteristic of the maturity of the cell than of the tissue itself (Showalter, 1993). The arabinogalactan proteins (AGPs) are also found in the extracellular space, but these are not generally considered to be integral components of the wall. Diverse forms of the AGPs are found in different tissues, either in the periplasmic space or associated with the plasma membrane, and they display tissue- and cell-specific distribution patterns (Pennell *et al.*, 1989; Serpe and Nothnagel, 1995). Although the AGPs are not a major structural component of the wall, they have been implicated in growth and other developmental processes (Pennell and Roberts, 1990). Walls of the Poaceae contain much lower amounts of these wall proteins and AGPs (Cassab and Varner, 1988).

As mentioned above, the non-cellulosic component of walls of the monocotyledonous Poaceae family, which includes the grasses and cereals, are further characterised by the presence of (1,3;1,4)-β-D-glucans. The (1,3;1,4)-β-D-glucans consist of unbranched glucosyl residues containing a mixture of (1,3)- and (1,4)- linkages in ratios of 1:2 to 1:4 in cereals (Figure 1-1)(McNab and Smithard, 1992; Read and Bacic, 1996; Li *et al.*, 2009). The (1,3;1,4)-β-D-glucans and other non-cellulosic components of walls in grasses are among the most studied cell wall polymers, due to the multiple benefits they provide to human nutrition and energy production.

1.1.3 The cell wall in human health

Cereal grains are an important economic commodity worldwide with rice and wheat alone providing at least half of the daily caloric intake of humans. In human nutrition the non-cellulosic components of the cell wall in cereals have been shown to have beneficial effects in reducing the risk of cardiovascular disease, colorectal cancer, obesity and non-insulin-dependent diabetes (Braaten *et al.*, 1994; Brennan and Cleary, 2005).

The (1,3;1,4)-β-D-glucans are abundant in the walls of cereals, specifically in the starchy endosperm of grain (Beresford and Stone, 1983; Read and Bacic, 1996). The average total (1,3;1,4)-β-D-glucan

content of cereals ranges from 1% in wheat grains, to 3-7% in oats and 5-11% in barley (Skendi *et al.*, 2003), with both soluble and insoluble (1,3;1,4)-β-D-glucans detectable. On average 80% of the (1,3;1,4)-β-D-glucan is soluble in oats and 54% in barley, which suggests that oats would provide the greatest benefit for use in human food, due to its higher soluble (1,3;1,4)-β-D-glucan content (Aman and Graham, 1987). However, oats (*Avena stavia*) are generally a minor cereal crop compared with barley (*Hordeum vulgare*), whose principal uses are currently as feed for animals and as grain for malting and brewing in the manufacture of beer and whiskey (Bathgate *et al.*, 1974; Bamforth, 1982; McNab and Smithard, 1992). The commercial value of cereal crops is not limited to the use of grain. Various tissues of the plant, such as the stems in crop plants that contain less of the non-cellulosic components and greater amounts of cellulose, are potentially valuable as sources of biomass for biofuel production.

1.1.4 Secondary cell wall

The secondary cell wall is defined as that component of the wall that is deposited inside the primary cell wall after the cell has ceased growing. It is mainly composed of cellulose, which can make up to 90% of the dry weight of these secondary walls (Taylor *et al.*, 2000), but also includes non-cellulosic polysaccharides. In some cells, lignin may also be incorporated, where it contributes to the wall's mechanical strength and resistance to compressive forces (Read and Bacic, 1996). The lignin also plays an important role in protecting the plant against invasion by pathogens and insects (Albersheim and Andersonprouty, 1975) and in reducing wall porosity (Donaldson, 2001).

More recent attention has been focused on the potential use of the plant cell wall for the production of bioethanol. The secondary cell wall accounts for the bulk of renewable biomass in crop residues that can be converted to fuel. The wall polysaccharides can be hydrolysed to provide fermentable sugars for bioethanol production. However, lignin is often the main barrier to such conversion because it limits the access of hydrolytic enzymes to wall polysaccharides. Many cell-wall deconstructing enzymes have been isolated and characterised, and many more are under investigation. To gain a better understanding of the processes involved in the breakdown of the cell wall we must explore the mechanics of cell wall growth and the potential role of expansins in this process.

1.2 CELL ELONGATION

All plant organs arise ultimately from a series of events that represent a developmental growth gradient and includes cell division, cell expansion and cell differentiation. Cell division occurs in the meristem or zone of division and involves the biogenesis and integration of new walls at the plane of

cell division. The zone proximal to the meristem is the zone of elongation, where cell division ceases but there is rapid cell expansion by elongation (Benfey *et al.*, 1993). As the cells cease expanding they progressively mature at greater distances from the meristem. Certain cell types that are subject to mechanical stress undergo additional cell wall synthesis after the cells have finished dividing and are fully expanded. As mentioned above, these additional walls are referred to as secondary cell walls. The secondary cell wall is deposited on the inner surface of the primary wall, which consists of the polysaccharides that are synthesised and deposited during growth and development. The focus of this section involves the mechanisms by which the primary cell wall is able to elongate.

1.2.1 Cell growth

The plant cell wall plays a determinative role in establishing the size and shape of the plant cell. In response to large osmotic gradients, plant vacuoles take up water and the resulting increase in turgor pressure gives rise to a stress in the load bearing parts of the wall and to compression of the cell contents. Turgor pressure exerted by the vacuole is essential in supporting plants in an upright position. However, growth of the cell is initiated when the load-bearing elements in the wall yield, thereby inducing a relaxation of the elastically distended wall elements and a reduction in wall stress (Fry, 1989). Turgor pressure is known to drive cell elongation in this manner but must be accompanied by loosening of the cell wall. The process of cell elongation is known to be induced by auxin (Cleland, 1971).

Growing plant cells also display a pH-dependent form of wall extension that is strongly promoted by wall acidification. Such growth stimulation comes about because the cell wall becomes more extensible at acidic pH. Cell growth at acidic pH is a phenomenon referred to as 'acid growth'. Under the influence of the plant hormone auxin, wall acidification is a result of the H^+ATPase in the plasma membrane 'pumping' H^+ ions from the cytoplasm and causing acidification of the cell wall space (Kutschera *et al.*, 1987; Hoson and Masuda, 1995). The pH of the wall drops and in turn stimulates the highly pH-dependent activity of proteins associated with matrix polymer rearrangement namely xyloglucan endotransglycosylases/hydrolyases (XTHs), cellulases and expansins. Cell wall expansion is considered to be a well coordinated process of wall polymer synthesis and secretion and, as such, induction of cell elongation by auxin leads to increased rates of polysaccharide synthesis (Brummell and Hall, 1985; Edelmann *et al.*, 1989).

1.2.2 Biosynthesis of the cell wall

During cell growth, plant cells enlarge by a factor of 10 to more than 100 times their original volume. The cell wall is able to accommodate this enormous expansion without losing mechanical integrity and generally without becoming thinner, through an effective means of intergrating new polymers into the wall without destablising the load-bearing network. Plant cells are thereby able to grow and change shape by controlling the direction and rate of wall expansion. Plant cells accomplish this by firstly depositing a biased (anisotropic) or isotropic wall, followed secondly by polymer rearrangement. Figure 1-3 is a diagrammatical representation of primary and secondary cell wall deposition.

Cellulose, as an essential component of the cell walls of higher plants, plays a major role in expansion in two ways. Firstly, the cellulose forms part of the structure that is considered to be the main load-bearing network that controls the extent of cell expansion. Secondly, the direction of cell expansion is determined by the orientation of cellulose microfibrils (Gardiner *et al.*, 2003). Cellulose is synthesised by putative cellulose-synthase (CesA) complexes in the plasma membrane (Doblin *et al.*, 2002) and is extruded as self-assembling microfibrils (Cousins and Brown, 1995). The orientation of the cellulose microfibril deposition has been shown to be regulated by the orientation of microtubules. Mutants that have disorganised microtubules have an abnormal orientation of cellulose deposition, as demonstrated by the *fragile fiber* mutants encoding a kinesin-like protein (*fra1*) and a katanin-like protein (*fra2*)(Burk and Ye, 2002; Zhong *et al.*, 2002). In contrast, the matrix polymers secreted by the Golgi apparatus become intergrated into the wall network by poorly understood mechanisms. Only recently have members from the Cellulose Synthase-like (Csl) gene family been shown to mediate the synthesis of (1,4)-linked β-D-mannan, (1,3;1,4)-linked β-D-glucans and (1,4)-linked β-D-glucans (Cocuron *et al.*, 2007; Burton and Fincher, 2009). None of the enzymes associated with the synthesis of xylan have yet been identified unequivocally. Despite the lack of information regarding the synthesis of the matrix polymers, their changing content in the growing cell wall has been measured during cell elongation.

The (1,3;1,4)-β-D-glucans have been found to be the prominent polysaccharide to undergo auxin-induced turnover in walls of the Poaceae (Hoson and Masuda, 1995). The (1,3;1,4)-β-D-glucans are virtually absent from the meristematic cells, but are synthesised at the onset of elongation (Carpita and Gibeaut, 1993; Buckeridge *et al.*, 2004). The (1,3;1,4)-β-D-glucans reach a maximum abundance during the most rapid phase of elongation, but decline once elongation has ceased. The (1,3;1,4)-β-D-glucan has until recently been shown to be largely absent in mature tissues where growth has ceased (Kim *et al.*, 2000a; Carpita *et al.*, 2001; Buckeridge *et al.*, 2004). In contrast, the

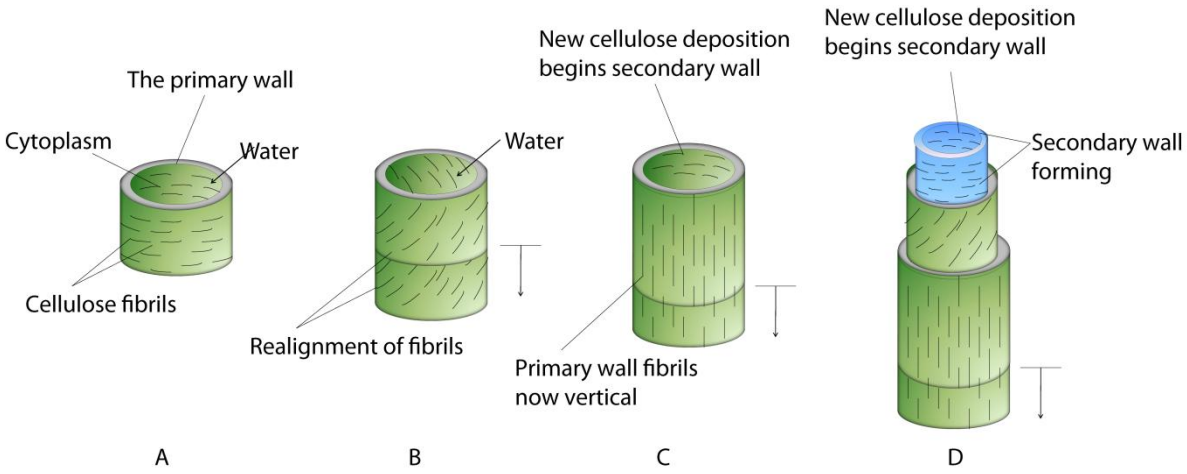


Figure 1-3 Diagrammatical representation of primary and secondary cell wall deposition.

A. Acid in young cell helps loosen cellulose fibres. B. Cellulose fibres begin to realign and elongation begins. C. Realignment completes and elongation continues. D. Elongation complete.

(1,3;1,4)-β-D-glucans identified in horsetail are abundant in both young and mature regions of stem (Sørensen *et al.*, 2008). However, in cereals the transient nature of the (1,3;1,4)-β-D-glucans is consistent with a developmental role and auxin has been shown to stimulate the breakdown of such glucans in coleoptile segments of oat, barley, rice and maize (Loescher and Nevins, 1972; Zarra and Masuda, 1979; Hoson and Nevins, 1989; Kotake *et al.*, 2000). The turnover of matrix polysaccharides is a result of polymer rearrangement via molecular creep and hydrolysis following cell wall deposition (Schopfer, 2001).

These models of polymer rearrangement allow cellulose microfibrils to break down, move apart or slip relative to each other to assist in wall loosening and cell elongation. Possible polymer rearrangement processes involve the cleavage of the backbone of the major matrix polymers (hydrolysis), disruption of putative covalent crosslinks between matrix polymers and weakening of the noncovalent bonding between polysaccharides. The mechanisms responsible for these types of cell wall modification can be classified into two types; enzymatic and non-enzymatic, as described below.

1.2.3 Enzymatic wall loosening

The enzymatic mechanisms of cell growth implicate cell wall proteins that hydrolyse components of the cell wall. They include cellulases, pectinases, xylanases, (1,3;1,4)-glucanases and xyloglucanases, all of which hydrolyse their corresponding polysaccharide substrate and potentially other substrates with similar glycosidic linkages. Both the cellulases and XTHs have been shown to be specifically enhanced during auxin stimulation of cell elongation (Hoson and Nevins, 1989; Inouhe and Nevins, 1991; Smith and Fry, 1991; Fry *et al.*, 1992).

There are four classes of cellulase enzymes based on the type of reaction they catalyse, namely the endo-(1,4)-β-D-glucanases, exo (1,4)-β-D-glucanases, β-glucosidases and exoglucosidases. Endoglucanases act randomly on internal linkages of cellulose chains to disrupt the crystalline structure of cellulose and expose individual microfibrils. Exoglucanases act progressively to preferentially liberate glucose or cellobiose from the non-reducing ends of the cellulose chain and these exoglucanases include the cellobiohydrolases (CBHs). The β-glucosidases and exoglucosidases liberate glucose from the cellobiose (Davies *et al.*, 1993; Warren, 1996; Teeri, 1997). Cellulases have also been shown to hydrolyse the 1,4-β-D-glycosidic linkages in the (1,3;1,4)-β-D-glucans and xyloglucans (Inouhe and Nevins, 1991; Wei *et al.*, 2010).

Similarly, the XTHs are a class of wall enzymes that hydrolyse linkages in xyloglucans and then reform the same linkage in a new position. This allows the cleavage of stress-bearing bonds in the wall, thus increasing extensibility, without impairing overall wall integrity (Pritchard *et al.*, 1993).

1.2.4 Non-enzymatic cell wall loosening

The hydroxyl radical (•OH) has been shown to cleave wall polysaccharides by non-enzymatically removing a hydrogen atom from polysaccharides (Fry, 1998). Liszkay and Schopfer (2003) were able to demonstrate the extension of isolated walls induced by artificially generated •OH. Despite the implication of •OH in auxin-induced cell growth, the total amount of wall extension caused by •OH was small before cell wall breakage was observed. However, as •OH is a highly reactive oxygen species, the action of cleaving polysaccharides may not be a specific event and the radical probably interacts with the first suitable molecule it encounters (Halliwell and Gutteridge, 1989).

In the early 1990's, McQueen-Mason *et al.* (1992) and Li *et al.* (1993) reported a class of wall proteins from cucumber (*Cucumis sativus*) and oat (*Avena sativa*) seedlings with the ability to induce extension of isolated plant cell walls. These were called expansins, later designated as the α-expansins. It was demonstrated by McQueen-Mason and Cosgrove (1994) that purified expansins can weaken hydrogen bonding between paper fibres without degrading the cellulose molecule and later established that these novel cell wall proteins did not exhibit hydrolytic activity on cell walls (McQueen-Mason and Cosgrove, 1995). After cloning and sequencing the cucumber expansins (Shcherban *et al.*, 1995), a search of the EST and genomic databases revealed a multitude of transcripts encoding proteins similar to expansins. Since the expansins' initial identification, extracts of maize pollen and purified maize group-1 pollen allergen *in vitro* were shown to have expansin activity when applied to wheat cell walls (Cosgrove *et al.*, 1997; Li and Cosgrove, 2001). The primary structure of the group-1 grass pollen allergens from timothy grass, ryegrass and maize were shown to be 20-25% identical to the original expansins giving rise to a subset of expansins stemming from the group-1 grass pollen allergens (Shcherban *et al.*, 1995). Analogues of the group-1 grass pollen allergen expansins were later detected in vegetative tissue both in grasses and other plant groups (Cosgrove, 2000b).

Expansins have been identified in a number of dicots and grasses. Studies of expansin activity have generally involved the use of highly purified native protein extracts, which reveal little about the molecular details of expansin action. Most of the expansins are known only from sequence homology, not from their biochemical or biological activities. It remains to be seen whether the functional definition of an expansin and its classification based on sequence homology are fully

congruent with each other, while the mechanism of non-enzymatic cell wall loosening still remains to be elucidated.

1.3 THE EXPANSIN SUPERFAMILY

As noted above, expansins are proteins that are believed to promote cell wall loosening. Expansins are encoded by a multigene family that can be divided into four sub-families based on phylogenetic sequence analysis: α-expansin (EXPA), β-expansin (EXPB), expansin-like A (EXLA) and expansin-like B (EXLB). The genes encoding the α-expansins and β-expansins are both found in all groups of land plants, from mosses to flowering plants (Kim *et al.*, 2000b; Li *et al.*, 2002; Schipper *et al.*, 2002; Colmer *et al.*, 2004; Jones and McQueen-Mason, 2004). In contrast the, expansin-like A and B proteins are only known from their gene sequence (Sampedro and Cosgrove, 2005a). It remains to be established whether expansin-like A and B proteins have cell wall loosening activity or not. The characteristic traits of the expansin family have been scrutinised in order to further understand their mechanism of action and role in cell growth.

1.3.1 Structural characteristics of the expansin proteins

The α- and β-expansins families share on average around 20-25% amino acid sequence identity, but contain underlying traits in that they are similar in size and have a number of conserved motifs. They are typically 250-275 amino acids long and are made up of two domains that are proceeded by a signal peptide, as shown in Figure 1-4 (Sampedro and Cosgrove, 2005a). The signal peptide of 17 to 25 residues at the NH_2-terminus directs the protein into the endoplasmic reticulum. The signal peptide is cleaved, leaving behind a mature protein of around 25-28 kDa. The mature protein contains two domains and their predicted secondary structures are up to 75% identical (Cosgrove *et al.*, 1997), despite the relatively low level of amino acid sequence identity.

The degree of amino acid sequence identity between the α-expansins and β-expansins is highest in Domain 1 at around 30% (Cosgrove, 2000a). Domain 1 has significant but distant homology to glycoside hydrolase family 45 (GH45) proteins, most of which are fungal (1,4)-β-D-endoglucanases (Henrissat *et al.*, 1998). A histidine, phenylalanine, aspartic acid (HFD) motif that makes up the catalytic site of the GH45 endoglucanases (Davies *et al.*, 1993) is also conserved in expansins, despite the apparent lack of hydrolytic activity in expansins. Domain 1 of the expansins also shares a number of conserved cysteines that form disulfide bridges in the fungal enzyme (Cosgrove, 1997). To further support the similarity of Domain 1 with GH45 proteins, Yennawar *et al*. (2006) determined the structure of maize EXPB1 by X-ray crystallography (Figure 1-5) to 2.75-Å resolution and was able to superimpose the structure of Domain 1 onto a GH45 enzyme (Protein Data Bank ID code

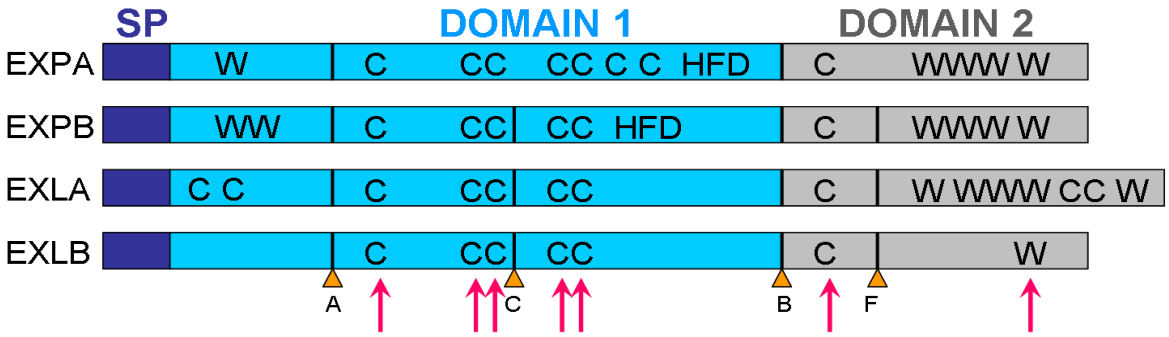


Figure 1-4 Domain structure of the expansin proteins.

The expansin proteins contain a signal peptide (SP) at the NH_2-terminus. The expansins also contain two conserved domains, a cysteine (C) rich region and a tryptophan (W) rich COOH-terminal segment. The degree of conservation between all groups is highest in domain 1. Domain 1 has significant but distant homology to family-45 endoglucanases including a histidine, phenylalanine, aspartic acid motif (HFD) that makes up part of the catalytic site of the Family 45 endoglucanases. This is absent in the expansin-like A and B proteins. Domain 2 has homology to a group of proteins found in grass pollen, named group-2 grass pollen allergens. The position and name of each intron (named A, C, B and F in the order in which they were identified) are indicated by the orange triangles and the amino acid residues conserved in all families are indicated by pink arrows. Figure adapted from Choi *et al.* (2006).

4ENG). With the assistance of a secondary structure algorithm program (Collaborative Computational Project Number 4, 1994), an overlap of 60% of the Domain 1 peptide backbone with GH45 4ENG was observed. Despite Domain 1 possessing the HFD motif of the catalytic site in GH45 4ENG, it was recognised that Domain 1 of *ZmEXPB1* is missing a catalytic residue that is required for (1,4)-β-D-glucan hydrolysis by GH45 enzymes (Davies *et al.*, 1993). Other observations include an exact superimposition of two of the three disulfide bonds in Domain 1 with GH45 4ENG and a match on all of the β-strands (Yennawar *et al.*, 2006). A diagrammatical representation of the two domain expansin protein structure is shown in Figure 1-5.

One of two unique differences between the α- and β-expansins in Domain 1 is a highly conserved 14 amino acid insert found in the α-expansins that includes two conserved cysteine residues, which suggests that an additional disulfide bridge is present in α-expansins. The second distinguishing feature is that potential N-linked glycosylation sites are found exclusively in the β-expansins. The β-expansins may possess one or two predicted N-linked glycosylation sites (consensus sequence Asn-X-Ser/Thr). The significance of the N-linked glycosylation motif for expansin function is unknown. In eukaryotes, N-glycosylation occurs in the secretory pathway as glycoproteins are transported from the endoplasmic reticulum to their final destination. β-Expansins are modified by posttranslational N-glycosylation and this glycosylation may be important to their function (Downes *et al.*, 2001). However, when considering the differences between the two families and the possible important impact on function, there still remains a gap in our understanding of the physiological basis for the glycosylation of one family but not the other.

Domain 2 of the group-1 grass pollen allergen subset of β-expansins also shares amino acid sequence identity of around 40% with a group of secreted proteins only found in the grasses, known in the immunological literature as the grass group-2/3 pollen allergens. These grass group-2/3 pollen allergens appear to have evolved from a truncated version of the β-expansin gene (Sampedro and Cosgrove, 2005a), but their biological function is unknown (De Marino *et al.*, 1999). However, it has been shown that a group-2/3 pollen allergen extracted from maize increases the accessibility of crystalline cellulose to enzymatic attack by cellulase, despite the fact that the pollen allergens themselves lack hydrolytic activity (Cosgrove and Tanada, 2007). A comparison of the Domain 2 structure of the crystallised maize expansin with the *Phleum pratense* (Phl p 2) protein, a group-2/3 grass pollen allergen (Protein Data Bank ID code 1WHO), shows that the eight β-strands in Domain 2 that are assembled into two antiparallel β-sheets form a β-sandwich similar to the immunoglobulin-like fold found in the Phl p 2 1WHO protein (Sampedro and Cosgrove, 2005a; Yennawar *et al.*, 2006). Domains with this fold characteristically have a binding function via two

Figure 1-5 Backbone and secondary structure of ZmEXPB1 (PDB ID code 2HCZ).

The protein fold in Domain 1 (green) is dominated by a six-stranded β-barrel flanked by short loops and α-helics as indicated by broad arrows in green. Domain 2 indicated in blue is composed of eight β-strands assembled into two antiparallel β-sheets indicated by broad arrows in blue (Yennawar *et al.*, 2006). The pink backbone represents the linker between the domains. This image was generated with PyMOL (DeLano Scientific).

surface aromatic residues aligned on the a plane on the surface of Domain 2 (Kerffa *et al.*, 2008). Examination of the structural features of the α- and β-expansins can only currently provide clues in relation to their functional mechanism of action. The characteristics of the expansin-like A and expansin-like B proteins have not been explored to the same extent as the α- and β-expansins and little is known about their 3D structure.

1.3.2 Characteristics of the expansin-like proteins

The expansin-like A proteins show high amino acid sequence identities within the group. In addition to the conserved cysteines found in the β-expansins, the expansin-like A's have a pair of conserved cysteines near the NH_2-terminus and a pair at the COOH-terminus (Figure 1-4). The expansin-like A also contains conserved tryptophan residues in the COOH-terminal region, but the spacing of the tryptophan residues differs from those found in the α- and β-expansins and include an additional tryptophan at the COOH-terminus. The expansin-like B contains only a single tryptophan at the COOH-terminus. The most notable difference between the expansins and the expansin-like proteins is the lack of an HFD motif in the central region of the expansin-like proteins (Choi *et al.*, 2006).

1.3.3 The evolution of expansins

Analysis of the *Arabidopsis* and rice gene structures and deduced amino acid sequences indicate that α- and β-expansin genes evolved from a common ancestral gene (Figure 1-6)(Lee *et al.*, 2001). Furthermore, the distribution of moss, fern and pine sequences throughout the expansin gene superfamily phylogenetic tree indicates that the expansin subfamilies arose very early in land plant evolution, and that the α- and β-expansin families were already present when vascular plants and mosses diverged while the expansin-like A and expansin-like B gene family appear after the angiosperms and gymnosperms split (Figure 1-6)(Li *et al.*, 2002; Schipper *et al.*, 2002). The overall exon/intron organisation is conserved between α- and β-expansins and expansin-like genes, but the number of introns and their lengths differ between individual genes (Lee *et al.*, 2001). Sequence analysis of the expansin genes lead to the identification of seven introns designated A, B, C, D, E, F and G in the order in which they were identified (Figure 1-4) (Sampedro *et al.*, 2005b). Intron D is located in the 5' untranslated region and introns A, B, C and F are widely distributed in the coding regions of the α- and β-expansins and the expansin-like A and expansin-like B genes. The positions of the introns in relation to the amino acid sequences are shown in Figure 1-4. Expansins often exhibit cell-specific expression patterns and such specific expression suggests that the evolutionary

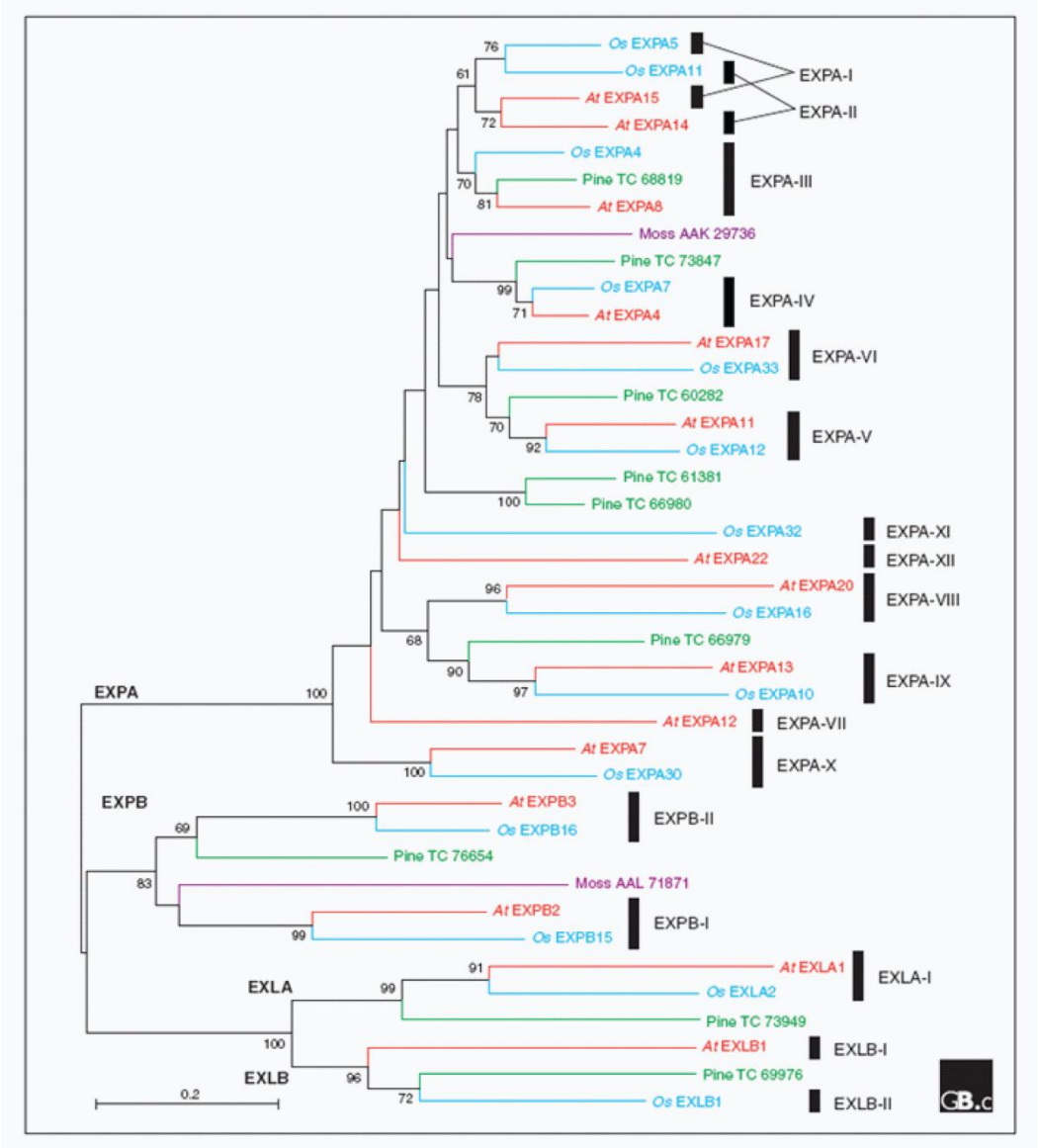


Figure 1-6 Phylogenetic tree of the expansin superfamily, including a selection of protein sequences from *Arabidopsis thaliana* (At), *Oryza sativa* (Os), *Pinus* species (pine) and *Physcomitrella patens* (moss).

These sequences were aligned with CLUSTALW. Bootstrap values above 60 are indicated next to the relevant node, and the four families are labelled at their roots. Clades, defined as all the descendants of the same ancestral gene in the last common ancestor of monocots and eudicots, are indicated by black bars to the right and given roman numerals as described in Sampedro et al. (2005b). EXPA, α-expansin; EXPB, β-expansin; EXLA, expansin-like A; EXLB, expansin-like B (Sampedro and Cosgrove, 2005a).

diversification of plant cell types may have involved a parallel duplication and specialisation of expansin genes. Still, little is known about the biological function of the expansin-like genes.

1.3.4 The multiple members of the expansins

In the fully sequenced *Arabidopsis* and rice genomes, 36 and 58 expansin genes, respectively, have been identified. In *Arabidopsis* this includes genes for 26 EXPA, six EXPB, three EXLA and one EXLB. By comparison, the rice genome contains genes for 34 EXPA, 19 EXPB, four EXLA and one EXLB (Sampedro and Cosgrove, 2005a). In wheat and maize the α-expansin count stands at 9 and 12, respectively, and 9 and 21 for β-expansins, respectively (Wu *et al.*, 2001a; Lin *et al.*, 2005; Muller *et al.*, 2007). No expansin-like genes have been reported for maize and wheat; but the numbers for maize and wheat includes some partial gene fragments and should be interpreted as minimum estimates. It has been estimated that the number of α-expansin genes in wheat could exceed 30 and the number of β-expansin genes could exceed 65 (Liu *et al.*, 2007). The large number of β-expansin genes in grasses is likely to be related to the fact that the cell wall matrix polysaccharides and structural proteins of grasses differ from those of most other angiosperms. The subset of β-expansins originating from the group-1 grass pollen allergens is known to be abundantly and specifically expressed in grass pollen (Ansari *et al.*, 1987). It has been suggested that these β-expansins aid in the loosening of the cell walls for pollen tube extension in the stigma (Cosgrove *et al.*, 1997; Pezzotti *et al.*, 2002). The grass pollen tube grows by tip growth to force its way between the cell walls of the stigma before entering the stylar track, where growth of the pollen tube involves further intrusive growth through and between cell walls (Heslop-Harrison *et al.*, 1984).

In other tissues the β-expansins are typically found in low abundance and are tightly bound to the cell wall, as shown by cell-fractionation studies (Li *et al.*, 1993; Lee and Choi, 2005). The β-expansins are suggested to act selectively on cell walls of the Poaceae. This hypothesis is based on the results of cucumber cell wall extract containing α-expansins having been shown to induce extension of isolated walls of dicots and monocots of the Amaryllidaceae, with a much smaller effect of cell wall extension observed in monocots of the Poaceae (McQueen-Mason *et al.*, 1992; Cho and Kende, 1997a). It is has been recently demonstrated that β-expansins act on wall components such as the (1,3;1,4)-linked β-D-glucans and glucuronoarabinoxylans found in walls of the Poaceae (Tabuchic *et al.*, 2011). This is supported by the greater number of β-expansins identified in rice and maize (Wu *et al.*, 2001a).

The functional significance of multiple expansin genes is unknown at this stage, but there is evidence that many of these genes show cell-specific expression and have been implicated in other developmental processes where cell wall loosening occurs, as described in the section below.

1.3.5 Functions of the expansins

The developmental processes in which expansins have been implicated include fruit softening (Rose *et al.*, 1997; Brummell *et al.*, 1999; Harrison *et al.*, 2001), xylem formation (Gray-Mitsumune *et al.*, 2004), leaf abscission (Belfield *et al.*, 2005), seed germination (Chen and Bradford, 2000) and rehydration of 'resurrection' plants (Jones and McQueen-Mason, 2004). These functions have been deduced from investigations in tomato (Rose *et al.*, 1997; Reinhardt *et al.*, 1998; Brummell *et al.*, 1999), cotton (Orford and Timmis, 1998), soybean (Downes *et al.*, 2001; Lee *et al.*, 2003), strawberry (Harrison *et al.*, 2001) and peach (Obenland *et al.*, 2003). The approaches taken to study the various roles and mechanisms of expansins includes immunolocalisation of proteins, analysis of gene expression patterns, antisense reduction of expansin gene expression, insertional mutagenesis of expansin genes and application of isolated expansin proteins to isolated cells and tissues.

Electron microscopy, combined with immunogold labelling of plant tissues using antibodies against expansins, indicates that expansins are dispersed throughout the cell wall and that they are not restricted to specific corners of the cell or to the plasma membrane-wall interface. Similarly the load-bearing part of the cell wall is not restricted to the membrane/wall junction or to cell corners, and this implies the expansins are widely distributed and could participate in wall loosening around the cell perimeter (Cosgrove *et al.*, 2002).

Studies of gene expression by Northern hybridisation analysis and in-situ hybridisation indicate that the different expansin genes are transcribed in different organs, tissues and cell types and that they respond differently to hormones, water stress and anoxia (submergence)(Downes *et al.*, 2001; Wu *et al.*, 2001b; Lee and Kende, 2002).

Downes *et al*. (2001) characterised a cytokinin-inducible β-expansin from soybean called Cim1. They were able to demonstrate that the hormones cytokinin and auxin act synergistically to induce the accumulation and proteolytic processing of Cim1 (β-expansin). Likewise Northern analysis by Harrison *et al*. (2001) showed that strawberry fruit development is associated with at least two different patterns of expansin gene expression and that expansin mRNA is also present in primarily expanding tissues of leaves and stolons in strawberry. The process of fruit softening involves cell wall breakdown, which is initiated by ethylene. Trivedi and Nath (2004) identified and characterised

an expansin gene *MaEXP1* from banana fruit that is ethylene-regulated and expressed only in the ripening banana fruit, while *Sambucus nigra* expansin activity was shown to be dramatically increased in abscission zones during ethylene-induced abscission (Belfield *et al.*, 2005).

Other environmental changes in which expansins participate include anoxia, which involves a reduction of O_2 in internode tissues, and has been studied in submerged deepwater rice. Deepwater rice has evolved the capacity to elongate very rapidly when it becomes submerged. The growth response is induced by an environmental signal and is mediated by at least three interacting hormones, namely ethylene, abscisic acid and gibberellin. Five rice expansin genes are expressed in the internode and their expression is induced by gibberellins (Cho and Kende, 1997a; Cho and Kende, 1997b; Kende *et al.*, 1998; Lee and Kende, 2002). A similar effect is observed in the semiaquatic fern *Regnellidium diphyllum*. An increase in the transcript levels of *RaEXP1* mRNA, accompanied by increases in wall extensibility of the rachis of *R. diphyllum,* was found when plants were submerged, and could also be initiated by ethylene treatment (Kim *et al.*, 2000b). However, not all expansin genes are responsive to hormones, because the level of mRNA transcript of *RaEXP2* in *R. diphyllum* did not change in response to submergence (Kim *et al.*, 2000b).

Expansin presence has also been observed under osmotic stress. Maize roots adapted to low water potentials are able to continue growing by increasing the extensibility of their cell walls (Wu *et al.*, 1996) and this is correlated with an increase in expansin transcription (Wu *et al.*, 2001b). A similar increase of expansin gene transcript levels was found in the leaf of osmotically stressed maize plants (Sabirzhanova *et al.*, 2005) and during the early stages of both dehydration and rehydration in the resurrection plant *Craterostigma plantagineum*. *C. plantagineum* displays extensive cell wall folding during dehydration and this is a unique characteristic of resurrection plants (Farrant, 2000). The transcript levels of three expansin genes were measured in *C. plantagineum* leaf during dehydration and rehydration, and each displayed different transcript profiles at each stage (Jones and McQueen-Mason, 2004). The widely different transcript profiles of the three expansin genes measured under the same stress condition further complicate our understanding of the biological function of expansins.

Gene silencing has been recognised as an efficient method to determine the biological function of a gene or its encoded protein. However, to study a gene family like the expansins with its multiple members and overlapping gene expression patterns, knockout or silenced mutations have limited value. The lack of phenotypic alteration in plants with reduced expansin expression may be explained by the ability of other family members to compensate; some genes might be functionally

redundant. Schipper *et al.* (2002) was able to generate four null knockouts for individual expansin genes in *Physcomitrella patens*, as indicated by a lack of transcript accumulation for the targeted expansin gene. However, none of the mutants showed observable phenotypes.

The overexpression of expansins has been shown to affect *Arabidopsis* leaves (Cho and Cosgrove, 2000), tomato plant growth and fruit softening (Brummell *et al.*, 1999; Rochange *et al.*, 2001), and soybean root growth (Lee *et al.*, 2003), but these effects can be counter-intuitive or unexpected because expansins belong to a large gene family with partly redundant functions. To study the mode of expansin action, an efficient expression system to produce biologically active recombinant expansin is needed. As noted previously, most experiments with expansin protein have been performed with highly purified native tissue extracts.

It has been observed that expression of active expansins in various recombinant systems has been unsuccessful due to improper folding, aggregation and hyperglycosylation (Downes *et al.*, 2001; Lee and Choi, 2005). As yet only partially purified native expansin protein has been utilised for activity assays and for study of the mechanism of action (McQueen-Mason and Cosgrove, 1994; Yennawar *et al.*, 2006).

1.3.6 Expansin mechanism of action

The mechanism of action of expansins has been widely debated for many years. Grobe *et al.* (1999) reported that the group-1 allergens (now known as β-expansins) were novel C1 cysteine proteinases and proposed that wall loosening by expansin was mediated by proteinase action. This was later refuted by Li and Cosgrove (2001). It has also been observed that the wall loosening of expansin is noticeably different from that caused by the endoglucanases that hydrolyse xyloglucan (Wei *et al.*, 2010). The ability of expansins to hydrolyse cell wall polysaccharides continues to be tested but no hydrolysis has yet been detected (Yennawar *et al.*, 2006). Binding studies of native maize β-expansin show that *ZmEXPB1* preferentially binds to xylans with some intermediate level of binding to xyloglucan (Yennawar *et al.*, 2006).

The current proposed mechanism of action involves the disruption of hydrogen bonding between cell wall polymers, which allows turgor pressure to drive cell expansion by molecular creep. McQueen-Mason and Cosgrove (1994) further demonstrated that partially purified expansins can weaken hydrogen bonding between paper fibres without degrading cellulose molecules. The application of exogenous expansin on isolated heat-treated cell wall specimens induces a stress relaxation and extension of the isolated cell walls in a pH-dependent manner (McQueen-Mason *et al.*, 1992).

Not all plant walls are susceptible to the action of expansins, however, and walls from the non-growing part of the plant do not extend when treated with expansins. In addition, they do not exhibit an acid-extension response (Cosgrove, 1996). This may be a result of the maturing cell wall forming tighter complexes with cellulose or other wall polymers, which makes the wall less susceptible to expansin activity (Carpita, 1984a). With no other proteins found to have this activity, and with little known of its mechanism of action, a challenge exists to resolve questions relating to the structure and functions of the expansin superfamily and its encoded proteins.

1.4 PROJECT AIMS AND OBJECTIVES

The overall aims of this project were to characterise the expansin family in barley. The expansin gene family has been investigated in *Arabidopsis*, rice, maize, tomato and, to a lesser extent, in wheat. In barley, however, no systemic identification of expansin genes has been reported. Barley was chosen as a diploid representative of the commercially important Triticeae, and because a good deal of genetic information is available. The work started before the release of the *Brachypodium* and sorghum genome sequence.

Expansins have been shown to participate in a diverse range of developmental processes and their mechanism of action is yet to be determined, but is believed to involve the disruption of hydrogen bonds between cellulose microfibrils and other glycans in the cell wall. In this study the function of the barley expansin family was explored at two levels. After identification of genes from the available EST databases, the mRNA transcript levels of barley expansins were measured in a barley developmental tissue series to provide evidence that many of these genes have cell-specific expression, with each cell type expressing a distinct set of expansins during growth. To characterise the functions of expansins in the cell wall, assays using expansin protein obtained via heterologous expression of three barley expansin genes in *E. coli* were trialled.

Thus, in order to explore the expansin family in barley and the proposed mechanism of action of expansins, the more specific objectives of this project were:

- To determine the number of expansin genes in barley.
- To classify each gene into an expansin subfamily based on phylogenetic analysis.
- To measure the mRNA transcripts of barley expansin genes in a range of barley tissues at various developmental stages, in order to gain a greater understanding of the role of particular expansins in developmental processes.
- To clone, express and purify three active expansins in *E. coli* for the purpose of examining their mechanism of action and/or substrate binding.
- To explore the effects of active recombinant expansin protein on cellulose in conjunction with other cell wall modifying proteins.

CHAPTER 2

CHARACTERISATION OF EXPANSIN GENES IN BARLEY

2 CHARACTERISATION OF EXPANSIN GENES IN BARLEY

2.1 INTRODUCTION

The first aim of this project was to characterise the expansin gene family in barley. Public databases have an abundance of ESTs corresponding to cell wall modifying gene transcripts including sequences that code for expansin genes. With the sequencing of the barley genome now being undertaken by an international consortium, the number of ESTs available has only increased with time, and to date, no systemic identification, description or characterisation of expansin genes has been reported in barley.

The first step was to analyse barley EST databases for putative expansins. There, the identification of expansin genes in barley was accomplished by carrying out a BLAST search of barley EST databases using putative rice and wheat expansin sequences as search templates. The public EST databases yielded 34 putative barley expansin and expansin-like genes. Multiple sequence alignments of the deduced amino acid sequences of the proteins encoded by barley expansins were performed with published plant expansins to confirm sequence characteristics and determine the subgroup to which each sequence belonged. Following classification into their subgroup of α- β- or expansin-like A, the numbering of the barley expansin genes were arbitrarily assigned, with exception to the published *HvEXPB1* (Kwasniewski and Szarejko, 2006).

By assembling the ESTs into tentative contigs, it was possible to predict full length sequences of 26 of the 34 barley expansin and expansin-like genes identified. The other eight sequences identified were only partial cDNAs. Of the three partial cDNA that was missing the downstream sequence, only one was successfully completed using 3' Rapid Amplification of cDNA Ends (RACE). The completion of five partial cDNAs via 5'RACE was met with limited success. Gene-specific PCR primers were designed to the 5' and 3' UTRs for the amplification of cDNA fragments from RNA preparations to successfully isolate eight cDNAs encoding barley expansins.

An *in silico* analysis of the *Brachypodium* and sorghum genomes was also conducted and identified 62 and 79 members of the expansin family respectively. A radial tree constructed of the identified expansins from these genomes with rice and barley expansins clearly illustrate the expansin orthologues within these genomes. The results of these analyses of the barley expansin genes are presented below.

2.2 MATERIALS AND METHODS

2.2.1 General materials, reagents and methods

All chemicals used for solutions were of analytical grade and were supplied by SIGMA-Aldrich (St. Louis, MO, USA) unless otherwise indicated. All oligonucleotide primers used were synthesised by GeneWorks (Adelaide, SA, Australia). Restriction enzymes and buffers were purchased from New England Biolabs (Beverly, MA, USA), and used in accordance with manufacturer's instructions unless otherwise indicated. Reagents used for agarose gel electrophoresis and ethidium bromide staining were purchased from Amresco (Solon, OH, USA). The Superscript III™ RT, RNaseOUT™, 1 Kb Plus DNA Ladder and Elongase® Amplification System were purchased from Invitrogen (Carlsbad, CA, USA). TURBO DNase-*free*™ and RNAase*Zap*® DNase-*free*™ were purchased from Ambion Inc (Austin, TX, USA). Taq PCR Core Kit and QIAprep Spin Miniprep Kit were purchased from QIAGEN GmbH (Hilden, Germany).

2.2.2 Plant material

Barley (*Hordeum vulgare* cv Sloop) seedlings were germinated on damp paper towels in the dark for 7 days at 20°C. The coleoptile, first leaf, germinating embryo and roots from 4 seedlings were collected and each tissue type was pooled and placed in 2 ml microfuge tubes with 3 mm stainless steel beads. Harvested tissues were immediately frozen in liquid nitrogen.

Barley stem RNA was supplied by Dr. Qisen Zhang (University of Adelaide, South Australia, Australia) and RNA from entire barley caryopsis at anthesis (Burton *et al.*, 2008) and mature grain RNAs were supplied by Assoc. Prof. Rachel Burton (University of Adelaide, South Australia, Australia).

2.2.3 RNA extraction

RNA was extracted from plant material with the guanidine/phenol TRIzol-like reagent (1 M guanidine thiocyanate, 1 M ammonium thiocyanate 10 mM sodium acetate pH 4.5, 38% (v/v) phenol pH 4.3, 5% (v/v) glycerol). Prior to the addition of the TRIzol-like reagent, the plant tissue was ground to a fine power using a Mixer Mill MM 300 (Retsch GmbH & Co. KG) with 30 s bursts at a frequency of 25 Hz. The germinated seed sample remained as fragments after multiple rounds in the mill, so was ground to a powder in a liquid nitrogen cooled mortar and pestle. TRIzol-like reagent was added to the frozen powder (1 ml/100 mg tissue), the mixture was vortexed until tissue was in suspension, and was left at room temperature for 5 min before the addition of 200 µl chloroform (0.2 ml per 1 ml TRIzol-like reagent). Samples were vortexed for 15 s and left at room temperature for a further 3 min. Phase separation was achieved by centrifugation at 12 000 rpm for 15 min at 4°C,

after which the upper aqueous layer was transferred to a fresh tube. Nucleic acid was precipitated with 750 µl isopropanol at room temperature for 10 min, and pelleted at 12 000 rpm for 10 min at 4°C. The supernatant was decanted and the remaining pellet was washed with 70% (v/v) ethanol prepared with DEPC treated water (DEPC 0.1% v/v, for 16 h at 37°C followed by autoclaving). RNA was stored at -80°C until required. RNA was recovered by centrifuging the pellet under 70% (v/v) ethanol at 10,000 rpm 4°C, 5 min, removing supernatant and drying the pellet in a Speedi-vac (3-5 min). RNA was resuspended in 100 µl DEPC treated water. The integrity and size distribution of total RNA purified was checked by running 5 µl RNA on a 1.6% (w/v) agarose gel containing 0.1 µl/ml ethidium bromide. The gel apparatus was treated with RNAase*Zap*® prior to use, to eliminate RNases.

RNA was DNase treated with TURBO DNase-*free*™ to remove contaminating DNA. To a maximum of 200 µg nucleic acid per ml, the equivalent of 0.1 vol 10x TURBO DNase Buffer and 2 units TURBO DNase-*free*™ was added. The DNase/RNA treatment mix was incubated at 37°C for 20 min. Following incubation, 0.1% (v/v) DNase Inactivation Reagent was added and the reaction incubated at room temperature for 2 min with occasional mixing. The RNA was recovered from the DNase treatment by centrifuging the mixture at 14,000 rpm for 90 s. The supernatant containing the RNA was transferred to a fresh tube.

2.2.4 cDNA synthesis for 3'RACE

Synthesis of 3' cDNA was accomplished using the Smart™ RACE cDNA Amplification Kit (Clontech Laboratories Inc, Mountain View, CA, USA). The 3' Smart cDNA synthesis was performed on 1 µg RNA with 1 µl 12 µM 3'-RACE CDS Primer A (5' AAGCAGTGGTATCAACGCAGAGTAC$(T)_{30}$V N 3' (N = A, C, G, or T; V = A, G, or C) to a volume of 3.5 µl. The RNA/primer mix was incubated at 70°C for 2 min. The equivalent of 2 µl 5x cDNA Synthesis Buffer, 1 µl 20 mM DTT and 1 µl 10mM dNTPs was added to each reaction from a master mix. PowerScript™ reverse transcriptase (1 µl, 50 U) was added to each reaction and mixtures incubated at 42°C for 90 min. A final incubation step of 72°C for 7 mins concluded the reaction after the addition of 90 µl of Tricine-EDTA (10 mM Tricine-KOH pH 8.5, 1 mM EDTA) buffer to each sample. The 3'RACE cDNA was stored at -20°C.

2.2.5 cDNA synthesis

Synthesis of cDNA was accomplished using the Superscript III™ RT (Invitrogen). Synthesis of cDNA was performed on 2 µg RNA with 1 µl 50 µM Poly $(dT)_{20}$ oligo and 1 µl 10mM dNTPs to a volume of 12 µl. The RNA/primer mix was incubated at 65°C for 5 min and immediately cooled on

ice slurry for 2 min. To each reaction 4 µl 5x cDNA Synthesis Buffer, 1 µl 0.1 M DTT, 1 µl RNaseOUT™ (40 U/µl) to a volume of 6 µl was added from a master mix. SuperScript™ III RT (50 units, 200 U/µl) was added to each reaction and mixtures incubated at 50°C for 60 min. Reactions were heat inactivated at 70°C for 15 min. The cDNA was stored at -20°C.

2.2.6 Barley expansin identification in silico

Tentative Contigs (TCs)/Transcript Assemblies (TAs) and ESTs of barley expansin cDNA that contained putative expansins were downloaded from the TIGR Plant Transcript Assemblies database (http://plantta.tigr.org). Further identification of expansin genes in barley was accomplished by carrying out a BLAST search of barley ESTs in the NCBI database (http://www.ncbi.nlm.nih.gov) using putative rice and wheat sequences as search templates. The homologous ESTs were assembled into contigs with putative open reading frames (ORFs) with ContigExpress (Invitrogen, Carlsbad, CA, USA) and adjusted manually. The deduced amino acid sequences of barley expansin proteins were aligned using CLUSTALW (http://clustalw.genome.ad.jp/). ClustalX2 (www.clustal.org/) was used for multiple sequence alignment and the drawing of phylogenetic trees. The TreeView software application (Dubessay *et al.*, 2004) was used to open the phylogenetic trees generated with ClustalX2 program. SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the signal peptide cleavage sites within the protein molecules.

2.2.7 Primer design

For partial cDNAs missing the 3' or 5' ends, gene-specific primers were designed on the basis of available sequences from partial ESTs. The oligonucleotide primers for 3' RACE were designed with the following features considered, 23-28 nucleotides, 50-70% GC rich and with an annealing temperature (T_m) ≥ 65°C. The oligonucleotides for generation of full-length cDNA were designed where both 5' and 3'UTR sequence was available. All primers were designed with the aid of the programs Primer3 (Rozen and Skaletsky, 2000), and assessed with NetPrimer (PREMIER Biosoft International, Palo Alto, CA, USA) for hair pin loops, primer dimers, palindromes and sequence repeats.

2.2.8 Generation of full-length cDNAs

2.2.8.1 3'RACE

PCRs were performed on cDNA generated from section 2.2.4. Reactions were carried out using the Advantage 2 Polymerase Mix (Clontech, Laboratories Inc., Mountain View, CA, USA). Reaction

components of the PCR were 1.25 µl cDNA, 1 µl 10 µM gene-specific primer (Appendix A, Table A-1) and 2.5 µl 12 µM Universal Primer A Mix (UPM 5' CTAATACGACTCACTATAGGGC 3'), 0.5 µl 10 mM dNTP's, 1x Advantage 2 PCR Buffer and 1x Advantage 2 Polymerase in a total volume of 25 µl. Cycling conditions for PCR were 40 cycles of 94°C 20 s, 68°C 30 s and 72°C 3 min. Samples were held at 25°C until 20 µl of the reaction mix was resolved on a 1% (w/v) agarose gel containing ethidium bromide, and viewed under UV light.

2.2.8.2 cDNA gel purification

Fragments of the predicted size were purified from the gel with the PCR clean-up Gel extraction NucleoSpin® Extract II kit (Macherey-Nagel, Duren, Germany), as per the manufacturer's protocol. The excised gel slice was incubated at 50°C with the addition of Buffer NT (200 µl per 100 mg of agarose gel) until dissolved. The dissolved gel slice and Buffer NT mix was loaded onto a NucleoSpin® Extract II and centrifuged for 1 min at 11,000 x g. The flow through was discarded and the silica membrane was washed with 600 µl of Buffer NT3 and centrifuged for 1 min at 11,000 x g. The wash was discarded and the column was further centrifuged for 1 min to remove remaining column wash. The column was transferred to a fresh collection tube and was placed in a heating block at 65°C for 2 min. The cDNA was twice eluted with 25 µl elution Buffer NE equilibrated to 65°C.

2.2.8.3 Cloning into pGEM®-T Easy Vector

Use of Advantage 2 Polymerase Mix resulted in amplicons with a single adenine overhang at the 5' ends of each nucleic acid strand. These 'A-tailed' PCR products were ligated into the pGEM®-T Easy Vector plasmid (Promega, Madison, WI, USA), which was provided as a linearised plasmid with T-overhangs, using T4 DNA ligase as per the manufacturer's protocol. Ligation reactions were performed with 1x Rapid Ligation Buffer, 3 Weiss units T4 DNA ligase, 10 ng pGEM®-T Easy Vector, made up to a 10 µl reaction with 3 µl of the purified cDNA product. Reactions were incubated at 4°C for 16 h to allow for ligation.

2.2.8.4 Transformation of Competent E. coli

Heat shock competent *E. coli* strain DH5α cells were prepared by the standard Hanahan (1985) method. pGEM®-T Easy ligation reaction (4 µl) was added to a 40 µl aliquot of thawed competent *E. coli* DH5α cells, mixed gently with a pipette and left on ice for a further 10 min. Samples were incubated for 45 s at 42°C and transferred back to ice for 5 min. Heat shocked cells were immediately resuspended in 200 µl Luria Bertani media (10 g/L tryptone, 5 g/L yeast extract, 10 g/L

NaCl) without selection and incubated at 37°C for 60 min. After this cell recovery period, samples were spread onto LB agar (15 g/L) plates containing 100 μg/ml ampicillin and 80 μg/ml 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) to allow blue/white colony selection according to standard methods (Sambrook *et al.*, 1989). Plates were incubated for 16 h at 37°C.

2.2.8.5 Colony screening

White colonies, indicative of the presence of an insert in the multiple cloning site (MCS), were picked using sterile pipette tips and inoculated onto a fresh LB agar plate supplemented with 100 μg/ml ampicillin. To confirm the presence of an insert, colony screening by PCR was conducted using two pGEM®-T Easy Vector specific primers, T7 (5' TAATACGACTCACTATAGGG 3') and SP6 (5' TATTTAGGTGACACTATAG 3') and PCR reagents from QIAGEN as follows. A small amount of the same colony was mixed in a 0.2 ml PCR tube containing 1.25 μl 10x CoralLoadPCR Buffer, 0.25 mM dNTPs, 0.75 μl 25 mM $MgCl_2$, 1.25x Q-Solution, 1 μM vector specific primers (T7 and SP6), and 0.5 units *Taq* polymerase (5U/μl) in a total volume of 10 μl. PCR cycling conditions were; 94°C for 2 min followed by 30 cycles of 94°C 30 s, 50°C 30 s, 72°C 2 min with a final step of 72°C for 5 min. The full amount of the reaction mix was resolved on a 1% (w/v) agarose gel containing 0.1 μg/ml ethidium bromide, and viewed under UV light.

2.2.8.6 DNA plasmid preparations

Colonies that produced positive fragments of the desired size were cultured for 16 h at 37°C in 5 ml LB supplemented with 100 μg/ml ampicillin. Plasmids were isolated from *E. coli* cultures using the QIAprep Spin MiniPrep Kit according to the manufacturer's protocols.

2.2.8.7 Restriction digest analysis of DNA plasmid inserts

DNA plasmid inserts were analysed using an *Eco*RI digest according to the manufacturer's instructions. *Eco*RI sites flank the MCS of pGEM®-T Easy, therefore releasing the cloned insert. Digested plasmids were analysed on a 1% (w/v) agarose gel containing ethidium bromide.

2.2.8.8 DNA sequencing and sequence analysis

Clones that displayed different insert sizes were analysed by DNA sequencing. All sequencing reactions were carried out using the Big Dye Terminator v3.1 sequencing reagent (Applied Biosystems, Foster City, CA, USA). Reactions contained 0.5 μl template DNA from a standard plasmid preparation (corresponding to about 50 ng), 1.5 μl 5x BigDye buffer, 1 μl Big Dye Enzyme mix and 1 μl primer (3.3 μm stock) made up to 10 μl with sterile H_2O. When sequencing the expansin inserts within the pGEM®-T Easy vector, the T7 and SP6 primers were used. PCR

sequencing reaction conditions were as follows: an initial 5 min denaturation step at 96°C was followed by 26 cycles of 96°C for 10 s, 50°C for 5 s and 60°C for 4 min. Following the PCR sequencing reaction, 75 µl wash buffer (0.2 mM $MgSO_4$, 70% EtOH) was added and samples were incubated at room temperature for 15 min, followed by a 15 min centrifugation at 4000 x g to pellet DNA. The supernatant was removed by tapping the tubes upside down on tissue paper and allowing the remainder of the liquid to air dry at room temperature. Samples were submitted to the Australian Genome Research Facility (AGRF, Adelaide SA, Australia) for service sequencing on an Applied Biosystems AB3730xl capillary sequencer. Resulting sequencing chromatograms were viewed, evaluated and aligned using ContigExpress. Sequence alignments and database searches were carried out using NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST).

2.2.8.9 5'RACE

Synthesis of cDNA was performed on 2 µg RNA with 1 µl 10 µM gene-specific primer 5'RACE (Appendix A, Table A-1) and 1 µl 10mM dNTPs to a volume of 12 µl. The RNA/primer mix was incubated at 65°C for 5 min and immediately cooled on ice for 2 min. The equivalent of 4 µl 5x cDNA Synthesis Buffer, 1 µl 0.1 M DTT, 1 µl RNaseOUT™ (40 U/µl) to a volume of 6 µl was added to each reaction from a master mix. SuperScript™ III RT (50 units, 200 U/µl) was added to each reaction and mixtures incubated at either 50°C or the annealing temperatures listed in Appendix A, Table A-1 for 60 min. Reactions were heat inactivated at 85°C for 5 min prior to the addition of 1 µl Ribonuclease H (Invitrogen) for a final incubation of 37°C for 20 mins. Reactions were directly cleaned with PCR clean-up Gel extraction NucleoSpin® Extract II kit (Macherey-Nagel, Duren, Germany) as described in section 2.2.8.2 with the alteration of elution volume to 20 µl. The dGTP tailing reaction was performed with reagents supplied by New England BioLabs (Beverly, MA, USA). The equivalent of 1 µl 10x NE Buffer 4, 1 µl 2.5 mM $CoCl_2$, 0.5 µl 10 mM dGTP and 1 µl Terminal Transferase (40 U/µl) was added to 7 µl clean cDNA and incubated at 37°C for 60 min. The tailing reaction was terminated by incubating at 75°C for 20 mins. Reactions were carried out using Elongase® *Taq* Polymerase Amplification System (Invitrogen). PCR was carried out in a total volume of 25 µl containing 2 µl of the tailed reaction as template, 1 µl 10 µM Poly $(dC)_{15}$ oligo, 1 µl 10 µM of the gene-specific primer (5'RACE PCR) listed in Table A-1 (Appendix A), 2.5 µl Elongase Buffer A, 2.5 µl Elongase Buffer B, 0.5 µl Elongase polymerase and 0.5 µl 10 mM dNTPs. Cycling conditions for PCR were; 94°C 5 min followed by 40 cycles of 94°C 30 s, 58-61°C (annealing temperature based on the T_m of the gene-specific primers -/+ 2°C) 30 s, 68°C 1 min and a final step

of 68°C for 5 min. Samples were held at 25°C until 20 µl of the reaction mix was resolved on a 1% (w/v) agarose gel containing ethidium bromide, and viewed under UV light.

2.2.9 Full length cDNA isolation and sequence analysis

2.2.9.1 Amplification of expansin cDNA

Full length coding sequences of eight expansin genes were amplified by PCR from cDNA generated from section 2.2.5. Reactions were carried out using the Elongase® *Taq* Polymerase Amplification System (Invitrogen). The gene-specific primers and cDNA templates used to amplify the eight expansin cDNAs are shown in Table 2-1. PCRs were carried out in a total volume of 25 µl containing 0.5 µl of the appropriate cDNA template listed (Table 2-1), 1 µl 10 µM of the gene-specific primers listed in Table 2-1, 2.5 µl Elongase Buffer A, 2.5 µl Elongase Buffer B, 0.5 µl Elongase polymerase and 0.5 µl 10 mM dNTPs. Cycling conditions for PCR were; 94°C 5 min followed by 30 cycles of 94°C 30 s, 58-61°C (annealing temperature based on the T_m of the gene-specific primers) 30 s, 68°C 1 min and a final step of 68°C for 5 min. The expected lengths of the eight predicted expansin cDNA and annealing temperatures are listed in Table 2-1. Samples were held at 25°C until visualised by running 5 µl PCR product on a 1% (v/v) agarose gel containing ethidium bromide.

2.2.9.2 cDNA gel purification

Full length fragments of the predicted size were gel purified as described in section 2.2.8.2.

2.2.9.3 DNA sequencing and sequence analysis

The DNA concentration of PCR fragments was measured on a spectrophotometer (NanoDrop ND-100 Spectrophotometer, NanoDrop Technologies, Wilmington, USA). For a PCR product between 500 – 1000 bp, 5 – 20 ng of template was used in the sequencing reaction. Sequencing and sequencing analysis was carried out as described in 2.2.8.8 using gene-specific primers.

2.2.10 In silico *analyses of expansins in* Arabidopsis thaliana*, rice (*Oryza sativa*),* Sorghum bicolor*,* Brachypodium distachyon *and barley*

The *Arabidopsis* and rice expansin protein sequences were downloaded from http://www.bio.psu.edu/expansins/other_species.htm. The full length coding region of *HvEXPA1, HvEXPB1* and *HvEXLA1* were used as queries to search the *Sorghum bicolor* and *Brachypodium distachyon* genome data available at http://gramene.org/ with an E-value cut off of e^{-25}. Introns were removed from nucleotide sequences by computational methods that rely on alignments of putative coding sequences with homologous genes from other species, with alignment gaps

Gene	Forward primer (5'—3')	Reverse primer (5'—3')	PCR size bp	Annealing temperature °C	cDNA template source
HvEXPA1	TCCCTCCCTCCCAGCAATG	TACAAACCAACCACGAGAGG	1171	52	coleoptile
HvEXPB1	ATAGATCATCAATGGCGGCTAG	CATCGATCCTTTGCTGTGACTACAA	1040	53	root
HvEXPB2	TACACTACCCCCACAAGCCT	TGCCTCTCCTCCATAGCCCA	1031	54	All cDNA
HvEXPB4	T GCGGCATGGGCTCCCTC T	GCCAATACACGACTCATCTCTCAC	1082	57	Root and leaf
HvEXPB5	GCAAGTATGGCTGGGGTCTC	GAATCTTGTGATAGTGGGCTGCGT	1061	56	All cDNA
HvEXPB9	CAATATGGCTGGCGTCTCC	ACTCCTCAGGACACACAATCG	1085	54	Root and leaf
HvEXPB10	GAGTCCACAGCAGAGCAACA	ATGACACCAGACAGACGGCA	1069	54	All cDNA
HvEXPB15	TCTTCAGTCTCTTCCTCCTTGTA	CACCAAAGTACCAAACTGACAGAT	1068	53	Developing endosperm

Table 2-1 Primers for isolation of full length barley expansin cDNA.

Gene-specific primers were designed in the 5' and 3' untranslated regions. Product sizes in base pairs, annealing temperature and templates used are listed.

identifying possible introns. Protein-protein BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were performed against the non-redundant protein sequences (nr) to check for incorrect predictions and sequences were manually adjusted. Sequences are annotated as their query location based on http://gramene.org/. Genomatix software (http://www.genomatix.de/cgi-bin/dialign/dialign.pl) was used for multiple sequence alignment whilst whilst ClustalX2 (www.clustal.org/) was also used for multiple sequence alignment followed by the drawing of phylogenetic trees. The TreeView software application (Dubessay *et al.*, 2004) was used to open the phylogenetic trees generated with the ClustalX2 program. SignalP 3.0 Server (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the signal peptide cleavage sites within the protein molecules. The numbers of homologues within each family were identified.

2.3 RESULTS

The aim of the work described in this Chapter was to characterise the expansin gene family in barley, using publicly available databases to estimate the number and classes of expansin genes. A search of the public barley EST databases yielded 34 putative barley expansin and expansin-like genes (Table 2-2). Of the 34 identified, it was possible to predict full-length sequences of 26 of these by assembling the EST sequences. The assembled full and partial length nucleotide sequences can be found in Appendix B. One of the cDNAs (*HvEXPB1* GenBank accession number AY351785) had previously been studied with respect to sequence and expression (Kwasniewski and Szarejko, 2006), but for completeness it is included in analyses undertaken in this project.

2.3.1 Characteristics of barley expansin proteins

Based on deduced amino acid sequences, a dendogram was constructed and the 34 barley expansin proteins clearly separated into three subfamilies, namely α-expansins (14 cDNAs, *HvEXPA1-HvEXPA14*), β-expansins (17 cDNAs, *HvEXPB1-HvEXPB16, HvEXPB18*) and expansin-like A (three cDNAs, *HvEXLA1-HvEXLA3*)(Figure 2-1). The amino acid sequence alignments of the predicted proteins encoded by these cDNAs show that the sequences contain key features of α- and β-expansins and expansin-like A, including a series of conserved cysteine (C) residues in the NH_2-terminal regions of the proteins and a series of conserved tryptophans (W) in the putative cellulose-binding domains in the COOH-terminal regions (Cosgrove, 1999; Cosgrove, 2000b; Li *et al.*, 2002) (Figures 2-2, 2-3 and 2-4). Analysis with the SignalP program (Bendtsen *et al.*, 2004) predicts a cleavable signal peptide at the NH_2-terminus. The length of this peptide varies from 20 to 30 residues amongst the α-expansins and 22 to 34 residues amongst the β-expansins. Only one of the expansin-like A proteins is full-length and has a predicted 35 residue signal peptide.

2.3.1.1 The barley α- and β-expansins

The typical characteristics of α- and β-expansin proteins, including a putative catalytic domain with a histidine, phenylalanine, aspartic acid (HFD) motif in the central portion of the protein, a 'GACG' motif in the cysteine-rich NH_2-terminal region and a tryptophan-rich COOH-terminal region, were observed in all the barley expansins. Of the cysteine (C) residues, eight were very well conserved in the α-expansins and six of the eight residues were conserved in the β-expansins. Some variation in the HFD motif is observed where the amino acid residue D is replaced by L in *HvEXPA6*, F is replaced by L in *HvEXPB9* and an F is replaced by I in *HvEXPB11*. An alignment of a selection of barley α- and β-expansin protein sequences clearly shows that there are two characteristic insertions, α and β in the central domain of the predicted proteins (Figure 2-5).

Gene ID	TC/EST	TA_4513	Unigene Hv.	ORF length (bp)
HvEXPA1	131916	32940	5784	756; Full*
HvEXPA2	131917	32938	2985/22560	750; Full
HvEXPA3	132916	36035	9096	864; Full
HvEXPA4	134956	46841	1667	768; Full
HvEXPA5	141369	38214	10758	717; Full
HvEXPA6	143385	41645	15860	780; Full
HvEXPA7	146182	52424/52690	1919	765; Full
HvEXPA8	152063	49015	15625	>429; Partial
HvEXPA9	BG368084	-	20874/23609	774; Full
HvEXPA10	BQ760682	-	15325	780; Full
HvEXPA11	BQ766938	-	26951/3'RACE	750; Full
HvEXPA12	BU973592	-	-	>120; Partial
HvEXPA13	BY848626	-	-	>369; Partial
HvEXPA14	BQ765644	-	-	>343; Partial
HvEXPB1	143572	45995	10452	849; Full*†
HvEXPB2	131611	33589	11421	798; Full*
HvEXPB3	131612	31856	6274/23921	855; Full
HvEXPB4	131613	31853	814	834; Full*
HvEXPB5	131614	47805	15553	822; Full*
HvEXPB6	131615/BM097618	-	16504	933; Full
HvEXPB7	134150	36987	16084	864; Full
HvEXPB8	134212	36986	873	870; Full
HvEXPB9	136089	44356	2106/21242	816; Full*
HvEXPB10	140385	33219	9123	798; Full*
HvEXPB11	141479	42532	12052	801; Full
HvEXPB12	144522/151633	46189	15275	>722; Partial
HvEXPB13	147696	36277	16330	834; Full
HvEXPB14	147697	43681	7312	1089; Full
HvEXPB15	148166	38645	7153	798; Full*
HvEXPB16	148426	39501	11576	789; Full
HvEXPB18	BY848215/BY869303	-	30903	825; Full
HvEXLA1	131209	28455	11566	842; Full
HvEXLA2	133975	42104	17851	>654; Partial
HvEXLA3	133976	-	20257	>330; Partial

†(Kwasniewski and Szarejko, 2006)

Table 2-2 List of characterised barley expansins.

Tentative Contigs (TC), Expressed sequence tags (EST) Transcript assemblies (TA) and Unigenes of the barley expansins. The numbering of the barley expansins is arbitrarily assigned. Sizes of the open reading frames (ORF) are indicated in base pairs (bp) and are shown as either full or partial. Asterisk indicates isolated full length cDNA.

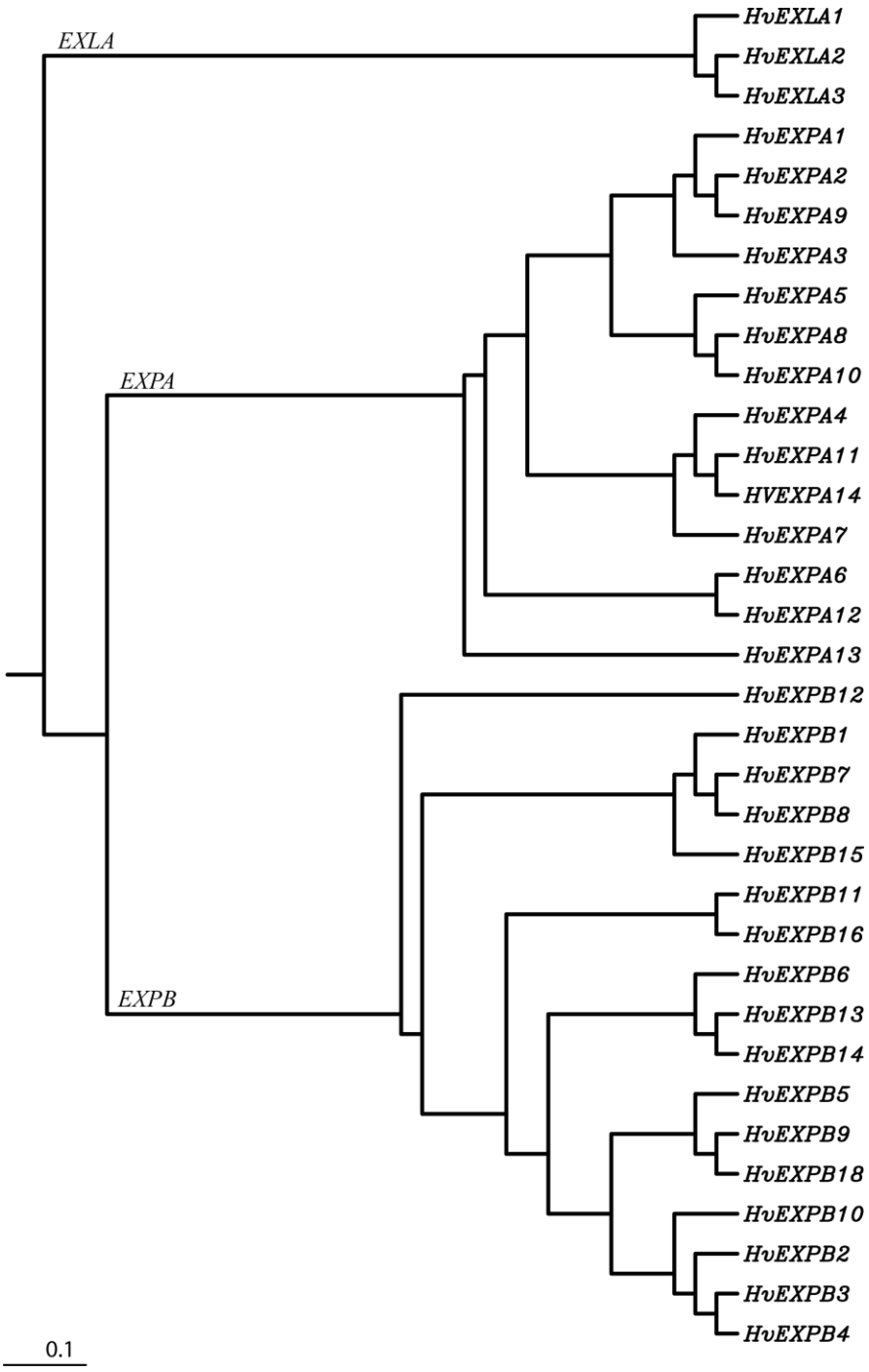


Figure 2-1 Dendogram of the barley expansin family.

The dendogram was generated using the CLUSTALW program. The dendogram clearly separates the barley expansins into three subfamilies, expansin-like A (EXLA), α-expansin (EXPA) and β-expansin (EXPB).

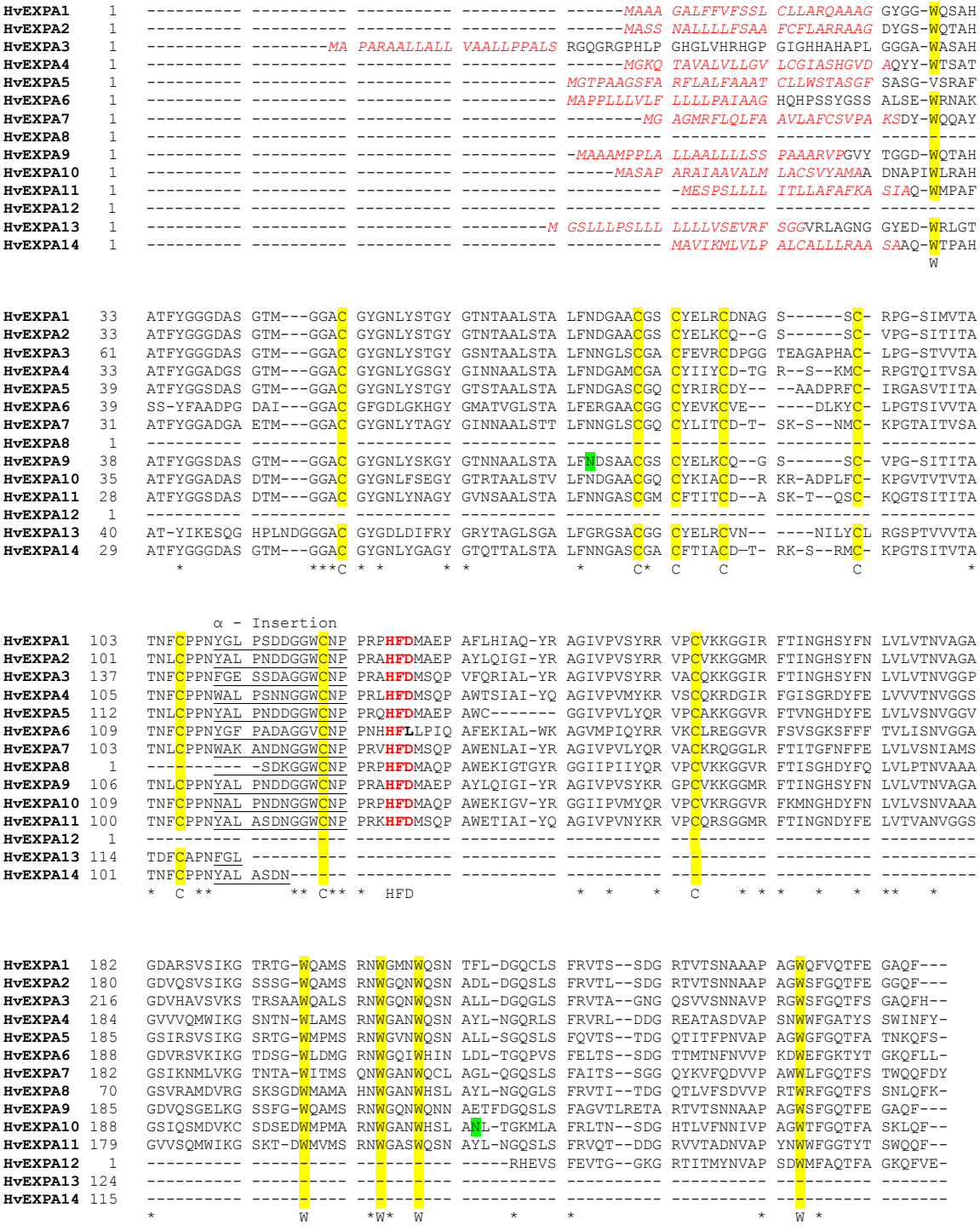


Figure 2-2 Alignment of barley α-expansin sequences.

The sequences in red *italics* represent the signal peptides predicted by the SignalP 3.0 Server program (http://www.cbs.dtu.dk/services/SignalP/). Potential N-linked glycosylation sites are highlighted in green. Capital letters on the bottom line indicate key amino acid residues that are conserved among the expansins and are highlighted with yellow. Asterisks denote conserved residues that are conserved amongst the α-expansins in this alignment. The histidine, phenylalanine, aspartic acid (HFD) motif is shown in red. The α-insertion unique to α-expansins is indicated.

```
HvEXPB1     1   ---------- ---------- ---------- ---------- ---------- ----MAASRG TLLLATVLVI LSLFVSPISG
HvEXPB2     1   ---------- ---------- ---------- ---------- ---------- ------MAPL SSKAVALVAL SSLLVTYAAA
HvEXPB3     1   ---------- ---------- ---------- ---------- ------MGRP QLTLLSAPAP ALVLAVLLLS SSYSLHSAEA
HvEXPB4     1   ---------- ---------- ---------- ---------- ---------- --------MG SLPSLAVLAA LLCFLAVGGA
HvEXPB5     1   ---------- ---------- ---------- ---------- ---------- -----MAGVS ANAVALAALV SVLLTYGCCA
HvEXPB6     1   ---------- ---------- ---------- ---------- ---------- -----MAATF SSYAIAVAFL CLLAANGCSG
HvEXPB7     1   ---------- ---------- ---------- ---------- ---------- ----MAKSFT SLALLSALVM LSLFVSPIAC
HvEXPB8     1   ---------- ---------- ---------- ---------- ---------- -----MANAC TLALLGALLV LSLLVSPIAC
HvEXPB9     1   ---------- ---------- ---------- ---------- ---------- ------MAGV STNAIALVAL LSVLFTSVRS
HvEXPB10    1   ---------- ---------- ---------- ---------- ---------- ----MAGVLS VKAVALAAVL AAAYVSSAAA
HvEXPB11    1   ---------- ---------- ---------- ---------- ---------- ------MGSL SYVLAAAVLA SLITGGACIA
HvEXPB12    1   ---------- ---------- ---------- ---------- ---------- -----MAPSR AAAAPLALCC ILLLASSSAA
HvEXPB13    1   ---------- ---------- ---------- ---------- ---------- ----MASSSS AVVAALILCI LIAHGHGCFA
HvEXPB14    1   ---------- -----MAPSS SSSFAVAAAL LLCILAAHGH GCCAKRSSAK KSHSHHGAPP PAPSPPATRP PSYGYGSPPP
HvEXPB15    1   ---------- ---------- ---------- ---------- ---------- ---MTFSVAR ISASIAVLMA LAFLLRPCAA
HvEXPB16    1   ---------- ---------- ---------- ---------- ---------- --------MA SSSALLVAAV LAVVVCGAHG
HvEXPB18    1   ---------- ---------- ---------- ---------- ---------- ---MAGVSAN AVAVVAVALL LSVLVAPVRC

HvEXPB1    39   ---------- ---------- ---------- ---------- YWKASRTRTR RPSSRRSWSS -GGATWYGSP YGAGSD--GG
HvEXPB2    36   ---------- ---------- ---------- ---------- ---GAGTFND SAFADPNWED -ARATWYGAP TGAGPMDDGG
HvEXPB3    37   ---------- ---------- ------AEEE ASAGLDTGVA GDPGLLNATA VSIGQSGV-- -ARATWYGAP NGAGPYDNGG
HvEXPB4    28   ---------- ---------- ---------- -VDLNATDAS PFDVDLNVTD ATKYWGPWTP -ARATWYGQP NGAGPDDNGG
HvEXPB5    40   ---------- ---------- ---------- ---------Q SPLNYTSLAK ASKASWSWLP -AKATWYGAP TGAGPDDNGG
HvEXPB6    54   -------CPW FLPATFCPEP TPDPIPTPNP APATPPPAPA TTSPGSGAGS TNGSTGGWLD -ARATWYGAP DGAGPLDNGG
HvEXPB7    53   ---------- ---------- -------SRK LPKPAMKVHK NHTAATPSSF AAYGSGGWSA -GGATYYGAP DGDGSD--GG
HvEXPB8    44   ---------- ---------- -SRKLAKAVG QHKPAAVKGH KNQTATNPSS SAAYGSGWLP -AGATYYGNP NGDGGE--GG
HvEXPB9    30   ---------- ---------- ---------- ---------- --AVNYDTAM ARSYNSGWLP -AKATWYGAP NGPGPDDNGG
HvEXPB10   31   ---------- ---------- ---------- ---------- ----VNLNTS AVSYSSAWLP -ARATWYGAP NGAGPDDNGG
HvEXPB11   27   ---------- ---------- ---------- ---------- --KVPPGPNI TTNYNMNWTP -AKATWYGKA TGSGPKDNGG
HvEXPB12   28   ---------- ---------- ---------- --FLLDGGKS AAKGKAKGNG KAAVDVEWRP -ATATWYGDA EGDGST--GG
HvEXPB13   34   ---------- ---------- ---------- ---------- -----KHQTP HPAANSSWLD -AKATWYGRP TGAGPDDNGG
HvEXPB14   72   PAASTPPPAP SPPATRPPPY GYGSPPPPAA STPPYGYGAP PPAANSSSNS TNVDAGGWLD -ARATWYGAP NGAGPDDNGG
HvEXPB15   34   ---------- ---------- ---------- ---------- ---------- ---------VE IHRKLSGWSN -GGATWYGGP NGAGSD--GG
HvEXPB16   27   ---------- ---------- ---------- ---------- IAKVPCGPNI TANYVSEWKD -AKTTWYGKP TGAGPKDNGG
HvEXPB18   30   ---------- ---------- ---------- ---------- --AANYDTSA ARSYNSGWLP -AKATWYGAP TGAGPMDNGG
                                                                                W       *W**           **

HvEXPB1    47   ACGYQSAVGQ RPFSSMIAAG GSSFFKSGKG CGACYQVKC- -TG--NKACS GRPVTVVITD SCPDGICASE D--HFDMSGT
HvEXPB2    43   ACGFKN-TNQ YPFSSMTSCG NEPIFKDGKG CGSCYQIRC- -TN--DQSCS GNPETVVITD M---NYYP-V AKYHFDLSGT
HvEXPB3    66   ACGFKN-VNR YPFMAMTSCG NQPLFKDGKG CGACYKIKC- -TK--HKACS GRQETVMITD M---NYYP-V APYHFDLSGT
HvEXPB4    52   ACGFKH-TNQ YPFASMTSCG NQPLFKDGKG CGSCYKIRC- -RK--DQSCS GRTETVIITD M---NYYP-V APYHFDLSGT
HvEXPB5    47   ACGYKH-TNQ YPFMSMTSCG NEPLYKDGMG CGACYRIRC- -VN--NKACS GKPETVMITD M---NYYP-V AKYHFDLSGT
HvEXPB6    79   ACGFKN-VNL PPFNAMTSCG NEPIFKDGKG CGSCYQIRC- -VGKVHPACS GDPETVIITD M---NYYP-V ARYHFDLSGT
HvEXPB7    60   ACGYQSAVGN RPFSSMIAAG NPSLYRGGKG CGACYEVKC- -TG--NQACS GQPATVVITD ECPGGAACLG EAAHFDMSGT
HvEXPB8    65   ACGYQTAVGH RPFSSMIAAG SSPLFMKGKG CGACYDVKC- -SS--NSACS GKPVTVVITD LSPGNLYP-G EPCHFDMSGT
HvEXPB9    43   ACGFKN-VNQ YPISSMGACG NEPIYADGEG CGMCYEIKCE YNN--NPSCS GQPRRIVIID M---NYYP-V ARYHLDLSGT
HvEXPB10   43   ACGFKH-VNQ YPFSSMTSCG NQPLFKDGKG CGSCYQIRC- -SG--DRSCS GNIETVMITD M---NYYP-V AQYHFDLSGT
HvEXPB11   43   ACGIKD-VNL APYNGMIACG NIPIFKDGKG CGSCYEVKC- -NK--PPPCS DKPITVFITD K---NYEP-I APYHIDLSGK
HvEXPB12   54   ACGYGTLVDV VPMKARVGSV SPVLFKGGEG CGACYKVRC- -LD--HGICS RRAVTVIT-- ---------- ---------
HvEXPB13   42   ACGFKN-VNL PPFSSMTSCG NQPLFKDGKG CGSCYQIRC- -LNSDHPACS GVPKTVIITD M---NYDP-V SRYHFDLSGT
HvEXPB14  126   ACGFKN-VNL PPFSAMTSCG NEPLFKDGKG CGSCYQIRC- -VSAGHPACS GVPETVIITD M---NYYP-V SRFHFDLSGT
HvEXPB15   40   ACGYQDAVDQ QPFSSMIAAG GPSIYQDGKG CGSCYQVKC- -TG--HGSCS SSPVTVVLTD ECPDGPCLD- EPVHFDLSGT
HvEXPB16   43   ACGYKE-VDK APFFGMTSCG NVPIFKDSRG CGSCFELKC- -TK--PEACS GEPTIVTITD K---NEEP-I AAYHFDLSGH
HvEXPB18   43   ACGFKN-VNK YPFSSMTSCG NEPLFDGGAG CGSCYEIRCV AAN--NPSCS GQPRTVVIID M---NYYP-V ARYHFDLSGT
                *C*         *   *             * C* C    C          C*          *                   **
```

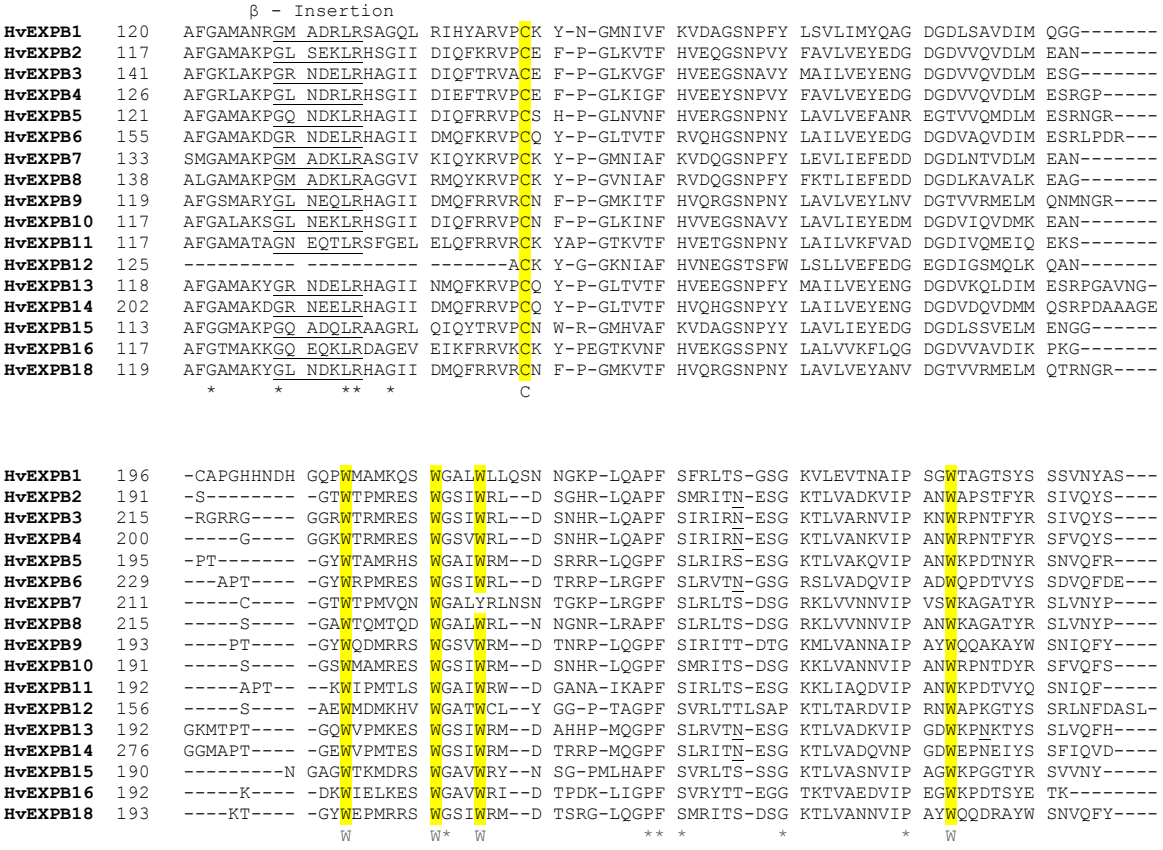


Figure 2-3 Alignment of barley β-expansin sequences.

The sequences in red *italics* represent the signal peptides predicted by the SignalP 3.0 Server program (http://www.cbs.dtu.dk/services/SignalP/). Potential N-linked glycosylation sites are underlined. Capital letters on the bottom line and highlighted in yellow indicate key amino acid residues that are conserved amongst the expansins. Asterisks denote residues that are conserved amongst the β-expansins in this alignment. The histidine, phenylalanine, aspartic acid (HFD) motif is shown in red. The β-insertion unique to β-expansins is underlined. The additional amino acids identified in the isolated *HvEXPB5* cDNA are shown in blue.

```
HvEXLA1     1   MAVSVCLGSS PSTPLLPPLL FLFFLFCSLP SLASACDRCA RHSKAAFYTS SLTLAAGSCG YGTAAASFNG GLLAAAGTAL
HvEXLA2     1   ---------- ---------- ---------- ---------- ---------- ---------- ----ARGLNG DLLAAAGPSL
HvEXLA3     1   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
                                                                                    *   **  ******  *

HvEXLA1    81   YRGGVGCGAC FQVRCKDKKL CSGAGARVVV TDRARTRTNR TDLVLSSPAF AAMARPGMAA RLTKLSAVDV EYKRVPCEYK
HvEXLA2    17   YRQGVGCGAC FQVRCKDEEL CSTAGVKVVV TDRASTKTND TELVLSSPAF AAMARPGMAA RLAKLGAVEV EYKRVPCEYE
HvEXLA3     1   ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
               ** *** **  **** **  *  * **  *** ** * * **  * ******** ********** ** ** ** * *********


HvEXLA1   161   GKNLSLRVEE RSRAPNKLAV RFLYQGGQTD IVALDVAKVG SSS-WKFMTR EHGPAWSTRQ APAGPLQFRV VVTGGYDGKW
HvEXLA2    97   GKNLSVRVEE RSRAPSELAI TILYQGGQTD IVEVDVAQVG SSSSWSSLTR DHGPAWSTSL APPGPLQLRA VVTSGFDGSW
HvEXLA3     1   ---------- --RAPNKLPS VXLYQGGQTD IVAVDVAQVG SSSSWRSLTR DHGPAWSTSL APPGPLQLRA VVTGGYDGKW
               ***** **** *****  *     ******** **  *** ** ***     **  *******   ** **** *  *** * **

HvEXLA1   240   VWADREVLPR RWRAGEVYDT GVQITDIAQE GCFPCDTHEW R
HvEXLA2   177   VFAEHEVLPR QWHAGEVYDT GVQITSIAQE ACFPCDTQEW K
HvEXLA3    69   VWADREVLPR GWRAGEVYDT GVQITDVAQE ACFPCDAQEW R
               * *  *****    ******* *****  ***   ** *  *
```

Figure 2-4 Alignment of barley expansin-like A sequences.

The sequence in red *italics* represents the signal peptide predicted by the SignalP 3.0 Server program (http://www.cbs.dtu.dk/services/SignalP/). Potential N-linked glycosylation sites are underlined. Key amino acid residues that are conserved amongst the expansins are highlighted in yellow. Asterisks denote conserved residues that are conserved amongst the expansins-like α in this alignment.

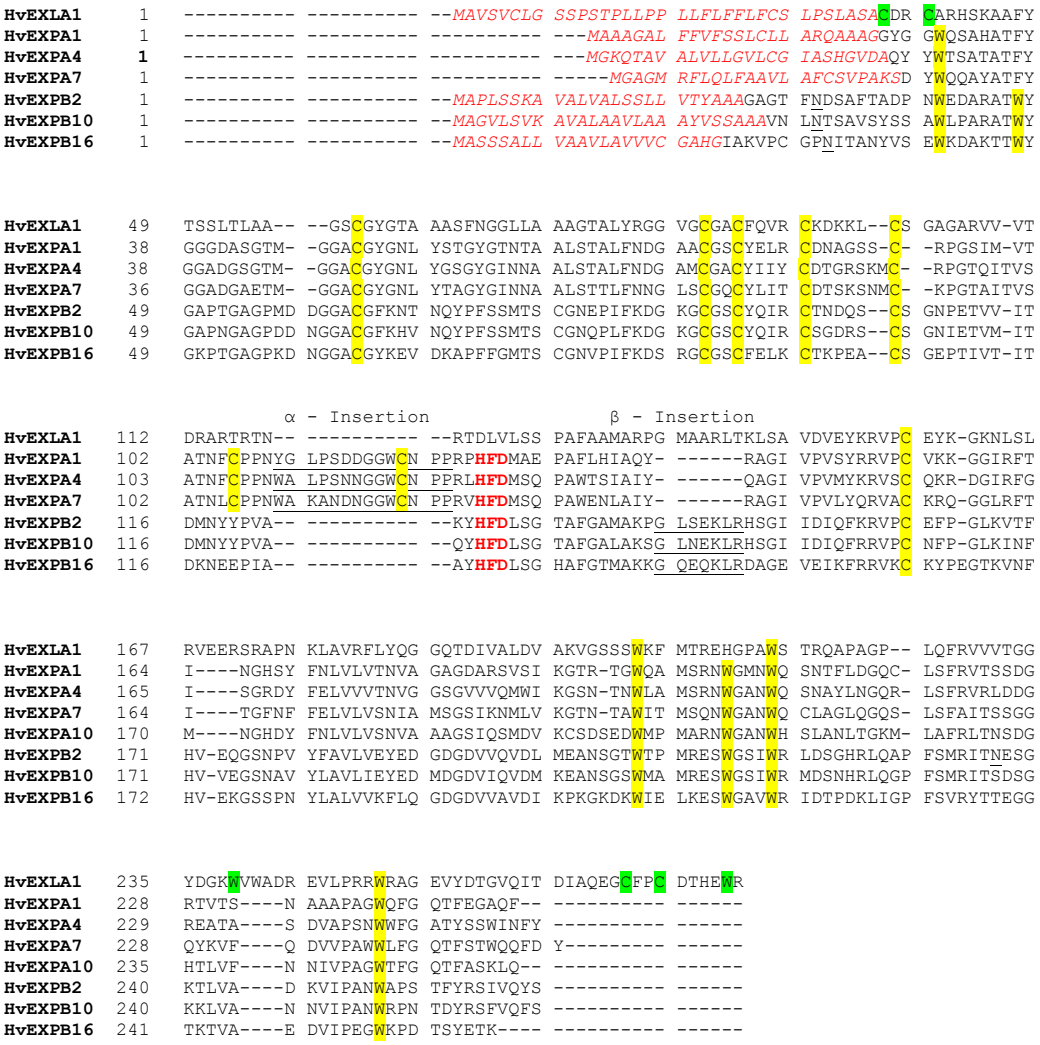


Figure 2-5 Alignment of selected members of each subfamily of the barley expansins.

The sequences in red *italics* represent signal peptides predicted by the SignalP 3.0 Server program (http://www.cbs.dtu.dk/services/SignalP/). Potential N-linked glycosylation sites are underlined. Key amino acid residues that are conserved amongst the expansins and highlighted in yellow, additional conserved residues amongst expansin-like A are highlighted in green. The histidine, phenylalanine, aspartic acid (HFD) motif is shown in red. The insertions unique to each of the α- and β-expansins are underlined and indicated.

The α-insertion is present in all α-expansins but absent in all β-expansins, it is approximately 14 amino acids in length and it contains four relatively conserved residues (WCNP). The β-insertion, consisting of seven residues, is not highly conserved. A distinguishing feature of the two families is the predicted N-linked glycosylation sites found exclusively in the β-expansins. Thus, *HvEXPB2-HvEXPB8, HvEXPB10, HvEXPB11, HvEXPB13, HvEXPB14* and *HvEXPB16* have predicted N-linked glycosylation sites near the NH_2-terminus, with the exception of *HvEXPB4, HvEXPB11* and *HvEXPB14* that have two sites while *HvEXPB2, HvEXPB3, HvEXPB6, HvEXPB13* and *HvEXPB14* have a single site near the COOH-terminal, with the exception of *HvEXPB13* that has two sites (Figure 2-3, underlined). In contrast to the literature, which indicates that N-linked glycosylation sites are found distinctly in all the β-expansins, *HvEXPB1* does not have a predicted N-linked glycosylation site (Kwasniewski and Szarejko, 2006). Four other barley β-expansins identified in this study also lack a predicted N-linked glycosylation site (*HvEXPB9, HvEXPB12, HvEXPB15* and *HvEXPB18*). There are also two putative predicted N-linked glycosylation sites in *HvEXPA9* and *HvEXPA10*. Percentage pairwise sequence similarities between full-length barley expansin proteins range from 40 to 86% identity in the α-group (Table 2-3) and from 29 to 80% identity in the β-group (Table 2-4). Between the two groups, identity values are 20 to 30%.

2.3.1.2 The barley expansin-like A genes

The barley expansin-like A cDNAs show significant amino acid sequence identity to the α- and β-expansins but lack some of the conserved features of expansins (Figure 2-5). They contain conserved C residues in the NH_2-terminus, including an additional two C residues in this and the COOH-terminal regions, and three conserved W residues in the COOH-terminal region plus an additional two W residues (Figure 2-4). The expansin-like A proteins also contain multiple potential N-linked glycosylation sites but lack the HFD motif.

2.3.2 Generation of full-length cDNAs

Of the 34 sequences identified as expansins, eight were partial cDNA sequences identified as putative barley expansins. One partial cDNA sequence was completed using 3' RACE (*HvEXPA11*). Five of the partial cDNAs contain a 3'UTR (*HvEXPA8, HvEXPA12, HvEXPB12, HvEXLA2 and HvEXLA3*) and efforts to extend the missing upstream sequences using 5'RACE were unsuccessful. Attempts at 3'RACE on two partial cDNAs containing a 5'UTR (*HvEXPA13, HvEXPA14*) were also unsuccessful. One partial cDNA (*HvEXPB12*) that appeared to be missing a conserved region in the middle of the cDNA was also unsuccessfully completed via PCR.

	HvEXPA2	HvEXPA3	HvEXPA4	HvEXPA5	HvEXPA6	HvEXPA7	HvEXPA8	HvEXPA9	HvEXPA10	HvEXPA11
HvEXPA1	**86 %**	75 %	61 %	68 %	51 %	55 %	57 %	76 %	62 %	61 %
HvEXPA2		72 %	62 %	69 %	51 %	57 %	58 %	**86 %**	65 %	63 %
HvEXPA3			56 %	61 %	47 %	55 %	57 %	65 %	56 %	57 %
HvEXPA4				57 %	43 %	61 %	55 %	55 %	56 %	72 %
HvEXPA5					49 %	58 %	59 %	62 %	67 %	63 %
HvEXPA6						**40 %**	43 %	43 %	44 %	45 %
HvEXPA7							56 %	51 %	58 %	62 %
HvEXPA8								51 %	71 %	57 %
HvEXPA9									55 %	57 %
HvEXPA10										58 %

Table 2-3 Percent pairwise sequence identity between members of the barley α-expansin subfamily.

Sequence alignments of available full length amino acid sequence with signal peptide removed of barley α-expansins were made using the Genomatix software (http://www.genomatix.de/cgi-bin/dialign/dialign.pl). The SignalP 3.0 Server program (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the signal peptide cleavage sites. The lowest identity is shown in red and highest in bold.

	HvEXPB2	HvEXPB3	HvEXPB4	HvEXPB5	HvEXPB6	HvEXPB7	HvEXPB8	HvEXPB9	HvEXPB10	HvEXPB11	HvEXPB12	HvEXPB13	HvEXPB14	HvEXPB15	HvEXPB16	HvEXPB18
HvEXPB1	46 %	42 %	43 %	44 %	42 %	58 %	55 %	40 %	46 %	40 %	37 %	44 %	44 %	60 %	40 %	40 %
HvEXPB2		73 %	78 %	68 %	70 %	51 %	51 %	58 %	74 %	53 %	35 %	68 %	66 %	54 %	49 %	65 %
HvEXPB3			76 %	65 %	62 %	41 %	46 %	56 %	72 %	50 %	35 %	63 %	62 %	50 %	50 %	59 %
HvEXPB4				67 %	61 %	45 %	45 %	55 %	76 %	50 %	34 %	62 %	60 %	51 %	47 %	56 %
HvEXPB5					65 %	48 %	49 %	61 %	69 %	54 %	35 %	64 %	66 %	49 %	52 %	69 %
HvEXPB6						47 %	46 %	58 %	65 %	55 %	36 %	74 %	72 %	48 %	51 %	62 %
HvEXPB7							72 %	41 %	50 %	40 %	37 %	45 %	43 %	61 %	37 %	44 %
HvEXPB8								41 %	52 %	42 %	42 %	47 %	43 %	57 %	37 %	43 %
HvEXPB9									64 %	51 %	**29 %**	57 %	59 %	47 %	45 %	**80 %**
HvEXPB10										51 %	35 %	65 %	66 %	51 %	49 %	67 %
HvEXPB11											34 %	53 %	52 %	44 %	60 %	51 %
HvEXPB12												37 %	34 %	39 %	32 %	32 %
HvEXPB13													79 %	48 %	50 %	61 %
HvEXPB14														47 %	50 %	63 %
HvEXPB15															42 %	47 %
HvEXPB16																48 %

Table 2-4 Percent pairwise sequence identity between members of the barley β-expansin subfamily.

Sequence alignments of available full length amino acid sequence with signal peptide removed of barley β-expansins were made using the Genomatix software (http://www.genomatix.de/cgi-bin/dialign/dialign.pl). The SignalP 3.0 Server program (http://www.cbs.dtu.dk/services/SignalP/) was used to predict the signal peptide cleavage sites. The lowest identity is shown in red and highest in bold and underlined.

2.3.2.1 3'RACE

A partial length barley EST clone of 523 bp (BG368084/*HvEXPA9*) was used as the basis for primer design for 3'RACE (5' CCACCAACTTCTGCCCGCCCAACTACGC 3') to obtain the 3'-end of the sequence. A single band was produced via 3'RACE and cloned. Sequences from three clones revealed high sequence identity with another partial putative barley expansin EST BQ766938 (*HvEXPA11*). Thus, this 3'RACE experiment completed the partial related cDNA of BQ766938 (*HvEXPA11*). A second primer was designed based on BG368084 (3'RACE 2nd 5' GACCTGAGGCTGGAGAAGACGAGGAG 3') downstream of the first primer with less sequence identity to any other known barley expansins. A second 3'RACE PCR with this primer on a mature grain 3'Smart cDNA template as described in section 2.2.8.1 produced a single band which was excised from the gel and cloned into pGEM-T Easy for sequencing. This revealed sequences of other barley expansins already characterised. A later search of the public database identified ESTs with high sequence similarity to BG368084 and revealed two unigenes Hv.20874 and Hv.23609 that could be assembled into a contiguous sequence that completed *HvEXPA9* (Figure 2-2). The gene-specific primers used for 3'RACE were designed on a single pass sequence of the EST (BG368084) and in this case did not completely match the Tentative Contig later obtained for *HvEXPA9*.

Primers designed to ESTs BY848626 (*HvEXPA13*) and BQ765644 (*HvEXPA14*) for 3'RACE were unsuccessful in obtaining the complete sequence. Semi-nested PCR was performed on target genes *HvEXPA13* and *HvEXPA14* with the Nested Universal Primer A (NUP 5' AAGCAGTGGTATCAACGCAGACT 3'; Clontech, Laboratories Inc.) and gene-specific primers (Appendix A, Table A-1) following unsuccessful 3'RACE PCR. Using a 1/100 dilution of the first round PCR as template for the semi-nested PCR, reactions were carried out using the Advantage 2 Polymerase Mix (Clontech, Laboratories Inc., Mountain View, CA, USA) as described in section 2.2.8.1 with an alteration to 40 cycles. Fragments of the predicted size were purified on a gel with the PCR clean-up gel extraction NucleoSpin® Extract II kit (Macherey-Nagel, Duren, Germany). Touchdown cycling conditions were also trialled to reduce non-specific binding of primers, because results from these 3'RACE PCRs often showed multiple bands or smearing. Cycling conditions for Touchdown PCR were 5 cycles of 94°C 30 s and 72°C 2 min, 5 cycles of 94°C 30 s, 70°C 30 s and 72°C 2 min and 30 cycles 94°C 30 s, 68°C 30 s and 72°C 2 min. Sequencing results from semi-nested 3'RACE PCR (including Touchdown PCR) failed to yield expansin sequences.

Further attempts to isolate the 3'end of *HvEXPA13* and *HvEXPA14* included PCR with nested gene-specific primer as listed in Appendix A (Table A-1) and the NUP. A 1/100 dilution of the first round

PCR generated (including templates generated from Touchdown PCR) was prepared as the template for nested PCR. Reactions were carried out as described above. Products of a 3'RACE touchdown PCR followed by nested touchdown PCR for *HvEXPA14* included bands of approximately 650 bp, 450 bp and <300 bp (data not shown). The expected size for *HvEXPA14* was 542 bp + 3'UTR. However, sequencing of the three fragments showed that they were not expansin cDNA products.

2.3.2.2 5'RACE

The method followed for 5'RACE required that the first-strand cDNA synthesis reaction was primed using an oligonucleotide complementary to a known sequence in the gene. After removing the RNA template, an anchor site at the 3'-end of the single-stranded cDNA is created, using terminal deoxynucleotidyl transferase, which adds a nucleotide tail, in this case a tail of Gs. A typical amplification reaction follows using a primer complementary to the newly added tail (Poly $(dC)_{15}$ oligo 5' CCCCCCCCCCCCCCC 3') and another primer complementary to a known sequence within the gene (5'RACE PCR).

Two complementary primers were designed to each of the TCs of *HvEXPA8, HvEXLA2* and *HvEXLA3* and the EST of BU973592 (*HvEXPA12*) for 5'RACE and subsequent PCR with a Poly $(dC)_{15}$ oligo. Conditions were optimised for the amplification of non-specific RNA and to maximise the amount of expansin derived cDNA. Reverse transcription was attempted at four different temperatures (T_m and -/+ 2°C T_m as listed in Appendix A, Table A-1) and 50°C.

Despite the first-strand cDNA synthesis being conducted at a number of annealing temperatures followed by a series of PCRs, all attempts to amplify a product corresponding to expansin sequences were unsuccessful.

2.3.3 Full length cDNA isolation and sequence analysis

Eight near full-length barley expansin cDNAs were amplified and sequenced, and comprised one α-expansin and seven β-expansins. All eight of these barley cDNAs are predicted to encode complete expansin proteins, as indicated in Table 2-2 and shown in Figures 2-1 and 2-2. Each of the eight cDNAs has a unique 3'UTR so it is concluded that they correspond to eight distinct genes in the barley genome.

2.3.3.1 Isolation of HvEXPA1

The full length *HvEXPA1* cDNA of 1171 bp in length including a 3'UTR of 395 bp, was obtained by PCR amplification of coleoptile cDNA using the primers listed in Table 2-1. A single band was

produced and after removing reaction components the cDNA was sequenced directly with the same gene-specific primers. The sequences had a single nucleotide difference in the coding region that resulted in an amino acid change of a conserved G to a V at position 243 in the contiguous sequence assembled by ESTs and a multiple sequence alignment (Figure 2-2). Repeat PCRs were conducted to determine if the single nucleotide change was the result of a PCR error but this nucleotide change was found in all sequencing reactions.

The *HvEXPA1* cDNA encodes a protein of 251 amino acids. Because α-expansins have lower activity on grass cell walls than on dicot walls (Cho and Kende, 1997b) and because it is suggested that the β-expansins have a dominant role in grasses, the full length *HvEXPA1* was the single barley α-expansin selected for isolation, sequencing and for heterologous protein expression.

2.3.3.2 Isolation of near full length barley β-expansin cDNAs

Since the gene family is large, the first ten barley β-expansins listed (Table 2-2) were initially selected for full length cDNA isolation. The gene-specific primers for generation of full-length cDNA were designed in the 5' and 3'UTR sequence and the cDNA templates were selected for PCR based on the tissues from which the EST libraries for corresponding ESTs were generated. A single round of PCR produced single bands of the predicted size for *HvEXPB1, HvEXPB2, HvEXPB4, HvEXPB5, HvEXPB9* and *HvEXPB10* and PCR products were sequenced directly after removal of excess oligonucleotides. Sequencing of the PCR products for *HvEXPB1, HvEXPB9* and *HvEXPB10* returned sequences with a few nucleotide changes when compared to the assembled contiguous sequences. However, the nucleotide changes in *HvEXPB1, HvEXPB9* and *HvEXPB10* did not result in any amino acid changes and the nucleotide changes could probably be attributed to the varietal or cultivar origin of the EST sequence in the database. *HvEXPB1*, *HvEXPB9* and *HvEXPB10* encode proteins of 282, 271 and 265 amino acids respectively.

The first sequences obtained for *HvEXPB2* and *HvEXPB4* included nucleotide changes that resulted in an amino acid change of a conserved C (*HvEXPB2*) at position 62. Subsequent PCR for *HvEXPB2* confirmed the presence of the conserved C and only single nucleotide changes elsewhere and no amino acid changes. The sequence for *HvEXPB4* showed a nucleotide change that changed a lysine (K) to glutamic acid (E) in position 75. In the multiple sequence alignment of β-expansins, this position is most often a K or neutral glutamine (Q) with *HvEXPB12* containing a glycine (G) in that position (Figure 2-3). A second PCR for full length generation of *HvEXPB4* confirmed the K in position 75 with the first nucleotide change likely to be a PCR error. *HvEXPB2* and *HvEXPB4* encode proteins of 265 and 277 amino acids respectively.

The contiguous sequence initially assembled for *HvEXPB5* was missing a conserved C in position 103. The isolation of a full length *HvEXPB5* cDNA revealed an additional six nucleotides that adjusted the reading frame to code for the conserved C in position 103, followed by two additional amino acids (Appendix B, Figure B-1). *HvEXPB5* encodes a protein of 273 amino acids.

In an attempt to isolate barley β-expansins that do not contain a predicted N-linked glycosylation site for later analysis by heterologous protein expression, *HvEXPB15* was also selected for full-length cDNA isolation. Based on the tissue from which the EST library was generated for the ESTs assembled into the contig assigned as *HvEXPB15*, the cDNA template used to isolate *HvEXPB15* was derived from entire caryopsis at anthesis. Cycling conditions for this PCR included an additional 10 cycles. A single PCR produced three fragments of around 550, 850 and 1000 bp. The expected size for *HvEXPB15* was 1068 bp. The largest fragment was purified and directly sequenced with gene-specific primer. The sequence was the same as the assembled contig sequence for *HvEXPB15*. Thus, *HvEXPB15* encodes a protein of 265 amino acids and was confirmed to lack an N-linked glycosylation site (Figure 2-3).

Attempts to isolate near full length cDNAs of *HvEXPB3, HvEXPB6, HvEXPB7 and HvEXPB8* were unsuccessful. Semi-nested gene-specific primers as listed in Appendix A, (Table A-2) were designed for a second round of PCR for *HvEXPB3, HvEXPB7 and HvEXPB8* but also failed to produce a fragment of the expected size. Conditions for semi-nested PCR were as described in section 2.2.9.1 on a 1/10 dilution of the first round PCR reaction.

2.3.3.3 Generation of a near full length HvEXPB12 cDNA

The contiguous sequence assembled for *HvEXPB12* appeared to be missing around 49 amino acids at the end of Domain 1. A number of PCRs were conducted with different amounts of an enhancer, dimethyl sulfoxide (DMSO) to increase yield, specificity and consistency. Three reactions were carried out using the Elongase® *Taq* Polymerase Amplification System (Invitrogen) as described in section 2.2.9.1 with 0, 4 and 10% DMSO in the reaction mixtures on a stem-derived cDNA template with 40 cycles of PCR and an annealing temperature of 54°C. No fragment was produced by any of the reactions with DMSO. A second set of primers was designed just upstream and downstream of the gap that had an expected size of approximately 300 bp. A repeat of the above conditions with the second set of primers was also unsuccessful at completing *HvEXPB12*. Thus, it is likely TC144522 (5'end) and TC151633 (3'end) represent different genes. However as both fragments have the same ortholog in rice, the two Tentative Contigs were treated as a single gene for alignments.

2.3.3.4 Isolation of HvEXLA1

With only one complete contig available for a member of the expansin-like A family, PCR was conducted to isolate *HvEXLA1* for further analysis. PCR as described in section 2.2.9.1 was performed on a root-derived cDNA template with two sets of gene-specific primers (Appendix A, Table A-3) but was unsuccessful. PCR was attempted with the addition of DMSO as described above (section 2.3.3.3) with cycling conditions 94°C 5 min followed by 40 cycles of 94°C 30 s, 48°C 30 s, 68°C 1 min and a final step of 68°C for 5 min. Conditions for semi-nested PCR forward primer was as described in section 2.2.9.1 on a 1/10 dilution with water of target gene cDNA generated in the first round of PCR. The seven unsuccessful combinations of PCRs attempted to isolate the full length sequence of *HvEXLA1* are summarised in Appendix A, Table A-3.

2.3.4 In silico *analyses of expansin genes in* Arabidopsis thaliana*, rice (*Oryza sativa*),* Sorghum bicolor *and* Brachypodium distachyon

An *in silico* analysis of the *Brachypodium* and sorghum genomes revealed the existence of expansin superfamilies containing 62 and 79 members respectively (Table 2-5). A radial tree constructed with the *Brachypodium*, sorghum and rice genes, together with the barley expansin cDNA sequences shows that both *Brachypodium* and sorghum have members in the α- and β-expansins and expansin-like A and expansin-like B families (Figure 2-6 and 2-7). Analyses of the *Brachypodium* and sorghum amino acid sequences show they contain all the key features of the α-, β- and expansin-like A genes and for *Brachypodium*, one sequence resembles an expansin-like B.

The number of expansin members in *Brachypodium* and sorghum is greater than in rice and the main source of the increase appears to be from tandem and segmental duplications, as indicated by the short branch lengths displayed on the radial tree (Figures 2-6 and 2-7).

2.3.4.1 Multiple sequence alignments

Although most of the predicted proteins appear to be typical for α- and β-expansins, some unusual features have been observed amongst the sorghum and *Brachypodium* expansins; these are also seen for the barley expansins. The predicted mature protein of *HvEXPA3* has an extension of 30 amino acids following the signal peptide that is rich in histidine (H), glycine (G), proline (P) and arginine (R) residues. While no significant sequence similarities were found in BLAST searches, a maize expansin has been identified with a similar extension (Wu *et al.*, 2001a). A multiple sequence alignment of the orthologues of *HvEXPA3* from rice, sorghum and *Brachypodium* (Figure 2-8) show that this extension is present in all the grass species. These sequences show more than 77% identity at the amino acid level.

Species	EXPA	EXPB	EXLA	EXLB
Arabidopsis	26	6	3	1
Rice	34	19	4	1
Sorghum bicolor	39	36	4	-
Brachypodium distachyon	32	24	5	1
Barley	14	17	3	-

Table 2-5 Sizes of the four expansin families in different plants

The number of genes in each family is listed for *Arabidopsis*, rice*, Sorghum bicolor* and *Brachypodium distachyon,* all of whose genomes have been sequenced. The numbers for barley are based on EST analysis and should be taken as a minimum estimate until the whole genome is sequenced. EXPA, α-expansin; EXPB, β-expansin; EXLA, expansin-like A; EXLB, expansin-like B.

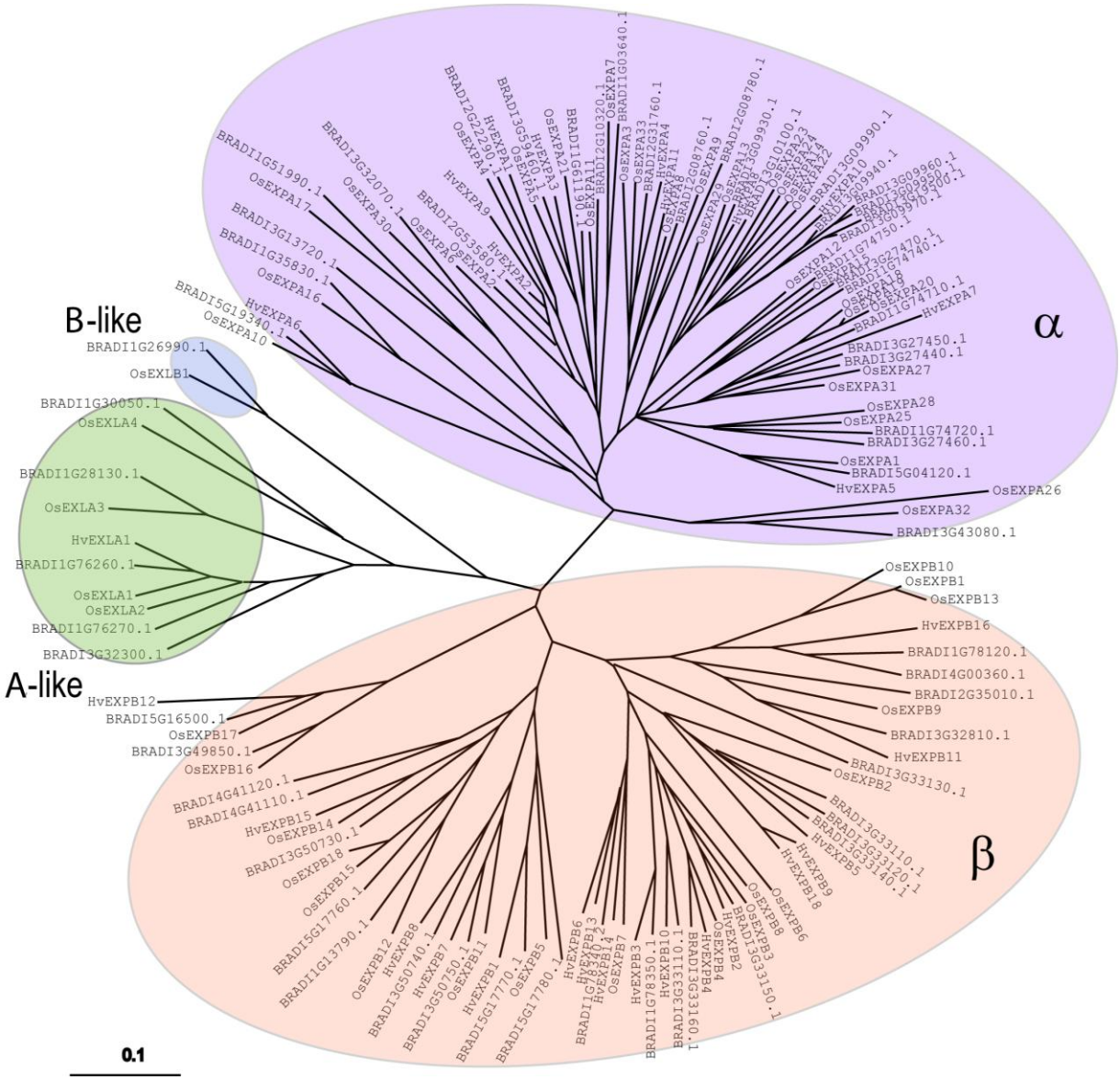


Figure 2-6 Radial tree of the *Brachypodium*, rice and barley expansin families

This tree was generated using ClustalX2 program and presented using the TreeView software application. The tree shows the orthologous expansin members of the three species and clearly indicates four subfamilies, which are α-expansin, β-expansin, expansin-like A and expansin-like B. To date no expansin-like B have been identified in barley. *Brachypodium* sequences are annotated as their query location based on http://gramene.org/.

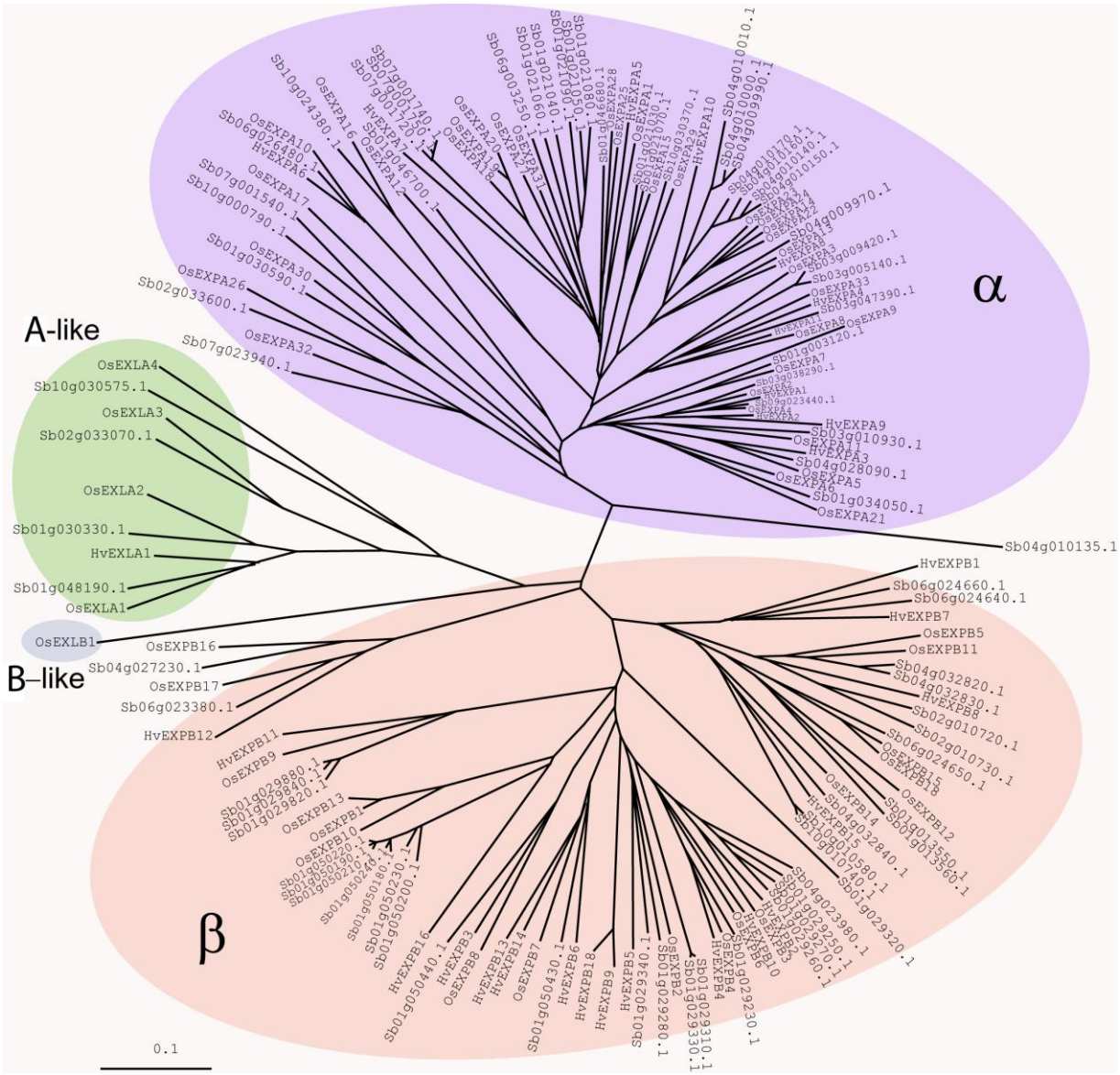


Figure 2-7 Radial tree of the Sorghum, rice and barley expansin families

This tree was generated using ClustalX2 program and presented using the TreeView software application. The tree shows the orthologous expansin members of the three species and clearly indicates four subfamilies, which are α-expansin, β-expansin, expansin-like A and expansin-like B. To date no expansin-like B have been identified in barley and no expansin-like B was identified in the sorghum genome. Sorghum sequences are annotated as their query location based on http://gramene.org/.

```
OsEXPA5                1   MSS--RRDVL AVVLVAALLP PALSRGLWLG H-------HG LGHG--HGRW RAPHVGGHGQ GQGPQQHAPL
Sb04g028090.1          1   MAP--RQAVL AAVVLAALFP LALSRGLGLV HGHVRPHAHG LGLG--HHHP R-PHPQ---- -QQPHGHAPL
HvEXPA3                1   MAP--ARAAL LALLVAALLP PALSRGQGRG -----P--HL PGHGLVHR-- -------HGP GIGHHAHAPL
BRADI3G59460.1         1   MAPVLRALLL AFLVAAQSPS SALSLGQGHG H-------HL PGHGLVHRHG N-VH-GGHG- ----HAHAPL

OsEXPA5               60   GGGGWSSAHA TFYGGGDASG TMGGACGYGN LYSQGYGTNT AALSTALFNN GLSCGACFEV RCDAGG---G
Sb04g028090.1         61   GGGAWSSAHA TFYGGGDASG TMGGACGYGN LYSQGYGTNT AALSTALFNS GLSCGACFEV RCDAAG---G
HvEXPA3               53   GGGAWASAHA TFYGGGDASG TMGGACGYGN LYSTGYGSNT AALSTALFNN GLSCGACFEV RCDPGGTEAG
BRADI3G59460.1        57   GGGAWSSAHA TFYGGGDASG TMGGACGYGN LYSTGYGSNT AALSTALYND GLSCGACFEV RCDPAGTEAG

OsEXPA5              127   GSHSCLPG-S VVVTATNFCP PNNALPSDDG GWCNPPRAHF DMSQPVFQRI ALFKAGIVPV SYRRVACQKK
Sb04g028090.1        128   GSHSCLPG-S VVVTATNFCP PNNALPSDDG GWCNPPRAHF DMSQPVFQRI ALYRAGIVPV SYRRVACNKK
HvEXPA3              123   APHACLPG-S TVVTATNFCP PNFGESSDAG GWCNPPRAHF DMSQPVFQRI ALYRAGIVPV SYRRVACQKK
BRADI3G59460.1       127   AAHACLPGTS VVITATNLCP PNNALPNDDG GWCNPPRAHF DMSQPVFQRI AIYKAGIVPV SYRRVACQKK

OsEXPA5              196   GGIRFTINGH SYFNLVLVTN VGGAGDVHAV AVKSERSAAW QALSRNWGQN WQSAALLDGQ ALSFRVTTGD
Sb04g028090.1        197   GGIRFTINGH SYFNLVLVTN VGGAGDVHAV AVKGERSAGW QALSRNWGQN WQSNTLLDGQ ALSFRVTTSD
HvEXPA3              192   GGIRFTINGH SYFNLVLVTN VGGPGDVHAV SVKSTRSAAW QALSRNWGQN WQSNALLDGQ GLSFRVTAGN
BRADI3G59460.1       197   GGIRFTINGH SYFNLVLVSN VGGPGDVHAV AVKSERSPSW QALSRNWGQN WQSNALLDRQ CLSFRVTAGD

OsEXPA5              266   GRSVVSNNAV PRGWSFGQTF SGAQFN
Sb04g028090.1        267   GRSVVSNNAA PRGWAFGQTF SGAQFN
HvEXPA3              262   GQSVVSNNAV PRGWSFGQTF SGAQFH
BRADI3G59460.1       267   GSSVVSSNAV PRGWAFGQTF SGAQFT
```

Figure 2-8 Multiple sequence alignment of plant orthologues of *HvEXPA3*

The Genomatix program was used to align rice (*OsEXPA5*), *Brachypodium* (BRADI3G59460.1), sorghum (Sb04g028090.1) and barley *HvEXPA3* sequences. The extension of 30 amino acids following the signal peptide that is rich in histadine (H), glycine (G), proline (P) and arginine (R) residues unique to this set of expansin sequences are underlined. The highlighted amino acids indicate the key features of the expansin family including a series of cysteins, tryptophans, and conserved histidine, phenylalanine, aspartic acid (HFD) motif. Other conserved amino acids are shaded. *Brachypodium* and sorghum sequences are annotated as their query location based on http://gramene.org/.

The predicted mature proteins of *HvEXPB14* and *HvEXPB6* contain extensions of 95 and 48 residues respectively following the signal peptide, similar to that of an extension found in a maize expansin that is rich in proline residues (Wu *et al.*, 2001a). A multiple sequence alignment was carried out with orthologous sequences from rice, *Brachypodium* and sorghum, and show that the sequence contained more than 69% identity at the amino acid level. These three genes also contain a long extension following the signal peptide that is rich in proline residues (Figure 2-9).

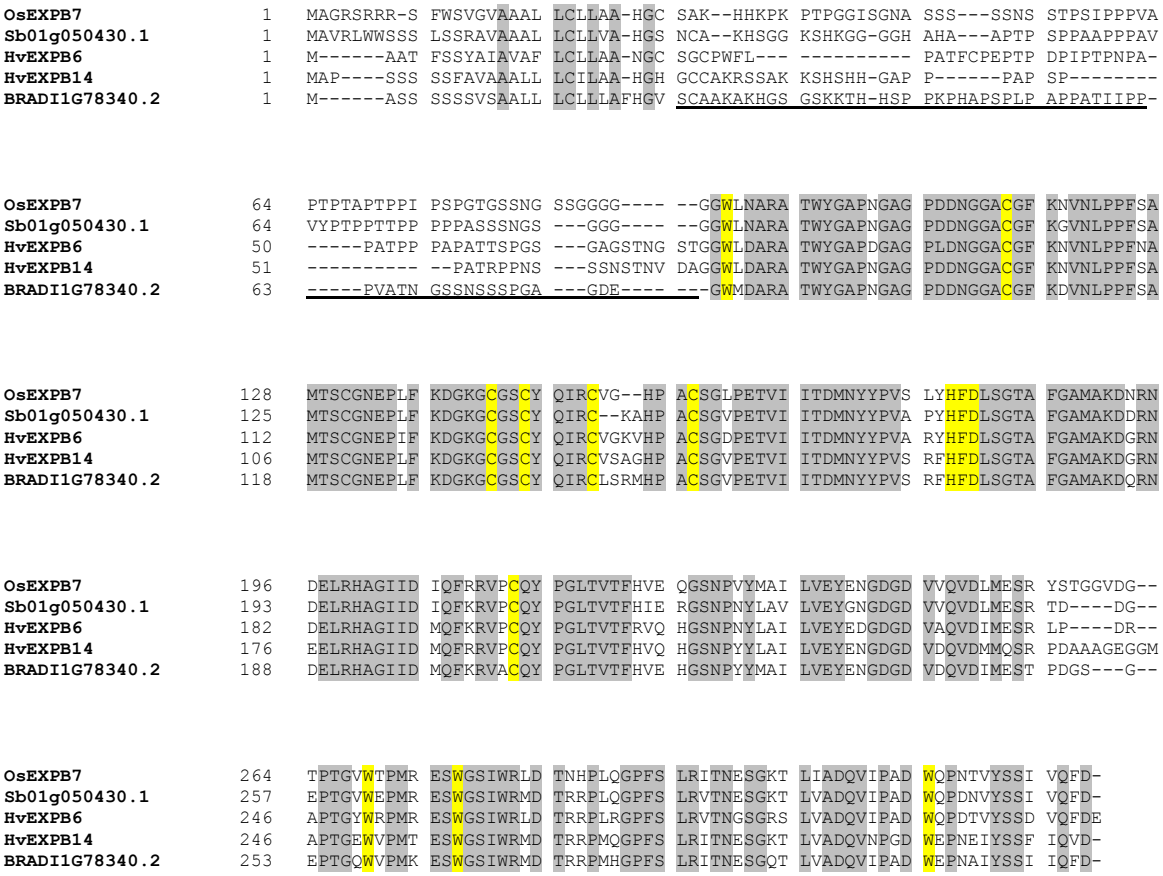


Figure 2-9 Multiple sequence alignment of plant orthologues of *HvEXPB6* and *HvEXPB14*

The Genomatix program was used to align rice (*OsEXPB7*), *Brachypodium* (BRADI1G78340.2), sorghum (Sb01g050430.1) and barley *HvEXPB6* and *HvEXPB14* sequences. The extension of 48 to 95 amino acids following the signal peptide that is rich in proline (P) residues unique to this set of expansin sequences is underlined. The highlighted amino acids indicate the key features of the expansin family including a series of cysteins, tryptophans, and the conserved histidine, phenylalanine, aspartic acid (HFD) motif. Other conserved amino acids are shaded. *Brachypodium* and sorghum sequences are annotated as their query location based on http://gramene.org/.

2.4 DISCUSSION

In this Chapter, experiments are described by which members of the expansin families from barley have been characterised and full length cDNAs from selected members isolated. A comparison of the barley expansin genes was made against grass species whose complete genomes have been sequenced, including rice, sorghum and *Brachypodium*. *Brachypodium* is believed to be an ideal model for studying barley because it is a small, easily propagated, temperate grass with a rapid life cycle, it has a relatively small genome and it is closely related to the Triticeae (Garvin *et al.*, 2008).

The *in silico* analysis of the sorghum and *Brachypodium* genomes identified 79 and 62 members of the expansin family, respectively, whilst the analysis of the barley public EST databases identified 34 members of the expansin family. This suggests that the barley gene family may contain at least twice the number of genes identified in this project. Although only eight full-length cDNAs were isolated here, it is likely that these represent the most highly expressed members of the expansin gene family. In the following sections the obstacles to generating and isolating full length barley expansins and the variation of the number of expansin members identified across the grass species are discussed.

The first aim of the project was to characterise the expansin family in barley. Rice and wheat expansin sequences were used to search the public EST databases to identify homologues. This resulted in the identification of nine near full length contiguous sequences and five barley ESTs of partial-length belonging to the α-expansins (Figure 2-10). One full length cDNA designated *HvEXPA1* was isolated and one partial EST was completed using 3'RACE. Three contiguous sequences of expansin-like A, one full length and two partial-lengths were found on the barley EST databases, all with unique 3'UTR sequences (Figure 2-10). Also identified were 16 near full length contiguous sequences belonging to the β-expansins and one partial (Figure 2-11). Seven full length barley β-expansin cDNAs were isolated.

Multiple sequence alignments of the newly identified protein sequences of barley expansins confirmed that these sequences contain all the key features of the expansin family, including a series of conserved cysteine (C) residues, eight in the α-expansins and six of the eight in the β-expansins, an HFD motif in both the α- and β-expansin sequences and four conserved tryptophan (W) residues near the COOH-terminus with additional tryptophan (W) residues found in the expansin-like A sequences (Figures 2-2, 2-3 and 2-4). The number of conserved cysteine (C) residues are similar to those observed in the glycoside hydrolase family 45 (GH45) protein, and the

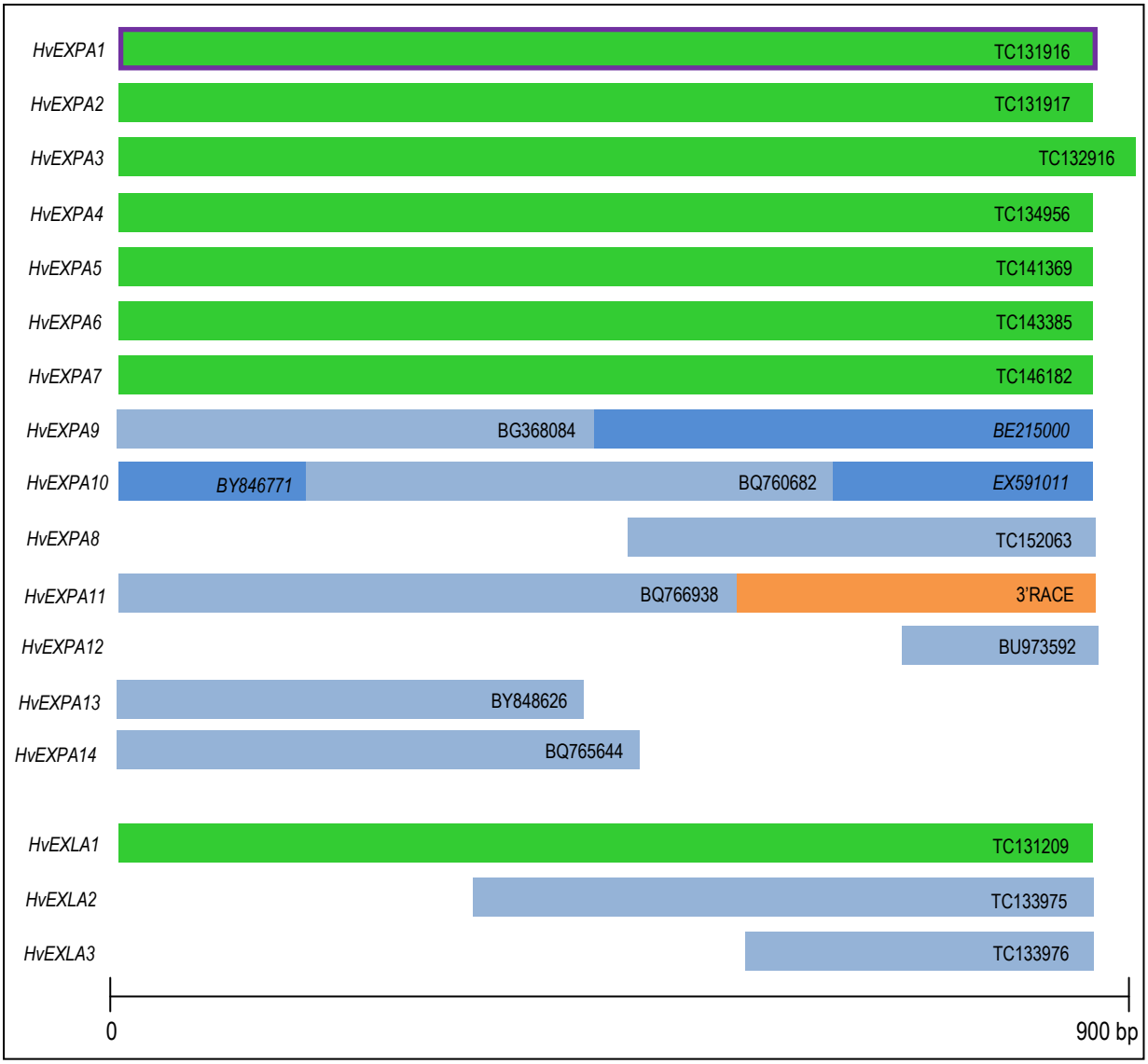


Figure 2-10 Initial results of open reading frame sequences of barley α-expansin and expansin-like A from database search and subsequent full length generation

Each bar represents an EST or contig. Green bars represent full length open reading frames and blue bars represent EST/partial sequence obtained from public database searches. The numbers within the bars to the right refer to the TIGR contig number (TC) as retrieved from the TIGR database, dark blue bars and *italicized* ESTs were obtained with further public database searches and assembled into full length open reading frames. The orange bar represents sequence obtained using 3'RACE. Purple outline represents isolated full length cDNA. Expansin gene names on the left were arbitrarily assigned.

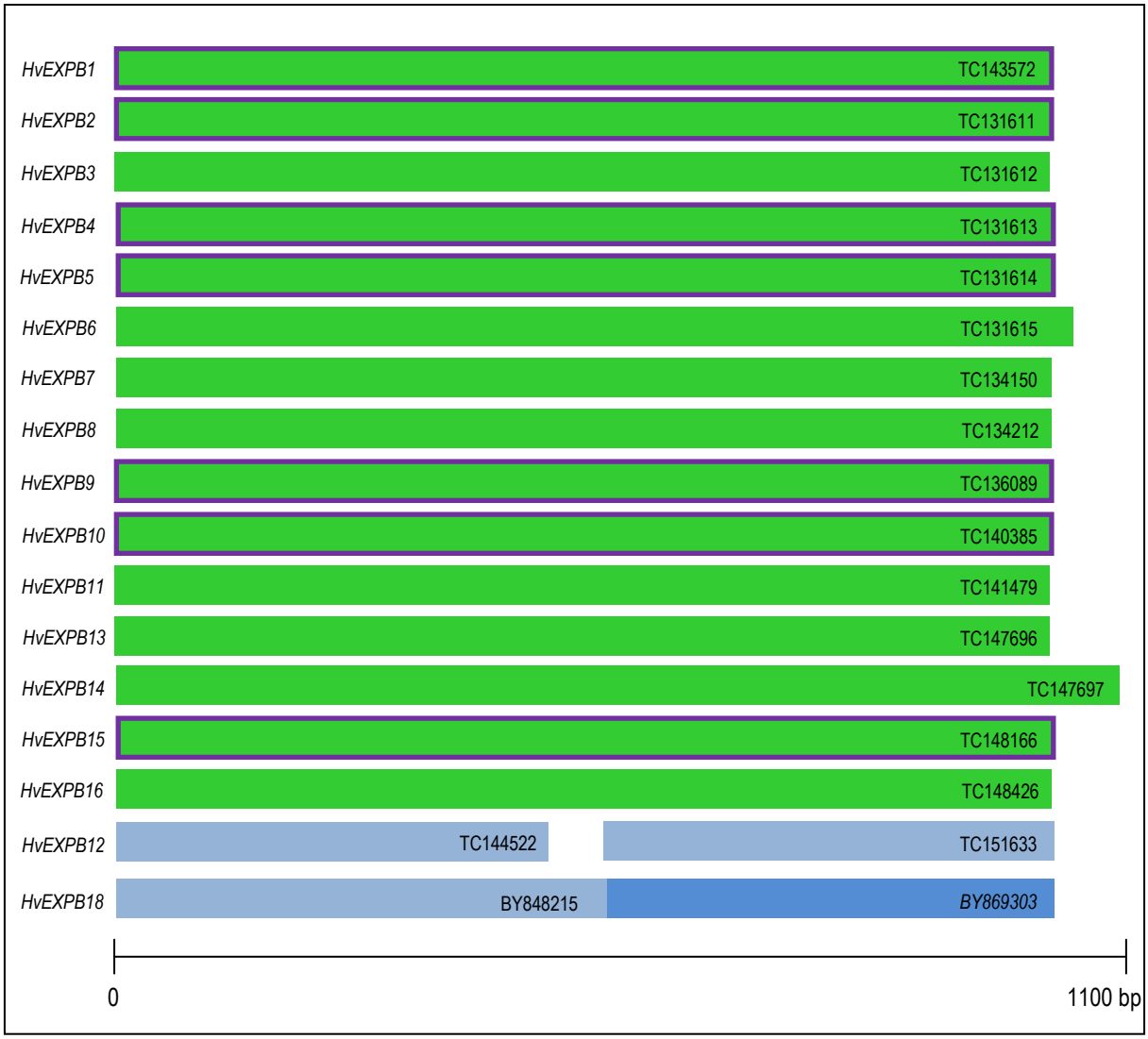


Figure 2-11 Initial results of open reading frame sequences of barley β-expansin from database search and subsequent full length generation

Each bar represents an EST or contig. Green bars represent full length open reading frames and blue bars represent EST/partial sequence obtained from public database searches. The numbers within the bars to the right refer to the TIGR contig number (TC) as retrieved from the TIGR database, dark blue bar and *italicized* EST was obtained with further public database searches and assembled into full length an open reading frame. Purple outline represents isolated full length cDNA. Expansin gene names on the left were arbitrarily assigned.

HFD motif that makes up the catalytic site of the GH45 endoglucanases. Despite the presence of the conserved GH45 motifs, no hydrolytic activity has been detected for expansin proteins.

Some variation in the HFD motif was observed in the tentative contigs of the barley expansins identified in this project. The isolation of the *HvEXPB9* cDNA confirmed that the phenylalanine (F) is replaced by lysine (L) in this motif (Figure 2-3). Variations of the HFD motif have been observed in *Arabidopsis* (Li *et al.*, 2002), wheat (Liu *et al.*, 2007), rice, sorghum and *Brachypodium*. The divergences observed in this motif include, HFL, HFE, YFD, HID, HLE and RFD. Other than YFD, each divergence in the motif is observed in at least two or more species including barley.

The percentage pairwise sequence similarities between the available full-length barley expansin proteins shown in Table 2-3 for the α-expansins and Table 2-4 for the β-expansins are similar to those found in *Arabidopsis* and rice with the mature β-expansins more divergent than the α-expansins (Choi *et al.*, 2006).

In general, attempts to complete partial cDNAs and isolate full-length sequences using PCR primers designed to ESTs met with limited success. In a gene family that displays high sequence conservation there are two limitations to efficient gene-specific primer design. In the instances of 3' and 5' RACE, the sequences used for primer design are either ESTs or partial contiguous sequences assembled from an overlap of two or more ESTs. These often short sequences to which primers have been designed is the first limitation to successfully amplifying more of the gene, because there is little choice or only potentially low quality sequences to work with. This reduces the efficacy of primers made in this region, because by designing the primers in an area of more reliable sequence, the risk of producing an expansin-specific primer increases due to regions of highly conserved areas in the gene family, therefore gene-specificity of the primer decreases, as observed from the results of the 3'RACE experiment to extend the *HvEXPA9/11* cDNA sequence.

For near full length cDNA isolation, the gene-specific primers were designed to the 5' and 3' UTRs, which are the most divergent regions of the gene. However, full length cDNA isolation failed for six of the 14 gene members attempted, including the amplification of a small insert missing from the central region of *HvEXPB12*. The attempts made with various alterations to PCR conditions are apparent in Appendix A, Table A-2. Even when gene-specific primers were designed in relatively conserved regions in the coding sequence, no additional expansin sequences were isolated. The same idea was rationalised with the experiments conducted to isolate expansin-like members. However, multiple attempts to isolate of full length *HvEXLA1* and full length generation of *HvEXLA2* and *HvEXLA3* cDNA were unsuccessful. The EST origin from which *HvEXLA3* contig was

assembled includes developing endosperm, a single EST from five day old seedlings under a dehydration stress and a single EST from a *Fusarium graminearum* infected spike. Replicating these exact conditions to harvest material to generate a suitable cDNA library can be difficult and hence only an endosperm cDNA library was used in the full length generation attempts of *HvEXLA2* and *HvEXLA3* cDNA. The ESTs origin for the *HvEXLA1* contig assembled gave more flexibility for cDNA libraries able to be used, however attempts at full length isolation was as equally unsuccessful as shown by the various conditions tested for PCR in Appendix A, Table A-3.

These above methods that require the expression of a gene in the initial phases of identification have a disadvantage that in order to be detected, the gene in question must be expressed in the cDNA library made from the appropriate tissue at the appropriate developmental time. Genes can also differ slightly in sequence between cultivars and thus the cultivar used for the cDNA library can also affect a positive outcome.

As a number of genes suitable for further analysis via heterologous expression had already been identified, the isolation of additional full-length clones was discontinued.

It has been noted previously that many of the expansin genes are tandemly repeated in the *Arabidopsis* and rice genomes (Li *et al.*, 2002; Sampedro *et al.*, 2005b). This phenomenon has also been observed in the *Sorghum bicolor* and *Brachypodium distachyon* genomes. The tandemly repeated genes are grouped together in the radial trees, indicating that some of these genes have undergone duplication relatively recently as shown by the short branches in Figures 2-6 and 2-7. The completed sequence of the sorghum genome reveals that expansin genes are located on all chromosomes but are concentrated on chromosomes I and IV. Five clusters of two or more genes are observed on chromosomes I for both α- and β-expansins. Both of the largest clusters contain seven genes, with one large cluster encoding α-expansins with a degree of amino acid sequence identity between 58 to 88%. The duplication of one of the genes in this cluster (Sb01g021030.1) appears to have occurred earlier due to the longer branch length and location on the radial tree. This would account for the large variation in amino acid sequence identity. The other cluster of seven genes spans a 46 kb region on Chromosome I, and encodes for members of the β-expansins. This cluster includes a pair of identical genes (paralogues) with the remaining five genes displaying an amino acid sequence similarity of between 96 and 99%. This is demonstrated by the short branch lengths illustrated on the radial tree in Figure 2-7 (genes Sb01g050180.1 to Sb01g050240.1). Most paralogues in sorghum are proximally duplicated including 5,303 genes in 1,947 families of ≥ 2 genes (Paterson *et al.*, 2009). In this case the nucleotide sequence of the 5'

and 3' untranslated regions of these duplicated genes also displays high sequence similarity indicating that the genomic neighbourhood of these expansin genes are preserved. Overall, the exon size distribution of orthologous sorghum and rice genes agree closely and intron position and phase show > 98% concordance (Paterson *et al.*, 2009) and intron size has been conserved between sorghum and rice. In addition, the exon/intron organisation in *Arabidopsis* and rice is conserved among α- and β-expansins and expansin-like genes.

The eight clusters on the *Brachypodium* genome are distributed across all five chromosomes. The largest two clusters are located on chromosome III and each contains seven members, one of α-expansins and the other a cluster of β-expansins. The degree of sequence identity at the amino acid level for the cluster of α-expansins ranges from 71 to 98% in contrast to the β-expansin cluster that only displays sequence identities of 47 to 81%.

Mapping of the *Arabidopsis* expansins revealed that the genes are scattered throughout all five chromosomes. A cluster of five α-expansin genes in tandem on Chromosome V shows a high degree of sequence similarity at the nucleotide and protein level (Li *et al.*, 2002). Similar results were observed during the analysis on the map-based sequence of rice (Sasaki and Burr, 2000), which revealed 58 expansin genes including two pairs of identical genes.

The process of gene duplication is often associated with the functional divergence or gene extinction, as generally the function of one of the duplicates is redundant (Paterson *et al.*, 2009). However, it was observed that two strawberry expansins (*FaEXP*) that share the greatest homology, are not only both expressed, but have different expression profiles. *FaEXP7* and *FaEXP2* have 96% amino acid identity and are expressed at different times during fruit development (Harrison *et al.*, 2001). An analysis of the sequenced *Arabidopsis* genome and public EST databases by Li *et al.* (2002) noted that half the *Arabidopsis* expansins identified were present as ESTs of full-length cDNAs. At this stage the functional purpose for the large number of members that make up the expansin family is unknown. There are several possible explanations for this. It could be related to the need to synthesise large amounts of expansin protein during various stages of normal growth and development, or it could be related to different functions of expansin sub-classes. In addition, it could also be a response to stress, or simply reflect the need for independent control of the expansin genes in different tissues.

In wheat and maize there have been 9 and 12 α-expansins and 9 and 21 β-expansins identified, respectively (Wu *et al.*, 2001a; Lin *et al.*, 2005; Muller *et al.*, 2007). The numbers for maize and wheat include some partial gene fragments and therefore should be interpreted as minimum

estimates, because the analyses were based on ESTs only. It has been suggested that the number of α-expansin genes in wheat could exceed 30 whilst the number of β-expansin genes could exceed 65 (Liu *et al.*, 2007). The hypothesis that the large number of β-expansin genes in grasses, compared with dicotyledons, is likely to be related to the fact that the cell wall matrix polysaccharides and structural proteins of grasses differ from those of most other angiosperms (Carpita and McCann, 2000), is supported by this study in barley. The observation here that asymmetrical expansion of gene members for the two subfamilies has occurred (Table 2-5) accounts for the larger number of β-expansin genes in rice. The greater number of β-expansin genes observed maybe related to the evolution of distinctive cell wall composition in grasses. The growth of these clades has probably involved both tandem and segmental duplications and it is likely that this began before the divergence of the cereal grasses (Paterson, 2004). This would account for sorghum and *Brachypodium* each recording an almost equal number of β-expansins than α-expansins, with close to even events of tandem duplication for both subfamilies.

2.5 CONCLUSION

The data presented in this Chapter details the analysis of barley EST data deposited in public databases for putative expansins. This resulted in the discovery of 34 partial or complete barley expansin genes. By assembling the ESTs into tentative contigs, it was possible to predict full length sequences of 26 of the 34 barley expansin and expansin-like genes identified. Of the 34 identified, eight near full-length barley expansin cDNAs were amplified and sequenced, and comprised one α-expansin and seven β-expansins. All eight of these barley cDNAs isolated are predicted to encode complete expansin proteins.

The barley expansin orthologs of rice, sorghum and *Brachypodium* are clearly illustrated on the radial trees generated in this Chapter and support the existence of further, so far unidentified members of the barley expansin family. The only member of the expansin gene family that has been identified by genomic sequence only is the expansin-like B. EXLB has been identified in rice and *Arabidopsis* genome, and now in the *Brachypodium* genome, but is absent in the sorghum genome. No expression of EXLB has been detected. This leads to the possibility that one member exists in barley.

Until the barley genome sequence is fully complete and annotated, the current analysis of the barley expansins is unlikely to differentiate between very closely related genes since the analysis of the barley expansins carried out here was based on ESTs.

The large number of expansin genes identified poses intriguing questions regarding the function of these proteins and the significance of their redundancy. Determining the pattern and control of expansin gene expression is the first step in elucidating the function of individual expansins. In Chapter 3 the transcription of expansins across a barley developmental tissue series is explored.

CHAPTER 3

TRANSCRIPTION OF EXPANSIN GENES IN BARLEY

3 TRANSCRIPTION OF EXPANSINS IN BARLEY

3.1 INTRODUCTION

The expansin gene family has been extensively investigated in *Arabidopsis*, rice, maize, tomato and wheat (Reinhardt *et al.*, 1998; Wu *et al.*, 2001a; Cho and Cosgrove, 2002; Lee and Kende, 2002; Lin *et al.*, 2005; Muller *et al.*, 2007). In the fully sequenced *Arabidopsis* and rice genomes, 36 and 58 expansin genes, respectively, have been identified. *In silico* analysis presented in Chapter 2 identified 79 expansin members in sorghum and 62 in *Brachypodium*. In wheat and maize the expansin count stands at 18 and 33 respectively, however the numbers for maize and wheat include some partial gene fragments and should be interpreted as minimum estimates. When the total count of expansin genes for each species is broken down, the monocots contain a larger number of β-expansins than dicots. It is predicted that the β-expansins may act selectively on cell walls of the Poaceae as the α-expansins have been shown to more effectively loosen the cell walls of dicots than those of monocots (Cosgrove *et al.*, 1997; Li and Cosgrove, 2001). As the composition of the cell wall changes during each stages of organ growth and development, profiling the expression of expansin genes is the first step towards investigating the specific functions of these genes in plant growth. The study of the expansin gene family in barley provides a good model to further explore the specialised role of the β-expansins on the synthesis and extension of Poaceae-type cell walls.

One method to investigate the role and process of an individual gene in a multi-gene family is to measure its mRNA transcript abundance in different tissues during development, to enable us to associate that gene with a particular tissue or organ, or growth stage. In itself, a change in mRNA transcript abundance is not confirmation that a gene is involved in a particular facet of tissue growth, but transcript profiling is widely used as a first step to investigate the potential role of genes in cellular processes. Therefore, the second objective of this project was the transcriptional profiling of barley expansin genes performed using either quantitative real-time PCR (Q-PCR) or *in silico* analysis of mircroarray data in a range of tissues from barley. It is acknowledged that some members of multi-gene families might only be expressed at high levels in response to abiotic or biotic stresses, but an investigation of the effects of stress on expansin gene transcription was not possible here.

It is also acknowledged that transcript levels do not necessarily reflect the final amounts of the expansin protein, and therefore that transcript levels and gene expression are not synonymous.

The individual expansin genes of *Arabidopsis*, rice and wheat appear to have evolved specialised functions, which require expression of different genes in different tissues. Previous methods utilised

to measure transcript levels have included semi-quantitative reverse transcriptase (RT) PCR, Northern hybridization analyses and cDNA microarrays. Northern hybridization analyses require a sequence specific probe for hybridisation with the mRNA of interest, and relatively large amounts of isolated RNA. In a multi-gene family, which sometimes contains members with high nucleotide similarity, this makes it difficult to select a specific probe for each gene member. Furthermore, genes with low transcript levels remain difficult to detect by Northern techniques. Screening of cDNA microarrays provides information about differentially expressed genes and the relative abundance of transcripts in a variety of tissues in a high throughput manner, but this technique requires sophisticated instruments to generate and screen the cDNA microarrays.

Public access to the results arising from the use of the 22K Barley1 GeneChip probe microarray by Close *et al.* (2006) has made transcript profiling using the cDNA microarray reference data for barley expansins achievable. The transcript profiles of 23 barley expansins were identified and downloaded from that barley Affymetrix chip database.

More recently Q-PCR has been adapted for measuring individual mRNAs, due to it's sensitivity, specificity and reliable quantification (Gachon *et al.*, 2004). The Q-PCR method used in this study relies on measuring the relative mRNA abundance of the genes of interest in comparison to mRNA transcripts of a number of control genes. The control genes are those for which transcriptional activity is not expected to change substantially under the conditions used, and they therefore provide internal experimental standards for the purpose of data normalisation.

The Q-PCR method relies on a measure of fluorescent dye associated with DNA amplified from the target sequence using gene-specific primers. Any amplification of non-specific products, such as primer dimers, reduces the efficiency of the measurement and produces systematic errors (Wilhelm *et al.*, 2003). Increasing the temperature at which the reaction is performed avoids the non-specific binding of primers to the template (Bustin, 2000). A melt curve for the amplicon, generated by the thermocycler by heating the amplicon at the end of PCR stock generation from 70 to 99°C, produces a plot of fluorescence as a function of temperature that is used to determine the optimal temperature at which fluorescence data is acquired, together with an indication as to the purity of the DNA product of the reaction.

The PCR stock that is generated during this process is used to prepare a dilution series to generate a standard curve to calculate mean transcript level and standard deviations for each set of replicates. In addition to the internal transcript concentration standards, the transcript abundances of four control genes, glyceraldehyde 3-phosphate dehydrogenase, heat shock protein 70,

cyclophilin and α-tubulin are calculated in each sample as described by Burton *et al*. (2004) to provide normalisation factors (NFs) for Q-PCR (Vandesompele *et al.*, 2002). The control genes are not functionally associated with the expansin genes of interest and are functionally independent of each other.

3.2 MATERIALS AND METHODS

3.2.1 cDNA Populations

3.2.1.1 General cDNA populations

The cDNA templates used for testing Q-PCR primer pairs are described in section 2.2.5.

3.2.1.2 Barley developmental tissue series

The two barley developmental tissue series used to measure the transcript levels of barley expansins were provided by Assoc. Prof. Rachel Burton (University of Adelaide, SA, Australia) as described in Burton *et al*. (2004; 2008). For the developmental tissue series, tissues were selected to represent the major stages of barley plant development. Leaf tip and leaf base were harvested from the top 7mm of the leaf and 3mm of base respectively, from 13 cm seedling leaves. For mature leaves, 1 cm sections were excised from the fifth leaf when it was 32 cm long and segments were 8, 16, 24 and 29 cm from the base of the leaf. A 1 cm section of root was harvested from the tip to include the elongating zone of the tissue and a 1 cm section about 6 cm behind the root tip containing the maturation zone was harvested for the root base. Anther and pistil were collected at 2 weeks before anthesis and the whole flower collected at anthesis. Stem tissue was harvested from the upper internode below the peduncle where cell elongation had ceased in this segment. The coleoptile was harvested 3 days after germination and the scutellum harvested 24 hours after germination. Whole developing grain was collected at two time points after anthesis (3-5 and 8-10 days) and the embryo dissected from the grain at 22 days after pollination.

3.2.2 Primer design

The oligonucleotide primers for the Q-PCR analysis were designed based on the available nucleotide sequences of then 3' untranslated region (UTR) of the corresponding genes to produce fragments of 80-300 bp (Tables 3-1 and 3-2). For individual genes, both forward and reverse primers matched sequences from within the 3'UTR; no primers to coding regions were used. All oligonucleotide primers were designed with the aid of the program Primer3 (Rozen and Skaletsky, 2000), and assessed with NetPrimer (PREMIER Biosoft International, Palo Alto, CA, USA) for the possibility of generating hair pin loops, primer dimers, palindromes and sequence repeats.

Primer pairs were initially tested on cDNA as described in section 2.2.5 for the generation of single products. Reactions were carried out using Elongase® *Taq* Polymerase Amplification System (Invitrogen). The gene-specific primers and cDNA template used to test Q-PCR primer pairs are shown in Tables 3-1 and 3-2. PCR reactions were carried out in a total volume of 25 µl containing

Gene	Forward primer (5'—3')	Reverse primer (5'—3')	PCR size bp	Annealing temperature °C	cDNA template	Acquisition temperature °C
GAPDH	GTGAGGCTGGTGCTGATTACG	TGGTGCAGCTAGCATTTGAGAC	198	-	-	80
HSP70	CGACCAGGGCAACCGCACCAC	ACGGTGTTGATGGGGTTCATG	108	-	-	83
α-Tubulin	AGTGTCCTGTCCACCCACTC	AGCATGAAGTGGATCCTTGG	248	-	-	80
Cyclophilin	CCTGTCGTGTCGTCGGTCTAAA	ACGCAGATCCAGCAGCCTAAAG	122	-	-	79
HvEXPA1	TACGCTGAGGCTGCTGATTA	TACAAACCAACCACGAGAGG	214	54	Coleoptiles	79
HvEXPA2	GCTATTCTGAGGCTGCTGCT	GGTTACAACGCAACAAGAGAA	245	50	Leaf	77
HvEXPA3	CATCTGGTTGTAGTAGTGGTTTTA	TCGGCGGGTGATACAAGGC	282	55	Stem	82
HvEXPA4	ATCCACGGCACTGCTTAATCTA	GTGGCGAATGCAACAGAGG	92	53	Root	74
HvEXPA5	TGGGGTGTACGGTATTTCTTG	AACCCACACAACACCTCGTC	103	54	Coleoptiles	79
HvEXPA6	CTCTGAGGAACGAGCTGATTAT	AAGCCTCTGGAGCAACTATCA	133	52	Root/leaf	76
HvEXPA7	TCAGTTCGCTTCGTTGAGGGC	TTGTTCCACGCCAAATAATCCT	147	56	Root/leaf	79
HvEXPA8	CGTGCGTGAAGATGAACTTAGAC	GCAAGCACACAATCCGATACAA	126	53	Root	76
HvEXPA10	ACCACTATCAACGGGACGAA	CAAGGATATTGGAGGTACACGA	150	54	Root	75
HvEXPA11	TCTCATCAGGGTACTCAAATAGGC	GCACAAGCAAAACAAAGATACAA	100	54	Root	75
HvEXPA12	GCCTTCGGACTGGATGTTTG	CTGGGTCACGGCATTTTAGT	175	52	Developing endosperm	80
HvEXLA1	AAGCAAATCGGGGATAGTAAACAA	GTGCGTGCGTGCGTAGCC	102	54	Root	81
HvEXLA2	GGGTGACGGTGGAGAAACAT	AGAACATCATTGCCGAAACC	207	52	caryopsis	80
HvEXLA3	AGGCAAGCCAAGAGGAAGAG	CGTGTCTCTGCGTCAGTTA	158	51	Developing endosperm	83

Table 3-1 Gene-specific α-expansin, expansin-like A and control gene Q-PCR primers

Q-PCR primers, product sizes in base pairs, annealing temperature for testing, template used and optimal acquisition temperature are listed.

Gene	Forward primer (5'—3')	Reverse primer (5'—3')	PCR size bp	Annealing temperature °C	cDNA template	Acquisition temperature °C
HvEXPB1	CCGACTGTATGCCCTTAGATCTGG	CATCGATCCTTTGCTGTGACTACAA	174	53	Root	81
HvEXPB2	TGCTGAACTCATCATCATTTGGAA	TGCCTCTCCTCCATAGCCCA	187	53	Root/leaf	80
HvEXPB4	TGCTACGGTCAAGTCATTGGGAGT	GCCAATACACGACTCATCTCTCAC	201	57	Leaf	83
HvEXPB5	TATGTTGCGTCGTCGTGCGT	GAATCTTGTGATAGTGGGCTGCGT	208	56	Leaf	81
HvEXPB7	CCGTGGGTTAATTTCTACTGC	ATCGACGACACGCATCACTA	165	51	Root	82
HvEXPB8	ATGGCTCCTGCGTGTGAAT	ACAAAGCAGAGGAAGGCAA	213	52	Root/leaf	81
HvEXPB9	CTTACCTGAGCACTTCTCCGA	ACTCCTCAGGACACACAATCG	148	54	Root/leaf	76
HvEXPB10	GCAGTTCATCACTCGGTTG	ATGACACCAGACAGACGGCA	98	54	Root/leaf	76
HvEXPB11	CGGATCCATTTAGCCCAGT	TTTTCAGCACATCGCAACCG	208	51	Caryopsis	76
HvEXPB12	GTGAGTAGTTTTGGAGCAGGAG	ACACGACAACAGGAGTGAAGC	163	55	Stem	83
HvEXPB13	TCGCCGACAAGGTCATCC	ATTACACGATGGACGGACACA	239	56	caryopsis	79
HvEXPB14	GGGTGATTTTAGTGCCTTACG	GGCTAAGCACAGGACTGGAGT	97	52	Stem	81
HvEXPB15	CGCCATCCTGGTTTATGTTTG	CACCAAAGTACCAAACTGACAGAT	153	53	Caryopsis	80
HvEXPB16	TGGCTCAAGAAGAAGAAGAAGAA	GCATGAATGACCCTCTCCCT	117	52	Caryopsis	77
HvEXPB18	GTTGATTCTCCCGCATTTCT	GGACAATACAAATAGACGCAGC	161	55	root	76

Table 3-2 Gene-specific β-expansin Q-PCR primers

Q-PCR primers, product sizes in base pairs, annealing temperature for testing, template used and optimal acquisition temperature are listed.

0.5 µl of the appropriate cDNA template listed, 1 µl 10 µM of the gene-specific primers listed in Tables 3-1 and 3-2, 2.5 µl Elongase Buffer A, 2.5 µl Elongase Buffer B, 0.5 µl Elongase polymerase and 0.5 µl 0.2 mM dNTPs. Cycling conditions for PCR were; 94°C 5 min followed by 30 cycles of 94°C, 30 s, 58-61°C (annealing temperature based on the T_m of the gene-specific primers) 30 s, 68°C, 30 s and a final step of 68°C for 5 min. The expected lengths of the Q-PCR test fragments and annealing temperatures used are listed in Tables 3-1 and 3-2. Samples were held at 25°C before separating 5 µl of the PCR product on a 1% (v/v) agarose gel containing ethidium bromide.

Initial primer testing serves a second purpose in the preparation of Q-PCR analysis, because it produces a suitable template to prepare stock solutions to generate a standard curve for quantifying PCR products.

3.2.3 Quantitative PCR Analysis of Transcript levels

Quantitative PCR experiments were performed using iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA) in an RG 6000 Rotor-Gene Real Time Thermal Cycler (Corbett Research, Sydney) as described in Burton *et al*. (2008)

3.2.3.1 Generation of PCR stock solution and determination of optimal acquisition temperature

PCR products of the genes of interest were amplified from cDNAs listed in Tables 3-1 and 3-2 in quadruplicate. PCR reactions were carried out in a total volume of 20 µl containing 1 µl of a 1:10 dilution of the cDNA template listed, 3 µl 4 µM of each forward and reverse gene-specific primers listed in Tables 3-1 and 3-2, 3 µl water and 10 µl iQ SYBR Green Supermix. Cycling conditions for PCR were; 95°C 3 min followed by 45 cycles of 95°C 1 s, 55°C 1 s, 72°C 30 s and a final step of 80°C for 15 min. At the end of amplification, a melt curve was produced by heating the sample from 70°C to 99°C and by collecting fluorescence data to determine the optimal temperature for product acquisition (Tables 3-1 and 3-2). The PCR products (4 replicates) were purified by HPLC and quantified as described by Burton *et al*. (2008). The stock solution (1 µl of a 20 ng/µl solution) was subjected to DNA sequencing to confirm product identity, as described in section 2.2.8.8 with gene-specific primers. A stock dilution of each standard containing 10^9 copies of the target sequence per microliter was prepared for the subsequent standard dilution series (10^7 – 10^1 molecules/µl).

3.2.3.2 Tissue transcript analysis

The Q-PCR experiment for each gene was conducted on tissue specific cDNAs (Schreiber *et al.*, 2008) in quadruplicate next to three replicates of each of the seven standard concentrations and two no-template negative controls. PCR reactions were carried out in a total volume of 10 µl containing

2 μl of a 1:10 dilution of the cDNA, 1.2 μL 4 μM of each forward and reverse gene-specific primers listed in Tables 3-1 and 3-2, 0.3 μl water, 5 μl iQ SYBR Green Supermix and 0.3 μl 10x SYBR green dye. Cycling conditions for PCR were; 95°C 3 min followed by 45 cycles of 95°C 1 s, 55°C 1 s, 72°C 30 s and 15 s at the optimal acquisition temperature (Tables 3-1 and 3-2). The Rotor-Gene V4.6 software (Corbett Research) was used to calculate the optimal cycle threshold from the dilution series, the mean expression level and standard deviations (SDs) for each cDNA quadruplet.

3.2.4 Transcript profiling using cDNA microarray reference data

Using a keyword search of 'expansin' on the Barley Base database (http://www.barleybase.org), the chip Contig numbers corresponding to expansins were retrieved and are shown in Table 3-3. Corresponding transcript profiles were downloaded from the 'Transcript patterns during barley development' experiment of the 22K Barley1 GeneChip probe array. All data were normalised using the Robust Multi-array Analysis (RMA) method (Irizarry *et al.*, 2003). Results are represented as expression intensity on a log base 2 scale. Information about the fifteen barley tissues prepared from two barley varieties (Morex and Golden Promise) can be found in Druka *et al*. (2006).

3.2.5 Transcriptional correlation of Q-PCR data of cell wall-related genes

The levels of the barley expansin transcripts were expressed as a percentage of maximal normalised levels for the specific gene (Burton *et al.*, 2004). When expressed as a maximum percentage, the value at 100% indicates the tissue in which a particular gene is found at its maximal level. In this way, coordinated transcript patterns of each gene can be detected and coordinated transcription might suggest similar functions or complementary relationships for some of the expansin genes.

The same treatment was applied to all the available cell-wall related genes that had been measured across the same barley developmental tissue series. Once expressed in these arbitrary units, a correlation matrix was prepared with the cell-wall related genes checked against the barley expansin transcript results. Transcript patterns with a correlation of > 0.90 were considered significant. The data and correlation analyses for cell wall-related genes were performed by Dr. Neil Shirley (University of Adelaide, SA, Australia).

Expansin	Contig number
HvEXPA1	Contig3674_at
HvEXPA2	Contig3675_at
HvEXPA3	Contig7055_at
HvEXPA4	Contig16079_at
HvEXPA5	Contig3676_at
HvEXPA6	Contig11609_at
HvEXPA7	Contig18422_at
HvEXPA8	HVSMEI0024B06r2_at → Contig45260
HvEXPA12	HB25G16r_at → Contig32912
HvEXPB2	Contig2877_at
HvEXPB3	Contig2876_at
HvEXPB4	Contig2873_s_at
HvEXPB5	Contig2878_at
HvEXPB8	Contig2967_at
HvEXPB9	Contig17140_at
HvEXPB10	Contig7394_at
HvEXPB11	Contig18714_at
HvEXPB13	HVSMEI0013G22r2_at → Contig45068
HvEXPB14	Contig8009_at
HvEXPB15	Contig7170_at
HvEXPB16	Contig8819_at
HvEXLA2	Contig12093_at
HvEXLA3	Contig12094_at

Table 3-3 Barley expansin Contig numbers from the Affymetrix chip

A keyword search of expansin on the Barley Base database (http://wwwbarleybase.org) recovered Affymetrix chip Contig numbers for tissue transcript studies of expansins during barley development.

3.3 RESULTS

3.3.1 Transcript analysis of the barley α-expansins via Q-PCR and Affymetrix data

The abundance of individual barley α-expansin transcripts was measured in a range of barley tissues via Q-PCR. The data from the Q-PCR experiments and select α-expansin transcript profiles from the Affymetrix data set are presented for each gene in the following sections. The 3'UTR sequences of *HvEXPA9*, *HvEXPA13* and *HvEXPA14* were not complete and thus not available for the generation of gene-specific primers. Due to high sequence conservation amongst expansins, primers in the coding region were not considered for Q-PCR. A search of the Affymetrix data showed that *HvEXPA9*, *HvEXPA13* and *HvEXPA14* were not represented on the chip.

3.3.1.1 HvEXPA1

HvEXPA1 was expressed in all tissues represented in the barley developmental tissue series. The data showed that of all the α-expansins profiled, the *HvEXPA1* transcript was the most abundant in anthers at pre-anthesis and in coleoptiles, and was the only one of high abundance in the leaf tip, where it was almost 5-fold higher than transcripts for the other α-expansins characterised (Figure 3-1, Panel A). There was some *HvEXPA1* transcript observed in the whole flower at anthesis, in the spike and in whole grain at 3-5 days after pollination (dap). The Affymetrix data showed the highest levels of *HvEXPA1* mRNA was in the anthers (Appendix C, Figure C-1, Panel A).

3.3.1.2 HvEXPA2

Of all the α-expansins characterised in this project, *HvEXPA2* showed the most abundant transcript levels overall in all tissues measured, except for in the embryo 22 dap (Figure 3-1, Panel B). These data show that the only other α-expansin that was highly transcribed in pre-anthesis anthers tissue was *HvEXPA2*. However, in contrast to *HvEXPA1*, the two tissues with the second highest amount of *HvEXPA2* mRNA were the root tip and leaf base, which represent the more metabolically active zones of these organs. *HvEXPA2* also displayed the highest transcript abundance amongst the α-expansins in the base of the root, which is considered the least metabolically active zone of the root. The transcript level for *HvEXPA2* was similar to, but higher than, *HvEXPA1* in the whole flower at anthesis, in the pistil at anthesis, in the spike and in whole grain 3-5 dap (Figure 3-1, Panel B). The Affymetrix data for *HvEXPA2* showed similar transcript patterns as the Q-PCR data in anthers, leaf, root and embryo 22 dap (Appendix C, Figure C-1, Panel B).

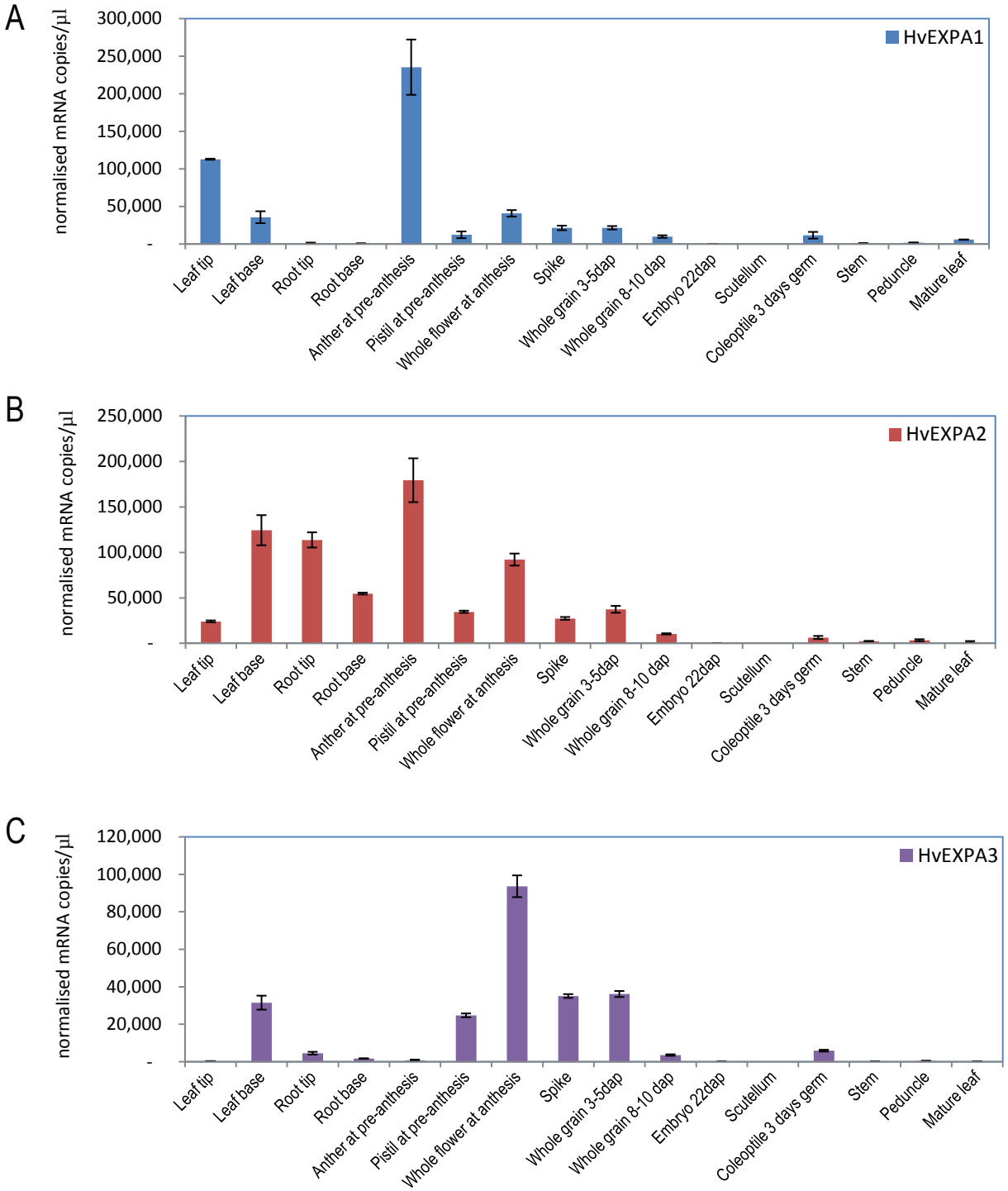


Figure 3-1 Normalised transcript levels for *HvEXPA1*, *HvEXPA2* and *HvEXPA3* in a barley developmental tissue series.

Levels of mRNA are presented as the number of copies per microlitre of cDNA after normalisation. Error bars = standard deviations for each mRNA. **A**, the transcript pattern for *HvEXPA1*, which is highly abundant in anthers at pre-anthesis. **B**, the transcript profile for *HvEXPA2*, which is most abundant in anthers at pre-anthesis and the metabolically active zones of the root and leaf. **C**, the transcript profile of *HvEXPA3*, which is most abundant in the whole flower at anthesis.

3.3.1.3 HvEXPA3

The data showed that *HvEXPA3* had the highest abundance of all the α-expansins in the whole flower at anthesis. The transcript pattern for *HvEXPA3* in the leaf base, spike and whole grain at 3-5 dap was similar to that of *HvEXPA1* (Figure 3-1, Panel C). The Affymetrix data for *HvEXPA3* showed high transcript intensities in most tissues, with the exception of anthers. This low abundance in pre-anthesis anther was also demonstrated by the Affymetrix data (Appendix C, Figure C-1 Panel C).

3.3.1.4 HvEXPA4

The highest amount of *HvEXPA4* mRNA was observed in the root tip, followed by the root base (Figure 3-2, Panel A). In all other tissues the transcript levels were low and in the whole grain 8-10 dap, embryo 22 dap and scutellum there was no *HvEXPA4* mRNA detected. These overall low values with higher transcript levels in the root were mirrored by the Affymetrix data for *HvEXPA4* (Figure 3-2, Panel B). The Affymetrix data also showed that *HvEXPA4* had high transcript levels in the germinating embryo (mesocotyl; Golden Promise) and radicle (seminal root)(Appendix C, Figure C-1, Panel D).

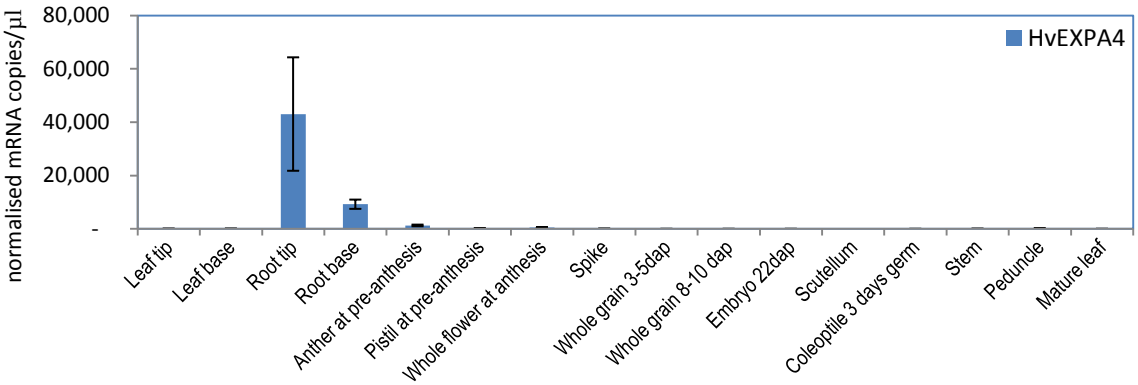
3.3.1.5 HvEXPA5

The coleoptile contained the highest level of *HvEXPA5* transcript, followed by the root base, root tip and whole grain at 3-5 dap (Figure 3-3, Panel A). The Affymetrix data showed that the highest intensity of *HvEXPA5* mRNA was in the radicle (seminal root; Morex), followed by the coleoptile (Golden Promise)(Appendix C, Figure C-1, Panel E). Equivalent radicle tissue was not included in the Q-PCR tissue transcript studies.

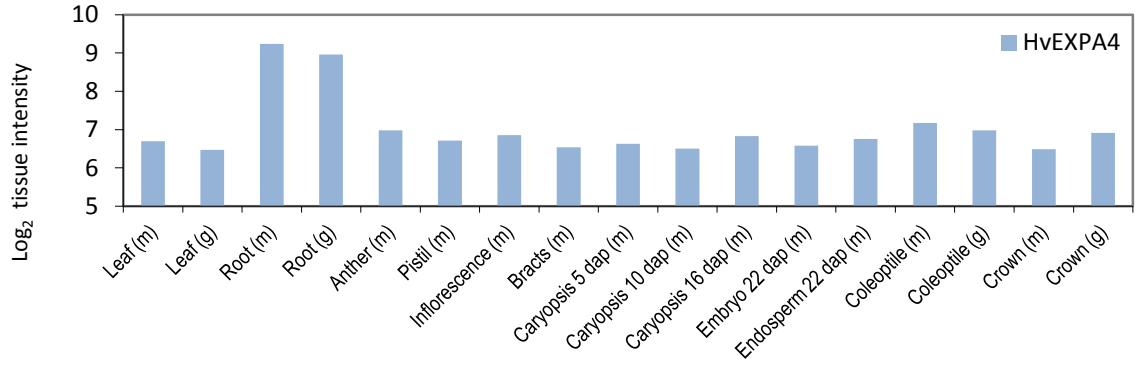
3.3.1.6 HvEXPA6

HvEXPA6 was expressed in all tissues of the barley developmental tissue series. The data showed that *HvEXPA6* mRNA was most abundant in the leaf base, followed by the root tip, which represent the most metabolically active zones of the leaf and root. However, amongst all the expansins characterised in this project, *HvEXPA6* had the highest transcript abundance in the embryo 22 dap, in the peduncle, stem and in the scutellum (Figure 3-3, Panel B). The Affymetrix data showed the highest intensity of *HvEXPA6* in tissues not measured in the Q-PCR transcript studies (radicle and germinating embryo, Golden Promise) followed closely by leaf and root (Appendix C, Figure C-1, Panel F).

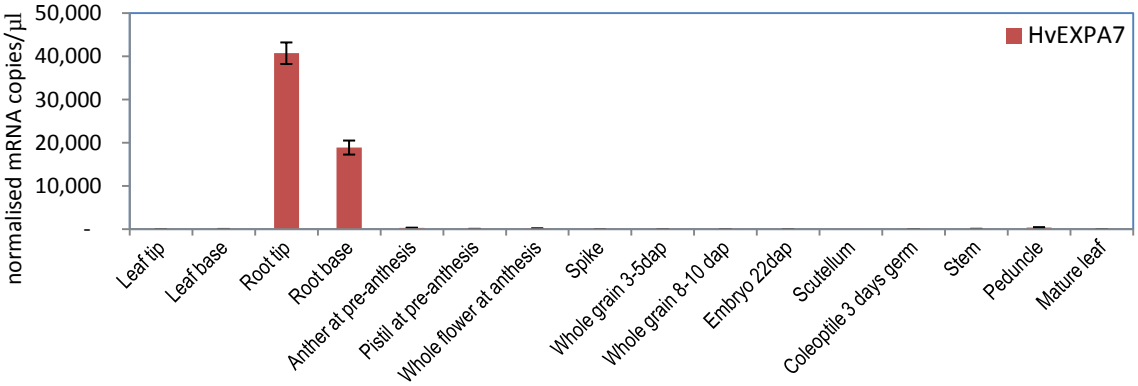
A


normalised mRNA copies/µl
80,000
60,000
40,000
20,000
-
HvEXPA4
Leaf tip
Leaf base
Root tip
Root base
Anther at pre-anthesis
Pistil at pre-anthesis
Whole flower at anthesis
Spike
Whole grain 3-5dap
Whole grain 8-10 dap
Embryo 22dap
Scutellum
Coleoptile 3 days germ
Stem
Peduncle
Mature leaf

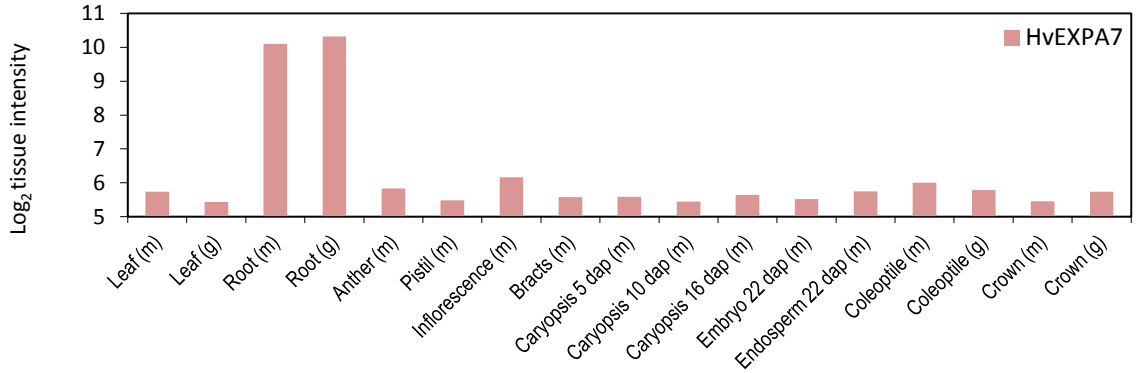
B


Log2 tissue intensity
10
9
8
7
6
5
HvEXPA4
Leaf (m)
Leaf (g)
Root (m)
Root (g)
Anther (m)
Pistil (m)
Inflorescence (m)
Bracts (m)
Caryopsis 5 dap (m)
Caryopsis 10 dap (m)
Caryopsis 16 dap (m)
Embryo 22 dap (m)
Endosperm 22 dap (m)
Coleoptile (m)
Coleoptile (g)
Crown (m)
Crown (g)

C


normalised mRNA copies/µl
50,000
40,000
30,000
20,000
10,000
-
HvEXPA7
Leaf tip
Leaf base
Root tip
Root base
Anther at pre-anthesis
Pistil at pre-anthesis
Whole flower at anthesis
Spike
Whole grain 3-5dap
Whole grain 8-10 dap
Embryo 22dap
Scutellum
Coleoptile 3 days germ
Stem
Peduncle
Mature leaf

D


Log2 tissue intensity
11
10
9
8
7
6
5
HvEXPA7
Leaf (m)
Leaf (g)
Root (m)
Root (g)
Anther (m)
Pistil (m)
Inflorescence (m)
Bracts (m)
Caryopsis 5 dap (m)
Caryopsis 10 dap (m)
Caryopsis 16 dap (m)
Embryo 22 dap (m)
Endosperm 22 dap (m)
Coleoptile (m)
Coleoptile (g)
Crown (m)
Crown (g)

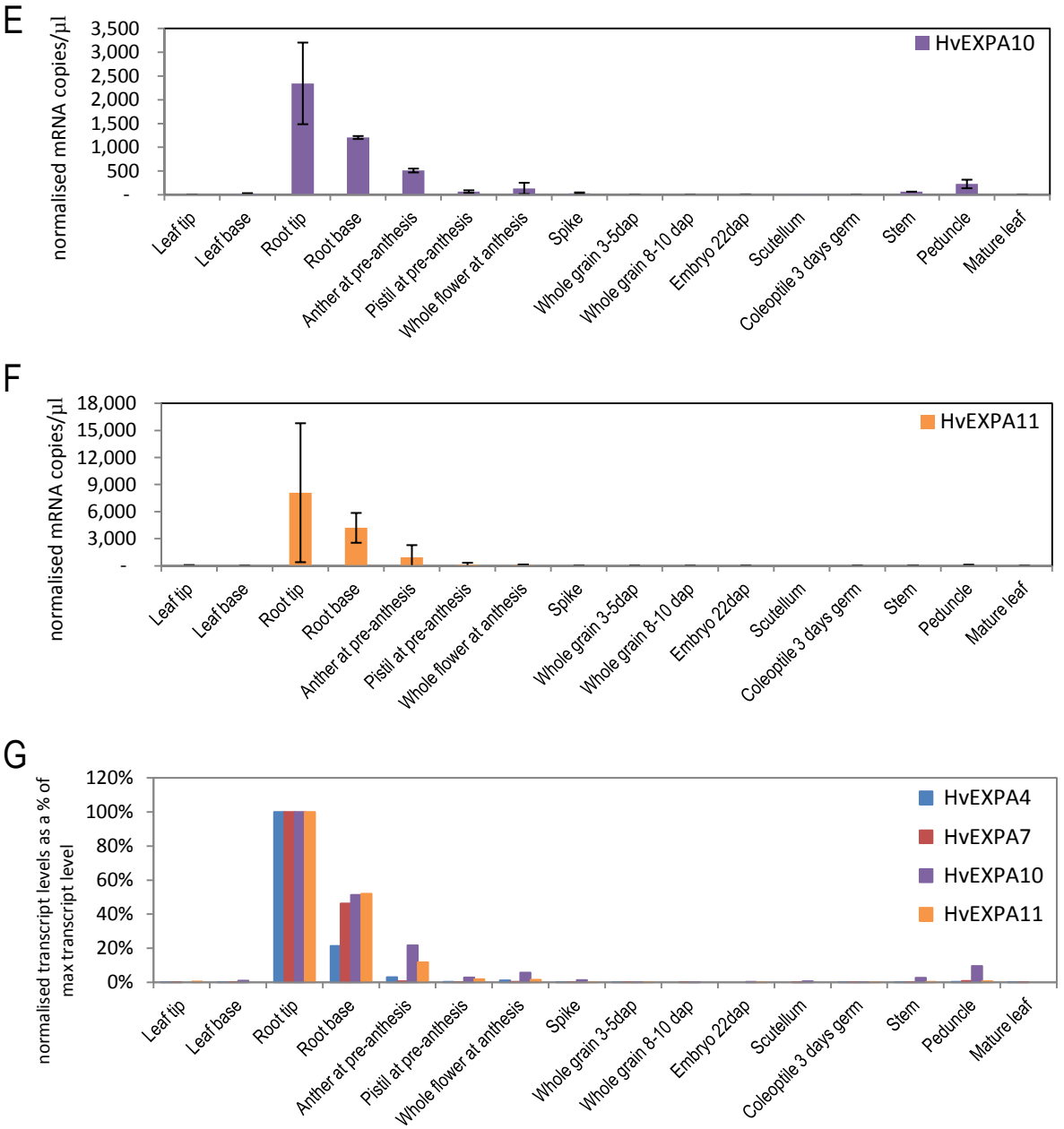


Figure 3-2 Tissue transcript profiles of *HvEXPA4*, *HvEXPA7*, *HvEXPA10* and *HvEXPA11* in a range of developing tissues by Q-PCR and microarray.

A, **C**, **E** and **F**, Q-PCR plots of *HvEXPA4*, *HvEXPA7*, *HvEXPA10* and *HvEXPA11* which are most abundant in the root tips. Levels of mRNA are presented as the number of copies per microlitre of cDNA after normalisation. Error bars on all Q-PCR plots = standard deviations for each mRNA. **B** and **D**, Affymetrix data of *HvEXPA4* and *HvEXPA7* which demonstrate highest transcript intensity in the roots. Affymetrix data intensity ratios are presented as log base 2, RMA normalised. A tissue intensity value < 5 is considered background. The tissues indicated on the *x*-axis correspond to samples taken from the cultivar 'Morex' (m) or 'Golden Promise' (g). **G**, Normalised transcript levels of four highly correlated barley *HvEXPA* genes in a range of tissues, expressed as a ratio of the maximal levels observed for the specific gene. Values of 100% indicate the tissue in which a particular gene is transcribed at its maximal level.

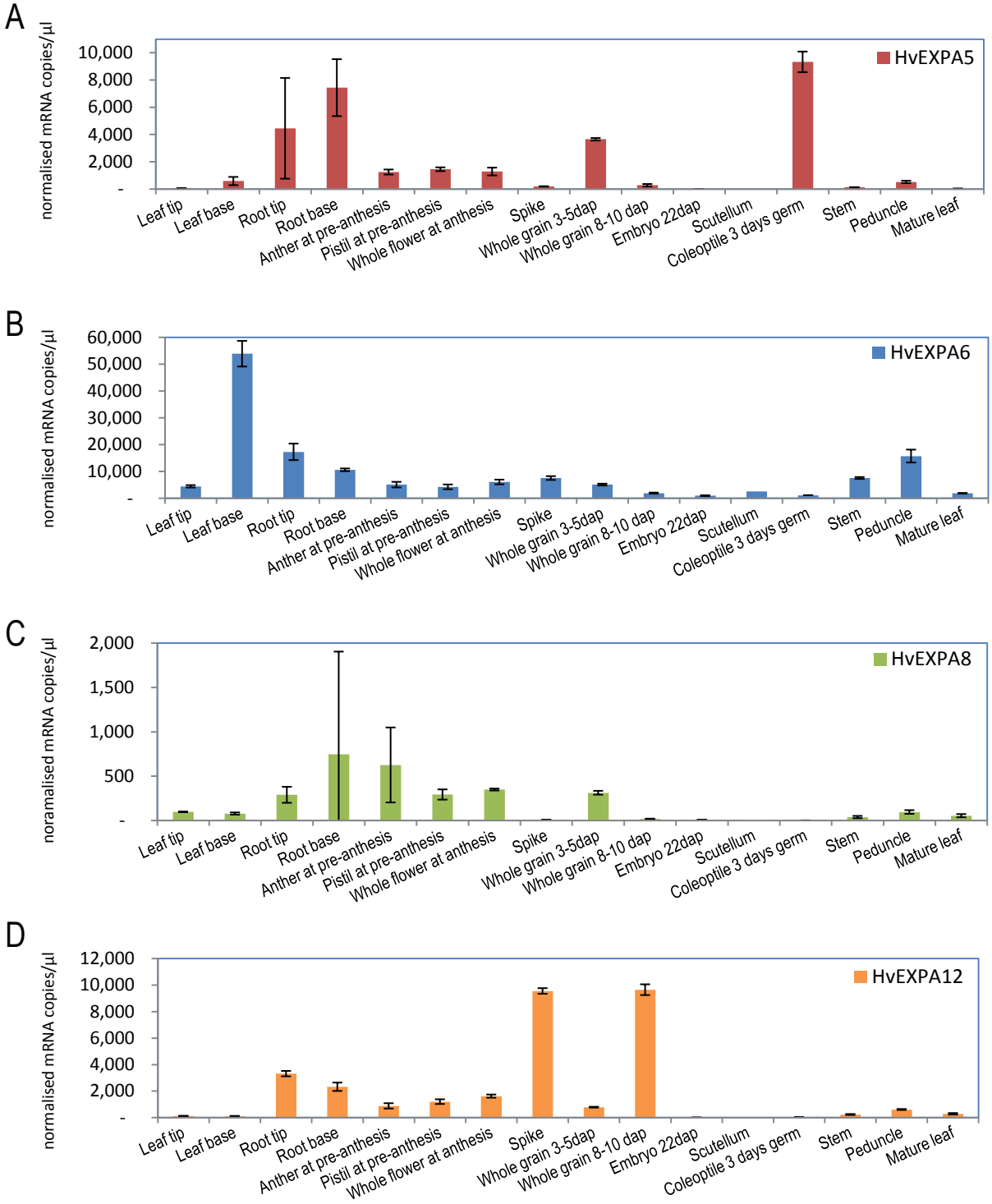


Figure 3-3 Normalised transcript levels for *HvEXPA5*, *HvEXPA6*, *HvEXPA8* and *HvEXPA12* in a barley developmental tissue series.

Levels of mRNA are presented as the number of copies per microlitre of cDNA after normalisation. Error bars = standard deviations for each mRNA. **A**, the transcript profile of *HvEXPA5*, which is most abundant in coleoptile. **B**, the transcript pattern for *HvEXPA6*, which is highly abundant in the leaf base. **C**, the transcript profile for *HvEXPA8*, which is most abundant in root base and anthers at pre-anthesis. **D**, the transcript profile of *HvEXPA12*, which is most abundant in the spike and whole grain 8-10 dap.

3.3.1.7 HvEXPA7

The overall transcript level for *HvEXPA7* was very low, with the exception of the root tip and root base (Figure 3-2, Panel C). These results were consistent with the Affymetrix data for *HvEXPA7* (Figure 3-2, Panel D). The transcript pattern for *HvEXPA7* was similar to *HvEXPA4* with high expression in the germinating embryo (mesocotyl, Golden Promise; Appendix C, Figure C-1, Panel G).

3.3.1.8 HvEXPA8

The transcript levels of *HvEXPA8* were low overall in most tissues analysed, with a peak in the root base, followed by the anther at pre-anthesis. Despite the relatively low abundance of *HvEXPA8*, it was detected in all tissues of the developmental tissue series (Figure 3-3, Panel C). A search of the Affymetrix data revealed highest levels of *HvEXPA8* in endosperm 22 dap, caryopsis 16 dap and the radicle (Morex) followed by root. Consistent with the Q-PCR transcript results, the transcript levels were low in the other tissues represented on the Affymetrix chip (Appendix C, Figure C-1, Panel H).

3.3.1.9 HvEXPA10

The root tip and root base were the tissues in which *HvEXPA10* transcript was highest. Besides these two tissues, *HvEXPA10* transcript was consistently found elsewhere in the plant at relatively low levels (Figure 3-2, Panel E). There was no *HvEXPA10* mRNA detected in the leaf tip or mature leaf. A search of the Affymetrix data showed that this gene was not represented on the chip.

3.3.1.10 HvEXPA11

HvEXPA11 mRNA was detected in the least number of tissues of the barley developmental tissue series and was the only α-expansin not expressed in the leaf base or mature leaf. A small amount was detected in the leaf tip (Figure 3-2, Panel F). The tissue with the highest transcript levels of *HvEXPA11* was the root tip followed by the root base. *HvEXPA11* had the same transcript profile as the three other expansins *HvEXPA4, HvEXPA7* and *HvEXPA10*. The graph representing all four highly correlated genes can be found in Figure 3-2, Panel G. A search of the Affymetrix data showed that this gene was not represented on the chip.

3.3.1.11 HvEXPA12

The two tissues in which *HvEXPA12* mRNA was found most abundantly were the whole grain 8-10 dap and spike. The tissues with the second highest transcript level of *HvEXPA12* were the root tip followed by the root base (Figure 3-3, Panel D). For the remaining tissues, *HvEXPA12* transcript levels were low. The Affymetrix data also reflected an overall low level of *HvEXPA12* mRNA in most

tissues. The two tissues with the highest amount of *HvEXPA12* mRNA were the germinating embryo and radicle from Golden Promise (Appendix C, Figure C-1, Panel I).

3.3.2 Transcript analysis of the barley β-expansins via Q-PCR and Affymetrix data

The abundance of individual barley β-expansin transcripts was measured in a range of barley tissues via Q-PCR. The data from the Q-PCR experiments and selected β-expansin transcript profiles from the Affymetrix reference data set are presented for each gene in the following sections. Q-PCR data for *HvEXPB3* and *HvEXPB6* are not presented due to unsuitable melt curves observed during generation of PCR stock solution and determination of optimal acquisition temperature. A search of the Affymetrix data showed that *HvEXPB1*, *HvEXPB6*, *HvEXPB7*, *HvEXPB12* and *HvEXPB18* were not represented on the chip.

3.3.2.1 HvEXPB1

The overall transcript level for *HvEXPB1* was low. The two tissues in which *HvEXPB1* mRNA was found most abundantly were the root base followed by the root tip. Some detectable transcript was also found in the anther at pre-anthesis and the peduncle (Figure 3-4, Panel A).

3.3.2.2 HvEXPB2

HvEXPB2 had the highest abundance of transcripts of all the β-expansins characterised in the scutellum and whole grain 8-10 dap. In most tissues analysed, *HvEXPB2* transcript levels were high, with peak amounts of transcript found in anther at pre-anthesis and the leaf base (Figure 3-5, Panel A). The Affymetrix data showed similarly high levels of *HvEXPB2* mRNA in the same tissues analysed by Q-PCR (Appendix C, Figure C-2, Panel A).

3.3.2.3 HvEXPB3

The Affymetrix data for *HvEXPB3* revealed highest transcript levels in the germinating embryo (Morex), radicle (Morex) and inflorescence. The group of tissues with the next highest intensities of *HvEXPB3* mRNA were the root, coleoptile and caryopsis at 5 dap (Figure 3-6).

3.3.2.4 HvEXPB4

The overall transcript level for *HvEXPB4* was high with the exception of the least metabolically active tissues; stem, peduncle, mature leaf, leaf tip and scutellum, which showed low levels, and in the embryo 22 dap, where *HvEXPB4* mRNA was not detected. Of the remaining ten tissues, *HvEXPB4* was found to be the most abundant of all the β-expansins in four of the tissues; pistil at

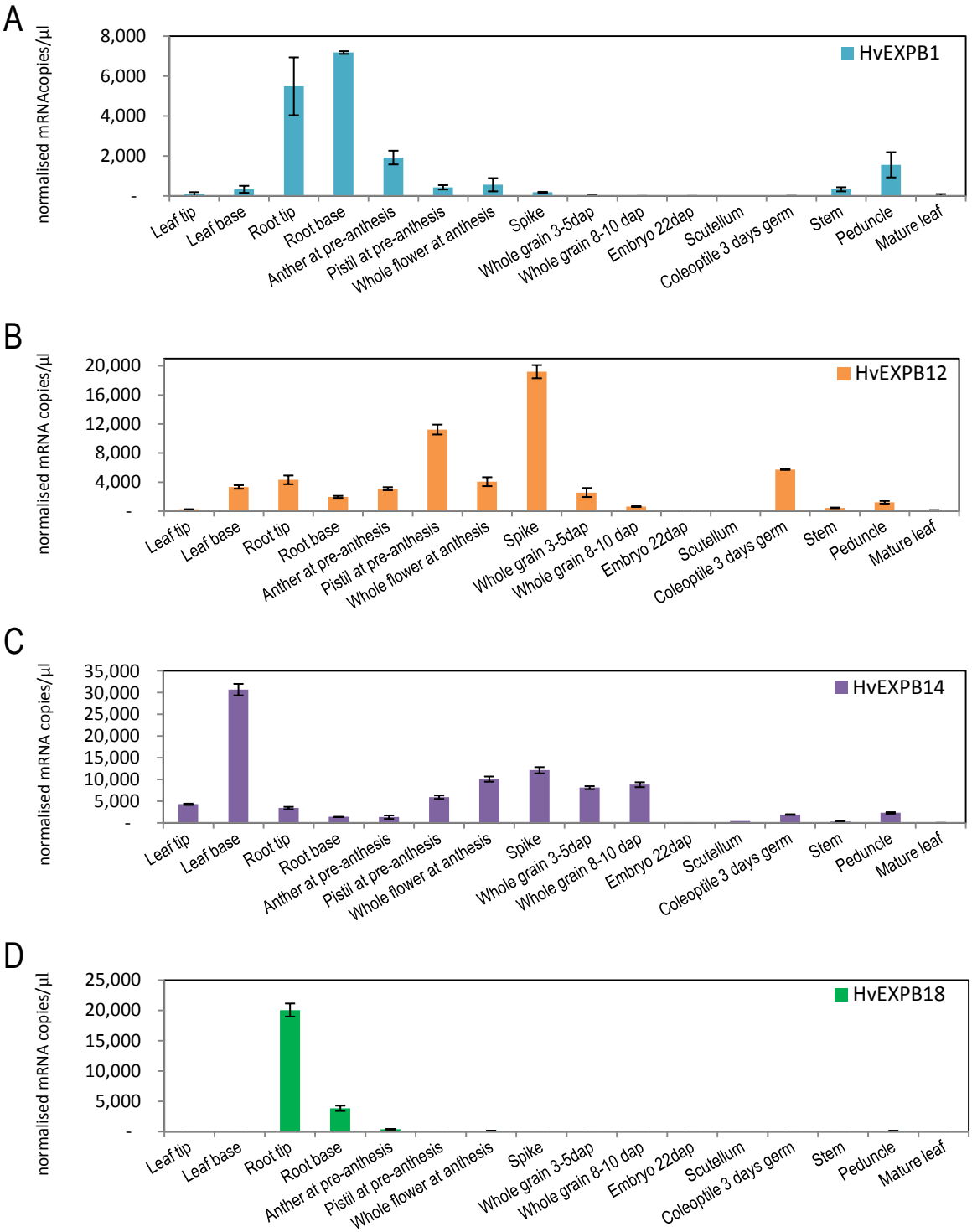


Figure 3-4 Normalised transcript levels for *HvEXPB1*, *HvEXPB12*, *HvEXPB14* and *HvEXPB18* in a barley developmental tissue series.

Levels of mRNA are presented as the number of copies per microlitre of cDNA after normalisation. Error bars = standard deviations for each mRNA. **A**, the transcript pattern for *HvEXPB1*, which is most abundant in root base. **B**, the transcript profile for *HvEXPB12*, which is most abundant in the spike. C, the transcript profile of *HvEXPB14*, which is most abundant in the leaf base and **D**, the transcript pattern for *HvEXPB18*, which is highly abundant in the root tip.

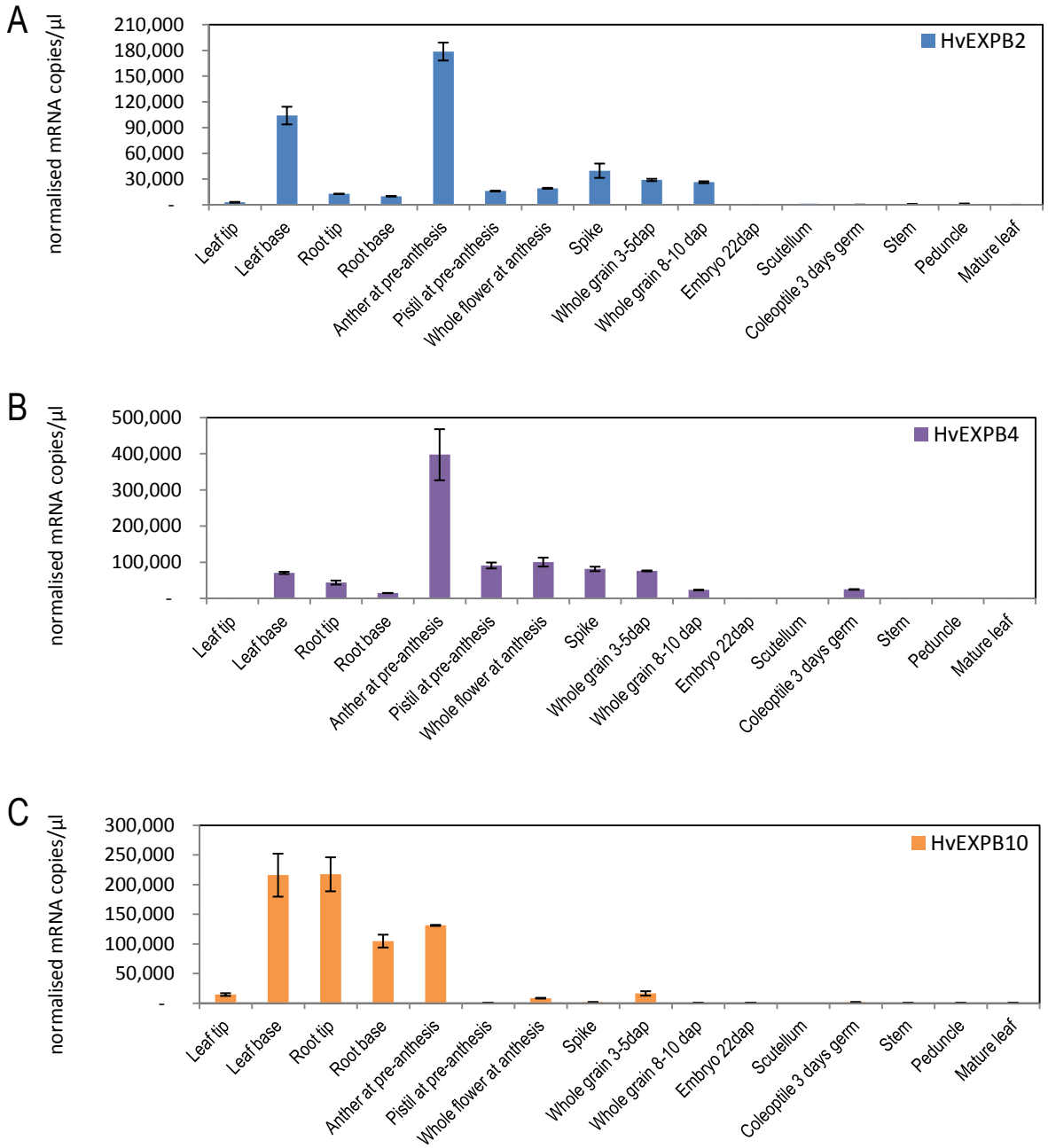


Figure 3-5 Normalised transcript levels for *HvEXPB2*, *HvEXPB4* and *HvEXPB10* in a barley developmental tissue series.

Levels of mRNA are presented as the number of copies per microlitre of cDNA after normalisation. Error bars = standard deviations for each mRNA. **A**, the transcript pattern for *HvEXPB2*, which is highly abundant in anthers at pre-anthesis and has the highest abundance of all β-expansins in whole grain 8-10 dap and scutellum. **B**, the transcript profile for *HvEXPB4*, which is most abundant in anthers at pre-anthesis and has the highest abundance of all β-expansins in pistil at pre-anthesis, spike, whole grain 3-5 dap and coleoptile. **C**, the transcript profile of *HvEXPB10*, which is the most abundant expansin in the root tip and leaf base and has the highest abundance of all β-expansins in root base, leaf tip, embryo 22 dap and mature leaf.

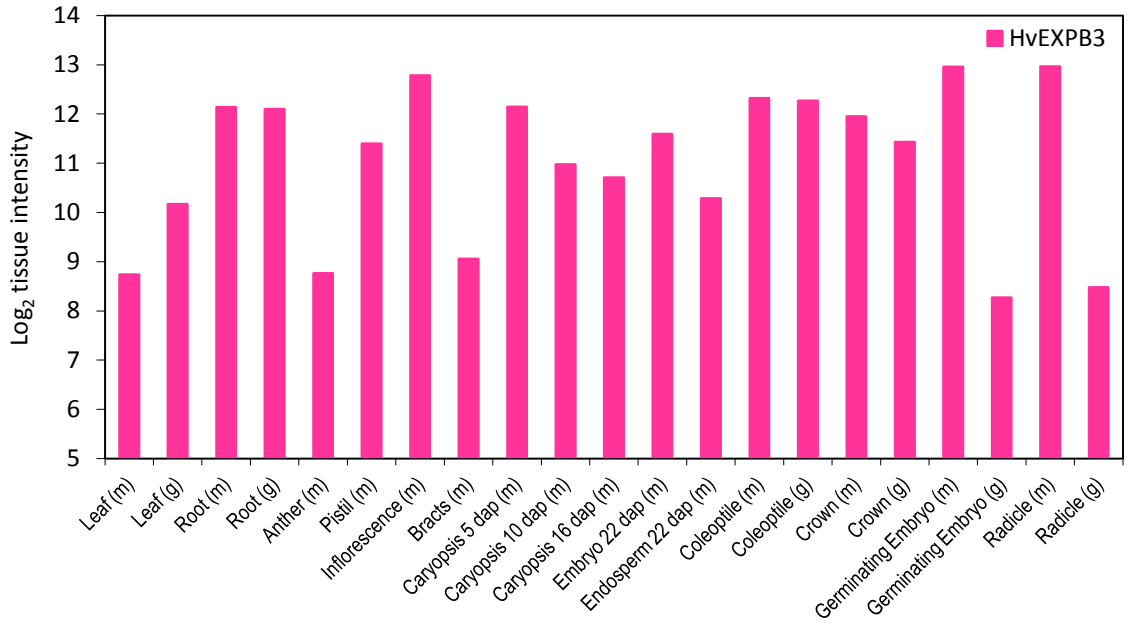


Figure 3-6 Transcription profile of *HvEXPB3* in a range of developing tissues by microarray.

Affymetrix data tissue intensity ratios are presented as log base 2, Robust Multi-array Analysis (RMA) normalised (Irizarry *et al.*, 2003). A tissue intensity value < 5 is considered background. The tissues indicated on the *x*-axis correspond to samples taken from the cultivar 'Morex' (m) or 'Golden Promise' (g).

pre-anthesis, spike, whole grain 3-5 dap and coleoptile. The Affymetrix data for *HvEXPB4* displayed consistently high intensity levels across all tissues analysed (Appendix C, Figure C-2, Panel B).

3.3.2.5 HvEXPB5

HvEXPB5 transcript was found in all tissues of the barley developmental tissue series. The data showed that *HvEXPB5* mRNA was most abundant in anther at pre-anthesis followed by root tip. However, among all the β-expansins characterised in this project, *HvEXPB5* had the highest transcript abundance in the peduncle and stem (Figure 3-7, Panel A). The Affymetrix data showed the highest intensity of *HvEXPB5* mRNA was in the anthers, followed by the crown, which was not measured in the Q-PCR tissue transcript studies (Figure 3-7, Panel E). There was also a three-fold increase of *HvEXPB5* detected in the radicle and germinating embryo of Morex compared with the same tissues from Golden Promise.

3.3.2.6 HvEXPB7

The leaf base and root tip were the tissues in which *HvEXPB7* mRNA was highly represented (Figure 3-8, Panel A) followed by the root base and spike. Despite the highest amount of *HvEXPB7* in the leaf base, there was no *HvEXPB7* mRNA detected in the mature leaf. For all other tissues analysed, *HvEXPB7* mRNA was found at low amounts.

3.3.2.7 HvEXPB8

The data showed that *HvEXPB8* mRNA was more abundant in leaf base than in other tissues, followed by the root tip and root base (Figure 3-8, Panel B). The mRNA level for *HvEXPB8* was higher than *HvEXPB7* in most of the tissues analysed but displayed a similar transcript pattern as shown in Figure 3-8, Panel C. The Affymetrix data for *HvEXPB8* confirms high transcript intensity for root, followed by leaf, in contrast to the Q-PCR results (Appendix C, Figure C-2, Panel C). The highest amount of *HvEXPB8* mRNA was observed in the radicle, a tissue not analysed by Q-PCR.

3.3.2.8 HvEXPB9

The two tissues in which *HvEXPB9* mRNA was found most abundantly were the anthers at pre-anthesis and root where *HvEXPB9* was found most highly in the root tip followed by the root base. For the remaining tissues analysed, *HvEXPB9* transcript levels were low. The Affymetrix data reflected overall low levels of *HvEXPB9* mRNA in most tissues. The two tissues on the Affymetrix chip for which *HvEXPB9* displayed a similar pattern to the Q-PCR results, and showed that *HvEXPB9* mRNA was highly represented were in root and anthers (Figure 3-9, Panel B), and in the radicle (Morex, Appendix C, Figure C-2, Panel D), a tissue not covered in the Q-PCR data set.

3.3.2.9 HvEXPB10

HvEXPB10 mRNA was found in all tissues measured in the barley developmental tissue series. The data showed that of all the β-expansins profiled, *HvEXPB10* transcripts were the most abundant in six of the sixteen tissues measured, including embryo 22 dap and mature leaf, which overall had very low or no expression of other β-expansins (Figure 3-9, Panel C). The highest amounts of transcript were found in the more metabolically active tissues such as the root tip, followed closely by the leaf base. The other two tissues with the highest amount of *HvEXPB10* mRNA were the root base and leaf tip. *HvEXPB10* mRNA was also highly represented in anther at pre-anthesis and whole grain 3-5 dap. The Affymetrix data for *HvEXPB10* transcripts exhibited similar results (Appendix C, Figure C-2, Panel E).

3.3.2.10 HvEXPB11

The transcript levels of *HvEXPB11* were not high with a peak in anthers at pre-anthesis (Figure 3-7, Panel B). Consistent with the Q-PCR results, the transcript levels for *HvEXPB11* were highest in anthers and were low in the other tissues represented on the Affymetrix chip (Figure 3-7, Panel F).

3.3.2.11 HvEXPB12

The spike and pistil at pre-anthesis were the tissues in which *HvEXPB12* mRNA was highly represented. *HvEXPB12* showed the second highest abundance of the β-expansins in the coleoptile (Figure 3-4, Panel B).

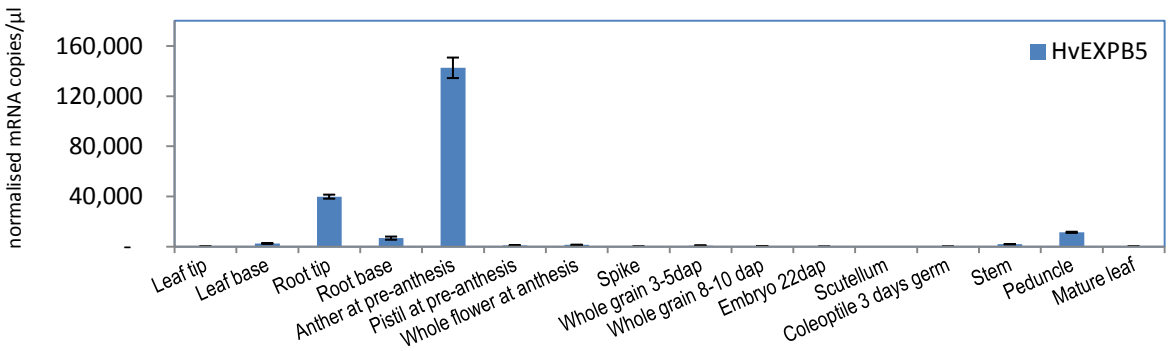
3.3.2.12 HvEXPB13

HvEXPB13 mRNA was detected in the least number of tissues of the barley developmental tissue series and is the only β-expansin not expressed in the leaf tip, leaf base and root base. The tissue with the highest abundance of *HvEXPB13* mRNA was the whole grain 8-10 dap, followed by whole grain 3-5 dap. For the remaining tissues, the transcript levels of *HvEXPB13* were very low (Figure 3-10, Panel A). The Affymetrix data mirrored the same results in the whole grain at 10 and 16 dap (Appendix C, Figure C-3, Panel A). *HvEXPB13* transcript levels peaked in three other tissues measured on the chip that were not covered in the developmental tissue series as shown in Appendix C, Figure C-3, Panel A.

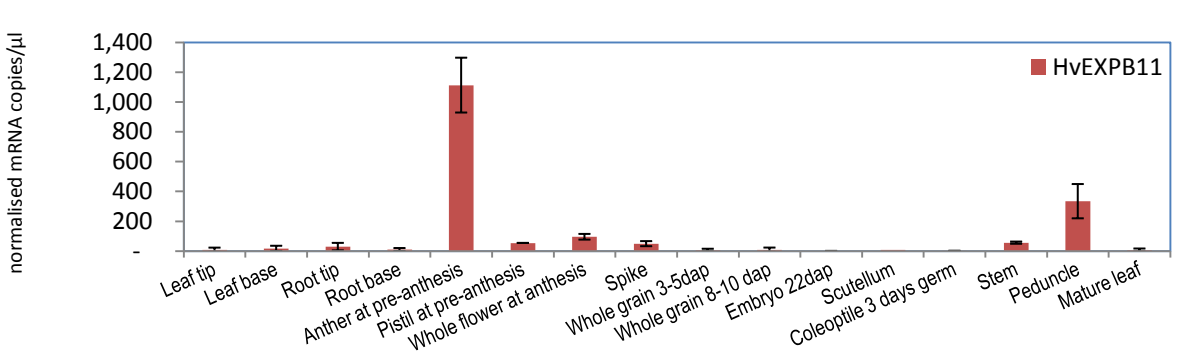
3.3.2.13 HvEXPB14

HvEXPB14 transcript was found in all tissues of the barley developmental tissue series. The data showed that *HvEXPB14* mRNA was most abundant in the leaf base (Figure 3-4, Panel C). The tissues that showed the next highest abundance of *HvEXPB14* were whole flower at anthesis and spike. There was a decrease of transcript levels of *HvEXPB14* in the whole grain 3-5 dap in

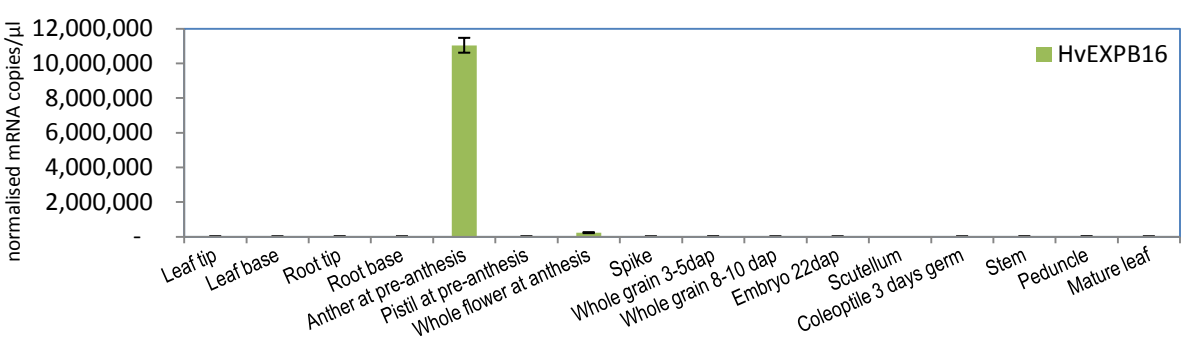
A


normalised mRNA copies/µl
160,000
120,000
80,000
40,000
-
HvEXPB5
Leaf tip
Leaf base
Root tip
Root base
Anther at pre-anthesis
Pistil at pre-anthesis
Whole flower at anthesis
Spike
Whole grain 3-5dap
Whole grain 8-10 dap
Embryo 22dap
Scutellum
Coleoptile 3 days germ
Stem
Peduncle
Mature leaf

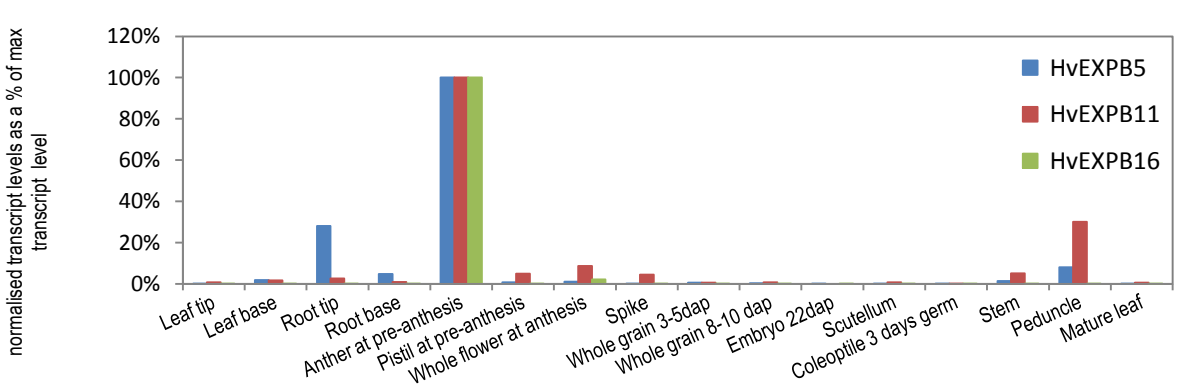
B


normalised mRNA copies/µl
1,400
1,200
1,000
800
600
400
200
-
HvEXPB11
Leaf tip
Leaf base
Root tip
Root base
Anther at pre-anthesis
Pistil at pre-anthesis
Whole flower at anthesis
Spike
Whole grain 3-5dap
Whole grain 8-10 dap
Embryo 22dap
Scutellum
Coleoptile 3 days germ
Stem
Peduncle
Mature leaf

C


normalised mRNA copies/µl
12,000,000
10,000,000
8,000,000
6,000,000
4,000,000
2,000,000
-
HvEXPB16
Leaf tip
Leaf base
Root tip
Root base
Anther at pre-anthesis
Pistil at pre-anthesis
Whole flower at anthesis
Spike
Whole grain 3-5dap
Whole grain 8-10 dap
Embryo 22dap
Scutellum
Coleoptile 3 days germ
Stem
Peduncle
Mature leaf

D


normalised transcript levels as a % of max transcript level
120%
100%
80%
60%
40%
20%
0%
HvEXPB5
HvEXPB11
HvEXPB16
Leaf tip
Leaf base
Root tip
Root base
Anther at pre-anthesis
Pistil at pre-anthesis
Whole flower at anthesis
Spike
Whole grain 3-5dap
Whole grain 8-10 dap
Embryo 22dap
Scutellum
Coleoptile 3 days germ
Stem
Peduncle
Mature leaf

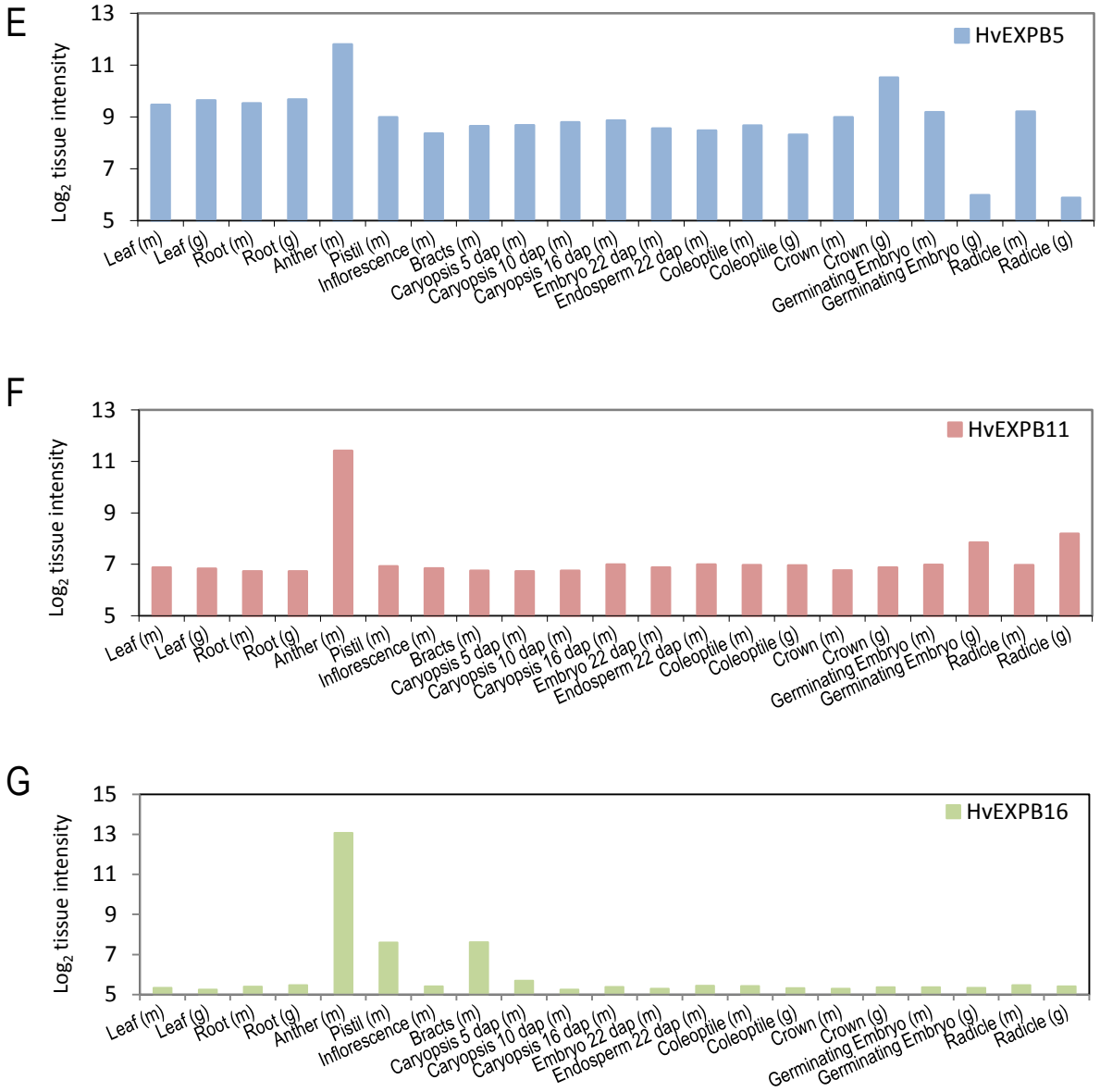


Figure 3-7 Tissue transcript profiles of *HvEXPB5*, *HvEXPB11* and *HvEXPB16* in a range of developing tissues by Q-PCR and microarray.

A, **B** and **C**, Q-PCR plots of *HvEXPB5, HvEXPB11* and *HvEXPB16* which are most abundant in anthers at pre-anthesis. Levels of mRNA are presented as the number of copies per microlitre of cDNA after normalisation. Error bars = standard deviations for each mRNA. **D**, Normalised transcript levels of three highly correlated barley *HvEXPB* genes in a range of tissues, expressed as a ratio of the maximal levels observed for the specific gene. Values of 100% indicate the tissue in which a particular gene is transcribed at its maximal level. E, F and G, Affymetrix data of *HvEXPB5, HvEXPB11* and *HvEXPB16* which show highest tissue intensity in anther. Affymetrix data tissue intensity ratios are presented as log base 2, RMA normalised. A tissue intensity value < 5 is considered background. The tissues indicated on the *x*-axis correspond to samples taken from the cultivar 'Morex' (m) or 'Golden Promise' (g).

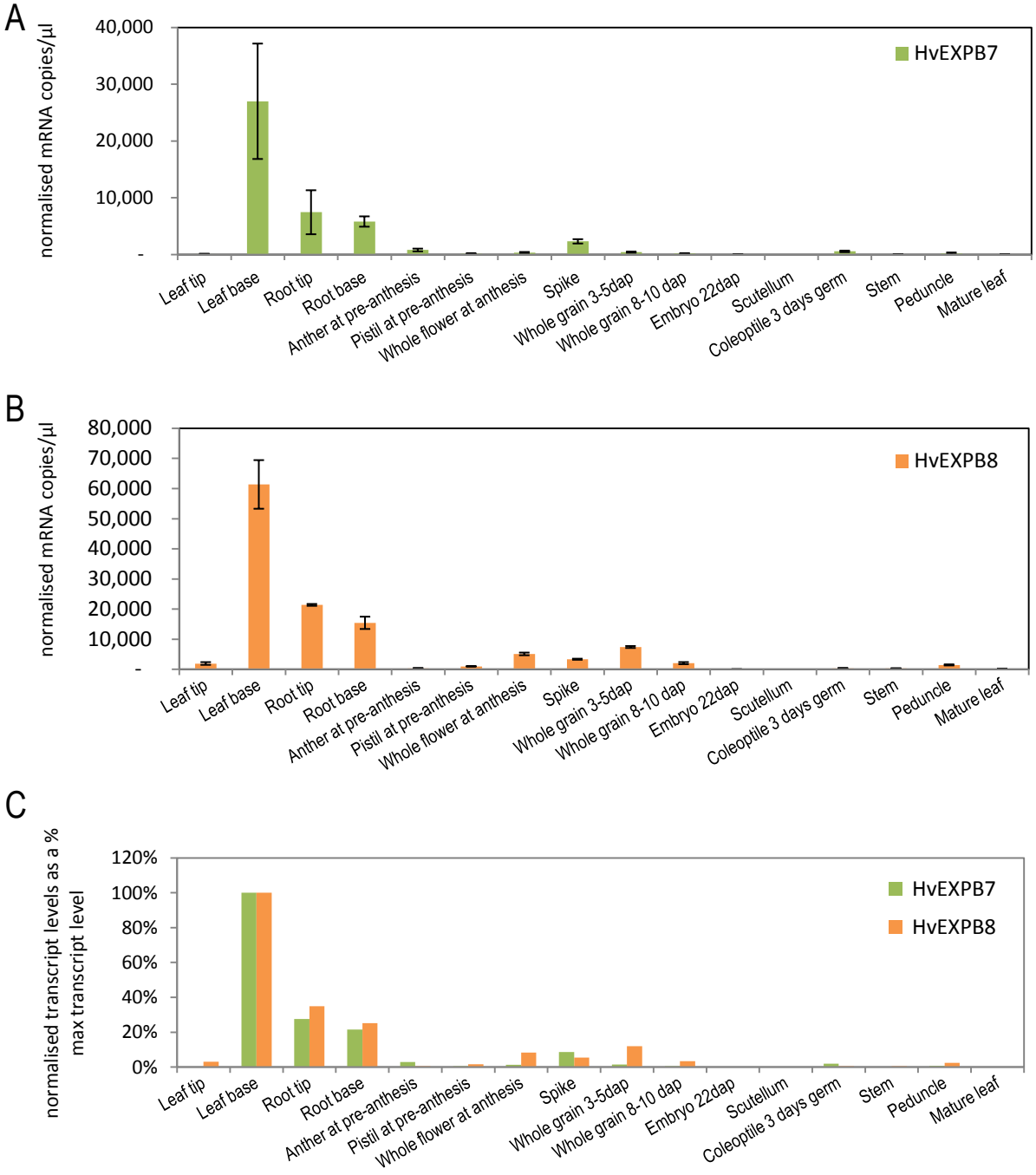


Figure 3-8 Tissue transcript profiles of *HvEXPB7* and *HvEXPB8* in a range of developing tissues by Q-PCR and microarray.

Levels of mRNA are presented as the number of copies per microlitre of cDNA after normalisation. Error bars = standard deviations for each mRNA. **A**, the transcript pattern for *HvEXPB7*, which is most abundant in leaf base. **B**, the transcript profile of *HvEXPB8*, which is highly abundant in leaf base. **C**, Normalised transcript levels of two highly correlated barley *HvEXPB* genes in a range of tissues, expressed as a ratio of the maximal levels observed for the specific gene. Values of 100% indicate the tissue in which a particular gene is transcribed at its maximal level.

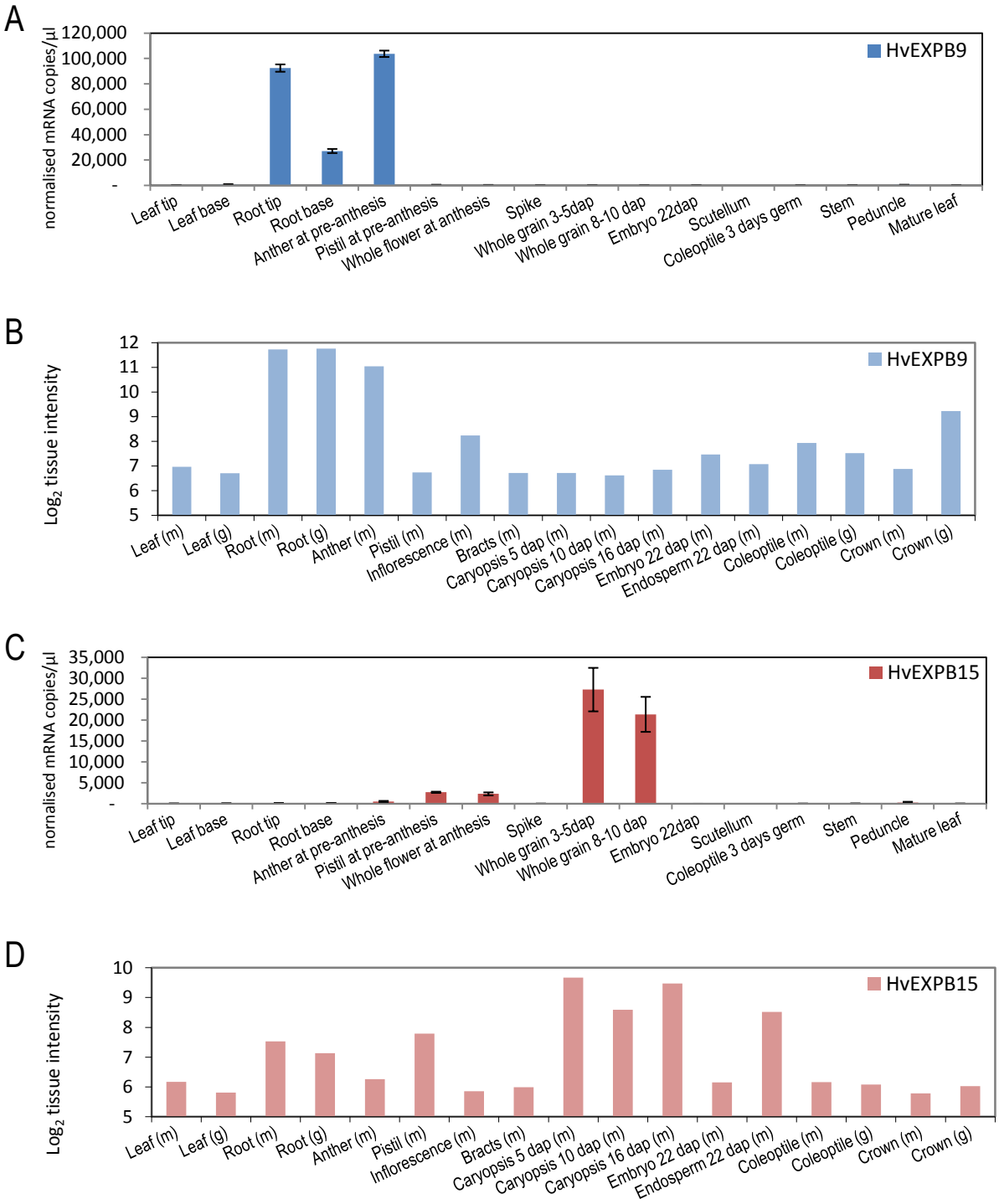


Figure 3-9 Tissue transcript profiles of *HvEXPB9* and *HvEXPB15* in a range of developing tissues by Q-PCR and microarray.

A and **C**, Q-PCR plots of *HvEXPB9* and *HvEXPB15*. Levels of mRNA are presented as the number of copies per microlitre of cDNA after normalisation. Error bars on all Q-PCR plots = standard deviations for each mRNA. **B** and **D**, Affymetrix data of *HvEXPB9* and *HvEXPB15* which reflect similar transcript profiles to the Q-PCR plots. Affymetrix data tissue intensity ratios are presented as log base 2, RMA normalised. A tissue intensity value < 5 is considered background. The tissues indicated on the *x*-axis correspond to samples taken from the cultivar 'Morex' (m) or 'Golden Promise' (g).

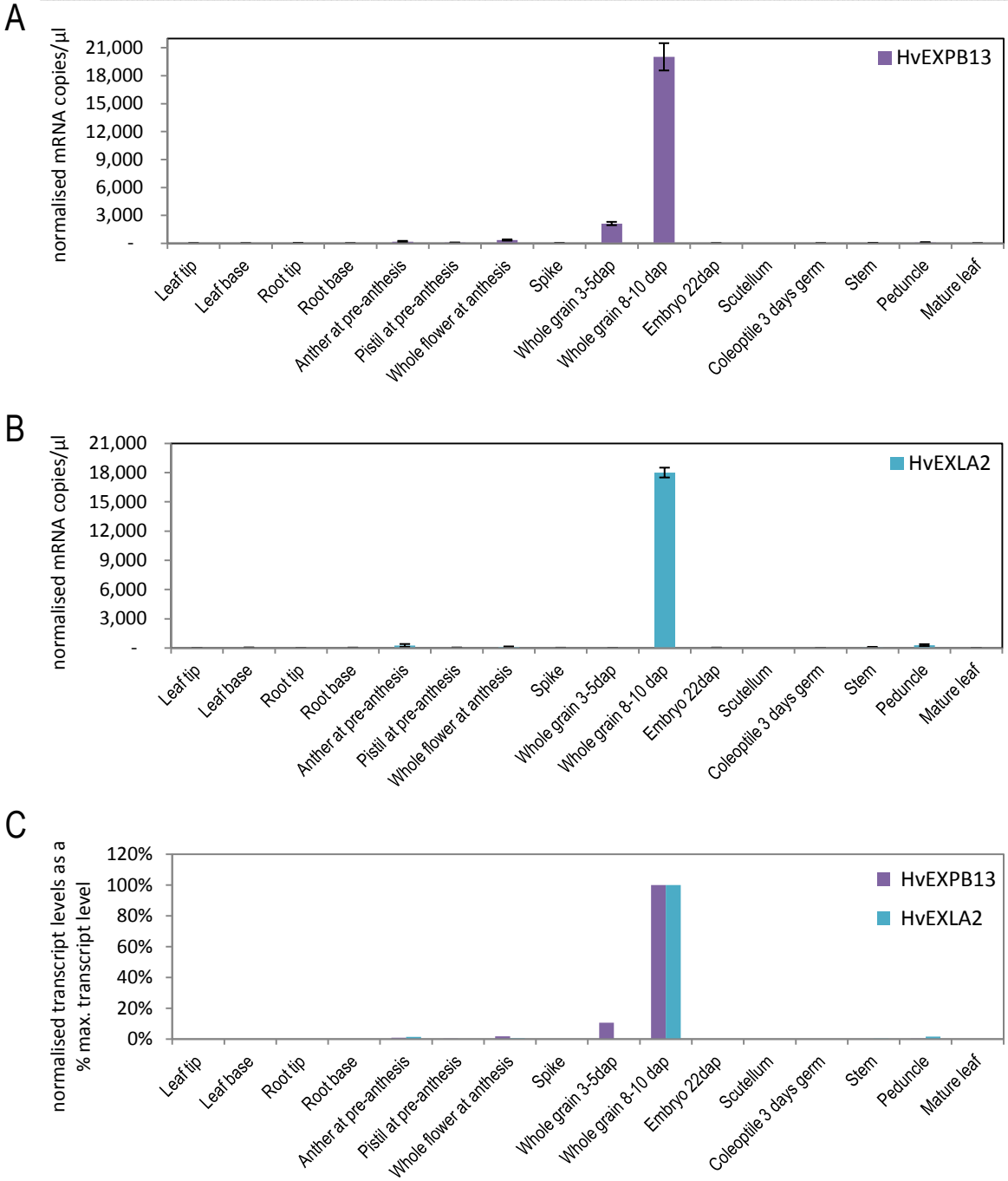


Figure 3-10 Tissue transcript profiles of *HvEXPB13* and *HvEXLA2* in a range of developing tissues.

Levels of mRNA are presented as the number of copies per microlitre of cDNA after normalisation. Error bars = standard deviations for each mRNA. **A**, the transcript pattern for *HvEXPB13*, which is most abundant in whole grain 8-10 dap. **B**, the transcript profile of *HvEXLA3*, which is highly abundant in whole grain 8-10 dap. **C**, Normalised transcript levels of two highly correlated barley *HvEXPB and HvEXLA* genes in a range of tissues, expressed as a ratio of the maximal levels observed for the specific gene. Values of 100% indicate the tissue in which a particular gene is transcribed at its maximal level.

comparison with the whole flower at anthesis, but then it increased slightly in whole grain at 8-10 dap. The Affymetrix data (Appendix C, Figure C-2, Panel F) verified this increase of *HvEXPB14* mRNA in the whole grain from 3-5 dap (caryopsis 5 dap) until 16 dap.

3.3.2.14 HvEXPB15

The whole grain 3-5 dap followed by whole grain 8-10 dap were the two tissues with the highest abundance of *HvEXPB15* mRNA. The second subset of tissues with some *HvEXPB15* mRNA detected was the pistil pre-anthesis and whole flower at anthesis. The remaining tissues analysed showed very low transcript levels of *HvEXPB15* (Figure 3-9, Panel C). The Affymetrix data confirmed highest levels in the caryopsis at 5 dap with a small decrease in caryopsis 10 dap (Figure 3-5, Panel D). The Affymetrix data included caryopsis 16 dap (not analysed for the Q-PCR experiments) and showed *HvEXPB15* levels increasing from caryopsis 10 dap. High amounts were also observed in the germinating embryo and radicle (Golden Promise; Appendix 3, Figure A3-2, Panel G)

3.3.2.15 HvEXPB16

The transcript levels of *HvEXPB16* peaked in the anthers at pre-anthesis followed by the whole flower at anthesis (Figure 3-7, Panel C). In the remaining tissues of the barley developmental tissue series, the representation of *HvEXPB16* was low. The Affymetrix data confirmed high levels of *HvEXPB16* mRNA in the anthers (Figure 3-7, Panel G) and overall low amounts in all other tissues analysed. The transcript pattern of *HvEXPB16* was similar to *HvEXPB5* and *HvEXPB11* as shown in Figure 3-7, Panel D.

3.3.2.16 HvEXPB18

The root tip and the root base were tissues in which *HvEXPB18* mRNA was highly represented (Figure 3-4, Panel D). Besides these two tissues, *HvEXPB18* was found at low levels elsewhere in the plant, with no transcript in the mature leaf. The transcript pattern of *HvEXPB18* was similar to *HvEXPA4*, *HvEXPA7*, *HvEXPA10* and *HvEXPA11* with high abundance in the root tip, decreasing in the root base and low abundance measured in the other tissues.

3.3.3 Transcript analysis of the barley expansin-like A via Q-PCR and Affymetrix data

The abundance of individual barley expansin-like A transcripts was measured in a range of barley tissues via Q-PCR. The data from the Q-PCR experiments and expansin-like A transcript profiles from the Affymetrix data set are presented for each gene in the following sections. A search of the Affymetrix data revealed that *HvEXLA1* was not represented on the chip.

3.3.3.1 HvEXLA1

The root tip, the metabolically active zone of the root, was the tissue in which *HvEXLA1* mRNA was highly represented, followed by the leaf tip, which is the least metabolically active zone of the leaf (Figure 3-11, Panel A). There was some *HvEXLA1* mRNA detected in the leaf base and mature leaf. The transcript level for *HvEXLA1* in the remaining tissues analysed was relatively low overall.

3.3.3.2 HvEXLA2

The overall transcript levels for *HvEXLA2* was low, and in root tip, leaf tip and mature leaf there was no *HvEXLA2* mRNA detected. The data showed that *HvEXLA2* was only abundant in whole grain 8-10 dap and shared a very similar transcript pattern with *HvEXPB13*. Affymetrix data (Appendix 3, Figure A3-3) confirmed that the highest intensities of *HvEXLA2* were found in the caryopsis 10 dap, decreasing slightly in caryopsis 16 dap (not measured on the Q-PCR developmental tissue series). The Affymetrix data also revealed high levels of *HvEXLA2* mRNA in the mesocotyl and radicle (both Golden Promise).

3.3.3.3 HvEXLA3

HvEXLA3 mRNA was detected in a similar pattern to the transcription of *HvEXPA1* in the barley developmental tissue series. Overall the transcript levels were low and were detected in the leaf tip and anther at pre-anthesis (Figure 3-11, Panel B). In contrast to the Q-PCR transcript studies, the Affymetrix data (Figure 3-11, Panel C) showed that *HvEXLA3* mRNA was found in a range of tissues, including the anthers, leaf and root. *HvEXLA3* displayed an increase in transcript intensity in the developing caryopsis between 5 dap and 16 dap.

A summary of the barley expansin transcript data can be viewed in a heat map (Figure 3.12)

3.3.4 Transcriptional correlations based on Q-PCR data of cell wall-related genes

The normalised transcript levels of the barley expansins across the barley developmental tissue series were expressed as a percentage of maximum transcript levels of each gene and assessed against other available cell wall gene transcript profiles. These analyses were performed in two ways. Firstly, transcriptional correlations were measured between the expansin genes themselves. Secondly, correlations were sought between individual expansin genes or groups of correlated expansin genes and other cell-wall related genes. The results are summarised in the brief sections below and discussed in detail in the discussion section.

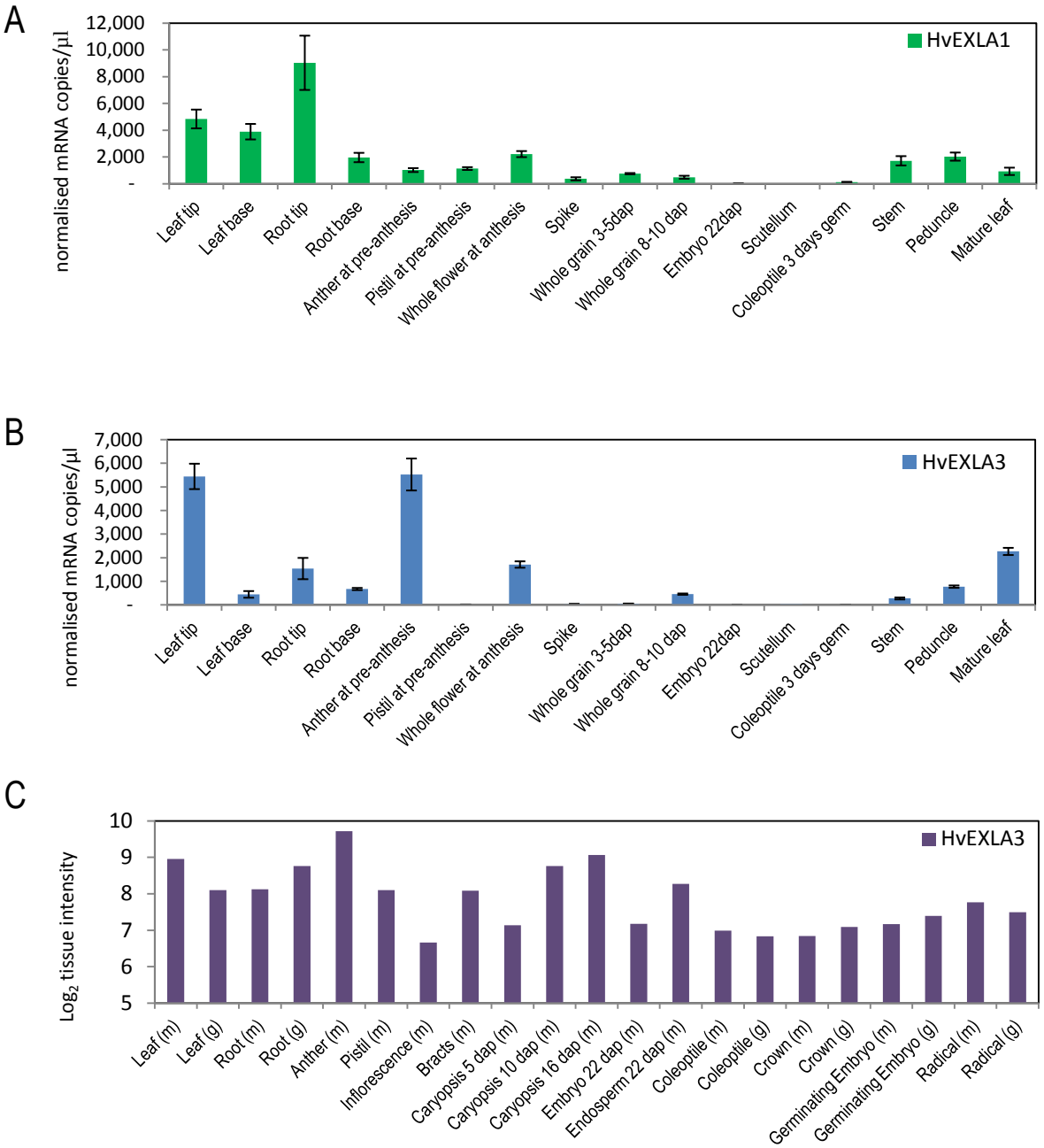


Figure 3-11 Transcription profiles of *HvEXLA1* and *HvEXLA3* in a range of developing tissues by Q-PCR and microarray.

A and **B,** Q-PCR plots of *HvEXLA1* and *HvEXLA3.* Levels of mRNA are presented as the number of copies per microlitre of cDNA after normalisation. Error bars on all Q-PCR plots = standard deviations for each mRNA. **C**, Affymetrix data of *HvEXLA3*. Affymetrix data tissue intensity ratios are presented as log base 2, RMA normalised. A tissue intensity value < 5 is considered background. The tissues indicated on the *x*-axis correspond to samples taken from the cultivar 'Morex' (m) or 'Golden Promise' (g).

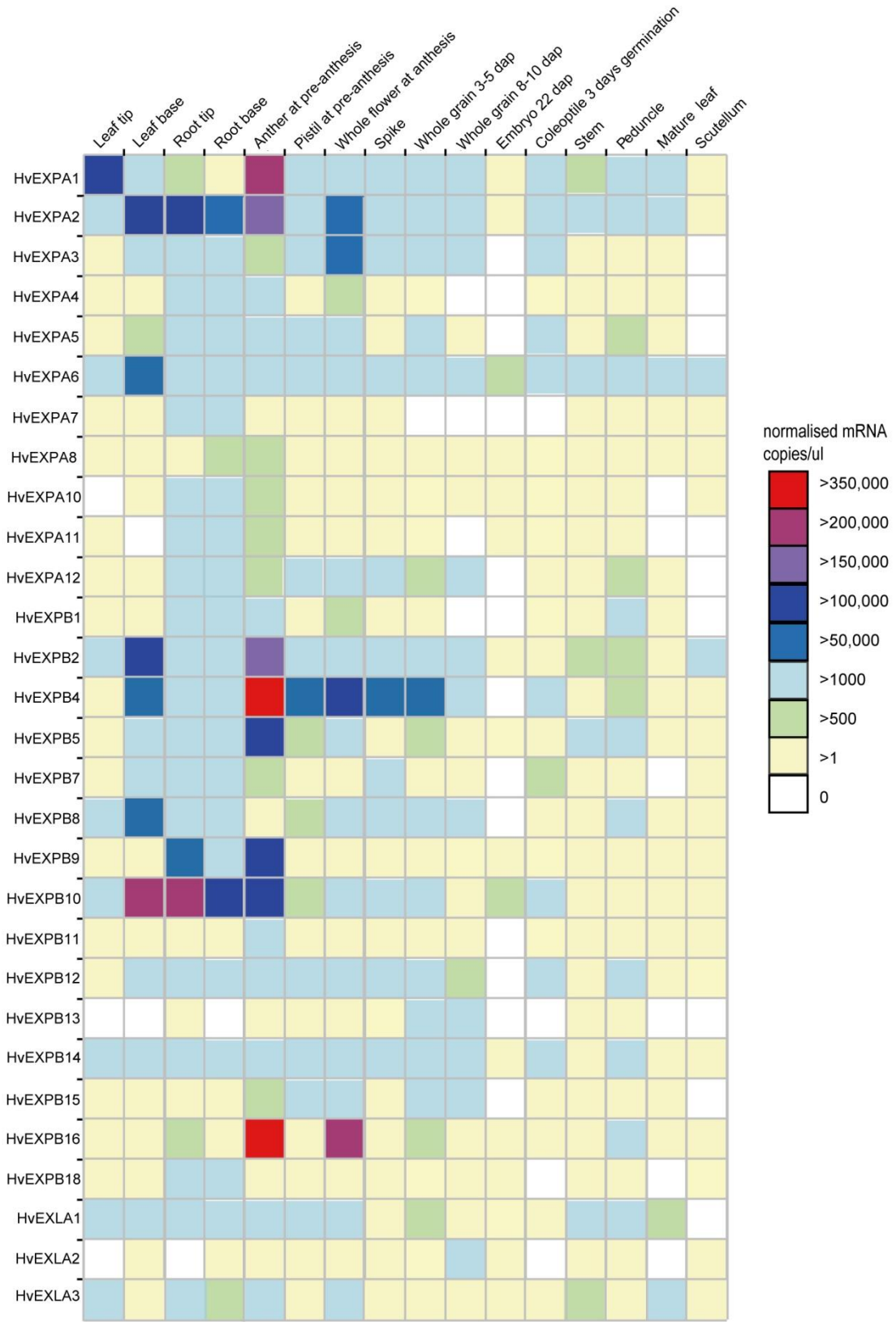


Figure 3-12 Heat map of barley expansin mRNA abundance in a developmental tissue series.

A colour gradient represents the mRNA transcript abundance of the barley expansin genes in the barley developmental tissue series.

Expansin transcript profiles with a correlation of >0.90 to other expansins or to cell wall-related genes are presented in this section.

3.3.4.1 Correlations between expansin genes

The correlation studies showed four groups of co-transcribed barley expansin genes. Figure 3-2, Panel G shows that *HvEXPA4, HvEXPA7, HvEXPA10, HvEXPA11* had similar transcript profiles. Further analyses showed that *HvEXPB18* transcript profile was highly correlated with this group (Figure 3-13, Panel A). The two co-transcribed β-expansin genes, *HvEXPB7* and *HvEXPB8* (Figure 3-8, Panel C) had a similar transcript pattern to *HvEXPA6* (Figure 3-13, Panel B). Three β-expansin genes, *HvEXPB5, HvEXPB11* and *HvEXPB16* had similar transcript patterns (Figure 3-7, Panel D). *HvEXPB13* had a similar transcript pattern as *HvEXLA2* (Figure 3-10, Panel C). A summary of these four groups can be viewed in Figure 3-14.

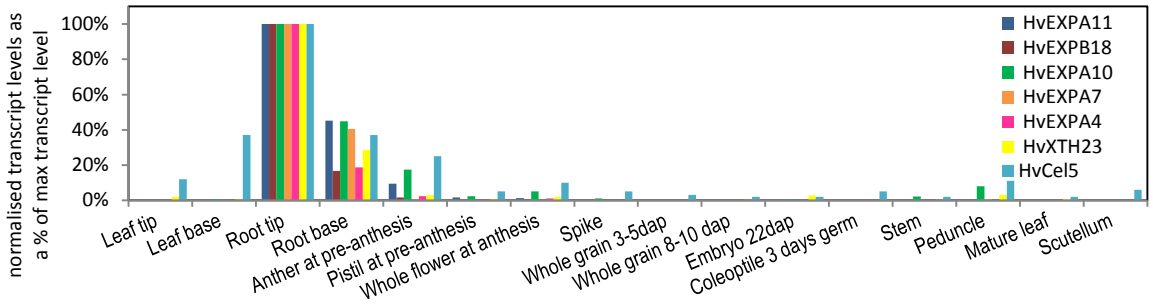
3.3.4.2 Correlations between expansins and other cell wall-related genes

Seven groups with similar transcript patterns were identified between barley expansins and other cell wall related genes (Figure 3-15). Analyses showed the highly correlated group of α-expansins *HvEXPA4, HvEXPA7, HvEXPA10, HvEXPA11* and one β-expansin *HvEXPB18* shared a similar transcript pattern with a xyloglucan endotransglycosylase (*HvXTH23*) and a cellulase (*HvCel5*), (Figure 3-13, Panel A) with the highest transcript levels observed in the root tip.

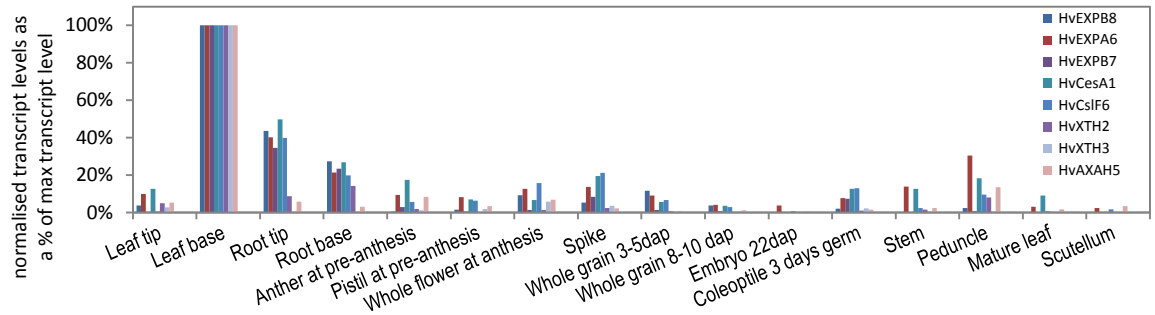
Correlation matrices revealed five genes with similar transcript patterns to three co-transcribed expansin genes. Figure 3-13, Panel B shows that a barley cellulose synthase (*CesA1*), cellulose synthase-like (*CslF6*), two *XTHs* (*HvXTH3; HvXTH5*) and an arabinoxylan arabinofuranohydrolase (*HvAXAH5*) had similar transcript patterns to *HvEXPA6*, *HvEXPB7* and *HvEXPB8*.

HvXTH5 had a similar transcript pattern to *HvEXPA5* as shown in Figure 3-13, Panel C. The root-specific *HvEXPB1* transcript pattern was highly correlated with *HvXTH24* (Figure 3-13, Panel D). A barley cellulose synthase-like D (*HvCslD4*) was shown to have a similar transcript pattern to *HvEXPB9* (Figure 3-13, Panel E). A cellulase (*HvCel10*) shared a similar tissue transcript pattern with *HvEXPB10* (Figure 3-13, Panel F). *HvEXPB15* had a similar transcript profile to *HvAXAH3* (Figure 3-13, Panel G).

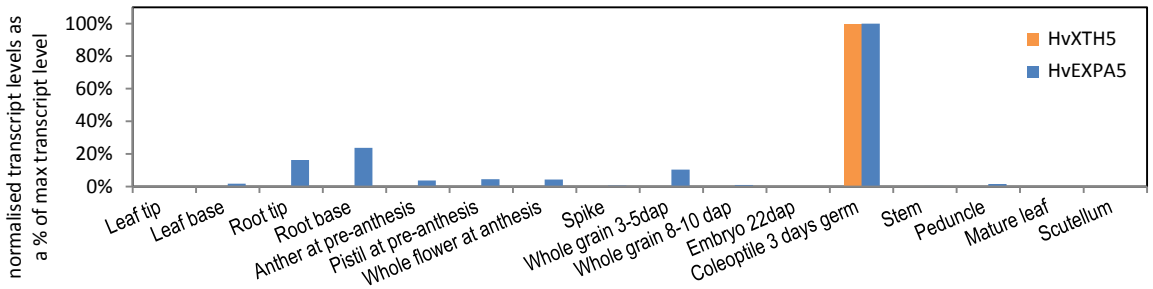
A


normalised transcript levels as a % of max transcript level
100%
80%
60%
40%
20%
0%
HvEXPA11
HvEXPB18
HvEXPA10
HvEXPA7
HvEXPA4
HvXTH23
HvCel5
Leaf tip
Leaf base
Root tip
Root base
Anther at pre-anthesis
Pistil at pre-anthesis
Whole flower at anthesis
Spike
Whole grain 3-5dap
Whole grain 8-10 dap
Embryo 22dap
Coleoptile 3 days germ
Stem
Peduncle
Mature leaf
Scutellum

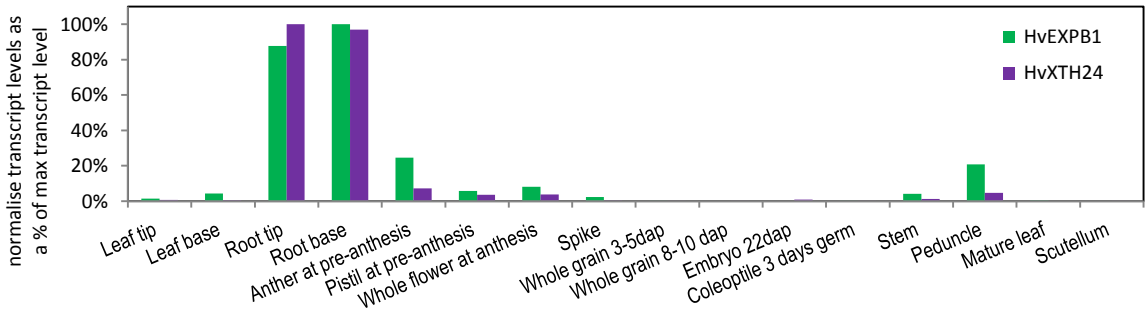
B


normalised transcript levels as a % of max transcript level
100%
80%
60%
40%
20%
0%
HvEXPB8
HvEXPA6
HvEXPB7
HvCesA1
HvCslF6
HvXTH2
HvXTH3
HvAXAH5
Leaf tip
Leaf base
Root tip
Root base
Anther at pre-anthesis
Pistil at pre-anthesis
Whole flower at anthesis
Spike
Whole grain 3-5dap
Whole grain 8-10 dap
Embryo 22dap
Coleoptile 3 days germ
Stem
Peduncle
Mature leaf
Scutellum

C


normalised transcript levels as a % of max transcript level
100%
80%
60%
40%
20%
0%
HvXTH5
HvEXPA5
Leaf tip
Leaf base
Root tip
Root base
Anther at pre-anthesis
Pistil at pre-anthesis
Whole flower at anthesis
Spike
Whole grain 3-5dap
Whole grain 8-10 dap
Embryo 22dap
Coleoptile 3 days germ
Stem
Peduncle
Mature leaf
Scutellum

D


normalise transcript levels as a % of max transcript level
100%
80%
60%
40%
20%
0%
HvEXPB1
HvXTH24
Leaf tip
Leaf base
Root tip
Root base
Anther at pre-anthesis
Pistil at pre-anthesis
Whole flower at anthesis
Spike
Whole grain 3-5dap
Whole grain 8-10 dap
Embryo 22dap
Coleoptile 3 days germ
Stem
Peduncle
Mature leaf
Scutellum

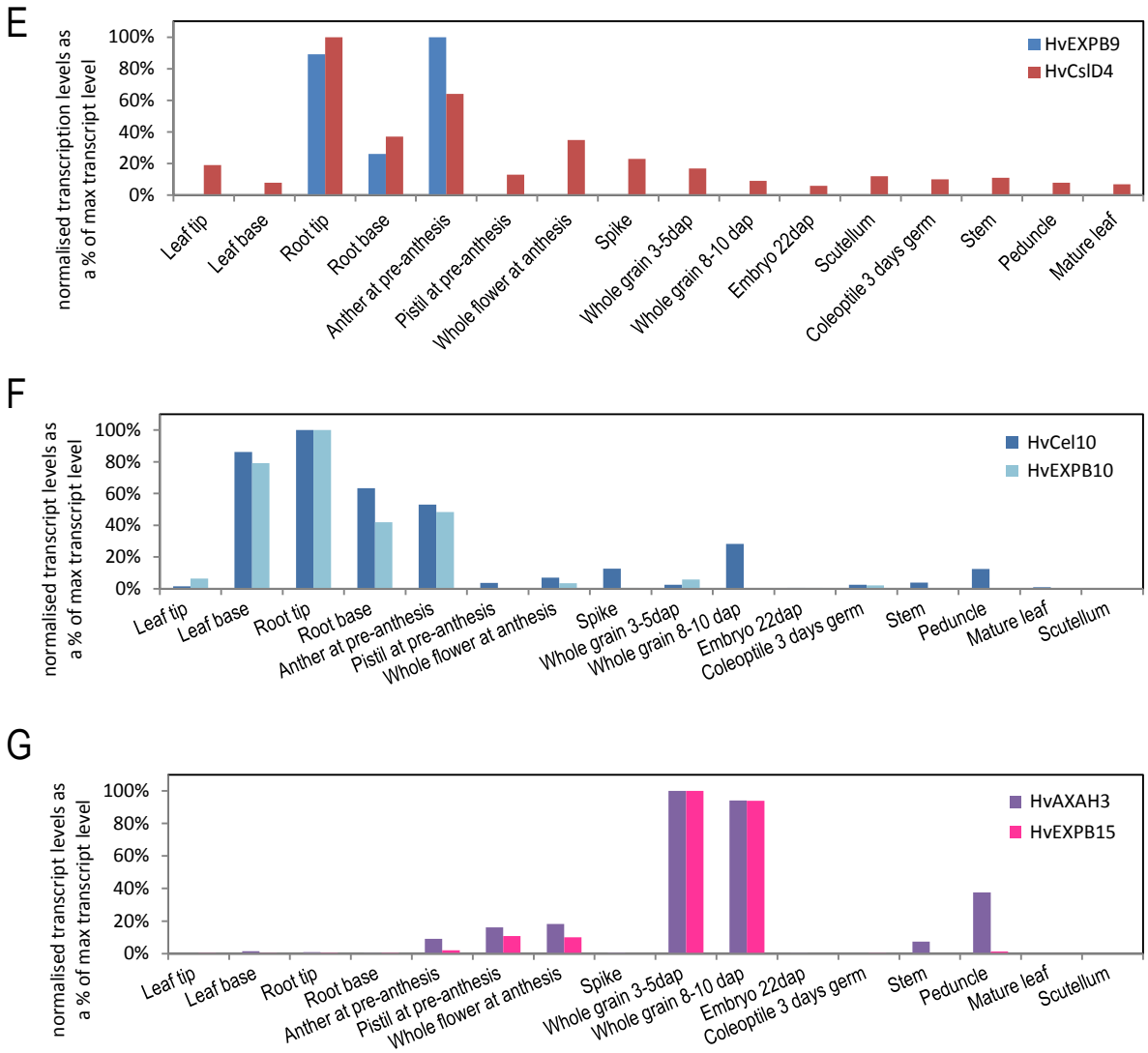


Figure 3-13 Tissue transcript profiles of highly correlated cell wall-related genes in a range of developing tissues.

Normalised transcript levels of highly correlated expansin genes with cell wall-related genes in a range of tissues, expressed as a ratio of the maximal levels observed for the specific gene. Values of 100% indicate the tissue in which a particular gene is transcribed at its maximal level. **A**, *HvEXPA4, HvEXPA7, HvEXPA10, HvEXPA11* and *HvEXPB18* with *HvXTH23* and *HvCel5.* **B**, *HvEXPA6, HvEXPB7* and *HvEXPB8* with *HvCesA1, HvCslF6, HvXTH2, HvXTH3* and *HvAXAH5*. **C**, *HvEXPA5* with *HvXTH5*. **D**, *HvEXPB1* with *HvXET24*. **E**, *HvEXPB9* with *HvCslD4.* **F**, *HvEXPB10* with *HvCel10*. **G**, *HvEXPB15* with *HvAXAH3.*

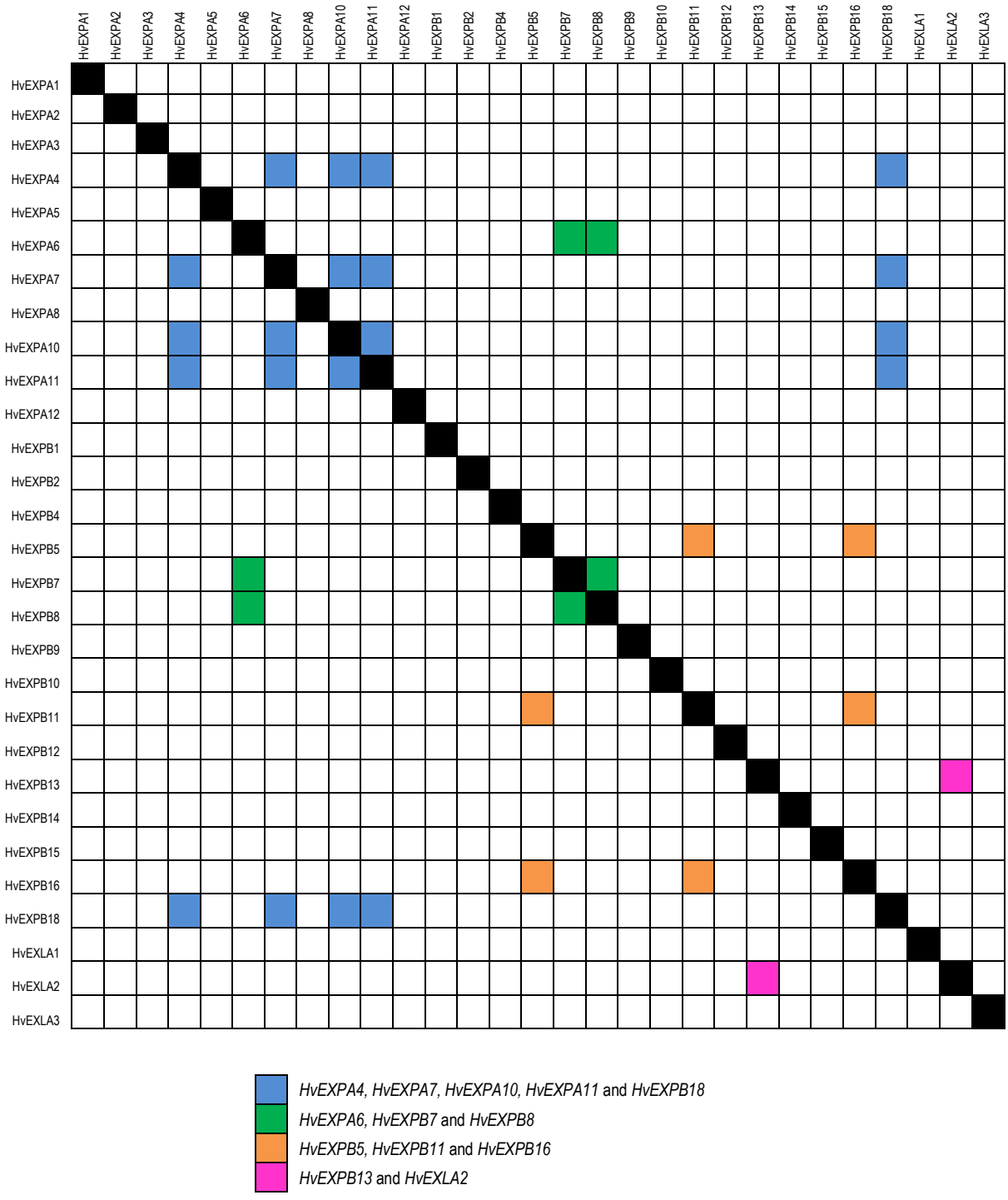


Figure 3-14 Summary of highly correlated transcription profiles between expansin genes.

The normalised transcript levels of barley expansin genes were assessed against each other. Four groups of co-transcribed barley expasins are shown. Each group of transcript profiles with a correlation of > 0.90 are demonstrated with common coloured squares.

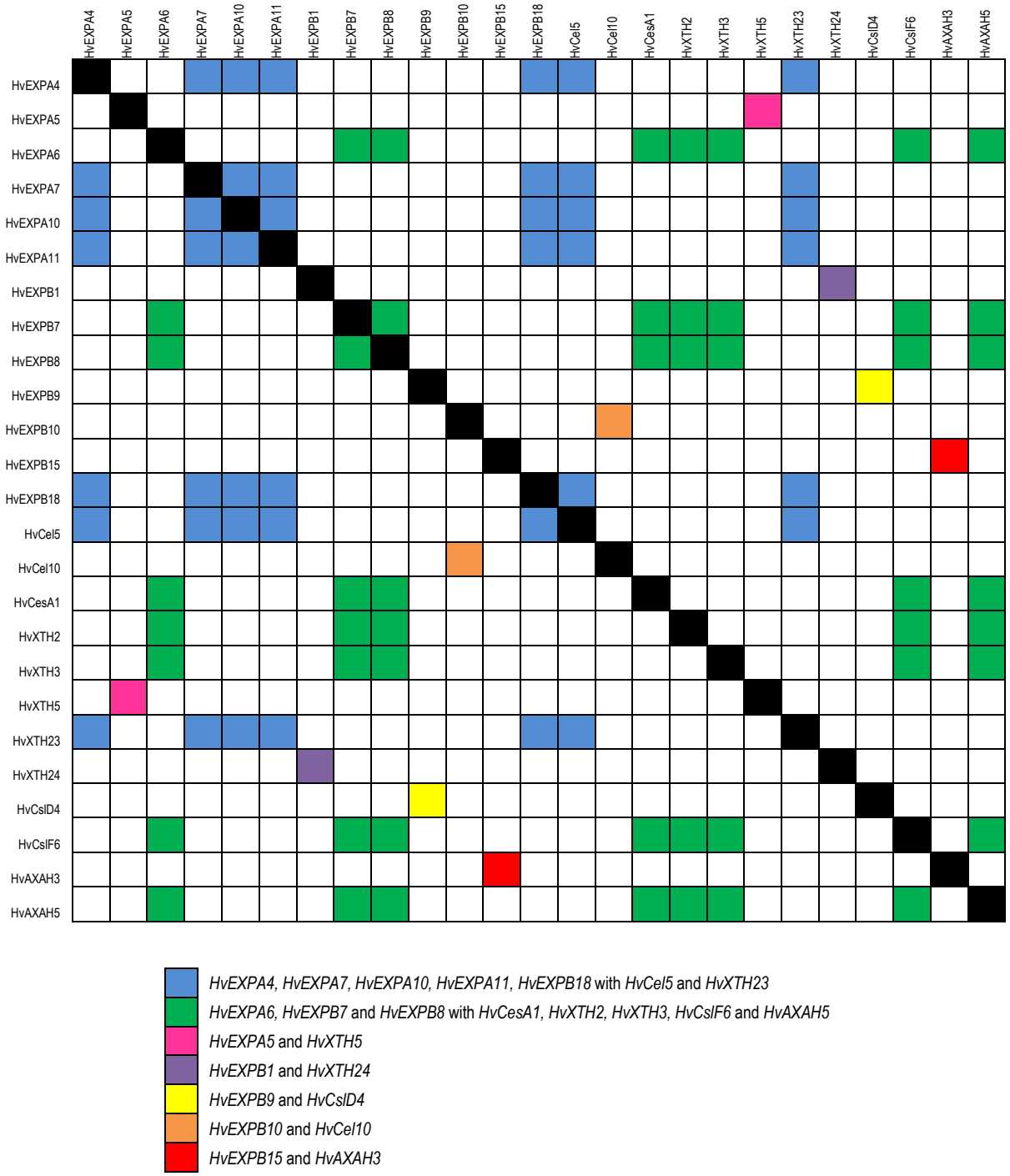


Figure 3-15 Summary of highly correlated expansin transcription profiles with other cell wall-related genes.

The normalised transcript levels of barley expansin genes were assessed against each other and transcript data from available cell wall-related genes. Each group of the seven transcript profiles with a correlation of > 0.90 are demonstrated with common coloured squares.

3.4 DISCUSSION

The results of the Q-PCR used to assess mRNA transcription levels of the barley expansin genes in a range of barley developmental tissues were presented side by side with Affymetrix data to allow a comparison of the transcript patterns obtained by the two techniques. The transcript patterns of the expansins were also assessed against available transcription data of cell wall-related genes and highly correlated co-transcribed gene sets.

A summary of these analyses is presented in the following sections. Firstly, the results of transcript levels for individual expansin genes in different tissues are presented. Secondly, the correlations between transcript levels of different members of the expansin gene families and the possible co-transcription of expansin genes are discussed. Thirdly, the correlations of expansin transcripts with those of other cell-wall related genes are explored and finally, the possible roles for some of the barley expansin genes as suggested by these Q-PCR results when linked to previous studies and existing knowledge of cell-wall related genes are discussed.

Microarray technology is a useful screening tool for examining expansin gene expression. The search of the 22K Barley1 GeneChip probe array revealed data for 23 barley expansin genes characterised in this project. Analysis of the transcript profiles downloaded from the 'Transcript patterns during barley development' Affymetrix data provided a general transcript profile of barley expansin genes across 15 different tissues and developmental stages from the cultivar Morex and five tissues from the cultivar Golden Promise. The Affymetrix data provided a foundation for further analysis of mRNA transcription levels by Q-PCR, which is often a more accurate and sensitive method of detection.

Among the barley expansins characterised, it was apparent that there was a major group within both of the α- and β-expansin families whose mRNA transcript levels were found to be the highest in the majority of tissues measured. The overall abundance of *HvEXPA1, HvEXPA2* and *HvEXPA3* transcripts was high in most tissues tested (Figure 3-1). The transcript levels of these three genes dominated in 12 of the 16 tissues measured as summarised in Figure 3-12. However, their distribution among the organ types did not clearly define their specific roles. In addition, *HvEXPA6* transcripts were consistently found in all tissues tested, including the scutellum and embryo 22 dap (Figure 3-3, Panel B). These two tissues contained little to no transcript of any other barley expansins, as shown in Figure 3-12.

The major members of the β-expansin subfamily whose mRNA transcript levels were found to be the highest in the majority of tissues included *HvEXPB2, HvEXPB4, HvEXPB5* and *HvEXPB10*

(Figures 3-5 and 3-7, Panel A). Collectively, these four were the most highly represented β-expansin transcripts in 14 of the 16 tissues analysed, with the exception being the anthers at pre-anthesis and the whole flower at anthesis (Figure 3-12). Despite *HvEXPB2, HvEXPB4* and *HvEXPB5* displaying the highest relative transcript abundance in vegetative tissues, their absolute highest transcript levels were found in the anthers at pre-anthesis (Figure 3-12). The β-expansin with the highest transcript abundance in the anthers at pre-anthesis and whole flower at anthesis was *HvEXPB16* (Figure 3-7, Panel C and Figure 3-12).

A second group of barley β-expansin genes characterised in this study was not highly transcribed and formed a unique group. As previously noted, a distinguishing feature of the β-expansins is the presence of one or two predicted N-linked glycosylation sites with the consensus sequence Asn-X-Ser/Thr. The significance of the N-linked glycosylation motif is unknown but glycosylation may be important for their function (Downes *et al.*, 2001). However, *HvEXPB1*, *HvEXPB9*, *HvEXPB12*, *HvEXPB15* and *HvEXPB18* all lacked the N-linked glycosylation site at the NH_2-terminal region of the protein. Of these, *HvEXPB1* has been associated with the initiation of root hair growth (Kwasniewski and Szarejko, 2006) so it is possible the other members also have specialised roles. An exception to this group was *HvEXPB14,* which has two N-linked glycosylation sites in the NH_2-terminus region. *HvEXPB14* mRNA was detected in all tissues of the barley developmental tissue series (Figure 3-12).

The transcript analyses of the barley expansin-like A genes did not provide any further insight into the possible role of this subgroup, as they each presented a distinct transcript profile. *HvEXLA1* was consistently found in most of the tissues in the barley developmental series (Figure 3-11, Panel A). *HvEXLA2* transcript data showed that the mRNA abundance of this gene was restricted to the developing grain at 8-10 dap (Figure 3-10, Panel B) and the transcript data for *HvEXLA3* showed that it was found in all tissues tested in various amounts (Figure 3-11, Panel, B). These distinct transcript profiles are summarised in Figure 3-12.

Based on the Affymetrix data and Q-PCR results, it appeared that four groups, comprising 13 members of the expansin family, are co-transcribed in the same tissues and therefore may overlap functionally. A summary of the correlated barley expansin genes is shown in Figure 3-14.

Further analysis of co-transcribed genes within the expansin family was made with all available cell wall-related genes that were measured on the same barley developmental tissues series were assessed against the expansin Q-PCR data. A correlation matrix was generated to determine gene

sets with similar transcript patterns. A summary of the barley expansin transcript correlations with cell wall-related genes can be viewed in Figure 3-15.

The growth and differentiation of plant cells are related to changes in extensibility of cell walls. The barley developmental tissue series provides an extensive platform to measure the transcript abundance of barley expansins during critical stages of cell differentiation and growth of the plant. It would appear that a gene family of a certain size is sufficient for fulfilling multiple roles *in planta*, but beyond a certain number of genes, function perhaps becomes redundant. The possible roles for some of the barley expansin genes as suggested by Q-PCR results are discussed below and related to previous studies and existing knowledge of cell-wall related genes.

The transcript profiles for four of the most highly represented members of the barley α-expansins (*HvEXPA1, HvEXPA2, HvEXPA3* and *HvEXPA6*; Figure 3-12) suggested roles in both elongation and cellular differentiation. However, tissue in which *HvEXPA1* mRNA transcription featured predominantly, when compared to all the barley expansins, is in the mature leaf. The presence of expansins in regions of the plant where elongation has ceased was consistent with reports in *Festuca pratensis* (Reidy *et al.*, 2001) and tomato (Caderas *et al.*, 2000), where a correlation between α-expansin gene transcription and tissue growth could not be demonstrated. It appears likely that the occurrence of expansin transcripts in slowly growing or non-elongating tissues reflects expansin functions related to cellular differentiation.

Both *HvEXPA2* and *HvEXPA3* showed high levels of mRNA transcripts in anthers at pre-anthesis and whole flower at anthesis respectively. This finding suggests a role in the elongation of anther filaments prior to pollination. Liu *et al.* (2007) identified two wheat α-expansins that were greatly and specifically induced in the rapidly elongating anther filament.

The transcript abundance of *HvEXPA6* as detected in the scutellum of germinated grain suggested it might act transiently to relax the cell wall architecture to assist with the release of the hydrolytic enzymes into the endosperm. Since *HvEXPA6* was consistently found in all tissues of germinated grain tested, this result suggests a unique role for *HvEXPA6* in grain germination.

The transcript profiles for three of the most highly represented members of the barley β-expansins (*HvEXPB2, HvEXPB4* and *HvEXPB10*; Figure 3-12) suggested a role in cell elongation. The relative transcript abundance of *HvEXPB5* was the highest of the β-expansins in the peduncle and stem, which are more mature tissues where cell elongation has mostly ceased. However, the absolute transcript abundance of *HvEXPB5* was highest in the anther at pre-anthesis. The

assessment of the transcript profile of *HvEXPB5* against the other expansin gene members found it to be similar to two members of the barley β-expansins, *HvEXPB11* and *HvEXPB16,* which showed the highest transcript abundance in the pollen-producing organ and the whole flower at anthesis (Figures 3-7 and 3-12). A similar observation in wheat was found by Jin *et al.* (2006), who characterised two wheat β-expansins that were found to be expressed in the early stages of male gametophyte development, from microspores to immature pollen. A third wheat β-expansin was identified from isolated premeiotic and immature anthers (Jin *et al.*, 2006). This example of co-expression of *HvEXPB5, HvEXPB11* and *HvEXPB16* based on similarity of the transcriptional profiles is one of several observed in this study.

The co-transcription of expansin gene members was not restricted within each subfamily. *HvEXPB13* and *HvEXLA2* shared similar tissue transcription profiles (Figure 3-10, Panel C) with their distribution mostly restricted to the whole grain at 8-10 dap. Neither the barley expansin-like A transcription studies or the work on rice expansin-like As by Lee and Kende (2002) or *Arabidopsis* (Cocuron *et al.*, 2007) suggest an obvious or specific function for this subgroup of the expansin superfamily. The three rice expansin-like A characterised by Lee and Kende (2002) showed that only one expansin-like A (*OsEXLA3*) was correlated with elongation. *OsEXLA1* and *OsEXLA2* had the highest mRNA levels in the non-elongating regions of the stem internode. Cocuron *et al*. (2007) presented results from microarray analyses that indicated that *AtEXLA1* transcription was strongly correlated with an *Arabidopsis* cellulose synthase-like C gene (*AtCslC4)* during pathogen attack. A further analysis of the library from which the ESTs for the contiguous sequence for each of the barley expansin-like A were assembled include a single EST from five day old seedlings under dehydration stress and two ESTs from a *Fusarium graminearum* infected spike. A BLAST search of the *Fusarium graminearum* (NCBI) and Munich Information Centre for Protein Sequences (MIPS) *Fusarium Graminearum* Genome database (www.mips.helmholtz-muenchen.de/gnre/proj/fusarium/) did not reveal any expansin-like sequences so there was low probability of contamination of the EST library. This may indicate that barley expansin-like As may be switched on in response to pathogen attack or other stresses.

The correlation analyses also revealed examples of co-transcribed barley expansin genes that were highly correlated with other cell wall-related genes. This type of analyses allowed the exploration of possible relationships of these genes with each other during plant development.

The largest group of co-transcribed barley expansin genes included four members from the α-expansins (*HvEXPA4, HvEXPA7, HvEXPA10* and *HvEXPA11*, Figure 3-2, Panel G) and one

member from the β-expansin family (*HvEXPB18*). The transcription of these genes appeared to be restricted mostly to the root, with highest transcript levels recorded in the root tip zone, which is recognised as the most metabolically active region of the root. A similar root-specific profile was observed in rice, which included six α-expansins and two β-expansins (Cho and Kende, 1997c; Lee and Kende, 2001; Lee and Kende, 2002), and in maize, with one α-expansin and two β-expansins (Muller *et al.*, 2007). The tissue expression studies for rice and maize did not cover an extensive tissue series such as the one used in this thesis, and it must be noted that the root-specific results were restricted due to the few organs that were tested.

There was also a xyloglucan endotransglycosylase (*HvXTH23*) and a cellulase (*HvCel5*) shown to be co-transcribed with this group of expansins (Figure 3-14, Panel A). Several studies have shown that expansin-induced wall extension is accompanied by the co-expression of endoglucanase and XTH genes. For example, Catala *et al*. (1997; 2000) showed that auxin directs characteristically high co-expression of endoglucanases, XTHs and expansins during tomato hypocotyl extension and fruit ripening. This was the only example of co-transcribed expansin genes identified in this study that were highly correlated with both a cellulase and an XTH. There were, however, other examples where individual members of the barley XTH and barley cellulase gene families were found to be highly correlated with transcripts from barley expansin genes.

The second group of expansins that was highly correlated with XTHs (*HvXTH2* and *HvXTH3*) included two β-expansins, which were not tissue-specific but were associated with the elongation zones of two organs. *HvEXPB7* and *HvEXPB8* showed the highest abundance of mRNA in the leaf base, followed by the root tip (Figure 3-8, Panel C). These two tissue segments are where primary cell wall synthesis would be expected to predominate and the correlation analyses also revealed a cellulose-synthase (*HvCesA1*), cellulose synthase-like gene (*HvCslF6*) and an arabinoxylan arabinofuranohydrolase gene (*HvAXAH5*) to have similar transcript profiles (Figure 3-14, Panel B). An additional expansin (*HvEXPA6*) was also shown to be highly correlated with this group.

The third group of expansins highly correlated with an XTH was *HvEXPA5,* which had the highest transcript level in the coleoptiles and was found to have a similar tissue transcript pattern to *HvXTH5,* which was coleoptiles-specific (Figure 3-14, Panel C). The rearrangement and re-modelling of xyloglucan in rapidly elongating coleoptiles is thought to be mediated through the activity of an XTH (Carpita and Gibeaut, 1993) and our data suggests that *HvEXPA5* might also assist in modifying xyloglucan during this growth stage.

The final XTH to share a similar transcription pattern to an expansin was *HvXTH24* with *HvEXPB1* (Figure 3-14, Panel D). *HvEXPB1* was shown to have a higher transcript abundance in the root base than in the root tip. The role of XTHs in roots has been studied in maize (Pritchard *et al.*, 1993; Wu *et al.*, 1994; Vissenberg *et al.*, 2003) and *Arabidopsis* (Vissenberg *et al.*, 2001; Osato *et al.*, 2006). Vissenberg *et al*. (2001) was able to demonstrate that root hair initiation is coupled with a highly localised increase of XTH. When linked with previous studies, this suggests that *HvEXPB1* has a role in root hair formation and it is possible that *HvEXPB1* and *HvXTH24* have a functional relationship.

Further support for a role for expansins in root hair growth comes from Cho and Kende (1997c) who identified a rice expansin (*OsEXPA2*) that continued to be expressed in the root hair zones where growth of the primary root has ceased. The difference in expression of *OsEXPA2* in that tissue, compared with other rice expansin genes, indicates a distinct role for the OsEXPA2 protein. To explore this further, Cho and Cosgrove (2002) examined transcript levels of two α-expansin genes, *AtEXPA7* and *AtEXPA18* in roots of *Arabidopsis* and found that expression of both was directly related to root hair formation. Previous work by Kwasniewski and Szarejko (2006) identified a barley expansin gene (*HvEXPB1*) was necessary for root hair development from an analysis of the spontaneous root hair mutant *brb* (*bald root barley*), a root hair-less form of the barley cultivar Pallas (Gahoonia *et al.*, 2001). In wild type Pallas plants, the initiation of root hairs was associated with an increase in *HvEXPB1* expression. There was a variation in transcript results from Kwasniewski and Szarejko (2006) compared with this study due to different analysis methods (semi-quantitative RT-PCR vs. Q-PCR) and different cultivars used. Also because *HvEXPB1* was transcribed at relatively low levels, it was not represented on the Affymetrix chip, so a major limitation of using microarrays is a decreased ability of the arrays to detect genes with low expression levels (Schreiber *et al.*, 2008).

The other barley cellulase that was shown to be highly correlated with an expansin tissue transcription profile was *HvCel10* with *HvEXPB10* (Figure 3-14, Panel F). *HvEXPB10* has been shown to be a predominant member of the barley expansins with its highest mRNA transcript abundance found in the leaf base and root tip, the metabolically active zones of the leaf and root respectively. Cellulases hydrolyse cellulose and potentially other substrates with similar glycosidic linkages (Inouhe and Nevins, 1991; Davies *et al.*, 1993; Warren, 1996; Teeri, 1997). Previous studies have suggested that expansins act by disrupting the non-covalent hydrogen bonds between cellulose fibers or cellulose-hemicellulose composites and thereby increase the accessibility of cellulose to cellulases (Cosgrove, 2001; Wei *et al.*, 2010).

The final two correlations of expansin with cell-wall related genes were *HvEXPB9* with *HvCslD4* and *HvEXPB15* with *HvAXAH3*. *HvEXPB9* transcription was highly correlated with a barley cellulose synthase-like D (*HvCslD4*; Figure 3-14, Panel E). The role of the CslDs in barley are yet to be determined. In *Arabidopsis* all CslD members exhibit diverse developmental roles in root hair and pollen tube elongation, and plant growth (Favery *et al.*, 2001; Bernal *et al.*, 2008). A member of the rice *CslD* family has also been demonstrated to play a role in root growth (Kim *et al.*, 2007). Further analyses of mutants involving the CslDs in rice and *Arabidopsis* have exhibited reduced growth and altered xylan levels, indicating a function in the biosynthesis of the non-cellulosic polysaccharide of the cell wall (Bernal *et al.*, 2007; Li *et al.*, 2009). This suggests that *HvEXPB9* has an involvement with xylan during cell elongation.

An AXAH (*HvAXAH3*) was found to be highly correlated with *HvEXPB15* (Figure 3-14, Panel F). The fine chemical structures of wall arabinoxylans are also subject to modification during cell growth. The hydrolysis of arabinofuranoside side chains from heteroxylans, which consist of a (1,4)-linked β-D-xylan backbone substituted primarily with arabinosyl residues (McNeil *et al.*, 1984), is attributed to AXAH (Lee *et al.*, 2001). The highest mRNA transcript abundance for both *HvAXAH3* and *HvEXPB15* occurs in the developing grain at 3-5 dap and 8-10 dap. At these developmental stages, the biosynthesis of the endosperm cell wall is occurring and because arabinoxylans constitutes 20% of the starchy endosperm cell wall (Fincher, 1975; Ballance and Manners, 1978), this would explain the transcript abundance of *AXAH*. More specifically, it has been shown that arabinoxylans deposited in the early stages of grain development are heavily substituted with arabinosyl residues, but that later in grain development the arabinoxylans carry fewer arabinosyl substitutes (Sarah Wilson, personal communication). The transcription of *AXAH* remains high at 8-10 dap when the aleurone layers become visible in the immature grain (Bosnes *et al.*, 1992). The aleurone walls are high in arabinoxylans, which constitute 70% of the aleurone walls (Bacic and Stone, 1981). The role of *HvEXPB15* may assist *AXAH* with the modification of arabinoxylans during grain development.

3.5 CONCLUSION

Transcript levels of expansin genes were determined by Q-PCR using gene-specific primers designed to the 3'UTR sequences and normalised against a series of internal control genes. The results of the transcriptional analysis of the barley expansins show that there are co-ordinately transcribed groups of genes within the α- and β-expansin groups, and that there are broader groups of cell wall-related genes that may function in a concerted fashion with these expansins, based on highly correlated tissue transcript patterns. The dominant group of barley α-expansins includes *HvEXPA1*, *HvEXPA2, HvEXPA3* and *HvEXPA6* and together they represent the highest transcript abundance in all tissues analysed. The dominant group of barley β-expansins includes *HvEXPB2*, *HvEXPB4*, *HvEXPB5* and *HvEXPB10,* which have the highest transcript abundance except in the anthers at pre-anthesis and the whole flower at anthesis. Although this latter dominant group shows high transcript levels in floral tissues, *HvEXPB16* transcript is even higher. The remaining members of the barley expansin family can either be grouped into similar tissue transcript profiles, where co-expression may occur and where their function may overlap, or with similarly transcribed cell wall-related genes. Or, in the case of the β-expansins, the remaining members can form a unique sub-group that lacks the usual distinguishing N-linked glycosylation site.

An *in silico* analysis of microarray data of the barley expansins in a range of tissues was also performed and the results mostly mirrored those from the Q-PCR analyses. Although traditional methods that measure gene expression (e.g., Northern blotting, RNase protection assays) are relatively labour intensive, they provide high resolution and can be used to validate or extend microarray data. Several limitations to microarrays are noted. A major limitation is a decreased sensitivity of the arrays to the detection of genes with low expression levels (low-abundance genes). Another drawback is that it is possible to confuse microarray results through a process of cross-hybridization in which specific components of the arrays will cross-hybridize because of sequence similarity of the probes, which is possible with a large gene family in an unsequenced genome such as barley, where members of the gene family contain highly conserved regions.

Q-PCR is another relatively high-throughput technique used for the quantification of steady-state mRNA levels. It provides high sensitivity so that rare sequences can be detected. It may also be used to detect mRNAs from small sections of tissue so that subsections of the grain or floral organs can be examined independently. Transcript level does not necessarily equate to protein level or enzyme activity and therefore these processes are only the first step in the investigation of gene function.

Further investigations could involve multiple strategies for studying function including transgenic and knockout lines. However, with over 30 genes identified, the possibility for redundancy and overlapping expression presents serious limitations to functional analysis by reverse genetics (Krysan *et al.*, 1999). The high number of observed correlated co-transcription of cell wall-related genes with the expansin gene family provides an opportunity to explore the functional relationship between them. Whilst the results of the correlation studies only provide testable hypotheses, they are still a valuable starting point. Therefore cloning, expressing and purifying three active expansins in *E. coli* for the purpose of examining their mechanism of action and/or substrate binding, and their behaviour when combined with other cell wall-related enzymes was the next approach taken in the current study.

CHAPTER 4

HETEROLOGOUS EXPRESSION OF BARLEY EXPANSINS

4 HETEROLOGOUS EXPRESSION OF BARLEY EXPANSINS

4.1 INTRODUCTION

The initial objective of the work described in this Chapter was to isolate sufficient quantities of purified expansins to allow their *in vitro* characterisation by experiments in which expansin action could be examined in the presence of cellulases and xyloglucan endotransglycosylases/hydrolase (XTH), both of which were available in the laboratory. In this way, any synergistic action involving expansins, cellulases and XTHs could be investigated. The inclusion of XTHs in this plan was based on observations that certain barley XTHs can use cellodextrins as substrates (Hrmova *et al.*, 2009) and because the high correlation of transcription abundance of expansins with XTHs presented in Chapter 3 suggested a functional relationship between XTHs and expansins.

In the past, the activity of native partially purified expansins has been measured by the induction of stress relaxation, or the extension of isolated cell walls in a pH-dependent manner, upon application of exogenous expansin on heat-treated cell walls (McQueen-Mason *et al.*, 1992; Li *et al.*, 1993). The proposed mechanism of action of expansins involved the disruption of hydrogen bonding between cell wall polymers (McQueen-Mason and Cosgrove, 1994), as demonstrated by the putative weakening of hydrogen bonding between paper fibres without the hydrolysis of cellulose molecules. Thus, no hydrolysis of the cell wall by expansins has been detected (Yennawar *et al.*, 2006). It is believed that expansins bind at the interface between matrix polysaccharides and cellulose (McQueen-Mason and Cosgrove, 1995) and weaken the non-convalent binding between wall polysaccharides, thus allowing cellulases greater access to cellulose microfibrils. This enhanced accessibility of cellulases for degrading or modifying cellulose should result in an increased hydrolysis of cellulose when expansin is included to the reaction mix with cellulase and cellulose, compared with cellulose treated with cellulase alone (Cosgrove, 2001).

As noted in Chapter 1, most experiments with expansin proteins have been performed with highly purified native tissue extracts (McQueen-Mason and Cosgrove, 1994; Yennawar *et al.*, 2006). In studies to determine the mechanism of action of expansins or in activity assays, only partially purified native expansins have been utilised (Li *et al.*, 1993; McQueen-Mason and Cosgrove, 1994; Yennawar *et al.*, 2006). So, whilst it is possible to extract and purify expansins from plants directly, the purification processes are usually time-consuming and it is relatively difficult in many cases to obtain the quantities required for detailed characterisation.

Yennawar *et al.* (2006) defined the crystal structure of a purified maize β-expansin, due to the abundance of a subset of the EXPBs in maize pollen. This subset of EXPBs is known in the

literature as group-1 grass pollen allergens (Ansari *et al.*, 1987; Cosgrove *et al.*, 1997) and can comprise up to 4% of the protein extracted from grass pollen (Li *et al.*, 2003). So in this instance, the extraction and purification of an expansin was possible. However, other genes in the EXPB family are expressed in a variety of tissues in the plant and are typically found in low abundance and/or are tightly bound to the cell wall (Lee and Choi, 2005). Whilst the α-expansins can be solubilised by extraction buffer for activity assays (McQueen-Mason *et al.*, 1992; Cho and Kende, 1997a), the β-expansins found in the vegetative tissues of the plant require harsh extraction conditions that result in denaturation of the protein (Lee and Choi, 2005). It has also been observed by Yennawar *et al.* (2006), where α-expansin protein has been purified from various plant tissues, that it is difficult to concentrate the protein to levels suitable for crystallization, although high concentrations were not required for the experiments envisaged here. For these reasons, expansin cDNAs were cloned from barley and attempts were made to express these cDNAs heterologously to produce pure expansin protein.

Heterologous expression of recombinant DNAs enables rapid production of exogenous proteins for functional studies. However, it is only successful if the host system is able to provide the conditions required for the production of a functionally active protein. One of the key requirements is to develop conditions that allow correct folding of the expressed protein, which might require the formation of disulphide bridges or specific post-translational modifications such as glycosylation or phosphorylation (Gustafsson *et al.*, 2004). A number of biological systems are available for the heterologous expression of recombinant proteins, including bacterial, fungal, viral, mammalian and insect cells, and more recently, cell-free expression systems (Kost, 1997; Sawasaki *et al.*, 2002). There are advantages and disadvantages for each of these systems that affect protein yield, correct folding, post-translational modification, cost and ease of use.

Bacterial expression systems are generally the cheapest, quickest and simplest for the synthesis of proteins. Heterologous expression of cDNAs in *Escherichia coli* can result in large quantities of protein expression relative to the amount that may be expressed in the organism from which the cDNA has been cloned. There are some disadvantages associated with the heterologous expression of plant proteins in *E. coli,* namely that incorrect protein folding can result in reduced or no activity (Kost, 1997) and that the bacterial expression system is unable to perform certain post-translational modifications of proteins, such as glycosylation and phosphorylation, which may also result in reduced or no activity (Dubessay *et al.*, 2004). Nevertheless, *E. coli* remains a popular first choice for the heterologous expression of plant proteins.

In general, fungi are excellent hosts for the production of recombinant proteins of eukaryotic origin due to their rapid growth to high cell densities on inexpensive media. As eukaryotes, they are able to perform protein modifications like glycosylation, thus producing complex foreign proteins that are identical or very similar to native products from plant or mammalian sources. Despite having the ability to perform modifications like glycosylation, a major disadvantage associated with the use of a fungal expression system is the risk of incorrect or over glycosylation, which can reduce activity or cause incorrect folding.

Not only is the choice of a suitable host important, but so is the choice of the expression vector. Successful protein expression is greatly increased when coupled with a vector that contains enhanced features that can assist with correctly folding of the expressed protein, based on the desired proteins' known characteristics. Therefore, the biological properties of the protein of interest dictate the type of expression system to use.

Following the cloning of three barley expansin cDNAs, attention was focused on the functional analyses of these genes. Candidates were selected from each of the two main sub-families; one α-expansin and two β-expansins. A unique characteristic of the β-expansins is a predicted N-linked glycosylation site. In eukaryotes, N-glycosylation occurs in the secretory pathway as glycoproteins are transported from the endoplasmic reticulum to their final destination. Analyses in Chapter 2 revealed that five of the barley β-expansins characterised in this study did not contain a predicted N-linked glycosylation site, including *HvEXPB1* (Kwasniewski and Szarejko, 2006). The implication of a possible N-linked glycosylation site is important when considering the host for protein expression. Therefore, one β-expansin with, and one without, a predicted N-linked glycosylation site were selected for heterologous expression. The significance of the N-linked glycosylation motif for expansin function is unknown (Downes *et al.*, 2001). When considering the differences between the two families and the possible impact on function, there remains a gap in our understanding of the physiological basis for the glycosylation of one family but not the other, and why there is a small group within the barley β-expansins that lacks N-glycosylation sites. With these features in mind, a bacterial expression system was chosen initially to express the three expansin cDNAs.

The second consideration when choosing a suitable expression system was an expression vector able to cater for the three disulfide bonds that are highly conserved in both EXPA and EXPB families. To encourage correct folding and thus active expansin protein, a Gateway-enabled vector (pTOOL7) based on pET32a was selected, because it includes a fusion tag that further enhances the formation of disulfide bonds in the cytoplasm, coupled with an Origami bacterial host that

contains mutations in both the thioredoxin reductase (*trxB*) and glutathione reductase (*gor*) genes. These two features greatly enhance disulfide bond formation in the cytoplasm. Studies have shown that expression in Origami cells (DE3) yielded 10-fold more active protein than in another host, even though overall expression levels were similar (Prinz *et al.*, 1997).

The pTOOL7 vector also contains a polyhistidine sequence, consisting of six histidine (His_6) amino acid residues at both the NH_2- and COOH-termini. The addition of the polyhistidine short affinity tag to each end of the expressed protein provides a purification strategy, usually *via* the affinity of the tag towards immobilised compounds such as metal resins, and allows for rapid, single step purification following protein expression. The purification process used for the heterologously expressed expansin proteins described in this Chapter employed an immobilised metal affinity chromatography (IMAC; Janknecht and Nordheim, 1991) resin charged with cobalt.

Following expression and purification of a recombinant protein, a suitable assay was required to determine both the activity of the expressed protein and its substrate specificity. The pilot activity assay described in this Chapter was based on previous experiments described by Cosgrove (2001) that showed an increased accessibility of cellulase to cellulose by the addition of expansin protein, which is measured by the amount of free sugars released from the degradation of cellulose. Following incubations of cellulose with recombinant expansin and a commercially available cellulase (*Trichoderma reesei* ATCC 26921, Sigma catalogue no. C2730), the total sugars liberated in the supernatant were measured by colorimetric microdetermination of absorbance at 490 nm in the presence of phenol and sulphuric acid (Dubois *et al.*, 1956).

A separate binding study is necessary to determine which cell wall polymer is bound to the expansin protein, in order to determine the site of action. In binding studies, Whitney *et al*. (2000) demonstrated that cucumber α-expansin induced extension of composites of cellulose microfibrils and xyloglucans but not of composites containing other cross-linking glucans. Yennawar *et al.* (2006) were able to show that a native maize β-expansin extract preferentially bound to a xylan substrate and also showed some binding affinity to a xyloglucan substrate. Both these studies indicate a degree of substrate binding specificity.

To further investigate the binding substrate of a barley β-expansin protein, the two domains of *HvEXPB5* were expressed separately. Domain 1 of the expansin protein contains disulfide bonds, so the pTOOL7 vector and Origami bacterial cell line were used to express this domain of the protein. Domain 1 has significant but distant homology to glycoside hydrolase family 45 (GH45) proteins, most of which are fungal (1,4)-β-D-endoglucanases (Henrissat *et al.*, 1998). Domain 2 of

expansin proteins contains fewer structural features and hence the simpler Gateway-enabled expression vector pDEST17 with T7 promoter and NH_2-terminus His_6-tag with the BL21 star bacterial cell line was chosen to express this domain of the protein. Domain 2 of the expansin proteins has some similarity to cellulose-binding domains found in certain microbial cellulases. It is Domain 2 that is predicted to be the binding domain of the expansin protein. The pilot assay used to determine the binding substrate involved the incubation of semi-purified recombinant protein of Domain 2 of *HvEXPB5* bound to resin with solutions of xylan, β-glucan and carboxymethyl cellulose.

The generation, expression, purification and analyses of three recombinant barley expansin proteins are presented in this Chapter.

4.2 MATERIALS AND METHODS

4.2.1 Preparation of expansin expression vectors

4.2.1.1 Amplification of open reading frame fragments of HvEXPA1, HvEXPB5 and HvEXPB15

Full length sequences of the three expansin cDNAs amplified by PCR, as described in section 2.2.9, were used as templates for PCR. Gene-specific primers used to amplify the three expansin cDNAs are shown in Table 4-1. The forward primers were designed to lie 3' to the predicted cleavage site of the signal peptide in order to exclude this region for heterologous expression. The reverse primers were designed to remove the native stop codon of the expansin cDNAs to enable incorporation of a COOH-terminus hexahistidine (His_6) tag sequence in the final expression vector. Replacement start and stop codons are carried on the pTOOL7 vector.

Reactions were carried out using the Elongase® *Taq* Polymerase Amplification System (Invitrogen). The annealing temperatures are shown in Table 4-1. PCRs were carried out in a total volume of 25 μl containing 0.5 μl of the appropriate PCR template, 1 μl 10 μM of the gene-specific primers listed in Table 4-1, 2.5 μl Elongase Buffer A, 2.5 μl Elongase Buffer B, 0.5 μl Elongase polymerase and 0.5 μl 10 mM dNTPs. Cycling conditions for PCR were; 94ºC for 2 min followed by 30 cycles of 94ºC 30 s, 53-57ºC (annealing temperature based on the T_m of the gene specific primers) 30 s, 68ºC 90 s and a final step of 68ºC for 2 min. The expected lengths of the three predicted expansin cDNAs and annealing temperatures are listed in Table 4-1. Samples were held at 25ºC and 5 μl of PCR products were separated on a 1% (v/v) agarose gel containing ethidium bromide.

4.2.1.2 Construction of entry vectors

Reaction components were removed with the clean-up Gel extraction NucleoSpin® Extract II kit (Macherey-Nagel, Duren, Germany) as per the manufacturer's protocol. Binding conditions of the PCRs were adjusted with 200 μl Buffer NT and loaded onto a NucleoSpin® Extract II column and centrifuged for 1 min at 11,000 x g. The flow-through was discarded and the silica membrane was washed with 600 μl Buffer NT3 and centrifuged for 1 min at 11,000 x g. The wash was discarded and the column was further centrifuged for 1 min to remove remaining column wash. The column was transferred to a fresh collection tube and placed in a heating block at 65ºC for 2 min. The PCR fragments were eluted twice with 25 μl elution Buffer NE equilibrated to 65ºC. The recovery of the fragments was determined by running 5 μl of PCR product on a 1% (v/v) agarose gel containing ethidium bromide. Use of Elongase polymerase, which does not have exonuclease activity, resulted in amplicons with a single adenine overhang at the 5' ends of each nucleic acid strand. The clean PCR fragments were cloned into the pCR®8/GW/TOPO® vector (Invitrogen), a linearised

Gene	Forward primer (5'—3')	Reverse primer (5'—3')	PCR size bp	Annealing temperature °C
HvEXPA1	GGCGGCTACGGCGGGTG	GAACTGGGCGCCCTCGAAG	683	57
HvEXPB5	CAGTCGCCGCTCAACTACA	ACGGAACTGGACGTTGGAGC	744	53
HvEXPB15	GTCGAGATCCACCGCAAGC	GTAGTTGACGACGGAGCGG	714	55

Table 4-1 Primers used to amplify the coding sequences of *HvEXPA1*, *HvEXPB5* and *HvEXPB15*

The forward primers were designed to exclude the signal peptide region for heterologous expression. The reverse primers were designed to anneal upstream of the stop codon to remove the native stop codon to enable inclusion of a COOH-terminus Hexahistidine (His_6) tag sequence in the expressed protein.

vector with overhanging 3' thymidine residues. The pCR®8/GW/TOPO® vector has a TOPO® cloning site for one-step efficient cloning of PCR products and contains *att*L1 and *att*L2 sites for recombination-based transfer of the gene of interest into a Gateway® enabled 'destination' vector for protein expression. The *att*L1 and *att*L2 sites in the vector, flanking the site of cDNA insertion, enables the use of the recombination properties of bacteriophage lambda using Gateway technology (Landy, 1989). This allows inserted sequences to be moved into appropriate 'destination' vectors, which contained *att*R1 and *att*R2 recombination sites, without the use of restriction enzyme digestion and ligation. The cloning reaction was carried out in a total volume of 6 µl containing 0.5 µl of the PCR fragment, 1 µl salt solution and 0.5 µl of the pCR®8/GW/TOPO® vector. Reactions were incubated at room temperature for 30 min and stored at -20°C until required for transformation. The vector maps of the entry vector constructs for *HvEXPA1*, *HvEXPB5* and *HvEXPB15* are shown in Figure 4-1.

4.2.1.3 Transformation of competent E. coli

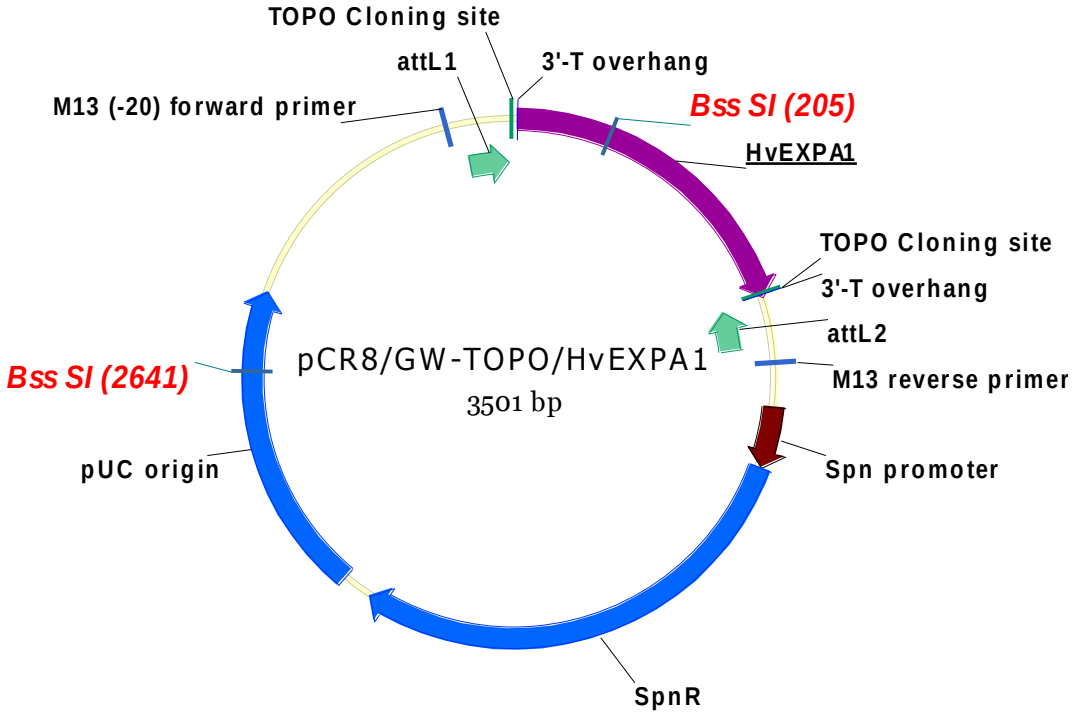
Heat shock competent DH5α *E. coli* cells, prepared by the standard Hanahan (1985) method were transformed with 3 µl cloning reaction as described in section 2.2.8.4 and plated onto LB agar plates containing 50 µg/ml spectinomycin. Plates were incubated for 16 h at 37°C.

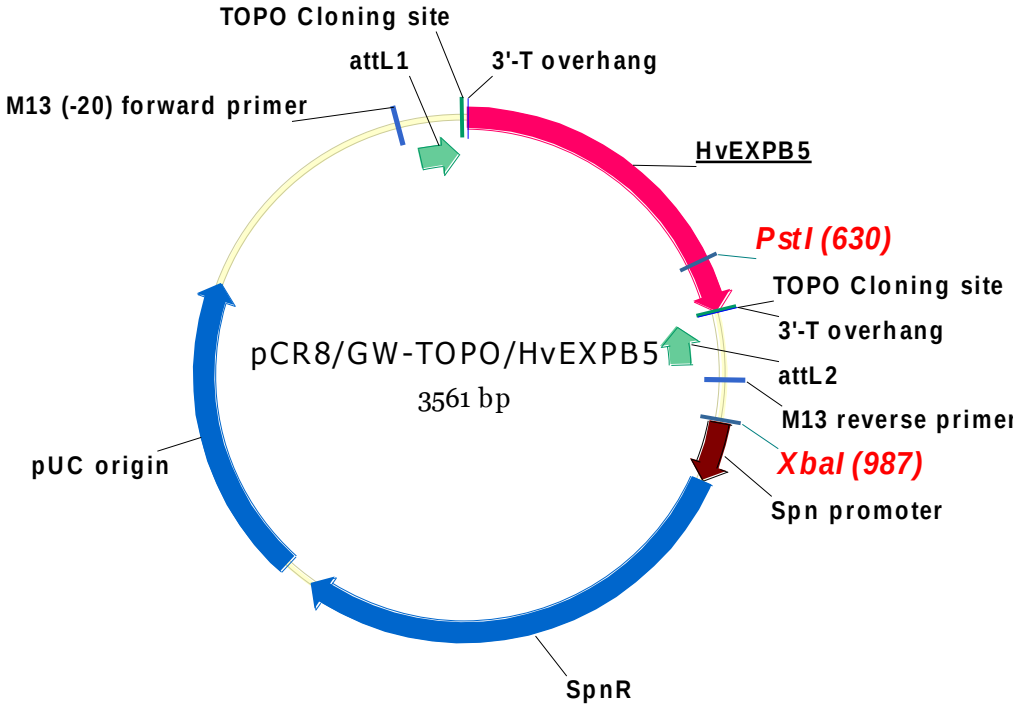
4.2.1.4 Plasmid DNA preparation

Colonies were cultured for 16 h at 37°C in 5 ml LB supplemented with 50 µg/ml spectinomycin. Plasmids were isolated from *E. coli* cultures using the QIAprep Spin MiniPrep Kit according to the manufacturer's protocols with an alteration to the eluting step where the spin column was placed into a fresh tube and incubated at 65°C for 1 min. Sample was eluted with 40 µl of elution buffer equilibrated to 65°C.

4.2.1.5 Directional restriction digest analysis of DNA plasmid for gene of interest insert orientation

Gateway recombination transfers the gene of interest into the destination vector in the same orientation as found in the entry vector. The TOPO® cloning reaction does not influence orientation of the gene of interest and therefore orientation was determined by restriction digest analysis of the DNA plasmid, which will give fragments of distinctive sizes in relation to direction of insert. The entry vector constructs were analysed for restriction sites by Vector NTI Suite Software version 10. The restriction sites, enzymes, buffer and fragment sizes for each construct are shown in Figure 4-1 and Table 4-2. Restriction digest reactions were carried out in a total volume of 10 µl according to the manufacturer's protocols and contained 2 µl DNA plasmid. Reactions were incubated at 37°C for 3 h. Digested plasmids were analysed on a 1% (w/v) agarose gel containing ethidium bromide.


TOPO Cloning site
attL1
3'-T overhang
M13 (-20) forward primer
Bss SI (205)
HvEXPA1
TOPO Cloning site
3'-T overhang
attL2
M13 reverse primer
Bss SI (2641)
pCR8/GW-TOPO/HvEXPA1
3501 bp
pUC origin
Spn promoter
SpnR


TOPO Cloning site
attL1
3'-T overhang
M13 (-20) forward primer
HvEXPB5
PstI (630)
TOPO Cloning site
3'-T overhang
attL2
M13 reverse primer
pCR8/GW-TOPO/HvEXPB5
3561 bp
pUC origin
XbaI (987)
Spn promoter
SpnR

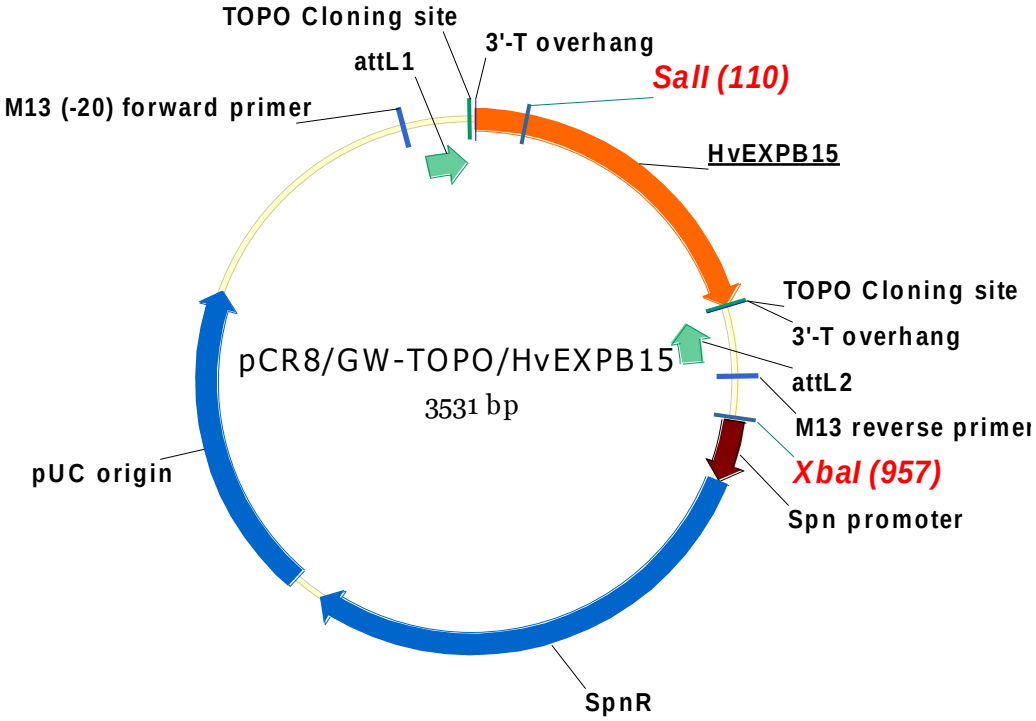


Figure 4-1 Maps of entry vector constructs of pCR®8/GW/TOPO® containing barley expansin cDNAs.

A: Map of pCR®8/GW/TOPO® with *HvEXPA1* cDNA. **B:** Map of pCR®8/GW/TOPO® with *HvEXPB5* cDNA. **C:** Map of pCR®8/GW/TOPO® with *HvEXPB15* cDNA. The cDNAs of *HvEXPA1, HvEXPB5* and *HvEXPB15* were inserted via the TOPO cloning site that allows for efficient cloning of PCR products. The entry vector contains *att*L1 and *att*L2 sites for recombination based transfer of the gene of interest into a destination vector *via att*R1 and *att*R2 sites for downstream analysis. The orientation of the gene of interest was determined and only plasmids containing the gene of interest in the forward orientation were selected for homologous recombination. Restriction sites used for determination of orientation are shown in red. The sequence of the inserts was checked using M13 (forward 5' GTAAAACGACGGCCAG 3' and reverse 5' CAGGAAACAGCTATGAC 3') primers located outside the cloning sites. The SpnR gene encodes spectinomycin resistance; pUC origin is for high-copy replication of the plasmid in *E. coli*.

Entry vector construct	Reaction components		Resulting fragment sizes (bp)	
	Restriction endonuclease	Buffer	Forward orientation	Reverse orientation
HvEXPA1	0.5 µl BssSI	1× NEB Buffer 3, 1× BSA	1065, 2436	1337, 2164
HvEXPB5	0.2 µl XbaI, 0.2 µl PstI	1× Roche Buffer H	357, 3204	867, 2694
HvEXPB15	0.2 µl XbaI, 0.2 µl SalI	1× Roche Buffer H	847, 2684	355, 3176

Table 4-2 Restriction sites used for determination of insert orientation

The enzymes, buffer and resulting fragment sizes for determining forward or reverse orientation for each gene of interest are shown. Prior to digestion the entry vector constructs were analysed for restriction sites using Vector NTI Suite Software version 10 (Invitrogen).

4.2.1.6 DNA sequencing and sequence analysis

Plasmids containing inserts in the correct orientation were sequenced to check the integrity of the insert within the entry vector as described in section 2.2.8.8, using M13 forward and reverse primers (Invitrogen), which anneal to the pCR®8/GW/TOPO® vector outside the Gateway cloning sites.

4.2.1.7 Generation of expression clones

For expression of near full length *HvEXPA1*, *HvEXPB5* and *HvEXPB15* in *E. coli,* the pTOOL7 vector was used. This vector was kindly supplied by Mrs Jodie Kretschmer (University of Adelaide, Australia), and had been adapted to the Gateway® cloning system by the addition of *att*R1-*CmR*-*ccd*B-*att*R2 recombination recognition sequences to the pET32a vector (Novagen, San Diego, CA; Figure 4-2). The presence of the *ccd*B (control of cell death) gene resulted in the production of a protein product toxic to most *E. coli* cells by binding to, and interfering with, DNA gyrase. The Gateway® entry clones harbouring expansin cDNA were inserted *via* an LR reaction into the pTOOL7 vector for *E. coli* expression as per the manufacturer's instructions (Invitrogen). LR reactions contained 1 μl 150 ng/μl pTOOL7 and 1 μl entry vector DNA plasmid. To these plasmids, 2 μl LR Clonase II enzyme mix (Invitrogen) was added and the volume was made up to 8 μl with 10 μM Tris-EDTA. Reactions were incubated at 25°C for 16 h, followed by the addition of 1 μl Proteinase K (Invitrogen) and incubated at 37°C for 10 min. To verify that recombination with pTOOL7 had occurred, 4 μl of the expression constructs were transformed into 50 μl One Shot® Mach1™ T1 Phage-Resistant Chemically Competent *E. coli* cells (Invitrogen) as described in section 2.2.8.4 and plated on LB agar plates containing 100 μg/ml ampicillin. Plates were incubated for 16 h at 37°C. Plasmids from colonies were prepared as described in section 4.2.1.4 with ampicillin selection. The presence of an insert was confirmed by a double digestion of 10 μl plasmid with 0.2 μl *Hind*III (New England Biolabs) 0.2 μl *Xho*I (Roche), 1x Super Duper Buffer restriction enzyme buffer (SDB, 33 mM Tris, pH 7.8 adjusted with glacial acetic acid, 65 mM potassium acetate, 10 mM magnesium acetate, 4 mM spermidine, 5 mM DTT) in a total of 20 μl. The integrity of the expression construct was confirmed by DNA sequencing with pTOOL7_F1 (5' GGTGCCACGCGGTTCTGGTA 3') that anneals to the pTOOL7 vector upstream of the start codon and a reverse gene-specific primer (Table 4-1) as described in section 2.2.8.8.

4.2.1.8 Transformation of Origami DE3 E. coli *for protein expression*

Heat shock competent Origami DE3 *E. coli* cells, prepared by the standard Hanahan (1985) method were transformed with 1 μl of isolated plasmid from section 4.2.1.7 as described in section 2.2.8.4 and plated on onto LB agar plates containing 12.5 μg/ml tetracycline, 100 μg/ml·ampicillin and 15 μg/ml·kanamycin. Plates were incubated for 16 h at 37°C.

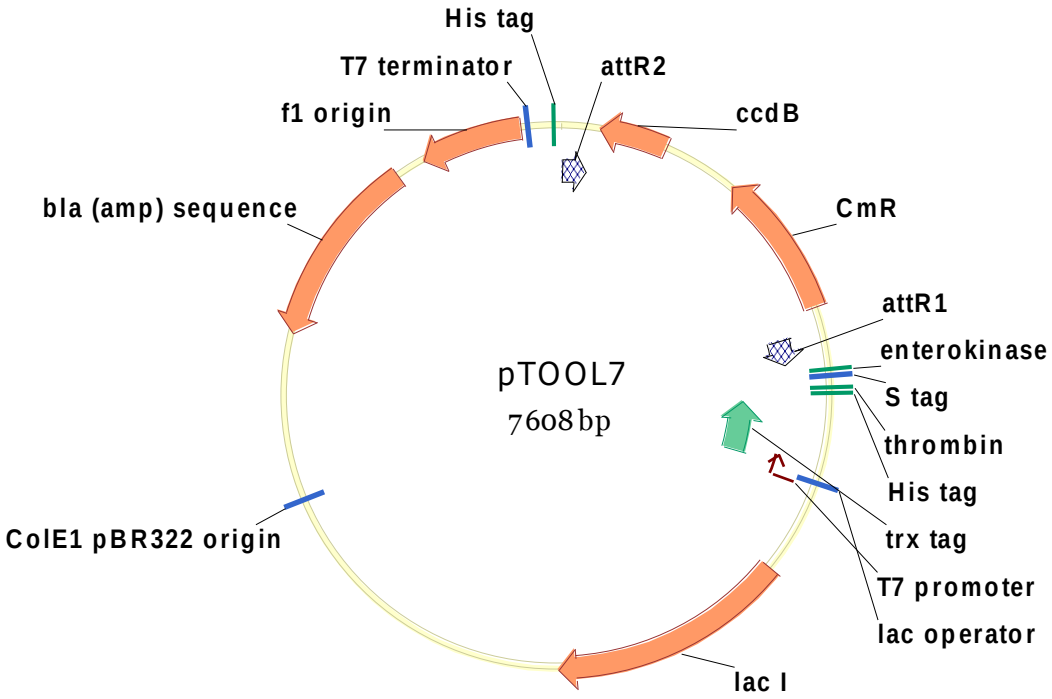


Figure 4-2 Map of the Gateway enabled pET32a (pTOOL7) destination vector used for protein expression.

Genes transferred into the pTOOL7 vector *via* Gateway-mediated LR recombination (Invitrogen) are inserted between the *att*R1 and *att*R2 sites. Expression of genes inserted into pTOOL7 is controlled by the T7 promoter. The pTOOL7 vector contains a fusion tag (109 aa) that enhances the formation of disulfide bonds in the cytoplasm when used with an Origami host that contains mutations in both the thioredoxin reductase (*trxB*) and glutathione reductase (*gor*) genes. The T7 promoter allows high-level IPTG-inducible expression of the recombinant protein. CmR encodes chloramphenicol antibiotic resistance, bla encodes the β-lactamase that confers ampicillin resistance. Negative selection of transformants is performed by the presence of the *ccdB* gene.

4.2.2 Generation of additional expression constructs

4.2.2.1 PCR addition of a 3' dodecahistidine (His_{12}) tag to HvEXPA1, HvEXPB5 and HvEXPB15

The near full length *HvEXPA1, HvEXPB5* and *HvEXPB15* cDNA (683bp, 744 bp and 714bp respectively) were amplified using Elongase DNA polymerase as described in section 4.2.1.1 with the reverse primers shown in Table 4-3. The integrity of the expression constructs were confirmed by DNA sequencing with pTOOL7_F1 only. The expression constructs were generated as described in sections 4.2.1.2 to 4.2.1.8 (construct not shown). The addition of a COOH-terminus dodecahistidine (His_{12}) tag sequence to *HvEXPA1, HvEXPB5* and *HvEXPB15* was to increase binding affinity of the expressed protein in the IMAC purification process.

4.2.2.2 Generation of expression constructs for the two domains of HvEXPB5

A set of PCR primers was designed and used to amplify selected fragments of *HvEXPB5* cDNA using Elongase DNA polymerase as described in section 4.2.1.1, with the alteration of a 60 s extension step. The amplified fragments were predicted to encode Domain 1 and Domain 2 of *HvEXPB5*. The nucleotide and deduced amino acid sequence of *HvEXPB5* are shown in Figure 4-9. PCR was performed at an annealing temperature of 54°C, with final predicted product sizes of 437 bp and 329 bp for Domain 1 and Domain 2, respectively. Each reverse primer contained a stop codon to utilise a single His_6-tag at the NH_2-terminus of the expression vector. The expression constructs were generated as described in sections 4.2.1.2 to 4.2.1.8, with the omission of section 4.2.1.5. In section 4.2.1.6 the orientation of the gene of interest and integrity of the construct were confirmed via sequencing before proceeding with section 4.2.1.7.

The Gateway expression system was also used to generate expression plasmid vectors in pDEST™17 (Invitrogen; Figure 4-3). The pDEST™17 expression vector contains a T7 promoter for high-level T7 RNA polymerase regulated expression of the gene of interest in *E. coli* and an integral His_6-tag at the NH_2-terminus for detection and purification of recombinant proteins. These expression constructs were generated as described in sections 4.2.1.2 to 4.2.1.4.

The presence of an insert was confirmed by an *Eco*RI (NEB) digest according to the manufacturer's instructions. *Eco*RI sites flank the insertion sites of pCR®8/GW/TOPO®, therefore releasing the cloned insert. Digested plasmids were analysed on a 1% (w/v) agarose gel containing ethidium bromide.

The orientation of the gene of interest in the entry vector was confirmed via sequencing as described in section 4.2.1.6. Plasmids containing the gene of interest in the correct orientation were

Gene	Reverse primer (5'—3')	Annealing temperature °C
HvEXPA1	**TCA**GTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGAACTGGGCGCCCTCGAAGGTCTGGCCGAACTGCCA	59
HvEXPB5	**TCA**GTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGACGGAACTGGACGTTGGAGCGGTAGTTTGTGTCGGG	53
HvEXPB15	**TCA**GTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTGGTAGTTGACGACGGAGCGGTAGGTGCCGCCGGGCTT	55

Table 4-3 Reverse primers for the addition of a 3' dodecahistidine (His_{12}) tag to sequences of *HvEXPA1*, *HvEXPB5* and *HvEXPB15.*

The reverse primers were designed to anneal upstream of the stop codon for the addition of a COOH-terminus dodecahistidine (His_{12}) tag sequence in the expressed protein. Underlined is a His_{12}-tag and bold text indicates a stop codon.

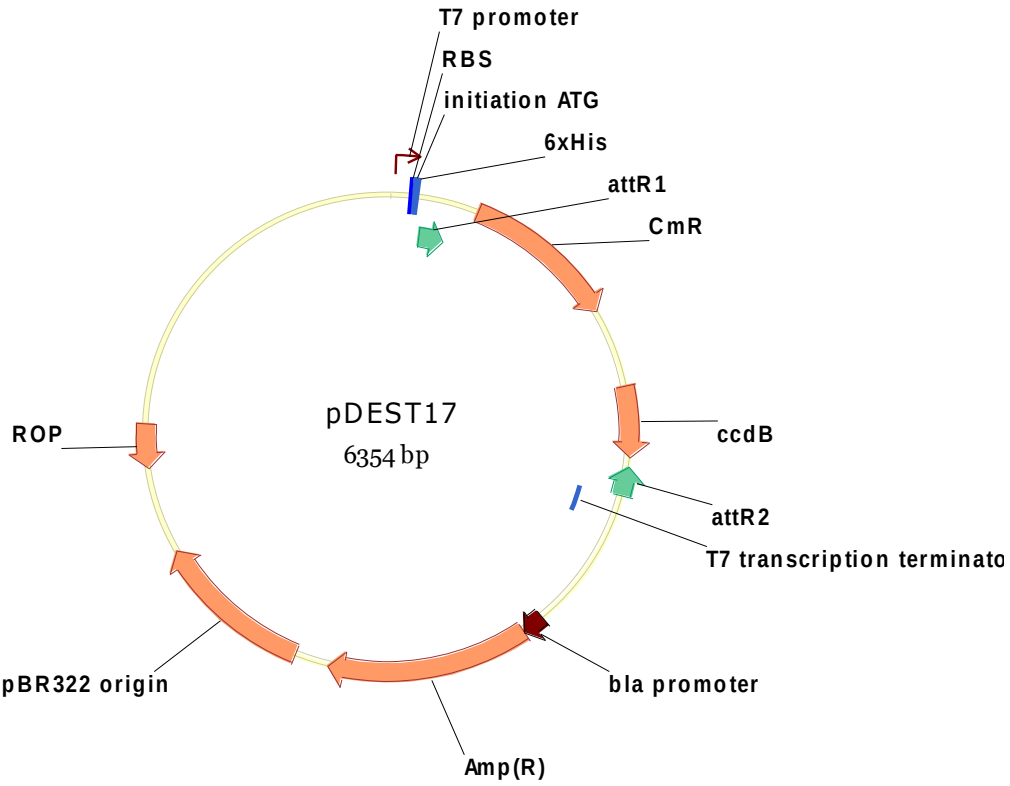


Figure 4-3 Map of pDEST™17 destination vector with an NH2-terminus His6-tag for protein expression.

Homologous recombination occurs between the entry vector pCR®8/GW/TOPO® bearing the gene of interest and the destination vector *via att*R1 and *att*R2 sites. As a result, the cassette bearing the gene of interest replaces the chlorampenicol and *ccdB* genes in destination vectors. The T7 promoter allows high-level IPTG-inducible expression of the recombinant protein and also drives the expression of chloramphenicol resistance and *ccdB* genes. Ampicillin and chloramphenicol resistance allow the selection and counter-selection of expression clones in *E. coli*, respectively. Rop is an open reading frame which interacts with the pBR322 origin to facilitate low-copy replication in *E. coli*. RBS is the ribsome binding site.

inserted *via* an LR reaction into the pDEST™17 vector for *E. coli* expression. The LR reaction (1 μl 100 ng/μl pDEST™17, 1 μl entry vector DNA plasmid, 2 μl LR Clonase II enzyme mix, to a final volume 8 μl with 10 μM Tris-EDTA) was performed at 25°C for 16 h, followed by the addition of 1 μl Proteinase K and incubated at 37°C for 10 min.

To verify that recombination with pDEST™17 had occurred, 4 μl of the expression constructs were transformed into 50 μl One Shot® Mach1™ T1 Phage-Resistant Chemically Competent *E. coli* cells (Invitrogen) as described in section 2.2.8.4 and plated on LB agar plates containing 100 μg/ml ampicillin. Plates were incubated for 16 h at 37°C. Plasmids from colonies were prepared as described in section 4.2.1.4 with ampicillin selection. The presence of an insert was confirmed by a *Eco*RI (NEB) digest according to the manufacturer's instructions in a total of 10 μl. The integrity of the expression construct was confirmed by DNA sequencing with a reverse gene-specific primer as described in section 2.2.8.8. The isolated plasmids were used to transform heat shock competent BL21 Star™(DE3)pLysS *E. coli* cells prepared by the standard Hanahan (1985) method. The transformation for protein expression was conducted as described in section 2.2.8.4 and plated onto LB agar plates supplemented with 100 μg/ml ampicillin. Plates were incubated for 16 h at 37°C.

4.2.3 Heterologous expression in E. coli

For expression of *HvEXPA1*, *HvEXPB5* and *HvEXPB15* in liquid media, a single colony of each construct was inoculated into 5 ml Luria Broth media containing 12.5 μg/ml tetracycline, 100 μg/ml ampicillin and 15 μg/ml kanamycin for Origami or 100 μg/ml ampicillin for BL21 Star™ cells and incubated at 37°C for 16 h with vigorous shaking. This 5 ml culture was used to inoculate 50 ml LB containing 100 μg/ml ampicillin for Origami cells or no selection for BL21 Star™ and incubated at 23°C to an optical density of 0.3–0.4 at 600 nm. The cells were induced at a final concentration of 1 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) and left shaking at 23°C until the culture reached an optical density of 0.6-0.7 at 600 nm (4 to 7 h). The bacterial cells were recovered by centrifugation at 4000 x g for 20 min at 4°C and the supernatant was removed. The pellet was resuspended in 1 ml chilled sodium phosphate buffer (50 mM NaPO4, 300 mM NaCl, pH 8) containing 1 mM phenylmethylsulphonyl fluoride (PMSF) and stored on ice for 20 min. The cells were ruptured using a Branson Sonifer B-12 sonic disintegrator (Danbury, CT, USA) with two 30 s pulses at an output intensity of 4. The ruptured cells were centrifuged at 16,000 x g for 20 min at 4°C to separate the soluble protein from the insoluble fraction (inclusion bodies). These fractions were used for direct determination of protein content *via* sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), or for purification via IMAC.

4.2.4 Purification of expressed proteins via immobilized metal affinity chromatography (IMAC)

4.2.4.1 Native conditions

The 1 ml soluble fraction from protein extracted in section 4.2.2 was added to 500 µl TALON® Metal Affinity Resin (Clontech) in a 10 ml tube. Prior to the addition of extracted proteins, the TALON resin was pre-equilibrated with same the native lysis buffer used to extract the protein (section 4.2.3). Samples were mixed gently on a rocking platform at 4°C for 2 h. The TALON resin was sedimented by centrifugation at 800 x g for 1 min and the supernatant (unbound fraction) was removed, leaving His-tagged proteins bound to the TALON resin. The resin was washed twice with 5 ml of the native lysis buffer. Washes involved the addition of buffer to the TALON resin, which were mixed on a rocking platform for 1 h at 4°C. The TALON resin was sedimented by centrifugation at 800 x g for 1 min and the buffer was collected between each successive wash. The resin was washed for a third time with 5 ml of lysis buffer supplemented with 5 mM imidazole to elute bound proteins. After mixing for 30 min on a rocking platform, the resin and wash buffer slurry were transferred to a Poly-Prep® Chromatography Column (BioRad) and the resin was allowed to settle before wash buffer was eluted and collected.

Bound protein was eluted from the column through the addition of 2 ml lysis buffer supplemented with 200 mM imidazole. Purified proteins used for the activity assay were concentrated and desalted using a 10 kDa molecular weight cut-off Vivaspin 20 Centrifugal Filter (Satorius Stedim Biotech GmbH, Goettingen, Germany) according to the manufacturer's instructions. The purified proteins were desalted in a solution of 200 mM NaCl and 10 mM sodium acetate buffer, pH 5.

4.2.4.2 Denaturing conditions

The pellets of the expressed proteins were resuspended in 2.5 ml denaturing lysis buffer (8 M urea, 20 mM NaH_2PO_4/Na_2HPO_4, 0.5 M NaCl at pH 7.8) and placed on a rocking platform for 5 min at room temperature. The cells were ruptured using a Branson Sonifer B-12 sonic disintegrator with three 5 s pulses at an output intensity of 4. The ruptured cells were centrifuged at 16,000 x g for 5 min. The supernatant was collected and added to 300 µl TALON resin that had been pre-equilibrated with denaturing lysis buffer. Samples were mixed gently on a rocking platform at room temperature for 1 h. The TALON resin was sedimented by centrifugation at 800 x g for 1 min and the supernatant (unbound fraction) removed, leaving His-tagged proteins bound to the TALON resin. Resin was washed with 2 ml denaturing wash buffer (8 M urea, 20 mM NaH_2PO_4/Na_2HPO_4, 0.5 M NaCl at pH 6) on a rocking platform for 2 min and supernatant removed as described above. Resin

was subjected to a second wash with 8 M urea, 20 mM NaH_2PO_4/Na_2HPO_4, 0.5 M NaCl at pH 5.3. Protein was eluted with 1 ml of elution buffer (8 M urea, 20 mM NaH_2PO_4, 0.5 M NaCl at pH 4) under the same conditions as the wash steps and supernatant collected.

4.2.5 Quantification of purified protein

The expressed proteins purified via IMAC were quantified using the Bradford assay (Bradford, 1976) with Coomassie Protein Assay Reagent (Pierce, Rockford, USA). A standard series of bovine serum albumin (BSA) solutions ranging from 0 to 2 μg/ml was prepared, and 500 μl aliquots were added to 500 μl of Assay Reagent. Similarly, 5 μl of purified protein was diluted with 495 μl water and added to 500 μl of Assay Reagent. Concentrations of proteins were measured at 595 nm after 5 min incubation at room temperature. A standard curve was prepared and the protein was quantified based on the standard curve and a best fit regression formula.

4.2.6 Polyacrylamide gel electrophoresis

The expansin proteins were predicted to have molecular weights of approximately 47-51 kDa. The fractions were prepared for electrophoresis by boiling in loading dye (0.125 M Tris-HCl buffer, pH 6.8, containing 4% w/v SDS, 20% v/v glycerol, 5% v/v β-mercaptoethanol, 0.0005% w/v bromophenol blue) for 10 min. The samples were separated on a 12% SDS-PAGE gel with an Xcell *SureLock*™ Mini-Cell gel system (Invitrogen) at 80 mA in 1x 2-(N-morpholino)ethanesulfonic acid (MES) running buffer (50 mM Tris base, 50 mM MES, 1 mM EDTA, 0.1% SDS at pH 7.3) with a SeeBlue® Plus2 Pre-Stained Standard (Invitrogen) until the loading buffer dye-front reached the lower edge of the gel. The resolving component of SDS-PAGE gels was prepared by mixing 3 ml 40% (v/v) acrylamide/Bis 29:1 solution (BioRad), with 2.5 ml 1.5 M Tris-HCl buffer, pH 8.8, containing 0.4% (w/v) SDS. The volume was made to 10 ml with sterile H_2O and polymerization was initiated by the addition of 70 μl 30% (w/v) ammonium persulphate and 20 μl Tetramethylethylenediamine (TEMED; Sigma). The 4% stacking gel component contained 500 μl 40% (v/v) acrylamide/Bis 29:1 solution, 1.25 ml 0.5 M Tris-HCl buffer, pH 6.8, containing 0.4% (w/v) SDS, to a total volume of 5 ml with sterile H_2O prior to polymerization with 35 μl 30% (w/v) ammonium persulphate and 10 μl TEMED.

Gels were stained with Coomassie Brilliant Blue R by placing them into a solution containing 40% (v/v) ethanol, 10% (v/v) acetic acid and 0.2 g Coomassie R and rocking gently for 2 to 12 h. Coomassie-stained polyacrylamide gels were destained with 25% (v/v) ethanol and 7% (v/v) acetic acid for approximately 5 h, with gentle shaking.

4.2.7 Western Analysis

For detection of poly-histidine tagged recombinant expansin proteins using anti-his antibodies, protein extracts were first resolved on a 12% (w/v) polyacrylamide gel as described in section 4.2.3 with the addition of BenchMark™ His-tagged Protein Standard (Invitrogen). Proteins from the SDS-PAGE were transferred onto 0.22 Micron GE Pure Nitrocellulose Transfer Membrane (GE Water and Process Technologies, Minnetonka, MN, USA) using the Mini Trans-Blot Cell system (BioRad, Hercules, CA, USA) in 1x Tris-glycine transfer buffer (25 mM Tris and 192 mM glycine, pH 8.3) at 100 volts for 80 min. The transfer of the protein was performed in an assembly of filter paper (2 pieces of 3 MM Whatman paper), nitrocellulose membrane, SDS-PAGE gel and filter paper (2 pieces the same size as the gel) piled on top of one another.

Once the protein transfer was complete, the nitrocellulose membrane was washed twice in 1x Tris-buffered saline (TBS; 150 mM NaCl and 10 mM Tris, pH 7.6) and incubated on a rocking platform for 60 min in 5% (v/v) milk diluted in 1x TBS. The blocking solution was rinsed off the membrane twice with 1x TBS. The membrane was incubated with a 6x His Monoclonal Antibody (Clontech) in a 1:5000 solution of 1% (v/v) milk in 1x TBS overnight on a rocking platform. The membrane was washed vigorously three times for 5 min in 1x TBS before incubating with a 1:30,000 dilution of Anti-Mouse IgG Alkaline Phosphatase antibody (Sigma) in 1% (v/v) milk in 1x TBS for 2 h. The secondary antibody was rinsed from the membrane in three washes of 1x TBS. To visualise location of poly-histidine tagged recombinant expansin protein, the membrane was incubated with 1 ml Western Blue® Stabilized Substrate for Alkaline Phosphatase (Promega) to develop a colorimetric reaction between the alkaline phosphatase and primary antibody. The reaction was stopped by rinsing the membrane with sterile H_2O after 1 to 5 min once the reactive areas had developed.

4.2.8 Expansin function assays

4.2.8.1 Activity assay

Expansin proteins have previously been shown to lack enzymatic activity (Yennawar *et al.*, 2006) but can still be assessed for activity. This activity assay measures the effect expansin protein has on the accessibility by cellulase to trigger the hydrolysis of crystalline cellulose. Reactions were carried out in a total volume of 1 ml and performed in triplicate. The reaction components were 2 mg/ml Avicel (Sigma), which is a microcrystalline cellulose, 50 mM sodium acetate buffer, pH 4.5 and 5 μg/ml cellulase from *Trichoderma reesei* (Sigma). These reactions were supplemented with

either 0.0225 μg/ml recombinant expansin protein or water for the control reaction. Reactions were incubated for 16 hr at 37°C with moderate shaking.

The cellulose suspension was allowed to settle. Following incubation at 0°C for 10 min the reaction was centrifuged for 2 min to clarify the supernatant. The supernatant was removed carefully and transferred to a fresh tube so the cellulose pellet was not disturbed and the total sugars released were measured via the phenol-sulphuric acid colorimetric method (Dubois *et al.*, 1956). In this order, 5.3 μl phenol and 666 μl concentrated sulphuric acid were added to 266 μl of the supernatant to a total reaction volume of 1 ml. A standard series of glucose solutions ranging from 0 to 375 μg/ml was prepared, and 266 μl aliquots were added to the same phenol, sulphuric acid reaction mix. The hot reactions were allowed to stand for 10 min, mixed and equilibrated to room temperature by incubating at 25°C for 10 min. Concentrations of total sugars were measured at 490 nm. A standard curve was prepared and the total sugars were quantified based on this and a best fit regression formula.

4.2.8.2 Binding Assay

Prior to the elution step of the native purification of HvEXPB5-Domain 2, the wash buffer was removed and resin resuspended in 1 ml sterile water. The resin was split into two fractions; one to elute purified protein and the remaining resin with bound protein was used in the binding assay. The resin/protein solution for the binding assay was washed and equilibrated with 20 mM sodium acetate pH 4.5. The reaction components for the binding study contained 50 mM sodium acetate, 2.5 mg substrate, barley (1,3;1,4)-β-glucan, birchwood xylan, oat spelt xylan or carboxymethyl cellulose in a total volume of 1 ml with sterile water. Samples were incubated at 25°C for 1 hr.

Unbound substrate was removed by washing with sodium acetate buffer. Any remaining substrate bound to the resin/expansin solution was resuspended with 666 μl concentrated sulphuric acid, 5 μl phenol and made up to a total volume of 1 ml with sterile water to be measured by colorimetric microdetermination of absorbance at 490 nm in the presence of phenol and sulphuric acid.

4.3 RESULTS

The aim of this work was to clone complementary DNAs in constructs that allowed expression of three expansin genes in *E. coli* with the expectation that they could be used for functional assays.

The BL21 Star™(DE3)pLysS *E. coli* cells were used in conjunction with the pDEST™17 expression vector because they are high performance BL21 hosts designed for improving protein yield in a T7 promoter-based expression system. Whilst it was probable that this system would be unsuccessful at expressing Domain 1 of *HvEXPB5* due to the disulfide bonds found in this domain, it was expected to be a useful system to express Domain 2, which has less complex features in its tertiary structure.

Expression constructs for *HvEXPA1*, *HvEXPB5* and *HvEXPB15* were successfully generated and soluble protein expressed. Limited protein was purified as it was observed during IMAC that protein was detected *via* Western analysis in the unbound fraction. The IMAC protocol was adjusted to limit the amount of protein lost in the early stages of purification. The altered parameters of the IMAC protocol are presented in this section as well as the generation of *HvEXPA1* and *HvEXPB15* expression constructs containing a COOH-terminus dodecahistidine (His_{12}) tag in addition to the NH_2-terminus hexahistidine (His_6) tag present in the pTOOL7 vector. The addition of a His_{12} tag was to increase the binding affinity of the protein during IMAC.

4.3.1 Generation of expression constructs for HvEXPA1, HvEXPB5 and HvEXPB15

The open reading frame minus the signal peptide and native stop codon of *HvEXPA1*, *HvEXPB5* and *HvEXPB15* were successfully cloned into the entry vector. The direction of the insert was checked in a double restriction enzyme digest that yielded fragments of distinctive sizes in relation to direction of insert Figures 4.1 and Table 4.2. Plasmids that contained inserts in the correction orientation were confirmed by DNA sequencing. The nucleotide sequences obtained for the expansin cDNAs after ligation into the entry vector were the same as those presented in Chapter 2 section 2.3.3. Constructs containing cDNAs in the correct orientation were transferred *via* an LR reaction into the Gateway-enabled pTOOL7 vector Figure 4.2. Resultant plasmids were sequenced using a primer located upstream of the artificial start codon to confirm that the cDNAs were in frame with the two poly-histidine epitope tags encoded by the expression vector. The first tag immediately follows the thioredoxin tag at the NH_2-terminus and the second tag lies at the COOH-terminus followed by an artificial stop codon. The thioredoxin tag fuses a 109 aa protein to expressed sequences in pTOOL7. Translations of the *HvEXPA1*, *HvEXPB5* and *HvEXPB15* cDNAs into their

predicted amino acid sequences showed that they consisted of 251, 273 and 265 amino acids respectively with size of the recombinant proteins predicted to be 47 kDa, 51 kDa and 48 kDa respectively. The finished constructs were used to transform Origami DE3 *E. coli* competent cells.

4.3.2 Heterologous expression of expansin proteins in E. coli

Proteins of the predicted sizes for *HvEXPA1, HvEXPB5* and *HvEXPB15* were expressed after induction by IPTG and were observed by Western blot analysis (Figure 4-4). Attempts were made to increase the amount of protein in the soluble fraction by decreasing the temperature and inducing expression with IPTG at a lower optical density. However, reducing the temperature to 23°C provided only a marginal improvement in the solubility of the protein, although there was a decrease in the amount of degraded protein, as shown in Figure 4-5.

4.3.2.1 Optimisation of conditions for expression and purification of expansin proteins via IMAC

Following expression of soluble protein, purification via IMAC did not appear to be efficient, because the proteins were detected in the unbound and wash fractions (Figure 4-6). The original purification conditions used the native lysis buffer described in section 4.2.6.1 with the addition of 5 mM imidazole. The subsequent washes (x2) contained 20 mM imidazole and the protein was eluted with the same lysis buffer supplemented with 200 mM imidazole. In the Western analysis in Figure 4-6 panel B, HvEXPB5 is present in the unbound fraction (lane 3) and the supernatant of the two washes in lanes 4-5, whilst it is only just detectable in the eluted (lane 5) and concentrated samples (lane 6). Similar results were seen for HvEXPA1 and HvEXPB15 (data not shown). The His_6-tagged protein detected in the unbound fraction indicated that the tags may have been inaccessible within the protein under native conditions and so were unavailable for binding to the resin. In addition to the protein detected in the soluble fraction, high levels of expression of recombinant proteins in a bacterial system can lead to the formation of insoluble aggregates. The insoluble aggregates, known as inclusion bodies, as seen in the insoluble fractions in Figure 4-5, can be solubilised by the use of denaturing buffers that will expose the His_6 tags to facilitate binding to the cobalt column. For biochemical studies, the protein has to be renatured and refolded after elution.

The pellets of the expressed proteins were purified under denaturing conditions (section 4.2.6.2). The results of the Coomassie and Western blot in Figure 4-7 still show His-tagged protein purified under denaturing conditions present in the unbound and wash fractions.

Following purification of the solublised proteins, refolding is initiated by the removal of the denaturant. Different methods for removing the denaturant and refolding the proteins include dialysis, dilution and chromatography. Due to the nature of the expansin protein and the likelihood

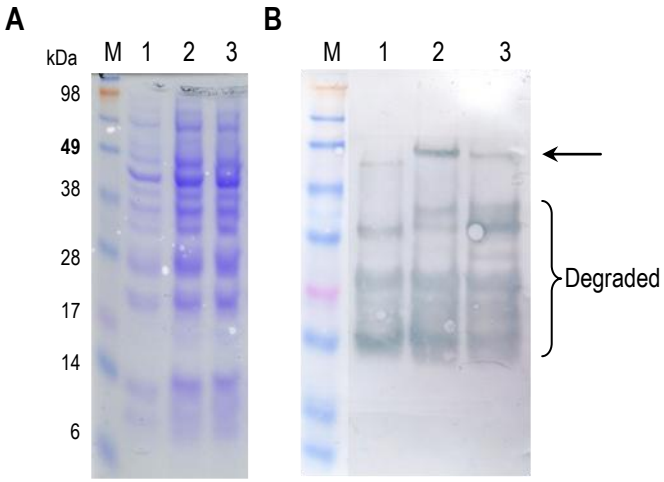


Figure 4-4 SDS-PAGE and Western analysis of the expression of HvEXPA1, HvEXPB5 and HvEXPB15 in *E. coli* at 37°C.

Total soluble protein extracts of HvEXPA1 (lane 1), HvEXPB5 (lane 2) and HvEXPB15 (lane 3) were resolved in duplicate on 12% (w/v) SDS-PAGE. Expected sizes of the proteins were 47, 51 and 48 kDa respectively. Equivalent samples were either stained with Coomassie Brilliant Blue (Panel A), or transferred onto a nitrocellulose membrane and subjected to immunoblotting using anti-his antibodies (Panel B). The expected size of the expressed proteins as determined using molecular weight markers is shown on the left hand side. Partially degraded protein is indicated by the anti-his signals observed at positions smaller than the expected size for the expected expansins.

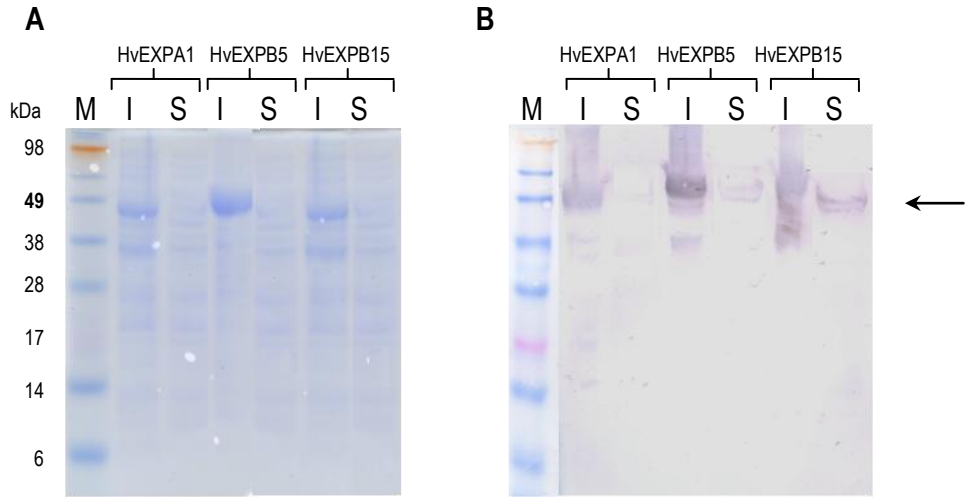


Figure 4-5 SDS-PAGE and Western analysis of expression of HvEXPA1, HvEXPB5 and HvEXPB15 in *E. coli* at 23°C.

Total soluble (S) and insoluble (I) protein extracts of HvEXPA1, HvEXPB5 and HvEXPB15 were resolved in duplicate on 12% (w/v) SDS-PAGE. Expected sizes of proteins were 47, 51 and 48 kDa respectively. Equivalent samples were either stained with Coomassie Brilliant Blue (Panel A), or transferred onto a nitrocellulose membrane and subjected to immunoblotting using anti-his antibodies (Panel B).

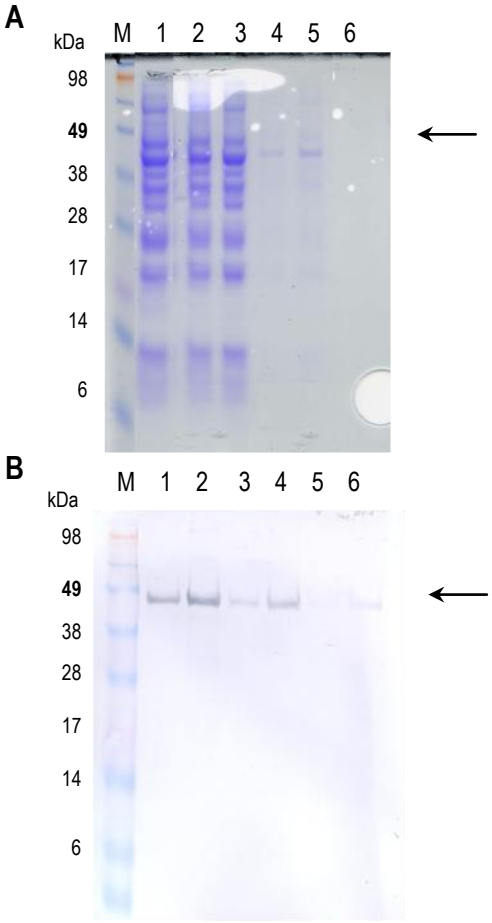


Figure 4-6 SDS-PAGE and Western analysis of purified HvEXPB5 protein.

Following extraction of soluble protein (lane 1), HvEXPB5 was purified via IMAC. Panel A represents Coomassie stained SDS-PAGE of samples from each of the purification steps. Panel B shows the same samples by Western analysis. The purification conditions used the same native lysis buffer used to extract the protein as described in section 4.2.6.1 with the addition of 5 mM imidazole. Lane 2 indicates protein present in the unbound fraction of the purification process. The subsequent washes (x2; lanes 3 and 4) contained 20 mM imidazole and the protein was eluted with the same lysis buffer supplemented with 200 mM imidazole in lane 5 and concentrated sample in lane 6. The size of the expressed proteins as determined using molecular weight markers is shown on the left hand side.

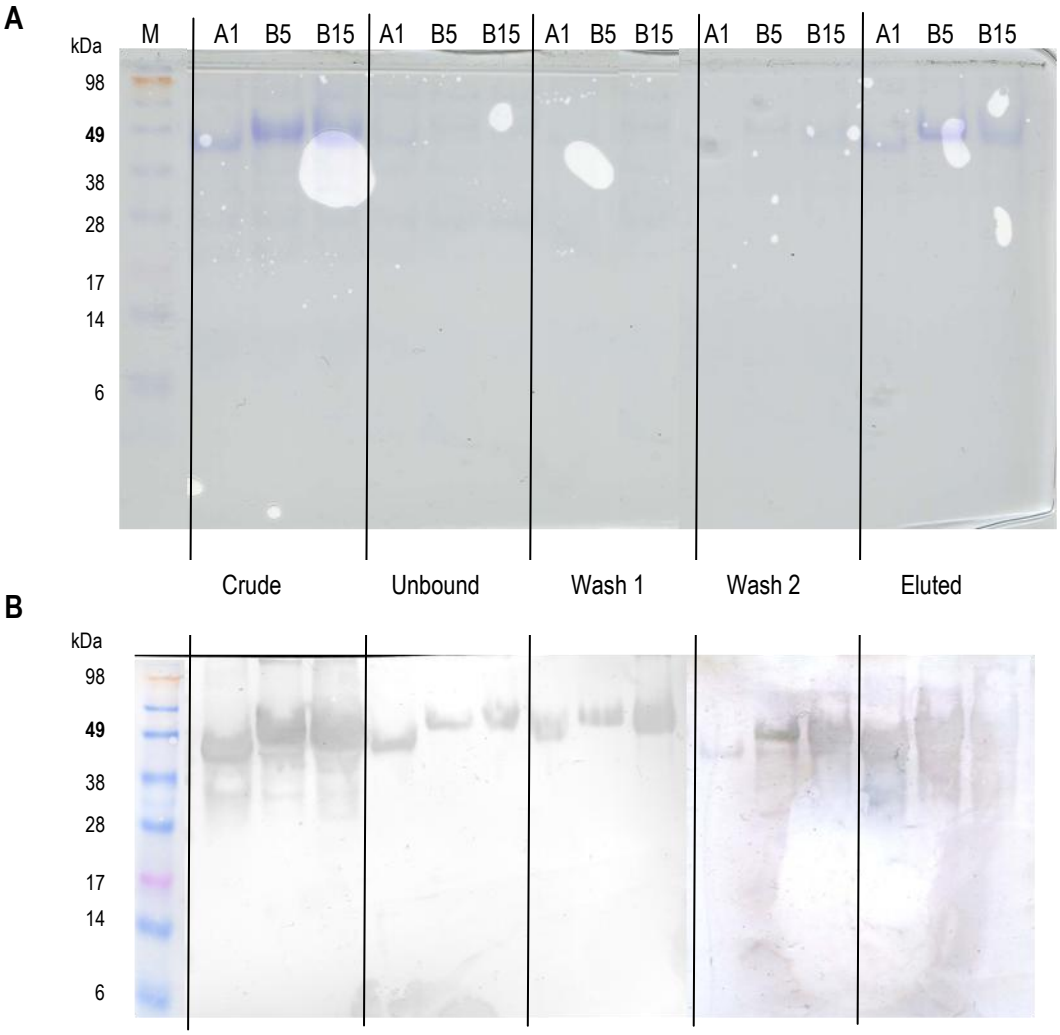


Figure 4-7 SDS-PAGE and Western analysis of purified expressed barley expansin proteins via IMAC under denatured conditions.

Expressed HvEXPA1 (A1), HvEXPB5 (B5) and HvEXPB15 (B15) proteins were solubilised under denaturing conditions and purified via IMAC under the same denaturing conditions as described in section 4.2.6.2. Expected sizes of expressed proteins were 47, 51 and 48 kDa respectively. Panel A represents Coomassie stained SDS-PAGE of samples from each of the purification steps. Panel B shows the same samples by Western analysis. The approximate size of the expressed proteins as determined using molecular weight markers is shown on the left hand side.

that it will bind to cellulose, dialysis tubing and chromatography columns that use cellulose or polysaccharide-based materials were not desirable for experiments to refold the solubilised proteins.

4.3.3 Generation of additional HvEXPB5 expression constructs

In an attempt to increase the exposure of the His tag to encourage binding during purification, expansin expression constructs containing a dodecahistidine (His_{12}) tag at the COOH-terminus were generated. HvEXPA1, HvEXPB5 and HvEXPB15 constructs with a His_{12} tag at the COOH-terminus were generated as described in section 4.2.2 with a final product size of 722, 777 and 753 bp respectively (data not shown). Sequencing results of the HvEXPA1-His_{12}-tagged construct revealed the addition of only eleven histidines (H) before the stop codon. Irrespective of this, both HvEXPA1 and HvEXPB15 were expressed as described in section 4.2.3. Initial analysis by Western blots showed no soluble protein expressed (data not shown) for these two constructs, whilst soluble HvEXPB5-His_{12}-tagged protein was detected by Western blot only (Figure 4.8). Purification of the soluble protein was as described in section 4.2.6.1, with a reduction of imidazole from 5 mM and 20 mM in the lysis and wash buffers, respectively, to no imidazole in the lysis and wash buffers and 5 mM in the third and final wash buffer respectively. The Western blot results of the purified protein with the reduction of imidazole in the lysis and wash solutions still detected protein in the unbound fraction (Figure 4-8, Panel B, lane 3). Despite this, attempts were made to elute (lane 6), further concentrate and desalt the protein (lane 7), but no purified protein was seen on the Coomassie stained SDS-PAGE (Figure 4-8, Panel A) after this procedure.

Purified HvEXPB5-His_{12} tagged protein was quantified using the Bradford assay as described in section 4.2.7. The concentration was determined to be 0.0225 μg/ml and the protein was tested for activity as described in section 4.2.8.1. A further attempt was made to increase the solubility of *HvEXPB5* by expressing each domain of the protein separately.

4.3.3.1 Heterologous expression of the two domains of HvEXPB5 in E. coli

Fragments predicted to encode Domain 1 and Domain 2 of *HvEXPB5* (Figure 4-9) were successfully cloned into the entry vector. The insert direction and sequence were confirmed. Constructs containing the cDNA in the correct orientation were transfered *via* LR reactions into both pTOOL7 and pDEST™17 vectors. The presence of an insert for both expression vectors were confirmed by restriction enzyme digest and integrity of expression constructs checked by DNA sequencing. Following confirmation of correct translational reading frame, the pTOOL7 constructs were used to transform Origami DE3 *E. coli* competent cells and pDEST17 constructs were used to transform BL21 Star™(DE3)pLysS *E. coli* cells. The predicted size of the HvEXPB5-Domain 1 protein was 37

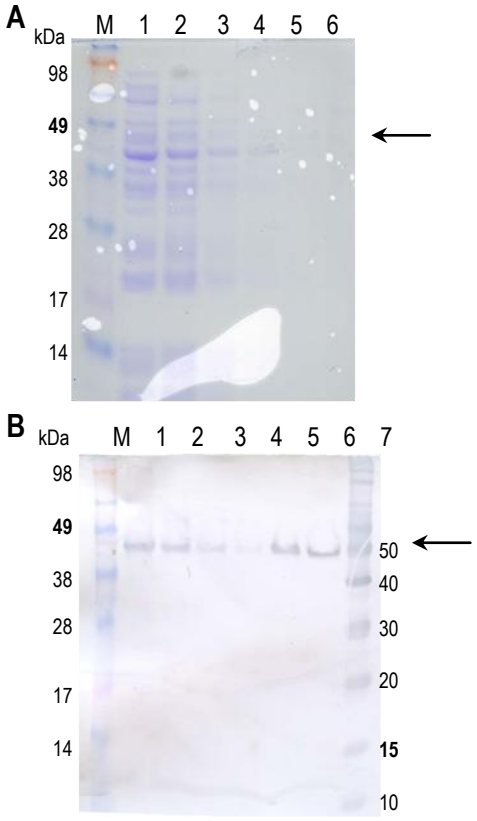


Figure 4-8 SDS-PAGE and Western analysis of purified expressed HvEXPB5-His12 tagged protein via IMAC.

Following extraction of soluble protein (lane 1), HvEXPB5 was purified via IMAC. Panel A represents Coomassie stained SDS-PAGE of samples from each of the purification steps. Panel B shows the same samples by Western analysis. The purification conditions used the same native lysis buffer used to extract the protein as described in section 4.2.6.1. Lane 2 indicates protein present in the unbound fraction of the purification process. The subsequent washes (x2; lanes 3 and 4) contained 5 mM imidazole and the protein was eluted with the same lysis buffer supplemented with 200 mM imidazole in lane 5 and concentrated sample in lane 6. The approximate size of the expressed proteins as determined using molecular weight markers is shown on the left hand side and a BenchMark His-tagged Protein standard in lane 7 of the Western blot

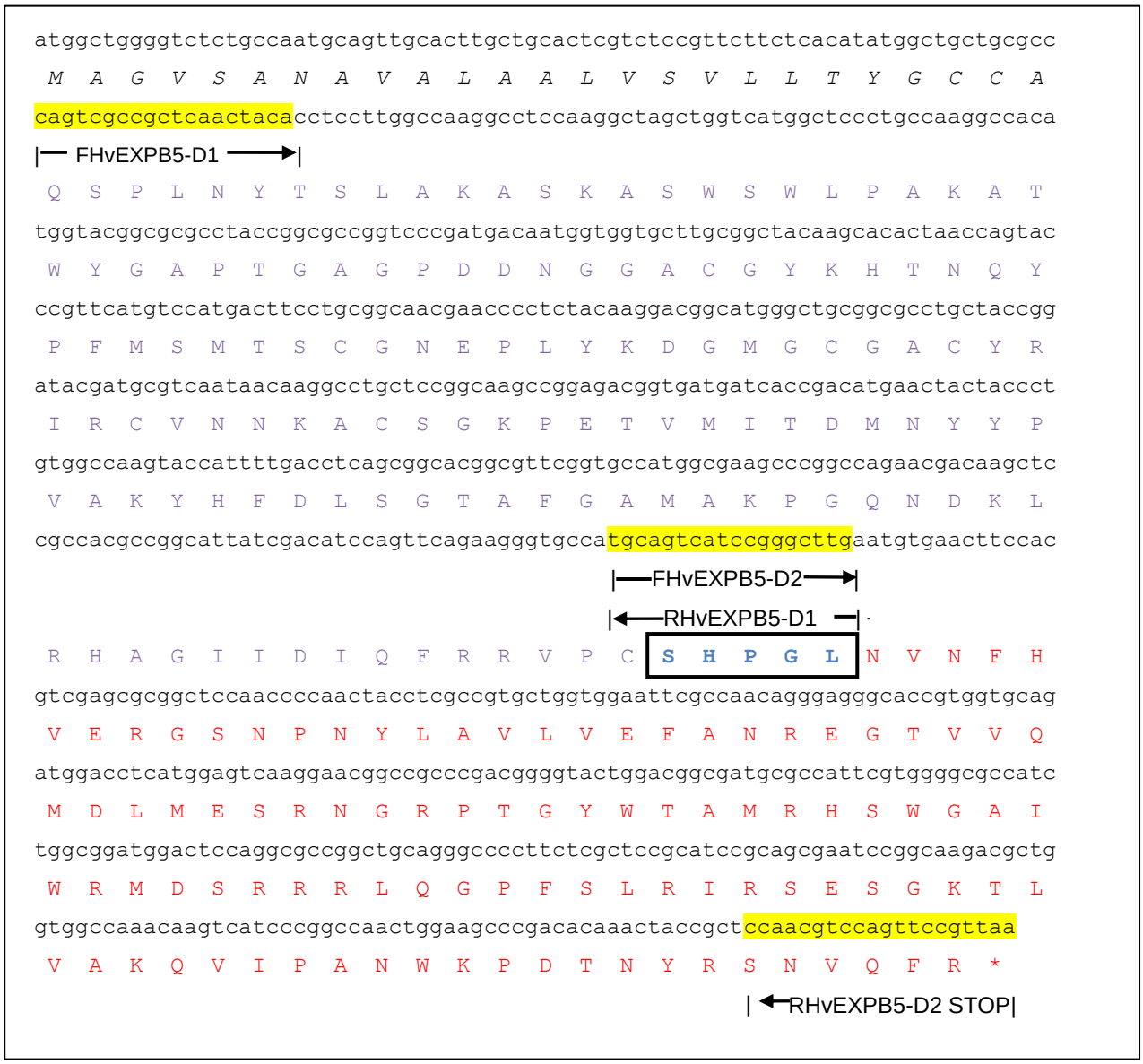


Figure 4-9 The nucleotide and deduced amino acid sequence of *HvEXPB5*.

The two domains of *HvEXPB5* are represented in purple (Domain 1) and red (Domain 2) text. Primers which were used to isolate each domain for protein expression are highlighted and arrows indicate their orientation. The linker between the domains is shown in blue and outlined. The *italicised* sequence represents the predicted signal peptide.

kDa and 19 kDa in pTOOL7 and pDEST17 vectors respectively and for HvEXPB5-Domain 2 the protein size was predicted to be 34 kDa and 16 kDa in pTOOL7 and pDEST17 vectors respectively.

The transformed cells were grown in LB media as described in section 4.2.2 and proteins viewed on a polyacrylamide gel (data not shown) and Western blot (Figure 4-10). Proteins of the predicted size for Domain 2 were expressed by both constructs and were soluble in the phosphate buffer as shown in Figure 4-10 lanes 5 and 9. Purification by IMAC and a pilot binding study were conducted on the HvEXPB5-Domain 2 protein expressed by the pTOOL7 construct.

4.3.3.2 Expansin Activity Assays

The results of the activity assay with the purified HvEXPB5-His_{12}-tagged protein were inconsistent. Overall, the reaction mix with the protein present gave a slightly higher reading of total sugars released than the control reaction containing cellulase only.

The single attempt at a binding assay with semi-purified HvEXPB5-Domain 2 failed to produce a colour change after the addition of sulphuric acid and phenol to various solutions of oat spelt xylan, birchwood xylan and barley (1,3;1,4)-β-glucan.

Figure 4-10 Western analysis of heterologously expressed Domain 1 and Domain 2 of HvEXPB5 in two *E. coli* cell lines detected using the anti-His antibody.

pTOOL7 and pDEST17 constructs containing fragments predicted to encode Domain 1 and domain 2 of *HvEXPB5* were expressed in one of two bacterial lines as described in section 4.2.3 and indicated below. Soluble protein of the predicted size is indicated by the red circles.

Lane	Sample (construct/bacterial line)	Fraction	Predicted protein size (kDa)
1	SeeBlue Plus2 Pre-Stained Standard	-	
2	HvEXPB5 Domain 1 in pTOOL7/origami	Insoluble	37
3	HvEXPB5 Domain 1 in pTOOL7/origami	Soluble	
4	HvEXPB5 Domain 2 in pTOOL7/origami	Insoluble	**34**
5	**HvEXPB5 Domain 2 in pTOOL7/origami**	**Soluble**	
6	HvEXPB5 Domain 1 in pDEST17/BL21 star	Insoluble	19
7	HvEXPB5 Domain 1 in pDEST17/BL21 star	Soluble	
8	HvEXPB5 Domain 2 in pDEST17/BL21 star	Insoluble	**16**
9	**HvEXPB5 Domain 2 in pDEST17/BL21 star**	**Soluble**	
10	BenchMark His-tagged Protein Standard	-	

4.4 DISCUSSION

In this Chapter experiments are described where constructs containing three members of the barley expansin family were generated for recombinant protein expression, purification and functional analysis. To date only partially purified native expansins have been used in studies for determining their mechanism of action. Advantages in using native protein ensure correct processing and posttranslational modifications and thus active protein. However, some members of the gene family that are found in low abundance are difficult to extract, or conditions of extraction from the plant are harsh enough to result in denatured protein (Lee and Choi, 2005). Previous attempts at recombinant protein expression have been unsuccessful due to issues associated with incorrect folding, aggregation and hyperglycosylation (Yennawar *et al.*, 2006). With careful consideration of the features present in the barley expansin members chosen for expression, a suitable expression system was selected to cater for these characteristics to yield a correctly folded and consequently active protein for functional analysis. These considerations are presented below.

Even though the α- and β-expansin families share on average only around 20-25% amino acid sequence identity, they are similar in size and have a number of conserved motifs. Their mature protein contains two domains and their predicted secondary structures are up to 75% identical (Cosgrove *et al.*, 1997). The degree of amino acid sequence identity between the α-expansins and β-expansins is highest in Domain 1 at around 30% (Cosgrove, 2000a). Domain 1 shares a number of conserved cysteines that form disulfide bridges (Cosgrove, 1997). One of two unique differences between the α- and β-expansins in Domain 1 is a highly conserved 14 amino acid insert found in the α-expansins (Figure 2-5). This includes two conserved cysteine residues, which suggests that an additional disulfide bridge is present in α-expansins. Therefore, the presence of a number of cysteines required a system that could promote the correct folding to allow the formation of disulfide bonds. The impact of an additional pair of cysteine residues in the insert unique to α-expansins thus adds to the complexity of expansin protein expression. Gateway enabled pET32a vector (pTOOL7) was used, as this vector carries a fusion tag that enhances the formation of disulfide bonds in the cytoplasm. This vector was coupled with the Origami bacterial host that contains features that greatly enhance disulfide bond formation (LaVallie *et al.*, 1993). These choices provided the greatest probability of expressing soluble, correctly folded protein that was likely to be active in the functional assay. In addition, the pTOOL7 vector contained a polyhistidine sequence consisting of six histidine residues at both the NH_2- and COOH-termini to allow for purification by IMAC.

The first recombinant expansin proteins produced at 37°C were partially degraded as indicated by a number of anti-his signals observed at positions smaller than the molecular mass expected for the

expansins (Figure 4-4, Panel B). Decreasing the temperature at induction has been known to increase solubility (Schein and Noteborn, 1988) and thus was the next approach taken. Soluble protein was successfully expressed for each of the three barley expansins at 23°C. The inclusion of the His_6 tag at both the NH_2- and COOH-termini enabled the protein to be detected with anti-His antibodies as shown in Figure 4.6 Panel B. Soluble HvEXPA1, HvEXPB5 and HvEXPB15 were easily detected *via* Western blot (Figure 4.6 Panel B). Despite the apparent strength of the anti-his signal on the Western blot bands and the correspondence with the expected molecular mass of the expansins, the proteins were never observed on polyacrylamide gels stained with Coomassie Brilliant Blue (Figure 4.6, Panel A), suggesting that expression of expansins in *E.coli* only occurred at relatively low levels.

Following successful expression, IMAC was used to purify the protein. Affinity tags facilitate the purification of proteins from heterologous expression systems but the presence of the tag does not always guarantee a successful purification of the protein of interest. For instance, the His_6-tag is sometimes buried within the protein, which prevents it binding to the metal affinity chromatography column (Terpe, 2003). Here, most of the expansin protein was detected in the unbound fraction and any bound protein was easily eluted by the less stringent wash buffers containing low concentrations of imidazole (Figure 4-6). The detection of the His_6-tagged protein in the unbound fraction indicated that the His_6-tags of the expressed protein may have been inaccessible within the protein and so were unavailable for binding to the resin. Optimisation of the purification protocol by reducing the imidazole concentrations did little to improve the purification process.

The next step was to expose the bacterial pellets to denaturing conditions to try and unravel the protein and expose the His_6-tags to facilitate binding during purification. This has the disadvantage that the purified proteins have to be renatured and refolded, thus returning the protein to an active state for the functional activity assays. There are a number of methods available to achieve this, including dialysis, where the concentrated protein is dialysed against a refolding buffer such that the concentration of the denaturant decreases with buffer exchange. The limitation of this method with these particular proteins is that the dialysis tubing is made of derivatized cellulose and therefore there is a real risk that the refolded expansin protein will bind to the dialysis tubing before recovery of the renatured protein. The same risks exist for other processes used for renaturing these proteins, such as using columns packed with cellulose, branched glucans or a beaded-form of a polysaccharide polymer.

Increasing the affinity tag to 12 histidine residues at the COOH-terminus of each protein construct met with some success as the HvEXPB5-His_{12} tagged protein was not only soluble, but was purified and used in an activity assay. However the HvEXPA1 and HvEXPB15 expression constructs failed to express soluble protein.

The activity assay was designed to test a hypothesis based on the observation that transcript patterns of certain barley expansins and cellulases are highly correlated (Chapter 3). Cosgrove (2001) also proposed that expansins increase the accessibility of cellulose to cellulases by weakening the non-covalent binding between wall polysaccharides. The assay for expansin activity failed to produce consistent outcomes. As with the protein expression work, this activity assay would have benefited from some optimisation. The Domain 2 protein for the binding studies was successfully expressed in both vector/bacterial systems used (Figure 4-10) relatively easily, providing a promising avenue for further work, as discussed in the next Chapter.

4.5 SUMMARY

The data presented in this Chapter details the isolation of the near full length cDNA of three barley expansins for heterologous expression and purification for the purpose of examining their mechanism of action and/or binding substrate. The results of the correlation studies of co-transcribed expansins with cellulase and XTHs in Chapter 3 provided a testable hypothesis for the design of the activity assay to examine the action of expansin in the presence of cellulase and XTH. However, the overall yields of soluble expansin protein were generally low and insufficient for the detailed characterisation of expansin activity.

Although the direct approach used in this Chapter towards the functional analysis of expansin proteins was via heterologous expression, the choice of expression system and the nature of the expansin protein made this a challenging task. To encourage correct folding and thus active protein, a Gateway-enabled pET32a vector (pTOOL7) paired with an Origami host was used, to facilitate the formation of disulfide bonds. This system only provided partially soluble expansin protein, which failed to bind to the metal affinity chromatography column.

Increasing the affinity tag proved successful for expression and purification for HvEXPB5 construct only of the three expansin cDNA selected for heterologous expression. The purified HvEXPB5-His_{12}-tagged protein was used in an activity assay, however, the inconclusive results from the activity assay with this recombinant expansin protein, indicated that a new approach to define the functions of expansins is required.

A set of constructs to allow expression of the two domains of HvEXPB5 for expression in a bacterial system were also tested to determine domain function. Domain 2 was successfully expressed and a single attempt to incubate the partially purified protein with solutions of xylan, (1,3;1,4)-β-glucan and carboxymethyl cellulose failed to produce a result.

Based on the mixed success of heterologous expression and purification of three barley expansins in various constructs and bacterial lines presented in this Chapter, there exists an opportunity for optimisation of heterologous expression conditions. These results also indicate that each member of the barley expansin family can produce varying results that present the possibility that the right candidate under the right conditions may express sufficient quantities of soluble protein suitable for purification and subsequent activity assays. The final Chapter further explores this option of heterologous expression of expansin proteins and presents some alternative methods for the functional analysis of the barley expansin genes.

CHAPTER 5

SUMMARY AND FUTURE DIRECTIONS

5 SUMMARY AND FUTURE DIRECTIONS

The overall aim of this project was to characterise the expansin gene family from barley. This family had been investigated in *Arabidopsis*, rice, maize and wheat, but no systemic identification, description or characterisation of expansin genes in barley had been reported. Emerging techniques of functional genomics provided new opportunities to confidently tackle the research objectives and to provide functional insights not possible in the previous studies. A summary of the results of the characterised barley expansin family in this thesis is presented in the following sections.

Firstly the identification of the barley expansin genes on the basis of sequence homology with putative expansins in the public EST databases (Chapter 2) was undertaken. Secondly, the mRNA transcript levels of barley expansin genes were measured in a barley developmental tissue series. The transcript analysis presented in Chapter 3 examined the pattern of expansin gene expression as part of the first step in elucidating the function of individual expansins. After demonstrating co-ordinately transcribed groups within the barley expansin family and the co-transcription of expansin genes with other genes such as cellulases and XTHs, the final stage of this project was the heterologous expression of isolated cDNA clones of three barley expansin genes in order to examine the function of their encoded proteins. The behaviour of the expansin proteins in the presence of proteins known to be involved in cell wall modification was presented in Chapter 4.

A total of 34 partial or complete barley expansin gene sequences were assembled from the public EST databases. By assembling the ESTs into putative contigs, it was possible to predict the full length coding sequences of 26 of the 34 barley expansin and expansin-like genes identified. The amino acid sequence alignments of these predicted proteins showed that the sequences contained key features of α- and β-expansins and the expansin like-α group as described by Cosgrove (1999; 2000b) and Li *et al*. (2002). The gene sequences from the barley were subsequently used for phylogenetic analysis with the sorghum and *Brachypodium* expansin genes identified by in this study, and with rice expansin genes. The *in silico* analysis of the sorghum and *Brachypodium* genome identified 79 and 62 expansin, members respectively. The comparison of the barley expansin genes against grass species whose complete genomes have been sequenced suggested that the barley gene family may contain at least twice the number of genes identified in this study. From the 34 barley genes identified, eight near full-length barley expansin cDNAs were amplified and sequenced; these encompassed one α-expansin and seven β-expansins.

Transcript studies were performed in an attempt to correlate transcript levels with possible functions of the gene. These analyses were performed using Q-PCR and *in silico* analysis of microarray data. The results of the transcript analysis of the barley expansins across a barley developmental tissues series showed that there was a dominant group of highly transcribed genes found within each of the barley α- and β-expansin subfamilies. This suggested that a core group of widely transcribed expansins provide stable and ongoing expression during plant growth and that cell-specific expression and specialised roles in different plants developed during the evolution of new cell types. Further tissue transcript analyses with cell wall-related genes identified seven co-ordinately transcribed groups of genes from within the α- and β-expansin groups. The observation of correlated co-transcription of cell wall-related genes with the expansin gene family provided a testable hypothesis to explore the functional relationship between expansin genes and available cell wall modifying enzymes such as cellulases and XTHs. Therefore, the final stage of this study was the heterologous expression and purification of three expansin cDNAs in a bacterial host for the purpose of examining their mechanism of action.

Partially soluble proteins were successfully expressed for each of the three barley expansins and products that corresponded to the expected molecular masses were easily detected *via* Western blot analyses. However, the expressed proteins failed to bind to the metal affinity column during purification. The introduction of a dodecahistidine tag to HvEXPA1 and HvEXPB15 expression constructs failed to produce increased amounts of soluble protein, but the addition of the same tag on the HvEXPB5 sequence did result in higher levels of soluble protein that was successfully purified and tested for activity. Protein activity was assayed by incubating purified protein with a solution of microcrystalline cellulose and a commercially available cellulase. The assay was designed to measure the effect expansin protein had on the accessibility of the cellulose to the cellulase, which would in turn be measured by increased hydrolysis of the crystalline cellulose. However, the results of this assay revealed no convincing difference between the samples containing expansin protein and those without.

To further investigate expansin substrate specificity, constructs were generated to express each domain of *HvEXPB5* separately, in particular Domain 2 that is predicted to be the binding domain of the expansin protein. The incubation of partially purified HvEXPB5-Domain 2 with solutions of oat spelt xylan, birchwood xylan, (1,3;1,4)-β-glucan and carboxymethyl cellulose failed to show any binding. Due to time constraints, optimisation of this binding assay was not completed. However as Domain 2 was expressed relatively easily in both vector/bacterial systems used, this provides a promising avenue for further work to determine the substrate specificity of this expansin.

The inconclusive results obtained using the bacterial heterologous systems and subsequent functional assays indicate that further optimisation is required or that a new approach needs to be developed to define the functions of the barley expansins. Some alternative approaches to facilitate the functional analysis of expansin proteins are discussed below.

The use of alternative heterologous expression systems such as yeast or cell-free systems may provide the right folding environment for activity and sufficient quantities for functional studies. Further work could include the trial of both these systems for the recombinant expression of α-expansins, which do not require post-translational modifications. Previous attempts to express active expansins in various eukaryotic recombinant systems have resulted in hyperglycosylation (Yennawar *et al.*, 2006). In addition to the yeast heterologous system, systems using vascular plants as the host (Voinnet *et al.*, 2003; Marillonnet *et al.*, 2005) may provide the right post-translation modification environment in further experiments to express barley β-expansins. Future work would include selecting alternative barley β-expansins with and without the predicted N-linked glycosylation site for expression in these systems.

Due to the high number of family members studied in this thesis, the tissue transcript analyses were in progress while near full length cDNAs were being isolated. Based on the observation that the β-expansins have a dominant role in grasses (Cho and Kende, 1997b), the focus of this study was concentrated towards the β-expansins, and the first ten arbitrarily assigned β-expansins were selected for cDNA isolation. *HvEXPB15* was specifically chosen for cDNA isolation because it lacked a predicted N-linked glycosylation site. The inclusion of *HvEXPA1* for cDNA isolation was to provide a good basis for comparison of the three barley expansins expression in a bacterial heterologous expression system, which is unable to perform post-translation modifications. However, with the completion of the comprehensive tissue transcript analysis and data from the subsequent correlations of expansin transcripts with those of other cell-wall related genes (Chapter 3), the selection of gene members for heterologous expression should now be re-addressed. Future work for examining the functional relationship between expansin genes and available cell wall modifying enzymes such as cellulase and XTH would focus on those members of the barley expansin famly identified to be co-transcribed with these enzymes (Chapter 3). Therefore the barley expansin genes that would be specifically targeted for heterologous expression in future studies are *HvEXPA4-HvEXPA7, HvEXPA10, HvEXPA11* and *HvEXPB1, HvEXPB7, HvEXPB8* and *HvEXPB18.*

Heterologous protein expression would also allow the investigation of amino acid residues in and around the possible catalytic site in Domain 1 of the expansin proteins by site-directed mutagenesis. The technique of using site-directed mutagenesis to facilitate functional analysis of a bacterial expansin proteins has been successful (Georgelis *et al.*, 2011) and could be applied to plant expansins. Domain 1 of the expansin proteins has a distant but significant homology to glycoside hydrolase family 45 (GH45) proteins, most of which are fungal (1,4)-β-D-endoglucanases (Henrissat *et al.*, 1998). A histidine, phenylalanine, aspartic acid (HFD) motif that is located in the catalytic site zone of the GH45 endoglucanases (Davies *et al.*, 1993) is also conserved in most expansins, despite the apparent lack of hydrolytic activity in expansins. Superimposing the crystal structure of Domain 1 of *ZmEXPB1* onto a GH45 enzyme (Protein Data Bank ID code 4ENG) suggested that Domain 1 of *ZmEXPB1* is missing a catalytic residue that is required for (1,4)-β-D-glucan hydrolysis by GH45 enzymes (Davies *et al.*, 1993). It is also noted that there was some variation of the HFD motif in the tentative contigs of the barley expansins identified in this project and confirmed in one cDNA isolated (*HvEXPB9*). Variations of the motif have been observed in *Arabidopsis* (Li *et al.*, 2002), wheat (Liu *et al.*, 2007), rice, sorghum and *Brachypodium*. The divergences observed in this motif include, HFL, HFE, YFD, HID, HLE and RFD. Other than YFD, each divergence in the motif is observed in at least two or more species, including barley. Further work could include site-directed modifications to candidate barley expansin proteins based on the crystal structure of members from the GH45 family to incorporate residues that may complete conservation of the possible catalytic site as identified in the GH45 enzymes. This work could be divided into two candidate groups. The first group would include barley expansin genes identified with the HFD motif and would test expansin protein for catalytic activity if the missing residue was re-introduced. The second group would include testing expansin protein for catalytic activity if the missing residue was re-introduced into candidates with variations in the HFD motif.

Homology modelling of Domain 2 may provide an alternative method to determine the substrate specificity of expansin proteins. Domain 2 has some similarity to cellulose-binding domains found in certain microbial cellulases particularly with respect to the spacing of the four highly conserved tryptophans that could be important in carbohydrate binding (Linder and Teeri, 1997). McQueen-Mason and Cosgrove (1995) showed that a native purified class of expansins from cucumber (*Cucumis sativus*) seedlings was capable of binding to cellulose *in vitro*. The binding of the *CsEXPAs* increased upon coating the cellulose with various non-cellulosic polysaccharide components of the wall including barley (1,3;1,4)-β-glucan. Native β-expansin extract from maize was shown to preferentially bind to a xylan substrate and was also shown to have some binding

affinity to a xyloglucan substrate. There are two areas in the binding assay described in this study that needed to be addressed if this work was continued. Firstly, the binding assay described in Chapter 4 to test the substrate specificity of Domain 2 of HvEXPB5 required some optimisation to produce consistent results. Secondly the tissue transcript analyses (Chapter 3) could be used to determine possible specific polysaccharide substrates to test in this assay. For example, arabinoxylan, which constitutes 20% of the starch endosperm on the cell wall during the stages of developing grain at 3-5 dap and increases to 70% in the aleurone walls at 8-10 dap when the aleurone walls become visible in the immature grain suggest that arabinoxylan may be a specific substrate to test for *HvEXPB15* which was found to be most abundant in those two tissues at those developmental stages. The same principle could be applied for members of the barley α-expansin family in future experiments.

Standard approaches for studying gene function using transgenic and knockout lines present serious limitations since there is the possibility for gene redundancy in a large family such as the expansins to compensate for over- or under-expression of a single introduced transgene. The *in silico* analysis of the sorghum and *Brachypodium* genome sequences (Chapter 2) found clusters of two or more expansin genes on chromosomes with degrees of amino acid sequence identity ranging from 47 to 99%. At the time this project was performed the barley genome sequence had not been released. However, it has since become available (Mayer *et al.*, 2011) and will provide a useful resource for future research on the barley expansin family. In the meantime, knowledge that some members of the barley expansin family are co-transcribed in a tissue-specific manner, as described in Chapter 3, can guide functional studies using simultaneous double or triple knockouts generated *via* crossing individual insertional mutants. For example, future work targeting any of the highly correlated groups such as *HvEXPB5, HvEXPB11* and *HvEXPB16,* or *HvEXPA6, HvEXPB7* and *HvEXPB8*, or members from the dominate group of highly transcribed genes found within each of the α- and β-expansin subfamilies could be investigated.

Recently attention has focused on certain members of the family 61 glycoside hydrolase (GH61) that were originally classified based on measurement of very weak endo-1,4-β-glucanase activity in one family member (Karlsson *et al.*, 2001). It has been demonstrated that certain GH61 proteins lack measurable hydrolytic activity by themselves but that have been shown to act synergistically with cellulases to hydrolyse lignocellulosic biomass in the presence of various divalent metal ions (Harris *et al.*, 2010). The proposed mechanism of action of certain members of the GH61 family is principally one of an oxidoreductase. Quinlan *et al*. (2011) further demonstrated that other GH61 proteins are copper-dependant oxidases and that in the presence of molecular redox-active

cofactors such as ascorbate and gallate, lignocelluloses hydrolysis is enhanced. It is possible that expansin proteins may display similar results if investigated under the same conditions. Future experiments could include the incorporation of metal ions and cofactors described by Harris *et al*. (2010) and Quinlan *et al*. (2011) to the activity assay used in Chapter 4.

The biological relevance of this study is related to previous studies that suggest expansins act by disrupting the non-covalent hydrogen bonds between cellulose fibres or cellulose-hemicellulose composites and thereby increase the accessibility of cellulose to cellulases (Cosgrove, 2001; Wei *et al.*, 2010). The commercial significance of this interaction lies with the production of cellulosic ethanol and in the pulp and paper industries. Current technology for the conversion of lignocellulosic biomass to ethanol requires chemical or enzymatic conversion of the biomass polysaccharides to fermentable sugars. The large amount of enzymes required for the enzymatic conversion of cell-wall polysaccharides to fermentable sugars increases the costs of production. At present, plant cell-wall hydrolysis enzymes such as cellulases are expensively produced in microbial reactors. If expansin protein could be produced in a similar process and used in conjunction with cellulases the efficiency of cellulase to breakdown the cell-wall polysaccharides would be increased. This could be done in a number of ways as described above with heterologous expression, particularly with members of the expansin family identified to be co-transcribed with barley cellulases (*HvEXPA4, HvEXPA7, HvEXPA10, HvEXPA11, HvEXPB10* and *HvEXPB18*). Alternatively, further work towards creating transgenic plants that overexpress both cellulases and expansins to enhance the conversion of cellulose into fermentable sugars, would improve the pretreatment of cellulosic biomass. Potentially any of the members from the dominant group of highly transcribed genes found within each of the barley α- and β-expansin subfamilies could be selected for this work as well as those co-transcribed with the cellulases.

APPENDIX

APPENDIX A

Primers

Target Gene	Primer purpose	Primer (5'—3')	T_m °C	cDNA Template
HvEXPA13	3'RACE	GCGGCGGCGTCAGGCTGGCGGGCAATGG	81	Root
	Nested 3'RACE	CGGTGCGTGAACAACATCCTCTATTGCCTGCG	90	-
HvEXPA14	3'RACE	GCGTCGTGCGGGGCGTGCTTCACCATCG	86	Root
	Nested 3'RACE	CACCAACTTCTGCCCGCCCAACTAC	71	-
HvEXPA12	5'RACE	CTGGGTCACGGCATTTTAGT	58	Root, stem
	5'RACE PCR	AATGTCTGCGCAAACATCCA	60	-
HvEXPA8	5'RACE	ATGGTGACCCTGAAGGAGAG	56	Root
	5'RACE PCR	GTTGAGGTAGGCGAGGGAGT	58	-
HvEXLA2	5'RACE	GGTTCTTGCCCTCGTACTCGC	63	Endosperm
	5'RACE PCR	ACGGCACCCTCTTGTACTCG	59	-
HvEXLA3	5'RACE	GTGTCGTAGACCTCGCCG	57	Endosperm
	5'RACE PCR	GCCCAGACCCACTTCCCGT	64	-

Table A-1 Barley expansin gene-specific primers for attempted 3' and 5'RACE and semi nested (3') RACE PCR to complete partial barley expansin sequences.

The oligonucleotide primers for 3' RACE were designed within the following parameters, 23-28 nucleotides, 50-70% GC rich and with an annealing temperature (T_m) ≥ 65°C. 5'RACE required that the first-strand cDNA synthesis reaction was primed using an oligonucleotide complementary to a known sequence in the gene (5'RACE) followed by PCR with a nested primer (5'RACE PCR). The annealing temperatures and templates used are listed. All attempts to complete partial barley expansin sequences were unsuccessful.

Gene	Forward primer (5'—3')	Reverse primer (5'—3')	PCR size bp	Annealing temperature °C	cDNA template
HvEXPB3	F1: CTACAAGCCGAGAGTCAGCC	AGCACCAAGACGAAGACGAAG	1181	56	Root, leaf, coleoptile
Semi-nested	F2: GTGAGGAGAATGGGGAGGC	AGCACCAAGACGAAGACGAAG	1132	55	1st round PCR
HvEXPB6	TAACCAACGAGACCAGCAAG	CACACCAGTGACATAATAAACTTG	1239	52	Root
HvEXPB7	F1: CTAGCTCGAAGAAGCAACTT	ATCGACGACACGCATCACTA	1097	50	Root
Semi-nested	F2: GCAGCAATGGCGAAATCTT	ATCGACGACACGCATCACTA	1075	49	1st round PCR
HvEXPB8	F1: GGAACTCACCCAACGAACAT	ACAAAGCAGAGGAAGGCAA	1219	50	Root, leaf, coleoptile
Semi-nested	F2: AGCAGCAGCAATGGCGAA	ACAAAGCAGAGGAAGGCAA	1131	49	1st round PCR
HvEXPB12	GTGAGTAGTTTTGGAGCAGGAG	ACACGACAACAGGAGTGAAGC	>722	51	stem
HvEXPB12$_2$	CGTGGTGCCGATGAAGGC	CGATGTTCTTCCCGCCGTA	~300	53	stem

Table A-2 Primers designed for the attempted isolation of full length barley β-expansin cDNAs.

Gene-specific primers were designed in the 5' and 3' UTR of the target sequence. Product sizes in base pairs, annealing temperature and templates used are listed. Template for semi-nested reactions was a 1/10 dilution of the first round PCR reaction. All attempts to isolate full length *HvEXPB3, HvEXPB6*, *HvEXPB7*, *HvEXPB8* and *HvEXPB12* cDNA were unsuccessful.

PCR no.	Forward primer (5'—3')	Reverse primer (5'—3')	PCR size bp	Annealing temperature °C	cDNA template
1	F1: ACGGAAACGAGAAGATGGC	R1: ACGCTACCGCTACGAGTCAT	1044	51 and 54	root
2	F2: TCGCTCGCCTCCGCCTG	R1: ACGCTACCGCTACGAGTCAT	939	54	Root, leaf
3	F1: ACGGAAACGAGAAGATGGC	R2: GTGCGTGCGTGCGTAGCC	956	51	Root, leaf
4 (semi-nested)	F2: TCGCTCGCCTCCGCCTG	R2: GTGCGTGCGTGCGTAGCC	851	57	PCR no.3
5	F2: TCGCTCGCCTCCGCCTG	R2: GTGCGTGCGTGCGTAGCC	851	50+DMSO	Root
6 (semi-nested)	F2: TCGCTCGCCTCCGCCTG	R2: GTGCGTGCGTGCGTAGCC	851	48+DMSO	PCR no.5
7	F1: ACGGAAACGAGAAGATGGC	R2: GTGCGTGCGTGCGTAGCC	956	48+DMSO	root

Table A-3 Primers designed for the attempted isolation of full length barley expansin-like A1 cDNAs.

Gene-specific primers were designed in the 5' and 3' UTR of the *HvEXLA1* sequence. Product sizes in base pairs, annealing temperature and templates used are listed. Template for semi-nested reactions was a 1/10 dilution of the first round PCR reaction from previous PCR no. All attempts to isolate full length *HvEXLA1* cDNA were unsuccessful.

APPENDIX B

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Tentative           1   ATGGCTGGGGTCTCTGCCAATGCAGTTGCACTTGCTGCACTCGTCTCCGTTCTT
Isolated            1   ATGGCTGGGGTCTCTGCCAATGCAGTTGCACTTGCTGCACTCGTCTCCGTTCTT
                          M  A  G  V  S  A  N  A  V  A  L  A  A  L  V  S  V  L

Tentative          55   CTCACATATGGCTGCTGCGCCCAGTCGCCGCTCAACTACACCTCCTTGGCCAAG
Isolated           55   CTCACATATGGCTGCTGCGCCCAGTCGCCGCTCAACTACACCTCCTTGGCCAAG
                      L  T  Y  G  C  C  A  Q  S  P  L  N  Y  T  S  L  A  K

Tentative         109   GCCTCCAAGGCTAGCTGGTCATGGCTCCCTGCCAAGGCCACATGGTACGGCGCG
Isolated          109   GCCTCCAAGGCTAGCTGGTCATGGCTCCCTGCCAAGGCCACATGGTACGGCGCG
                          A  S  K  A  S  W  S  W  L  P  A  K  A  T  W  Y  G  A

Tentative         163   CCTACCGGCGCCGGTCCCGATGACAATGGTGGTGCTTGCGGCTACAAGCACACT
Isolated          163   CCTACCGGCGCCGGTCCCGATGACAATGGTGGTGCTTGCGGCTACAAGCACACT
                          P  T  G  A  G  P  D  D  N  G  G  A  C  G  Y  K  H  T

Tentative         217   AACCAGTACCCGTTCATGTCCATGACTTCCTGCGGCAACGAACCCCTCTACAAG
Isolated          217   AACCAGTACCCGTTCATGTCCATGACTTCCTGCGGCAACGAACCCCTCTACAAG
                          N  Q  Y  P  F  M  S  M  T  S  C  G  N  E  P  L  Y  K

Tentative         271   GACGGCATGGGCTGCGGCGCCTGCTACCGGATACGAGGA------GAAACAGCC
Isolated          271   GACGGCATGGGCTGCGGCGCCTGCTACCGGATACGATGCGTCAATAACAAGGCC
                          D  G  M  G  C  G  A  C  Y  R  I  R  G        E  T  A
                          D  G  M  G  C  G  A  C  Y  R  I  R  C  V  N  N  K  A

Tentative         319   TGCTCCGGCAAGCCGGAGACGGTGATGATCACCGACATGAACTACTACCCTGTG
Isolated          325   TGCTCCGGCAAGCCGGAGACGGTGATGATCACCGACATGAACTACTACCCTGTG
                          C  S  G  K  P  E  T  V  M  I  T  D  M  N  Y  Y  P  V

Tentative         373   GCCAAGTACCATTTTGACCTCAGCGGCACGGCGTTCGGTGCCATGGCGAAGCCC
Isolated          379   GCCAAGTACCATTTTGACCTCAGCGGCACGGCGTTCGGTGCCATGGCGAAGCCC
                          A  K  Y  H  F  D  L  S  G  T  A  F  G  A  M  A  K  P

Tentative         427   GGCCAGAACGACAAGCTCCGCCACGCCGGCATTATCGACATCCAGTTCAGAAGG
Isolated          433   GGCCAGAACGACAAGCTCCGCCACGCCGGCATTATCGACATCCAGTTCAGAAGG
                          G  Q  N  D  K  L  R  H  A  G  I  I  D  I  Q  F  R  R

Tentative         481   GTGCCATGCAGTCATCCGGGCTTGAATGTGAACTTCCACGTCGAGCGCGGCTCC
Isolated          487   GTGCCATGCAGTCATCCGGGCTTGAATGTGAACTTCCACGTCGAGCGCGGCTCC
                          V  P  C  S  H  P  G  L  N  V  N  F  H  V  E  R  G  S

Tentative         535   AACCCCAACTACCTCGCCGTGCTGGTGGAATTCGCCAACAGGGAGGGCACCGTG
Isolated          541   AACCCCAACTACCTCGCCGTGCTGGTGGAATTCGCCAACAGGGAGGGCACCGTG
                          N  P  N  Y  L  A  V  L  V  E  F  A  N  R  E  G  T  V

Tentative         589   GTGCAGATGGACCTCATGGAGTCAAGGAACGGCCGCCCGACGGGGTACTGGACG
Isolated          595   GTGCAGATGGACCTCATGGAGTCAAGGAACGGCCGCCCGACGGGGTACTGGACG
                          V  Q  M  D  L  M  E  S  R  N  G  R  P  T  G  Y  W  T

Tentative         643   GCGATGCGCCATTCGTGGGGCGCCATCTGGCGGATGGACTCCAGGCGCCGGCTG
Isolated          649   GCGATGCGCCATTCGTGGGGCGCCATCTGGCGGATGGACTCCAGGCGCCGGCTG
                          A  M  R  H  S  W  G  A  I  W  R  M  D  S  R  R  R  L

Tentative         697   CAGGGCCCCTTCTCGCTCCGCATCCGCAGCGAATCCGGCAAGACGCTGGTGGCC
Isolated          703   CAGGGCCCCTTCTCGCTCCGCATCCGCAGCGAATCCGGCAAGACGCTGGTGGCC
                          Q  G  P  F  S  L  R  I  R  S  E  S  G  K  T  L  V  A

Tentative         703   AAACAAGTCATCCCGGCCAACTGGAAGCCCGACACAAACTACCGCTCCAACGTC
Isolated          757   AAACAAGTCATCCCGGCCAACTGGAAGCCCGACACAAACTACCGCTCCAACGTC
                          K  Q  V  I  P  A  N  W  K  P  D  T  N  Y  R  S  N  V

Tentative         805   CAGTTCCGTTGA
Isolated          811   CAGTTCCGTTGA
                          Q  F  R  -
```

Figure B-1 The tentative nucleotide, isolated nucleotide sequence and deduced amino acid sequences of *HvEXPB5.*

The tentative nucleotide sequence obtained for *HvEXPB5* from public EST databases and the cDNA sequence isolated in this project were aligned and show the isolated cDNA sequence contains six additional nucleotides that code for the conserved cysteine in position 103 as highlighted.

Appendix B

Assembled full and partial length nucleotide barley expansin sequences

Red indicates start codon and bold underlined indicates stop codon.

Barley α-expansin

HvEXPA1

>TC131916

CGGCACGAGGGCCATTTCGAGTCTAGCTAGCTAGCTTTAGACACCATATCCCTCGAAAGCTAAGCTAGCTTGCTGAGGAT
ACAAGGAAGCTCCCTCCCTCCCTCCCAGCAATGGCCGCTGCCGGCGCTCTCTTCTTCGTCTTCTCCTCCTTGTGCCTCCT
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GGCAAGGACAAGTGGATCGAGCTCAAGGAGTCGTGGGGAGCCGTTTGGAGGATCGACACACCTGACAAGCTCATCGGCCC
ATTCTCCGTCCGCTACACCACCGAGGGCGGCACCAAGACCGTCGCCGAGGACGTCATCCCCGAGGGCTGGAAGCCCGACA
CCTCCTACGAGACCAAG**TGA**AGTCAAGAACAAGATGCATGACTCTTCTCTTCCGGCCACCTTAATTCATTCATTCATTTT
GGCTCAAGAAGAAGAAGAAGAAACCAGCTAGACGCACATATCATATAGGAGTCCTGCATCTGCATGCATGACGAAGGTCA
TTCGTGCTGTCTGTATAGGGAGAGGGTCATTCATGCATGGACACCTAATACTTTGAGGTCCCTAACGAAGGAGGCAACCA
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CAGATTTCGTTAGATGTTTTAAACATTAATGAGGTCCTTTGTTCAAGAGTAATACATTATTTAGACCTC

HvEXPB18

>BY848215/BY869303/Hv.30903

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CGTGCTCGTCGCGCCCGTCCGTTGTGCGGCCAACTACGACACCTCCGCCGCCAGATCCTACAACTCCGGCTGGCTCCCCG
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AAGTACCCCTTCTCCTCCATGACCTCCTGCGGCAACGAGCCTCTCTTCGACGGCGGCGCCGGCTGCGGCTCATGCTACGA
GATCCGATGCGTGGCCGCCAACAACCCTTCCTGCTCCGGCCAGCCGCGGACGGTGGTGATCACCGACATGAACTACTACC
CCGTGGCCAGGTACCACTTCGACCTCAGCGGCACCGCGTTCGGAGCCATGGCCAAGTACGGCCTCAACGACAAGCTCCGC
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CGCGGGCTGCAGGGGCCCTTCTCCATGCGCATCACCAGCGACTCCGGGAAGACGCTGGTGGCCAACAATGTCATCCCGGC
CTACTGGCAGCAGGACAGAGCATACTGGTCCAACGTACAGTTCTATTGATTTTGGCCTAATTACCTATTTCTGTGATCGA
TCGTTGATTCTCCCGCATTTCTCCTCTCGTTCATTGTGAGTAATAAGGCCTTGTACAATGTAATGCAAGGTCTATAGGAA
CGTGTTTGTAAAAAGAAACCGGTACTTAGCTGCGTCTATTTGTATTGTCCTAGCACATAAGCTTACTTAC

Barley Expansin-like A

HvEXLA1

>TC131209

CTGGCTAGCTCCTCTCTCTTTCTCTCTCTCTCTCTCTCTGAGTTTCCCTCTGCTTCATCAAGCAAGCGAACGACCCACCG
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CGCCGCTCCTCTTCCTCTTCTTCCTCTTCTGCTCCCTCCCGTCGCTCGCCTCCGCCTGCGACCGCTGCGCCCGCCACTCC
AAGGCCGCCTTCTACACCTCCTCGCTCACCCTCGCCGCCGGCTCCTGCGGGTATGGCACGGCGGCCGCCTCCTTCAACGG
CGGCCTGCTCGCCGCCGCCGGCACGGCCCTGTACCGCGGCGGCGTCGGCTGCGGCGCGTGCTTCCAGGTGAGGTGCAAGG
ACAAGAAGCTCTGCAGCGGTGCCGGCGCCAGGGTGGTCGTGACGGACCGCGCGAGGACGAGGACGAACCGCACGGACCTT
GTGCTGAGCAGCCCGGCGTTCGCCGCCATGGCCCGCCCCCGGCATGGCCGCGCGGCTCACCAAGCTCAGCGCCGTCGACGT
CGAGTACAAGAGGGTGCCGTGCGAGTACAAGGGCAAGAACCTCTCGCTGCGTGTGGAGGAGCGGAGCCGCGCGCCGAACA
AGCTCGCCGTCCGGTTCCTCTACCAGGGCGGCCAGACCGACATCGTCGCCcTCGACGTCGCGAAGGTGGGGTCGTCGAGT
GGAAGTTCATGACGCGGGAGCATGGGCCGGCGTGGAGCACGCGGCAGGCGCCGGCGGGGCCGCTGCAGTTCAGGGTGGTG
GTGACCGGCGGGTACGACGGCAAGTGGGTGTGGGCCGACCGCGAGGTGCTGCCGCGACGGTGGCGCGCCGGCGAGGTCTA
CGACACGGGGGTCCAGATCACCGATATCGCGCAGGAGGGCTGCTTCCCGTGCGACACCCACGAGTGGAGG**TGA**AAGCAAA
TCGGGGATAGTAAACAAACAAACGATCGCCAACATGGAGCCGAAGAAGATGCTGCTTCTGGCTCCTCGTTTTGGCCGGGC
TACGCACGCACGCACGAAGGATCGCAGCACAGCACCACACCAGACAGGGGAGGTGATTAGTTTAGTTTCACACGAGATGA
TGACTCGTAGCGGTAGCGTCCTGCTGATCCATCGGCAACTTTGGCTGCCGCCTTTTCTTATTTTCTTTTCTCCTTGTTTT
GTGAGCCTTGTGTATATACTCACATACTTACATGAGCTGCTGACGTGAATGAAGAGAAGATAAGTAAGTAAATTAAGCAG
AGCATATCCTGGGGAGTGGTAATATCAGAGGACGGCGTCACGGCGACGATAGGATATGTGTGTCGTAGCTTTGCTGCTTC
AACAGCTTGTCCCCATGTATGACTATAAGAATATCTCCAACCATGGACTAAGCC

HvEXLA2

>TC133975

CGGCACGAGGCCTCAACGGCGACCTGCTCGCCGCTGCGGGCCCGTCACTGTACAGACAAGGCGTCGGCTGCGGCGCGTGC
TTCCAGGTGCGGTGCAAGGACGAGGAGCTCTGCAGCACCGCCGGCGTGAAGGTCGTCGTCACGGACCGCGCCAGCACGAA
GACGAACGACACCGAGCTCGTGCTCAGCAGCCCGGCGTTCGCCGCCATGGCCCGCCCCGGCATGGCCGCGCGGCTAGCCA
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CGGAGCCGGGCGCCCAGCGAGCTGGCCATCACGATCCTCTACCAGGGCGGCCAGACGGACATCGTCGAGGTCGACGTCGC
GCAGGTCGGATCGTCGTCGAGCTGGAGTTCGCTGACACGCGACCACGGGCCGGCGTGGAGCACGAGCCTGGCGCCGCCGG
GGCCGCTGCAGCTCAGGGCGGTGGTGACCAGCGGGTTCGACGGGAGCTGGGTCTTTGCCGAACATGAAGTCCTGCCGCGC
CAGTGGCACGCCGGCGAGGTCTACGACACCGGCGTCCAGATCACCAGCATCGCGCAAGAAGCCTGCTTCCCCTGCGACAC
GCAGGAGTGGAAG**TGA**CGGGTGACGGTGGAGAAACATGCATGAGCTGCACTGCTGCAGCCTGCAGCTAAGGTGTGAGATG
CTACGTAGTACTGTATGTAGTATCAACATGTAGTGATAACGAAGGCGAGGCCGTGGCCTGAATCGCCGGACCTGCAAAAT
ACTACTCGATGAATAACGTCGCATGCATGTCGGGGCCCCGTTGGTTTCGGCAATGATGTTCTTTGCTCGATG

HvEXLA3

>TC133976

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GTCGGGTCGTCGTCGAGCTGGAGGTCCCTGACACGCGACCACGGGCCGGCGTGGAGCACGAGCCTGGCGCCGCCGGGGCC
GCTGCAGCTCAGGGCGGTGGTGACCGGCGGGTACGACGGGAAGTGGGTCTGGGCCGACCGGGAGGTCCTGCCGCGCGGGT
GGCGCGCCGGCGAGGTCTACGACACCGGCGTGCAGATCACCGACGTCGCCCAAGAAGCCTGCTTCCCCTGCGACGCGCAG
GAGTGGAGG**TGA**GGTGACCACCGACGACGACGAGGCAAGCCAAGAGGAAGAGCTAGGCTCCAAAATCATGGCTGGGCATC
GAACGAAATGGCAAGCCTCGTCACTGCTGCTGCCCGTTTCGGCTTGGCTTGGAATTGCAATTGGAAGGATTGGTAGATAG
ATAGAGATTAGTAACTGACGCAGAGACACGTTGAGTCCTTGTTGCTTATCCAACTCTTGTGGACAAACGTGCATGAGCTG
CAGCTGACGTGTGAGATACTAGTAGTAGTATGTACGAGTAGCAACCATGAAGCGATAATGAGTAGACGACGGCGGCGAGG
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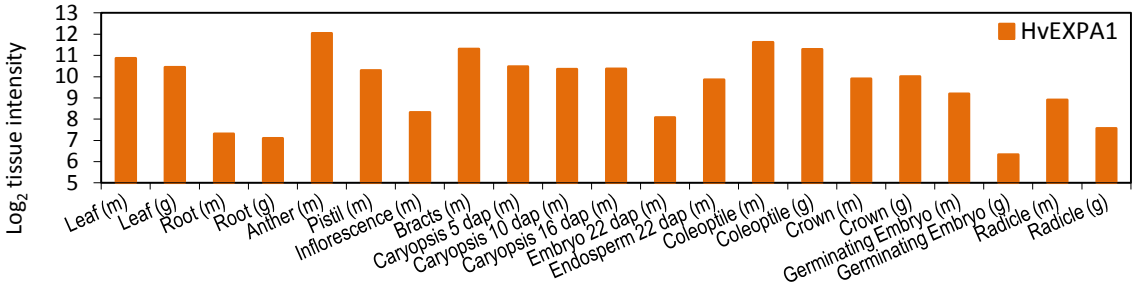
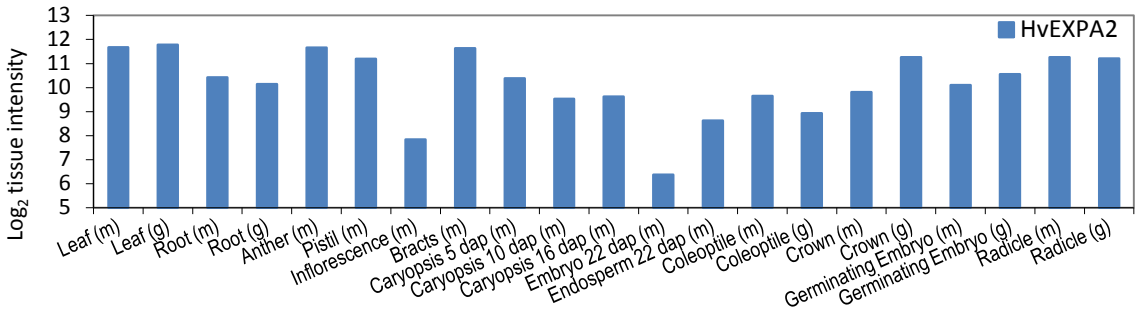
APPENDIX C

Barley Expansin Affymetrix Data

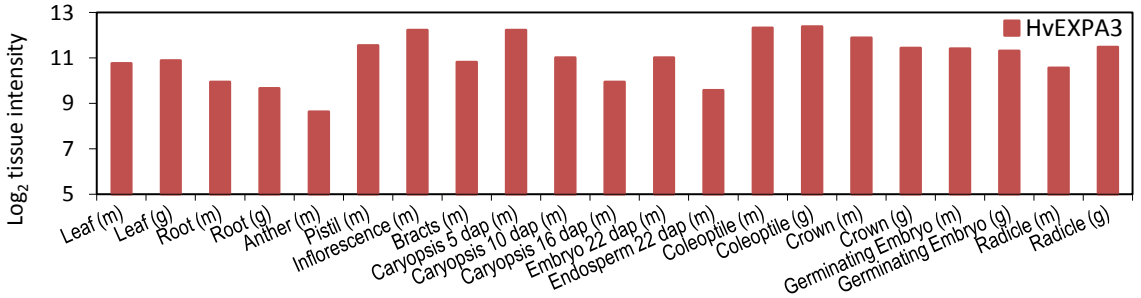
A



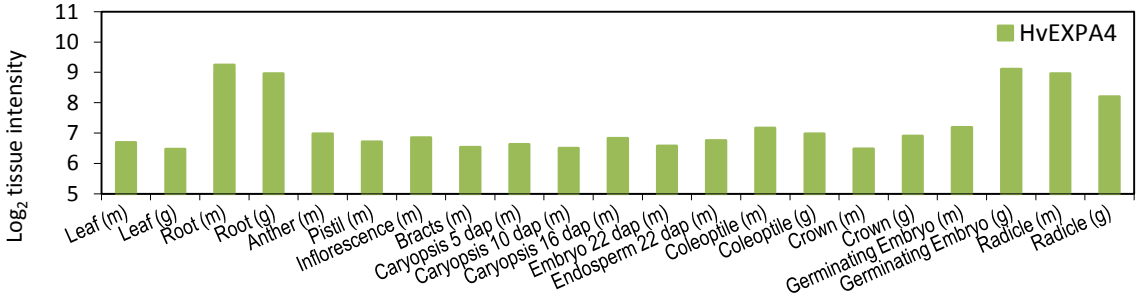
B



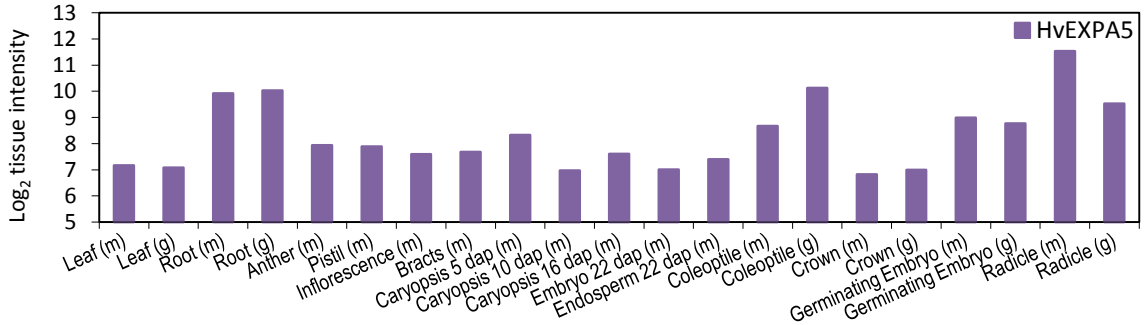
C



D



E



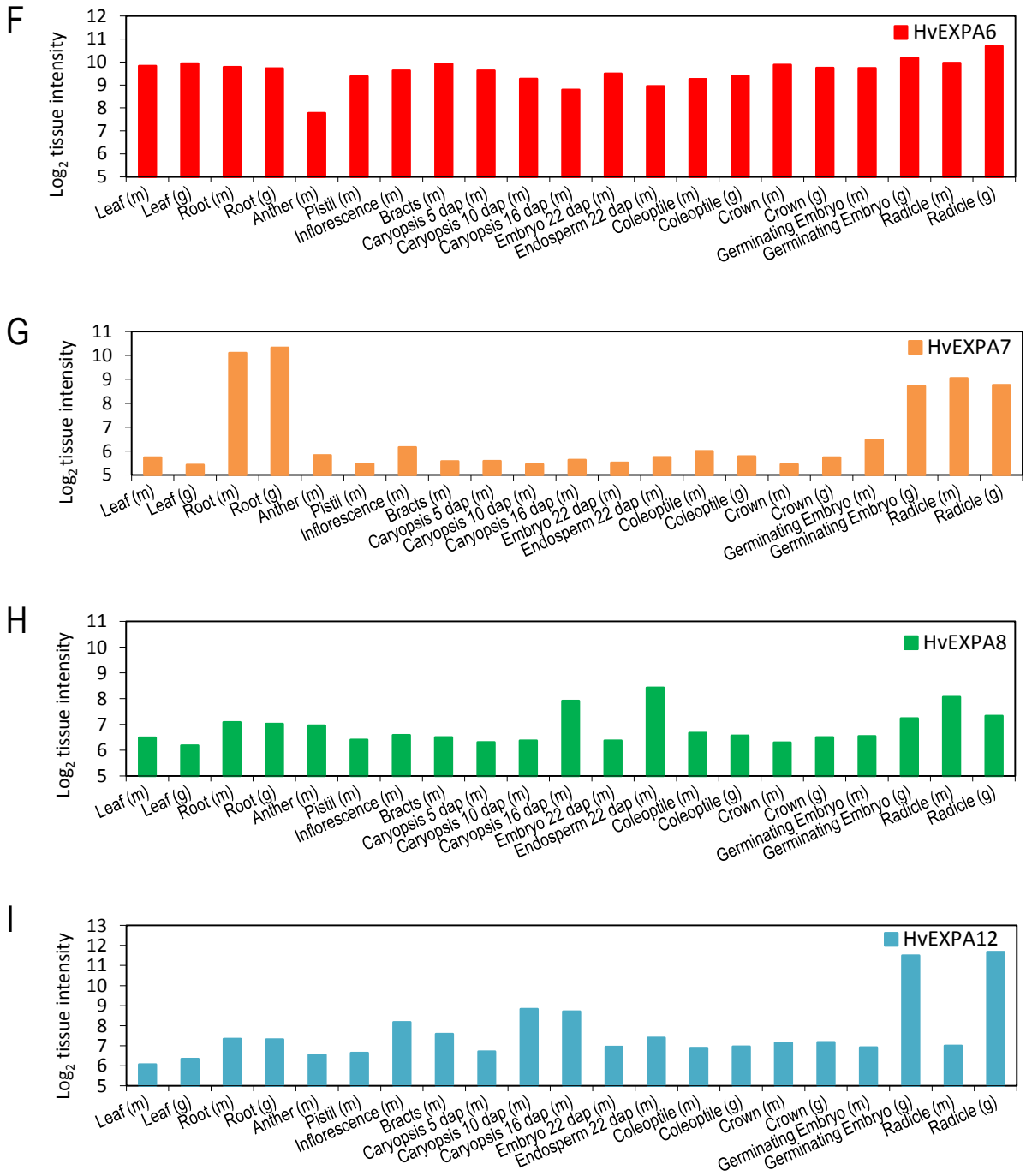
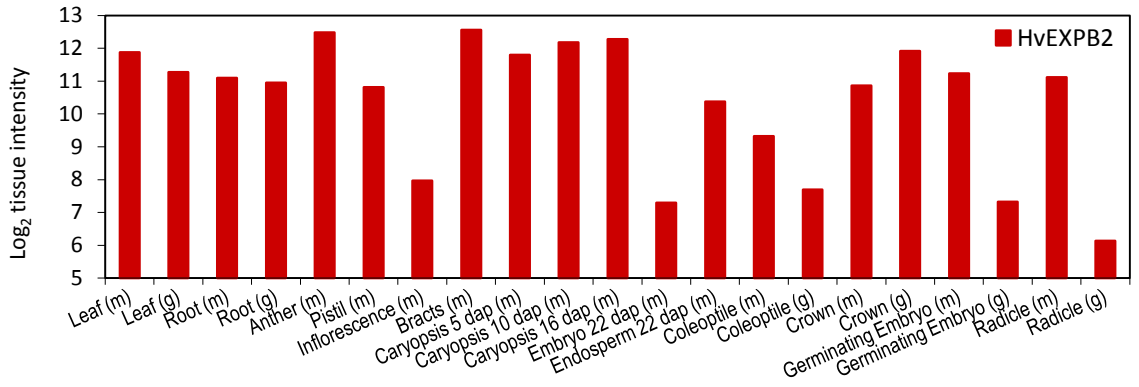


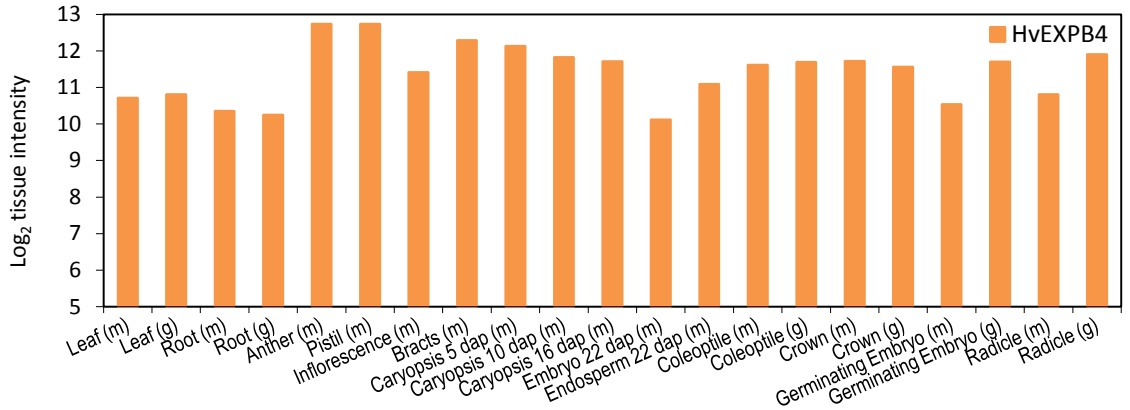
Figure C-1. Tissue transcript profiles of all *HvEXPA* genes present on the barley Affymetrix chip.

Affymetrix data tissue intensity ratios are presented as log base 2, RMA normalised. A tissue intensity value < 5 is considered background. The tissues indicated on the *x*-axis correspond to samples taken from the cultivar 'Morex' (m) or 'Golden Promise' (g). **A**, *HvEXPA1*. **B**, *HvEXPA2*. **C**, *HvEXPA3*. **D**, *HvEXPA4*. **E**, *HvEXPA5*. **F**, *HvEXPA6*. **G**, *HvEXPA7*. **H**, *HvEXPA8*. **I**, *HvEXPA12*.

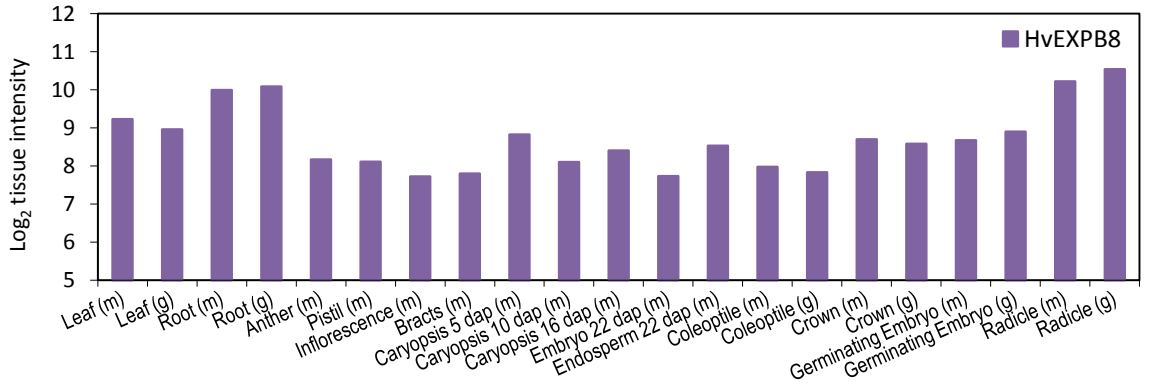
A


HvEXPB2
Log2 tissue intensity
13
12
11
10
9
8
7
6
5
Leaf (m)
Leaf (g)
Root (m)
Root (g)
Anther (m)
Pistil (m)
Inflorescence (m)
Bracts (m)
Caryopsis 5 dap (m)
Caryopsis 10 dap (m)
Caryopsis 16 dap (m)
Embryo 22 dap (m)
Endosperm 22 dap (m)
Coleoptile (m)
Coleoptile (g)
Crown (m)
Crown (g)
Germinating Embryo (m)
Germinating Embryo (g)
Radicle (m)
Radicle (g)

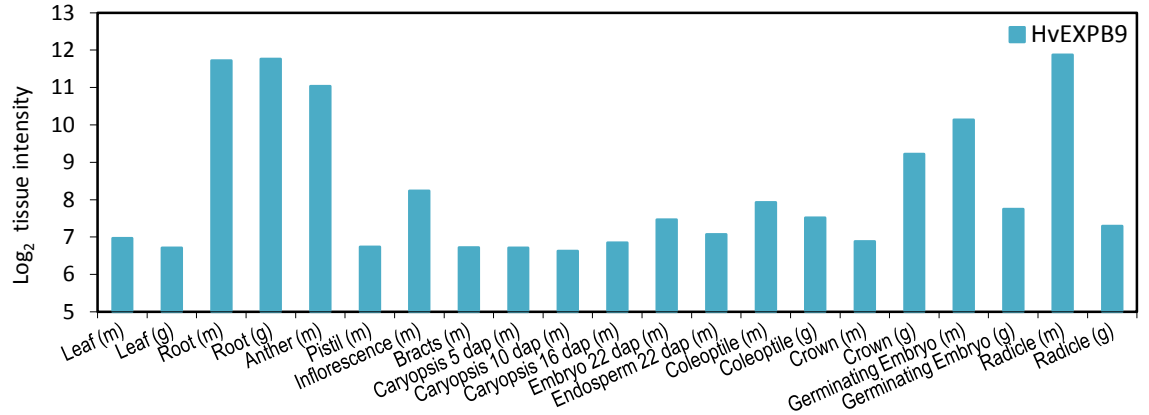
B


HvEXPB4
Log2 tissue intensity
13
12
11
10
9
8
7
6
5
Leaf (m)
Leaf (g)
Root (m)
Root (g)
Anther (m)
Pistil (m)
Inflorescence (m)
Bracts (m)
Caryopsis 5 dap (m)
Caryopsis 10 dap (m)
Caryopsis 16 dap (m)
Embryo 22 dap (m)
Endosperm 22 dap (m)
Coleoptile (m)
Coleoptile (g)
Crown (m)
Crown (g)
Germinating Embryo (m)
Germinating Embryo (g)
Radicle (m)
Radicle (g)

C


HvEXPB8
Log2 tissue intensity
12
11
10
9
8
7
6
5
Leaf (m)
Leaf (g)
Root (m)
Root (g)
Anther (m)
Pistil (m)
Inflorescence (m)
Bracts (m)
Caryopsis 5 dap (m)
Caryopsis 10 dap (m)
Caryopsis 16 dap (m)
Embryo 22 dap (m)
Endosperm 22 dap (m)
Coleoptile (m)
Coleoptile (g)
Crown (m)
Crown (g)
Germinating Embryo (m)
Germinating Embryo (g)
Radicle (m)
Radicle (g)

D


HvEXPB9
Log2 tissue intensity
13
12
11
10
9
8
7
6
5
Leaf (m)
Leaf (g)
Root (m)
Root (g)
Anther (m)
Pistil (m)
Inflorescence (m)
Bracts (m)
Caryopsis 5 dap (m)
Caryopsis 10 dap (m)
Caryopsis 16 dap (m)
Embryo 22 dap (m)
Endosperm 22 dap (m)
Coleoptile (m)
Coleoptile (g)
Crown (m)
Crown (g)
Germinating Embryo (m)
Germinating Embryo (g)
Radicle (m)
Radicle (g)

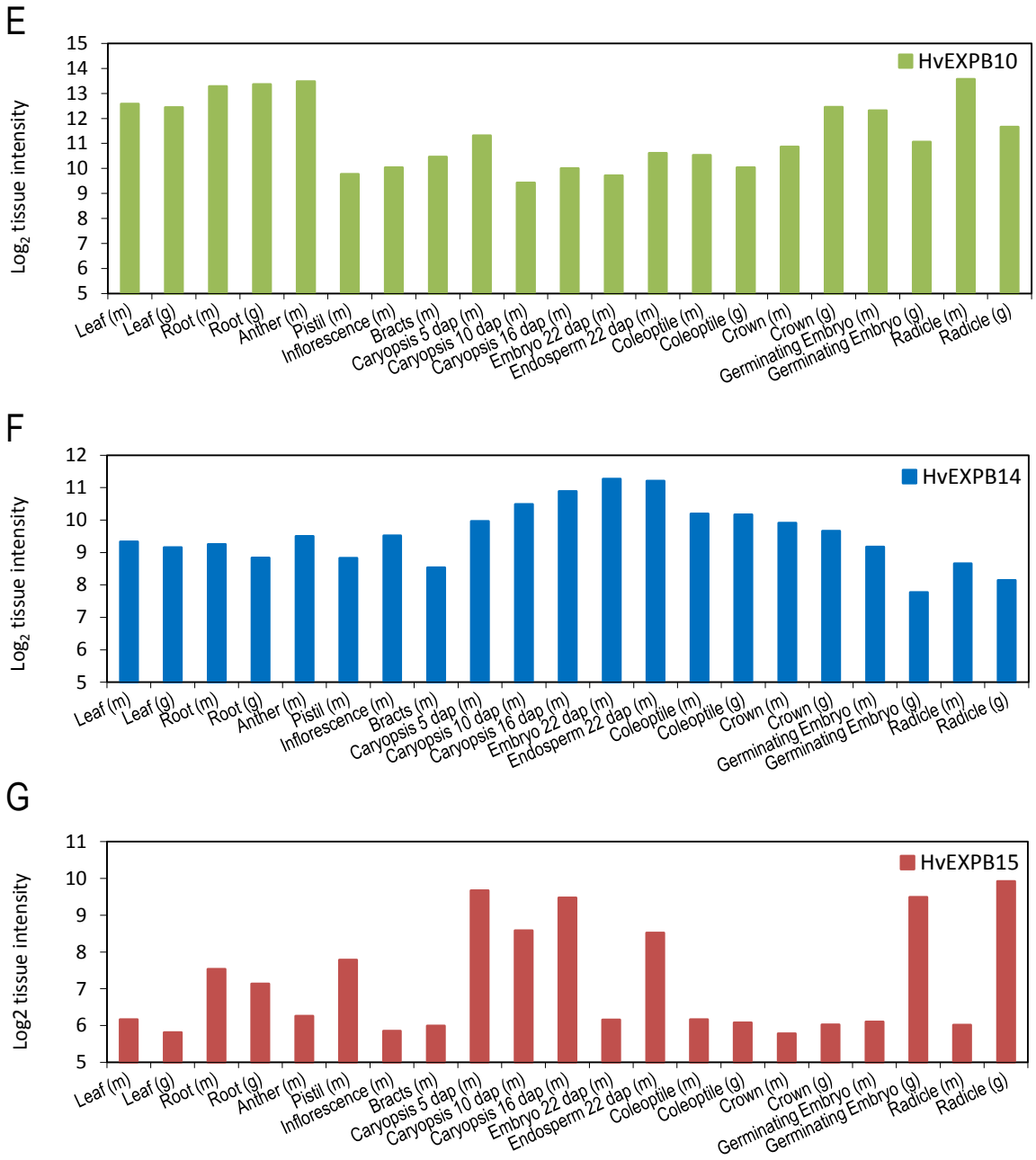


Figure C-2. Tissue transcript profiles of seven *HvEXPB* genes present on the barley Affymetrix chip. Affymetrix data tissue intensity ratios are presented as log base 2, RMA normalised. A tissue intensity value < 5 is considered background. The tissues indicated on the *x*-axis correspond to samples taken from the cultivar 'Morex' (m) or 'Golden Promise' (g). **A**, *HvEXPB2*. **B**, *HvEXPB4.* **C**, *HvEXPB8*. **D**, *HvEXPB9*. **E**, *HvEXPB10*. **F**, *HvEXPB14*. **G**, *HvEXPB15.*

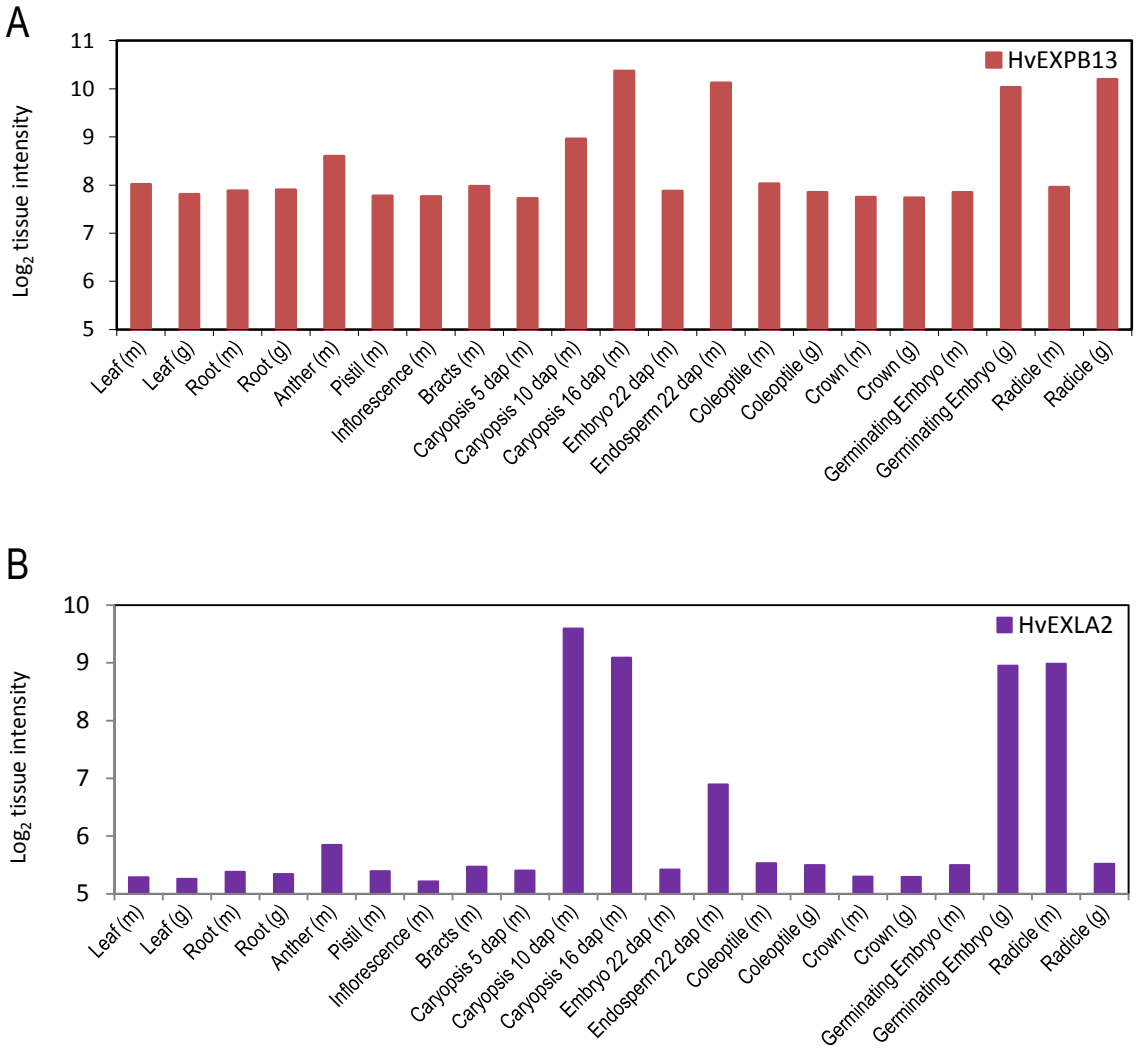


Figure C-3. Affymetrix data of *HvEXPB13* and *HvEXLA2*.

Affymetrix data tissue intensity ratios are presented as log base 2, RMA normalised. A tissue intensity value < 5 is considered background. The tissues indicated on the *x*-axis correspond to samples taken from the cultivar 'Morex' (m) or 'Golden Promise' (g). **A**, *HvEXPB13*. **B**, *HvEXLA3.*

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REFERENCES

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