The expression of hydrolytic enzymes in germinating barley grain

By Catherine Elizabeth Gibson

The University of Adelaide

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Publications from this thesis

1. Gibson C. E., Burton R. A., Sedgley M., Logue S., MacLeod L. and Fincher G. B. (1999). The localization of hydrolytic enzymes in germinated barley grain. Proceedings of the 8th Australian Barley Technical Symposium; Melbourne, Australia

2. Poster presentations (1998);
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Acknowledgement

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To everyone, I HAVE FINISHED.

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List of Abbreviations

Ab	Absorbance
aa	Amino acid
ABA	Abscisic acid
Amp	Ampicillin
α-amylase	Alpha amylase
ATP	Adenosine triphosphate
bp	Base pair
β-amylase	Beta amylase
β-glucan	(1→3,1→4)-D-β-glucan
β-glucanase	(1→3,1→4)-D-β-glucanase
BSA	Bovine serum albumin
CCL	Crushed cell layer
cDNA	Complementary DNA
CTP	Cytidine triphosphate
dATP	2'-Deoxyadenosine triphosphate
dCTP	2'-Deoxycytidine triphosphate
dGTP	2'-Deoxyguanosine triphosphate
dTTT	2'-Deoxytriphidine triphosphate
DEAE	Diethylaminoethyl
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPs	Deoxyribonucleotide triphosphates
DP	Diastatic power
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
et al.	and others
EtBr	Ethidium bromide
GA	Gibberellic acid
HWE	Hot water extract
In situ	*In situ* hybridization
IPTG	Isopropylthio-β-D-galactoside
kb	Kilobases
KDa	Kiladalton
LB medium	*Luria-Bertani* medium
LD	Limit Dextrinase
LDIN	Limit Dextrinase Inhibitor
LM	Light microscopy
mg	Milligram
μg	Microgram
mRNA	Messenger ribonucleic acid
Oligo(dT)	Oligodeoxythymidylic acid
PAS	Periodic acid/Schiffs
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
Pers. comm.	Personal communication
pI	Isoelectric point
PMSF	Phenyl methyl sulphonyl fluoride
Poly(A)	Polyadenylic acid
PVP	Polyvinylpyrrolidine

RACE	Rare amplification of complementary DNA ends
RNA	Ribonucleic Acid
rpm	Revolutions per minute
RT	Reverse transcriptase
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SSC	Sodium saline citrate
TAE	Tris-acetate EDTA
TBO	Toluidine blue O
TE	Tris EDTA
v/v	Volume/volume
w/v	Weight/volume
X-gal	5-Bromo-4-choro-3-indoyl-b-D-galcatoside
$(1{\rightarrow}4)$-β-xylanase	$(1{\rightarrow}4)$-β-xylan endohydrolase

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(Below are unique point of interests marked on figures by S for Sloop and H for Himalaya followed by a number which is cross referenced in the text)

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Figure 4.6: H1

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Chapter 6: Identification and location of specific Limit Dextrinase and Limit Dextrinase Inhibitor mRNAs in *Hordeum vulgare* L. (barley) grain during germination

Abstract

Modification of the barley grain endosperm in germination is fundamental to successful plant growth but also has important ramifications for down-stream industrial uses of barley, particularly in the malting and brewing industries. There are a battery of enzymes that are involved in the modification process but the sites of synthesis and action of only of a few of these have been described in detail and most have only been studied in isolated tissues.

The development of a sensitive and robust *in situ* mRNA hybridization (ISH) procedure, which allows the detection of specific transcripts representing proteins in grain sections is described here. This first required the optimization of grain fixation, embedding and sectioning procedures as well as the adaptation of a staining method using calcofluor white to accurately assess the modification state of each grain prior to processing. Once these technical parameters were established, the non-radioactive ISH protocol was developed to allow the detection of transcripts of $(1,3;1,4)$-β-glucanases, $(1\rightarrow4)$-β-endo-xylanases, limit dextrinase and limit dextrinase inhibitor in grain sections of the two barley cultivars Sloop and Himalaya. The panel of enzymes selected for study covers several aspects of grain modification, such as $(1,3;1,4)$-β-glucan, xylan and starch breakdown, which are all important for successful grain germination.

The successful ISH technique proved sensitive enough to discriminate between the $(1,3;1,4)$-β-glucanase and xylanase isoenzymes and clearly defined whether the transcripts for these enzymes were synthesized in both a tissue-specific manner and a fixed temporal sequence during grain germination. The use of monoclonal antibodies specific for the two $(1,3;1,4)$-β-glucanase isoforms in a related immunolabelling procedure, using the same fixed grains, also allowed the patterns of transcript and protein appearance to be correlated.

As expected, use of the ISH method showed that the transcripts of the $(1,3;1,4)$-β-glucanase, xylanase and limit dextrinase inhibitor genes are variously found in the aleurone cells, the starchy endosperm tissue and the scutellum. However, there were also substantial amounts of transcript detected in the tissues of the growing embryo which suggests that these enzymes may also contribute substantially to early seedling development.

Chapter 1

General Introduction

1.1: Barley as a crop plant

Barley (*Hordeum vulgare* L) was one of the first crops to be domesticated and used in fermentable beverages, probably around 3,000-5,000 B. C. (Bamforth, 1998; Young, 2001). Barley production is a significant component of agricultural income in Australia, with an annual value of around one billion dollars. While Australian barley production is below 6% of the world barley market (about 6.8 metric tonnes of barley annually, ABARE, 2005), the Australian malting trade accounts for 30% (1.5-3 million tonnes annually) of export markets (Barley Australia website, 2016; MacLeod *et al.,* 2000). Following the Second World War when the demand for malted barley in alcohol production dramatically increased, the largest use for barley became, and is still today, for the brewing and distilling industries. Much of the information available about barley grain germination and malting is derived from research that was carried out decades ago. There have been incremental additions to this but no big advances in the study of events or enzymes in particular tissues. This project is set against this backdrop.

1.2: Barley grain structure

Barley is a member of the Poaceae grass family, of the tribe Triticeae and genus Hordeum. The grain of this genus is characterised by a one-flowered spikelet and awn, with unusually long subulate and scabrid-hairy rachilla. The awns of the lemma of the central spikelets are very long and stiff, remaining straight in the fruiting stage (Bothmer and Jacobsen, 1985). The features presented in Figure 1.1 are generally used as a guide in the identification of barley varieties, as subtle differences in the crease shape and in the form of the barbs and rachilla are observed between varieties.

The average barley grain has a dry weight in the range 30-40 mg, is 6 to 12 mm long, 2.7-5 mm wide and 1.8 to 4.5 mm in thickness between the dorsal and ventral sides (Briggs, 1987). The barley grain has an elongated body (Figure 1.2) that tapers towards the ends, with a roughly oval cross-section, and a groove or furrow running longitudinally along what, by convention, is called the ventral side. The barley grain can be divided up into many sub-sections (Figure 1.2 and 1.3). These include the embryo from which grow the rootlets, acrospire (early shoots) and the coleorhiza (root shoots) and lying laterally to the embryo is the scutellum and scutellum epithelium. Adjacent to the scutellum is the crushed cell layer, which borders the large area of the starchy endosperm surrounded by the aleurone layers. The pericarp and testa are two extremely thin layers lying just underneath the husk and above the aleurone layer.

The husk of a barley grain consists of two outer layers that surround the entire grain and which adhere firmly to the grain in most cultivated barleys, but in some varieties separate during threshing. These latter varieties are termed hulless or naked barleys. The palea is the husk layer on the ventral side of the grain, while the lemma is located on the dorsal or backside of the grain.

Figure 1.1: Barley grain appearance Modified diagram taken from the American Malting Barley Association, "Know your malting variety" (2005) as a guide to identification of barley varieties.
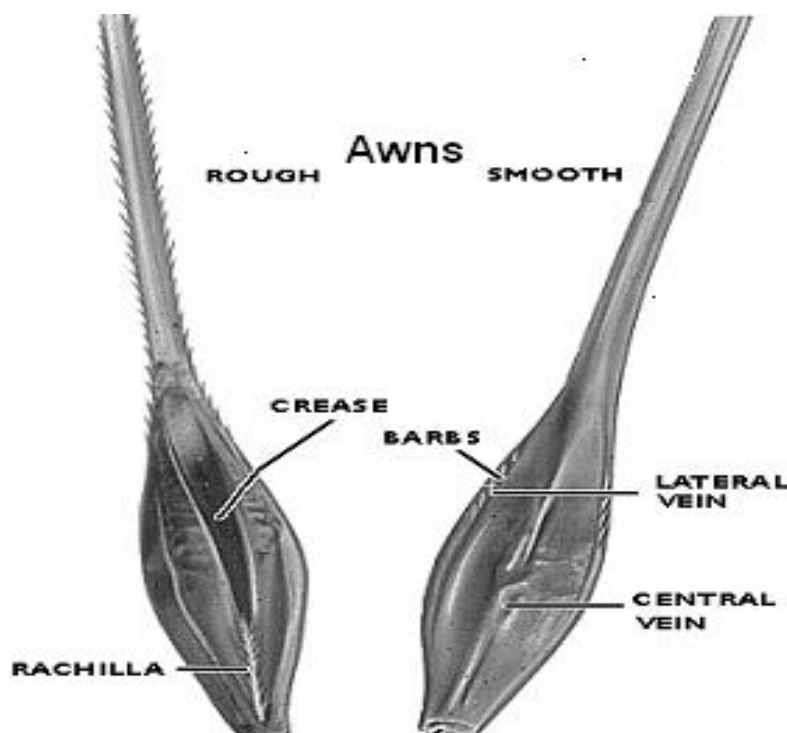
During germination the husk serves to maintain water stability and control water access into the embryo (Collins, 1918; Bradbury *et al*., 1956; Palmer, 1989; Briggs, 1978; MacGregor, 1991; Cu *et al.,* 2016) whilst also protecting the developing acrospire. The embryo, scutellum and aleurone tissue of the barley are metabolically active during germination, and are described in more detail in the next sections.

1.2.1: The embryo

The barley embryo comprises about 3-4% of the dry weight of the grain and contains all the juvenile tissues, which expand into the emergent seedling. In the early stages of germination it contains mainly storage proteins, lipids, sucrose, raffinose, fructans and minerals (Palmer, 1989b). The embryo is heterotrophic and thus requires a source of energy to sustain growth until the seedling reaches photosynthetic autotrophic status. In barley this energy is provided by the starchy endosperm and the scutellum. The

germinated barley embryo (Figure 1.3) can be separated into six distinct areas, 1) the rudimentary root system containing the root initials (five usually present), also called the coleorhiza (Barrero *et al*., 2009), with the potential metaxylem covered by the root cap; 2) the acrospire, which includes the coleoptile, a cylindrical organ protecting the apex and the leaf primordia; 3) the ventral trace region, which includes the node and procambial strands; 4) the cellular scutellum surrounding both area 1 and 2 and 5) the single layer of the epithelial cells (scutellum epithelium) at the interface of the scutellum and 6) the crushed cell layer (Figure 1.2), (Bowes, 1996; Eneri and Sopanen, 1986; Palmer, 1989).

1.2.2: The scutellum

The scutellum (sc) lies between the embryo and the starchy endosperm and is a shield-shaped organ. Scutellum tissue comprises parenchyma cells and the scutellar epithelial cells (Palmer, 1982). It is generally accepted that the scutellum has a dual function; the secretion of hydrolytic enzymes into the starchy endosperm and the absorption of nutrients from the endosperm as the grain germinates (Fincher, 1992).   During germination the columnar cells of the scutellar epithelium expand and increase their surface area, projecting further into the endosperm (Figure 1.3; Neiwdorp, 1963; Neiuwdorp and Buys, 1964).

Figure 1.2: The structure of a typical barley grain is shown. Modified from a diagram taken from Briggs and Hough (1981).
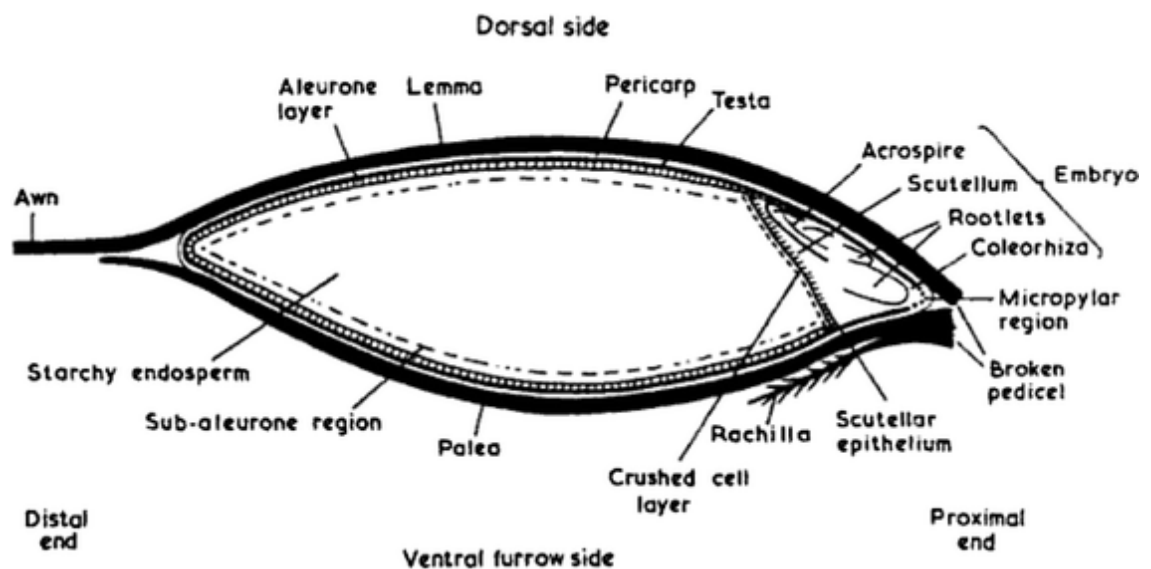
This expansion is believed to facilitate the transfer of nutrients from the starchy endosperm to the embryo, by providing a larger surface area. Although Palmer (1982) stated that the potential of the barley embryo to secret α-amylase is not linked to the elongation properties of the epithelial cells, recent studies on scutellum epithelium peptide transporters (Waterworth *et al.,* 2000; 2005) along with documented evidence of the uptake of amino acids, small proteins and sugars from the scutellar epithelium (Sopanen *et al.,* 1980; Higgins and Payne, 1981; Sopanen and Vaisanen, 1985; reviewed in Eneri and Sopenan, 1986; Salmenkallio and Sopanen, 1989; West *et al.,* 1998) dispute this.

The site of gibberellin production is located within the scutellum tissue (Yan *et al.,* 2014). There is no evidence that hormones act on scutellar tissue; it is more likely that the hormone just moves through the tissue to its target, the aleurone (Palmer, 1982; 1989b). Another interesting feature of scutellar tissue in germinated grain is the presence of starch granules in the sub-epithelial and parenchyma cells of the embryo base and at the junction between the developing acrospires and roots, as observed by Smart and O' Brien (1972) and Neiuwdorp and Buys (1964). The most logical purpose of this starch is for embryo growth and respiration, as the starch disappears from the epithelial cells in the later stages of germination but persists for longer in scutellar parenchyma cells (Smart and O' Brian, 1972; Van der Meulen *et al.,* 2000), particularly in embryos from dormant grains (Van der Meulen *et al.,* 2000).

1.2.3: The crushed cell layer

The crushed cell layer (CCL, Figure 1.2) or intermediate layer can be found in the starchy endosperm adjacent to the scutellar epithelium (Figure 1.3). Comprising principally of remnant endosperm cell wall material, at grain maturity it appears to represent a barrier between the embryo and its nutrient supply (Brown and Morris, 1890; MaGregor and Dushnicky 1989; Palmer, 1982; 1998). The layer is starch free (Brown and Morris, 1890; MacLeod and Palmer, 1966; Palmer, 1974) but rich in (1,3;1,4)-β-glucan as expected from its origin between the developing embryo and the endosperm tissue (MacGregor *et al.,* 1994; MacGregor and Dushnicky, 2013). Water uptake and retention in the region of the CCL (Collins 1918), and the CCL position between the embryo and the endosperm (MacGregor, 1991) provides no insight into the role of the CCL in barley germination.
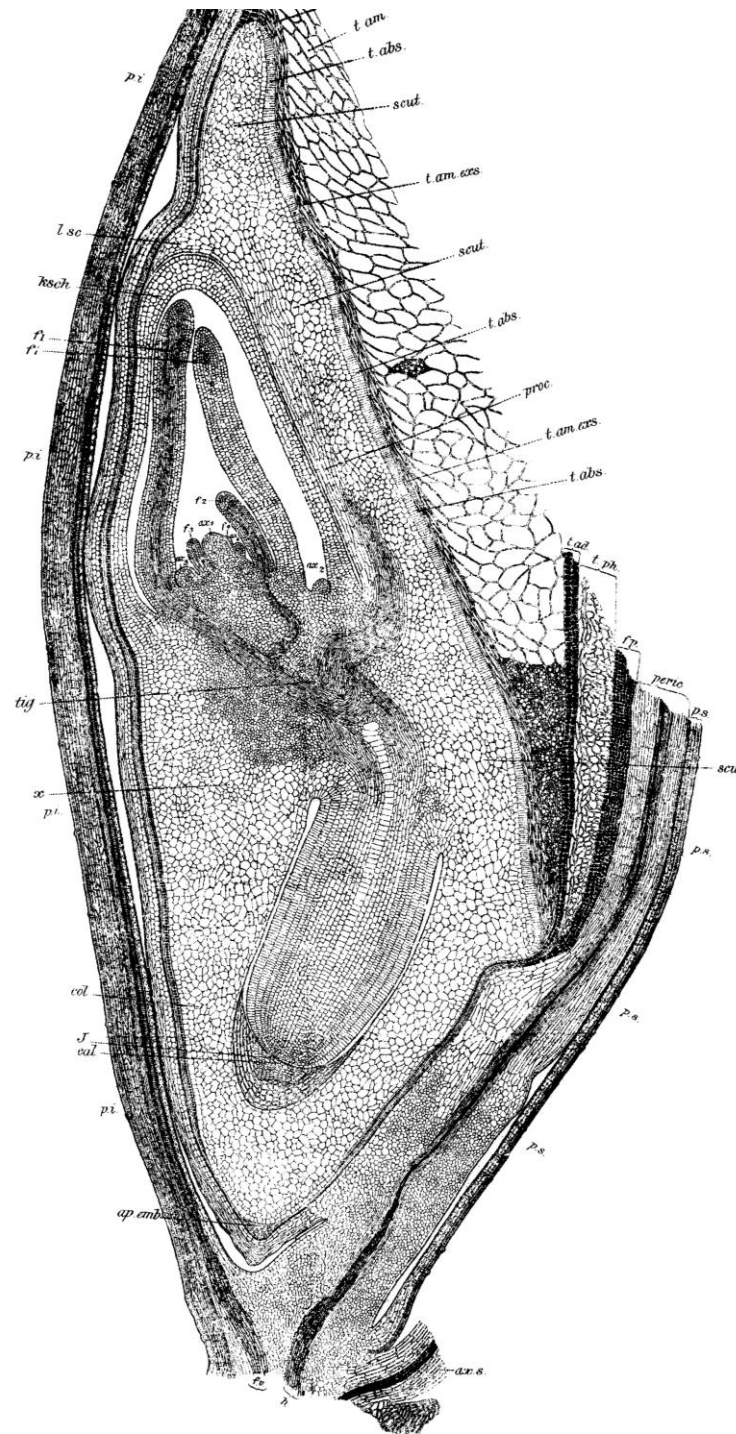
Figure 1.3: Longitudinal cross section of a barley grain embryo. A modified diagram of a longitudinal embryo section from a barley grain taken from the Brown and Morris (1890).

pi. Inferior palea, consisting of *ep.e.* External epidermis, *sch.* Schlerenchyma ; sub-epidermal bast, *fu.* Vascular bundle, *chl.* Chlorophyll layer and *ep.i.* Inner epidermis. *per.e.* Outer layers of pericarp, *per.* **i.** Inner layers of pericarp, *teg.* Testa layers of pericarp, *ep.nucell.* Epidermis of nucellus*, t.ad.* **Aleurone**-cells of endosperm, *t.am.exs.* Depleted and compressed cells of endosperm (**crushed cell layer**), *t.am.* Starch-containing cells of parenchyma of endosperm, *scut.* **Scutellum**, *t.ahs.* Absorptive and secretory epithelium (**scutellar epithelium**), *proc.* Elongated parenchymatous cells of scutellum, *ksch.* Plumule-sheath (**coleoptile**), *f.1, f.2, f-3, f.4.* Leaves of plumule (coleoptile leaves), *ax1.* Primary axis, *ax2, ax3, ax4.* Secondary axes (shoot primordia), *tig.* Point of origin of the procambium strands which enter the scutellum, leaves, and radicles (**ventral trace**), *J.* Growing point of primary radicle, *cal.* Calyptrogen. (**Root-cap**), *coz.* **Coleorhiza**. (Root-sheath), *ap.emb.* Appendage of embryo, *t.ph.* Sheaf-like cells: the remnant of the nucellus, *p.peric.* Dorsal portion of pericarp (residue of suspensor), *p.s.* Superior palea, *h.*

A strand of tracheides, *ax.s.* Basal bristle, Meristematic portion of the hypocotyledonary axis, which is in greater part absorbed by the secondary radicles during their development, *f.p.* Funiculus.

Microscopic studies demonstrating (1,3;1,4)-β-glucan degradation have indicated channel formation through the layer, early in germination (Walwork, 1997; MacGregor, 1994), which is a possible sign of enzyme mobility between the embryo and starchy endosperm.

1.2.4: Sheaf cells

Sheaf cells are thought to be modified aleurone cells. They lie within a channel distinct from the surrounding starchy endosperm on the ventral side of the grain (ventral groove) surrounded by the remains of the nucellar projection and crease aleurone cells. Sheaf cell wall polysaccharides are laden with highly fluorescent phenolic acids (Briggs and MacDonald, 1983). It has been observed that endosperm modification (section 1.3.3) is more rapid in the endosperm cells adjacent to the sheaf cells, which could be due to enzymes moving relatively more freely through this tissue. There is evidence that sheaf cells are involved in nutrient transfer during development and function in nutrient storage and hydrolase synthesis (Cochrane and Duffus, 1980).

1.2.5: The starchy endosperm

The starchy endosperm occupies a large percentage (70-80%) of the total cereal grain dry weight, representing the nutrient store that is degraded during germination to support seedling growth (Fincher, 1989). It has also been reported that the endosperm can act as a water reservoir for the embryo tissue in the early stages of germination if water is restricted (Allen *et al.,* 2000). The endosperm is surrounded by the cellular aleurone layer and components of the husk (Figure 1.2). The endosperm is metabolically inactive; comprising starch granules embedded in a protein matrix, surrounded by cell walls made up of (1,3;1,4)-β-glucan (70%), arabinoxylan (26%), ferulic and other phenolic acids (Fincher, 1975; Palmer, 1987; 1989b). The fine structure of the endosperm cell walls has not been well characterised. The most reliable molecular weight estimations for cell wall (1,3;1,4)-β-glucans ranges from 150,000 to 300,000, as they are heterogeneous with respect to size, solubility and molecular structure (MacGregor and Fincher, 1993).

Barley (1,3;1,4)-β-glucan is found in a wide range of molecular sizes (150-300 kDa) and its intrinsic viscosity is related to both molecular size and axial ratio (length/width) or asymmetry of the molecule in solution. The properties of (1,3;1,4)-β-glucan, which consists of glucose molecules bound together in straight chains by either β-(1→4) (70%) or β-(1→3) (30%) linkages, cause kinks in the chain reducing intermolecular

associations (Edney *et al.,* 1991; MacGregor and Fincher, 1993; Johansson *et al.*, 2007). It is believed that (1,3;1,4)-β-glucan is not synthesized in dividing cells, but accumulated specifically during grain maturation (Carpita and Gibeaut, 1993; Kin *et al.*, 2000; Wilson *et al.*, 2006). The final β-glucan content is influenced by the genotype and the environment (Wang *et al.* 2004; Swanston *et al.* 2006; Anker-Nilson *et al.* 2008).

Arabinoxylans are complex carbohydrate polymers with many physicochemical similarities to β-glucans, such as their tendency to produce highly viscous aqueous solutions (Schwartz and Han, 1995). The β -(1→4) linked xylopyranose backbone is the most highly conserved feature of arabinoxylan (Izydorozyk and Biliaderis, 1993), as there appears to be significant variability in xylose to arabinose ratio and substitution patterns between varieties (Ballance *et al.,* 1986; Vietor *et al.,* 1994). The degree of branching of arabinoxylan can be represented by the d-xylanopyranosyl/l-arabinofuranose ratio, differing between endosperm and aleurone tissues (Li *et al.* 2005).

The release and mobility of degraded starch, carbohydrate and protein through endosperm cell walls is size dependent, as the pore size predicted for the general cell wall is limiting (Carpita, 1982; Carpita *et al*., 1979). The breakdown of endosperm cell walls is therefore a critical aspect of endosperm degradation and it is believed that the absence of secondary thickening and lignin in the walls of the starchy endosperm renders them more susceptible to rapid enzymatic degradation during endosperm mobilization (Fincher, 1989). This process is termed grain modification, and will be described in greater detail in section 1.7.

The starchy endosperm-embedded starch is the major carbohydrate reserve of cereal grains (Aman *et al*., 1985) and is composed mainly of the linear (1→4)-α-glucan amylose and the branched (1→4, 1→6)-α-glucan amylopectin (MacLeod and Wallwork, 1991; MacGregor and Fincher, 1993; Ellis *et al.*, 1998). In barley there are two morphologically distinct types of starch granules; the larger (A) granules are 15-50 μm in diameter and the smaller (B) granules are only 5-10 μm (Palmer, 1972b). Up to 90% of the total starch granule numbers are of the small type, although they only make up 10% of the total starch weight (Palmer, 1972b). It is widely accepted that variation of starch content occurs between grains with the major causes either due to environmental conditions (Morgan and Briggs, 1981) or genetics (Kenn *et al.*, 1993; Allan *et al*., 1995).

The terms mealiness or steeliness have been used to classify density variations in areas within the starchy endosperm. Under the light microscope mealy areas appear to contain loosely packed cells with air spaces between the starch granules, while steely areas appear densely packed with small starch granules in a dense protein matrix (Chandra *et al*., 1999). Mealy and steely patches can occur randomly across the starchy endosperm and there appears to be an association of steely areas with higher concentrations of (1,3;1,4)-β-glucan and/or protein (Noots *et al*., 2001).

Two thirds of the total barley protein reserve is present in the starchy endosperm. The barley grain protein matrix (storage protein) of the starchy endosperm contains a mixture of hordein (prolamin), albumin, globulin and glutelin, which are generally characterised by high levels of glutamine and proline (Palmer, 1989b). The hordeins are the major storage proteins associated with endosperm starch (Enari and Sopanen, 1986), and are generally categorised into B, C or D groups, with their distribution through the endosperm wide-spread (Millet *at al.,* 1991). During germination, proteins are hydrolysed into a mixture of di- and tri-peptides by endopeptidases (cleave within the peptide chain) and carboxypeptidases (cleave at the carboxyl end) further degrade these to produce single amino acids (Higgins and Payne, 1977; Sopanen, 1979; Fincher, 1989; O'Rourke, 2002). Peptides and amino acids show no competition and are absorbed by independent transport systems, but peptides of widely different structures do complete through a single system capable of handling both di and oligopeptides. The uptake of amino acids into the embryo from the endosperm most likely occurs through at least four amino acid uptake systems identified in barley scutellum epithelium (Higgins and Payne, 1978; Sopanen, 1979; Salmenkallio and Sopanen, 1989; Fincher, 1989).

During the late stages of barley germination the starchy endosperm is acidified by the accumulation of malic acid (Macnicol and Jacobsen, 1992; Drozdowicz and Jones, 1995), to produce a low pH favourable to enzyme secretion and activity (Green, 1994), transport across the scutellum to the embryo (Bush, 1993) and the proteolytic activation of several hydrolases (Guerin *et al*., 1992).

1.2.6: Aleurone cells

Lying beneath the husk and surrounding the starchy endosperm is the aleurone (Figure 1.2 and 1.3). Barley aleurone layers are normally two to three cells deep, although aleurone tissue consisting of only one cell layer does occur, especially near the scutellum (Palmer, 1989b; Bacic and Stone, 1981a). Aleurone adjacent to the embryo/scutellum (germ aleurone) has also been identified as containing more lipid and

protein but less polysaccharide (Raju and Walther, 1988; Cochrane, 1994) than aleurone surrounding the starchy endosperm. The aleurone does appear to be one of the limiting factors in germination, since it has been demonstrated that in dormant barley grains, not only is the embryo unable to initiate the germination process but also the aleurone is impaired in its activities (Schuurink *et al.,* 1992; Wang *et al.,* 1995).

The walls of aleurone cells are much thicker than starchy endosperm cells, and are composed mainly of arabinoxylan (70%) and (1,3;1,4)-β-glucan (26%) with small amounts of cellulose (Bacic and Stone, 1981; Fincher, 1989; Palmer, 1989). Autio *et al.* (1996) suggested that arabinoxylan and (1,3;1,4)-β-glucan exist in separated macroscopic layers. The aleurone cell walls are composed of a thin inner layer that resists digestion and a thicker outer layer that is progressively hydrolysed during germination (Jones, 1969; Taiz, and Jones, 1970, 1973; Bacic, and Stone, 1981a; Gulber *et al.,* 1987; Bacic *et al,* 1988; Fincher 1992). Like the scutellum, the aleurone cells are a source of hydrolytic enzymes for the degradation of the starchy endosperm during germination (Palmer, 1989b), but unlike the scutellum, the aleurone does not reabsorb nutrients. The most prominent organelles in aleurone cells are the aleurone grains, which are membrane units containing protein, phytic acid, carbohydrates (Jones, 1969) and amino acids for the *de novo* synthesis of enzymes (Fath *et al.,* 2001).

Isolated aleurone tissue and cultured cells (protoplasts) are widely used systems for studying hormonal signaling in plant development (Fath *et al*., 1999). A large number of published research findings, including those on programmed cell death (section 1.8.5), stem from use of these assay system (as discussed in section 1.9).

Plasmodesmata are lateral canals that transverse the aleurone cell walls, and are believed to allow the movement of enzymes from the aleurone tissue to the starchy endosperm (Taiz and Jones, 1973; Ashford and Jacobsen, 1974; Gubler *et al*., 1987; Palmer, 1987).

1.3: Barley varieties used in this study

One of the two varieties used in this study is Himalaya, a naked (hulless) feed variety that has been widely studied since 1960, primarily because its hulless nature makes the sterilization and isolation of aleurone layers easy for research. The excellent response to GA$_3$ in terms of α-amylase secretion of this variety probably accounts for why over two thirds of the published studies with sectioned grain, dissected tissue or protoplasts use Himalaya. These include studies by Stuart *et al.* (1986); Higgins *et al. (*1982); Taiz and Jones (1973); Fath *et al. (*1999); Deikman and Jones (1985); Gubler *et al. (*1986);

Jacobsen and Beach (1985); Bethke *et al. (*1999); Ranki *et al.* (1990); (1994); McFadden *et al*. (1988) and Wang *et al. (*1998). The use of germinated Himalaya by McFadden *et al.* (1988) in measuring total glucanase expression with *in situ* hybridization was the basis for including it in this study.

The variety Sloop is adapted to growing in South Australian conditions, and was considered an ideal variety to include as a comparison to Himalaya, due to its superior malting quality and hulled nature. Experimental conclusions using Sloop will also be of more relevance to the malting and brewing industry as this variety is currently in wide use in Australia for malting. A summary of the quality characteristics of these two varieties is presented in Appendix 1.

1.4: Barley grain germination

The germination of barley is a complex process involving several different cell types and organs, specifically those described in section 1.2 such as the embryo, scutellum and aleurone layers. Progress of germination is generally characterised by the production of hydrolytic enzymes and progressive changes in the structure of the starchy endosperm.

1.4.1: Imbibition

Germination of a non-dormant barley grain is initiated by the imbibition or uptake of water. Mature seeds are dry, normally containing only 10% (w/w) moisture. After exposure to water a seed will start absorb it within one to two hours (Hunter and Erickson, 1952; Simon, 1984). The uptake of water is crucial to the activation of the embryo, the production of hydrolytic enzymes and the reactivation of metabolic processes including renewed cell expansion and cell division that had ceased upon maturation of the grain (Lin and Ho, 1986; Simons, 1984; Mayer and Marbach, 1981; Brookes *et al.,* 1976; Kirsop *et al.,* 1967). Gamlath *et al*. (2008) believe that endosperm cell wall components may have significant impact on water uptake, linked to kernel hardness. The proximal half of the grain has been observed to absorb water faster than the distal region (Brookes *et al.,* 1976). A study by McEntyre *et al*. (1998) has shown, using magnetic resonance imaging (MRI), that water accumulates under the surface of the entire husk tissue within one hour of imbibition, and then migrates into the aleurone, eventually penetrating through to the starchy endosperm tissue. Transient water distribution was also observed through the embryonic tissue (scutellum), into the ventral furrow with the crushed cell layer playing a critical role in attracting moisture (McEntyre *et al.*, 1998), probably due to the hydroscopic nature of the (1,3;1,4)-β-glucan present in the crushed cell layer. However, a study by Cu *et al*., (2016)

indicates that variations in water uptake rates between cultivars is genetically controlled and not influenced by either starchy endosperm cell wall thickness and nor the (1,3;1,4)-β-glucan content. Over the germination period, the starchy endosperm will eventually contain approx. nine times as much water as the embryo (Allen *et al*., 2000). The movement of water across the grain has been described as a front, initiating from the embryo and spreading into the starchy endosperm (Brookes *et al.,* 1976), correlating strongly with starchy endosperm enzymic degradation and grain modification (Cole *et al.,* 1998; Davies, 1990; 1992; Landau *et al.,* 1995; Reynolds and MacWilliam, 1966; Colin, 1918). However Moyolle *et al.,* (2010) has recently presented a non-limiting relationship between water diffusion and enzyme activity. There is a linear relationship between hydration of the barley starchy endosperm in the steeping stage and the final malting grade, which is sometimes used as a malting index to predict the quality of the barley (Davies, 1992; Collins *et al*., 1999).

1.4.2: Malting (controlled germination)

Malting is the term used to describe the controlled germination of barley, during which the aim is to maximise the amount of extractable carbohydrate, limit proteolysis, while at the same time ensuring that an adequate amount of hydrolytic enzymes are activated or synthesised (Longstaff and Bryce, 1993). The process of malting is controlled through manipulation of the environmental conditions under which the grain is germinated.

Under controlled conditions the grain is totally immersed in water (steeped), with intermittent air rests. The aim is to imbibe the seed to a moisture content of between 35-45% in an adequate oxygen supply (Burger and LaBerge, 1985). Following steeping, the grain is allowed to germinate, with both air temperature and humidity carefully controlled to maintain temperatures between 12-25°C. Grain moisture is generally maintained at 41-45%. This results in the growth of the embryo, which is signified by the emergence of the acrospire (the first shoot developing from the seed) and the rootlets (termed chitting). At 4 to 5 days of germination the process is arrested using controlled kilning of the grain. The grain is heated over a 24 h cycle with temperatures increasing to 80°C (Palmer, 1989b). This ensures that the germination process is halted and the moisture of the grain decreased to about 4-5%, while retaining some enzymic activity, required later for brewing (Burger and LaBerge, 1985). The term "malted grain" is given to grain undergoing this process of controlled germination.

1.5: Modification of the barley starchy endosperm

The term modification is used to include all the physical and chemical changes that occur in the starchy endosperm when barley grain is malted (Briggs, 1987, O'Brian and Fowkes, 2005). Patterns of modification within a barley grain have been of interest for a long time, with various techniques developed, as described by Gibbons and Neilsen (1983); Gibbons (1980a); Aastrup and Erdal (1980), Briggs and MacDonald (1983) and reviewed by Jamar *et al.,* (2011).. Recognised organisations, such as the IGB (Institute and Guild of Brewing), EBC (European Brewing Convention), and ASBC (American Society of Brewing Chemists) have all a documented analysis method/procedure for assessing starchy endosperm modification.

The general pattern of modification within the endosperm during germination has been established as a sequence of events involving the activation and/or secretion of hydrolytic enzymes, cell wall breakdown and the hydrolysis of endosperm protein and starch (Fretzdorff *et al*., 1982; Gibbons, 1980), basically to provide sugars to be used as a carbon source for the germination of the embryo. Cell wall modification is highly associated with the enzyme activity of (1,3;1,4)-β-glucanase (Collins *et al.,* 2004). To achieve uniform modification across the barley grain during malting, even hydration of the endosperm is required (Axcell *et al*., 1983). A minor factor influencing this process can be the structure of the grain, particularly its mealy and steely nature, as referred to in section 1.2.4.

1.5.1: Hydrolytic enzymes involved in barley grain modification.

Enzymes produced by the aleurone and scutellar epithelium during germination can be classified into three groups; 1) enzymes involved in ongoing intermediary metabolism within the cells, 2) enzymes that mobilize the reserves of the aleurone and scutellar cells themselves and 3) hydrolytic enzymes that mobilize reserves of the starchy endosperm (Fincher, 1989; reviewed by Evans *et al.,* 2010). The appearance of hydrolytic enzymes is one of the most important aspects of early germination. Some enzymes are synthesised *de novo* and secreted into the starchy endosperm, while others pre-exist in a latent form and are activated at this stage (Gibbons, 1979; Lauriere *et al*., 1986). The final products of starchy endosperm degradation (glucose and maltose) are translocated to the embryo to support seedling growth (Bewley and Black, 1994). Morrall and Briggs (1978) calculated that hydrolysis of endosperm cell wall material contributed the equivalent of 18.5% of the carbohydrate supply to the embryo with the starch providing the remaining 81.5%.

The synthesis of these hydrolytic enzymes for the mobilisation of starchy endosperm reserves occurs in two barley tissues, the scutellum and aleurone (Neiuworp and Buys, 1964; Briggs, 1972; Gibbons, 1981a; Akazawa, 1988; McFadden *et al.,* 1988; Okamato *et al*., 1980; Stuart *et al.,* 1986; Gubler *et al*., 1986; 1987; Ranki and Sopanen, 1984; Mundy and Munck, 1985; Palmer, 1982; 1989). The levels of hydrolytic enzymes secreted by cereal aleurone and scutellar tissue are influenced by phytohormones (gibberellins) produced by the embryo and appear to also be strongly influenced by Ca^{++} ions (Fincher, 1989). A regulatory feedback control mechanism may also exist via sensing of levels of sugars produced in the endosperm (Yu *et al*., 1996). The level and expression of enzymes from both organs (aleurone and scutellum) has been the focus of a considerable amount of research. The general consensus is that both tissues are involved; yet there is still uncertainty among some authors on the relative significance of each type.

1.5.2: *Specific enzymes in barley modification.*

Degradation of aleurone and endosperm cell walls represents an important event in early germination, removing the physical barrier to hydrolytic enzymes reaching carbohydrate and protein reserves in the starchy endosperm (Taiz and Jones, 1970; Ashford and Jacobsen, 1974; Simons, 1984; Gubler *et al*., 1987; Slade *et al*., 1989; Fincher, 1989; Benjavongkulchai and Spencer, 1989; reviewed by Burton *et al,* 2010 and Fincher, 2011). A β-glucan solubilase is believed to initiate the hydrolysis of (1,3;1,4)-β-glucan, rendering the (1,3;1,4)-β-glucan soluble in the starchy endosperm cell walls (Bamforth *et al.,* 1979; Bamforth 1981; Walker *et al*., 2001; Kuntz and Bamforth, 2007). The soluble (1,3;1,4)-β-glucan is hydrolyzed further by a group of glycosidic hydrolases including (1,3;1,4)-β-glucan endohydrolases, β-D-glucan exohydrolases and β-D-glucosidases/(1,4)-β-glucan glucohydrolases (Hrmova *et al*., 2001; Gianinetti *et al*., (2007). It has been found that the more (1→4) linkages present in the molecule the lower the solubility of the polymer (Izydorezyk *et al,* 1998; Lambo *et al*, 2005). Gianinetti, (2009) has experimentally demonstrated that during malting β-D-glucan degradation follows first order kinetics and that β-D-glucan content and solubility levels play a negative regulating role in the spread of modification across the starchy endosperm (Jin *et al*., 2004; Brennan and Cleary, 2005).

Two isoenzymes of (1,3;1,4)-β-glucanase (endo-hydrolase) responsible for the depolymerisation of (1,3;1,4)-β-glucan have been isolated from extracts of germinated barley (Woodward and Fincher, 1982a). These two isoenzymes (EI and EII) exhibit

almost identical substrate specificities and kinetic properties (Woodward and Fincher, 1982b). A third putative $(1,3;1,4)$-β-glucanase isoenzyme was also detected immunologically in isolated scutellar tissue (Stuart and Fincher, 1983) but was dismissed as endo $(1,3)$-β-glucanase (Stuart *et al.*, 1986). Differential expression of the glucanase isoenzymes has been demonstrated by northern blot analysis (Slakeski and Fincher, 1992). The Isoenzyme EI is found predominantly in the scutellum of the barley grain early in germination, while both isoenzymes can be found in the aleurone layer later in germination (Slakeski and Fincher, 1992).

Another important component of the cell walls is arabinoxylans. A number of decades ago it was reported by Preece and MacDougall (1958) that the complete breakdown of the branched arabinoxylan requires the action of several enzymes (Figure 1.4). The enzymes α-L-arabinofuranosidase and α-glucuronidase are involved in liberating the side chain sugars (Preece and MacDougall, 1958).

The enzyme $(1{\rightarrow}4)$-β-xylan endohydrolase (EC 3.2.1.8, xylanase) hydrolyses the polysaccharide backbone in essentially a random fashion once the arabinosyl side chains are removed. Xylan chains are further degraded by exoxylanase to produce xylobiose, to then be hydrolysed by a fourth enzyme, xylobiase (Preece and MacDougall, 1958). The role of esterases has also been examined due to the strong association of arabinoxylan and ferulic acid in barley cell walls (Moore *et al.*, 1996). Around 300 xylanases in all plants have been identified to date (Brenda, 2008; Azy, 2008). Three barley endoxylanases have been purified with a molecular mass of 41,000, and an isoelectric point of 5.2 (Slade *et al.*, 1989). The distribution of closely linked $(1{\rightarrow}4)$-β-xylanase genes have been mapped to the long arm of chromosome 7(5H), in a tight cluster that suggests that they have arisen by tandem duplication of a single ancestral gene (Banik *et al.,* 1997). Caspers *et al.* (2001) found different forms of the major endo-xylanase (termed XYN-1) in the aleurone of germinating barley grain, believing it to be synthesized as an inactive enzyme in the cytoplasm which becomes active only in the later stages of germination.

In extracts of germinating barley, most authors report that xylanase activity appears several days later than $(1,3;1,4)$-β-glucanase (Slade *et al.*, 1989); in fact the endo-xylanase genes are transcribed approx 24 h after those of $(1,3;1,4)$-β-glucanases (Banik *et al.*, 1997). The presence of endo-xylanase inhibitors in barley may also play a role in this delay (Goeaert *et al.*, 2001) although Kuntz and Bamforth, (2007) have reported xylanase activity earlier in germination. It has also been suggested that along with delayed protein expression the endo-xylanase proteins are further delayed during export

into the endosperm due to a strong binding with the aleurone cell walls (Benjavongkulchai and Spencer, 1989; Slade *et al*., 1989). It was noted by Van Campenhout *et al.,* (2007) that the N terminal domain of 61.5kda xylanase (Casper *et al.,* 2001) encodes a carbohydrate binding module that binds heteroxylans, making up 70% of the aleurone cell walls. Van Campenhout *et al.,* (2007) also believe that the xylanases might be more important in providing material for the growing seedlings than accessibility to the starchy endosperm.

Several of the starch degrading enzymes involved in endosperm modification have been characterised and their role in the germination process is well documented (Figure 1.5) and is reviewed by Evans *et al.,* (2010) and Fauconnier *et al.,* (2011). These include, α-amylase, β-amylase, α-glucosidase and limit dextrinase. The general consensus is that α−amylase (E.C. 3.2.1.1) is synthesised *de novo* in both aleurone layers and scutellar epithelium (Filner and Varner, 1967; Gibbons 1981a; Hardie, 1975). Alpha-amylase rapidly hydrolyses internal α-1,4-glucosyl linkages of amylose and amylopectin in a random fashion (Dunn, 1974; MacGregor and Ballance, 1980; Sun and Henson, 1991; Sissons and Macgregor, 1994).

Figure 1.4: Sites of arabinoxylan hydrolysis. Enzymatic hydrolysis of cell wall arabinoxylans (this figure is adapted from Lee *et al.*, 1999; taken from Jamar *et al.*, 2001)
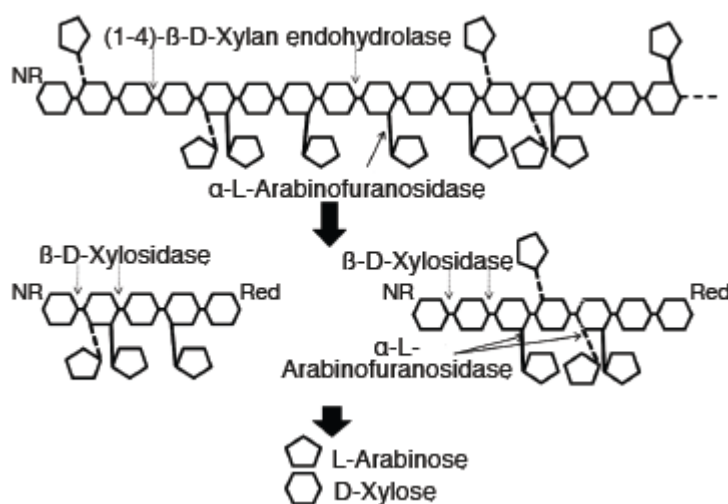
There are two isoenzyme groups of α-amylase (Jacobsen and Knox, 1973; Akazawa and Miyata, 1982); the low isoelectric point (4.5-5.0) isoenzymes, classified as α-amylase I, and the higher isoelectric point (5.9-6.4) isoenzymes classified as α-amylase II. Each group consists of multiple isoenzymes (Muthukrishnan *et al*., 1984). Alpha-amylase II has been demonstrated to be 2.3 times more active per unit protein than α-amylase I (McGregor and Morgan, 1992; Mitsui, 1997).

Barley α-glucosidase has been shown to degrade oligosaccharides (α-1,4 and α-1,6 linkages), although at a slower rate than α-amylase (Sissons and MacGregor, 1994; Sun and Henson, 1991). Alpha-glucosidase is synthesised *de novo* during germination (Hardie, 1975). A higher proportion of the enzyme activity is present in scutellum tissue, compared to aleurone layers (MacGregor and Lenoir, 1987). The enzyme can also be found in the pericarp tissue of un-germinated cereal grains (MacGregor and Lenoir, 1987).

The exo-enzyme β-amylase (E. C. 3.2.1.20) works only on hydrolysed dextrins released by α-amylolytic attack on the starch granules (Maeda *et al*., 1978). Beta-amylase cleaves alternate 1→4-glucosyl linkages to release the sugar maltose (Sun and Henson, 1991). Beta-amylase is not secreted by aleurone or scutellum tissue but is synthesised in the barley endosperm during development of the grain (LaBerge and Marchylo, 1986). Existing in a bound (latent) form, β-amylase is believed to associate with the periphery of starch granules (Lauriere *et al*., 1986). During germination bound β-amylase is released and activated by the action of proteolytic enzymes (Hejgaard, 1979). Over the past few years there have been a number of reports identifying isoforms and alleles of β-amylase. The forms are identified by their isoelectric focusing point and/or thermostability and are described as starch degrading (SD) SD1, SD2H or SD2L (Ma *et al.*, 2001, 2000a, 2000b, 2002; Eglington *et al*., 1998; Evans *et al.,* 2010) or Bmy1, 2, and 3 (Li *et al*., 2000), or as A, B and C (Kihara *et al*., 1998).

Another starch degrading enzyme is limit dextrinase (EC 3.2.1.41, LD) (Jespersen *et al*., 1993; Nakamura *et al*., 1996; Renz *et al*., 1998). This enzyme is also referred to as pullulanase, R-enzyme or starch debranching enzyme. It has the ability to hydrolyse the α-1→6-glucosyl linkages in amylopectin and branched dextrins, which are not hydrolysed by amylases (Kneen and Spoerl, 1948; Lee and Plyer, 1984). LD falls into the starch debranching enzyme group, capable of hydrolysing (1→6)-α-linkages in pullulan (Lee *et al.,* 1971) and its crystal structure has been defined (Vester-Christensen *et al.,* 2010). The enzyme is thought to be synthesised *de novo* during germination (Hardie, 1975; Burton *et al.,* 1999), although the rate of synthesis appears to be much lower than other hydrolytic enzymes (MacGregor *et al*, 1994c). There is evidence that a 'bound form' of LD exists in the mature grain, which may be activated by proteases *in vivo* or *in vitro* with several hours' incubation in the presence of reducing agents (Serre and Lairier, 1989; McCleary, 1991; Lee and Pyler, 1984). Even without the

addition of endogenous reducing agents the enzyme will eventually become activated over a period of time (5 to 10 days) during germination (Longstaff and Bryce, 1993).

Little is known about the proportions of bound to free limit dextrinase during seedling growth in barley or about the mechanism of its release from a bound form *in vitro* under the influence of reducing agents (Longstaff and Bryce, 1993; Yamada *et al*., 1981). The bound enzyme is found in the grain earlier than the free enzyme (Macgregor *et a*l., 1994).

Figure 1.5: Starch hydrolysis.
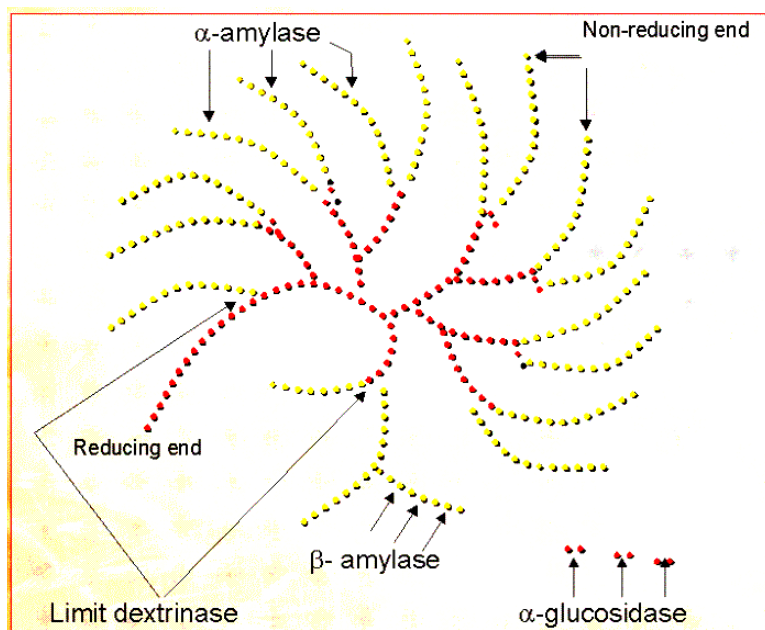
Studies in barley have also detected limit dextrinase activity during grain development (Kruger and Marchylo, 1978; MacGregor and Dushnicky, 1989; Burton *et al.,* 1999, Sissons, 1993; Kristensen *et al*., 1998; MacGregor *et al.,* 1994).   A debranching enzyme at this stage in grain development would be capable of producing primers for the starch synthase reaction if required (Duffus and Cochrane, 1993).

Two limit dextrinase inhibitors (LDIN) have been identified (Marci *et al.,* 1993). They appear structurally different to either hordothionins (Jones, 1982) or the barley α amylase inhibitor (Weselake, 1983), although a cDNA sequence labelled as barley α amylase inhibitor (Lazaro *et al*., 1988) was found to encode LDIN by Macgregor *et al.* (2000). The two inhibitors differ in molecular weight, with a different pI (cysteine or glutathione), differences arising through post-translation modification (Macgregor *et al.,* 2000; 2004), and are more heat labile than the active LD enzyme at 60°C (Marci *et al*., 1993; Longstaff and Bryce, 1991). It takes a short period of time for purified LDIN

to completely inhibit the activity of limit dextrinase (MacGregor *et al*., 1994), and the activation can be reversed by warming the complex to $40^{o}C$ in the presence of reducing agents (MacGregor *et al*., 1994c). Bound LD is thought to be complexed in a 1:1 molar ratio (MacGregor *et al.,* 2003) with LDIN and is present in the mature barley kernels, but it disappears several days after the onset of germination (MacGregor, 2004). However, it is inconclusive that LDIN alone inhibits limit dextrinase activity as levels of detectable LDIN are not sufficient to account for all inactive limit dextrinase when released by reducing agents *in vitro* (MacGregor, 1995).

1.5.3: *Hormonal control and regulation of enzyme mobilization*

Hormonal control of hydrolytic enzyme secretion and/or activation has been extensively documented and reviewed (Palmer, 1972; Briggs, 1972a: Macleod and Palmer, 1966; Dominguex and Cejudo, 1999; Mundy and Fincher, 1986; Banik *et al.*, 1996; Chandler *et al*., 1984; Schuurink *et al*., 1997; Richie *et al.*, 1999; Jiang *et al*., 1996; Woodger *et al.,* 2010). Over the last two decades numerous components of signal transduction pathways of various plant hormones have been identified. There appears to be a lot of cross interaction among different hormone signal transduction pathways. The predominant hormone in barley appears to be Gibberellins (GAs) implicated in the regulation of various developmental processes throughout the life cycle of the plant, particularly promoting the rapid expression of genes encoding hydrolyses (α amylase) (Lovegrove and Hooley, 2000; Olszeluski *et al.,* 2002). There are more than 72 known gibberellins and 61 of these are present in higher plants (Mayer and Poljakoff-Mayber, 1989). In barley, most metabolic studies on the mechanism of gibberellins have been made with GA_3, the predominant gibberellin in barley (Palmer, 1971). A nucleotide sequence element in the promoter region of several barley genes has been implicated in GA_3 induction (Chandler *et al*., 1984; Mundy and Fincher, 1986; Gubler and Jacobsen, 1992; Banik *et al.,* 1997). When the embryo is removed from a barley seed, external or exogenous supplies of gibberellic acids are needed to initiate endosperm modification (Radley, 1967; Palmer, 1971; 1970). Gibberellin biosynthetic inhibitors do not appear to inhibit the increase in amylase production during germination, suggesting the embryo is mobilizing stored GA_3 precursors rather than synthesizing GA_3 *de novo* (Grosselindemann *et al.,* 1991).

A GA_3 feedback control loop was initially proposed in germinating barley seeds (Briggs, 1992) and several studies reported evidence supporting this mechanism with sugars (Yu *et al*., 1996; Perata *et al*., 1997), calcium chloride (Jones and Jacobsen, 1991; Bush, 1995), guanosine 3', 5'-cyclic monophosphate (cGMP) (Penson *et al*.,

1996), a regulated protein GAMYB and related genes (Gubler *et al.,* 1995*,* 1999, 2002; Robertson *et al.,* 1998; Zentella *et al.,* Woodger *et al.,* 2004; 2002; Rubio-Somoza *et al.,* 2006; Murray *et al.,* 2006) or a GA-insensitive/repressor of ga1-3 (GAI/RGA) (Richards *et al.,* 2001). H_2O_2 activation of GA signaling has also been suggested (Bahin *et al.*, 2011). Some authors believe that the association of GA_3 and endosperm modification is not a simple process with many factors needed to be considered, such as grain sensitivity and varietal differences (Fincher, 1989; Grosselindernann *et al.,* 1991; Wang *et al.*, 1996). In addition to the process of GA synthesis, inactivation is also important in regulating levels as has been found in immature sorghum (Cantoro *et al.,* 2012). Studies using genetic approaches in barley have identified positive and negative signaling components (Sun and Gulber, 2004). A class of nuclear proteins (DELLA proteins) have been characterized and appear to act as a suppressor of GA signaling and responsive processes such as seed germination (Sun and Gulber, 2004; Hedden and Philips, 2000; Olszewski *et al.,* 2002). A *trans-cis* acting factor regulating down stream GA responsive genes in higher plants appears to induce proteolysis of the DELLA proteins (Ritchie and Gilroy, 1998; Sun and Gulber, 2004). And there is suggestion that the DELLA protein complex may restrict GA promoted processes by modulating both GA and Abscisic acid (ABA) pathways (Zentella *et al.,* 2007). Several studies have attempted to identify the site of hormome action, which may act either at the mRNA transcriptional level (Zwar and Hodey, 1986; Skriver *et al.*, 1991) or with a soluble GA receptor (GA insensitive DWARF1; GID1) (Griffiths *et al.,* 2007). The critical link between cytoplasmic signalling events and gene transcriptional regulators are largely unknown (Bethke and Jones, 1998; An and Lin, 2011).

The nucleotide sequences linked to GA_3 stimulation have been identified in α-amylase gene promoters, but not in the (1,3;1,4)-β-glucanase genes. However, expression studies have highlighted a difference in phytohormonal sensitivity between the EI and EII isoenzyme promoter regions (Stuart *et al.,* 1986; Slakeski and Fincher, 1992a; Hrmova *et al.,* 1997).

The movement of gibberellins through the barley grain is likely to occur by active diffusion through cytoplasmic and plasmodesmata connections (Palmer and Scattler, 1996). Bruggeman *et al.* (2001) has produced a mathematical model on the diffusive transport of hormones through the aleurone layer of barley. The model consists of two partial differential equations describing the accumulation of phytohormone in the apoplastic and symplasmic compartments of the aleurone layer.

ABA is also believed to play a major role, both in the control of embryo maturation and in the prevention of precocious germination in the morphologically mature but still unripe grain, technically termed dormancy (King, 1976; Chandler *et al*., 1987; Schuurinnk *et al*., 1993; Bewley, 1997), and is a key phytohormone responsible for stress sensing and signal transduction processes (reviewed by Takezawa *et al*., 2011 and Staroske *et al.,* 2016). The mode of action of ABA is believed to be either in the suppression of the expression of germination specific genes, particularly the ABA 8'-hydroxylase gene or in the restriction of the availability of energy and metabolites that may be necessary for embryo maturation (Garciarrubio *et al.,* 1997; Williamson and Quatrano, 1988; Van den Wijngaard *et al.,* 2005; Millar *et al.,* 2006). ABA may simultaneously raise the levels of proteins such as the α-amylase substilisin inhibitor (BASI), which inhibits the activity of the hydrolytic enzyme α-amylase (Jacobsen, 1979; Mozer, 1980; Mundy and Fincher, 1986; Mundy, 1984). Multiple receptor sites and internal or external binding systems have been suggested (McCarty, 1995; Ritchie and Gilroy, 1998), but only a single binding protein has been found (Razem *et al.,* 2006). The association of white light and ABA embryo content has been suggested (Gubler *et al.,* 2008) with a strong association between dormancy, coleorhizae growth and the balance between ABA and its metabolized precursor phaseic acid (Jacobsen *et al*., 2002; Kushiro *et al*., 2004; Okamoto *et al.* 2006; Barrero *et al.,* 2009).

The starchy endosperm pH (optima around 4.5) may also play a role in the regulation of enzyme activities and is believed to help facilitate movement across the scutellum to the embryo in an osmotic fashion (Bush, 1993; Green, 1994).

1.5.4: *Methods for measuring modification of the starchy endosperm.*

Palmer (1989b), Briggs (1987) and reviewed by Fincher (2011) have detailed the chemical and physical analyses used to assess modification. They include; hot water extract, which gives a measure of available products (protein, carbohydrates) brought into solution when a malt is mashed under controlled conditions; wort viscosity; the Kolbach index, which is the ratio of soluble nitrogen over total nitrogen; β-glucan content as a measurement of undegraded cell wall material and diastatic power (DP). DP is defined as the collective activity of all the enzymes involved in the hydrolysis of starch to fermentable sugars during malting and the mashing stage of brewing (Moll and Flayeaux, 1975). The key enzymes are α-amylase, β-amylase, α-glucosidase and limit dextrinase. The combined activity of these enzymes in malt is measured under standard conditions (Delcour and Verschaeve, 1987; Evans and MacLeod, 1993). Limit dextrinase may be measured as part of DP, because its activity is seen as critical

to complete starch degradation, although most limit dextrinase is produced late in germination and therefore does not feature predominately in commercial assessments where germination is completed within five days (Longstaff and Bryce, 1993; Lee and Pyler, 1984).

Physical indices are also used to assess malt modification. These include friability, milling energy (Swanston *et al.,* 1995), endosperm comparison (steely/mealy), endosperm hardness (Walker *et al,* 2011), gas displacement measurements (Walker and Panozzo 2011) or grain sectioning and staining using reagents such as calcoflour white which binds to cell walls (Briggs and MacDonald, 1983, Mundy *et al*, 1981; Palmer 1974). The chemical analyses of grain modification involve the homogenisation of the milled grain, while physical assessment is at an individual grain level. Variation between individual grains can cause problems in physical assessment, and so is overcome by assessing large sample numbers. Problems defining the physical changes occurring in barley during malting using sectioned material have given rise to much controversy (Mundy *et al*., 1981). Inadequate microscopic tools, poor specimen preparation and uneven sectioning have hindered definitive conclusions. Briggs and MacDonald (1983) concluded that many sectioning and staining procedures were invalid and unrepresentative, particularly when the loss of tissue integrity in modified areas of endosperm prevents accurate visualization of the area. This was also highlighted by Briggs in 1989, who examined different sections through the barley grain to compare the morphology differences when sectioning either mid grain or at an angle to the ventral line. Sectioning is a critical aspect to any study examining the physical characteristics of a barley grain and was therefore an essential part of this study and is subsequently discussed in greater deal in Chapter two.

Chemical analysis, on the other hand, may be seen as an indirect assessment (Johnson and Dyer, 2000) and works on an average that can conceal heterogeneity. Ideally the chemical data should relate to the actual changes observed within the grains but generally correlates to only (1,3;1,4)-β-glucan breakdown and endo-β-glucanase activity. But the relationship between the two have been observed by some researchers, such as McFadden *et al.* (1988) and Sigmoto *et al. (*1998), who demonstrated with *in situ* hybridization that the expression of endo-(1→4,1→3)-β-D-glucanase and amylase mRNA respectively, followed the pattern of endosperm cell wall breakdown and α-amylase enzyme expression during germination.

Maltsters have also been known to use the length of the acrospire as an indication of the degree of modification (Aastrup and Erdal, 1980), although it has since been shown to have a poor correlation to the stage of grain germination.

1.5.5: *Assessment of starchy endosperm cell wall breakdown in barley grains during modification*

1.5.5.1: *Calcofluor staining*

Both (1→3, 1→4)-β-D-glucan content and β-glucanase activity have been observed to correlate well with cell wall modification (Aastrup and Erdal, 1980; Mundy *et al*., 1981; Collins *et al*., 2004). Tissue sectioning and staining with specific dyes is a common technique used to measure endosperm modification, and one of the most commonly used dyes is the UV fluorescent dye calcofluor M2R (CW), which binds to β-glucosidic linkages (cellulose) and (1,3;1,4)-β-glucans (Fulcher and Wong, 1980). In 1983, Wood and Fulcher developed a method of sectioning grain and staining with calcofluor to locate and quantitate (1,3;1,4)-β-glucan in grains, thus giving a structural assessment for the degree of cell wall degradation. Calcofluor analysis of half grain sections is classified as an official assessment modification method by the European Brewing Convention, and has been used extensively to monitor the progressive breakdown of the endosperm cell walls in malted grains (Munck *et al*., 1981; Aastrup and Endal, 1980; Gibbons, 1980; Jacobsen and Chandler, 1987).

The major disadvantage with the calcofluor dye is that it does not have the ability to bind to (1,3;1,4)-β-glucans of low molecular weight (*i.e.* molecules containing fewer than 60 glucose residues), thus it cannot detect complete breakdown (Gomez *et al.,* 2000; Foldager and Jorgensen, 1984; MacGregor *et al*., 1994b; Pomeranz, 1974). In addition, as the Calcofluor dye binds largely but not solely to (1,3;1,4)-β-glucan it is not completely specific and cannot tell us anything about other cell wall components (Selvig *et al.*, 1986), such as arabinoxylan and phenolic acids. Palmer (1983) believed that measuring (1,3;1,4)-β-glucan levels alone is not a reliable prediction of the degree of modification. However, the CW assay is routinely used in malting laboratories and is the best method developed to date.

1.5.5.2: *Hydration assay.*

Grain hydration is a measure of the ability of the water to move through the endosperm during grain germination. Endosperm water uptake and distribution can be measured either by iodine vapour or boiling intact grain methods (Landau *et al.,* 1995). Both techniques rely on treatment, sectioning and examination of individual grains. For the boiling method, a score is given based on the amount of white endosperm still visible

following boiling, because sufficiently hydrated starch will gelatinise, making the area appear translucent (1-4, Table 1.1), while the total hydration score of a sample is determined by the equation given in Table 1.1. There can be enormous variability between individual endosperm hydration scores within a sample group (Oh and Briggs, 1989; Chandler and Proudlove, 1998; MacGregor, 1994; Palmer, 1982; H. Collins, personal communication 2003; data presented in Chapter two), and this is most likely due to the mealy/steely nature of the endosperm (Allison *et al*., 1979; Palmer, 1989; Schildbach *et al*., 1990: Jacobsen and Chandler, 1987), variation in grain size within a heterogeneous population, nitrogen content (Brookes *et al.,* 1976; Axell *et al.,* 1994) hulless nature (Summer and Schildback, 1970; Ulonska, 1976; Chandra *et al*., 1999) or endosperm levels of (1,3;1,4)-β-glucan and protein acting as a barrier. A high hydration value is usually found in grain with a mealy endosperm, along with significant levels of (1,3;1,4)-β-glucanase detected early in the embryo, while a low hydration value is linked to a steely endosperm and all (1,3;1,4)-β-glucanase activity occurring later from the aleurone (Chandler and Proudlove, 1998). This variation within a sample population received considerable attention in the experimental planning of this study, as discussed in Chapter two.

Table 1.1: Degree of hydration observed in the endosperm of sectioned grain.

Category	Less quarter*	Quarter to half*	Half to three quarters*	Over three quarters*
Score	1	2	3	4

Final score = Number of grains in each category X score for category.

*Classification from Cole *et al*. (1997).

1.5.6: Secretion of hydrolytic enzymes from scutellum and aleurone tissue.

Despite suggestions that the scutellum plays no part in endosperm modification (Palmer, 1982; 1989; 1996), several authors have emphasised the role of the scutellum during germination (Briggs, 1972; Okamoto *et al*., 1980; Gibbons, 1981; Ranki and Sopanen, 1984; Mundy *et al.,* 1985; Stuart *et al*., 1986; McFadden *et al*., 1988b; MacGregor *et al.,* 1994b; Sugmioto *et al.,* 1998). Many believe that the scutellum initiates the secretion of enzymes for starchy endosperm modification in the early stages of germination (Munck *et al*., 1981; Akawazwa and Miyata, 1982; Briggs and MacDonald, 1983; McFadden *et al.,* 1988). The amylases, the glucanases, proteases and ribonucleases detected by several procedures such as mRNA expression, immunofluorescence and *in situ* hybridization assays, have all been reported to be initially secreted during germination from the scutellar region, and later from aleurone cells (Okamoto *et al*., 1980; Gibbons, 1980; 1981a; Ranki and Sopanen, 1984;

McFadden *et al*., 1988; Sugmioto *et al*., 1998). Alpha amylase activity (between 5 to 10% of total α-amylase activity) has been detected in both scutellar epithelial cells (Mundy *et al*., 1985; Ranki, 1990), and the scutellum in germination (Gibbons, 1981a; MacGregor *et al.,* 1984; Ranki and Soparen, 1984), with the low pI α-amylase form predominant (MacGregor and Marchylo, 1986; MacGregor 1978). Studies on the involvement of the scutellum tissue in hydrolytic synthesis with immunostainng and *in situ* hybridisation studies have been more convincing. McFadden *et al*. (1988) used the technique of *in situ* hybridisation and a specific cDNA probe to (1→4, 1→3)-β-D-glucanase isoenzyme EII to demonstrate mRNA first in the scutellum (day one of germination) and at day two in the aleurone layer. This confirmed the earlier work of Mundy *et al*. (1985) and Stuart *et al*. (1986), who demonstrated the presence of 1→4, 1→3)-β-D-glucanase mRNA in isolated aleurone and scutellum tissue. These studies also detected differences in the expression of the endo-(1→4,1→3)-β-D-glucanase isoenzymes, where isoenzyme EII is secreted predominantly from the aleurone, while isoenzyme EI originates from the scutellum (Stuart *et al.*, 1986).

Several authors have located enzyme activity and mRNA expression of hydrolytic enzymes in aleurone tissue (Chrispeels and Varner, 1966; Gubler *et al*., 1986), particularly after hormonal induction (Jacobsen and Knox, 1973; Gubler, 1987). An immuno-staining study (Gulber, Ashford and Jacobsen, 1987) with gold-labeled antibodies against α-amylase, demonstrated that this particular enzyme is released from barley aleurone cells via channels formed in the outer wall layer (Van der Eb and Nieuwdorp, 1967). It has been deduced from the many studies using aleurone layers that the hydrolytic enzymes are synthesized on rough ER and targeted to the lumen of the ER, most likely through the recognition of signal peptides (Fincher, 1989). Golgi-derived vesicles then appear to be implicated in the transport of the secretary products to the plasma membrane (Fernandez and Staehelin, 1985), both in the aleurone and scutellar epithelium (Nieuwdorp and Buys, 1964; Gram, 1982). Such leader sequences have been identified on cDNA nucleotide sequences of α–amylase (Fincher, 1989). The involvement of the aleurone in the production of hydrolases during germination is believed to reach a maximum activity at about 3 to 5 days of germination (Gibbons, 1979; Gibbons, 1980b; Okamoto *et al*., 1980; MacFadden *et al.,* 1988; Sugmoto *et al.,* 1998), slightly delayed compared to the scutellum.

1.6: Previous studies on enzyme mobilization during germination
The sites of hydrolytic enzyme synthesis and secretion in barley germination have been researched quite extensively. Evidence indicates that the hydrolytic enzymes are

probably secreted from both the scutellum and aleurone layers (Briggs, 1972; Okamoto *et al*., 1980: Briggs and MacDonald, 1983; Gibbons, 1980, 1981; Munck *et al.*, 1981; MacGregor *et al.*, 1984; Mundy *et al*., 1985; Stuart *et al.*, 1986; McFadden *et al*., 1988). In general, endosperm degradation begins adjacent to the scutellum and progresses from the proximal to distal end of the grain as a front moving away from, and parallel to, the face of the scutellum across the aleurone layer (Gibbons, 1980, 1981; Astrup and Erdal, 198l; Briggs and MacDonald, 1983; Selvig *et al.*, 1986; McFadden *et al*., 1988). However, Palmer (1975; 1987; 1990; 1996) has cast doubt on this with his observation that enzymic modification occurs mainly from the aleurone on the dorsal surface of the endosperm rather than at the scutellum-endosperm interface. Palmer (1974) tried to highlight the role that the aleurone has in the production of hydrolytic enzymes in endosperm modification with his concept of gentle seed abrasion. Seed abrasion increases endosperm modification by promoting the access of externally supplied gibberellins to the aleurone layers, thus producing modification from both ends of the grain (Palmer, 1969). Smith and Briggs (1979), disputed the two-way modification concept, finding only a one-sided pattern of modification using the abrasion technique, although Mundy *et al.* (1981), showed evidence that abrasion did increase the level of modification in the grain. The abrasion process has been adopted in some cases by the malting industry to accelerate barley modification, although grain shattering during milling and increased bacterial counts are drawbacks of the process.

Tissue isolated from barley, particularly protoplasts made from aleurone layers, have also provided a vast amount of information on enzyme synthesis and secretion during germination. Problems exist with these types of studies, as tissue obtained can be contaminated with adjacent tissue types, and most manipulations are performed in aqueous media which allows the loss of water-soluble components (Mares and Stone, 1973; Palmer, 1982; Aishen *et al*., 1983). Preparation of the scutellum to ensure it is free of contaminating tissue has been reported (Stuart *et al*., 1986) although this is difficult to achieve. Most research has concentrated on measuring a quantitative level; either protein or mRNA secreted and extracted from the tissue (Slakeski *et al.,* 1990; Slakeski and Fincher, 1992) or *in vitro* translated products of mRNA isolates (Mundy *et al*., 1985). Data obtained from extracted mRNA can only provide an average level of gene expression and may not detect changes occurring at the cellular level, such as differentiation or atrophy (Penschow 1987; Chandler *et al*., 1991). This raises the problem that there is a fundamental assumption that the scutellum and aleurone are

similarly affected by the treatment they have received during preparation (Palmer and Duff, 1986).

The possibility of microbial contamination in tissue extracts stored for periods of time in nutrient medium could affect the results obtained. Higgins *et al*. (1982) are one of the few authors who tested for microbial contaminates in the medium of their excised tissue, allowing for misleading results due to contamination. Others have tried to maintain sterile conditions by using laminar flow units in dissection procedures (Eastwell *et al*., 1982) or maintaining the tissue in medium containing antibiotics (Chrispeels and Varner, 1966; Stuart *et al.*, 1986; McFadden *et al*., 1988). The effect of antibiotics used in solutions of isolated tissue sections has also been questioned (MacLeod *et al*., 1963; Palmer, 1986).

The time at which tissue is dissected constitutes additional problems. Many researchers use methods that involve a 3-day imbibition prior to isolation of the tissue (Chrispeels and Varner, 1966; Stuart *et al.*, 1986; Muthukrishnan, 1979; Eastwell, 1982; and Gibbons, 1979). When researching the early expression of hydrolytic enzymes this time delay could be critical, as MacGregor (1984) noted that alpha amylase was already detectable in the underlying endosperm after dissection of the scutellum at two days post steeping, indicating that the enzyme had already been activated and secreted from the scutellum before excision. Variations in the preparation of the grain before tissue excision can also affect the results obtained and makes it hard to compare results between different research groups. Examples include germination temperatures ranging between 15 and 28°C, germination carried out on either filter paper (Slakeski and Fincher, 1992), agar (Ranki, 1990) or a commercial malting schedule and the use of different barley cultivars.

Immunocytochemical and hybridization techniques have found increasing application in the measurement of secretion (mobilization) of hydrolytic enzymes in barley. These are favorable techniques due to their high resolution, and the application to intact grains avoids the interpretative problems associated with tissue disruption in isolated samples (Fincher, 1989). However, there are difficulties in observing structural changes using whole grain sections, as fixation, infiltration and preservation of whole grain structures requires particular care (to be discussed in section 1.11). The direction of sectioning is also critical when determining the pattern and degree of endosperm degradation (Selivg *et al.*, 1986).

1.7: Endosperm heterogeneity

Physical heterogeneity (variation in physical properties) and physiological heterogeneity (variation in chemical properties) of the endosperm have major impacts on cereal grain processing. As discussed earlier, there is significant variation between varieties and within a population of grain of the same variety (Andersson *et al*., 1999). In a single batch of barley for example, heterogeneity exists as grain hardness, chemical composition and the potential for generating enzymes.

1.8: Aleurone programmed cell death

The appearance of DNA fragmentation in the aleurone layer has been observed to start near the embryo and extend along the aleurone cells away from the embryo in a time dependent manner, similar to the pattern of hydrolytic enzyme expression (Jones and Bush, 1991; Wang *et al*., 1996a). DNA fragmentation was dramatically accelerated by the application of cell wall degrading enzymes to the aleurone *in vitro* (Wang *et al*., 1996a). The emerging picture is that cell death in aleurone tissue is stimulated by gibberellins and blocked by ABA (Kuo *et al*., 1996; Bethke *et al*., 1999). An inhibitor of gibberellin signaling in barley aleurone (LY83583) has been found to prevent aleurone cell death (Bethke *et al*., 1999; Fath *et al*., 2001). Aleurone cells induced by gibberellin have been shown to synthesize a 33kDa nuclease protein, with ribonuclease, deoxyribonuclease and 3' nucleotidase activity (Brown and Ho, 1986) and exhibiting features typical of a nuclease I (Wilson, 1975). The protein appears related to the decreased production of non-secreted hydrolases (Holsten *et al*., 1991, Bethke *et al*., 1999; Londsdale *et al*., 1999) and/or fusing of aleurone cell plasmodesmata (Bethke *et al*., 1998; Swanson *et al*., 1998). Fath *et al*. (2001) also implied that GA treated cells lose their ability to scavenge ROS (reactive oxygen species) and that this loss ultimately results in oxidative damage and cell death, while ABA treated cells, on the other hand, maintain their ability to scavenge ROS and remain viable. These studies suggest that cell death may occur due to the massive production of hydrolytic enzymes, the simultaneous loss of the cell walls and an increase in osmotic pressure and production of sugars caused by degradation of the endosperm.

1.9: Summary of factors affecting barley germination and modification

Germination in barley is a complex process involving several different tissue types, and is characterised by the production of hydrolytic enzymes and progressive changes in the structure of the starchy endosperm. In the early stages of germination, embryonic hormones induce the secretion of hydrolytic enzymes from the scutellum and aleurone. Aleurone, endosperm and possibly scutellum cell walls are degraded, allowing access

of the hydrolytic enzymes to the endosperm starch and protein reserves. Low molecular weight sugars, amino acids and peptides are then transported via the scutellar epithelium to the embryo as nutrients for the growing seedling. Malting of barley involves controlled germination of the grain, such that hydrolytic enzymes are produced while starch and protein degradation in the endosperm is kept to a minimum for use later in the brewing process. A detailed understanding of the production and action of the various hydrolytic enzymes is fundamental to successful malting and brewing.

Modification of barley during germination is the result of a number of complex interactions, thus several means of assessment should be used to ensure an accurate determination as concluded by Munck *et al.* (1981), both at the chemical and physical (structural) level in order to best evaluate the degree of modification. Controversy exists as to the importance of aleurone and scutellum tissue in the enzymic process of germination. The movement of the enzymes through the barley grain in germination has not been definitively described. It is difficult to judge, from the endosperm cell wall modification pattern alone, where enzyme expression initiated (Selvig *et al*., 1986). Looking at specific enzyme expression and secretion in individual tissues is necessary. A combination of techniques including the established chemical assays and more recently developed *in situ* hybridization and immunocytochemical methods open the way to clearly document in detail areas participating in hydrolytic enzyme secretion and their subsequent mobilization across the germinating barley grain.

1.10: Brewing

The main aim of brewing is simply described as the extraction of fermentable sugars from malted barley and the anaerobic fermentation of these by yeast into ethanol, which also generates molecules with agreeable flavour and aromas. After milling or grinding, malted barley is mashed into warm water to extract fermentable sugars, dextrins, peptides, amino acids and hydolyse starch (gelatinization at 65°C). The final liquid product is referred to as wort and the brewer's quality requirement of the wort stems from an optimal endosperm modification of the barley during germination (Scheer, 1990).

1.11: Industry related problems

Cell wall material in the starchy endosperm of barley must be sufficiently degraded to avoid problems with extract yield, beer filtration and wort (malt extract) clarity (Ahluwalia and Fry, 1986; Bamforth, 1981; 1982). For grain to be considered optimal

for brewing or distilling it must be at malting grade standard and achieve specific quality parameters, such as protein composition, enzyme activity and extract.

The synthesis, secretion and migration of a large number of enzymes through the grain during germination are prerequisites for successful malting and subsequent brewing and distilling. As most enzymes are heat labile and destroyed rapidly by mashing temperatures in brewing, the $(1,3;1,4)$-β-glucan degradation that occurs during malting is important, as further breakdown in the mash is limited (MacGregor, 1990). Residual $(1,3;1,4)$-β-glucans in the molecular weight range of 7 X 10^4- 6 X 10^5 daltons have been found both in wort and beer, and are believed to contribute to viscosity, haze and filtration problems in the brew house (Bamforth, 1982; 1985; 1994; MacGregor and Fincher, 1993; Manzanaries and Sendra, 1996). In fact, high molecular weight $(1,3;1,4)$-β-glucan is possibly the foremost cause of wort viscosity problems (Biermann *et al.,* 1990; Lewis and Young, 1995).

There are parallels between the content of $(1,3;1,4)$-β-glucan and arabinoxylan in barley, where both produce highly viscous aqueous solutions. Therefore the two together most likely compound the problems encountered in brewing. There is not a great deal of data reported on the influence of arabinoxylan in the brewing industry, but a study of wheat arabinoxylan degradation (Schmiz *et al.*, 1974) described the release of free xylose from the xylan backbone decreasing the viscosity of a solution and another study has shown that arabinoxylan constitutes 88% w/w dry basis of a haze derived from a commercial beer (Coote and Kirsop, 1976). In addition, microbiological exogenous endoxylanase was able to reduce viscosity, improve extract and increase beer filtration (Ducroo and Frelon, 1989). The addition of exogenous β-glucanase and xylanases to the mash or fermenting beer is implemented by some brewers, but does have a cost consideration (Ducroo and Frelon, 1989; Bamforth, 1994). Thus, a better understanding of the endogenous presence and action of such enzymes at all stages in germination warrants further investigation (Henry, 1985; MacGregor, 1990; MacGregor and Fincher, 1993).

High $(1,3;1,4)$-β-glucan and arabinoxylan levels generally reflect partial modification of the grain, where cell wall breakdown is incomplete. This has implications for the restriction of enzyme accessibility to starch and protein within the endosperm matrix (MacGregor and Fincher, 1993). This includes the capacity to degrade protein to peptides and amino acids by proteases and peptidases, which occurs primarily during the malting process, and not in the brewing mash due to enzyme instability at the higher temperatures (Palmer, 1983). The B and D hordeins are more successfully modified

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than the other storage proteins during malting, with 35-40% of malt protein solubilised (Skerritt and Henry, 1988; O'Rourke, 2002) for yeast nutrients in the brewing fermentation process. The brewing process demands a critical measure of protein, as high protein in malt (wort) can cause haze (Biermann, 1990; Nischwitz *et al.*, 1998; Asano *et al.*, 1982; George, 1989; Siebert, 1999; Gunkel *et al.*, 2002) and/or affect wort filtration and brew house performance such as foam formation and taste (Woodward and Fincher, 1983; Bamforth, 1985; Woodward *et al.,* 1988; Mikola and Enary, 1970).

Barley endosperm starch also has implications in the brewing process; with the starch content of the barley remaining at approx. 10% (mostly small granules) un-hydrolysed during malting (Bathgate and Palmer, 1973). The importance of starch degrading enzymes (section 1.8.3) cannot be overlooked and these are therefore discussed in greater detail in section 1.8.3.

1.12: Technical aspects of this study

To explore the concept of assessing mRNA and protein expression in barley tissue sections two main procedures were considered; *In situ* hybridization and immunocytochemistry.

1.12.1: *In situ hybridisation (ISH)*

In situ hybridisation (ISH) was first described in Brown (1953) and has since been used in all fields of biological research (Guide to In Situ, 1994). *In situ* hybridisation allows us to study gene function within different tissues (Schwarzacher *et al.*, 1989; Coghlan *et al.,* 1985*)* and it is specific enough to distinguish between the expressions of related genes in gene families (Braissant *et al.*, 1996). The use of ISH on plant tissue is considerably less than that reported in the area of medical research, particularly with the non-radioactive procedure. Some plant studies have been reported using Arabidopsis (Haslekas *et al.,* 1998; Verelst *et al.,* 2004; Bruno *et al.,* 2011), maize (Bih *et al.*, 1999; Trevisan *et al*, 2011) rice (Sigmoto *et al.,* 1999; Kikuchi *et al.,* 1999; Yuan *et al.,* 2011; Sun *et al.,* 2011) wheat (Lizana *et al*, 2010) and barley (Martinez *et al.*, 2004; Rae and Smith, 2002; Hause *et al.,* 1996; McFadden *et al.,* 1988a) for example and these form the basis for development of the assay used in this study. The technique is an important tool for the location of genomic sequences on chromosomes which is generally called FISH (Trusk, 1991). It is an extension of northern blotting (Nygarard and Hall, 1963), although in this case naturally occurring DNA or RNA is immobilized in the cell or tissue of origin. The two types of ISH that are in general use are hybridization to nuclear DNA and hybridization to cellular RNA (Pardue, 1985), which can be applied to tissue sections or used for the whole mount technique (de

Almeida Engler *et al*., 1998). The threshold levels for standard *in situ* hybridisation detection of a target sequence within a single cell are approximately 10 to 20 copies per cell for target mRNA and approx. 10 copies per cell for target DNA (reviewed by Komminoth and Long, 1993). The technique is possible because two complementary strands in a DNA double helix, a DNA/RNA duplex or single stranded RNA (riboprobes) can be separated/denatured and applied to a tissue specimen under conditions that favor the annealment to a complementary sequence within the tissue. Development of ISH has been influenced by the availability of specific probes from recombinant DNA technology (Gerhard *et al.*, 1981; Robins *et al.*, 1981; Venezky *et al.*, 1981). Certain critical parameters and use of appropriate controls must be addressed for successful application of ISH. These include treatment and quality of the tissue preparation through to length, concentration and detection of the probe (revised in Chapter three). Figure 1.6 is a diagrammatic simplification of the steps and theory involved in the procedure of *in situ* hybridisation, provided for reference over the next few sections.

1.12.2: Immunocytochemistry (ICC)

Technically the concept is very similar to ISH, except that immunocytochemistry (ICC) uses antibodies as a direct probe for detecting specific macromolecules in cells and tissues (Haues, 1994). Antibodies are a large family of glycoproteins that share key structural and functional features, working as precise molecular recognition tools (Haues, 1994; Harlow, 1989).

The interaction of an antibody with a protein/polysaccharide (antigen) within the tissue forms the basis of all immunochemical techniques (Harlow, 1989). Detection of this tissue bound antigen-antibody complex is usually with either radioactivity or enzyme and fluorochrome labelling that has been coupled to the primary or secondary antibody (Rasmussen, 1987). Variations in ICC include; 1) specificity of the primary antibody in detection of the tissue-specific antigen, to influence the accuracy of the assay, 2) susceptibility of the antigen (macromolecule) to tissue fixation, and 3) the choice of label on the antibody to visualise the site of attachment in the tissue (Haues, 1994). The critical parameters that need to be considered with this technique are; 1) problems with cross reactivity of mouse antibodies to plant tissue products, 2) the ability of the technique to recognise any protein, and not necessarily active enzyme, and 3) that the technique only indicates if the product is located in the cell, not whether the gene for the protein is being expressed in that particular cell (Coghlan *et al*., 1985; Penshow *et al.,* 1987). However in combination with ISH on serial sections this is potentially a

powerful detection technique. Successful use of ICC in barley tissue has been documented by Gibbons (1980; 1981) demonstrating the expression of α-amylase and by Wittich and Vrengdenhil (1998) for sucrose synthesis isoenzymes, but to date the specific (1,3;1,4)-β-glucanase isoenzymes have not been examined using this technique in plant tissue.

1.12.3: Whole tissue examination

There are a number of advantages in using whole tissue sectioning as opposed to excised tissue samples when applying the ISH technique to barley grain tissue; 1) RNA is notoriously difficult to isolate from some plant tissues (Lawerence and Singer, 1985), and whole tissue sectioning avoids the need for this isolation. 2) Spatial location of mRNA or its products within tissue is only possible using whole tissue sections. 3) Lower cell numbers are required per sample than for extracted tissue transfer experiments, and 4) double localization of targets within a tissue can be achieved by using adjacent sections (2 to 3 μm apart).

1.12.4: Detection of messenger RNA and protein in tissue sections

To apply ISH to plant tissue sections, it is fundamental to understand the dynamics of messenger RNA within tissue itself. The decay rate of transcripts in plants appears to be similar to those observed in other eukaryotes. Half-lives range from one hour for unstable species to several days for stable transcripts, with the average being several hours (Gutierrez *et al.,* 1999) and stability probably plays a fundamental role in the regulation of gene expression. It is believed that the poly A tail and 7-methyl-G cap protect the mRNA in plants from degradation (Gutierrez *et al*., 1999).

The most popular means of measuring extracted mRNA has been by northern blotting (Nygarard and Hall, 1963). Extracted mRNA is fractionated electrophoretically and immobilized onto nitrocellulose for detection. Secreted or extracted protein can also be detected in a similar procedure using western blotting. DNA is detected with Southern blotting. Methods now available such as microarrays, RNAseq and real time PCR (RT-qPCR) have opened the way to semi-quantitative or quantitative analysis of mRNA expression. Other methods of detection include pulse labeling tissue with radioactive amino acids, *in vivo* translation of mRNA (Mundy *et al*., 1985) and detection of RNA in tissue sections with dyes such as metachromatic fluorochrome acridine orange (McFadden *et al*., 1988; Pearce, 1972). Germinated wheat embryos have been shown to synthesize both ribosomal (rRNA) and messenger RNA (mRNA) at the earliest time after the onset of germination (Spiegel *et al.,* 1975). A number of researchers have

relied only on northern blotting to detect specific mRNA expression in barley tissue extracts (Schwarzacher *et al.*, 1989; Slakeski *et al.*, 1990; Slakeski and Fincher, 1992; Banik *et al.*, 1996). Therefore a more detailed visual exploration of the expression of particular enzymes through germination using sectioned grain would contribute significantly to the knowledge already reported.

1.12.5: *Tissue fixation*

As many of the procedures for ISH and ICC tend to dissociate cellular components in the tissue or dislodge the section from the slide, the type of tissue fixative is critical for any procedure reliant on tissue integrity (Graig and Goodchill, 1982). Experience has shown that selection of the best fixative, most appropriate for the systems being used, is critical, particularly in the case of barley grain, with its many diverse layers and array of constituents. Both glutaraldehyde and formaldehyde are more effective at reducing the protein and RNA loss from the tissue than acetone or Carnoy's (3 ethanol: 1 acetic acid) for ISH and ICC studies (Godard *et al*., 1980; Angerer and Angerer, 1981; Gee *et al*., 1983; Lawrence and Singer, 1985; Moench *et al*., 1985; Shivers *et al*., 1986). The reduced loss and superior morphological preservation with glutaraldehyde and formaldehyde occurs because they are highly effective protein cross-linkers producing abundant intra- and intermolecular bridges (Hopwood, 1972).

Up to 75% or more of total cellular RNA can be lost from tissue after Carnoy's fixation (Angerer and Angerer, 1981; Lawrence and Singer, 1985). However, a disadvantage with glutaraldehyde and formaldehyde fixation in ISH is that excessive protein cross-linking can make the tissue impervious and either limit the accessibility of longer probes, or non-specifically entrap the probe (Moench *et al*., 1985; Lawrence and Singer, 1985; Penschow *et al.,* 1987). So, when using these cross-linking fixatives, it is necessary to include a proteolytic treatment of the fixed tissue in the ISH or ICC procedure (Angerer and Angerer, 1981; Gee *et al*., 1983; Lawrence and Singer, 1985). But generally glutaraldehyde is not recommended for fluorescent work in ICC studies as it can have an effect on tissue by either inactivating certain protein antigens or rendering them inaccessible to antibody staining (Craig and Goodchill, 1982) along with the well documented autofluorescence side effect (Lee *et al*., 2013).

Figure 1.6: Theory of *in situ* hybridisation.

**No bound antibody
No positive signal**

**Black staining from
mRNA-bound DIG-antibody
indicates a positive signal**
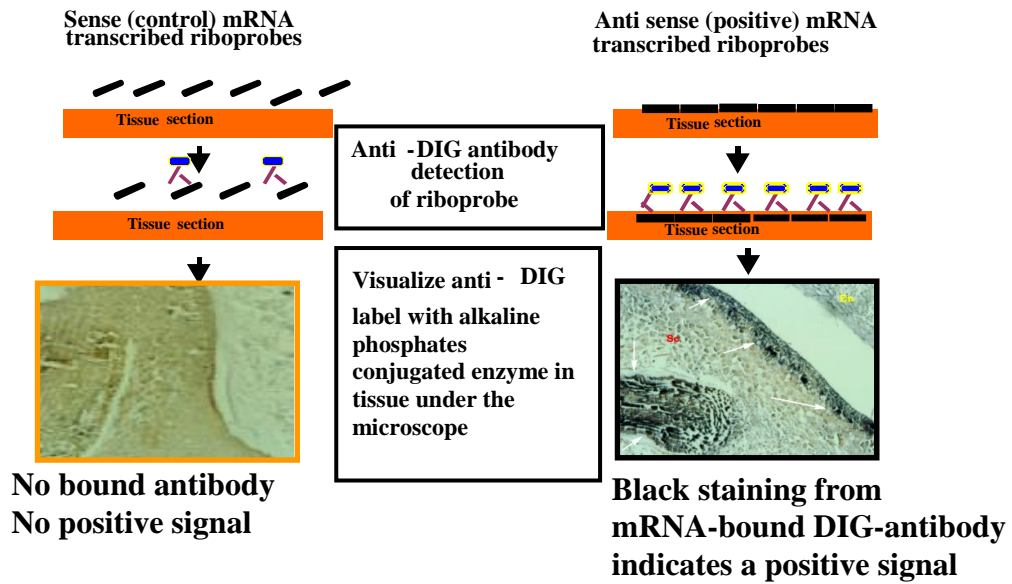
The use of an enzyme linked immunosorbent assay (ELISA) to determine the effect of the fixative on a particular macromolecule can assess this first and is a common practice (Brahie *et al*., 1984).

A mixture of periodate, lysine and paraformaldehyde (PLP) is also a commonly used cross linking fixative by virtue of the reactivity of lysine amino groups with periodate-activated cellular carbohydrate moieties (McLean and Nakane, 1974). Many researches believe that PLP is the best fixative for superior presentation of a wide range of cellular and viral antigens in ICC and ISH (Moench *et al*., 1985; Rasmussen *et al*., 1985; Gibbons, 1979; 1980; McLean and Nakane, 1974) and ideal for cereal seeds containing large amounts of carbohydrates (McLean and Nakane, 1974).

Another popular tissue fixative, paraformaldehyde (4%) is seen as being superior to ethanol acetic acid, glutaraldehyde or Carnoy's in providing excellent morphology and minimal autofluorescence in ICC studies (Lawerence and Singer, 1985). Paraformaldehyde (2-5%) is the most popular fixative for light microscopic ICC (Haues, 1994), although, for ISH studies, it is not ideal in preserving nucleic acid within the tissue. The combination of fixatives, such as glutaraldehyde and paraformaldehyde have been commonly reported in the literature for use with barley grain (Sugimoto *et al*., 1998; Gubler, 1987; McFadden *et al.,* 1988b).

There is a fine line between under and over fixation of tissue (Panoskaltsis-Mortari, 1995). A commonly used incubation time scale is 5 min to 20 h, carried out at 4°C to

reduce endogenous enzyme activity. Transverse cuts (furrow) and removal of the distal end of the grain can help to accelerate penetration of the fixative solution. When under-fixation occurs, there is a reduction or leaking out of mRNA and tissue fragments from the section during the steps prior to ISH. On the other hand, prolonged exposure to fixatives tends to reduce the hybridization signal (Wilcox *et al.,* 1986; Brigati *et al.,* 1983) most likely due to the extensive over cross-linking within the tissue, which will be discussed in Chapter three in more detail.

1.12.6: Tissue embedding

Following a review of the literature, it does appear that most grain-based studies have selected paraffin embedding as the method of choice. Paraffin sectioning can provide excellent preservation of tissue structure and enables the collection of serial sections (Leitch *et al.*, 1994). Fixed tissue is dehydrated prior to embedding in paraffin wax (Leitch *et al.*, 1994) and then the paraffin wax is removed before both ISH and ICC procedures. This can be done with sodium methoxide dipping (Leitch *et al.*, 1994; Haues, 1994), or xylene washes (Angerer and Angerer, 1981). A disadvantage to paraffin embedding is the loss or damage of tissue and nucleic acids during the whole process, thus target mRNA or DNA needs to be abundant so that a small degree of hybridization signal can be sacrificed (Penschow *et al.*, 1987). Fresh tissue should be fixed as soon as possible to minimise endogenous nuclease activity (Leitch *et al.*, 1994). There is less nuclease breakdown problems with paraffin embedding tissue than those fixed as an intermediate step such as in cryopreservation (Guide to In Situ, 1994). Cryosectioning is one of the best methods to preserve tissue antigenicity; it avoids tissue dehydration through fixation and chemical-induced protein denaturing. However, morphological preservation is often poor and RNA loss substantial but as an embedding medium it allows good accessibility of the probe to the target site compared to other embedding procedures and successful ISH was achieved by McFadden *et al.* (1988b) using cryo-preserved grain, fixed in 2% glutaraldehyde. Generally, antigens that are particularly susceptible to fixation and heat during resin and paraffin preparation are recommended for cryopreservation and cryostat sectioning (Wilcox *et al.*, 1986; Shivers *et al.*, 1986).

The morphology of resin embedded tissue is far superior to that of paraffin or cryopreserved material. Materials for examination at high resolution using either light microscopy or electron microscopy are generally embedded in acrylic resins following fixation (Leitch, 1994). Lowicryls is an acrylic resin, formulated for low temperature

embedding, resulting in superior preservation of antigenicity for ICC (Armbruster *et al*., 1983; Carlemalm *et al*., 1982; Roth *et al*., 1981; Acetarin *et al.,* 1986). Barley tissue has successfully been prepared for sectioning using Lowicryl resin (Gubler *et al*., 1986; McFadden *et al*., 1988b). As described in Chapter two of this study we used the resin GMA for preservation of barley grain in order to obtaining structurally superior tissue sections, as described by Walwork (1997). However, a major disadvantage in the use of these resins for ISH and ICC is the inability to subsequently completely remove the resin from around the tissue sections, causing masking and background problems.

1.13: Project Overview

The cereal barley is central to the brewing and malting industries, with germinated grain a key component in the production of beer and whisky type beverages. During commercial malting, barley is germinated under controlled conditions. The synthesis, secretion and migration of a large number of enzymes through the grain during germination are prerequisites for successful malting. The term modification is used to describe events in the early stages of malting, particularly the degradation of endosperm cell walls. This removes the physical barriers between secreted hydrolytic enzymes, such as α-amylase, β-amylase, and limit dextrinase, and their polysaccharide and protein substrate reserves that are stored within the cells of the starchy endosperm. The major constituents of endosperm cell walls are (1,3;1,4)-β-glucans and arabinoxylans. Two (1,3;1,4)-β-glucan endohydrolases are important in the hydrolysis of (1,3;1,4)-β-glucans whilst the enzyme (1$\rightarrow$4)-β-xylanase is necessary for the degradation of arabinoxylans.

There has been a large amount of interest in the enzymes involved in barley germination, with the majority of studies aimed at defining the location of enzyme synthesis and secretion during germination. Isolated scutella and aleurone layers have been used in the majority of these studies creating intrinsic problems, firstly, because the tissue may be contaminated with other tissue types (Mares and Stone, 1973), and secondly because it is likely that such systems do not truly reflect *in vivo* events (Chrispeels and Varner, 1966). It would clearly be more appropriate to define the locations of enzyme expression within intact grain, using techniques such as *in situ* hybridization and immunostaining.

In situ hybridization allows the detection of mRNAs encoding particular enzymes, through the use of specific complementary DNA (cDNA) or RNA probes, and the cells

in which the corresponding genes are expressed can therefore be identified (McFadden *et al*., 1988b). Immunostaining techniques allow the locations of the enzymes themselves to be defined, through the use of specific monoclonal antibodies. It must be remembered, however, that while immunostaining will reveal where an enzyme is located at the time of tissue sectioning, it might not allow the identification of cells from which secreted enzymes originate.

1.14: Experimental plan

The overall aim of this study was to examine the expression and tissue location of some key enzymes involved in barley grain modification, namely $(1,3;1,4)$-β-glucanase EI and EII isoforms, the $(1\rightarrow4)$-β-endo-xylanase isoforms, limit dextrinase and the limit dextrinase inhibitor.

Objectives arising from these aims were to:

a) Develop a working non-radioactive *in situ* hybridisation procedure using labeled probes and sectioned barley grain, based on the successful research conducted by McFadden *et al.* (1988b) who demonstrated the distribution of total glucanase mRNA.

b) Develop a way of sectioning barley grain that has been preserved at different stages during germination for the *in situ* hybridisation assay.

c) Develop an immuno-staining procedure with $(1,3;1,4)$-β-glucanase EI and EII isoform specific antibodies.

d) Develop specific probes from cDNAs encoding limit dextrinase, limit dextrinase inhibitor, $(1\rightarrow4)$-β-endo-xylanase isoforms and $(1,3;1,4)$-β-glucanase isoforms.

e) Observe the expression patterns of the mRNA and protein in barley grain through the germination process for all the above-mentioned enzymes.

Achievement of these objectives may allow exploration of the following points:

a) Observation of the differential expression patterns of the $(1,3;1,4)$-β-glucanase EI and EII isoenzymes, as opposed to the general "glucanase" picture provided by McFadden *et al.* (1988).

b) Document the pattern of expression of the $(1\rightarrow4)$-β-endo-xylanase isoforms throughout the germination process.

c) Establish whether the limit dextrinase and limit dextrinase inhibitor proteins are found in the same tissue/cell type.

d)   Determine whether the limit dextrinase enzyme remains in the aleurone cells throughout the germination process or whether it moves into the endosperm.

Chapter 2

Development of a robust procedure to allow accurate assessment of microscopic structure and modification patterns of the *Hordeum vulgare* L (barley) grain throughout germination

2.1: Introduction

2.1.1: Modification patterns

During barley germination, a sequence of events leads to endosperm modification, including the synthesis and activation of hydrolytic enzymes in the scutellum (embryo) and aleurone layers, the sequential breakdown of cell wall material and protein in the endosperm and the hydrolysis of starch granules (Briggs, 1978; Palmer, 1982; 1989; Fretzdorff *et al*., 1982; MacGregor and Matsuo, 1982; Briggs and McDonald, 1983; Selvig *et al.,* 1986). It is the understanding of these morphological changes that take place during germination that forms an important and integral part of the study of hydrolysis of the barley grain endosperm.

The pattern and rate of modification in relation to the production of the hydrolyzing enzymes in germinating grain have long been of academic and commercial interest (Palmer, 1982, O'Brian and Fowkes, 2005). Studies have employed both chemical and physical analysis of grain to characterise modification during germination. Using chemical analyses produces average values from bulked grain samples, while physical or structural methods can define the characteristics of individual grains. Physical indicators of endosperm modification range from measuring acrospire length (Aastrup and Eral, 1980), protein matrix and starch breakdown (Briggs, 1972; Fretzdorff *et al*., 1982; MacGregor and Matsuo, 1982; Wallace and Lance, 1988), to understanding enzyme secretion (Chrispeels and Varner, 1966; Jacobsen, 1973; Mozer, 1980) and noting grain hydration rates via histochemical observations made under either the light or electron microscope (see reviews by Briggs and MacDonald, 1983; Gibbons, 1981 and Fox, 2010).

In general terms, patterns of modification have been placed into two groups; the "symmetrical" pattern of modification which proceeds from the scutellum to the distal end of the grain in a wave-like fashion through the endosperm (Briggs and MacDonald, 1983; Gibbons, 1980; 1981), whilst the other is an "asymmetric" pattern in which modification occurs first (and only) from the aleurone layers (Palmer, 1982) progressing to the distal end of the grain.

Modification pattern, malting quality (Davies, 1992) and water distribution through the endosperm are strongly correlated (Colin, 1918; Reynolds and MacWilliam, 1966). Uniform water uptake by the grain in the early stages of germination is critical to achieve efficient and homogenous endosperm hydration. As discussed in Chapter 1, a simple assay is routinely used by the malting industry to assess grain hydration and to predict the grain's probable modification stage (Collins *et al.,* 1999). As highlighted in

Chapter 1, this assay is more likely to predict and correlate to grain modification stages than most widely used visible non-destructive procedures. However, there is still substantial variation in the hydration patterns of grains collected and examined at any particular time point.

The status of endosperm structure during germination of sectioned grain has also been explored by Briggs and MacDonald (1983) using different stains. These included Congo Red, Trypan blue and Calcofluor White M2R (CW). CW is a dye that stains (1→3, 1→4)-β-glucan and cellulose, therefore binding up to 70-75% (w/v) of the barley endosperm cell wall material, mainly as (1→3, 1→4)-β-D-glucan content, as minimal cellulose exists in the endosperm (Thompson and LaBerge, 1977). The correlation between cell wall breakdown as measured by CW and β-glucanase activity is well documented (Aastrup and Erdal, 1980). Gianinetti (2009) showed that endosperm pre-mobilization to macromolecules coincides with the reaction front for (1→3, 1→4)-β-D-glucan degradation and this equates to cell wall breakdown as shown by FITC dextrin perfusion experiments. CW dye does not bind to (1→3, 1→4)-β-D-glucan under the molecular weight size of 10, 000 daltons (Aarnes and Lie, 1985), and therefore does not indicate complete (1→3, 1→4)-β-D-glucan degradation. A major advantage in the use of CW staining over other modification assessment assays is that the stain can be easily applied to grain either cut as half blocks and mounted in modeling clay (Calcofluor method, Aastrup and Erdal, 1980) or to very thin 7 μm sections on glass slides (Gibbons, 1979).

2.1.2: Grain Fixation and sectioning

Tissue fixation is an important step when considering microscope-based techniques, impacting on the success of many downstream experimental procedures. It must retain the maximum level of cellular target DNA or RNA in the tissue, while maintaining optimal morphological detail and allowing sufficient accessibility of the probe (Maki *et al*., 1997; Leitch, 1991). For each given tissue specimen, experimental determination of the optimal fixative and its concentration is required (Rasmussen *et al.,* 1987). There is often a tradeoff between preservation, retaining antigenic reactivity and structural morphology (Larsson, 1988).

The angle of sectioning is also a critical consideration in this type of study. Palmer (1982) highlighted a connection between researchers reporting different modification patterns and variations in the angle of longitudinal cuts through sectioned grain, which is exacerbated by the fact that the barley grain is seldom structurally symmetrical.

Indeed, descriptions of the structural changes occurring in barley during germination have often been conflicting (Mundy *et al*., 1981) as discussed in section 1.6.1. The aims of the work presented in this chapter were, (1) to determine a consistent method for assessing patterns of endosperm modification for Sloop (a hulled malting barley variety) and Himalaya (a hulless feed barley variety) during germination, and (2) to subsequently define specific stages in modification for each variety in order to best characterise and compare enzyme activity between each stage and between the two barley varieties.   To achieve this, considerable investigation was undertaken to optimally preserve and section the barley gain during different stages of germination for assessment. This provides confidence that differences displayed between cultivars and between germination stages are valid and not simply the result of biological variation shown by individual grains.

2.2: Materials and methods

2.2.1: Plant material

Barley varieties Sloop (*Hordeum vulgare* L. cv. Sloop) and Himalaya (*Hordeum vulgare* L. cv. Himalaya) were used throughout this study. Sloop is a two-row malting variety released in 1997 by the South Australian Barley Improvement Program, at the University of Adelaide. Himalaya is a six-row hulless feed variety, developed at Pullman University (Washington State, USA) and kindly supplied by Dr Jake Jacobsen (grown at the CSIRO Plant Industry, Canberra, Australia, 1997). All grain was collected at the beginning of the study and stored for the duration at 12^0C in the dark, to minimize environmental variations. As shown in appendix 1, Sloop is adapted to growing conditions in South Australia, characterized by moderate levels of malt extract (HWE), low wort viscosity, high diastatic power (DP), high soluble nitrogen (Kolbach), very low malt and wort β-glucan (H. Collins, The University of Adelaide, Waite Campus, personal communication) and good grain size. In comparison, hulless Himalaya has lower diastase activity (which involves the testing of any one of a group of enzymes which catalyses the breakdown of starch into maltose), half the Kolbach, high wort β-glucan (Jorgensen and Aastrup, 1988) and four times the wort viscosity of Sloop.

2.2.2: Germination of barley grains

All grains were screened (between 2.5-2.8 mm) to eliminate size variability (Davies, 1992). Grains were germinated as outlined in McFadden *et al.* (1988) with some modification. Specifically, the grains were surface-sterilized by immersion in 0.2 % (w/v) sodium hypochlorite for 20 min, and washed thoroughly with 0.05 M phosphate buffered saline, pH 7.2 (800 mM NaCl; 20 mM KCI; 216 mM Na_2KHPO_4 pH 7.2; PBS) and sterile distilled water. They were steeped in Petri dishes in sterile water containing an antibiotic solution of 100 $g.mL^{-1}$ neomycin, 10 $g.mL^{-1}$ chloramphenicol, 100 $units.mL^{-1}$ penicillin and 100 $units.mL^{-1}$ nystatin (Sigma-Aldrich, Missouri, USA; Hoy *et al*., 1981) for 8 h in the dark at room temperature. The grain was exposed to air without drying for 8 h and re-submerged in the antibiotic solution for a further 8 h until a moisture content of 43-46 % was achieved. Grain moisture content was determined by oven drying at 100^0C for 3 h. Grains were incubated on filter paper moistened with the antibiotic solution, in darkness, at 20^0C for a total of up to 112 h, with the crease side of the grain facing downwards. This schedule is shown in Figure 2.1. Note that time zero h corresponds to the immersion of the grain in the antibiotic solution.

2.2.3: Grain sampling for hydration assay

Grain hydration was assessed using a modified Capon boiled grain hydration method as described by Cole *et al*. (1997).

Figure 2.1: Germination schedule.

Germination procedure   Grain sampling

```
Mature
grain
  |
  v
Surface
sterilisation
  | (20 minutes)
  v
Antibiotic          <-------->     0h
solution                            |
  | (8 hours)                       v
  v
Air dry             <-------->     8h
  | (8 hours)                       |
  v                                 v
                    <-------->    16h
                                    |
  v                                 v
Antibiotic
solution            <-------->    24h
  | (8 hours)                       |
  v                                 v
Temperature controlled   ----->  32h, 40h, 48h, 56h,
germination                      64h, 72h, 80h, 88h,
                                 96h, 104h and 112h
```
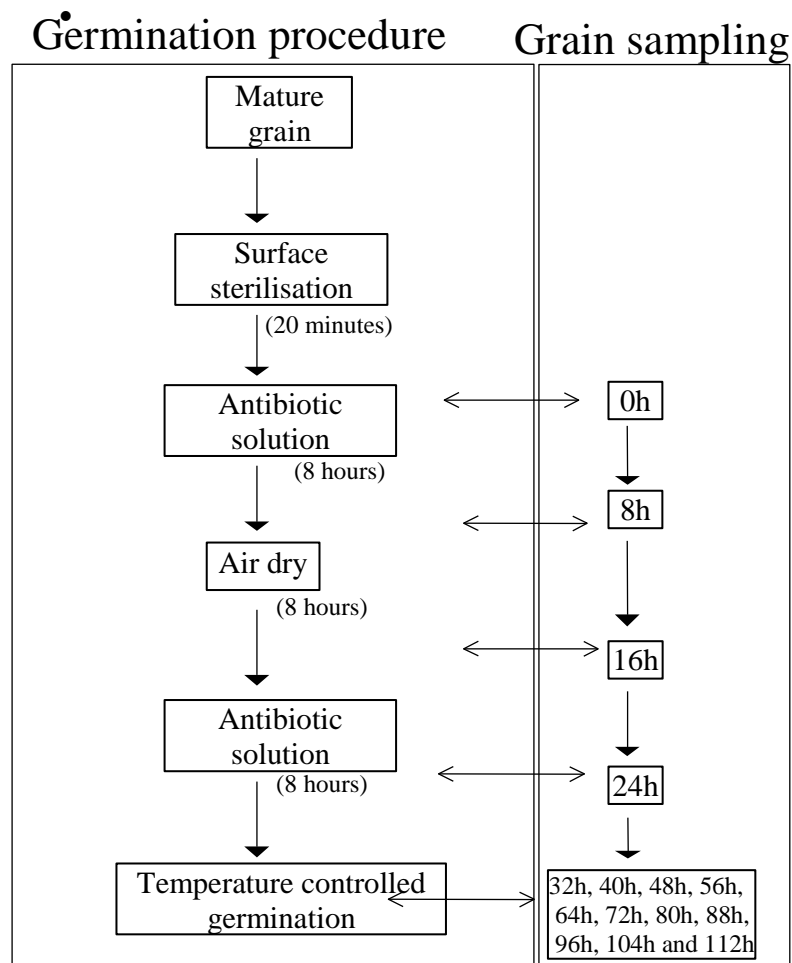
A tea strainer containing 30 grains was held in boiling water for 30 s, moved into cold water for 60 s, and each grain cut with a razor blade longitudinally through the crease. The extent of hydration is documented by the change in endosperm starch from white to translucent and scored based on a pre-determined classification (Table 1.1).

2.2.4: Grain sampling and preparation for sectioning

Initially, 24 h germinated grains were halved longitudinally through the crease and each half-grain was placed in each of the fixatives as listed in Table 2.1. All fixations were conducted at 4^0C for 12, 24 or 30 h. Paraformaldehyde (Sigma) was prepared by weighing out the specific volume for the desired concentration in PBS, heating to 65^0C for 30 min and clearing with 5 µL of 5 M sodium hydroxide (Sigma). Glutaldehyde (EM grade, Ajax-Finechem, Australia) was diluted as per Table 2.1 (h to j) in PBS. Fixed half-grains were embedded in paraffin (Paraplast Plus, Sigma), glycol methacrylate (GMA, Sigma) polyethylene glycol (PEG; Brown *et al*. 1989) or OCT (Cryostat specimen matrix, ProSciTech, OLD Australia). PEG was prepared by melting two parts PEG 1450 (Sigma) to one part PEG 3500 (Sigma) at 60^0C and infiltration into the barley grain sections occurred at 60^oC. OCT was applied to freshly sectioned grain at room temperature, snap frozen in liquid nitrogen and stored at -80^0C.

Half grains for embedding in paraffin were placed in cassettes (Tissue Tek II, ProSciTech, QLD Australia), washed in PBS, dehydrated through a graded ethanol series and embedded in Paraplast Plus (Paraffin; Sigma) using a commercial embedder (Shandon–Citadel 2000, Tissue Tek II). The paraffin embedding schedule as listed was designed by Natasha Penno, Animal Science, The University of Adelaide; 1 h in 70 % (v/v) ethanol, 1 h in 80 % ethanol, 1 h in 95 % ethanol, 2 h in 100 % ethanol (two changes), 1 h in histolene (Sronine): ethanol (1:1), 2 h in 100 % histolene (two changes) and finally 2 h in paraffin at 65^0C. To ensure correct orientation each longitudinally cut half-grain was placed at the base of a small metal capsule so that the cut surface was orientated towards the front of the polymerised block.

Half grains for embedding in GMA were initially processed at 4^0C through an alcohol series of methoxyethanol (BDH, Merck Pty Ltd), ethanol, propanol (BDH, Merck Pty Ltd) and butanol (BDH, Merck Pty Ltd). Half grains were positioned cut side down prior to polymerization, GMA infiltration proceeded with submersion of the grains in butanol:GMA (1:1) followed by butanol:GMA (1:3) to several changes in 100 % GMA and placed at 60^0C for a minimum of 2 days in gelatin capsules (Size 00, Panmedica, Sydney). GMA was prepared by mixing 93 mL of 2- hydroxtethyl methacrylate

polyethylene glycol (Sigma), 7 mL polyethylene glycol 400 (Sigma) and 0.6 g benzoyl peroxide (Sigma).

2.2.5: Sectioning and staining

OCT prepared grain halves were sectioned at 5 μm on a Cryostat (SDR clinical Technology) at -20^0C. Sectioned material was treated as suggested by McFadden *et al*. (1988) by adherence onto glass slides using thermal changes from -20^0C to room temperature and immediately fixed according to the method in Table 2.1j.

Paraffin and PEG embedded samples were sectioned at 7 μm with a rotary microtome (Leitz, 1512) and applied to Superfrost Plus (Menzel-Glaser) glass slides with the aid of adhesive tape (3 M Highland 371, Wayne Richardson Sales Ltd, Adelaide, Australia) and sterile water. Tape was placed across the face of the paraffin block prior to sectioning. Ribbons of sections with tape attached were placed on clean white paper, and the sections cut out with a razor blade. These tape-section pieces were adhered to the slide with sterile water. Each glass slide (with 3 longitudinal sections) was incubated at 42^0C for a minimum of two days prior to use and stored at room temperature in a humidified container for up to one month. The adhesive tape and paraffin were removed just before assaying by successive 10 min washes in chloroform (99.5%, BDH, Merck Pty Ltd), xylene (>0.055% impurities, BDH, Merck Pty Ltd) and a graded ethanol series, followed by a final rinse in sterile water.

GMA embedded grains were sectioned at 3 μm with a Reichert Jung 2050 Super Cut Microtome. Sections were collected onto droplets of water and slides dried at 60^0C for 1-2 days prior to use.

Morphology and preservation of grain sections was assessed following staining of grain sections with Periodic Acid-Schiffs/Toluidine blue (PAS/TBO; O'Brien and McCully, 1981), with reduced time (15-20 min) in Schiff's reagent (BDH, Merck Pty Ltd) to avoid over-staining the large quantities of starch present in cereal endosperms. To display the cell contents, sections were counterstained with 0.05% Toluidine blue (Sigma) in 1% sodium benzolate buffer (Sigma) pH 4.5. Following staining, sections were air dried and mounted under cover slips in Micromount (Surgipath Medical Industries, USA). Freshly prepared fluorescent stain Calcofluor M2R (Polysciences, CW) at 0.05% w/v in sterile water was applied to the sections on the slide for 5 min and then rinsed in serial water washes. Staining was observed with sections glass-cover-slipped under 80% glycerol (BDH, Merck Pty Ltd).

2.5.6: Microscopy and Photography

Observations of all sections followed staining with PAS/TBO and CW (O'Brien and McCully, 1981) using a Zeiss Axiophot microscope in light (tungsten) or fluorescence mode (360 nm excitation, 415 nm emission) respectively. The blue tinge present in a majority of colour photographs presented in this study is a product of the tungsten light source. Images were recorded on Kodak 400 ASA Film or Kodak 160 T slide Film and digitized using Adobe Photoshop (Microsoft). CW positive tissue displayed a blue fluorescence under UV light. Regions of reduced fluorescence were evident where hydrolysis of (1→3, 1→4)-β-D-glucan had occurred.

2.2.7: Experimental procedure

Individual grains of both Sloop and Himalaya were collected every eight hours from the beginning of steeping until approximately 112 h and preserved in a combination of 4 % (v/v) paraformaldehyde and 0.25% (v/v) glutaldehyde in PBS for 24 h followed by paraffin embedding and sectioning as outlined in sections 2.2.4 and 2.2.5. In all cases, true longitudinal orientation was confirmed by observing the shape of the cells of the scutellar epithelium. Only those half-grains in which these cells appeared columnar, indicating a correct vertical longitudinal plane, were considered to be true in cross-section (Wallwork, 1997).

Table: 2.1: Fixation combinations (A + B) attempted on germinated barley grain.
Germinated grain, once fixed, was either paraffin or PEG preserved. Fixation was also attempted on grain following cryostat sectioning.

A +	B
a) 0 or 2 or 4 or 6% paraformaldehyde in PBS	0% glutaraldehyde in PBS
b) 0 or 2 or 4 or 6% paraformaldehyde in PBS	0.1% glutaraldehyde in PBS
c) 0 or 2 or 4 or 6% paraformaldehyde in PBS	0.25% glutaraldehyde in PBS
d) 0 or 2 or 4 or 6% paraformaldehyde in PBS	0.5% glutaraldehyde in PBS
e) 0 or 2 or 4 or 6% paraformaldehyde in PBS	1% glutaraldehyde in PBS
f) 0 or 2 or 4 or 6% paraformaldehyde in PBS	2% glutaraldehyde in PBS
g) Acetone (BDH, Merck Pty Ltd) alone	
h) PLP (Periodate-lysine-paraformaldehyde, Sigma-Aldrich)	
i) HistoChoice® Tissue Fixative (Sigma-Aldrich)	
J) 2% glutaraldehyde, 33% ethylene glycol in 0.15M $NaPO_3$ buffer at pH 7.2 (McFadden *et al.* 1988).	

Figure 2.2: Demonstration of grain sectioning. Dissecting microscope photo of barley grain cut longitudinally through the crease (10 X magnification). Diagram 2.2B: hand drawing of barley grain cut in the longitudinal direction; A1: longitudinally cut through the crease, B.A2 and B.A3, the separation of the grain (a+b); B.B represents the longitudinal cut through the crease looking horizontally at the grain. Figure B.C and B.D represent the early attempts at cutting grain at a 20 degree angle to the crease, as discussed in section 2.4.2. Drawings were made under direction from Beth Chambers, Adelaide, Australia.
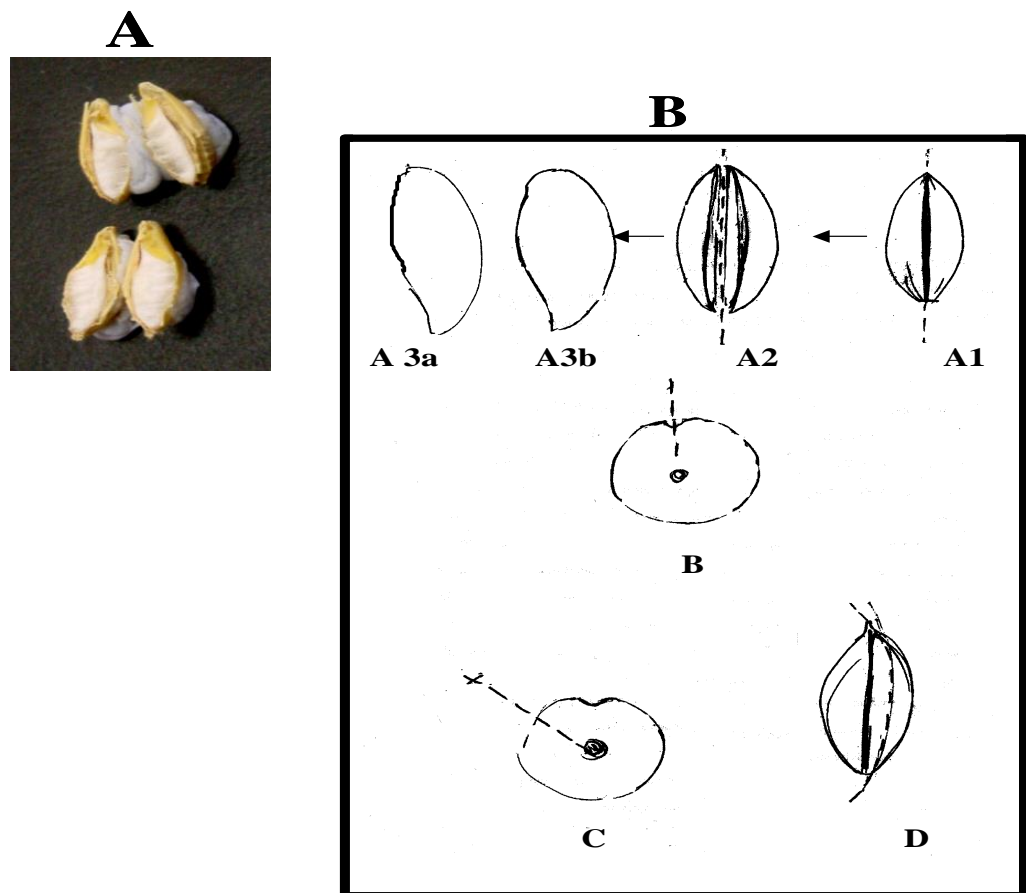
2.3: Results

2.3.1: Hydration assay

A hydration assessment, as explained in section 1.6.2.2 was made on Himalaya and Sloop grain following the germination schedule outlined for this study, with the aim of documenting modification stages appropriately (Table 2.2). The results in Table 2.2 only show the hydration values of grain collected at 48 h from the start of steeping. Significant variation in hydration scores can be seen for each variety, and this was observed at all collection points (data not shown).

Table 2.2: Example of hydration results for the two varieties presented in this study at 48 h from the start of steeping. Grain placed in categories A to D as per Cole *et al*. (1997) and presented in Table 1.1. Values determined from modification patterns of 100 grains.

Category	A (% less quarter hydrated)	B (% Quarter to half hydrated)	C B (% Half to three quarter hydrated)	D B (% Over three quarters hydrated)
Sloop	4	24	40	32
Himalaya	9	8	31	52

2.3.2: Fixation, embedding and sectioning

Experimental grain samples germinated to 24 h were used to determine the most effective fixation scheme (Table 2.1) for barley grain preservation necessary to achieve success in the assay procedure used throughout this study. Combinations and times of fixation were deduced from those chosen by past researchers (Table 2.3).

Tissue preservation following fixation was determined visually using observations at a whole structural and cellular level with periodic acid-schiffs/toluidine blue stains and Calcofluor White. The best fixation was achieved using 4 % paraformaldehyde and 0.25 % glutaraldehyde. Examples of poor fixation are shown in Figures 2.3 (A to E). When tissue was presented in an under-fixed condition, it generally resulted in poor preservation at a cellular level leading to a reduced image of specific areas and often with fragmented sections present on the slide (Figure 2.3 A). Over fixation resulted in a more brittle tissue preservation, which could not be successfully sectioned due to crumbling (not shown). Eventually a combination of paraformaldehyde and glutaraldehyde, particularly at the lower end of the glutaraldehyde concentration range, was found to preserve the barley grain at a level for acceptable ISH assessment.

Attempts to section grain which had been PEG or cryostat preserved were unsuccessful, mainly due to poor adhesion to the glass slides, independent of the treatment or type of glass slides used (Table 2.3; results not shown). Paraffin-embedded grain could only be

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successfully fixed to the Superfrost Plus glass slides with the aid of the adhesive tape as described in section 2.2.5.

A time course using the chosen fixative was undertaken to explore optimal preservation under the conditions needed for this study. Tissue preservation was dramatically reduced when the time of exposure was under 18 or over 30 h, with 24 h found to be consistently ideal. The temperature for the fixation was predetermined and maintained at 4^oC on the basis that RNA degradation was dramatically reduced when the temperature was kept low, due to reduced activity of RNases.

Table 2.3: Fixation protocols as reported in previous research.

Authors	Fixation procedure
McFadden *et al*. (1988 [a and b])	4% paraformaldehyde + glutaraldehyde in phase partition fixation [a] 2% glutaraldehyde + 33% ethylene glycol pH 7.2 [b]
Marttila (1999); Sugimoto *et al. (*1998)	3% paraformaldehyde + 0.25% glutaraldehyde
Van der Meulen *et al. (*2000)	2.5% glutaraldehyde + 2% paraformaldehyde
Gubler *et al*. (1986)	2% glutaraldehyde; embedded in Lowicryl K4
Rasmussen *et al*. (1990)	2% paraformaldehyde + 1.25% glutaraldehyde,
Wang *et al*. (1998)	4% paraformaldehyde + 0.25% glutaraldehyde in PBS
Braissant and Wahli (1998)	4% paraformaldehyde; frozen embedding
Godard and Jones (1980)	1% glutaraldehyde only
Brigati *et al.* (1983); Hafen *et al.* (1983); Lawrence and Singer (1985)	Various % of paraformaldehyde only

2.3.3: Classification of modification stages

It was decided that a microscopic technique to monitor and display grain modification rates and patterns was best for this study. A favorable consideration was the non-invasive nature of the procedure. The use of sectioned material from embedded grain allows the grain to be assessed on an individual basis and then the rest of the tissue in the block used for other applications, such as *in situ* hybridisation for mRNA detection and immunostaining for studying protein expression. Initially, sampled grain was sectioned and observed under the microscope after CW staining. Each grain was placed into one of 9 stages that represented a point in cell wall changes, seen during the course of germination until 112 h after steeping (Figure 2.1). These original 9 stages were then refined to 5 stages and called modification pattern "1 through to modification pattern 5". The approximate time spanned by each modification (M) stage was variable (8-30 h, data not shown) as predicted by previous literature and discussed later. The development of this classification system entailed standardisation of grain-to-grain variation under these experimental conditions.

2.3.4: Modification of endosperm during grain germination

The pattern of loss of cell wall fluorescence was documented through the germination procedure and is presented as illustrations representing each stage (Figure 2.4 A to 2.5 A). Whole longitudinal sections of both Sloop (Figure 2.4 B) and Himalaya (Figure 2.5 B) embedded in paraffin provide images representing each modification stage, 1 to 5, during germination.   Images of GMA embedded tissue have also been included to provide finer details of selected regions.   In this study all images are presented with the endosperm crease beneath the grain.

2.3.4.1: Modification stage M1

At this early stage in germination there was a very slight loss of CW fluorescence in the crushed cell layer of Sloop sections that did not extend into the adjacent endosperm area of the grain. This was consistently seen on sections at this stage, as illustrated in detail by Figure 2.6 B, M1 (white arrows). Himalaya sections placed in the M1 stage showed no visible $(1{\rightarrow}3, 1{\rightarrow}4)$-$\beta$-D-glucan degradation in any region (Figure 2.7 B, M1).

2.3.4.2: Modification stage M2

The Sloop M2 modification stage displayed $(1{\rightarrow}3, 1{\rightarrow}4)$-$\beta$-D-glucan degradation in the crushed cell layer and the endosperm cell walls adjacent to the crushed cell layer in an area towards the center of the grain near the embryo (solid white arrows, Figures 2.4 B, M2 and 2.6 B, M2). Himalaya sections displayed a slightly less extensive pattern of loss of fluorescence to that of Sloop (solid white arrows, Figure 2.5 B, M2 and 2.7 B, M2) seen generally through the crushed cell layer but in only the endosperm cells very close to this region.

2.3.4.3: Modification stage M3

The modification pattern for Sloop sections at the M3 stage showed a substantial loss of florescence in cell walls at the crushed cell layer interface extending into the endosperm adjacent to the aleurone layer on the ventral side of the grain (Figures 2.4 A and B, M3, white arrows), with no observed degradation of the cell walls near the aleurone layers at the distal end of the grain (Figure 2.7 A, M3 white arrows). This pattern is consistent for all sections placed into this modification stage. The Himalaya grain at M3 showed a similar pattern of reduced CW fluorescence but to a lesser extent than that observed for Sloop. This is demonstrated in Figure 2.5 A and B, M3 where the cell walls adjacent to the crushed cell layers still stained positive for $(1{\rightarrow}3, 1{\rightarrow}4)$-$\beta$-D-glucan as compared to the corresponding regions in Sloop sections (Figures 2.4 A and B, M3 and 2.6 A and B, M3).

Figure 2.3: Examples of poor tissue preservation to demonstrate fixation protocols.
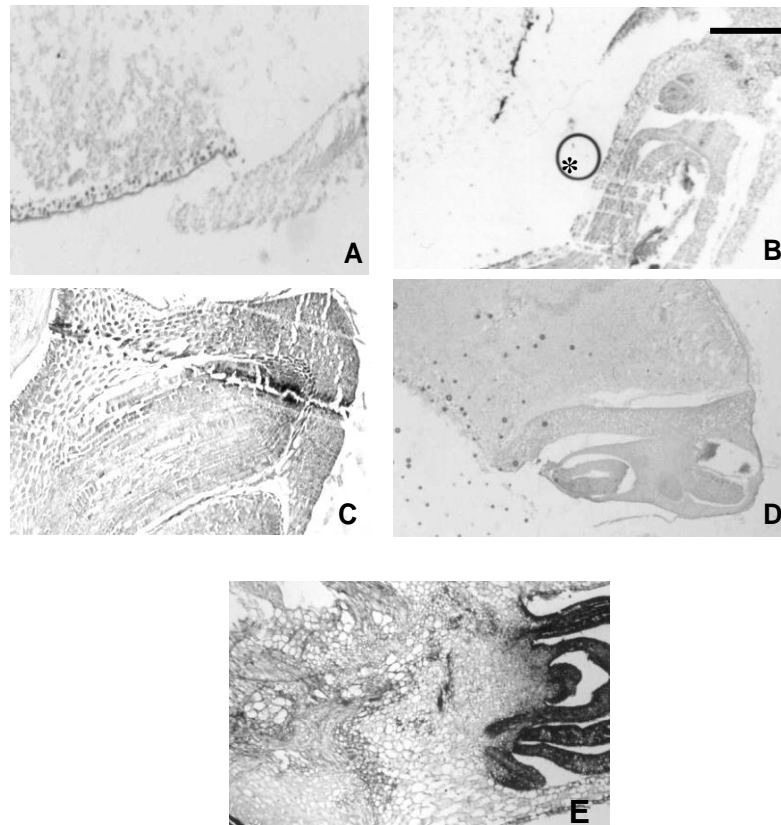
Figure 2.3 A: Example of tissue preservation on glass slides observed when germinated barley grain is under-fixed in either time or concentration with paraformaldehyde and glutaraldehyde as per section 2.2.4. 2.3 B: Example of germinated barley grain preserved in acetone and embedded in paraffin. *air bubble. 2.3 C: Example of barley grain over-fixed in either time or concentration with paraformaldehyde and glutaraldehyde as per section 2.2.4. 2.3 D: Example of germinated barley grain preserved in 2 % glutaraldehyde, 33 % ethylene glycol in 0.15 M $NaPO_3$ buffer at pH 7.2 and embedded in paraffin. 2.3 E: Example of barley grain fixed at optimal time and temperature with paraformaldehyde and glutaraldehyde as per section 2.2.4. Scale bar is 400 μm.

As indicated by the fluorescence there is still a large amount of the endosperm that remains intact at this stage for both varieties (Figure 2.4 A and B, M3 for Sloop and 2.5 A and B, M3 for Himalaya).

2.3.4.4: Modification stages M4 and M5

A considerable amount of the starchy endosperm cell wall did not stain with CW by the M4 stage. This was consistent for both varieties. The M4 stage Sloop sections showed significantly reduced cell wall fluorescence across the whole endosperm, particularly in areas directly adjacent to the scutellum/crushed cell layer interface and in proximity to the encircling aleurone layers (Figures 2.4 A and B, M4 and 2.6 A and B, M4). In contrast, the loss of cell wall fluorescence close to the scutellum/crushed cell layer interface for Himalaya sections was less, as seen in Figures 2.5 A and B, M4 but both varieties showed a consistent degradation of $(1{\rightarrow}3, 1{\rightarrow}4)$-$\beta$-D-glucan, as judged by CW fluorescent staining, in regions adjacent to the aleurone layers surrounding the endosperm (Figures 2.4 A and B, M4, 2.5 A and B, M4 and 2.7 A and B, M4).

Sections of grain chosen to represent the M5 stage for both varieties showed advanced degradation of the endosperm cell walls as detected by CW staining, except for remnant areas within the center of the endosperm (Figures 2.4 A and B M5 and 2.5 A and B M5, black arrow). Himalaya sections consistently presented a larger area of non-degraded walls within the center of the endosperm than the Sloop sections, although the area differences were not quantitated. Beyond this stage (at 112 h), all grain displayed a completely modified endosperm, where the remnants of the cell wall material treated with CW did not fluoresce under UV (data not shown).

In summary, a consistent pattern of modification was observed for both varieties. Endosperm changes were initially observed at the scutellum/crushed cell layer interface on the ventral side of the grain, progressing towards the proximal end of the endosperm, with degradation eventuating from the surrounding aleurone layer.

2.4: Discussion

Extensive work was considered necessary in this study to develop an optimal fixative and sectioning procedure, pivotal to the projects' final outcome. A general fixation procedure as used by others was adopted with slight modification along with a novel sectioning and adhesion technique.

Aspects of the work presented in this chapter were; 1) to develop reliable fixation and embedding protocols, 2) to maintain consistency in the region chosen for observation by ensuring that the angle of sectioning is symmetrical, and 3) to overcome the inherent errors associated with single grain selection.

2.4.1: Grain germination conditions

There are numerous possible combinations of water and air resting techniques employed to activate the embryo and evenly hydrate the endosperm during controlled germination experiments. Due to the small sample numbers used in this study, micro-malt germination was not an option, therefore a laboratory Petri dish germination method as described by McFadden *et al.* (1988) and Stuart *et al*. (1986) was modified to germinate grain to a moisture content of 43-46 %, close to industry specifications (Brookes *et al.,* 1976; Macey and Stowel, 1961; Pollock and Kirsop, 1956; Kirsop *et al.,* 1966). This included combinations of exposure to oxygen or "air rests" and water submergence during steeping. The inclusions of air rests during steeping promotes aerobic conditions that are important to help water infiltration into the endosperm, linked to increased respiration and metabolic activities in the scutellar region (Yin *et al.,* 2000; Wilhelmson *et al.,* 2006) and α-amylase enzyme levels (Briggs, 1968; Raynes and Briggs, 1985). This generally improves the malt quality of the grain (Cole *et al*., 1998; Brookes *et al*., 1976; Davies, 1990; Briggs and MacDonald, 1983).

A standard temperature was adopted during grain germination as suggested in the review by Sommer (1971) where steeping at temperatures between the range of 12-20^{0}C has no significant effect on malting quality, only on germination time. At temperatures over 20^{0}C, effects were noted (Sommer, 1971; Sung *et al.,* 2005).

2.4.2: Justification for fixation

Others have demonstrated the usefulness of aldehydes (such as glutaraldehyde) as fixatives for hybridisation (Lawrence and Singer, 1985; Hoefler, 1986; Table 2.4).
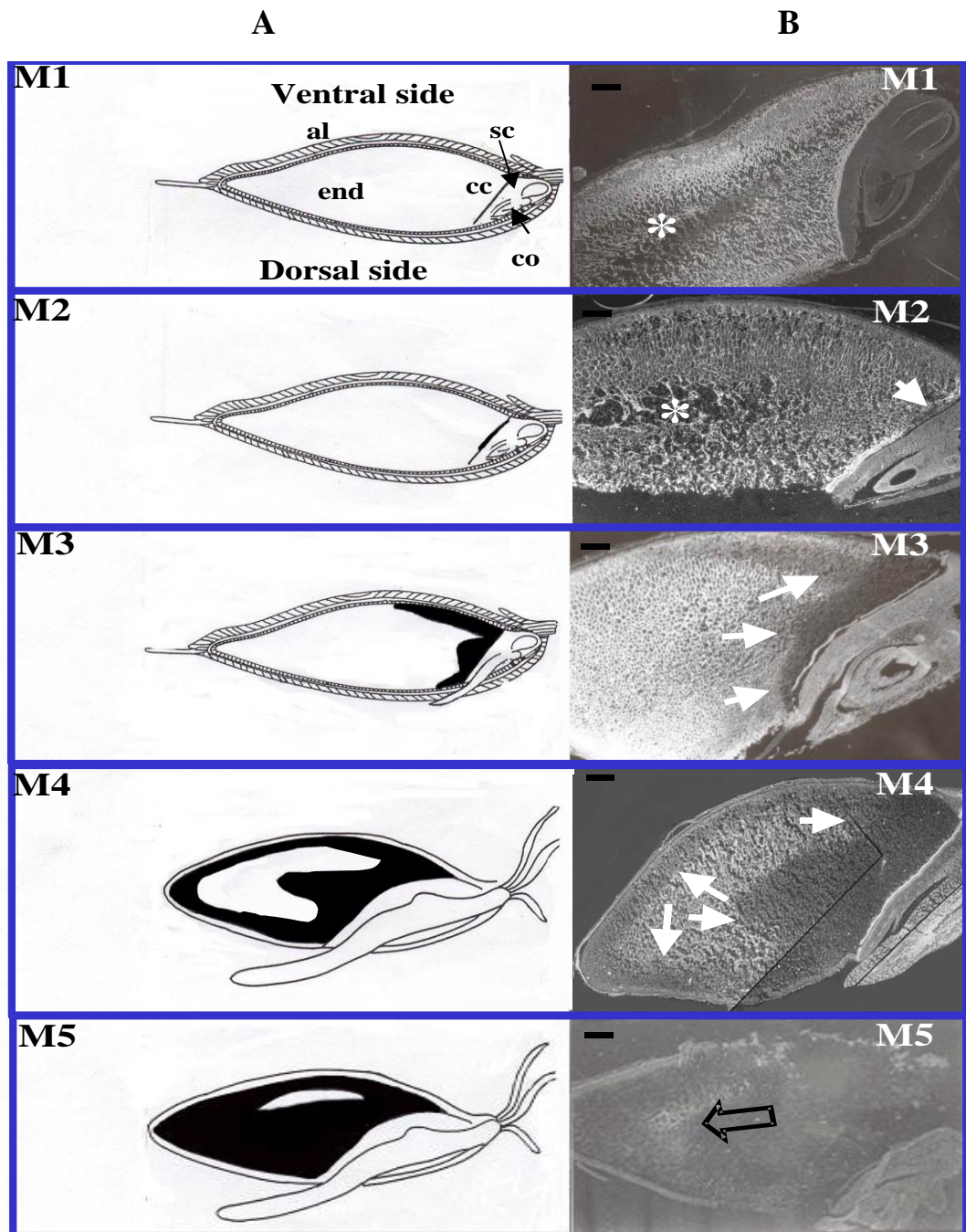
Figure 2.4: Modification patterns of paraffin-embedded Sloop grain.

Figure 2.4 A: Individual hand-drawn diagrams representing modification patterns for Sloop barley grain in the M1 to M5 stages. Black regions are areas of reduced CW fluorescence indicating loss of cell wall (1→3, 1→4)-β-D-glucan. co, coleoptile; sc, scutellum; al, aleurone layer; cc, crushed cell layer; and end, starchy endosperm. Figure 2.4 B: Paraffin-embedded longitudinal sections of Sloop grain stained with CW, corresponding to the M1 to M5 stages. White arrows indicate regions of loss of CW staining. The black arrow in M5 is an area of remnant CW stained cell wall (1→3, 1→4)-β-D-glucan. *Indicates areas of endosperm damage due to grain preparation and not CW fluorescence loss. Scale bar is 400μm
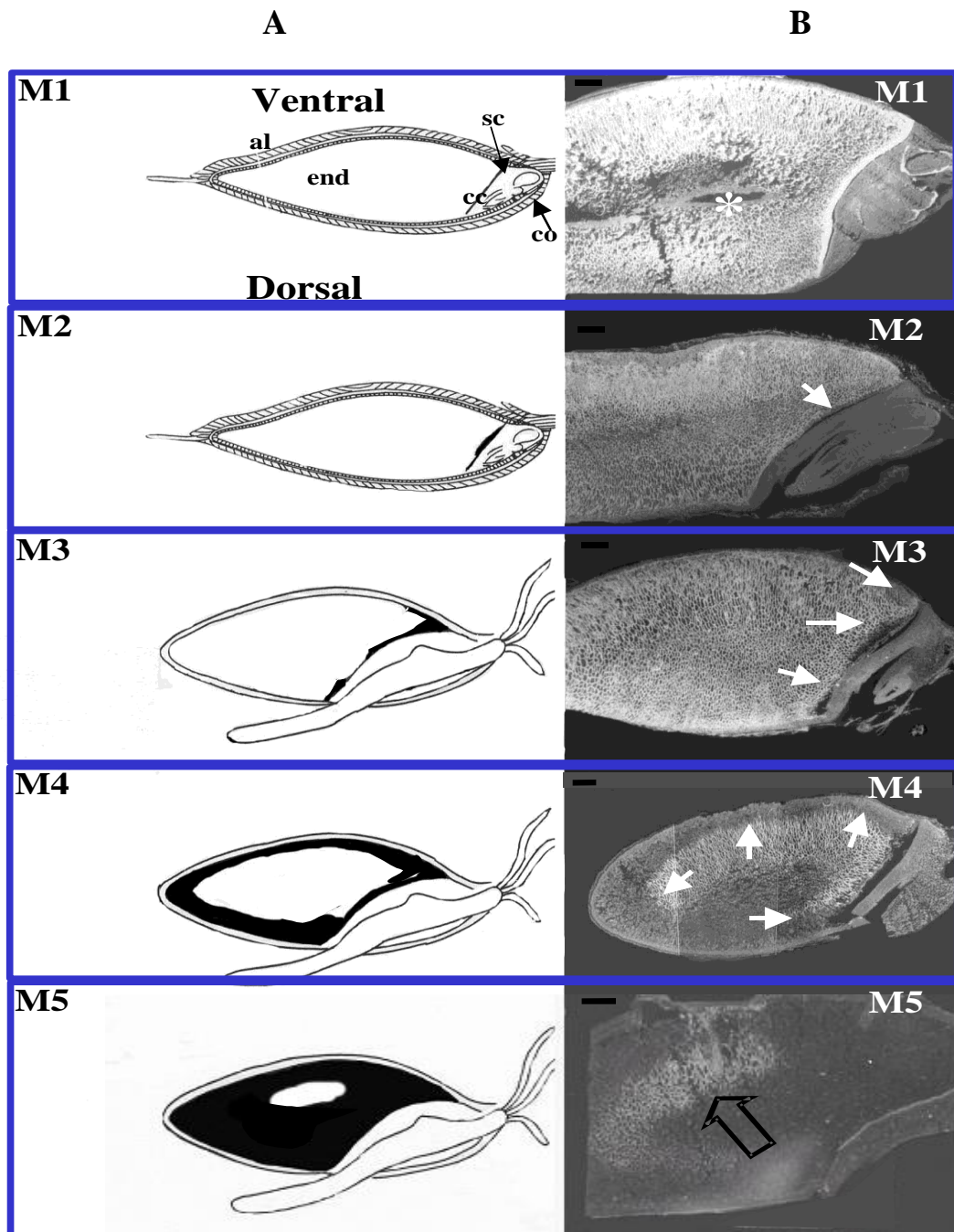
Figure 2.5 A and B: Modification patterns of paraffin-embedded Himalaya grain.

Figure 2.4 A: Individual hand-drawn diagrams representing modification patterns for Himalaya barley grain in the M1 to M5 stages. Black regions are areas of reduced CW fluorescence indicating loss of cell wall $(1\rightarrow3, 1\rightarrow4)$-β-D-glucan. co, coleoptile; sc, scutellum; al, aleurone layer; cc, crushed cell layer; and end, starchy endosperm. Figure 2.5 B: Paraffin-embedded longitudinal sections of Himalaya grain stained with CW, corresponding to the M1 to M5 stages. White arrows indicate regions of loss of CW staining. The black arrow in M5 is an area of remnant CW stained cell wall $(1\rightarrow3, 1\rightarrow4)$-β-D-glucan. *Indicates areas of endosperm damage due to grain preparation and not CW fluorescence loss.

Scale bar is 400μm

Figure 2.6: Modification patterns of GMA-embedded Himalaya and Sloop grain at the embryo end.
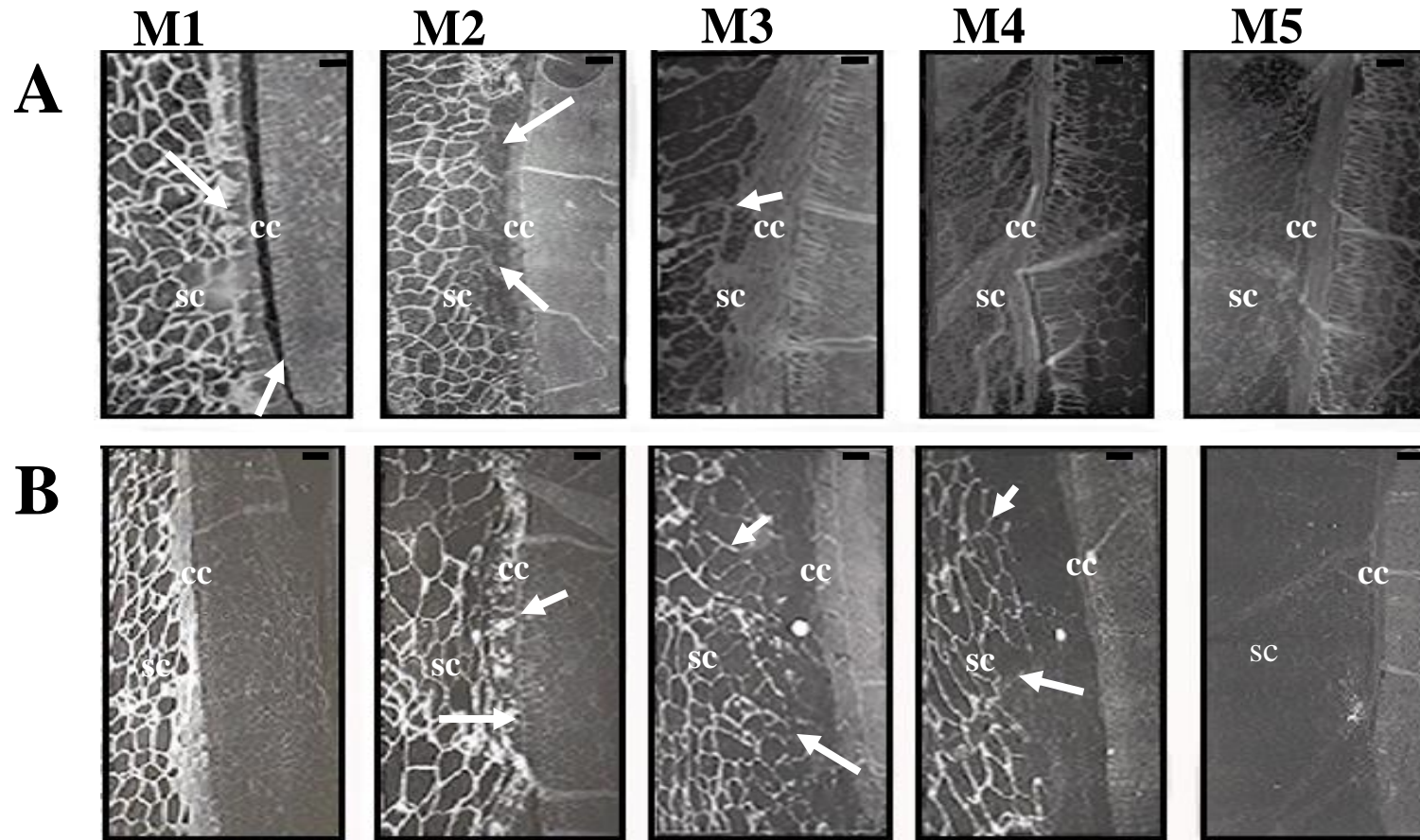
Figure 2.6 A: Embryo end of a Sloop grain shown at each M stage. GMA-embedded sections stained with CW.

Figure 2.6 B: Embryo end of a Himalaya grain shown at each M stage. GMA-embedded sections stained with CW.

White arrows indicate regions of loss of CW staining. al; aleurone layer; sc, scutellum and end, starchy endosperm.

Scale bar is 25 μm
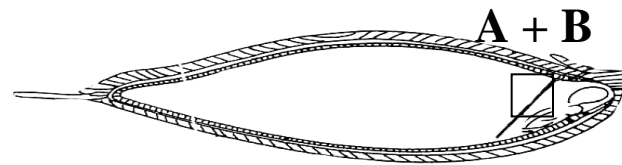
Figure 2.7: Modification patterns of GMA-embedded Himalaya and Sloop grain at the distal end.
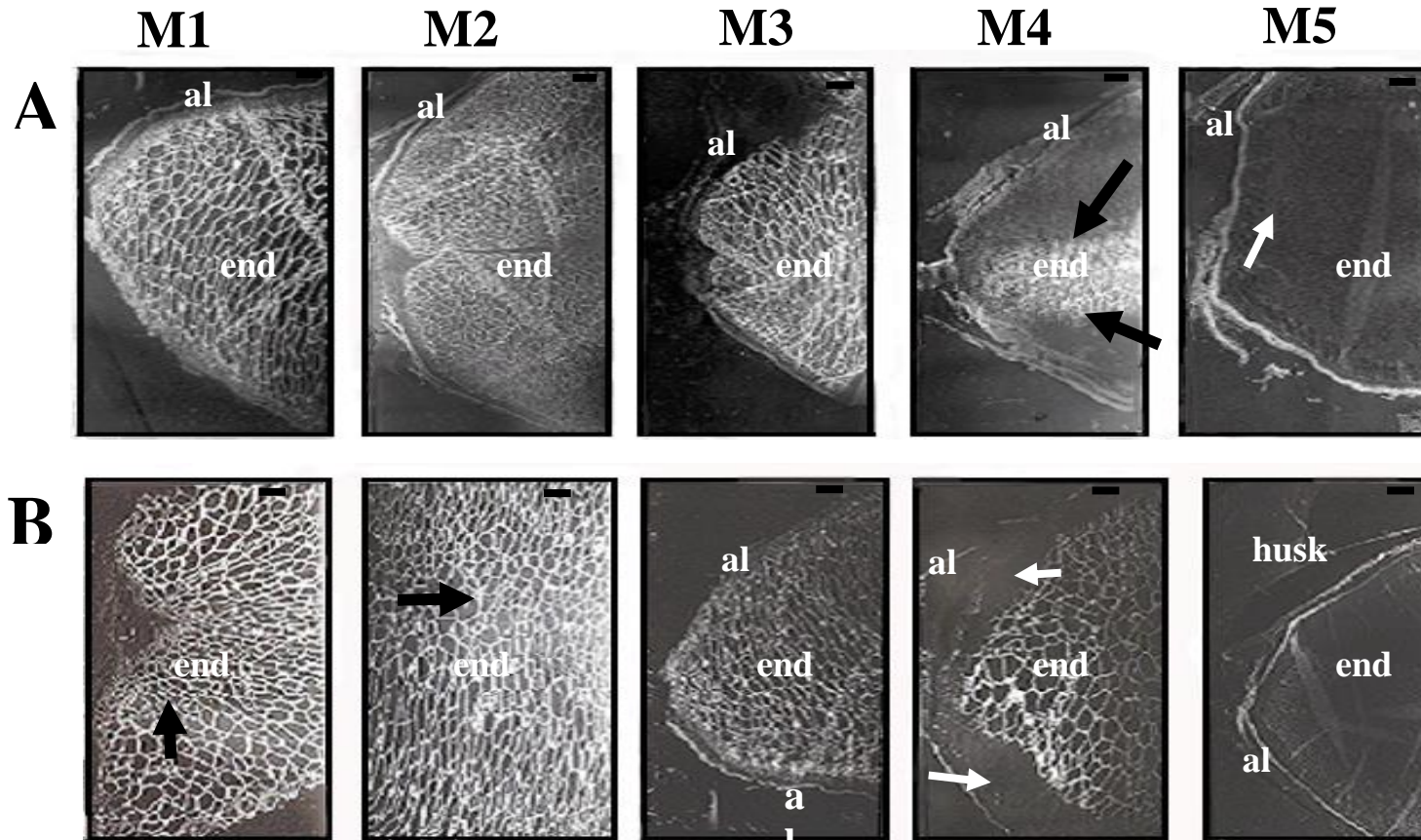
Figure 2.7 A: Distal end of a Sloop grain shown at each M stage. GMA-embedded sections stained with CW. White arrows indicate regions of loss of CW staining.

Figure 2.7 B: Distal end of a Himalaya grain shown at each M stage. GMA-embedded sections stained with CW. Black arrows are the areas of CW staining showing cell wall β-glucan still present.

al, aleurone layer; sc, scutellum and end, starchy endosperm. Scale bar is 25 μm
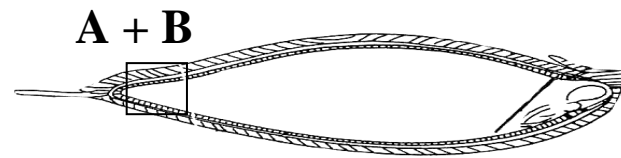
They are considered to be one of the best fixatives for preserving tissue quality and keeping RNA within the tissue, whilst still allowing a good recognition of the RNA by the probes. Paraformaldehyde alone was not ideal, and it has been reported to produce lower ISH signals than glutaraldehyde (Binder *et al.*, 1986; Moench *et al.*, 1985). Glutaraldehyde, as reviewed in Chapter 1, is capable of providing more structural integrity, although in barley tissue high concentrations of glutaraldehyde can create background autofluorescent problems. If future work required more detailed cellular studies, perhaps by TEM, better preservation could be achieved by inclusion of an extra step in the process, such as the perfusion of tissue under vacuum or phase partition fixation (Zalokar and Erk, 1977) where fixatives such as paraformaldehyde (4 % w/v) and glutaraldehyde (0.25 %) are mixed in n-heptane gas.

The other critical aspect at this stage was to maintain a large volume of fixative to tissue ratio and repeated changes of fixative were necessary to ensure maximum saturation and osmotic perfusion of the tissue. Fixative infiltration is a major problem in cereal grains owing to the presence of cell walls and a complex endosperm that is all enclosed by a cellular husk (O'Brien and McCully, 1981; Hayat, 1981). Transverse cuts (furrows) and the removal of the distal end of the grain can help to accelerate penetration of the fixing solution (Panoskaltsis-Mortari, 1995), or as we chose, to section the grain longitudinally at collection to allow optimal access.

Over fixation can also lead to the generation of background ISH problems (Dagerlind *et al.*, 1998; Schaeren-Wiemers and Gerfin-Moser, 1993). Either over-fixation or under-fixation can reduce the ISH signal obtained, as blocking or leaking out of the RNA from the tissue respectively can occur during the steps prior to ISH (Dagerlind *et al.,* 1998; Schaeren-Wiemers and Gerfin-Moser, 1993; Wilcox *et al.*, 1986; Brigati *et al.*, 1983). The best combination was considered to be 4 % (v/v) paraformaldehyde and 0.25 % (v/v) glutaraldehyde in PBS buffer, not dissimilar to the fixation process used by other researchers (Table 2.4) with plant tissue.

2.4.3: *Justification for paraffin embedding*

The final media chosen to embed and section the barley grain was paraffin. Paraffin is the ideal medium for embedding tissue; it allows small ribbons of tissue to be sectioned (7 μm) whilst retaining the structural integrity of the grain. Maintaining the complete structure of an entire longitudinal half of a barley grain section was a challenge, although it was not unexpected when you consider the composition of the many layers

through the grain, ranging from a rich cellulosic husk layer, to the aleurone with its thicker walls, then into a starch and polysaccharide rich matrix within the endosperm. But the main advantage of the paraffin medium compared to other products used for embedding, such as polyethylene glycol (PEG), a polystyrene film painted on the surface of the section (Briggs, 1979) or OCT as used by McFadden *et al*. (1988), is the ease of its removal from the tissue specimen on the slide. Xylene dissolves paraffin from around the tissue without interference to the tissue structure. This is critical for successful non-radioactive ISH assays where tissue integrity is vital. Although more recent studies have successfully used Steedmans wax and PEG, both are similar to paraffin in ease of use for tissue embedding and cutting, but can be difficult to de-wax in xylene and ethanol, leading to high levels of background. PEG was not a successful embedding medium for this study.

2.4.4: Grain sectioning

The issue raised in point two of section 2.4, has been addressed by a number of past researchers. In previous studies some authors attempted to cut the tissue transversely (Chrispeels and Varner, 1967; Palmer, 1974; 1967; 1972), along the equatorial axis or crease (Varner and Chandra, 1964; MacGregor and Matsuo, 1982; Gibbons, 1980; MacGregor *et al.,* 1994), or they do not mention the exact angle of sectioning (Ranki, 1990; McFadden *et al.,* 1988a). Preliminary efforts concentrated on sectioning the grain at a 20 degree angle from the crease (Figure 2.2), the aim being to avoid the presence of the sheaf cells located at the crease which would interfere in the interpretation of the results with respect to aleurone staining (Briggs, 1972). Several problems arose from trying to cut at a 20 degree angle. Firstly, the lack of a consistently accurate site to cut each grain led to different amounts of the embryo and scutellum epithelium being exposed on each individual section and therefore interpretation of the results became difficult. The direction was changed to cut straight down through the crease as a symmetrical cut through the center of the grain. To overcome the slight variation that may occur using this approach, all sections were examined under the microscope to ensure that the columnar cells in the embryo at the crushed cell layer interface were of similar size and appearance.

2.4.5: Mounting of sections onto slides

Adherence of the tissue to the slides for assessment was also a major challenge in the development of the ISH assay. Listed in Table 2.4 are some of the referenced and

general methods documented to facilitate adhesion. All documented adhesion procedures were attempted with material fixed with glutaraldehyde and paraformaldehyde followed by paraffin embedding. It was found that the commercially purchased glass slides (Table 2.4 h) maintained consistency and were the most reliable adhesion method for tissue used within this study, particularly in the ISH assays where a combination of washing steps and incubations place tissue sections constantly in solution for almost two days.

Table 2.4: Different methods recorded in past published research to adhere sectioned tissue to glass slides.

a) Baking the tissue on the slide for 2 h at 60^0C (Clavel *et al.*, 1991).
b) Coating the slides with polyvinyl alcohol-vinyltriethaxysilane (polyvinyl) adhesive (Fink *et al*, 1987).
c) Using only perfectly flat sections for ISH (Personal communication, E. A. McGregor, Grain Research Laboratory, Canadian Grain Commission, Canada).
d) Coating slides with 1% gelatine and 0.1% (w/v) CrK $(SO4)_2$ x H2O and 0.25% formaldehyde (Coghlan *et al*, 1985; McFadden *et al.,* 1988a).
e) Coghlan *et al*. (1985) placed slides on a dry ice block for 30 min.
f) Nitric acid (8%) coating of slides prior to use (Sigma)
g) 3-aminopropyl triethanxyl silane coating of slides (Sigma)
h) Commercially produced superfrosted plus slides (Laboratory supplies)
i) Vectabond coated slides (Sugmioto *et al.,* 1998)

A novel approach to aid in adhesion of barley grain sections to the glass slides (Table 2.4 h) was used in this study. Adhesive tape was laid across the paraffin section at the time of sectioning to aid in maintaining the integrity of the grain structure and allow easy transfer to the glass slides. The original use of commercially available adhesive tape in paraffin sectioning came from work on bone tissue (Mee *et al*., 1997), and was modified for use on the barley grain sections. Numerous adhesive tapes were tested to ensure that the glue on the tape could be completely dissolved in chloroform and xylene washes during the paraffin removal stages. Only tape with glue that will dissolve in xylene was useful, creating little damage to the tissue and visual hindrance to the *in situ* hybridization assay.

A distinct difference was noted between the preservation and sectioning of the two varieties in this study. Sections cut from embedded Himalaya grain were better both in

ease of sectioning and in the degree of preservation, so the properties of Himalaya appeared to be more favorable for the fixation process. The most likely reason for the better sectioning and preservation of Himalaya compared to Sloop is the hulless nature of the grain, although the chemical nature of the two varieties also differs, particularly the nitrogen content (Himalaya has a Kolbach of 22, compared for 41 of Sloop, appendix 1) and the endosperm content (Himalaya with approx. five times the malt viscosity of Sloop, appendix 1, Chapter 1). Subsequently, a considerable amount of the photographic work presented in this thesis is of Himalaya, with images of Sloop presented when differences were noted at comparable modification stages. Early in the study, attempts were made to de-husk Sloop grain by peeling (Gibbons, 1979; Palmer, 1982) and acid treatment (MacGregor *et al*., 1984; Lauriere *et al.,* 1986), but on sectioning both treatments were seen to have physically damaged the aleurone layers of the grain.

2.4.6: Modification pattern of germinated grain

The development of a classification into modification stages (section 2.3) was to minimize sampling variation. This variation stems from the heterogenous nature of a barley grain and its uneven germination. It is well documented that a range of modification patterns can be observed at any point in germination (Collins *et al.,* 2003; Landau *et al.,* 1995; Cole *et al.,* 1997; Gianinetti *et al.,* 2007) and this was duly noted in a preliminarily assessment of the grain using hydration studies at each collection point (Table 2.2) through the germination schedule. As discussed in section 2.1.1, the Calcoflour White staining of sectioned grain was chosen as providing the best visual display of endosperm modification, whilst at the same time allowing embedded grain, once classified, to be utilised in further studies.

The Calcoflour White staining pattern seen at M1 and M2 for Sloop (Figures 2.3 and 2.5) indicates the initial digestion of $(1\rightarrow3, 1\rightarrow4)$-β-D-glucan occurs in the crushed cell layer, and endosperm cell walls closest to the crushed cell layer, on the ventral side of the grain (Figure 2.6 B solid white arrows). This pattern of degradation is suggestive of $(1\rightarrow3,1\rightarrow4)$-β-D-glucanase activity originating from the scutellum and moving into the adjacent endosperm tissue to degrade the $(1\rightarrow3, 1\rightarrow4)$-β-D-glucan in its path (MacGregor *et al.,* 1994). This is in agreement with other researchers such as Gibbons (1980); Aastrup and Erdal (1980); Pogsen *et al*. (1989); Briggs and MacDonald (1983); Davies (1990); Macgregor *et al*. (1994). Wallwork (1997) observed hydrolysis or a

breakdown in the crushed cell layer walls not long after grain hydration and noted that the degradation was on the ventral side of the grain. This result is not surprising, as the process of cell wall degradation is presumed to start as soon as the grain is exposed to water (Bamforth and Martin, 1981), and water can be detected in the endosperm 2 h from first exposure (Schildback and Rath, 1994). In hydration studies water was also observed to enter the ventral side of the endosperm more rapidly than the dorsal (Ridenour *et al*., 1996; Schildback and Rath, 1994; Axell *et al*., 1994; Davies, 1990 and Yin *et al.,* 1996). The variety Himalaya showed the same pattern as Sloop where the crushed cell layer walls on the ventral side of the grain were the first to degrade but this appeared to occur at a slightly slower rate than in Sloop grain (Figures 2.3 B and 2.5 B).

The pattern of degradation on both Sloop and Himalaya sections at the M3 and M4 stages shows extensive breakdown of $(1{\rightarrow}3, 1{\rightarrow}4)$-β-D-glucan in the endosperm cell walls lying adjacent to the aleurone layers of the grain that surround the whole endosperm. At the same stages the advancement of the cell wall breakdown moving away in parallel to the scutellum was reduced in the Himalaya sections compared to those from Sloop. Himalaya therefore displays a modification pattern that implies a reduced action of the scutellum early in germination, either in relation to overall enzyme activity or in the ease with which these hydrolytic enzymes penetrate the crushed cell layer and endosperm tissue (Briggs and MacDonald, 1983), although structural differences in the varieties such as cell wall thickness and total $(1{\rightarrow}3, 1{\rightarrow}4)$-β-D-glucan content cannot be ruled out.

2.4.7: *Varietal differences in cell wall breakdown modification patterns*

The observed delay in the rate of modification in Himalaya grain sections compared to Sloop has also been noted by others (MacGregor *et al.,* 1994) and many reasons have been put forward to explain this. These include a possible difference in enzyme expression levels between the two varieties, particularly the $(1{\rightarrow}3,1{\rightarrow}4)$-β-D-glucanase enzyme, as indicated by the malt DP enzyme level difference for the two varieties (appendix 1) or a difference in endosperm structure such as cell wall thickness or starch and protein composition. Himalaya has a higher starch and $(1{\rightarrow}3, 1{\rightarrow}4)$-β-D-glucan endosperm content (appendix 1). The delayed endosperm cell wall breakdown pattern of Himalaya contradicts the higher hydration values of Himalaya when compared to Sloop (appendix 1) which is believed to result from the hulless nature of

the grain and which has been reported by others (Chandler *et al*., 1998).

By the M5 stage both Sloop and Himalaya show an advanced degree of cell wall $(1\rightarrow3, 1\rightarrow4)$-β-D-glucan degradation across the whole endosperm except for in the very central regions, as was also noted by Gibbons (1979). This was slightly less expansive in Himalaya but the differences were not quantified.

The progression of endosperm modification presented in this study therefore follows a pattern in general agreement with a number of other authors (Gibbons, 1980; Aastrup and Erdal, 1980; MacGregor and Matsuo, 1982; Jensen and Heltved, 1982; Briggs and MacDonald, 1983; 1972; Gibbons and Neilsen, 1983), although Fretzdorff *et al,* (1982) noted a pronounced modification breakdown in the dorsal (nonfurrowed) parts of the grain. On the other hand, Palmer (1974; 1975; 1967; 1972; 1972) and MacLeod and Palmer (1966; 1967) reported a totally different pattern of degradation beginning at the dorsal apex of the scutellum and moving adjacent to the edge of the aleurone that is nowhere near the center of the scutellum and crushed cell layer interface. Indeed, this study does show degradation of the endosperm areas close to aleurone layers in both varieties but only in the later stages of germination.

As discussed in Chapter 1, modification patterns signify the presence of secreted enzymes (Munck *et al*., 1981; Briggs and MacDonald, 1983; Benjavongkulchai and Spencer, 1987), and a similar profile as seen here for modification has been shown for α−amylase expression (Briggs, 1973; Gibbons, 1979; 1980; Pogsen, 1998 and Mathews *et al.,* 2002), which also follows endosperm protein and starch degradation (Gram, 1982; MacGregor *et al.*, 1984; Fretzdorff, 1982). The data presented in this chapter does suggest that the scutellum is the main tissue involved in enzyme production and secretion early in germination, particularly of $(1\rightarrow3,1\rightarrow4)$-β-D-glucanase, while the aleurone layers are involved at a later stage. Palmer (1982) has argued that it is difficult to decide whether the detected enzymes involved in endosperm modification in the starch endosperm have moved directly from the scutellar tissue into the aleurone to be then secreted or whether they are secreted from the scutellar epithelial cells and the aleurone tissue during germination. This study strengthens the implication, as suggested by others, that the $(1\rightarrow3,1\rightarrow4)$-β-D-glucanase enzymes can move across the crushed cell layers through defined channels early in germination, but confirmation by more specific means, possibly at the electron microscope level, is needed.

2.5: Conclusion

In order to balance both morphological preservation with a functional assay in this study, fixation combinations with germinated barley grain were tested and it was determined that the optimal preservation was obtained with a combination of glutaraldehyde at low concentration (0.25 %) and paraformaldehyde (4 %), as also used by a number of other barley researchers including Sugimoto *et al.* (1998) and Gubler (1987). The choice of which embedding technique is employed can be as simple as considering the equipment available within the laboratory. As discussed earlier, the direction and consistency of sectioning is critical and this can, to some degree, influence the embedding material and subsequent ease of use.

The general pattern of endosperm cell wall breakdown as assessed by CW fluorescent staining was consistent between the two varieties assessed in this study, except for a delayed, and subsequently slightly reduced, degradation emigrating from the scutellum area of Himalaya grain. The general pattern of cell wall degradation was observed to be reasonably consistent with the results presented by Briggs and MacDonald (1983) who described the breakdown pattern as a front moving down towards the distal end of the grain, extending parallel from the face of the scutellum. This therefore supports the "symmetrical" pattern of modification.

The designation of paraffin-embedded longitudinally cut germinated grain into modification stages as assessed by CW staining was used to place grain sections into groups, hence reducing biological variability. This is important in relation to further studies on these tissue samples when the expression patterns of specific enzymes using *in situ* and immunostaining were examined and assessed in relation to the modification stage of the grain. The non-destructive CW staining technique was successfully used to both map endosperm modification and allow further assays to be performed on sections originating from the same embedded grain halves. The development of *in situ* hybridisation and immunostaining detection techniques is outlined in the next chapter.

Chapter 3

Development of *In situ* hybridisation methods to locate hydrolytic enzyme mRNA and proteins in germinated barley grain sections.

3.1 Introduction

At the initiation of this study the majority of research on enzyme expression in barley grain had concentrated on measuring either protein or mRNA extracted from isolated tissues (Slakeski *et al*., 1990; Slakeski and Fincher, 1992), or the *in vitro* translated products of mRNA samples extrapolated to provide a general picture at a whole grain level (Mundy *et al*., 1985).   Limitations in these studies have been documented, particularly with respect to the fact that the metabolically distinct aleurone cells are in such close proximity to the scutellar tissue, complicating the interpretation of experimental data (Palmer, 1982; Aishen *et al*., 1983).   While preparation of the scutellum to ensure it is free of contaminating aleurone tissue has been reported (Stuart *et al*., 1986), it is difficult to achieve and isolation of certain cell types has been very difficult.   Ranki *et al*., (1990) stated that they removed only 90% of contaminating aleurone cells with their dissection procedure, whilst Slakeski *et al*., (1990) had to use light and scanning electron microscopy to demonstrate that scutellar tissue was free from adhering pericarp, testa, starchy endosperm and other embryonic tissue, but may have overlooked a few adhering cells.   The need for pure tissue specimens also has implications with regard to mRNA concentrations, as extracted mRNA encoding the hydrolytic enzymes may be diluted by mRNA from other sources surrounding the scutellum epithelium and aleurone tissue (Palmer and Duff, 1986).   Recently the use of real time quantitative reverse transcription polymerase chain reaction to enhance mRNA from tissue sections has become popular in various plant types and does overcome the contamination problem to some degree (Nemati and Navalqoour, 2012; Burton *et al.*, 2011; Wang *et al*., 2010; Koltai and Bird, 2000).

We also need to consider that data obtained from extracted mRNA can only provide an average level of gene expression and is not able to show changes that may be occurring at the cellular level, such as during differentiation or cell death (Penschow, 1987). Microbial contamination in tissue extracts stored for periods of time in nutrient medium could also affect the results obtained.   Higgins *et al*. (1982) is one of the few authors who tested for microbial contaminates in the medium of their excised tissue, allowing for misleading results due to contamination, although others have tried to maintain sterile conditions by using laminar flow units in dissection procedures (Eastwell *et al*., 1982).   Standardising and allowing for loss of RNA by enzyme degradation is also important as RNA loss is known to be more rapid in tissue homogenates than from whole tissue sections (Penschow *et al*., 1987).

Taking into account the possible limitations of past studies examining enzyme expression, and the successful research conducted by McFadden *et al*., (1988a) demonstrating $(1{\rightarrow}3,1{\rightarrow}4)$-β-D-glucanase mRNA expression in tissue sections of germinated barley grain using a radioactive *in situ* hybridisation assay (ISH), ISH was considered to be an ideal procedure to demonstrate the presence of mRNA of specific enzymes during this study, including adaptation of the method into a more user-friendly non-radioactive method with paraffin embedded grain tissue.

As outlined in Chapter 1, ISH enables the precise location of RNA targets and provides an avenue to study the temporal and spatial expression patterns of specific genes (Engler *et al*., 2001; Braissent *et al.,* 1992).   The resolution obtained is such that distribution gradients of mRNA present at less than 30 copies within a cell can be detected (Cox *et al*., 1984).   A flow diagram (Figure 3.1) outlines the progression of steps taken in this study to develop a successful and robust ISH method using RNA single stranded probes (riboprobes) on barley grain pieces fixed in 4 % glutaraldehyde and 0.25 % paraformaldehyde and paraffin embedded. This follows the general steps intrinsic to any ISH, including optimal tissue preservation and sectioning of the germinated grain pieces, through riboprobe labelling, to the development of a non-radio active digoxigenin (DIG) detection technique on barley grain sections. Information from previous researchers, particularly those using barley or plant tissue such as McFadden *et al.* (1988); Sugmioto *et al.* (1998) and Braissant and Wahli (1998), as discussed in Chapter 1, formed the basis for this procedure but a number of the methods reported here are also novel to this study.

3.2: Materials and Methods Development

3.2.1: Grain germination and sampling
Samples of both Sloop and Himalaya grain were collected every 8 h from the beginning of steeping until approx 110 h into germination.

3.2.2: Grain fixation
As described in Chapter 2, individual grains were sectioned longitudinally through the crease and both halves immediately preserved in fixative; 8 mL of 4% paraformaldehyde and 0.25% glutaraldehyde in PBS for 12 h at 4^0C, followed by a change of fixative and another 12 h incubation. Grain was washed in a large volume of cold PBS and transferred to a paraffin embedding case for successive 24 h periods in 70% and then in 100% (v/v) ethanol in preparation for paraffin embedding (Chapter 2).

Figure 3.1: Process chart in the development of a non-radioactive ISH assay on germinated barley grain

Grain germination

Fixation

Tissue embedding

Sectioning

Slide adhesion

Pre-hyb treatment (de-wax)

Hydration

Riboprobe preparation   ISH method development

Template preparation   Probe concentration

Probe assembly + labelling   Temperature washes

Purification   Modification of established techniques
                                            and reagents

Determine concentration   Substrate incubation

                                                    Cover-slipped

ISH stained tissue

3.2.3: *Grain embedding and sectioning*

Grain halves fixed as described were embedded in paraffin as outlined in Chapter 2 and were sectioned with a microtome (section 2.2.4). Paraffin sections could only be successfully anchored to the Superfrost Plus glass slides with the aid of adhesive tape and sterile water. The procedure is outlined in Chapter 2, section 2.2.4. These glass slides supporting the paraffin sections were incubated at 37^0C for a minimum of two days prior to use and stored in a humidity controlled box until use. Each glass slide could accommodate longitudinal sections of three half grains.

3.2.4: *Restriction enzyme digestion*

Specific cDNA fragments for riboprobe transcription were selected (described in detail in each relevant chapter) and cut with restriction enzymes to obtain cDNA fragments of interest. Restriction enzyme maps were considered for each cDNA (data not shown) and restriction enzymes were chosen to optimise cDNA fragment length and specificity with regard to riboprobe transcription. A linear pBluescript vector (Stratagene, La Jolla, CA, USA) was also prepared by cutting with specific restriction enzymes within the multiple cloning site. The restriction enzyme procedure for both the cDNA of interest and pBluescript was as follows; 1 µg DNA; 1 µL of restriction enzyme (Gibco, Life Technologies, USA or BioLabs, USA; 2.5 µL of 10 X buffer (Gibco); 2.5 µL of 10 X BSA (Gibco) to a total volume of 25 µL in sterile water. This was incubated for 1 h at 37^0C and a small aliquot was run on a 1% agarose gel to ensure complete digestion. Restriction-cut DNA was extracted with phenol:choroform and precipitated with 1 vol 3 M sodium acetate buffer, pH 5.2 and 2.5 vol ethanol. The DNA was collected by centrifugation at 13,200 rpm in a microcentrifuge for 15 min. The pellet was washed with 70 % ethanol, dried and dissolved in 50 µL TE (10 mM Tris HCl, Sigma).

3.2.5: **E.Coli** *competent cells*

Competent cells for DNA transformation were prepared using *E.coli* XL-1-Blue (P2) cells (Stratagene). XL-1 Blue cells were grown overnight at 37^0C with shaking at 250 rpm in Millers LB broth (10 g/L Luria-Bertiaini (DIFCO Laboratories); 5 g/L Bacto yeast (DIFCO Laboratories); 5 g/L NaCl (Sigma), pH 7.5) containing 15 µg/mL of tetracycline (Sigma). One ml of the overnight culture was aseptically transferred to a 500 mL Erlenmeyer flask containing 100 ml Miller's LB broth and 15 µg/mL tetracycline, and the flask was incubated at 37^0C with shaking at 250 rpm until the culture reached an absorbance of 0.5 at 600 nm. Cells were transferred to 50 mL polypropylene tubes and centrifuged at 3,000 rpm for 15 min at 4^0C. The supernatant was discarded, and cells resuspended in one-third of the original volume of glycerol

buffer (60 mM CaCl (Sigma); 15% glycerol (Sigma); 10 mM PIPES (piperazine-1, 4-bis (2-sulforic acid) pH 7) and incubated on ice for 1 h. The cells were centrifuged at 3000 rpm for 15 min at 4^0C and the supernatant again discarded. The final cell pellet was resuspended in glycerol buffer and left on ice for 15 min. The competent cells were quick-frozen using liquid N_2 and stored in 100 μL aliquots at -80^0C.

3.2.6: DNA Ligation and E. Coli *transformation*

The purified digested cDNA (3.2.5) was ligated into the complementary digested pBluescript vector (3.2.5) for 16 h at 16^oC using T4 DNA ligase (New England BioLabs, USA). The recombinant plasmid was transformed into competent XL-1 Blue cells (3.2.6).

3.2.7: E.Coli *transformation*

Frozen competent cells (100 μL; section 3.2.6) were thawed on ice and mixed gently, transferred to pre-chilled microcentrifuge tubes and the ligation reaction (10 μL from section 3.2.7) was gently added. Tubes were incubated on ice for 30 min, at 42^0C for 90 s and incubated again on ice for 2 min. LB broth (700 μL) was added, incubated for 90 min at 37^0C, with shaking at 180 rpm. The tubes were centrifuged and all but 100 μL of the superannuant was removed, the cell pellet was resuspended in the remaining media and spread onto LB plates (1 g Bacto agar to 1 litre LB broth) containing 100 μg/mL ampicillin (Boehringer-Mannheim), 40 μL/mL X-gal (5-Bromo-4-Chloro-3-Idolyl-β-D-Galactose) and 12.5 μg/mL IPTG (Isopropyi-β-D-Thiogaloctoside). The plates were incubated at 37^0C for approximately 16 h. The presence of a DNA insert in the pBluescript vector could be identified as white colonies whereas clones with no insert remain blue since the successful ligation of a DNA fragment into the cloning site of pBluescript disrupts β-galactosidase expression. White colonies were picked and transferred to LB containing 100 μg/mL ampicillin using sterile pipette tips. The cultures were shaken at 250 rpm overnight at 37^0C.

3.2.8: Isolation of plasmid DNA

The sub-cloned pBluescript DNA was recovered from overnight cultures of the competent cells using a mini-prep reaction (GTE buffer; 1% SDS; 0.2 M NaOH;1 M potassium acetate) followed by ethanol precipitation and resuspension in R40 (10 mM Tris HCL; 1 mM EDTA (Sigma), pH 7.4; 5ng/L RNAase). To check the identity of the insert, the plasmid DNA was digested with relevant restriction enzymes as described in section 3.2.5 and run on a 1% agarose gel (DNA grade, Progen Industires) in TAE (10 mM Tris-HCI, 0.1 mM EDTA, pH 7.5) buffer. To run on the agarose gel, the

restriction cut DNA sample was mixed with 5 X DNA loading buffer (0.25% w/v bromophenol blue (BDH chemicals), 0.25% w/v xylene cyanol, 40 % w/v sucrose, Sigma) and separated by 1% (w/v) agarose gel and 10 ug/mL ethidium bromide (Amresco) electrophoresis as described by Sambrook *et al*. (1989). A 1 kb ladder (Invitrogen) was used as a DNA standard.

3.2.9: DNA sequencing

The insert was also sequenced using Big Dye reagent version 2 (ABI) on an ABI 3700 capillary sequencing machine at the Institute of Medical and Veterinary Science (IMVS, Adelaide SA). All sequences were subsequently checked via an advanced BLAST (version 2.0.10, Altschul *et al.,* 1996) using the NCBI web site.

3.2.10: Riboprobe production

Single-stranded RNA fragments (riboprobes) were prepared and diogoxigenin (DIG) labelled for use as the detection system in the ISH assay. The sub-cloned pBluescript plasmid was linearised for riboprobe production with specific restriction enzymes within the linker region of the clone as per section 3.2.5. Riboprobes were prepared from linear cDNA using the following reaction; linear cDNA (1 µL, 1 µg/uL); 2 µL 10 X Transcription buffer (Boehringer-Mannheim), 1 µL RNasin^R ribonuclease inhibitor (Promega 40 units/µL); 2 µL 10 X DIG-RNA Labelling mix (µL/mL dUTP, Boehringer-Mannheim); 2 µL of either T3 or T7 RNA polymerase (Boehringer-Mannheim) and deoxyribonuclease free water to a final reaction volume of 20 µL. Reactions were performed for 2 h at 37^0C and terminated by the addition of 2 µL of DNase 1 (10 units/µL) for 15 min at 37^0C, 1 µL of 0.5 M EDTA pH 8 (Sigma). Riboprobes were purified by overnight precipitation at -20^0C with 2 µL tRNA (10 mg/ml, Promega); 2.5 µL 3 M Sodium Acetate (Sigma); 75 µL 100% absolute grade ethanol (BDH), followed by centrifugation at 16,000 rpm for 30 min, removal of the supernatant and re-hydration in 100 µL sterile water. Once rehydrated, probes were stored at -20^0C. Figure 3.2 outlines the process of riboprobe generation and, depending on the orientation of the specific cDNA of interest within the pBluescript plasmid, sense (control) or anti-sense riboprobes can be obtained from each of the cDNA strands.

3.2.11: DIG quantification

Riboprobe DIG-labelling can be semi-quantitatively determined using DIG test-strips (Boehringer Mannheim). The efficiency of the labelling reaction was tested via a dot

blot analysis by comparing the intensity of labelled probes with known concentrations of a control label on strips supplied by the manufacturer (Boehringer Mannheim).

Figure 3.2:   DIG labelled riboprobe production using T7 or T3 polymerase. Process of riboprobe generation. Depending on the orientation of the specific cDNA of interest within the pBluescript plasmid, sense (control) or anti-sense riboprobes can be obtained from each of the cDNA strands.
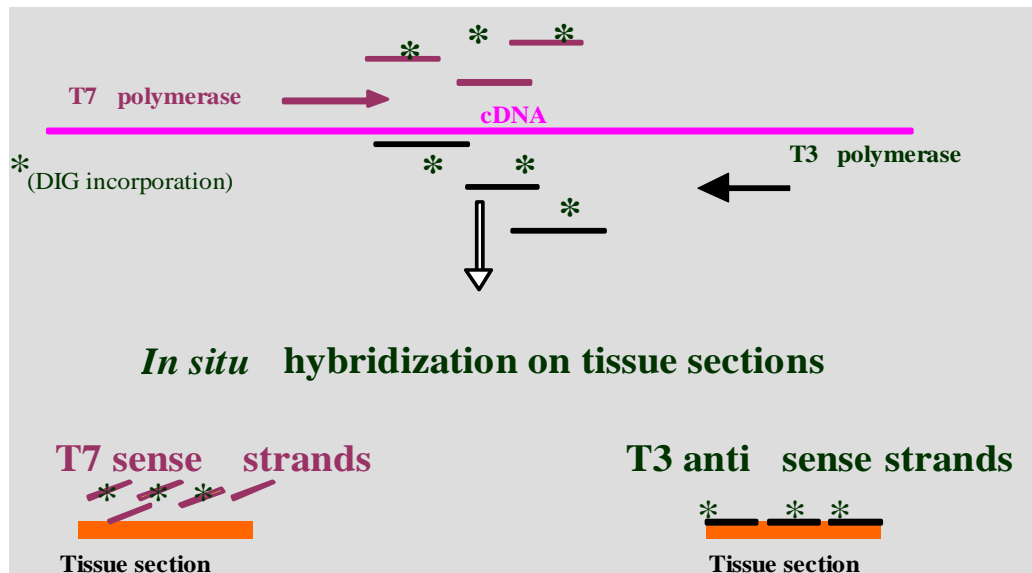
Following the manufacturer's instructions synthesised riboprobes (as per section 3.2.10) were pre-diluted 1 μL to 39 μL in sterile DNA dilution buffer (1 mM EDTA; 10 mM Tris-HCl, 50 μg/mL DNA from herring sperm, pH 8.0), termed dilution A and further diluted as listed in table 3.1.

Table 3.1: Dilution procedure to determine riboprobe concentrations as per Boehringer Mannheim DIG test strips.

A	1 μL to 39 μL DNA dilution buffer
B	10 μL to 23 μL DNA buffer
C	5 μL to 45μL DNA buffer

Aliquots (1 μL) of each dilution (A, B and C) were spotted onto the designated areas on the Boehringer Mannheim test strips and air-dried. One control strip and all test strips were incubated in 10 mL maleic buffer (0.1 M maleic acid; 0.15 M NaCl, pH 7.5, Sigma) containing 1% BSA for 30 min at room temperature, then transferred to maleic buffer containing 1% BSA and anti-digoxigenin alkaline phosphatase Fab fragments (Boehringer Mannheim, 1/2000 dilution) for 30 min. The strips were washed in fresh

maleic buffer then transferred to the detection butter (0.1 M tris-HCl; 0.1 M NaCl, 50 mM $MgCl_2$, pH 9.5) containing 40 μL/2mL NBT/BCIP substrate (section 3.2.18, Boehringer-Mannheim). The spot colour intensities of the control and experimental dilutions were compared to estimate the concentration of labelled riboprobe. Figure 3.3 is an example of the test strip results observed using this procedure. A historical procedure for assessing riboprobe production is presented in Figure 3.4 showing the RNA levels in a 1% agarose electrophorsis gel as described in section 3.2.9 following DEPC treatment of the apparatus.

3.2.12: In situ hybridisation

A standard *in situ* hybridization procedure using DIG-labelled riboprobes was developed and followed for all tissue sections used in this study, as outlined below.

3.2.13: Pre-hybridisation preparation

Mounted paraffin sections were deparaffinized with chloroform (100%) for 10 min; xylene (100%) for 10 min and put through a graded ethanol sequence of 100% for 10 min; 70% for 10 min; 30% for 10 min to finally rest in sterile water. Between the choroform and xylene incubations sterile forceps were used to remove the dislodged tape from the tissue sections.

Figure 3.3:   Example of Boehringer-Mannheim DIG labelled riboprobe quantitation test strips. DIG control strips indicate the colour intensity expected for the respective ng/µL probe concentration in the test solution.   Samples A to D are tested riboprobes.   A: Measured at 300 ng/µL DIG labelled probe; B: 100 ng/µg; C: 10 ng/µL and D: 3 ng/µg.
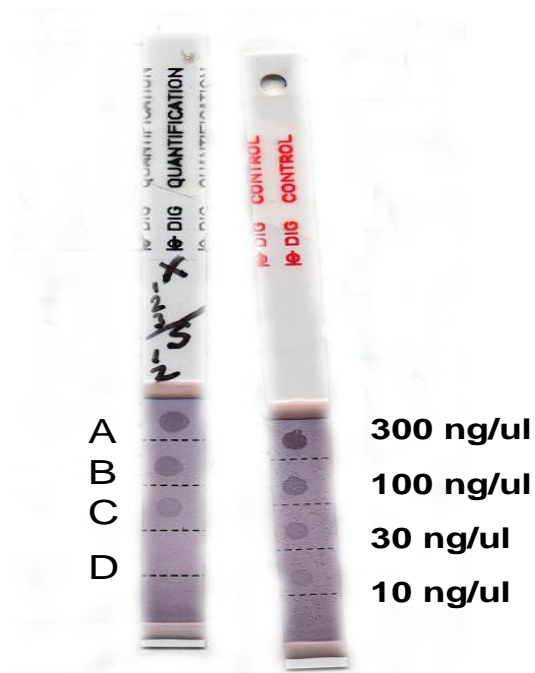
Slides were incubated at room temperature in fresh 4% paraformaldehyde (Sigma) in PBS for 10 min to provide extra RNA preservation, washed twice for 15 min in PBS, then incubated in a continually stirred solution of PBS containing 0.1% DEPC (Sigma) for 20 min. The slides were placed in two 10 min incubations of 5 X SSC buffer (5 X-150 mM sodium chloride and 15 mM sodium citrate). This was followed by a 2 h incubation at 45^0C in a pre-hybridisation solution (4 X SSC; 20 μg/mL salmon sperm DNA (5 mg/ml), 50% formamide (BDH) in a humid chamber containing a sponge soaked in 4 X SSC and 50% formamide.

3.2.14: Hybridisation

Following the pre-hybridisation incubation, the pre-hybridisation solution was removed by tipping the slide and allowing the solution to drain away. The riboprobe was added to the tissue sections in hybridisation buffer (4 X SSC; 20 μg/mL salmon sperm DNA and 50% formamide) at a volume of 40 μL per slide, with three sections present on each slide. Using the Boehringer DIG labelling determination method (section 3.2.11) 5μl of each riboprobe (at a concentration of 3 μg/mL) in 40ul of hybridisation buffer was added to each incubation. Before addition to the slide the hybridization mix was vortexed, heated to 80^0C for 5 min and placed on ice. Small pieces of parafilm were used to seal the solution onto the slide during the overnight incubation in the humid chamber at 45^0C.

3.2.15: Post hybridisation

Following careful removal of the parafilm strips with forceps, stringent post hybridisation washes were performed with serial dilutions of washing buffers starting at 2 X SSC for 1 h down to 0.1 X SSC and 50% formamide, for 45 min. All incubations were performed at 45^0C with gentle agitation.

3.2.16: Pre-DIG antibody detection blocking

The slides were incubated in antibody buffer (100 mM Tris-HCl (sigma), 150 mM NaCl (Sigma), pH 7.5) for 5 min. At this stage the slides are laid out on a vertical slide staining rack and incubated with 50-100 μL per slide of antibody buffer containing 1% bovine serum album (BSA, Sigma) in PBS for 30 min, at room temperature.

3.2.17: Incubation with anti-DIG antibody

Antibody buffer was drained from the slide by tapping, and the anti-digoxigenin alkaline phosphatase antibody was applied at 50 μL per slide (1/1000, Fab fragments;

Figure 3.4: Example of RNA DIG labelled riboprobes on a non-denaturing electrophoresis mini gel. The gel was prepared and run according to section 3.2.9. Samples of RNA were loaded on to the gel at approx. 1 μg lane as per section 3.2.9. Mk, DNA markers; A to D are riboprobes, the same shown in Figure 3.3 and E is a tRNA control sample.
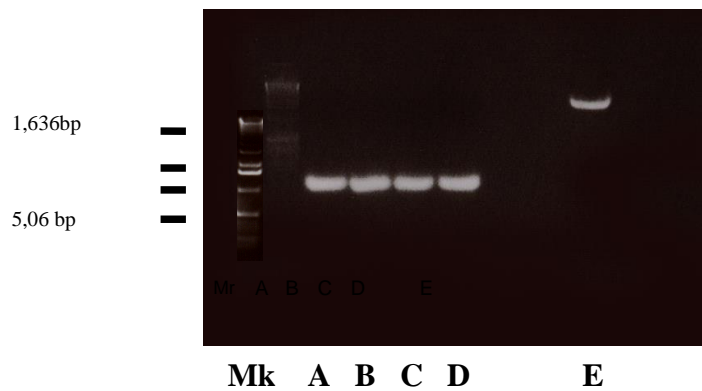
Boehringer Mannheim) in the recommended antibody buffer (1% BSA and 0.05% tween in PBS). The slides were incubated in a sealed container for 2 h at room temperature.

3.2.18: Post anti-DIG antibody washes

Whole slides were transferred to fresh antibody buffer (1% BSA in PBS) containing 0.05% Tween 20 and incubated at room temperature for 60 min with two buffer changes.

3.2.19: Anti- DIG colour reaction detection

Following the washes, whole slides were transferred to a detection buffer containing alkaline phosphatase (AP) substrate NBT (nitro-blue tetrazolium chloride) and BCIP (5-bromo-4-chloro-3'-indolyl-phosphate p-toluidine salt), Boehringer-Mannheim) in 0.1 M Tris-buffer pH 9.5; 0.05 M $MgCI_2$; 0.1 M NaCl and left undisturbed in the dark at room temperature. The colour development was monitored (approximate time was 2-3 h) by observing the individual sections under a microscope. The reaction was stopped by tipping off the reaction solution and washing the slides in 2 X SSC for 30 min. Final washes included several changes of distilled water to remove the salt crystals which otherwise hampers histological observation. Sections on the slides were air dried, and sealed with UV active glue (Loctite 349), which was not alcohol-based and compatible with the alcohol sensitive alkaline phosphate substrate.

The BCIP is the AP-substrate that reacts further after dephosphorylation to produce a dark-blue indigo dye as an oxidation product, whilst the NBT serves as the oxidant (Boehringer Mannheim).

3.2.20: Additional information

Most reagents and equipment were maintained RNAase free by washing in 0.1 N NaOH and/or autoclaving. Glass containers and cylinders were washed with sterile water after use, and baked overnight at 180^0C. Slides were handled with gloved hands at all times and maintained under sterile conditions where possible until the reaction was completed.

3.3: Results and Discussion

Initially, whilst searching through the literature it became obvious that a non-radioactive ISH procedure on barley grain had not been reported. Therefore, a critical aspect of this study was to develop a straightforward non-radio-labelled ISH protocol which would work on half sectioned barley grain sections with maximum sensitivity, with almost non-existent background, while maintaining tissue integrity. Historically, radioactive probes and autoradiography have been favoured for ISH assays, but the need for long exposure times, low resolution and safety issues are among the many reasons to choose a non-radioactive probe detection system (Panoskaltsis-Mortari, 1995; Braissant and Wahli, 1998; Farquharson *et al*., 1990; Morris *et al*., 1990).

Sugmioto *et al.* (1998) has used non-radioactive ISH on paraffin-embedded rice grain sections and the paper by Braissant and Wahli (1998) played a significant role in the developmental process of the ISH procedure presented in this thesis, providing advice on the fundamental application and use of each step in the procedure.

3.3.1: The ISH assay

When the papers by Panoskaltsis-Mortari and Bucy (1995) and Braissant and Wahli (1998) were published, they questioned the previously established ISH assays. Most ISH assays, including the previous ISH method used for $(1\rightarrow3, 1\rightarrow4)$-$\beta$-D-glucan, were based on a radio-labelled detection method (McFadden *et al*., 1988a). Modifications suggested by the authors above were systematically applied during the development of the ISH assay as discussed in the following sections. The final ISH method established here was used exclusively in this study and constitutes a simplified assay completed in less time with reduced background, particularly on difficult tissue sections.

3.3.1.1: First ISH step: Proteinase K treatment

The initial ISH procedure followed in the early stages of this study included the application of 1 μg/mL proteinase K for 20 min in pre-warmed 1 M Tris, pH 7.6 and 1 ml of 0.5 M EDTA, at 37^0C, followed by a post fixation step using 4% paraformaldehyde for 10 min and a 0.1 % glycine wash. The use of proteinase digestion on glutaraldehyde fixed tissue has been documented to increase the hybridisation signal, almost to the same level as for results obtained from unfixed tissue (Lawerence and Singer, 1985; Biochemica, 1990). The proteinase K enzyme produced by the fungus *Tritirachium album* Limber is an endolytic protease that cleaves peptide bonds at the carboxyl side of aliphatic aromatic or hydrophobic amino acids. A special

feature of the proteinase K enzyme is its ability to digest the protein in its native or functional state.

But the exact timing of the proteinase K application to the tissue is critical. Over-incubation with proteinase K can cause a loss of more than half of the total cellular RNA and be detrimental to cell morphology (Lawerence and Singer, 1985). Denatured cellular debris from the digestion can remain within the tissue, contributing to background problems with the hybridisation (Coghlan *et al*., 1985) and the nucleic acid physically entrapped in the cell debris has reduced accessibility to the probe. In contrast, under-treatment restricts probe access to its hybrid target within the tissue, generally due to the tissue fixation process. Therefore, many protocols include a post fixation step in the form of paraformaldehyde after the proteolytic digestion to stabilise the tissue structures (Brigatic *et al.*, 1983). Following a suggestion by Braissant and Wahli (1998), the proteinase K treatment was omitted from the procedure in this study, without any noticeable effect on the ISH signal. In fact the tissue integrity increased, which outweighed any benefits of the protease treatment. The paraformaldehyde post fixation step was maintained as it was not determinantal to the assay and could only strengthen the tissue preservation on the slide.

3.3.1.2: Second ISH step: Acetylation treatment

Pre-hybridisation treatment of the sections with acetic anhydride after proteinase K (0.25% acetic acid in 100 mM triethanolamine) for 20 min, followed by a dehydration of the tissue in a series of ethanol steps (35- 100%) was part of the original method, as employed by numerous past researchers. The acetylation treatment neutralizes any free amine groups and prevents electrostatic binding of the probe (Angerer and Angerer, 1981). During the course of assay development and on the advice of Braissant and Wahli (1998) carbethoxylation with DEPC was implemented as a replacement for acetylation and, as reported, was found to be less time consuming and just as effective. The frequently-employed serial ethanol dehydration, ranging from 70, 95 to 100% ethanol followed by air-drying prior to the hybridisation of the probe (Brahic *et al*, 1984) was also examined in this study.

Lawerence and Singer (1985) and Braissant and Wahli (1998) were sceptical about the need for this step, reporting a two or three fold increase in background when incorporating it into the ISH method. Fink (1987) also reported wrinkling effects on the tissue from the ethanol dehydration. Substitution of this step with salt washes (4 X SSC) was successful (Braissant and Wahli, 1998) and so was maintained throughout.

3.3.1.3: Third ISH step: Hybridisation of the nucleic acid probe
3.3.1.3.1: Choice of probes

There is not a single probe or label in ISH which is optimal for every requirement (Penschow *et al*, 1987). The historical pros and cons for using either RNA or DNA as probes are well documented. Single-stranded RNA probes (riboprobes) were chosen for this study as they offer the advantage that there is no reannealing in solution, they provide high efficiency, withstand higher stringency (Cox *et al.,* 1984; Panoskaltsis-Mrtari and Bucy, 1995) and have the advantage of producing an ideal control through the use of both sense and anti-sense strands from the same template. Additionally, antisense RNA probes do not contain vector sequences that can cause non-specific hybridisations as encountered with DNA probes (Angerer and Angerer, 1981), although RNA probes are more susceptible to degradation by nucleases than DNA, therefore requiring a more stringent working environment. In retrospect the use of real time quantitative reverse transcription polymerase chain reaction for probe construction may have allowed more probes to be prepared and tested and is a consideration in further studies (Burton *et al.*, 2011; Wang *et al*., 2010; Koltai and Bird, 2000).

3.3.1.3.2: Probe labelling

The type of labelling for ISH depends largely on the specificity, sensitivity, and resolution required. The labelling of either RNA or DNA probes for use in ISH assays can be achieved through many processes. These include enzymatic incorporation of either radioisotope, digoxigenin (DIG), biotin or fluorescein dUTP or UTP onto the nucleic acid by processes such as nick-translation or random primer methods which ultise the *E. coli* DNA polymerase I enzyme.   Other options use a terminal deoxynucleotide transferase or the use of synthetic olignucleotides where the label is incorporated during manufacture.

In this study, bacteriophage RNA polymerases were employed with DNA templates (Symons, 1986) to produce riboprobes (Figure 3.1). RNA polymerases are DNA-dependent RNA polymerases with strict specificity for their respective double stranded promoters. They catalyze the 5' to 3' synthesis of RNA on either single stranded or double stranded DNA downstream from their promoters and incorporate modified or labelled nucleotides during the reaction. One unit of the enzyme incorporates 1 nmol of AMP into a polynucleotide fraction in 60 min at 37^0C. RNA riboprobes made this way are considered the best choice for tissue sections (Cox *et al.,* 1984), as both control and test probes are the same length, having the same guanine (G) and cytosine (C)

content and DIG incorporation, which thus require identical hybridisation conditions. Such probes are also documented to have less background problems as they have only short flanking sequences (part of the multiple-cloning site of the plasmid vector) producing single-stranded RNA probes at about 10 μg for 1 μg of input DNA (Boenhringer-Mannheim, 1996).

The decision to use non-radioactive ISH labelling offers advantages over the use of radio-labelled probes such as;

a)   Stability of the probe

b)   Improved safety

c)   Reduced waste disposal problems

d)   Better morphology and spatial resolution of the enzyme reaction (Farquharson *et al.*, 1990; Panoskaltsis-Nrtari and Bucy, 1995).

e)   Ease of use

f)   Under optimal conditions, better sensitivity (Singer, 1986; Clavel, 1991), although this is not agreed by all researchers.

g)   Lower overall cost (Pinkel *et al.*, 1986; Schwarzacher *et al.*, 1989).

The type of non-radio label is a personal choice, influenced by expertise and familiarity within the laboratory situation. The most commonly used procedure has been to label with fluorochrome dyes that are illuminated by light of an appropriate wavelength, which is very favorable when double labelling is desirable. Disadvantages of fluorescence labelling include the fact that labels become unstable upon excitation, the signal fades quite dramatically and there can be background problems caused by non-specific binding of the probe and antibodies to cytoplasm and cell walls (particularly when several amplification steps are required (Brigati, *et al.*, 1988; Schwarzacher *et al.,* 1991). This is a major problem in barley, particularly when fixed using glutaraldehyde as in this study. The fluorochrome CY3, which is in the far red spectrum and can be visualised under the confocal microscope, can be used when auto-fluorescence is a problem and was considered in this study, but the cost and access to the confocal was limiting and would have hampered assay development.

The most commonly used non-radioactive haptens for ISH are biotin and digoxigenin. Digoxigenin-II-2' deoxy-uridine-5'-triphosphate (DIG-11-dUTP) was chosen in this study to label the RNA riboprobes. Digoxigenin (DIG) is a steroid hapten from *Digitalis vanata*, and the labelled and bound hybrids are detected using a digoxigenin-antibody interaction, as described in more detail in section 3.3.2.

3.3.1.3.3: Probe hydrolysis

The rate of renaturation of DNA in solution is proportional to the square root of the (single-stranded) fragment lengths; therefore longer probes should be better. But it appears that smaller probes are more successful for intracellular work (Brachic, 1984; Branhic and Haase, 1978, Angerer and Angerer, 1981; Gee and Roberts, 1983). Although authors such as Lawerence and Singer (1985) believe that hybridisation obtained with probe fragments below 1000 nucleotides is not as effective than that with those above, and variability in the data increases. This higher signal for longer probes is probably due to networking (Cox *et al*., 1984: Gee *et al.*, 1983, Godard *et al.*, 1980, Lawrence and Singer, 1985). Probe networking is where sections of the larger probes anneal to each other in a sandwich fashion, thus amplifying the signal and making them less quantitative, and this does not occur as readily with small probes (Lawerence and Singer, 1985). The likelihood of repetitive sequences is increased in longer probes and this can also give rise to additional unwanted signal (Leitch *et al.,* 1994).

Braissant and Wahli (1998) advised against the hydrolysis of probes, as the hydroloysis treatment can lead to elevated background signals. In the ISH developed here, both type of probes were assessed and no difference was noted between hydrolysed or un-hydrolysed riboprobes using the carbonate hydrolysis method (Table 3.2), over a size range of 1.6 to 0.2 kb, therefore all data presented in this study is from the use of un-hydrolysed riboprobes.

Table 3.2: Probe hydrolysis formula. $T^{\$}$; time in min, Lo; original length of probe transcript; Lf; desired length of probe transcript. Constant is 0.11

Alkaline hydrolysis	$T^{\$}$= Lo-Lf / 0.11.Lo.Lf
Carbonate Hydrolysis	160 μL H_20, 20 μl 0.6M Na_2CO_2, 20 μL 0.4 M NaCO, 60^0C in desired time. Reaction stopped; 1.6 μL glacial acid, 6.6 μL 3 M NaAC, 2 μL tRNA then precipated out of solution with 500 μL 100% EtOH overnight at -20^0C

3.3.1.3.4: Hybridisation

The concept and application of ISH was described in great detail in Chapter 1. More specific details of a successful ISH assay are outlined in this section.

The melting temperature (Tm) of the probe refers to the temperature at which the probe is annealed to 50% of its exact complement. Accurate estimation of the Tm of a probe is vital for successful application. Historically, several formulas have been developed to provide an estimate of probe Tm values with respect to use in ISH assays.

In the absence of a destabilizing agent such as urea or formamide, the denaturation Tm of the probe will depend on three major factors.

a)   The GC content of the probe sequence. The Tm will differ according to the percentage of G or C base pairs within the sequence of interest; a high percentage results in a higher melting temp (Guide to *in Situ*, 1994; Braissant and Wahli, 1998).

b)   The probe concentration, as high concentrations are more favourable for hybrid links than lower (see section 3.3.2.3.5).

c)   The salt concentration of the hybridization buffer. Monovalent cations (sodium ions) interact electrostatically with nucleic acids, so that the electrostatic repulsion between the two strands in a duplex decrease with increasing salt concentration (Bohringer-Mannheim, 1996), therefore a high salt concentration in the hybridisation buffer influences the hybridisation rate and stability of mismatched hybrids.

Table 3.3 details equations used to determine the optimal temperature to perform the *in situ* hybridization assay using specific probes, considering salt concentration, probe length and the addition of formamide. The formula is applicable to longer probe sequences, but has been documented as having limitations since it does not take into account the nearest neighbor interactions of the probe. A more detailed thermodynamic analysis (IDT) of the melting process could be employed if necessary but will not be presented here in great detail. It has also been suggested that the coefficient of 16.6 in the formula (Table 3.3) used to adjust the stringency effects on the Tm of longer probes is too high and more accurately should be set at 12 (SantaLucia *et al*., 1996).

By incorporating a helix destabilising reagent such as formamide, optimal conditions for annealing may be maintained at lower temperatures such as $37^{0}C$ instead of using higher calculated Tm temperatures that could be detrimental to tissue specimens. Figure 3.5 highlights how the temperature of the ISH assay can influence tissue morphology and structure and finally, preservation on the slide. As with all stages in this assay development, sequential temperature conditions were assessed with regard to the ISH signal, tissue integrity, preservation and laboratory convenience. It was very obvious early in this study that the barley grain, independent of fixation could not handle high ISH temperatures. Barley is one of those susceptible tissues composed of heat sensitive carbohydrates. Subsequently, formamide use reduced tissue denaturation (Cox *et al.,* 1984).

Figure 3.5: Example of Boehringer-Mannheim DIG labelled *in situ* hybridisation results under different temperature conditions. ISH incubation performed at A: 65^0C, B: 45^0C and C: 55^0C, all with the inclusion of formamide. (B) at 45^0C gave the best results with regard to tissue morphology, structure and preservation on the slide.
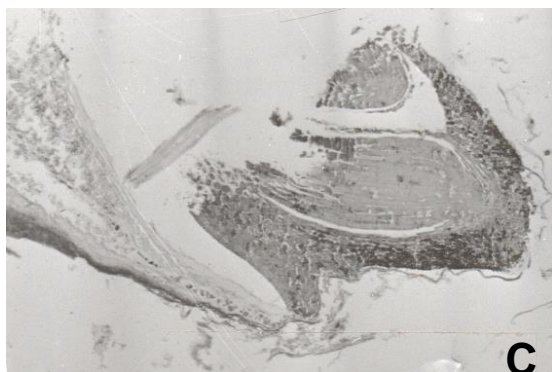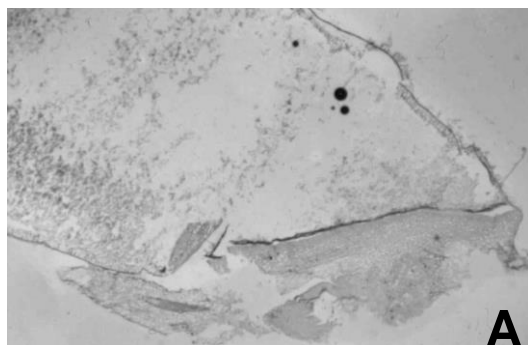
Table 3.3: Useful equations for calculating ISH temperature. Tm*, the temperature at which half the DNA is present in a single stranded (denatured) form. % GC[&], the molar percentage of guanine plus cytosine; salt concentration[^], between 0.01 and 0.20 M; (M), salt concentration (molar). stringency[@], where M_f, mismatch factor (1 for probes >150 bp, 5 for probes <20 bp);ta, temperature at which ISH mix or washing conditions used (Hybaid). Howley *et al.* (1979).

Tm* when considering the DNA GC[&] content and salt concentration[^] of the hybridization mix.	Tm* = 16.6 log (M)+ 0.41 (% GC) + 81.5
Tm* when also considering the addition of formamide	Tm* = 16.6 log (M) + 0.41 (%GC)+81.5-0.72 (% formamide)
Stringency	Stringency[@] (%) = 100-M_f(tm-ta)

The initial procedure used in this study involved adding the probe to the sections in a hybridisation buffer consisting of 4 X SSC, 1 X Denhardts, 9 µg/mL transfer (t) RNA, 10 mM DTT, 5% Dextran sulphate and 50% formamide and incubating overnight in the humid chamber at 45^0C. Subsequent modifications to this included the removal of the DTT, as this is really only needed for radiolabelled ISH assays using ^{35}S (Braissant and Wahli, 1998). Substitutions of the Denhardts and tRNA for salmon sperm DNA at a 100 fold concentration of the probe was used to avoid background problems (Braissant and Wahli, 1998), which substantially reduced the cost of reagents used in the assay and gave a cleaner more reproducible ISH assay. Most investigators use hybridization times ranging from one to four days (Venezky *et al.,* 1981; Brahic *et al.,* 1984; McFadden *et al.,* 1988; Panoskaltsis-Mrtari and Buy, 1995), but this depends on the probe label and detection method. The procedure developed here could produce a signal within 2 h and an advantage of using the alkaline phosphatase (AP) system was that the reaction could be carried out under a light microscope and stopped at the desired stage to avoid major background problems.

3.3.1.3.5: Probe concentration

A sequential procedure, similar to that employed by Braissant and Wahli (1998) was used to optimize the probe concentration on the test tissue. Braissant and Wahli (1998) tested increasing concentrations of riboprobes (100, 200, 400, 800 ng/mL and 2 µg/mL) on paraffin embedded tissue sections.

The best specific versus unspecific signal ratio was at 400 µg/mL. This equates to a probe range of 5 to 10 ng RNA (Boehringer Mannheim). The hybridisation signal in an ISH assay can increase as a function of probe concentration up to a saturation point, above which only the background signal increases.

Figure 3.6: Diagram of the DIG labelled RNA and alkaline phosphatase (AP) detection system.
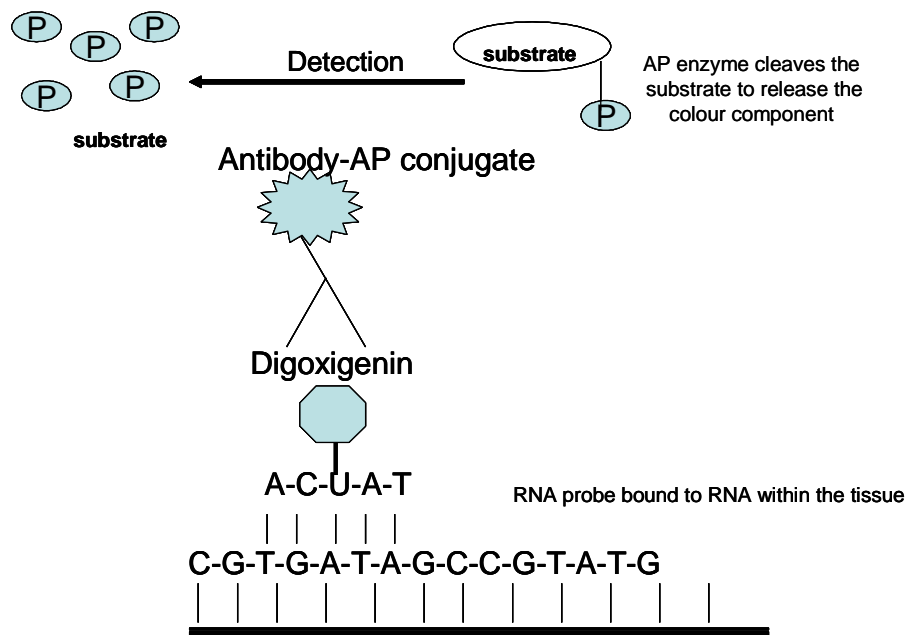
In sea urchin embryo sections the saturation point is around 0.3 ng probe/uL hybridization solution/kb (Cox *et al*., 1986; Jackson, 1991). The optimal concentration to use with the DIG AP detection probes on barley tissue was determined to be 0.3 ng/uL probe as deduced from experimental trials.

3.3.2: Post hybridisation (detection of the attached probe)

The bound RNA-riboprobe complex was detected within the tissue by indirect labelling, where the site of hybridization is indicated by a secondary reaction, in this case immunologically (Landegent *et al.,* 1985). The AP immunological detection system used in this study was chosen for its stability and colour complementation to barley tissue, although the detection systems of horseradish peroxidase (HRP), or β-glucosidase enzyme could also easily have been chosen. The reaction is schematically shown in figure 3.6.

3.4: Conclusions

It took a methodical assessment of the ISH process to develop a highly sensitive assay compatible with germinated barely grain tissue. Options were considered from the stage of pre-fixation through to the sealing of the final product on the slide. Due to this careful process, a robust, reliable, and valuable assay has been developed. The paraffin-embedded grain had many advantages, one in particular being the ability to take sequential sections for the analysis of the grain's modification state followed by the expression of many of the key enzymes in barley germination. It is this work that will be presented in the following chapters.

Chapter 4

Identification and location of specific (1,3;1,4)-β -glucanase isoenzyme mRNA and protein in germinating *Hordeum vulgare* L. grain

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4.1: Introduction

The breakdown of cell walls, predominantly in the starchy endosperm, is a significant event in barley grain germination and has been the subject of numerous studies (MacLeod *et al*., 1964; Gibbons, 1981; 1980; Briggs and MacDonald, 1983; MacGregor *et al*., 1984; Ranki, 1984; McFadden *et al.,* 1988a; Slakeski *et al*., 1990; Wolf, 1992; Freeman and Palmer, 1984; Palmer, 1989; Hrmova and Fincher, 2001; Jin *et al*., 2004; Kuntz *et al*., 2007; Faucomier *et al*., 2011). Living tissue (aleurone and scutellum) adjacent to the dead starchy endosperm is known to contribute to the production and secretion of enzymes involved in this degradation. As discussed in previous chapters, there is debate as to the importance of each tissue for enzyme expression and secretion during the various stages of germination. Until the work of McFadden *et al*. (1988a) only circumstantial evidence existed that enzymes were produced from the scutellum during grain germination (Figure 4.1).

There are several enzymes that act on the (1,3;1,4)-β-glucans of the cell walls (Figure 4.2), namely endohydrolases, β-D-glucan glucohydrolase and possibly β-D-glucosidases (Fincher, 1986; 1989; Leach *et al*., 1995; Hrmova and Fincher, 1998; Hrmova, 1998). The combined action of these enzymes leads to the specific conversion of (1,3;1,4)-β-glucan to glucose, an important energy source for the developing seedling (Fincher, 1989). Up to 18.5% of the total glucose released during endosperm mobilisation in barley has been attributed to (1,3;1,4)-β-glucan degradation (Morrall and Briggs, 1978). There are two (1,3;1,4)-β-glucan endohydrolases (EC 3.2.1.73), termed (1,3;1,4)-β-glucanase EI isoenzyme (referred to as EI) and (1,3;1,4)-β-glucan glucanase EII isoenzyme (referred to as EII). Originally described by Woodward and Fincher (1982a, b), the genes encoding EI and EII were subsequently isolated and characterised by Fincher (1987); Loi *et al*. (1988); Litts *et al*. (1990); Slakeski *et al*. (1990) and Wolf (1991). The (1,3;1,4)-β-glucanase isoenzymes are known to be encoded by separate genes (Fincher, 1987; Loi *et al*., 1988; Skakeski *et al*., 1990), which probably arose by duplication of a common ancestral gene (Woodward *et al*., 1982). EI is encoded by the G*Ib* 1 gene, located on chromosome 5(1H) (Skakeski and Fincher, 1992a), while EII is encoded by G*Ib* 2 gene, loacted on chromosome 1 (7H) (Loi *et al*., 1988).

The two (1,3;1,4)-β-glucanase isoenzyme proteins display identical enzyme substrate specificity and share similar kinetic properties (Woodward and Fincher, 1982a, b), but have different molecular weights as shown by SDS PAGE studies, with EI at

Figure 4.1: Location of mRNAs encoding (1,3;1,4)-β-glucanase in germinating barley grains detected using a ^{32}P labelled cDNA probe. Location of mRNA encoding (1,3;1,4)-β-glucanase in germinating barley grains as shown by McFadden *et al*. (1988a). Near-median longitudinal sections counter-stained with toluidine blue representing stages of germination are presented in upper panels (a: Completion of imbibition (24 h), b: 2 days from start of imbibition, c: 3 days from start of imbibition, d: 5 days from start of imbibition). Bound ^{32}P labelled cDNA probe encoding (1,3;1,4)-β-glucanase as detected by X-ray film autoradiography is shown in the lower panels e: Completion of imbibition (24 h), f: 2 days from start of imbibition, g: 3 days from start of imbibition, h: 5 days from start of imbibition. em, embryo; sc, scutellum, en, endosperm. Black arrows indicate area of probe labelling, open arrow indicates labelling of aleurone in the embryo (germ aleurone). Boxed sections indicate areas of interest discussed in the published paper.

28 and EII at 30 kDa (Stuart *et al*., 1987), along with different carbohydrate contents and thermal stabilities (Woodward and Fincher, 1982a; Loi *et al.,* 1987).

Figure 4.2: (1,3;1,4)-β-glucan hydrolysis. Enzymatic hydrolysis of cell wall (1,3;1,4)-β -glucan, with accessibility hindered by arabinoxylans (this diagram is adapted by Hrmova *et al.,* 2001; taken from Jamar *et al.,* 2011).

Northern and Western blot analyses demonstrate that EI mRNA transcripts and protein appear to be more abundant in the scutellum early in germination, while both isoenzymes can be found in the aleurone layer later in germination (Mundy *et al.,* 1985a; Stuart *et al.,* 1986; Slakeski and Fincher, 1992a; George and Kiraemer *et al*., 2004). The sites of (1,3;1,4)-β-glucanase mRNA gene expression in germinated barley grains were confirmed using *in situ* hybridisation by McFadden *et al*. (1988a) as initiating in the scutellum of the grain, but transferring to the aleurone later in germination, as shown in Figure 4.1. However, the inability to distinguish between the two (1,3;1,4)-β-glucanase isoenzymes was a limitation to this study. When full-length cDNAs for both isoenzymes were subsequently isolated (Fincher *et al*., 1986; Slakeski *et al*., 1990), it was noted that the nucleotide sequences were 92% identical over the coding regions of the two genes (Figure 4.3a). However, the 3' untranslated regions of the genes showed considerable sequence divergence (Slakeski *et al*., 1990), thus allowing the preparation of probes from the two (1,3;1,4)-β-glucanase isoenzymes that would enable identification of the individual isoenzymes (Slakeski *et al.,* 1990). In addition, specific

monoclonal antibodies have been raised against the two (1,3;1,4)-β-glucanase isoenzymes from barley (Høj *et al*., 1990) and these antibodies have been shown by Western blot analysis and ELISA to be specific for each isoenzyme (Høj *et al*., 1990; Gibson *et al*., 1997, Figure 4.3b).

Although many biochemical and molecular biological aspects of the expression of the (1,3;1,4)-β-glucanase isoenzyme genes have been reported, at the time of this study no detailed observations had been made on the progress of individual isoenzyme gene expression during germination. Only from *in vivo* studies has there been a suggestion that the two genes are expressed at different rates in different tissues (Fincher, 1987; Slakeski and Fincher, 1992b). The study presented in this chapter further explores the expression of the two glucanase isoenzymes during barley germination in an attempt to address two main questions. Firstly, is there tissue specificity for isoenzyme expression during germination? Secondly, is there a temporal pattern of expression unique to each isoenzyme? At an experimental level *in situ* hybridisation was used to locate the mRNA, whilst immunostaining was used to confirm the presence of the protein in a series of barley grain sections collected through germination.

Figure 4.3a: Nucleotide sequence of the cDNAs encoding barley (1,3;1,4)-β-glucanase isoenzymes EI and EII. EI begins at nucleotide 1 (NCBI accession number M62740; Wolf, 1991; 1450 total base pair total length). EII begins at nucleotide 51 (GenBank/EMBL accession number X56260; Slakeski *et al.,* 1990, 1303 base pair total length). Blue and green blocks indicate designed primers as per table 4.1.

```
EI     51  cacccacaccctcaccctccaacgcagctagagagagaaagagaatggca 100
                                          ||  |    ||||||
EII     1  ..............................gagtttcgagacccaatggcg 21
               .         .         .         .         .
EI    101  ggccaaggcgttgcctccatgttggctctggcattgctcctcggagcctt 150
           ||||||||||||||||||||||||||  ||||  |||||||||  |||||||||||
EII    22  agccaaggcgttgcctccatgttcactctcgcattgcttctcggagcctt 71
               .         .         .         .         .
EI    151  cgcctccatcccacaaagtgtggagtccatcggggtgtgctacggcatga 200
           |||  ||  ||||||| |  |  |||||||   ||||||||||||||||||||
EII    72  cgcgtctatcccaccaggcgtggagtcgatcggggtgtgctacggcatga 121
               .         .         .         .         .
EI    201  gcgccaacaatctgccggcggcgagcaccgtggtcaacatgttcaagtcc 250
           ||||||||||||  |||||||||||||||||||||  ||  |  ||||||||||||||
EII   122  gcgccaacaacctgccggcggcgagcaccgtcgtgagcatgttcaagtcc 171
               .         .         .         .         .
EII   572  ttgcccgcaccaacgcgccgctcatggccaacatttacccgtacctggcc 621
               .         .         .         .         .
EI    701  tgggcctacaacccgagcgccatggacatgagctacgcgctcttcaccgc 750
           |||||  |||||||||||||||||||||||||||  ||||||||  |||||||||  |||
EII   622  tgggcttacaacccgagcgccatggacatgggctacgctctcttcaacgc 671
               .         .         .         .         .
EI    751  ctccggcaccgtggtccaggacggctcctacgggtaccagaacctgttcg 800
            |||||||||||||||  |||||||  |||||||||||||||||||||||||
EII   672  gtccggcaccgtggtcagggacggcgcctacgggtaccagaacctgttcg 721
               .         .         .         .         .
EI    801  acaccaccgtggacgccttctacacggccatggccaagcacggcggctcc 850
           |||||||||||||||||||||||||||||||||||  |||||||||||||||||||||
EII   822  ggcggcgactccggccaacgctaggttctacaaccagcacctcatcaacc 871
               .         .         .         .         .
EI    951  acgtcgggcgcggcaccccccgccacccgggcgccatcgagacctacgtc 1000
           |||||||||||||||||||||  ||||||||||||||||||||||||||||||  ||
EII   872  acgtcgggcgcggcaccccacgccacccgggcgccatcgagacctacatc 921
               .         .         .         .         .
EI   1001  ttctccatgttcaacgagaaccagaaggacaacggcgtggagcagaactg 1050
           |||  ||||||||||||||||||||||||||||  ||||||||||||||||||||||||
EII   922  ttcgccatgttcaacgagaaccagaaggacagcggcgtggagcagaactg 971
               .         .         .         .         .
EI   1051  ggggctcttctaccccaacatgcagcacgtctaccccatcagcttctgat 1100
           |||  ||||||||||||||||||||||||||||||||||||||||||  |||||||
EII   972  gggactcttctaccccaacatgcagcacgtctaccccatcaacttctgac 1021
               .         .         .         .         .
EI   1101  gaggtagcagcta|cctagtgcccgtatgtccgtacgtac|gcgcgcgcgta 1150
                         Oli 17 →
            |   |      |||    ||    |||    |      ||  |  |     ||
EII  1022  ggagctcgtgctcgttaag|tccctact|.......|tgttcttgttaacg|ag 1064
                              Oli 18 →
EI   1151  taagagcgtgtacgccgtacgtatgcgcacattatgtattgtacaggct 1200
            |||  |      |     ||      ||     |   |   |    |||  |  ||
EII  1065  taaaaagtcatgttacgcgaacttgacgagctactcgtttggagagcctc 1114
               .         .         .         .         .
EI   1201  tgggttgggaacttgggatgcgaccgctgaggcagctcagatcgtacgcg 1250
           |    ||     ||       |    |       |    | | |  |||  ||||  | |
EII  1115  ttaattacctcct.......ctttccacatgagggatgagaacgtatgag 1157
               .         .         .         .         .
```

```
EI   1251 agtagtggcttgctatactagtgtaccagtacgtatgattttcgatggaa 1300
               |    |    |   | || |||   | |      |||    |||||
EII  1158 ttaataaccagaccccattactgt....gaattccccattgagattggaa 1203
                    .           .         .         .         .
EI   1301 gggaagcatatgcaaacgctcccccttcctcgattgatcatgcacttgat 1350
               ||||       |  |   | |   || ||||       |
EII  1204 tacttatgtatgtgttgtataacttgtattgacgtggtcataacgaagca 1253
                    .           .         .         .         .
EI   1351 acgtacacgcatgtgtgcgtacctaggaactatattgtagggttcaaaat 1400
               ||    |   | | | |  ||  | | | |
EII  1254 accgtaaatcttttttagttcctt                            1303
                    .           .         .         .         .
EI   1401 ttcgtcaaaacttgacgaaatttgtccaaaataataaagtatgaaatata 1450
                    .           .         .         .         .
EI   1451 tcggcgaagaccgaatattcctatatataatttt. 1484
               ← EI3AS
```

Figure 4.3b: Amino acid sequence of barley (1,3;1,4)-β glucanase isoenzymes EI and EII.

```
EI     1 MAGQGVASMLALALLLGAFASIPQSVESIGVCYGMSANNLPAASTVVNMF 50
           || ||||||  |||||||||||| |||||||||||||||||||||||.||
EII    1 MASQGVASMFTLALLLGAFASIPPSVESIGVCYGMSANNLPAASTVVSMF 50
                   .           .         .         .         .
EI    51 KSNGINSMRLYAPDQAALQAVGGTGVNVVVGAPNDVLSNLAASPAAAASW 100
           ||||| |||||||.||||||||||:|||||||||||||||||||||||||
EII   51 KSNGIKSMRLYAPNQAALQAVGGTGINVVVGAPNDVLSNLAASPAAAASW 100
                   .           .         .         .         .
EI   101 VRSNIQAYPKVSFRYVCVGNEVAGGATQNLVPAMKNVQGALASAGLGHIK 150
           |:||||||||||||||||||||||||.|||||||| ||| .|||||||
EII  101 VKSNIQAYPKVSFRYVCVGNEVAGGATRNLVPAMKNVHGALVAAGLGHIK 150
                   .           .         .         .         .
EI   151 VTTSVSQAILGVYSPPSAGSFTGEADAFMGPVVQFLARTGAPLMANIYPY 200
           ||||||||||||:|||||||||||| ||||||||||||| ||||||||||
EII  151 VTTSVSQAILGVFSPPSAGSFTGEAAAFMGPVVQFLARTNAPLMANIYPY 200
                   .           .         .         .         .
EI   201 LAWAYNPSAMDMSYALFTASGTVVQDGSYGYQNLFDTTVDAFYTAMAKHG 250
           |||||||||||| |||| ||||||.||.|||||||||||||||| |||
EII  201 LAWAYNPSAMDMGYALFNASGTVVRDGAYGYQNLFDTTVDAFYTAMGKHG 250
                   .           .         .         .         .
EI   251 GSNVKLVVSESGWPSAGGTAATPANARIYNQYLINHVGRGTPRHPGAIET 300
           ||.||||||||||| |||||||||| |||:||||||||||||||||||||
EII  251 GSSVKLVVSESGWPSGGGTAATPANARFYNQHLINHVGRGTPRHPGAIET 300
                   .           .         .
EI   301 YVFSMFNENQKDNGVEQNWGLFYPNMQHVYPISF 334
           |:|.|||||||.||||||||||||||||||||.|
EII  301 YIFAMFNENQKDSGVEQNWGLFYPNMQHVYPINF 334
```

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4.2: Materials and Methods

4.2.1: Barley grain samples.

The barley (*Hordeum vulgare* L.) varieties Sloop and Himalaya were used in this study. Section 2.2.2 in Chapter 2 details the qualities of each variety.

4.2.2: Tissue preservation

The schedule of barley grain germination used in this study is outlined in section 2.2.3 of Chapter 2. All results presented in this chapter are from grain sections fixed in 4% paraformaldehyde and 0.25% glutaraldehyde, embedded in paraffin and microtome sectioned, as described in Chapter 2, sections 2.2.4; 3.2.5 and 2.2.6.

4.2.3: Modification pattern

Longitudinal sections of barley grain collected at set time points during germination were stained with the calcofluor white (CW) dye, as described in section 2.2.7 of Chapter 2. The degree of (1,3;1,4)-β-glucan degradation, as indicated by the CW dye under UV light, determined the grain's modification stage. As described in section 2.2.3, from a total of 12 collection points over the germination schedule for each variety the grain was placed into five modification stages, M1 to M5.

4.2.4: Riboprobe preparation

Single-stranded DIG-labelled RNA riboprobes were used to detect the presence of specific mRNAs in longitudinal barley sections, as previously described in section 3.2.5 of Chapter 3. Dependent on the orientation of the DNA within the cloning vector, either sense strands (control) or anti-sense strands (positive) can be transcribed and labelled as outlined in section 3.2.5 of Chapter 3.

Since there is 92% nucleotide identity between the coding regions of EI and EII, cDNA sequences of the two (1,3;1,4)-β-glucanase isoenzymes from the variable 3' untranslated regions were prepared for riboprobe transcription (Figure 4.3, NCBI BLAST; Slakeski *et al*., 1990). To prepare EI DIG labelled riboprobes, cDNA encoding the 3' untranslated region of EI was sub-cloned using *Eco*R1 and *Bam*H1 restriction enzymes from a pUC19 (Genesis Bioventures) clone originally made by Professor G. Fincher (1986, at the Sainsbury Laboratory, Norwich, UK) into pBluescript SK+ (Stratagene), to ensure optimal riboprobe transcription. This construct was transformed into competent *Escherichia coli* XL1-Blue (Stratagene) cells and plasmid DNA was isolated as described in section 3.2.3. For sense riboprobe production plasmid constructs were linearised with the restriction enzyme *Eco*RI (NEB) and transcribed using T7 RNA polymerase (Boehringer-Mannheim), or cut with *Bam*HI (NEB) and transcribed with T3

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RNA polymerase (Boehringer-Mannheim) for antisense probes. The final EI-DIG-labelled riboprobes were 400 nucleotides in length.

The 3' untranslated region of EII cDNA was isolated by PCR from the original EII cDNA contained in the pUC19 vector (Fincher, 1986). The PCR primers Oli 18 (5'-TCCCTACTTGTTCTTGTTAACG -3'; Fincher, 1987, Figure 4.3a) and reverse primer (Geneworks, 5'-TGACCGGCAGCAAAATG -3') at 0.5 μL each (1-0.1 μg. μL^{-1}) were added to EII mini-prep plasmid DNA (1 μl) with 2.5 μL 10 X PCR buffer (Invitrogen); 1.5 μL 250 mM MgCl$_2$ (10 X, Sigma); 1 μL 5 mM dNTPs (Promega); 1 μL Taq DNA polymerase (Invitrogen) and deoxyribonuclease free water to a final reaction volume of 25 μL. Reactions were performed in a Perkin-Elmer Cetus DNA Thermal Cycler as follows; 94^{0}C, 2 min initial denaturation; 35 cycles of denaturation at 94^{0}C, 40 s, annealing at a primer specific temperature of 50^{0}C, 40 s, extension at 72^{0}C, 1 min; followed by a 2 min final extension at 72^{0}C.

The PCR products were excised from a 1.2% (w/v) agarose gel (DNA grade, FMC BioProducts) in 1 X TAE buffer (40 mM Tris, 1 mM EDTA, pH 8.0 adjusted with acetic acid) containing 0.1 μg.mL^{-1} ethidium bromide (Sambrook *et al*., 1989), purified using Geneclean (Bresatec, Adelaide, Australia), then sub-cloned into pBluescript SK+. Subsequently, a modified 3' untranslated EII isoenzyme cDNA clone was made by shortening the original clone to remove the 23 bp poly A tail at the 3' end, thus reducing the potential for non-specific background binding. The ends of the coding region were modified during the PCR to create suitable restriction sites with the primer T3 (Stratagene) 5'-AATTAACCCTCACTAAAGGG-3' and EII3AS (Geneworks), 5'-GGGTCTGGTTGAATTCTCATACGTTCTC-3', incorporating an *Eco*R1 restriction site. The PCR reagents were as described above with the PCR reaction as follows; 94^{0}C, 2 min initial denaturation; 35 cycles of denaturation at 94^{0}C, 40 s, annealing at a specific primer temperature of 48^{0}C, 40 s, extension at 72^{0}C, 1 min; followed by a 2 min final extension at 72^{0}C.

The PCR products were purified with a PCR clean-up kit (UltraClean, MoBio Laboratories), ligated into a pGEM T vector (TEASY, Promega), where single 3' T nucleotide overhangs improve the ligation rate and then sub-cloned into pBluescript SK+ using *Eco*RI and *Bam*HI sites and DNA was isolated as described previously (section 4.2.3). For sense riboprobe production plasmid constructs were linearised with the restriction enzyme *Eco*RI and transcribed using T7 RNA polymerase (Boehringer-Mannheim) or *Bam*HI and T3 RNA polymerase (Boehringer-Mannheim) for antisense probes. The final EII DIG labelled riboprobe product was 230 nucleotides long.

Table 4.1: Sequences of primers used in RT-PCR reactions
(refer back to Figure 4.3a). [#]10 X (250 mM) MgCl per PCR reaction; bp is base pairs; Tm, annealing temperature for the PCR reaction; fw, forward primer; rev, reverse primer. 3' nucleotide primers are complementary to the cDNA sequence.

5' nucleotide primer (5' to 3')	3' nucleotide primer (5' to 3')	Primer specific Tm	PCR product size
Oli 17: CCTAGTGCCCGTA TGTCCGTACGTAC	EI3AS: GAATATTCG GTCTTCGCCGA	[#]51^0C	360 bp
Actin rev: CGACACGGAGC TCATATAGAA	Actin fw: GTCTTTCCCAG CATTGTAGG	53^0C	197 bp
Oli 18 TCCCTACTTGTT CTTGTTAACG	Reverse primer: TGACCGGCA GCAAAATG	50^0C	90 bp
GAPDH rev: GCCTTAGCATC AAAGATGCTGG	GAPDH fw: CCACCGGTGTCTT CACTGACAAGG	65^0C	550 bp

The total glucanase riboprobe (ET) was included to validate the method. This is essentially a repeat of previous experiments carried out by McFadden *et al*. (1988a) but using a novel method. These riboprobes were obtained from the coding region of the (1,3;1,4)-β-glucanase EII isoenzyme cDNA (Fincher *et al.,* 1986) contained in pUC19, and subsequently sub-cloned into pBluescript SK+ using the restriction enzymes *Eco*RI and *Bam*HI. For sense riboprobe production plasmid constructs were linearised with the restriction enzyme *Eco*RI and transcribed using T7 RNA polymerase (Boehringer-Mannheim) or *Bam*HI and T3 RNA polymerase (Boehringer-Mannheim) for antisense probes. The total glucanase DIG-labelled riboprobes were 1,200 nucleotides long and contained both the coding region and 3' UTR of EII, which thus possessed 92% identity to EI.

All inserts used as templates in riboprobe transcription were sequenced to verify the insert as described in section 4.2.6. All sequences were subsequently checked on an advanced NCBI BLAST (version BLASTN 2.2.3) search (Altschul *et al*., 1996).

4.2.5: In situ hybridisation assay on sectioned barley grain
The *in situ* hybridisation procedure as developed and outlined in section 3.2.5 of Chapter 3 was used with the three (1,3;1,4)-β-glucanase riboprobes; EI, EII and ET.

4.2.6: Total RNA isolation from whole barley grain
Whole grain with attached coleoptiles and roots were sampled at intervals throughout germination at 32, 56 and 96 h from the start of steeping. This material was immediately frozen in liquid N_2 for long-term storage at -80^0C.

For assaying, frozen whole barley seedlings were immersed in RNA Later (Ambion, USA) for several minutes to soften the grain and reduce RNA degradation, prior to being ground to a fine paste in 1 mL TRIzol reagent (Life Technologies) using a mortar and pestle, previously baked at 180^0C for 4 h to eliminate ribonuclease contamination. The starchy endosperm from the Himalaya grain was squeezed out from the husk and discarded prior to seedling storage in RNA Later to reduce starch contamination. The mixture was transferred to a 2 mL Eppendorf tube and allowed to stand at room temperature (22-25^{0}C) for 10 min prior to centrifugation at 12,000 g for 15 min at 4^0C. Supernatants were removed and RNA was isolated by phase separation with the addition of 200 μL of chloroform, mixed and centrifuged at 12,000 g for 15 min at 4^0C. After centrifugation, the upper aqueous phase containing the total RNA was removed and RNA precipitated by the addition of 0.25 mL isopropanol and 0.25 mL 1.2 M sodium chloride. Following centrifugation at 12,000 g for 10 min at 4^0C, the RNA pellet was

washed with 1 mL 75% (v/v) ethanol, centrifuged at 7,500 g for 5 min at 4^0C and air dried. RNA samples were resuspended in 30 μL ribonuclease free water, vortex mixed and incubated at 65^0C for 5 min. Centrifugation at 12,000 g for 15 sec separated the RNA solution from the pellet of insoluble starch and other polysaccharide material. The purity of the isolated RNA was quantitated spectrophotometrically by determining the A260/280-absorbance ratio (Sambrook *et al.,* 1989). An A260/280 absorbance ratio of 2.0 indicated that the RNA preparation was of high purity (Sambrook *et al.,* 1989). Aliquots of the RNA sample were also run on a non-denaturing 1.2% (w/v) agrose gel (DNA grade, FMC BioProducts) in 1 X TAE buffer (40 mM Tris, 1 mM EDTA, pH 8.0, adjusted with acetic acid) containing 0.1 μg.mL^{-1} ethidium bromide (Sambrook *et al.*, 1989) to confirm the presence of the highly expressed ribosomal RNA bands, 28s and 18s.

4.2.7: Complementary DNA synthesis and RT-PCR

To complement the *in situ* and immunostaining results described in this chapter, reverse transcription Polymerase Chain Reaction (RT-PCR) was employed to screen germinated barley grain samples for the presence of specific (1,3;1,4)-β-glucanase isoenzyme mRNA. The term reverse transcription refers to the purified mRNA being transcribed into single stranded cDNA, which acts as a template for the Taq polymerase during PCR. The combination of reverse transcription (RT) and PCR is RT-PCR.

First strand cDNA from the germinated whole grain RNA samples was made using the Thermoscript RT-PCR system (Invitrogen) as described by the manufacturer. Total RNA (approx. 2 μg) and oligo (dT) primer (0.5 μg; 5'-GACTCGAGTCGACATCGAT$_{17}$-3'; Invitrogen Life Technologies) were annealed in a 12 μL volume by denaturation at 65^0C for 5 min followed by snap cooling on ice. To begin cDNA synthesis further reagents were added, consisting of 4 μL of 5 X first strand buffer (125 mM Tris-HCl buffer pH 8.3; 100 mM KCl; 40 mM MgCl$_2$; 4 mM sodium pyrophosphatase; 1 μL 0.1 M DL-dithiothreitol; 2 μL 10 mM dNTPs (Invitrogen Life Technologies); 1 μL RNaseIN (40 units, ribonuclease H inhibitor, Promega); 0.5 μL ThermoScript reverse transcriptase (RT**,** 200 units, Invitrogen Life Technologies); and 1.5 μL ribonuclease-free water, and incubated at 55^0C for 60 min. On completion of first strand synthesis, the RNA template was digested by incubation with ribonuclease H (RNase H, 2 U/μL, Invitrogen Life Technologies) at 37^0C for 20 min, with a final addition of 30 μL sterile water.

PCR reactions were performed to detect the presence of the relevant cDNAs encoding either EI or EII with primers designed to the 3' UTRs as listed in Table 4.1 (all manufactured by Geneworks, Adelaide, South Australia, except for Oli 18 and Oli 17 which were supplied by Professor G. Fincher, University of Adelaide, Waite Campus). The cDNAs encoding β-actin and the constitutive glycolytic pathway enzyme glyceraldehyde 3-phosphate dehydrogenase (GAPDH, accession no. U36650) from barley were used as internal controls for the PCR reactions. The negative control included all reagents except the first strand cDNA population while the positive control for these PCR reactions included either EI or EII isoenzyme mini-prep plasmid DNA as used for riboprobe production in section 4.2.3. PCR reactions consisted of the following reagents; cDNA (3 μL); 2.5 μL 10 X PCR buffer, 1.5 μL 25 mM $MgCl_2$; 1 μL 5 mM dNTPs; 0.5 μL each of 0.1-1.0 μg. $μL^{-1}$ primer; 1 μL Taq DNA polymerase; deoxyribonuclease free water to a final reaction volume of 25 μL. Reactions were performed in a Perkin-ElmerCetus DNA Thermal Cycler; 94^0C 2 min initial denaturation; 35 cycles of denaturation at 94^0C, 40 sec annealing at primer specific temperature, 40 sec extension at 72^0C, 1 min; followed by 2 min final extension at 72^0C. Amplified products were detected by electrophoresis at 100 V on 1.2 % (w/v) agarose gels in 1 X TAE buffer containing 0.1 μg.mL^{-1} ethidium bromide (Amresco) (Sambrook *et al*. 1989).

4.2.8: (1,3;1,4)-β -glucanase monoclonal antibodies

Monoclonal antibodies specific to EI and EII were produced in the laboratories of Professor N.J. Hoogenraad, Latrobe University, Melbourne, Australia. The specificity of EI and EII monoclonal antibodies has been documented (Høj *et al*., 1990; Edmunds *et al*., 1994; Gibson *et al*., 1997).

4.2.8.1: Western analysis of (1,3;1,4)-β -glucanase monoclonal antibodies.

Sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed followed by Western blot immunostaining to document specific (1,3;1,4)-β-glucanase isoenzyme protein expression in the varieties selected for this study. Grains were germinated under the schedule described in section 2.2.3 and collected at 32, 56 and 96 h from the start of steeping. According to a modified method of Lai *et al.* (1993) 28 grains per time point were ground to a paste in 28 mL of cold extraction buffer using a mortar and pestle. The extraction buffer included; 100 mM sodium acetate pH 5.0; 10 mM sodium azide; 10 mM EDTA; 3 mM 2-mercaptoethanol (14.3 M); and 3 mM phenylmethylsulphonyl fluoride. The extract was kept on ice for 30 min then centrifuged at 4000 rpm for 20 min at 4^0C. The supernatant of the extracts (10 μL) and

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pure EI and EII protein (1 μg/10 μL kindly provided by Prof Maria Hrmova, School of Agriculture, Food and Wine, Waite Campus, University of Adelaide) were heated to 95^0C for 5 min with 5 μL loading buffer (10 mM EDTA pH 8.0; 1% methionine; 0.5 M sucrose; 0.05% bromophenol blue; 0.1 M DL-dithiothreitol and 1% (w/v) sodium dodecyl-sulphate, SDS, pH 8.8). This sample was loaded onto a pre-cast 4-20% acrylamide gel (1 mm in diameter), and run in SDS-running buffer (25 mM Tris-HCl; 0.192 M glycine; 0.1 % w/v) SDS, pH 8.3) at 120 volts in a Bio-Rad gel system. Protein markers (10 μL, Bio-Rad) were loaded in the first wells of each gel comprising phosphorylase β (Mr 95,000), BSA (Mr 68,000), ovalbumin (Mr 43,000), carbonic anhydrase (Mr 30,000), trypsin inhibitor (Mr 20,000), and alpha-lactalbumin (Mr 14,400).

4.2.8.2: Transblot of (1,3;1,4)-β-glucanase SDS-PAGE

The protein gel was transferred to nitrocellulose using a Bio-Rad transblot system. The sandwich in the transblot cassette was as follows: supplied foam, 3 MM Whatman filter paper; protein gel, Hybond^{TM}-C nitrocellulose membrane (Amersham); 3 MM paper; supplied foam, assembled from the negative (cathode) to positive (anode) electrodes. The transblot buffer consisted of 3 M Tris, 14.5 M lysine and 2% methanol, run at 100 v for 1 h with a cooling block. On transfer completion the protein-imprinted nitrocellulose filters were blocked in a PBS solution containing 5% bovine serum albumin for several hours at room temperature. Gels that were preserved for loading level determination were not transferred to nitrocellulose and were instead stained with the red dye, Ponceau S.

4.2.8.3: Antibody staining of the transblot

The nitrocellulose filters were taken from the blocking solution, washed in PBS, and incubated overnight at 4^0C in PBS containing 1% Tween 20 and an antibody at the pre-determined concentration of 1:30 for the EI or 1:50 for the EII monoclonal antibody. Serial dilutions of each monoclonal were performed to determine the optimal working concentration and were found to be consistent with the published literature (Høj et al., 1990; Gibson et al., 1997). Excess primary monoclonal antibody was removed by washing with agitation in PBS containing 1% Tween 20 for two 15 min intervals. Filters were incubated at room temperature for 2 h in PBS containing 1% Tween 20 and secondary alkaline phosphatase conjugated goat anti-mouse IgG at a dilution of 1/1000 according to the manufacturer's instructions. On completion, filters were washed with agitation in PBS containing 1% Tween 20 for two 15 min intervals. Supersignal® chemiluminescence substrate for Western blots (Pierce, Rockford, IL USA) was used to

detect the secondary antibody. Western membranes were placed in a luminol mix (15 ml luminol solution and 150 μL p-coumaric acid) for 1 min, wrapped in cling wrap and placed against a x-ray film for an optimised time (1 min) in the dark room. Lumiol solution contained 1.2 mM lumiol (5-amino-2, 3-dihydro-1.4-phthalazinedione in H_2O_2 /Tris-HCl pH 8.6 while the p-coumaric acid solution consisted of 7 mM p-coumaric acid in dimethyl sulphoxide (DMSO).

4.2.9: (1,3;1,4)-β -glucanase immunostaining on sectioned barley grain

The development of a reproducible immunostaining method with the glucanase monoclonal antibodies on sections of germinated barley grain involved assessment of different fixation regimes as described in section 2.2.4 of Chapter 2. Various fixatives, as listed in Table 2.3 of Chapter 2, were assessed in the development of a robust barley grain immunoassay. Grain fixed in 0.25% glutaraldehyde and 4% paraformaldehyde, paraffin embedded and sectioned with adhesive tape as described for the *in situ* hybridisation assay, was also found to be ideal for the immunostaining study. As discussed in Chapter 2, the glutaraldehyde/paraformaldehyde fixation was found to be the best for preserving whole grain sections during the assay procedure without compromising antibody-antigen detection.

In a plastic vertical holder, mounted paraffin sections were deparaffinised and separated from the adhesive tape by treating with chloroform for 10 min, xylene for 10 min and a graded ethanol (analytical grade) series of 100% for 10 min; 70% for 10 min; 50% for 10 min; 30% for 10 min to finally rest in sterile water. Between the chloroform and xylene incubations forceps were used to remove the dislodged tape from the tissue sections. Following incubation in PBS for 10 min, slides were laid out on a humidity-controlled staining rack and covered with 50-100 μL blocking solution (PBS containing 1% BSA) at room temperature for 2 h.

After removal of the blocking solution by tapping the slides on absorbent paper, either the EI at a 1/150 dilution, or the EII monoclonal antibody at a 1/50 dilution was applied in fresh blocking solution (100 μL) to the relevant slides. Optimal dilutions were pre-determined by serial analysis (data not shown). Control tissue sections were incubated with normal mouse serum (40-75 mg/mL, Sigma) as recommended by the supplier. Sections were covered with strips of parafilm and incubated overnight at 4^0C. As previously documented (Høj *et al*., 1990; Stuart *et al*., 1986; Gibson *et al.,* 1997) it was found that the addition of SDS at 0.05% was required for successful binding of the EII monoclonal antibody. The reason is not clear, but may indicate that the epitope of

isoenzyme EII is not normally accessible to the antibody in untreated samples (Stuart *et al*., 1986).

After incubation the parafilm strips were removed and slides transferred to a vertical holder and washed with 0.05% Tween 20 in PBS for two 15 min intervals. In the humidity controlled staining holder 50-100 µL of alkaline phosphatase conjugated goat anti-mouse IgG (Sigma) at a dilution of 1/200, according to the manufacturer's instructions, was added. The slides were incubated for 2-3 h at room temperature whilst covered with strips of parafilm.

Following antibody incubation the parafilm strips were removed and slides washed with 0.05% Tween 20 in PBS for two 15 min intervals. In a dark humidity controlled staining rack 50-100 µL 0.1 M Tris-buffer pH 9.5 (0.05 M magnesium chloride; 0.1 M sodium chloride) and 1-2 µL nitroblue tetrazolium chloride and 5-bromo-4-chloror-3-indolylphosphate p-toludine stock solution were applied, and left to incubate for 2-3 h. The reaction was stopped by removing the substrate solution and washing the slides in PBS for 30 min and finally washing with several changes of distilled water to remove the salt crystals. Sections were air dried and coverslipped with UV active glue as described in section 3.2.19.

4.2.10: (1,3;1,4)-β -glucanase enzyme analysis

Total malt (1,3;1,4)-β-glucanase was measured reductometrically. Extracts (20 µL) prepared for the SDS-PAGE Western blot (section 4.2.9) were incubated at 40^0C in tall glass tubes for 2 h with 50 µL substrate (0.2% w/v (1,3;1,4)-β-glucan from barley flour made by Deltagen dissolved in 50 mM sodium acetate buffer, pH 5.0 containing 5 mM sodium azide and 400 µg/mL BSA) and 180 µl buffer (50 mM sodium acetate (pH 4.7). Standard glucanase controls included 0, 25 and 50 nM and 1 mM glucose. The reaction was stopped with 250 µL alkaline copper reagent (4 parts Somogyi I and 1 part Somogyi II (Somogyi, 1952). After boiling for 10 min, the tubes were cooled and 250 µL 2 parts 0.75 M H_2SO_4 and 1 part arsenomolybdate (Nelson, 1944) added to develop the blue colour that is indicative of reducing sugar product. The tubes were vortexed for 30 s, centrifuged for 2 min at 2000 rpm and the supernatants read at 660 nm. Controls included for optimal spectrometry reading included a buffer/enzyme and buffer/barley glucan substrate sample alone. The enzyme assay is expressed as one unit of enzyme activity, defined as the amount of enzyme required to release 1 µmol reducing equivalents per minute.

4.2.11: DNA blot analysis of (1,3;1,4)-β-glucanase riboprobes

DNA blot analysis was used to confirm the specificity of the DIG labelled riboprobes. The transfer procedure was similar to a Southern blot analysis as follows. The pBluescript SK+ clones containing the cDNA fragments of the 3' untranslated regions of EI, EII and ET used for riboprobe transcription were digested with specific enzymes (*Eco*R1 and *Bam*H1) as described in Chapter 3 (section 3.2.5) and aliquots of 20 ng run on a 1.2% (w/v) agarose DNA gel in 1 X TAE buffer containing 0.1 $\mu g.mL^{-1}$ ethidium bromide (Sambrook *et al*., 1989) to separate the specific fragments from the pBluescript SK+ vector. Barley β-D-glucan glucohydrolases cDNAs, also referred to as (1→3)-β-D-glucanase G1 and G5, were kindly supplied by Dr. Andrew Harvey (School of Ag, Food and Wine, Waite Campus, University of Adelaide) and were loaded onto the gel for cross reactivity assessment. Ladder markers (10 μL, I kbPlus GIBCO BRL) were loaded in the first well of the gels.

A piece of clean sponge was soaked in 0.4 M NaOH and placed in a plastic tray, and two sheets of Whatman 3 MM filter paper were placed on top of the sponge. The edges of the filter paper were sealed with parafilm strips and the gel was placed upside down on the paper, without trapping air bubbles. Hybond N+ membrane was placed on top of the gel and any air bubbles removed by rolling a glass pipette over the surface of the membrane. Two additional sheets of Whatman 3 MM filter paper were soaked in 0.4 M sodium hydroxide, placed on top of the membrane and overlaid by a stack of dry paper towels. A glass plate was placed on top of the stack and the tray filled with approx 100 mL of 0.4 M NaOH. The DNA was allowed to transfer overnight at room temperature. The membrane was washed in sterile 2 X SSC (1 X SSC contains 150 mM sodium chloride and 15 mM sodium citrate pH 7).

The membrane was pre-hybridised for 4 h in a hybridisation bag at 45^{0}C with DIG Easy hybrisidation buffer (Boehringer-Mannheim). The pre-hybridisation solution was replaced by DIG Easy hybridisation buffer containing 50 ng of the specific DIG-labelled riboprobe; to incubate at 45^{0}C for 16 h. After hybridisation, the filter was washed with 2 X SSC; 0.1% SDS for 30 min, at 45^{0}C, followed by 1 X SSC, 0.1% SDS and 50% formamide for 30 min at 45^{0}C.

The detection procedure was carried out as recommended by the Boehringer-Mannheim manual. The membranes were transferred to a washing buffer (0.1 M maleic acid and 0.15 M NaCl) for 10 min, followed by the addition of anti-digoxigenin (DIG) alkaline phosphatase Fab fragment antibodies at a concentration of 1/5000 in washing buffer and 0.3% Tween 20 for 2 h at room temperature (23^{0}C).

Membranes were washed in washing buffer containing 0.3% Tween 20 for 30 min, and rinsed in detection buffer (1 M Tris HCl; 0.1 M NaCl, pH 9.5). Membranes were developed in the dark, without shaking, in detection buffer (200 ml) containing 10 mL NBT/BCIP (Nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl-phosphate toluidine salt Roche Diagnostics, Australia). The reaction was stopped by immersing the membranes in TE (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.5) buffer

4.3: Results

4.3.1: Specificity of the (1,3;1,4)-β-glucanase riboprobes used in the in situ hybridisation assay

The specificity of the riboprobes was tested by Southern blot analysis against (1,3;1,4)-β-glucanase cDNAs (Figure 4.4 a). Bands were observed in lanes containing the respective cDNAs encoding either EI (Plate 4.4 b), EII (Plate 4.4 c) and ET (Plate 4.4 d) when screened with their respective anti-sense riboprobe. Bands were not observed in the controls when the (1,3;1,4)-β-glucanase cDNA encoding EI (Figure 4.4 e), EII (Figure 4.4 f) and ET (Figure 4.4 g) were screened with their respective sense riboprobes. To rule out cross-reactivity to other glucanase genes, no bands were observed with any of the three (1,3;1,4)-β-glucanase riboprobes against the $(1\rightarrow3)$-β-D-glucanase cDNAs of GI (cDNA of $(1\rightarrow3)$-β-D-glucanase G1) and G5 (cDNA of $(1\rightarrow3)$-β-D-glucanase G5; Figure 4.4 d). Cross reactivity was also not observed with other cDNA sequences used to generate other riboprobes in this study, such as limit dextrinase, limit dextrinase inhibitor, and endo-$(1\rightarrow4)$-β-xylanase (data not shown).

4.3.2: In situ hybridisation assay of the (1,3;1,4)-β-glucanase isoenzymes on sectioned barley grain

Grain sections collected through germination and categorised into stages M1 to M5, as described in Chapter 2, were assessed for specific (1,3;1,4)-β-glucanase isoenzyme mRNA expression. Sense riboprobes were routinely run in parallel with the anti-sense riboprobes as internal controls. The control staining generated by these sense riboprobes was minimal for all three riboprobes, although a slight background was observed in the starchy endosperm and crushed cell layer on some tissue sections, particularly at the later time points (Figure 4.7 a and c). This staining was sporadic and considered to be artefactual. All control checkpoints discussed in Chapter 3 for *in situ* hybridization assessment were observed. The presence of mRNA complementary to the

Figure 4.4: Southern blot analysis of the (1,3;1,4)-β- glucanase riboprobes. Specificity of the (1,3;1,4)-β- glucanase isoenzyme riboprobes as measured against (1,3;1,4)-β- glucanase cDNAs by Southern blot analysis. Arrows indicate specific bands. a) - fragments of (1,3;1,4)-β- glucanase cDNAs encoding EI, EII and ET separated by gel electrophoresis. b), c) and d) - Southern blot filters probed with DIG labelled anti-sense riboprobes specific for EI (b), EII (c) and ET (d). e), f) and g) - control filters probed with sense riboprobes specific for EI (e), EII (f) and ET (g). Bound riboprobe fragments were detected with anti-DIG antibodies and NBT/BCIP. Mr (bp), molecular weight ladder; GI, cDNA of (1→3)-b-D-glucanase G1; G5, cDNA of (1→3)-b-D-glucanase G5.
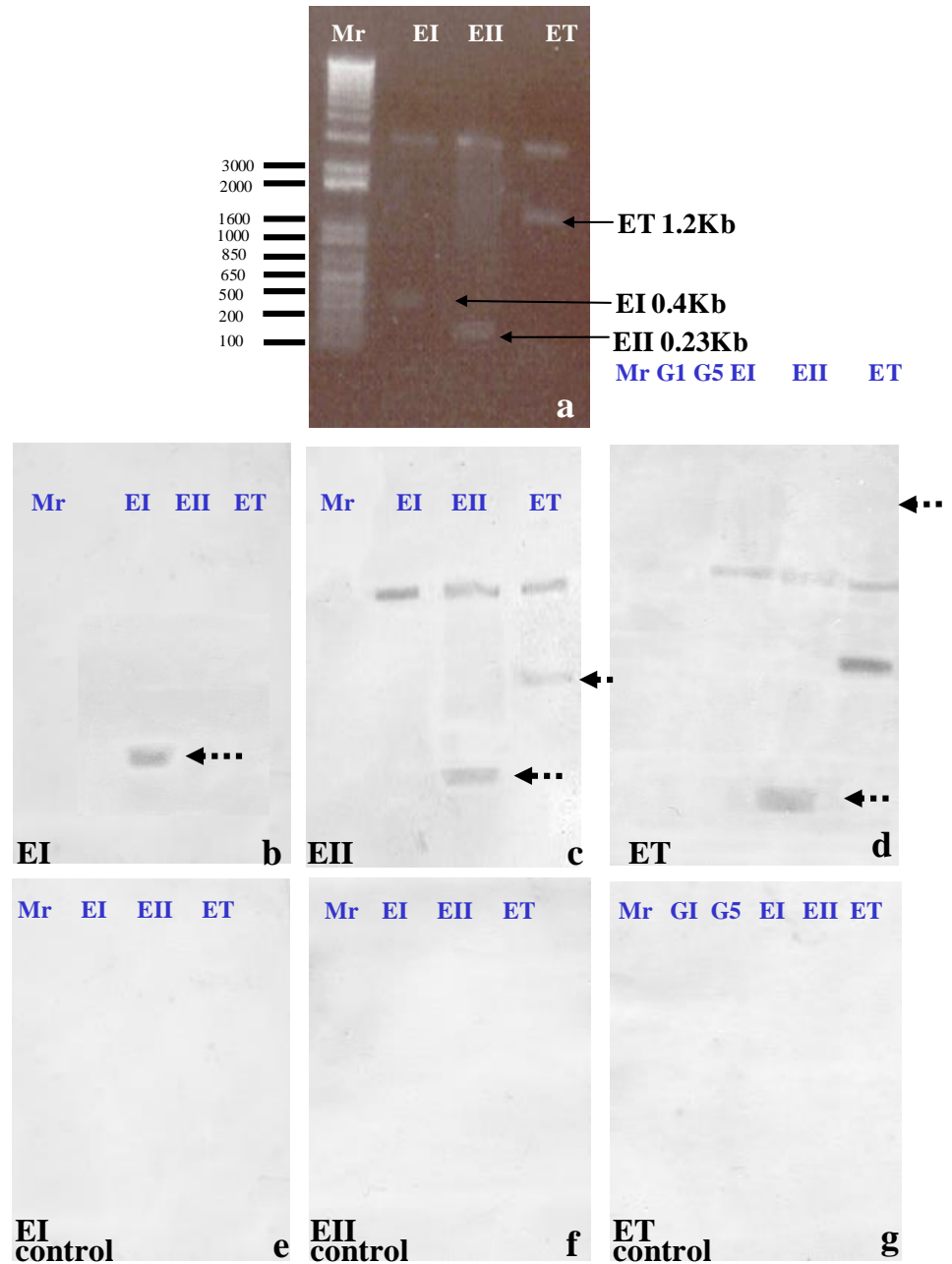
sections. On the presented figures, S indicates points of interest on Sloop sections whilst H represents Himalaya sections.

Un-germinated grain or that analysed immediately before the start of imbibition was shown to have no detectable mRNA or protein present for the (1,3;1,4)-β-glucanase isoenzymes (data not shown).

4.3.2.1: Modification stage M1 for Sloop

The *in situ* hybridisation results for the variety Sloop at M1 are shown in Figure 4.5. The appearance of mRNA transcripts early in germination for ET (Figures 4.5 a and d), EI (Figures 4.5 b, e and f) and EII (Figures 4.5 c) was observed. Figure 4.5 a, illustrates staining in the parenchyma tissue (S1) of the embryo, particularly around the rootlet (S2) and acrospire (S3). A higher magnification of EI (Figure 4.5 e and f) shows the cytoplasmic nature of the mRNA staining in the tissue above the root (coleorhiza S4). An interesting observation was the appearance of mRNA transcripts early in germination in the rootlet tip for EI (S2, Figure 4.5 e) which was not observed using the EII riboprobe (Figure 4.5 c). Staining observed for the ET riboprobe (Figure 4.5 a and d, S2 and S4) showed the combined mRNA pattern of both EI and EII. Control sections incubated with respective sense riboprobes were clear of staining (Figure 4.5 g for ET and h for EI).

4.3.2.2: Modification stage M1 for Himalaya

Low levels of both EI and EII mRNA were seen at this early time point in Himalaya sections (Figure 4.6). An interesting observation was the expression of EI (Figure 4.6 a and c, H1) on the ventral side of the scutellum parenchyma and the absence of staining in the coleorhiza as observed for Sloop (S4). The ET results presented in Figure 4.6c, H1 represent the additive signals. Control sections incubated with respective sense riboprobes were clear of staining (Figure 4.6 d).

4.3.2.3: Modification stage M2 for Sloop and Himalaya

Staining patterns at the M2 stage (data not shown) were not significantly different to the results observed for the M1 stage, indicating that mRNA in the embryo was present until approx. 32 h into germination. RNA of either isoenzyme in the aleurone was not visible at the M1 or M2 stages.

4.3.2.4: Modification stage M3 for Sloop

The most striking feature of the mRNA staining seen at the M3 stage for the variety Sloop is the difference between the two isoenzymes in the embryo region of the grain. A higher magnification of the EI staining is seen in Figure 4.7 a, showing the pattern of

Figure 4.5: Messenger RNA of (1,3;1,4)-β- glucanase in Sloop grain at the M1 stage of germination.

In situ hybridisation on longitudinal sections of Sloop at the M1 stage of germination probed with the following; a) and d) – ET anti-sense riboprobe (entire coding region of (1,3;1,4)-β- glucanase EII) b),e) and f) – EI anti-sense riboprobe (3' untranslated region of (1,3;1,4)-β- glucanase EI). c) – EII anti-sense riboprobe (3' untranslated region of (1,3;1,4)-β- glucanase EII). Control sections were treated with DIG labelled sense riboprobes corresponding to EI (h) and ET glucanase (g). Bound riboprobes were detected with anti-DIG antibodies and NBT/BCIP staining. sc, scutellum; al, aleurone layer; rt, rootlets; co, coleoptile (acrospire), vt, ventral trace; hs, husk and end, starchy endosperm. S1 – scutellum parenchyma, S2 – rootlet, S3 – acrospire, S4 – coleorhizaScale bar represents 400 mm.

* Staining artifact
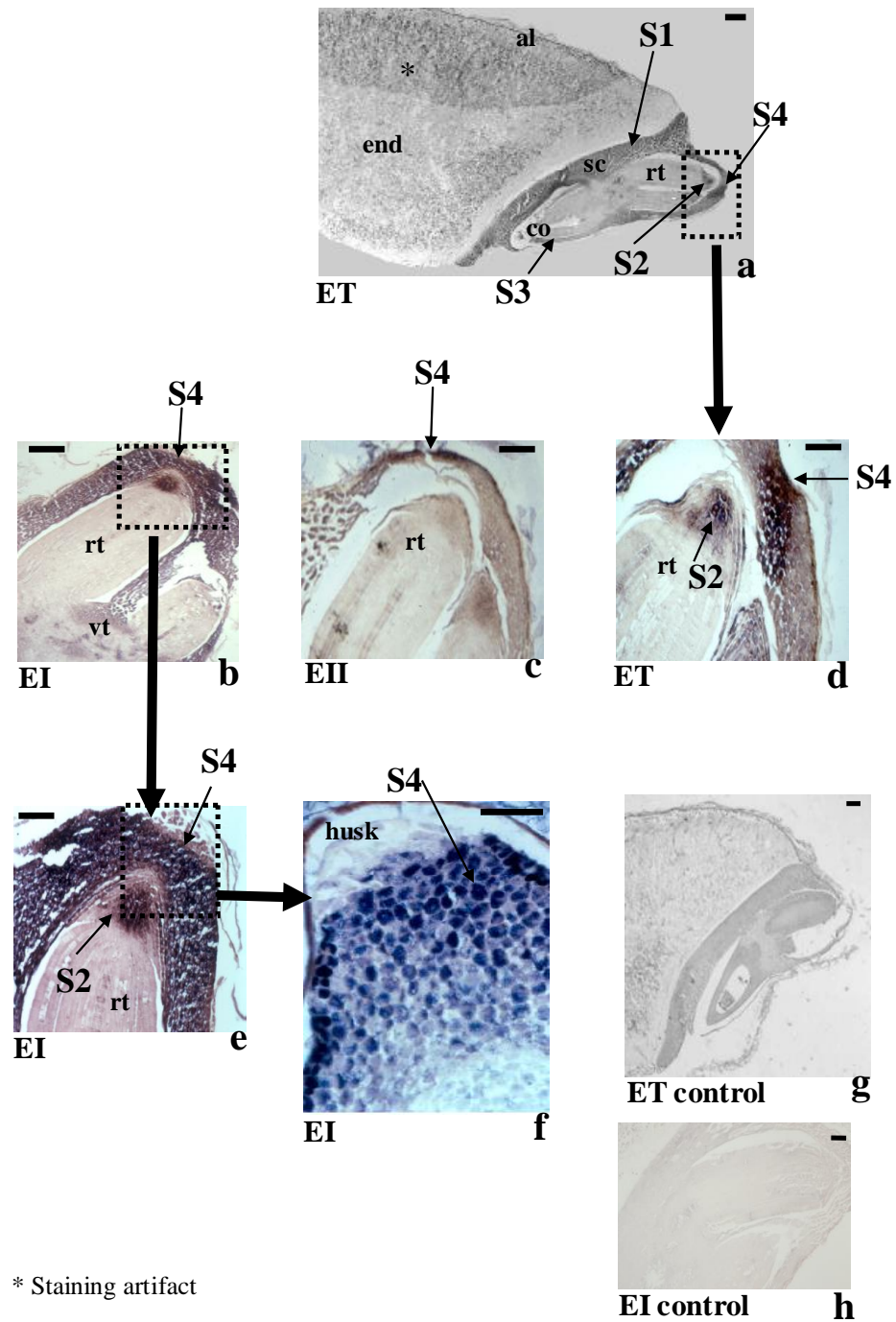
Figure 4.6: Messenger RNA of (1,3;1,4)-β- glucanase in Himalaya grain at the M1 stage in germination.

In situ hybridisation results on longitudinally cut sections of Himalaya at the M1 stage probed with the following; a) and b) – EI anti-sense riboprobe. c) – ET anti-sense riboprobe. Control sections were treated with DIG-labelled sense riboprobes. Presented is the control section for ET glucanase (d).Bound riboprobes were detected with anti-DIG antibodies and NBT/BCIP staining. sc, scutellum; al, aleurone layer; vt, ventral trace; rt, rootlets; co, coleoptile and end, starchy endosperm. H1 – scutellum parenchymaScale bar represents 400 mm
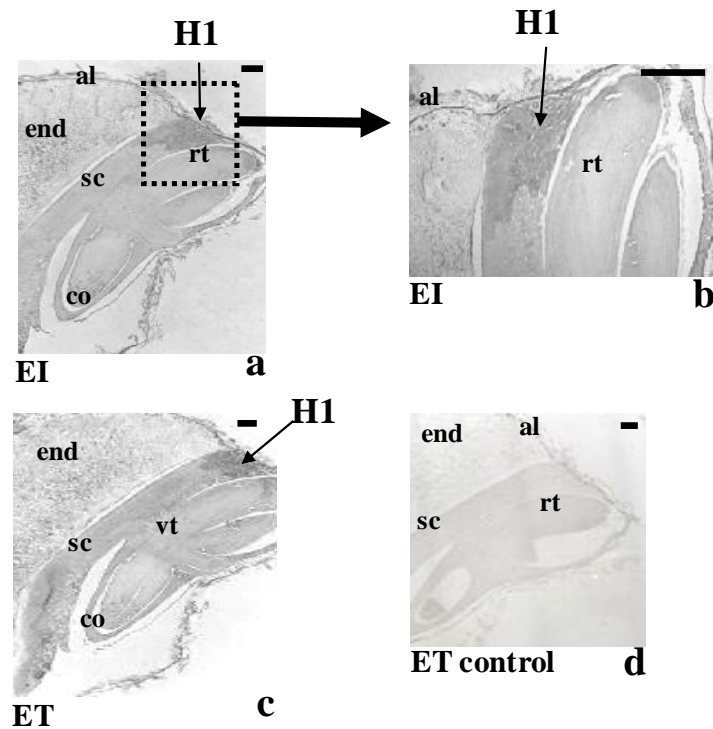
mRNA accumulation in the ventral trace region and surrounding parenchyma tissue (S6) but not actually in the rootlet or coleoptile. In contrast, the EII mRNA was concentrated to a specific region of the ventral trace area of the embryo (Figure 4.7 b, c and d, S7). This is most likely the point of origin of the vascular strands that enter the scutellum at the base of the emerging rootlets and coleoptile, also referred to as the nodal area. Slight staining was also consistently present around the edge of the expanding coleoptile (shoot primordium, S8) and rootlets (S9) for the EII mRNA (Figure 4.7 b). The *in situ* hybridisation results for the variety Sloop at M3 (Figure 4.7) produced staining along the dorsal side of the scutellar epithelial layer when probed with either EI (data not shown) or EII (Figure 4.7 b, S5).

A higher magnification of the staining is shown in Figure 4.7 e, which represents the scutellum epithelium of the ventral side of the grain section probed with ET (S5). The ET result presented in Plate 4.7 (f) for the M3 stage is dominated by the contribution of the EII mRNA (S7 and S9). Control sections incubated with the sense riboprobes were clear of staining (Figure 4.7 g).

At this stage mRNA in the aleurone could be observed for all probes (Figures 4.8a for EII and 4.8c for ET, S10 and H2) in both cultivars. Stronger expression was close to the scutellar epithelium on both sides of the grain, but slightly more advanced on the dorsal side (Figure 4.8 b, S10). At this stage the advancement of staining was observed along the length of the aleurone tissue for Sloop (data not shown), showing that transcription was now stronger and found in almost all cells of the aleurone layer.

Control sections incubated with respective sense riboprobes were clear of staining (Figure 4.8 d).

Expression in the aleurone was not observed as a progression from cell to cell along the length of the tissue, as a wave might be imagined, but more as a scattered pattern with positive cells within close proximity of each other as seen in Figure 4.8 f, S10 (black arrows).

4.3.2.5: Modification stage M3 for Himalaya

Differences in mRNA were observed between the isoenzymes at M3 (data not shown). EI mRNA expression appeared to be very strong at the base of the coleoptile, rootlet and surrounding scutellum (data not shown), as also seen on Sloop sections (Figure 4.7 a, S6). The mRNA for EII (data not shown) appeared concentrated in the ventral trace area of the embryo, along with a more general expression throughout the entire scutellum

Figure 4.7: Messenger RNA of (1,3;1,4)-β- glucanase in Sloop grain at the M3 stage in germination.

In situ hybridisation results on longitudinal sections of Sloop at the M3 stage in germination probed with the following; a) – EI anti-sense riboprobe. b), c) and d) – EII anti-sense riboprobe. e) and f) – ET anti-sense riboprobe.

Control sections were treated with DIG labelled sense riboprobes. Presented is the control section for ET (g).

Bound riboprobes were detected with anti-DIG antibodies and NBT/BCIP staining.

sc, scutellum; sce, scutellum epithelium; al, aleurone layer; rt, rootlets; co, coleoptiles; vt, ventral trace and end, starchy endosperm. S5 – scutellum epithelium, S6 – ventral trace, S7 – nodal area, S8 – shoot coleoptile, S9 – rootletsScale bar represents 200 mm.

Blue colour a product of the tungsten light on the microscope

Figure 4.8: Messenger RNA of (1,3;1,4)-β- glucanase in Sloop and Himalaya aleurone at the M3 stage in germination.

In situ hybridisation results on longitudinally cut sections of Sloop and Himalaya aleurone at the M3 stage in germination probed with the following; a) - EII anti-sense riboprobe. b) and c) - ET anti-sense riboprobe. Control sections (d and e) were treated with the corresponding DIG-labelled sense riboprobes.

a), c), e) and f) – Sloop tissue b), and d) – Himalaya tissue. Bound riboprobes were detected with anti-DIG antibodies and NBT/BCIP staining. al, aleurone layer; and end, starchy endosperm. S10 – aleurone, H2 – aleurone. Scale bar represents 100 mm.
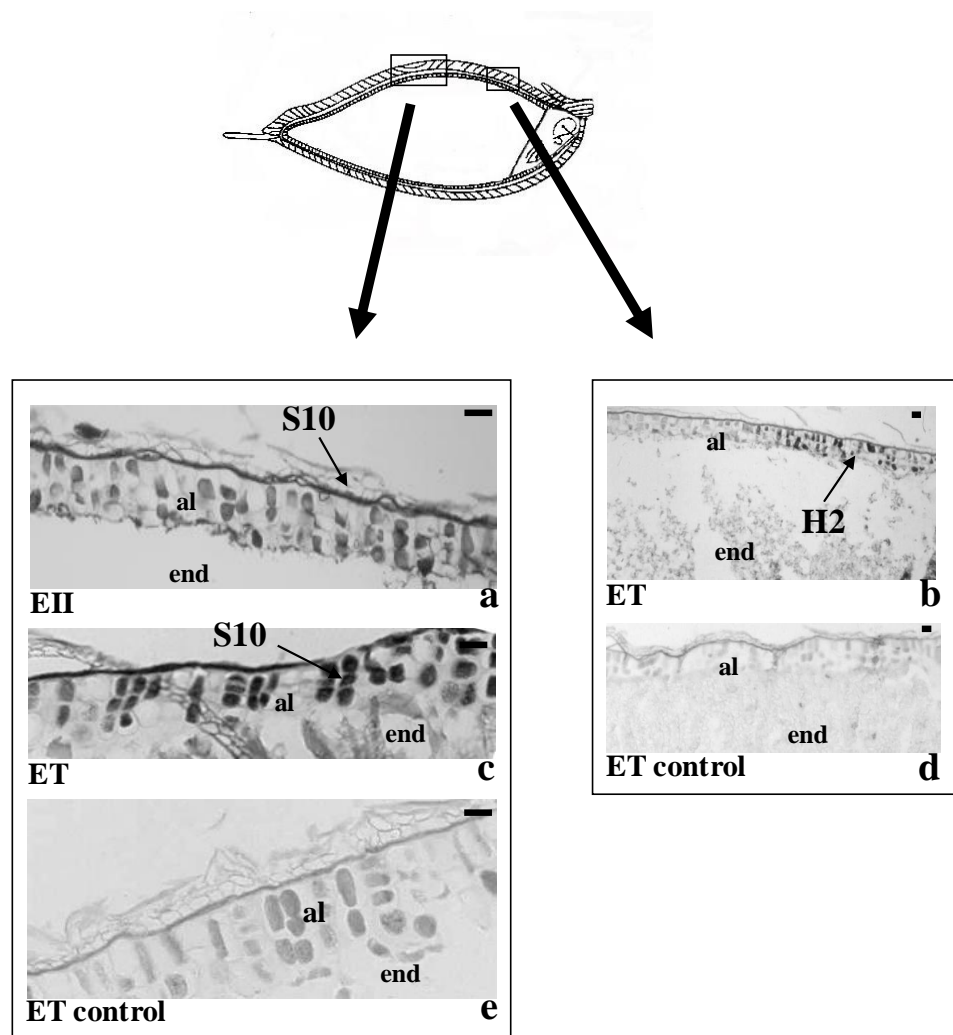
tissue area and the scutellum epithelium as also seen for EII in Sloop sections (Figure 4.7 S5, S7 and S9). Staining for ET (data not shown) represented the combined mRNA expression of both the EI and EII 3' UTR riboprobes.

4.3.2.6: Modification Stage M4 for Sloop

There appeared to be a reduced presence of (1,3;1,4)-β-glucanase mRNA in the scutellum of Sloop at the M4 stage, with more signal along the length of the shootlet leaves, particularly at the base in the shoot primordium (Figure 4.9e and f, S12) for both EI (Figure 4.9a, S11) and EII (Figure 4.9c, e and f). EII mRNA was still evident in the scutellum epithelium (Figure 4.9 e, S13).

4.3.2.7: Modification stage M4 for Himalaya

There are a few points of interest to note in relation to the staining of the embryo/scutellum for the M4 stage Himalaya grain sections (Figure 4.10). The ET mRNA (Figure 4.10a and b) is concentrated at the ventral trace area (H3) scutellum epithelium (H4) and adjacent scutellum (H5), shoot primordium (H6) and throughout the rootlet tissue (H7).

The mRNA staining of ET Himalaya at (M4) showed similarities to the staining seen for EI with Sloop at an earlier stage (M3) Figure 4.7 (EIb, S6). A common area of staining between the two varieties is that of the ventral trace region (S7 and H6) and growing shoot primordium (H6). In contrast, the mRNA staining for EI (Figure 4.10 d and e) can only faintly be observed in the ventral trace region near the base of the coleoptile (H6) and developing shoot primordium, within the rootlet tissue (H7) and the scutellum adjacent to the epithelium (H4) on the dorsal side of the grain. It was deduced by examination of other sections at this collection point that the pattern indicated by ET was most likely due to expression predominantly of EII, similar to that seen for Sloop in Figure 4.7. Control sections incubated with respective sense riboprobes were clear of staining (Figure 4.10 c).

4.3.2.8: Modification stage M5 for Sloop

By M5 (approx. 90 h from the start of steeping) most of the mRNA for the two isoenzymes had disappeared in the scutellum/embryo area but is still present at high levels throughout the whole aleurone layer, particularly along the dorsal side of the grain, and along the expanding coleoptile (data not shown).

Aleurone expression in Himalaya at this stage was very similar to that seen in Sloop (data not shown), where both isoenzymes showed mRNA in the aleurone tissue surrounding the entire endosperm.

Figure 4.9: Messenger RNA of (1,3;1,4)-β- glucanase in Sloop grain at the M4 stage in germination.

In situ hybridisation results on longitudinally cut aleurone sections of Sloop at the M4 stage in germination probed with the following; a) – EI anti-sense riboprobe. c) – EII anti-sense riboprobe.Control sections were treated with the corresponding DIG labelled sense riboprobes EI (b) and EII (d).Bound riboprobes were detected with anti-DIG antibodies and NBT/BCIP staining. sc, scutellum; sce, scutellum epithelium; al, aleurone layer; co, coleoptile; end, starchy endosperm; vt, ventral trace and sp, shoot primordium. S11 – shootlet leaves, S12 – shoot primordium, S13 – scutellum epithelium. Scale bar represents 100 mm.
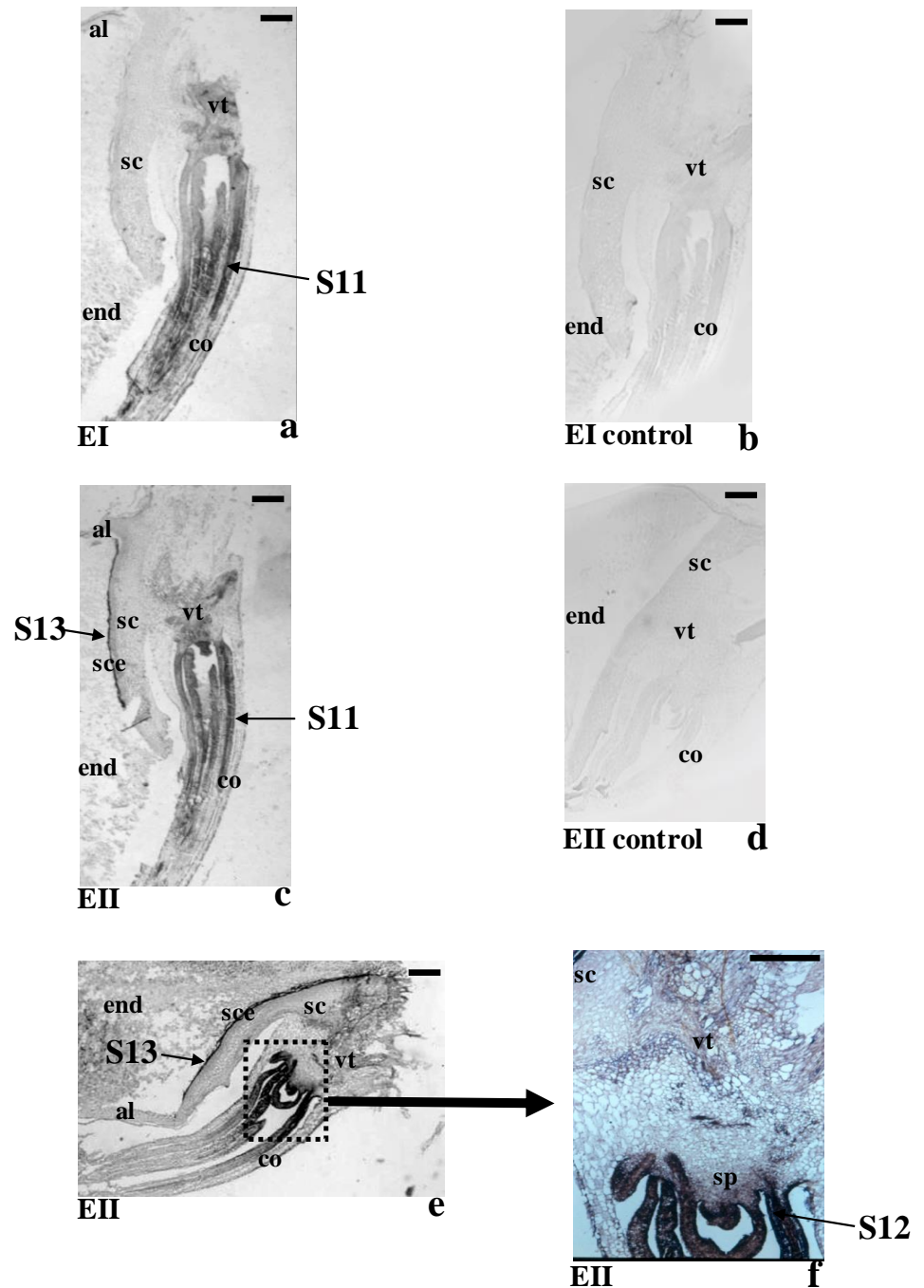
Figure 4.10: Messenger RNA expression of (1,3;1,4)-β- glucanase in Himalaya grain at the M4 stage in germination.

In situ hybridisation results on longitudinally cut sections of Himalaya at the M4 stage in germination probed with the following; a) and b) - ET anti-sense riboprobe. d) and e) – EI anti-sense riboprobe. Control sections (e) were treated with corresponding DIG labelled sense riboprobes. Bound riboprobes were detected with anti-DIG antibodies and NBT/BCIP staining. sc, scutellum; sce, scutellum epithelium; vt, ventral trace; rt, rootlets; co, coleoptile and end, starchy endosperm. H3 – ventral trace, H4 – scutellum epithelium, H5 – scutellum, H6 – shoot primordium, H7 – rootlets Bar represents 100 mm
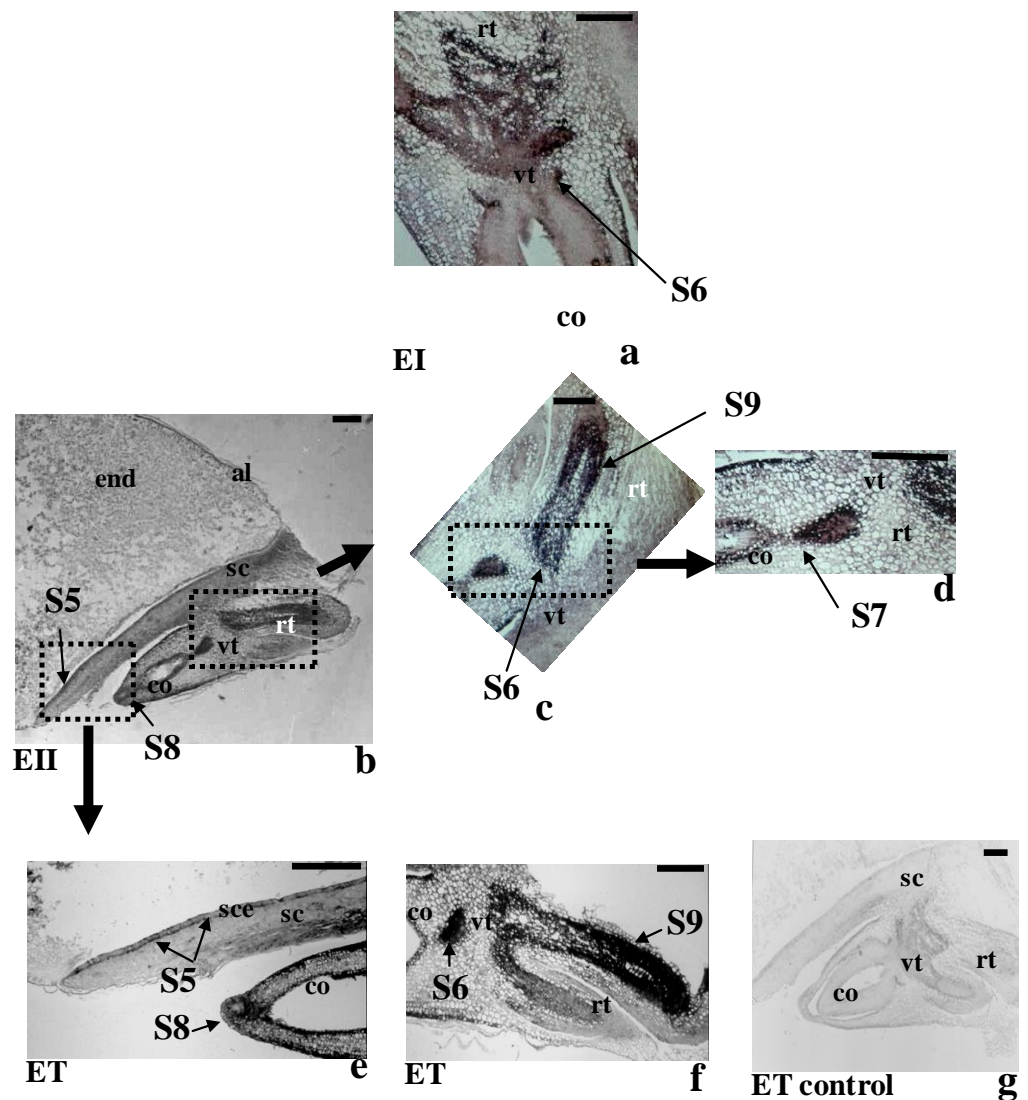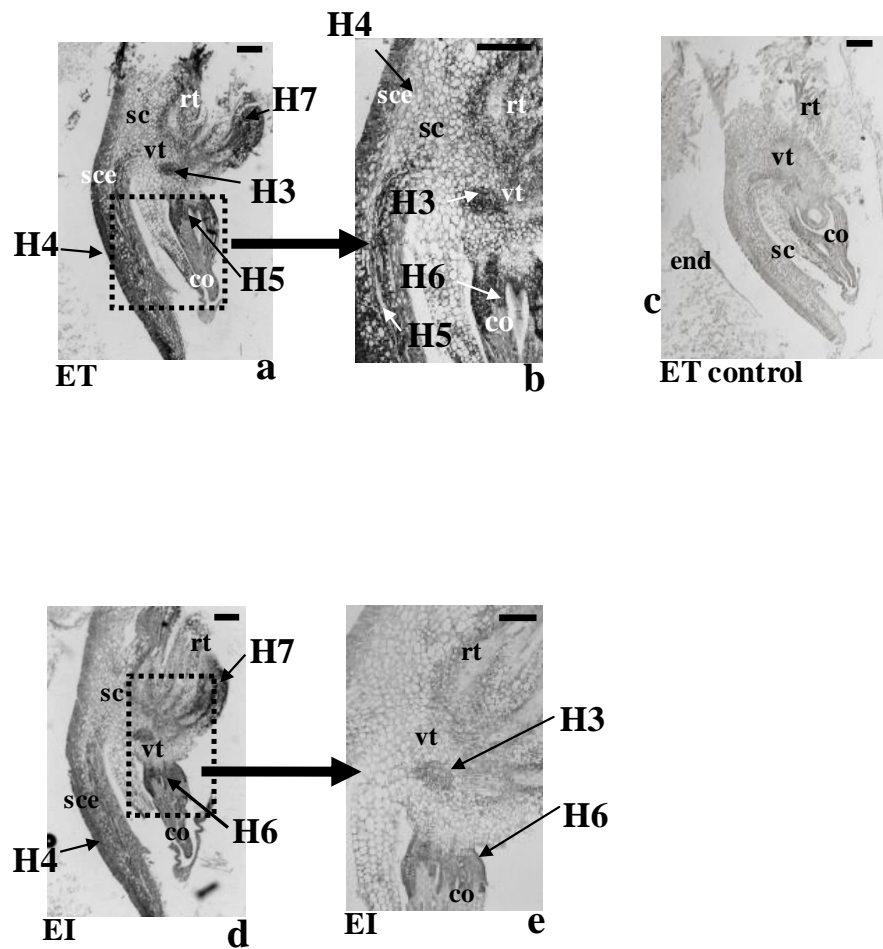
Figure 4.11: Messenger RNA of (1,3;1,4)-β- glucanase in Himalaya grain at the M5 stage in germination.

In situ hybridisation results on longitudinally cut sections of Himalaya at the M5 stage in germination probed with the following; a) - EI anti-sense riboprobe. c) - EII anti-sense riboprobe. Control sections (d) were treated with corresponding DIG labelled sense riboprobes.Bound riboprobes were detected with anti-DIG antibodies and NBT/BCIP staining. end, starchy endosperm and al, aleurone layer. H8 – aleuroneBar represents 400 mm
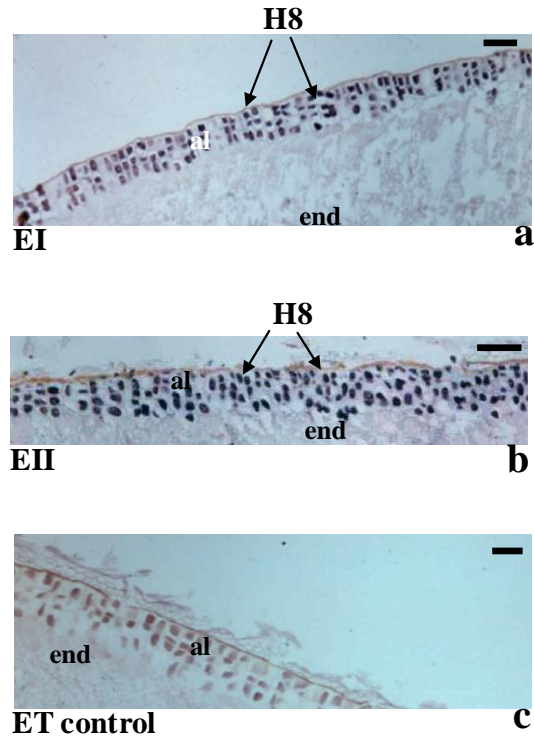
4.3.2.9: Modification stage M5 for Himalaya

As observed with Sloop M5 sections, most of the mRNA for the two isoenzymes is reduced in the scutellum/embryo area but is still present at high levels throughout the whole aleurone layer, particularly along the dorsal side of the grain (Figure 4.11). All cells in the layers of the aleurone are involved (Figure 4.11a and b, H8) including the central nucellar sheaf cells on the ventral side of the grain (data not shown). Glucanase mRNA located in the coleoptile, scutellum epithelium and rootlet was still observed in grain sections at this final assessment stage, predominantly arising from EII expression (data not shown).

An overall summary of the mRNA expression patterns observed for the two (1,3;1,4)-β-glucanase isoenzymes by *in situ* hybridisation staining on longitudinally cut Sloop barley grain sections is presented in Figure 4.12. Himalaya grain section staining was not dissimilar to that of Sloop (data not shown).

4.3.3: Western analysis of (1→3,1→4)-β-D-glucanase monoclonal antibodies

As reported in past studies (Høj *et al*., 1990), the (1,3;1,4)-β-glucanase isoenzyme monoclonal antibodies specifically detected the corresponding pure protein (EI or EII lanes; Figure 4.13 and Figure 4.14) at the correct molecular weight band size in germinated grain extracts (isoenzyme EI, 28 kDa and isoenzyme EII 30 kDa; Stuart *et al*., 1987). An SDS-PAGE Western blot was performed to test the (1,3;1,4)-β-glucanase monoclonal antibodies on whole grain extracts collected during the barley germination schedule. The Western transfer of whole grain extracts screened with (1,3;1,4)-β-glucanase isoenzyme monoclonal antibodies is shown in Figure 4.13.

Close examination of the Western transfer indicates a developmental increase in the appearance of the (1,3;1,4)-β-glucanase isoenzymes in germinating Sloop, consistent with earlier studies by Høj *et al*. (1990); Stuart and Fincher (1983) and Edmunds *et al*. (1994) who used ELISA determination. The presence of (1,3;1,4)-β-glucanase protein in whole grain for the Himalaya variety is shown to be considerably lower for both isoenzymes (Figure 4.14), but does show a trend to increase as germination progresses (compare 96 H to 96 S for both isoenzymes, Figure 4.14). The (1,3;1,4)-β-glucanase proteins were sought on longitudinally sectioned barley grain at the corresponding M points presented for the *in situ* hybridisation (mRNA expression) study. A limitation to the work presented here is the level of starchy endosperm preservation; this may affect the clarity of the staining, and makes the pattern of enzyme movement through the endosperm hard to document accurately, particularly for the Sloop variety as was discussed in section 1.3.

Figure 4.12: Hand drawn representations of the mRNA patterns observed for (1,3;1,4)-β-glucanases in Sloop. Red is representative of the place on the longitudinal grain section where an *in situ* hybridisation signal was identified at the particular stage of germination.
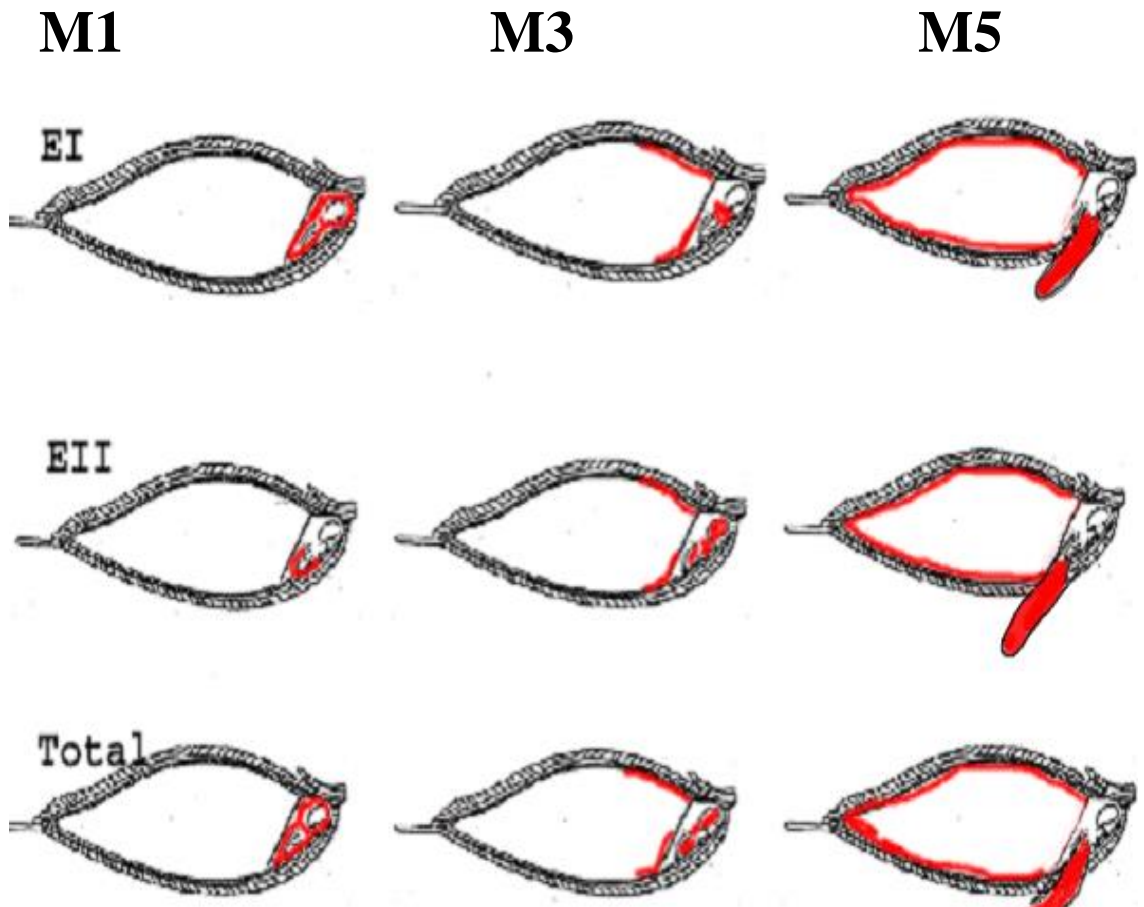
Figure 4.13: Specificity of the (1,3;1,4)-β-glucanase isoenzyme monoclonal antibodies as shown by Høj *et al*. (1990). Panel A from Høj *et al*. (1990): Purified barley (1,3;1,4)-β-glucanase EI and EII and (1→3)-β−D−glucanase GI and GII proteins (1 ug each), were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis, blotted onto nitrocellulose and probed with the monoclonal antibodies used in this study. Bound antibodies were detected with (^{125}I)-protein A.

Figure 4.14: Identification of (1,3;1,4)-β- glucanase isoenzyme proteins in germinated barley extracts by Western transfer analysis. Presence of (1,3;1,4)-β-glucanase isoenzyme proteins in germinating barley grains as detected by Western blot analysis. The blot shows extracts of grain germinated and collected at the times shown (32, 56 and 96 h) run beside purified EI and EII protein (lanes as indicated) after separation of the grain extracts and standards by SDS-PAGE and Western transfer. Filters were probed with monoclonal antibodies specific for either EI (higher panel) or EII (lower panel). Bound antibodies were detected with goat anti-mouse alkaline phosphatase, Lumiol substrate and X-Ray film development. Mk, markers as described in methods; Gap, space in gel; H, Himalaya grain extractions; S, Sloop grain extractions; EI or EII pure protein.

Isoenzyme EI

Isoenzyme EII
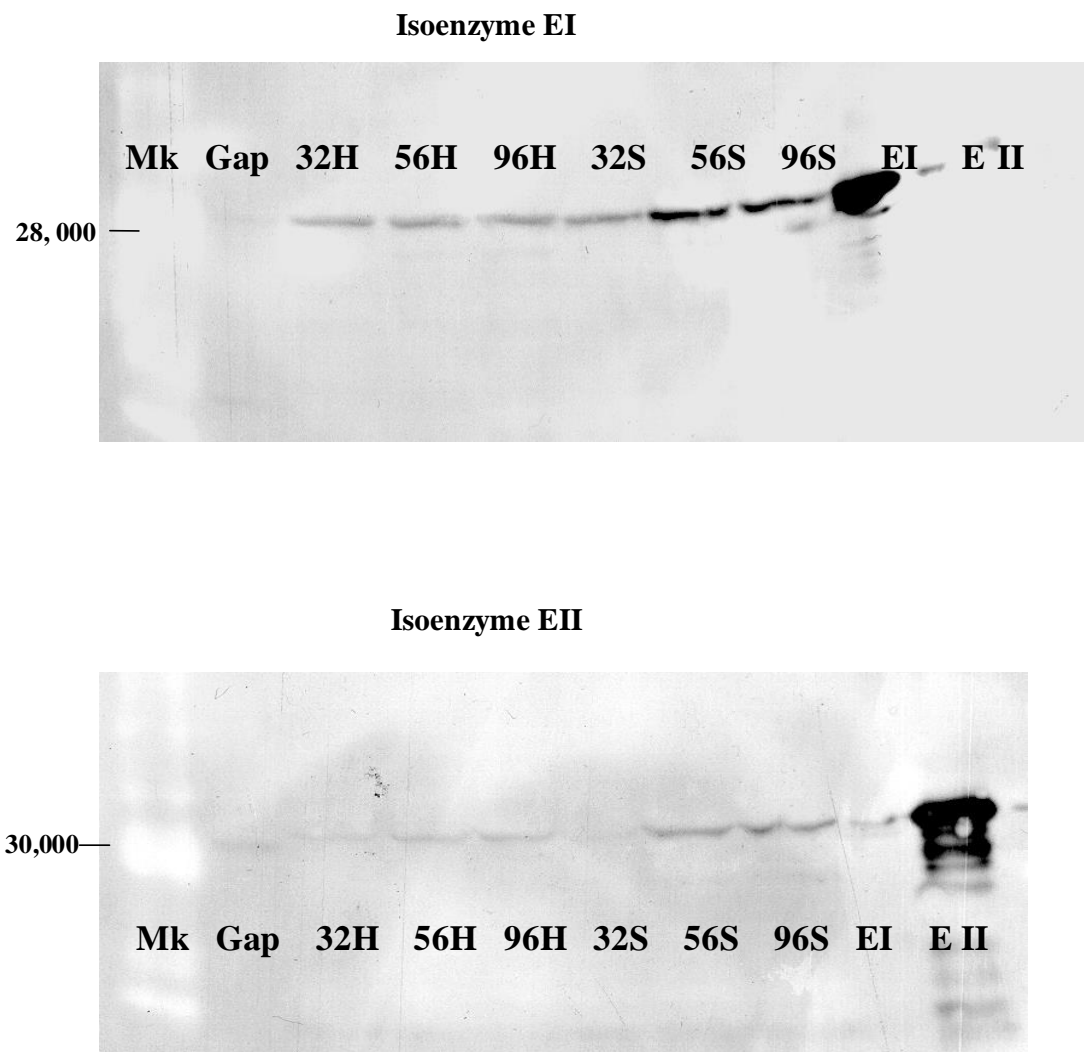
4.3.4: Immunostaining of sectioned barley grain with (1,3;1,4)-β-glucanase monoclonal antibodies

The inclusion of an immunostaining assessment in this study was for two reasons; 1) to observe the differences between the location of the mRNA as defined by ISH and the protein product as detected by the binding of specific antibodies, and 2) to examine possible protein secretion of the isoenzymes in the two varieties under the germination conditions used in this study.

4.3.4.1: Modification stage M1 for Sloop and Himalaya

Typical immunostaining results on tissue sections from the M1 stage with monoclonal antibodies against (1,3;1,4)-β-glucanase isoenzymes are shown in Figure 4.15. The protein staining appeared to concentrate in the scutellum tissue just surrounding the rootlets and coleoptile (Figure 4.15c and d, S14). Higher magnification of the scutellum area near the dorsal side of the grain clearly shows the presence of EI and EII positively stained cells (Figure 4.15b and d, S14). The serum control (Figure 4.15e and f) was clear of signal. No protein staining in the aleurone was observed for either isoenzyme in both varieties at this stage (data not shown).

4.3.4.2: Modification stage M3 for Sloop and Himalaya

On Sloop sections at M3 the EI protein was detected throughout the whole embryo and scutellum region, extending into the starchy endosperm near the scutellum epithelium, predominantly on the ventral side of the grain (data not shown).

The staining for EII followed a similar pattern (data not shown). A comparable, but less advanced, progression of protein into the endosperm was observed for Himalaya (data not shown).

4.3.4.3: Modification stage M4 for Sloop and Himalaya

The overall pattern of (1,3;1,4)-β-glucanase protein expression did not change as germination progressed. At the M4 stage in the Himalaya variety, EI protein expression in the scutellum was still concentrated in specific areas close to the scutellum epithelium (Figure 4.16 a and b, H9), with a small quantity of protein found in the rootlets (H10) and expanding coleoptile (H11). A similar pattern had been observed for mRNA expression in these tissues. The EII protein distribution at this stage was not as predominant in the scutellum area, concentrated more in the cellular layers between the husk and the embryo, (data not shown). Strong staining was observed at this stage in the aleurone on both the ventral and dorsal sides (Figure 4.16a, H12) for both isoenzymes.

Figure 4.15: Immunostaining at the M1 stage following the application of either EI (a and b) or EII (c and d) monoclonal antibodies to Himalaya sections.

Immunostaining at the M1 stage following the application of either EI (a and b) or EII (c and d) monoclonal antibodies to Himalaya sections. Control sections were treated with anti-mouse serum only (e and f). Bound antibodies were detected with alkaline phosphatase conjugated goat anti-mouse IgG antibodies and NBT/BCIP staining which produces a blue precipitate. sc, scutellum; sce, scutellar epithelium and rt, root sheath. S14 – scutellum parenchymaBar represents 400 mm
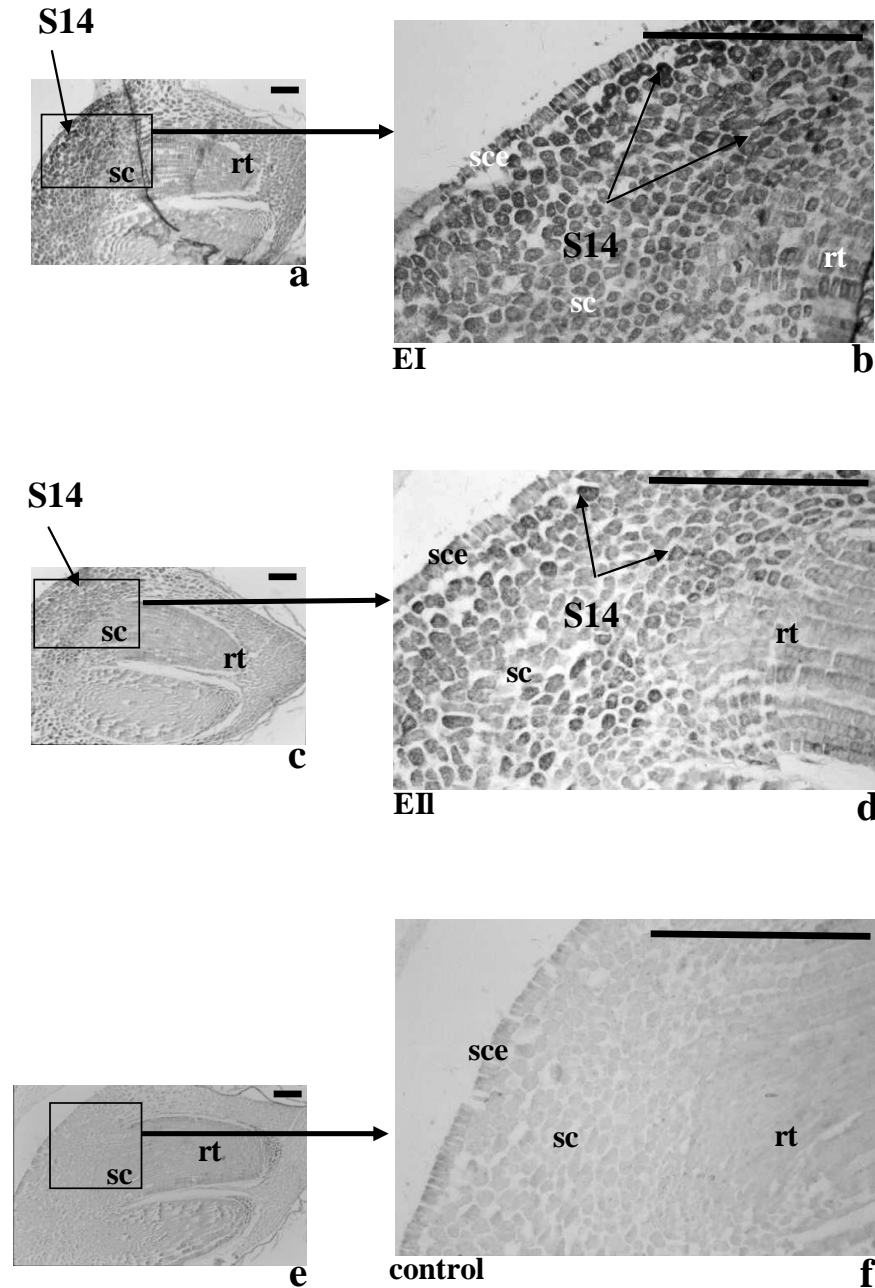
Figure 4.16: Immunostaining at the M4 stage following the application of either EI (a and b) monoclonal antibodies on Himalaya sections. Immunostaining at the M4 stage following the application of EI (a and b) monoclonal antibody on Himalaya sections. Control sections were treated with anti-mouse serum (c). Bound antibodies were detected with alkaline phosphatase conjugated goat anti-mouse IgG antibodies and NBT/BCIP staining. sc, Scutellum; co, coeoptile; sce, scutellar epithelium; rt, root sheath; al, aleurone layer; vt, ventral trace area, end, starchy endosperm. H9 – scutellum parenchyma, H10 – rootlets, H11 – coleoptile, H12 – aleurone. Bar represents 400 mm
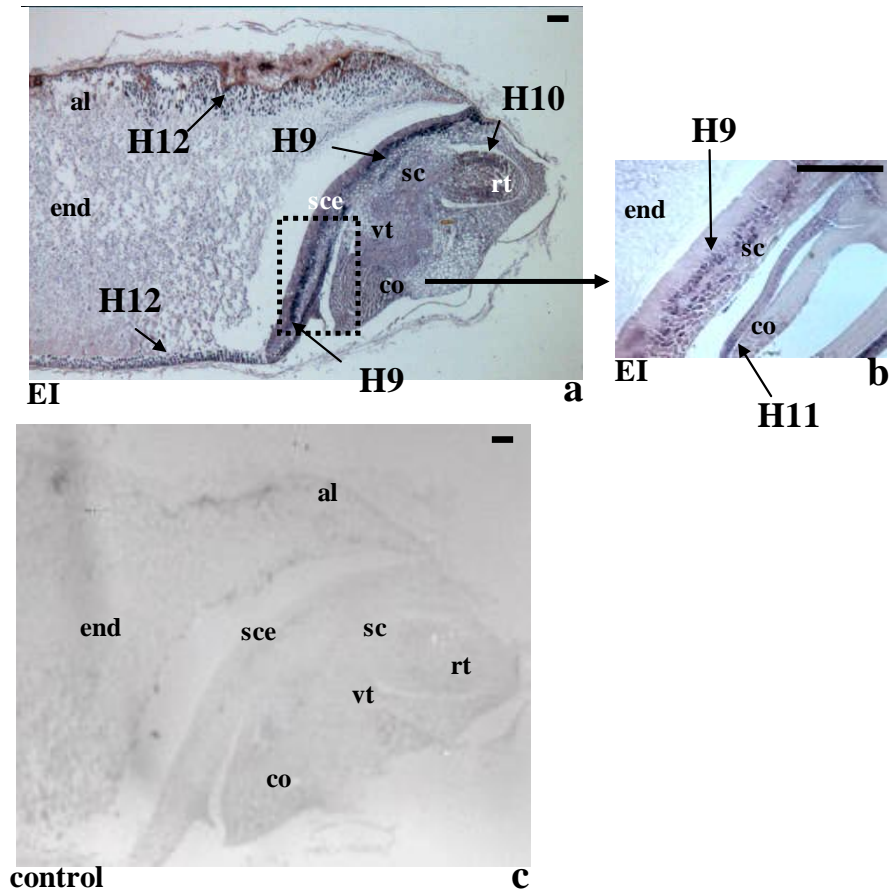
4.3.5.4: Modification stage M5 for Sloop and Himalaya

By the M5 stage, EI protein staining has reduced in the embryo of the grain, while still being evident in the dorsal scutellum/epithelial tissue and tip of the growing coleoptile for EII. The protein staining for both EI and EII is strongest in the aleurone (data not shown).   A similar pattern is seen for both varieties. At this stage it is obvious that the starchy endosperm is well advanced into degradation and cell wall breakdown.

4.3.5: Total (1,3;1,4)-β-glucanase enzyme levels

Total glucanase enzyme activity was measured in extracts of grain at similar stages to those used for Western protein analysis (32, 56 and 96 h). A positive relationship between the presence of mRNA (detected by RT-PCR), protein (shown by Western analysis) and the detection of enzyme activity was demonstrated. Results in Figure 4.17 are consistent with increasing protein levels as demonstrated by the Western analysis (Figure 4.14) and previously suggested by others (Stuart *et al*., 1987; Høj *et al*., 1990; MacFadden *et al.,* 1988; Skakeski *et al*., 1990; Wolf, 1992; Stuart and Fincher, 1983, 1987). Enzyme activity for total (1,3;1,4)-β-glucanase increased as germination proceeded, being slightly higher for the Sloop variety (Figure 4.17 S) when compared to Himalaya (Figure 4.17 H). The assay used here did not allow for discrimination between the isoenzymes, nor possible (1→3)-β-D-glucanase and cellulase activity (microbial origin) (Stuart and Fincher, 1983).

Figure 4.17: Total (1,3;1,4)-β- glucanase enzyme levels. Extracts of grain germinated and collected at the times shown (32, 56 and 96 h) were analysed using the Somogi/Nelson method to determine total active glucanase enzyme levels. Values are expressed as specific activity (units per mg) according to Somogyi (1952). H, Himalaya; S, Sloop.
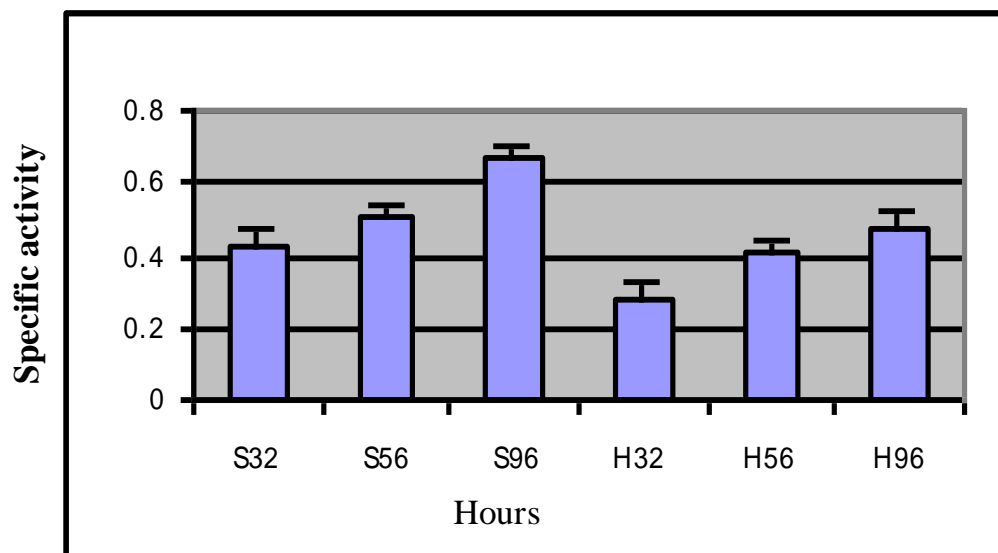
Grain samples

4.4: Discussion

The primary objective of the work described in this Chapter was to identify the tissue location and time in expression of the (1,3;1,4)-β-glucanase isoenzymes EI and EII in germinated grain by detecting mRNA and protein. As such, a specific aim was to take the work of McFadden *et al*. (1988a) a step further and document tissue specific expression of the two (1,3;1,4)-β-glucanase isoenzymes separately in barley during germination. Similar to the study presented by McFadden *et al*. (1988a) *in situ* hybridization on sections of whole grain was used to visually document expression patterns, concentrating on the two sites containing living tissue in the germinating grain, namely the aleurone and the embryo/scutellum regions.

4.4.1: Verification of riboprobe and antibody specificity

The riboprobes used in this chapter for the (1,3;1,4)-β-glucanase isoenzymes in barley, as shown by Southern blot (Figure 4.4), were specific for their cDNA templates. BLAST analysis was also performed using the cDNA sequences presented in Table 4.1 which did not identify any other strong similarities even to the β–D-glucan glucohydrolases, β-glucan exohydrolases and endo-(1→3)-β-D-glucanase members of this family of enzymes. The most likely cross contamination candidates were the endo-(1→3)-β-D-glucanases, as individual endo-(1→3)-β-D-glucanase isoenzymes exhibit 50-60% positional identity with the amino acid sequence of barley (1,3;1,4)-β-glucanase. Screening of the (1,3;1,4)-β-glucanase riboprobes by Southern blot discounted cross reactivity to the cDNAs of endo-(1→3)-β-D-glucanase G1 and G5 (Figure 4.4), so reactivity to the other (1→3)-β-D-glucanases is unlikely. The presence of a third (1,3;1,4)-β-glucanase isoenzyme has previously been discussed by Stuart and Fincher (1983) but eventually ascribed by Stuart *et al*. (1986) to be endo-(1→3)-β-D-glucanase activity.

The specificity of the two glucanase isoenzyme antibodies used in this study has been well documented by others (Figure 4.15) and was reiterated by the Western analysis (Figure 4.14) carried out in this study.

4.4.2: In situ hybridization and immunostaining of germinated barley grain for
(1,3;1,4)-β-glucanase isoenzyme expression

Using the *in situ* technique, no mRNA was detected in either ungerminated or grain samples taken immediately after the start of imbibition, a result not unexpected since (1,3;1,4)-β-glucanase proteins or mRNAs have also not been detected at this stage by other workers (Stuart and Fincher, 1983; 1987; Edmunds *et al*., 1994).

This observation was also made by McFadden *et al*. (1988a), leading to the conclusion that the isoenzymes are produced *de novo* early in germination. This study confirms that mRNA and protein was not observed until 24 h into germination so it is unlikely that residual mRNA persists from expression in the developing grain, as discussed by Palmer in 1981 and as demonstrated for the limit dextrinase protein by MacGregor *et al*. (1998).

4.4.2.1: Modification stage M1

The pattern of mRNA and protein as determined by riboprobe and antibody labelling shown in Figures 4.5 and 4.15 confirm that the scutellum is the main expression site of both (1,3;1,4)-β-glucanase isoenzymes early in germination. This was also observed by McFadden *et al*. (1988a) using a probe for the (1,3;1,4)-β-glucanases that was unable to distinguish between the isoenzymes but more recently Betts *et al*. (2017) could see both EI and EII transcripts in the first 24 h of germination. Many other reports of active enzyme expression early in germination from the scutellum tissue have been made. These include Enari and Sopanen (1986), Sugimoto *et al*. (1998), Matthews *et al*. (2002) and Van der Meulen *et al*. (2000) who measured α-amylase mRNA expression. Okamoto *et al*. (1980) who detected proteinase RNA and Ranki *et al*. (1990), Gonzalea *et al*. (1998) and Mundy *et al*. (1986b) who reported carboxypeptidase and phosphoenolpyruvate carboxylase (PEPC) mRNA and protein. Gonzalea *et al*. (1998) demonstrated localisation of PEPC protein and Matthews *et al.* (2002) used a GFP α-amylase fusion clone to show proteins in a very similar location to that observed in this study; the coleorhiza and scutellum tissue surrounding the radicle.

The mRNA and protein levels of EI appear more abundant than for EII at this early stage (Figures 4.5 and 4.16), although determining a true quantitative level is not possible and therefore represents a limitation of these assays. The presence of EI at higher levels in the scutellum early in germination has previously been documented at the level of mRNA (Slakeski and Fincher, 1992) and protein (Høj *et al*., 1990; Stuart *et al*., 1986 and Edmunds *et al*., 1994), although expression has not been previously documented as early in germination as found in this study until the more recent study by Betts *et al*. (2017). The observations made here are therefore novel since intact tissue sections were collected earlier at only 8 h after the initiation of imbibition and this has been combined with the more sensitive method of non-radioactive ISH detection. Results presented here also demonstrate particular staining in distinct areas of the scutellum (Figure 4.5), such as the scutellum tissue surrounding the emerging rootlet and coleoptile, particularly for Sloop. Researchers such as McFadden *et al*. (1988a) using liquid emulsion on X-ray

film to visualize *in situ* signals were unable to relate expression patterns to a detailed cellular location on their grain sections.

When reviewing the literature, β–D-glucan glucohydrolase, β-glucan exohydrolase, endo-$(1\rightarrow3)$-β-D-glucanase and cellulase enzyme expression found in the scutellum area close to the emerging coleoptile and rootlets have been implicated in loosening cell wall polysaccharides during cell expansion to facilitate tissue or organ elongation (Hoson and Nevins, 1989; Sakurai and Matsuda, 1978; Taiz and Jones, 1973; Slakeski and Fincher, 1992a). In fact, the spatial expression pattern of ABA genes associated in coleorhizae in Arabidopsis and barley suggested that the tissue has a major role in controlling cereal dormancy (Millar *et al*., 2006; Okamoto *et al*., 2006; Gubler, 2009). The enzymes listed above have been suggested to be key in this role (Harvey *et al*., 2001; Labrador and Nevins, 1989; Hrmova and Fincher, 1998; Inouhe and Nevins, 1998; Varghese, 1999; Hohl *et al*., 1991), but the results presented in this study suggest that (1,3;1,4)-β-glucanases should also be included in this group. An alternative explanation for the expression of (1,3;1,4)-β-glucanase in the embryonic tissue sites of barley early in germination was suggested by Slakeski and Fincher (1992). They suggest that the enzyme may participate in the formation of intercellular airspaces necessary for the diffusion of carbon dioxide, oxygen and water vapour into parts of the leaf that initially have no direct access to the atmosphere. This was subsequently dismissed by these authors as the presence of (1,3;1,4)-β-glucanase protein could not be proven (Slakeski and Fincher, 1992, unpublished data), but this study suggests that they may be correct. As discussed in Chapter 7, more specific expression studies are needed to confirm the authenticity of this result, which may include micro-dissection of particular tissues, followed by real time RT-qPCR or RNAseq experiments.

4.4.2.2: Modification stage M3

As germination progressed from the M1 to M3 stages, the sites of mRNA expression of the two isoenzymes changed. Expression of both the isoenzymes at the M3 stage is now concentrated in the scutellum epithelium, and surrounding scutellum, indicative of a tissue which is actively transcribing large amounts of mRNA to make protein to be secreted into the starchy endosperm. This time point in germination is equivalent to the point where EI and EII protein secreted from the scutellum into the starchy endosperm has been detected by others (Stuart *et al*., 1986) using less sensitive methods. Their presence is not surprising given that these enzymes play a major role in breaking down the cell walls of the starchy endosperm. However, it is possible that the glucanase enzymes have other more subtle roles to play in ensuring the successful establishment of

a young seedling and clues are given that this may be the case using the ISH technique where expression is seen in a number of precisely defined locations away from the starchy endosperm.

For example, the presence of EI and EII mRNA in the ventral trace region and the embryo rootlet early in germination has not been previously documented for glucanase but was noted by Mathews *et al.* (2002) with α-amylase expression. The ventral trace or nodule area of the scutellum during barley germination has been implicated as the site of GA_3 synthesis. The promoter of the gene encoding EI has been shown to drive a low level of reporter gene expression in barley aleurone protoplasts which is unaffected by GA_3 (Wolf, 1992), whilst EII is GA_3 responsive and expressed at a higher level in the presence of this hormone. The effect of GA_3 on glucanase expression was not assessed in this study, but may be worthy of future consideration as the levels of secreted EII (1,3;1,4)-β-glucanase enzyme are enhanced by GA_3 (Stuart *et al.*, 1986).

The presence of glucanase expression in the rootlet early in germination was noted by Slakeski and Fincher (1992) for EI at day 3 of germination, but was not as obvious in the work of McFadden *et al*. (1988a). Litts *et al*. (1990) has shown that a rice β- glucanase gene (*Gns1*) with 82% amino acid similarity to the barley β-glucanase EI isoenzyme is expressed at high levels in root tissue. Therefore, there could be a potential role for glucanase in early rootlet development, once again possibly in cell wall loosening or cell expansion during root growth.

4.4.2.3: Modification stage M4

The pattern of expression observed at this stage by ISH was more consistent with the classically reported mRNA distribution in the scutellum epithelium as assessed by radioactive methods for glucanase (McFadden *et al*., 1998a). There is a clear distinction in the expression patterns between the two varieties at this point. Himalaya grain displays an expression pattern previously observed for Sloop in the M3 stage; while Sloop has clearly progressed beyond this point to show expression predominantly in the aleurone. This is not surprising since Sloop is considered an elite malting variety, which therefore will modify more quickly than Himalaya, which is not used for malting.

The EI protein, as shown by immunostaining, appears to be concentrated close to the scutellum epithelium, more so than the EII protein at this stage (Figures 4.15 and 4.16). This could explain why the majority of past literature cites EI as originating from the scutellum whilst EII is secreted from the aleurone, particularly the germ aleurone as shown in Figure 4.5 and referred to by Cochrane (1992) during the post–chitting phase

and more recently Betts *et al*. (2017).   Indeed, McFadden *et al*. (1988a) showed (1,3;1,4)-β-glucanase gene expression in a monolayer of the aleurone cells covering the adaxial face of the scutellum.

The pattern of mRNA expression at the scutellum epithelium, advancing along the aleurone layers, differs from the CW pattern of (1,3;1,4)-β-glucan degradation as seen in Chapter 2 (Figure 2.4). It also differs to the immuno-protein staining pattern shown for the two isoenzymes in the starchy endosperm for Sloop (Figure 4.15) as it advances along the ventral side of the grain. However, the mRNA signal agrees with past literature (Briggs and MacDonald, 1983; McFadden *et al*., 1988a; Sugimoto *et al*., 1997), as being more advanced on the dorsal side of the grain, parallel to coleoptile elongation. A possible explanation for this discrepancy in endosperm wall (1,3;1,4)-β-glucan breakdown and the location of (1,3;1,4)-β-glucanase mRNA could be due to the angle of sectioning employed and the fact that the mRNA shown by the ISH is not mobile whilst the protein is. However, it may also be as suggested by others, that the presence of specific activators (Inouhe and Nevins, 1997) or inhibitors of these enzymes in these areas would result in an apparent discrepancy between mRNA levels and the location of enzyme activity. This observation and the fact that proteins may be secreted whilst mRNA is static adds to criticisms relating to why protein detection may not necessary correlate to the location of transcription.

In Sloop sections at this stage, distinct expression in the coleoptile could be seen. Others have noted expression of EI in vegetative tissues physically separated from the scutellum and scutellum epithelium, such as the young seedling leaves of barley (Slakeski and Fincher, 1992a; Kotake *et al*., 2000; Jin *et al.,* 2004) or wheat (Roulin and Feller, 2001). Hence, this study is the first to report the presence of (1,3;1,4)-β-glucanase mRNA and protein in germinating grain tissue other than the coleoptiles before day 3 of germination, particularly EI. This has also recently been reported by Betts *et al*. (2017) where EI transcript is three times the levels of EII in a malting variety with the suggestion that EII transcription is restricted to the aleurone. The role of glucanase in the coleoptile during grain germination could be such as that suggested by Meikle (1994) and others who report that in coleoptiles the (1,3;1,4)-β-glucans make a transient appearance during the elongation phase of cell growth (Carpita, 1984; Gibeaut and Carpita, 1993; Luttenegger and Nevins, 1985; Kotake *et al*., 1997). This was confirmed by Gibeaut *et al*., (2005) who documented a 10% increase in (1,3;1,4)-β-glucan levels over a five-day growth schedule. In fact the (1,3;1,4)-β-glucan content of 4-day old barley coleoptiles is 19% by weight (Sakurai and Masuda, 1978). In addition,

Inouhe and Nevien, (1997) and Thomas *et al.* (2000) were able to demonstrate an interference of *in situ* (1,3;1,4)-β-glucan degradation and reduced cell wall autohydrolytic reactions in the cell walls of maize and rice coleoptile tissue in the presence of (1,3;1,4)-β-glucan antibodies. One might conclude that the coleoptile cell wall (1,3;1,4)-β-glucan is being hydrolysed by both endo and exo hydrolases (Gibeaut *et al.*, 2005; Gibeaut and Carpita, 1991; Roulin *et al.*, 2002).

4.4.2.4: Modification stage M5

By the M5 stage, most of the mRNA and protein of the two isoenzymes was detected in the aleurone layers, particularly along the dorsal side of the grain. Expression had all but gone from the scutellum for EI and was much reduced for EII. This is not unexpected, since it follows the well-documented pattern of cell wall breakdown in germinated barley grain (Briggs and MacDonald, 1983), α-amylase secretion (Gibbons, 1980; 1981; Palmer, 1982) and also reflects the (1,3;1,4)-β-glucan cell wall breakdown as measured by calcofluor white (CW) presented in Chapter 2 (Figure 2.4). Unlike the different mRNA expression patterns of the (1,3;1,4)-β-glucanase isoenzymes seen in the scutellum, both isoenzymes could be detected in most cells of the aleurone layers in agreement with George-Kraemer *et al.* (2004) and Jin *et al.* (2004). Pogsen (1989) and Matthews *et al.* (2002), examining α-amylase I and II expression, also found that all the cells in the rows of the aleurone appear to be involved in expression, even the germ aleurone. In contrast, Sugimoto *et al.* (1998) noted that the site of α-amylase mRNA expression in the aleurone layers was positioned towards the seed coat, where the induction signal is supposed to be transmitted through the cavity between the aleurone layer and seed coat. These authors also calculated that mRNA expression of α-amylase takes 11-13 min from the onset of expression in one cell before it commences in the adjacent cell.

The mode of secretion of the (1,3;1,4)-β-glucanase isoenzymes from scutellum and aleurone tissue into the starchy endosperm (the site of their substrate) was not addressed in this study but is believed to be similar to the release of other hydrolytic enzymes as discussed in Chapter 1, section 1.3. Sequencing of the signal peptide region of both the glucanase genes has shown a target sequence for the endoplasmic reticulum and hence to the extracellular space (Slakeski and Fincher, 1992a). The movement of enzymes through the aleurone cells into the endosperm is well documented, but how the enzymes move through the embryo into the scutellum is not clear, although a breakdown of the crushed cell layer, as observed in Chapter 2 with CW dye, implies a possible transport route.

4.4.3: *Varietal differences in (1,3;1,4)-β-glucanase expression*

Varietal differences between Sloop and Himalaya were noted in this study with respect to (1,3;1,4)-β-glucanase isoenzyme mRNA (summarised in Figures 4.12, 4.18 and 4.19) and protein expression patterns and also, as discussed in Chapter 2, in the (1,3;1,4)-β-glucan cell wall breakdown (CW) rate, hydration pattern and malt quality. This is not a surprising result considering that past literature reports varietal differences for (1,3;1,4)-β-glucanase (Edmunds *et al*., 1994; Stuart *et al.,* 1986; Betts *et al*., 2017) and other barley enzymes such as α-amylase (Karner *et al*., 1991). It is quite obvious when comparing Figures 4.10 and 4.7 that the expression intensity and pattern of mRNA in Himalaya at M4 is similar to the M3 stage of Sloop. It appears from this result that it is the timing of mRNA expression and not the tissue location that is different between the two varieties. In contrast, the work of Edmunds *et al*. (1994) who measured isoenzyme protein levels by ELISA during germination concludes that varieties could vary in their expression levels of EI, whilst EII showed only a similar time delay as reported in this study.

4.5: Summary and future directions

Optimal endosperm modification during barley germination is fundamental to successful malting. Undigested (1,3;1,4)-β-glucan has been documented to seriously interfere with the brewing process (Bamforth, 1982, 1985). Therefore, understanding the expression profiles of the (1,3;1,4)-β-glucanase isoenzymes is of interest to maltsters, brewers and barley researchers. This also has implications for the treatment of grain with respect to handling prior to commercial germination for both feed and malting barley, specifically ensuring that the embryo is not damaged and that the scutellum remains intact, as this has been shown to be a major site of glucanase production early in germination.

The ISH technique developed here has been successful and sensitive enough to discriminate between the EI and EII isoenzymes of (1,3;1,4)-β-glucanase at the cellular level which has not been achieved previously. These hybridization and immunostaining studies revealed a temporal and spatial co-ordination of (1,3;1,4)-β-glucanase isoenzyme gene expression in specific areas of barley tissue which is not possible to observe using homogenised grain extracts or dissected segments

The main objective of the work presented in this Chapter relates to the two (1,3;1,4)-β-glucanase isoenzymes with the aim of establishing whether they are expressed in a tissue specific manner and/or different time sequence throughout the germination of a barley

grain. This may then relate to different functions of the two genes which potentially affecting malting of the grain and early seedling vigour.

The main conclusions from the work presented in this chapter are that;

a) The presence and wide distribution of (1,3;1,4)-β-glucanase isoenzyme mRNA in barley grain was established much earlier in germination than suggested in current literature and the location could be pinpointed to novel sites within the embryo.

b) The pattern of (1,3;1,4)-β-glucanase during germination did support the view that both the embryo and the aleurone are involved in enzyme expression, at different stages of germination. The ISH technique was also able to highlight the differences between the two varieties used in this study, opening the way towards varietal examination.

c) The function of (1,3;1,4)-β-glucanase in barley grain germination is to facilitate rapid access to the nutrients located in the starchy endosperm by degrading cell walls, but as indicated by this study an additional important role may be in initiation or successful emergence of the coleoptiles and root system, particularly with respect to EI, and as alluded to in past studies (Slakeski *et al.*, 1992a). What, for example, would be the consequence of over expressing these glucanase genes through the now routine procedure of barley transformation? This type of modification may be highly desirable to those involved in the brewing industry but may be a disaster for farmers if grain germinates poorly in the field due to unforeseen alterations in the coleoptile or rootlet tissue.

d) The results presented in this Chapter have implications for important processes correlated to early seedling vigour not just grain germination.

Research into isoenzyme expression has attracted much interest lately, continuing to build the picture of the role played by these enzymes in germination. Clearly there are several factors affecting expression of isoenzymes (Slakeski *et al.*, 1990) and this study raises questions as to why this pattern of tissue specific expression has evolved. Presumably a concerted effort by all hydrolytic enzymes is needed, such as endohydrolases, exohydrolases and β-glucosidase in the cell wall degradation process and detailed expression site studies of each would be interesting. The ISH assay presented in this thesis can be used not only to assess specific native gene expression patterns but is also sensitive enough to be used on transgenic material, where a range of genes have been down regulated by dsRNAi approaches and some are over-expressed

(Stewart *et al*., 2001; Burton *et al.,* 2011). It therefore provides another tool for functional assessment of genes and their products.

The assay would also work very well on developing grain sections where the (1,3;1,4)-β-glucan and other cell wall constituents are being laid down. Deposition and hydrolysis has the potential to make substantial changes to the way in which grain behaves during malting and information about the enzymes during these stages of development may be a vital part of this process.

Figure 4.18: Summary of (1,3;1,4)-β-glucanase isoenzymes EI and EII expression in Himalaya grain as detected by mRNA. M2 and M4 not included.

Tissue	M1 I	M1 II	M1 Total	M3 I	M3 II	M3 Total	M5 I	M5 II	M5 Total
Tip of scutellum (edge of the rootlet)	▦		▦	▦	▦	▦	▦	▦	▦
Tip of scutellum (edge of the coleoptile)				▦	▦	▦	▦	▦	▦
General scutellum tissue				▦	▦	▦			
Scutellum epithelium				▦	▦	▦	▦	▦	▦
Rootlet tissue							▦	▦	▦
Ventral trace region					▦	▦	▦	▦	▦
Coleoptile							▦	▦	▦
Shoot primordium							▦	▦	▦
Aleurone				▦	▦	▦	▦	▦	▦

Figure 4.19: Summary of (1,3;1,4)-β-glucanase isoenzymes EI and EII expression in Sloop grain as detected by mRNA.

Tissue	M1 I	M1 II	M1 Total	M3 I	M3 II	M3 Total	M5 I	M5 II	M5 Total
Tip of scutellum (edge of the rootlet)	▦	▦	▦	▦	▦	▦			
Tip of scutellum (edge of the coleoptile)	▦	▦	▦		▦	▦			
General scutellum tissue	▦	▦	▦						
Scutellum epithelium				▦	▦	▦		▦	▦
Rootlet tissue	▦		▦		▦				
Ventral trace region	▦		▦	▦	▦	▦	▦	▦	▦
Coleoptile					▦	▦	▦	▦	▦
Shoot primordium							▦	▦	▦
Aleurone				▦	▦	▦	▦	▦	▦

Chapter 5

Identification and location of specific (1→4)-β-xylan endohydrolase mRNA in germinated *Hordeum vulgare* L. grain

5.1: Introduction

During germination, cell walls constitute a physical barrier that limits the access of hydrolytic enzymes secreted from the aleurone layer or the scutellum to their substrates within the cells of the starchy endosperm. Arabinoxylans represent up to 20% of barley endosperm cell walls by weight and 67% to 85% of aleurone cell walls (Bacic and Stone, 1981; McNeil *et al.,* 1975) with the highest concentration close to the plasma membrane in the aleurone layer (Guillon *et al.*, 2004). Therefore, degradation of cell wall arabinoxylans is believed to be an important factor contributing to successful endosperm modification and is mediated, in part at least, by the action of (1→4)-β−xylan endohydrolases (EC 3.2.1.8, endoxylanase) and exohydrolases, with endoxylanases as the prevalent form. In 1958, Preece and MacDougall described an inter-dependent scheme of enzymes present in various cereals that degrade arabinoxylan including arabinosidase, endoxylanase, exoxylanase, and xylobiose. Synthesis of such enzymes during germination of barley is reported to be slow and therefore the ultimate extent of arabinoxylan breakdown is minimal (Preece and MacDougall, 1958; Slade *et al*., 1989; MacGregor, 1990). The arabinoxylan wall content has recently been found to remain essentially constant at 25-30% during barley germination (Gibeaut *et al*., 2005), although a change in the fine structure of arabinoxylans has been reported, with the ratio of substituted to unsubstituted 4- linked xylosyl units changing from about 4:1 to 1:1 (Gibeaut *et al*., 2005). About 80% of backbone xylopyranosyl residues in newly synthesised arabinoxylan are substituted with arabinosyl residues but these were progressively removed during coleoptile growth (Gibeaut *et al*., 2005). The removal of the arabinosyl residues is probably mediated by the action of arabinoxylan arabinofuranohydrolases (Lee *et al.,* 2001; Laidlaw *et al*., 2012).

Endoxylanases (xylanases), some of which contain various domains in addition to the catalytic domain, have been reported in various micro-organism (Gilkes *et al.,* 1991, Tomme *et al*., 1995; review by Juturu and Chuan Wu, 2014), and in plants have been reported in barley, Arabidopsis and Maize (Bih *et al*., 1999; Zhang *et al.,* 2013; Kanauchi et al., 2013). The role that barley xylanase plays in aleurone and endosperm cell wall breakdown is still inconclusive, as xylanase is detected in the medium of *in vivo* extractions somewhat later than other hydrolytic enzymes (Taiz and Honigman, 1976; Ashford and Gulber, 1984; Casper *et al*., 2001). Some believe that xylanase is not responsible for early cell wall hydrolysis (Taiz and Honigman, 1976), although Kuntz and Bamforth (2001, 2007) have reported the presence of xylanase in grain tissue during steeping of the grain and an arabinoxylan degradation product has been

detected some 16 h before the release of the xylanase protein itself (Dashek and Chrispeels, 1977). The common explanation amongst the majority of authors is that the xylanases are tightly bound to the aleurone wall during the formation of channels and are only released when degradation of the outer wall layer is complete (Fincher, 1989). Fusion of GFP to *Arabidopsis thaliana* xylanase (AtXyn1), which has similarities in its deduced amino acid C terminal end to the catalytic domain of barley xylanase isoform I (X-I), showed it was a cell wall protein (grain extracellular), predominantly present in the vascular bundles (Suzuki *et al.*, 2002). The timing coincides with cellular breakdown linked to programmed cell death (PCD) in aleurone cells (Wang *et al*., 1996; Kuo *et al.,* 1996). Indeed, Casper *et al.* (2001) demonstrated a strong association with the release of xylanase isoenzyme I from aleurone cells and PCD. However, these observations still do not provide a definite role for the xylanase enzymes during germination.

A number of barley (1→4)-β-endoxylanase isoenzyme cDNAs have been isolated (Banik *et al*., 1996; Casper *et al*., 2001). Banik *et al*. (1996) initially isolated a near full length sequence for xylanase I (pMXI, Figure 5.1) which encodes a polypeptide containing 427 amino acid residues, including a putative signal peptide of 32 amino acids (Figure 5.2). Casper *et al.* (2001) completed the full-length open reading frame (ORF) and presented evidence of a 5' intron located in the xylanase isoenzyme I gene, which leads to alternative splicing. This provides an explanation for inconsistency in the literature with regard to an estimated molecular weight of between 34 to 44.6 kDa's and an isoelectric point of either 5.2 or 6.1 (Slade *et al*., 1989; Banik *et al*., 1996; Benjavongkvlchai and Spencer, 1986). Immuno-staining with a specific xylanase antibody (XYN-I) has shown that a large protein precursor (61.5 kDa) exists early in germination (day 4), which is reduced to 40 kDa around day 8 of germination and by day 10 this has been cleaved further down to 34 kDa (Casper *et al.,* 2001). This series of processing steps is mediated in part by aleurone cysteine endoproteases which attack the protein at the carboxy terminal (Casper *et al.,* 2001), in a similar way to that seen for the activation of β-amylase (Hejgaard, 1979).

Banik *et al*. (1996) also identified an incomplete cDNA for xylanase isoenzyme II (pMXII) derived from a separate gene to xylanase isoenzyme I (Figure 5.1). There is sequence identity of 91% at the nucleotide level between xylanase isoenzyme I and xylanase isoenzyme II, which represents a 13% divergence in the deduced amino acid sequences (Figure 5.2). Southern blot analysis suggests that these two xylanase cDNAs correspond to two of the three or four genes in the xylanase family (Banik *et al*., 1996),

which is also supported by the purification of three barley xylanases as described by Slade *et al*., (1989).

Both exo- and endo- xylanases have been demonstrated in germinating barley grain, specifically in the aleurone layers and root tissue (Dashek and Chrispeels, 1977; Taiz and Honigham, 1976), but not in the shoots of the growing seedling (Slade *et al*., 1989; Benjavongkvlchai and Spencer, 1986). Northern blots indicate that xylanase mRNA is present at high levels in GA_3 treated aleurone layers, but is low or absent from scutellum, coleoptiles, roots and young leaves of germinated grain (Banik *et al*., 1996). Ions, such as Ca^{2+}, Sr^{3+} or Mg^{3+}, in association with GA_3, are essential for the *de novo* synthesis and secretion of xylanase in barley (Dashek and Chrispeels, 1977; Taiz and Honigmam, 1976; Eastwell and Spencer, 1982; Deikman and Jones, 1985; Benjavongkvlchai and Spencer, 1989; Casper *et al*., 2001). A GA_3 response complex in the xylanase gene was identified (Banik *et al.,* 1996).

The present study was aimed at elucidating a clearer role that xylanase might play in barley germination, in an effort to expand on data already reported, and with regard to the publication by Casper *et al.* (2001). Given the substantial amount of arabinoxylan present in the both the endosperm and aleurone cell walls of a barley grain, the role of xylanase is presumed to be as important as that of (1→3,1→4)-β-D-glucanase (Chapter 4) for successful modification of grain. In fact, the degradation of arabinoxylan and xylanase levels is positively correlated (Schwartz and Han, 1995) to malt extract values and to the process of grain modification involving the breakdown of endosperm cell wall (1→3,1→4)-β-D-glucan. Using the techniques of *in situ* hybridization (ISH, as described in Chapter 3) the location of endoxylanase via mRNA levels in germinated barley grain was examined. In this Chapter, ISH experiments are described in which riboprobes transcribed from a cDNA encoding xylanase isoenzyme I (Banik *et al*., 1996) were used.

As stated previously, the nucleotide identity between xylanase isoenzyme I and xylanase isoenzyme II is extremely high (Figure 5.1). Therefore in order to discriminate between the two xylanase isoenzymes, attempts were made to isolate the divergent 3' untranslated region of the xylanase II cDNA by screening a number of libraries, both genomic and cDNA, and by using a number of different PCR based techniques with different sets of primers on a range of templates, made from various tissues including GA_3-treated aleurone and 5 or 10 day old leaf tissue. All attempts to isolate this sequence wwere unsuccessful. Instead, an indirect approach was used whereby the 3' untranslated region of the xylanase isoenzyme I cDNA, which shows

the highest sequence divergence from xylanase II, was isolated by PCR and subcloned into pBluescript SK+. This was used as a specific probe against the xylanase isoenzyme I (XYN-I), whilst the whole coding region of xylanase isoenzyme I (total XYN) was used as a second probe which was assumed to detect both xylanase isoenzyme I and xylanase isoenzyme II and possibly mRNAs from other as yet uncharacterised (1→4)-β-xylanase genes.

The specific aims of the work presented in this Chapter were to record the presence of combined total XYN and XYN-I specific mRNAs through ISH and RT-PCR during barley germination. This would allow documentation of the specific loaction of combined total xylanase and specific xylanase I mRNA as germination proceeds, whilst also correlating the transcript to the presence of active enzyme. These approaches were used in the hope of further clarifying the role of the xylanase enzymes in barley grain germination.

5.2: Material and methods
5.2.1: Tissue preservation

The schedule of barley grain germination used in this study is outlined in section 2.2.3 of Chapter 2. All the results presented in this chapter are from grain sections fixed in 4% paraformaldehyde and 0.25% glutaraldehyde, embedded in paraffin and sectioned with the microtome, as described in Chapter 2, sections 2.2.4; 2.2.5 and 2.2.6.

5.2.2: Modification pattern analysis

Longitudinal sections of barley grain collected at set time points during germination were stained with the CW dye, as described in section 2.2.5 of Chapter 2. The degree of (1→3, 1→4)-β-D-glucan degradation as detected by the amount of binding of CW dye under UV light determined the modification stage of the grain. As described in section 2.2.2, a total of 12 collection points were made over the germination schedule for each variety, and the sampled grains were then placed into five modification stages, M1 to M5.

As xylan production in the grain and accumulation of xylanase mRNA transcript both have been linked to a pathogenesis response (Fuchs *et al*., 1989; Aauini *et al*., 1994; Peltonen, 1995), grain used in this study was surface sterilized according to Hoy *et al.* (1990) and Banik *et al.* (1996).

5.2.3: Riboprobe preparation

To prepare the total xylanase (TXYN) DIG-labelled riboprobes, cDNA encoding the entire coding region of xylanase isoenzyme I (1.6 kb, Figure 5.1) was kindly provided by Mitali Banik (University of Adelaide) in pBluescript SK+. For riboprobe production, plasmid constructs were linearised with *Hind*III (NEB) and transcribed using T3 RNA polymerase (Boehringer-Mannheim) for sense probes or *Spe*I (NEB) followed by transcription with T7 RNA polymerase (Boehringer-Mannheim) for the anti-sense probes as described in section 3.2.5. The final total xylanase DIG labelled riboprobes (TXYN) were 1600 nucleotides long. Consideration was given to the length of this probe, and trials using an alkaline hydrolysed probe, as discussed in section 3.2.5 of Chapter 3, were performed. Use of a hydrolysed probe did not alter the staining pattern or the intensity of the signal in the *in situ* hybridization assay so full-length un-hydrolysed riboprobes were used.

The 3' UTR of xylanase isoenzyme I (XYN-I) was isolated by PCR from the original xylanase isoenzyme I cDNA kindly provided by Mitali Banik (University of Adelaide). The PCR primers are as indicated in Table 5.1. Primers Xieco (GeneWorks, incorporating an *Eco*R1 restriction site) and Xibam (GeneWorks, incorporating a *Bam*

HI restriction site) at 0.5 μL each (1-0.1 μg.μL^{-1}, 25 pmol), were added to Xy-1 mini-prep plasmid DNA (1 μL, 0.3 μg.mL^{-1}); with 2.5 μL 10 X PCR buffer (22 mM Tris-HCI (pH 8.4) 55 mM KCI, Invitrogen); 1.5 μL 25 mM MgCl$_2$ (10 X, Sigma); 1 μL 5 mM dNTP's; 1 μL Taq DNA polymerase (Invitrogen) and deoxyribonuclease free water to a final reaction volume of 25 μL. Reactions were performed in a Perkin-ElmerCetus DNA Thermal Cycler as follows; 94^{0}C, 2 min initial denaturation; 35 cycles of denaturation at 94^{0}C, 40 s, annealing at a primer specific temperature of 53^{0}C, 40 s, extension at 72^{0}C, 1 min; followed by a 2 min final extension at 72^{0}C. The PCR products were excised from a 1.2% (w/v) agarose gel (DNA grade, FMC BioProducts) run in 1 X TAE buffer (40 mM Tris, 1 mM EDTA, pH 8.0 adjusted with acetic acid) containing 0.1 μg.mL^{-1} ethidium bromide (Sambrook *et al.*, 1989). The fragments were purified from the gel using Geneclean (Breastec, Adelaide, Australia) according to the manufacturer's instructions and ligated overnight at 16^{0}C into the pGEM-T Vector (TEASY, Promega). T-vector ligation reagents included: l μL T vector, 5 μL 10 X supplied buffer; 1 μL ligase and 3 μL PCR fragments. Following transformation into *E.coli* competent cells (as described in section 3.2.6) the correct insert size was checked by PCR using the SP6 5'-TATTTAGGTGACACTATAG-3' and T7 5'-CGGGATATCACTCAGCATAATG-3' primers, Stratagene, Table 5.1) with annealing at 44^{0}C and PCR reagents as described above. All fragments used as templates for riboprobe transcription were sequenced to determine insert orientation and to verify the sequence as described in section 3.2. All sequences were subsequently checked on an advanced NCBI BLAST (version BLASTN 2.2.3) search (Altschul *et al.*, 1996) to eliminate cross-reaction with other sequences.

A positive clone containing the correct cDNA fragment was purified by plasmid mini-prep (section 3.2.9), the insert excised from the T vector with the restriction enzymes *Eco*RI and *Bam*H1, (section 3.2.5) and run on a 1.2% agrose gel as previously described. DNA of the correct size was cut from the gel and purified with the Geneclean procedure (Bresatec, Adelaide, Australia) according to manufacturer's instructions, then sub-cloned into *Eco*RI and *Bam*HI cut pBluescript SK+ (prepared as per section 3.2.3). For XYN-I riboprobe production plasmid constructs were linearised with *Bam*HI and transcribed using T3 RNA polymerase (Boehringer-Mannheim) for sense probes or digested with *Eco*R1 and transcribed using T7 RNA polymerase (Boehringer-Mannheim) for the antisense probes, as described in section 3.2.5. The resultant XYN-I DIG-labelled riboprobes were 136 nucleotides long.

Figure 5.1: Nucleotide sequence of the cDNAs encoding the full length of the barley (1→4)-β-endoxylanase XI (TXYN) and XII (XYN-I). XI: Begins at nucleotide 1 (NCBI accession number; U73749, Banik *et al.*, 1996, 1630 base pair total length). XII: Begins at nucleotide 1 (NCBI accession number; U59312, Banik *et al.*, 1996, 1550 base pair total length). Blue and green blocks indicate designed primers as per table 5.1.

```
XI    101 gcggtgtgcgccaaggagggcgggtggtcggagatcatgggcgccttccg 150
                                    | ||||||||||||||||||
XII     1 ...........................acagagatcatgggcgccttccg 23
              .         .         .         .         .
XI    151 gctcaggacggagccgcgcagcgccgcggtttacgtccacggcgcccccg 200
          ||||| |||||||||||||||||||||||||||||||| ||||||||||
XII    24 actcagcacggagccgcgcagcgccgcggtttacgtccagggcgcccccg 73
              .         .         .         .         .
XI    201 ccggcgtcgacgtcaaggtcatggatctccgcgtctacccggtggaccac 250
          ||||||||||||||||||||||||||| || |||| || | |||||| |
XII    74 ccggcgtcgacgtcaaggtcatggatcttcgtgtcttccacgcggaccgc 123
              .         .         .         .         .
XI    251 aaggcgcgcttcaggcagctcaaggacaagactgacaaggcgcgcaagag 300
          |||||||||||| ||| |||||||||||||||||||||||||||||||||
XII   124 aaggcgcgcttcacgcaactcaaggacaagactgacaaggcgcgcaagag 173
              .         .         .         .         .
XI    301 ggacgtgattctcaagctggggcacgccggcgggagcgggagcgggcgcgg 350
          |||||||| |||||||||||||| || || |||||                |
XII   174 ggacgtggttctcaagctgggcgcggcgacggga...............g 208
              .         .         .         .         .
XI    351 cggcgtccgtgcgcgtggtgcagttggacaacgccttcccccttcgggaca 400
          ||||| ||||||||| |||||||||||||    ||||| |||||||||
XII   209 cggcgcgcgtgcgcgtcgtgcagttggacaacagtttcccctttcgggaca 258
              .         .         .         .         .
XI    401 tgcatcaacacgtccgtcatccagaagccggccttcctcgacttcttcac 450
          |||||||||||| | |||||||||| || |||||| ||||||| |||||||
XII   259 tgcatcaacacgacggtcatccagaacccagccttcgtcgacttcttcac 308
              .         .         .         .         .
XI    451 caaccacttcgactgggccgtcttcgagaacgagctcaagtggtaccaca 500
          ||||||||| ||||||||||||||||||||||||||||||||||||||||
XII   309 caaccacttggactgggccgtcttcgagaacgagctcaagtggtaccaca 358
              .         .         .         .         .
XI    501 cggaggtgcagcagggccagctcaactacgccgacgccgacgcgctgctc 550
          ||||||| ||||||| || ||||||||||||||||||||||||||| |||
XII   359 cggaggcgcagcaagggcagctcaactacgccgacgccgacgcgctcctc 408
              .         .         .         .         .
XI    551 gcgttctgcgaccgcctgggcaagaccgtccgcggccactgcgtcttctg 600
          |  |||||||||||||||||||||||||| || ||||||||||||||||||
XII   409 gacttctgcgaccgcctgggcaagcgcgcccgcggccactgcgtcttctg 458
              .         .         .         .         .
XI    601 gtccgtggacggcgacgtgcagcagtgggtcaagaacctcaacaaggacc 650
          ||||    ||||||||| |||||||||||||||||||||||| ||   ||||
XII   459 gtccacggacggcgttgtgcagcagtgggtcaagaacctcgaccgcgacc 508
              .         .         .         .         .
XI    651 agctcaggtcctccatgcagagccgcctcgagggcctcgtctcccgctac 700
          |||||||||| || | || |||||| || ||||||| || |||||||||
XII   509 agctcaggtccgccgtccaaagccgcatccagggcctggtgtcccgctac 558
              .         .         .         .         .
XI    701 gccggcaggttcaagcactacgacgtcaacaacgagatgctgcacggccg 750
          |||||||| |||   ||||||||||||||||||||||||||||||||||
XII   559 gccggcagattcccgcactacgacgtcaacaacgagatgctgcacggccg 608
              .         .         .         .         .
XI    751 cttcttccgggaccgcctcggcgacgaggacgtcccggcgtacatgttca 800
          ||||||||||||||||||||||||||||||||||||||||||||||||||
XII   609 cttcttccgggaccgcctcggcgacgaggacgtcccggcgtacatgttca 658
              .         .         .         .         .
```

```
XI    801 aggaggtggcgcggctggacccggagcccgtgctcttcgtcaacgactac 850
          |||||||||||||||||||||||||| ||| ||||||||||||||||||||
XII   659 aggaggtggcgcggctggacccggaggccgcgctcttcgtcaacgactac 708
                .         .         .         .         .
XI    851 aacgtggagtgcggcaacgaccccaacgcgacgccggagaagtacgccga 900
          ||||||||||||||||||||||||||||||||||||||||||||||||||
XII   709 aacgtggagtgcggcaacgaccccaacgcgacgccggagaagtacgccga 758
                .         .         .         .         .
XI    901 gcaggtcgcatggctgcagagctgcggcgcggtggtgcgcggcatcgggc 950
           |||||||||||||||||||||||||||||||||||||||||||||||||
XII   759 ccaggtcgcatggctgcagagctgcggcgcggtggtgcgcggcatcgggc 808
                .         .         .         .         .
XI    951 tgcagggccacgtgcaaaacccggtcggggaggtcatctgcgccgcgctc 1000
          |||||||||||| |      ||||| |||||||||||||||||| ||| ||
XII   809 tgcagggccacatcagcaaccccgtcggggaggtcatctgcggcgcccta 858
                .         .         .         .         .
XI   1001 gacaggctcgccaagacggggggtgcccacctggttcaccgagctcgacgt 1050
          |||||||||||||      |||||  ||||||  ||||||||||||| ||||||
XII   859 gacaggctcgccgccacgggcgtgcccgtctggttcaccgagctggacgt 908
                .         .         .         .         .
XI   1051 gccggagtacgacgtgggcctccgcgccaaggacctggaggtggtgctcc 1100
          |   ||| ||||||| |||||||||||| ||||||||||||||||||||||
XII   909 gtgcgaggccgacgttggcctccgcgcccaggacctggaggtggtgctcc 958
                .         .         .         .         .
XI   1101 gggaggcgtacgcgcacccggccgtggagggcatcgtgttctggggcttc 1150
          ||||||||||||||||||||||||| |||||||||||||||||||||| ||
XII   959 gggaggcgtacgcgcacccggcggtggagggcatcgtgttctggggcatc 1008
                .         .         .         .         .
XI   1151 atgcagggcacaatgtggcgccagaacgcttggctcgtcgacgccgatgg 1200
          |||||||||| |||||||||| || |||| ||||| |||||||||| ||
XII  1009 atgcagggcaaaatgtggcgcaaggacgcctggcttgtcgacgccgacgg 1058
                .         .         .         .         .
XI   1201 caccgtcaacgaggcgggccagatgttcctgaatctgcagaaggagtgga 1250
          |||||| ||||||||| ||||||||| || ||||||| || ||||||||||
XII  1059 caccgtgaacgaggcaggccagatgctcatgaatctccacaaggagtgga 1108
                .         .         .         .         .
XI   1251 agacggacgcgcgggggaacttcgacggcgacgggaacttcaagttcagg 1300
          ||||||||||||||| |||||| ||||| || |
XII  1109 agacggacgcgcgcgggaacgtcgacaacgcc                   1158
                .         .         .         .         .
XI   1301 ggcttctacggcagatacgtcgtggaggttacgacggcgaagcggaagca 1350
                .         .         .         .         .
XI   1351 gatgctcaagaccttcacggtggagaaaggggacaacacacctgtcgtcg 1400
                                                      Xieco →
XI   1401 tggatttggctgacgcctgacggtgaatctatctaagaaactatttattt 1450
             Xieco →              X13S →
XI   1451 atacctatctaattacatgcaacacgtcaagggataattggttgtataat 1500
                .         .         .         .         .
XI   1501 tttcacatttctaaggtaacgggtattgtattttgtaagagaagtgtatg 1550
                .   ← Xibam .         .         .         .
XI   1551 gtatttgtactcctaaatctgatgaacatgattgaagcaaaatgcctatt 1600
                                            ← X13AS
XI   1601 ggtcttactagcaaaaaaaaaaaaaaaaaa.................... 1630
            ← X13AS
```

5.2.4: In situ hybridisation assay on sectioned barley grain

The *in situ* hybridisation procedure, developed as outlined in section 3.2.5 of Chapter 3, was used with the TXYN and XYN-I riboprobes.

5.2.5: Total RNA isolation from whole barley grain

As previously discussed in section 4.2.6, whole seedlings were sampled and total RNA isolated at intervals throughout germination at 32, 56 and 96 h from the start of steeping. A detailed procedure is outlined in section 4.2.6.

5.2.6: ComplementaryDNA synthesis and RT-PCR

To complement the *in situ* hybridization data, reverse transcription Polymerase Chain Reaction (RT-PCR) was employed to screen total RNA extracted from germinated barley grain. Synthesis of cDNA from the RNA samples using the ThermoScript RT-PCR system is outlined in section 4.2.7. PCR reactions were performed to detect the presence of cDNAs encoding XYN-I with the primers XI3S and XI3AS as listed in Table 5.1 (supplied by Geneworks, Adelaide, South Australia). The negative control included all reagents except cDNA template, while the positive control for this PCR reaction included TXYN mini-prep plasmid DNA as described for riboprobe production in section 5.2.4. PCR reactions contained the following reagents; cDNA (3 µL); 2.5 µL 10 X PCR buffer, 1.5 µL 25 mM $MgCl_2$; 1 µL 10 mM dNTP's; 0.5 µL each of 1-0.1 µg µL^{-1} primers; 1 µL Taq DNA polymerase; deoxyribonuclease free water to a final reaction volume of 25 µL. Reactions were performed in a Perkin-ElmerCetus DNA Thermal Cycler as follows; 94^{0}C, 2 min initial denaturation; 35 cycles of denaturation at 94^{0}C, 40 s, annealing at primer specific temperature, 40 s, extension at 72^{0}C, 1 min; followed by 2 min final extension at 72^{0}C. Amplified products were detected by electrophoresis at 100V on a 1.2% (w/v) agarose gel in 1 X TAE buffer containing 0.1 µg.mL^{-1} ethidium bromide (Sambrook *et al*., 1989).

5.2.7: Total xylanase enzyme analysis

Samples (250 µl) of extract as prepared in section 4.2.10 for (1→3, 1→4)-β-D-glucanase Western blot analysis, once centrifuged, were added to 250 µL sodium phosphate buffer (25 mM, pH 6.0) containing 10 mM sodium azide according to the instructions supplied with the Megazyme endo-1,4-β−xylanase assay kit (McCleary, 1992). A Xylazyme AX tablet (Megazyme, Bray Co, Wicklow, Ireland) was added to each sample and incubated for 10 min at 40^{0}C. The assay was stopped by the addition of 10 mL 2% Trizma base (pH 8.5, Sigma). The samples were vortexed, incubated at room temp for 5 min, vortexed again and filtered through Whatman 9 cm filter circles.

Figure 5.2: Amino acid sequence of XI (TXYN) and XII.
Complete XI, aligned against partial XII.

```
XI       1 MGAFRLRTEPRSAAVYVHGAPAGVDVKVMDLRVYPVDHKARFRQLKDKTD 50
           ||||||  ||||||||||  ||||||||||||||||:   |  |||| |||||||
XII      1 MGAFRLSTEPRSAAVYVQGAPAGVDVKVMDLRVFHADRKARFTQLKDKTD 50
               .            .         .         .         .
XI      51 KARKRDVILKLGTPAGAGAGAAASVRVVQLDNAFPFGTCINTSVIQKPAF 100
           |||||||:||||     |      || |||||||.||||||||.||| |||
XII     51 KARKRDVVLKLGAATG.....AARVRVVQLDNSFPFGTCINTTVIQNPAF 95
               .            .         .         .         .
XI     101 LDFFTNHFDWAVFENELKWYHTEVQQGQLNYADADALLAFCDRLGKTVRG 150
           .|||||| |||||||||||||| ||||||||||||||| ||||||| ||
XII     96 VDFFTNHLDWAVFENELKWYHTEAQQGQLNYADADALLDFCDRLGKRARG 145
               .            .         .         .         .
XI     151 HCVFWSVDGDVQQWVKNLNKDQLRSSMQSRLEGLVSRYAGRFKHYDVNNE 200
           |||||| || ||||||||.:|||||..|||::|||||||||| |||||||
XII    146 HCVFWSTDGVVQQWVKNLDRDQLRSAVQSRIQGLVSRYAGRFPHYDVNNE 195
               .            .         .         .         .
XI     201 MLHGRFFRDRLGDEDVPAYMFKEVARLDPEPVLFVNDYNVECGNDPNATP 250
           ||||||||||||||||||||||||||||| |||||||||||||||||||
XII    196 MLHGRFFRDRLGDEDVPAYMFKEVARLDPEAALFVNDYNVECGNDPNATP 245
               .            .         .         .         .
XI     251 EKYAEQVAWLQSCGAVVRGIGLQGHVQNPVGEVICAALDRLAKTGVPTWF 300
           ||||:|||||||||||||||||||||: ||||||||| |||||| |||| ||
XII    246 EKYADQVAWLQSCGAVVRGIGLQGHISNPVGEVICGALDRLAATGVPVWF 295
               .            .         .         .         .
XI     301 TELDVPEYDVGLRAKDLEVVLREAYAHPAVEGIVFWGFMQGTMWRQNAWL 350
           ||||| |  |||||||.|||||||||||||||||||||||| ||| |||..|||
XII    296 TELDVCEADVGLRAQDLEVVLREAYAHPAVEGIVFWGIMQGKMWRKDAWL 345
               .            .         .         .         .
XI     351 VDADGTVNEAGQMFLNLQKEWKTDARGNFDGDGNFKFRGFYGRYVVEVTT 400
           |||||||||||| :|| |||||||||| |
XII    346 VDADGTVNEAGQMLMNLHKEWKTDARGNVDNA                  395
               .            .         .         .         .
XI     401 AKRKQMLKTFTVEKGDNTPVVVDLADA...................... 427
```

The absorbence of the filtrate were measured at 590 nm against a reaction blank and activity determined by reference to the standard curve supplied with the Xylazyme AX tablets to convert absorbance to milli-Somogyi units of activity per assay. Xylanase activity units are defined as the amount of enzyme required to release one micromole of reducing sugar equivalents (as xylose by the Somogyi reducing-sugar method) from arabinoxylan per min under standard assay conditions (40^0C and pH 6) (Megazyme, McCleary, 1992). Values are expressed as units/ml of original preparation. Duplicate analyses were performed on each extract.

5.2.8: *Southern blot analysis of total XYN and XYN-I riboprobes*

As previously discussed in section 4.2.11, Southern blot analysis were performed to confirm the specificity of the DIG-labelled riboprobes (data not shown). The pBluescript SK+ clone containing the XYN-I cDNA were restriction cut with *Spe*I and *Hind*III to obtain the TXYN cDNA region, while a 3'untranslated cDNA fragment was obtained with restriction enzymes *Pst*I and *Eco*RI, according to the restriction map in Banik (1997), as described in Chapter 3 (section 3.2.3).

Table 5.1: Sequences of primers used in PCR and RT-PCR reactions.

All primers were manufactured by Geneworks, Adelaide, South Australia, *$EcoRI$ restriction site, &$BamHI$ restriction site. Bp, base pairs; Tm, annealing temperature for the PCR reaction.

Primer	Sense primer (5'nucleotide primer)	Primer	Antisense primer (3" nucleotide primer)	Tm	Product
Xieco*	CTGTCGTCGTGAATTCGGCTG	Xibam&	CAATACCCGTTACCTTAGAAATG	53^0C	**136bp**
XI3S	CCTGACGGTGAATCTATCTAAGAAA	XI3AS	GCTAGTAAGACCAATAGGCATTTT	50^0C	**200bp**
T7	CGGGATATCACTCAGCATAATG	SP6	TATTTAGGTGACACTATAG	44^0C	**150bp**

5.3: Results

5.3.1: *In situ hybridization of xylanase on sectioned barley grain throughout germination*

The grain sections collected throughout germination and categorised into stages M1 to M5 as described in Chapter 2 were assessed for TXYN and XYN-I mRNA expression by ISH. Sense riboprobes, as internal controls, were routinely run in parallel with the anti-sense riboprobes. The signal generated by the sense riboprobes was considered minimal, although a higher intensity of staining was observed in the starchy endosperm and crushed cell layer of a number of tissue sections, as seen with the (1→3,1→4)-β-D-glucanase and limit dextrinase *in situ* hybridisation results, and was therefore regarded as artifactual. Ungerminated grains of both varieties, immediately after imbibition, were shown to contain no TXYN and XYN-I mRNA since the staining was not above the background level of the control slides (data not shown).

5.3.1.1: Modification stage M1 for Sloop and Himalaya.

The *in situ* hybridization results for the variety Sloop at the earliest M1 stage (Figure 5.3) demonstrated that the mRNA for TXYN (Figure 5.3 a to d) first appears in embryo tissue early in germination, specifically in the ventral trace region (procambial strands, Figure 5.3 c and d, S16) and the coleorhiza (tissue surrounding the root-cap Barrero *et al.,* 2009; Figure 5.3 a and b, S17), and concentrated more on the ventral side of the grain near the growing rootlets and scutellum (Figure 5.3 a, S18). A higher magnification of the region (Figure 5.3 b) shows the cytoplasmic nature of the staining in the coleorhiza and surrounding parenchyma tissue at this stage for the TXYN mRNA (S17). Staining observed for the XYN-I riboprobe (Figure 5.3 e) was similar to that observed for total xylanase, including staining in the scutellum parenchyma tissue of the embryo, particularly around and at the tip of the rootlet (Figure 5.3 e, S17 and S20). The slight difference in the staining between Figure 5.3 a and e is because section a is from the outer part of the root and has missed the meristem/root cap.

The presence of XYN-I and TXYN mRNA was minimal in the growing coleoptile and surrounding scutellum tissue at this stage (Figure 5.3 c, S19). Sense control sections were clear of staining (Figure 5.3 f). The mRNA patterns detected by both riboprobes in Himalaya sections at M1 (Figure 5.4) were similar to that observed for Sloop sections, with mRNA concentrated in the root cap (H12) and the coleorhiza (Figure 5.4 a and b, H13). A surprising result from this early point in germination, for both varieties, was the presence of TXYN and XYN-I mRNA, as detected with ISH, in aleurone tissue (Figure 5.4 c, H14). Quite distinct expression in aleurone cells scattered

Figure 5.3: Messenger RNA for XYN-I and TXYN in Sloop grain at the M1 stage in germination. *In situ* hybridisation on longitudinal sections of Sloop at the M1 stage in germination with the following riboprobes. a), b), c) and d) – TXYN anti-sense riboprobe. e) – XYN-I (3' UTR of total xylanase) anti-sense riboprobe. Control sections were treated with DIG labeled sense riboprobes complementary to the anti-sense riboprobe TXYN (f).Bound riboprobes were detected with anti-DIG antibodies and NBT/BCIP staining. sc, scutellum; al, aleurone layer; rs, rootlets; co, coleoptile; vt, ventral trace and end, starchy endosperm. S16 – procambial strands, ventral trace region, S17 – coleorhiza, S18 – scutellum, S19 – coleoptile ,S20 – root cap. Scale bar represents 400 mm.

Blue colour a product of the microscope tungsten light
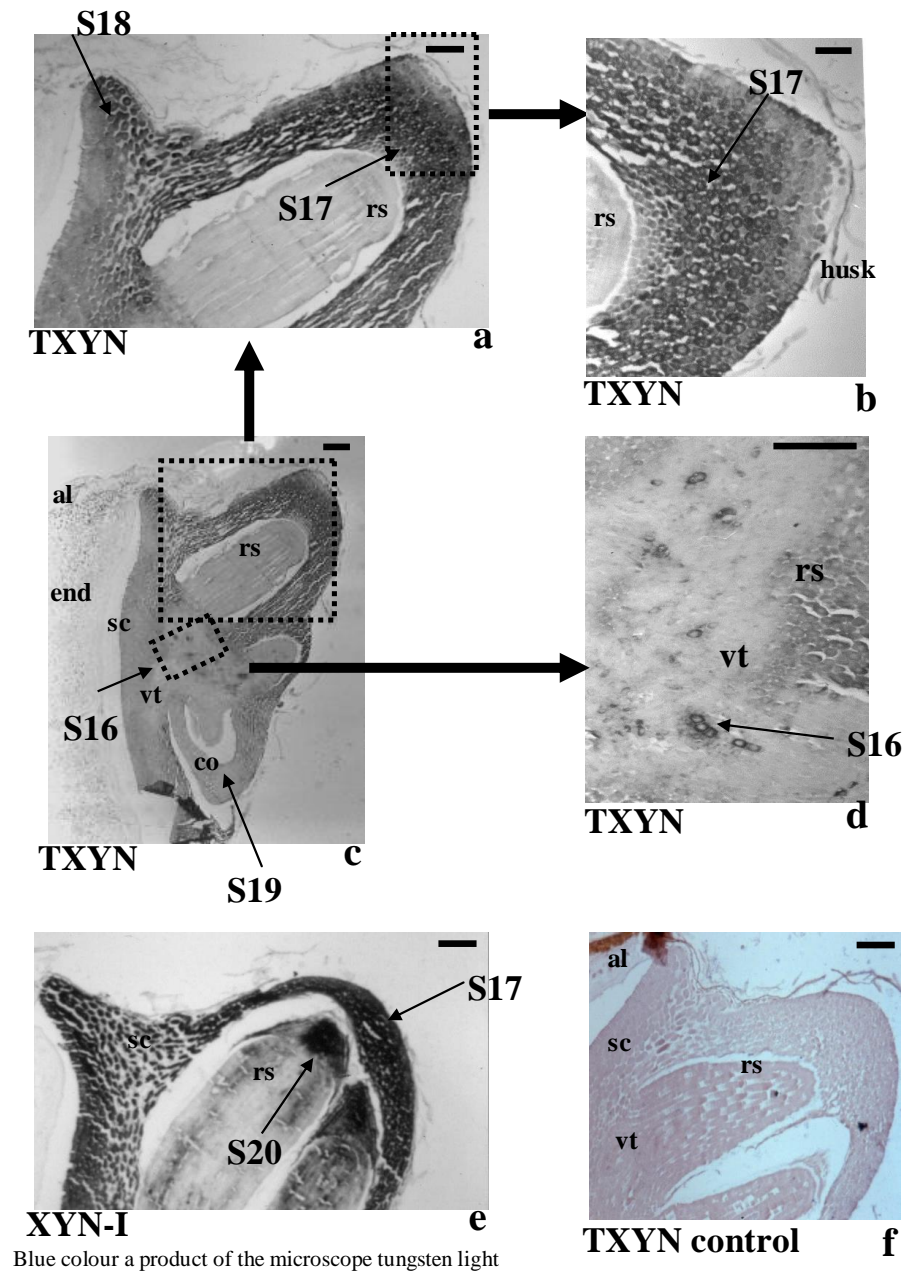
through the layers close to the embryo/scutellum on both the ventral and dorsal side of grain can be seen. The germ aleurone and ventral trace cells also appear to show positive staining for both Sloop and Himalaya (not shown). Sections stained with the control sense riboprobes were clear of signal (Figure 5.4 d).

5.3.1.2: Modification stage M2 in Sloop and Himalaya.

The M2 stage (data not shown) was not significantly different to the M1 stages.

5.3.1.3: Modification stage M3 in Sloop and Himalaya.

TXYN and XYN-I mRNA at the M3 stage on Sloop grain sections was found in the root cap (Figure 5.5 a, b and d, S20) and young embryo leaves (Figure 5.5 a and b, S21). Minimal xylanase mRNA is now present in the scutellum parenchyma tissue close to the scutellum epithelium (S22), and around the ventral trace and germ aleurone regions (Figure 5.5 a, b and d, S23). This similarity in mRNA at the M3 stage on Sloop sections for both probes was consistent throughout all sections. Sections treated with xylanase sense control riboprobes were clear of signal/staining (Figure 5.5 c). Aleurone labelling for both riboprobes in Sloop sections at this M3 stage was quite evident (data not shown), with mRNA now seen in all cells throughout the entire layer. Similar to the M3 stage for Sloop grain sections (Figure 5.5), Himalaya grain at M3 shows less parenchymal expression, with mRNA now concentrated in the growing rootlets and coleoptile of the seedling for both riboprobes (Figure 5.6 a, H16). Distinct XYN-I and TXYN mRNA expression in the scutellum epithelium on the ventral side of the grain is also evident (Figure 5.6 a, H17). A feature of the xylanase riboprobe signal on Himalaya grain sections at this stage was the specific pattern of mRNA expression at the ventral trace region (Figure 5.6 a and b, H18).

In contrast to staining seen in Sloop sections at this stage, Himalaya coleoptile has not yet developed to the size seen in Sloop, although when examining earlier Sloop sections with coleoptiles of a smaller size (Figure 5.5 b) staining of the distinct ventral trace node is not present.

At this stage, a slight delay in aleurone expression was noted between the Himalaya and Sloop sections; cells staining positive in the aleurone of Himalaya sections at M3 (data not shown) were only scattered through the layer along most of the ventral side of the grain, whilst all aleurone cells on both sides of the grain in Sloop sections at M3 displayed positive staining (data not shown). All Himalaya sections at this stage treated with the relevant xylanase sense (control) riboprobes were clear of staining (data not shown).
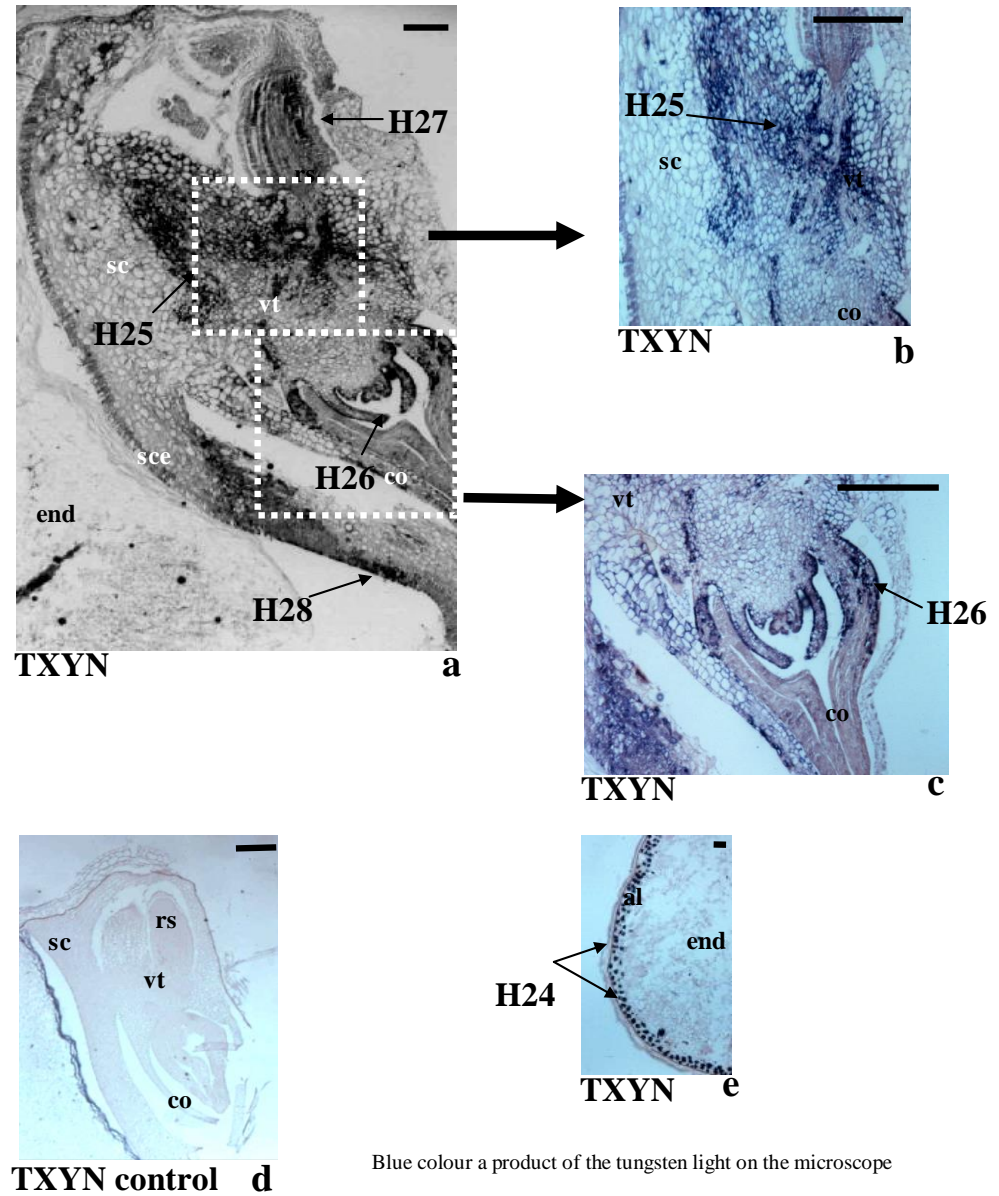
**Figure 5.4: Messenger RNA for TXYN in Himalaya grain at the M1 stage in germination.** *In situ* hybridisation on longitudinal sections of Himalaya at the M1 stage in germination with the following riboprobes. a), b) and c) - TXYN anti-sense riboprobes. Control sections were treated with DIG labelled sense riboprobes corresponding to the anti-sense riboprobes TXYN (d).Bound riboprobes were detected with anti-DIG antibodies and NBT/BCIP staining. sc, scutellum; al, aleurone layer; vt, ventral trace; rs, rootlets; co, coleoptile and end, starchy endosperm. H13 – scutellum. H14 – aleurone Scale bar represents 400 mm.
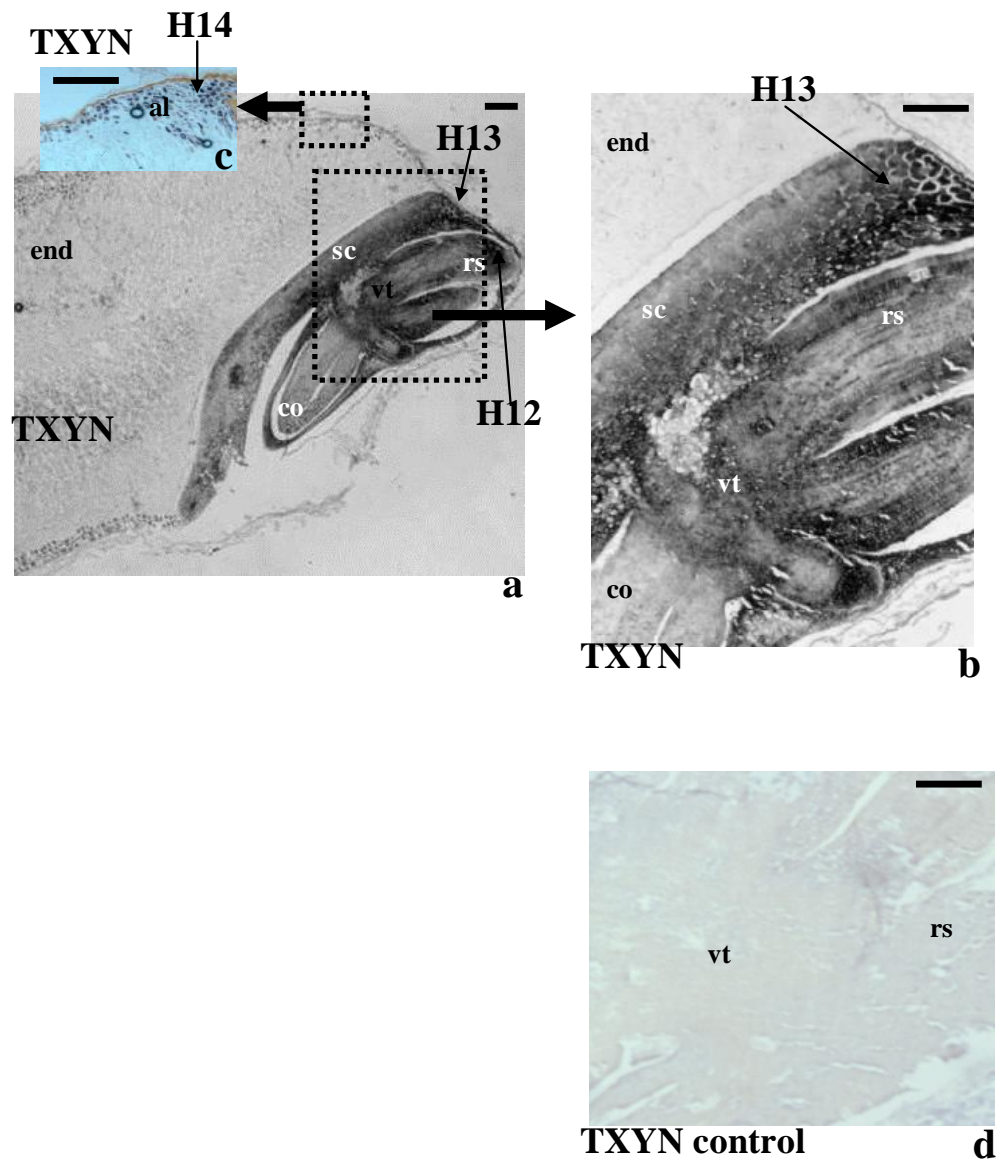
Figure 5.5: Messenger RNA for TXYN and XYN-I in Sloop grain at the M3 stage in germination. *In situ* hybridisation on longitudinal sections of Sloop at the M3 stage in germination with the following riboprobes. a)– TXYN anti-sense riboprobe. b) and d)- XYN-I anti-sense riboprobe.Control sections were treated with DIG labelled sense riboprobes corresponding to the anti-sense riboprobes TXYN (c).Bound riboprobes were detected with anti-DIG antibodies and NBT/BCIP staining. sc, scutellum; al, aleurone layer; vt, ventral trace; sce, scutellum epithelium; rs, rootlets; co, coleoptile and end, starchy endosperm; cc, crushed cell layer. S20 – root cap, S21 – coleoptile, S22 – scutellum epithelium. S23-ventral trace and germ aleurone. Scale bar represents 400 mm
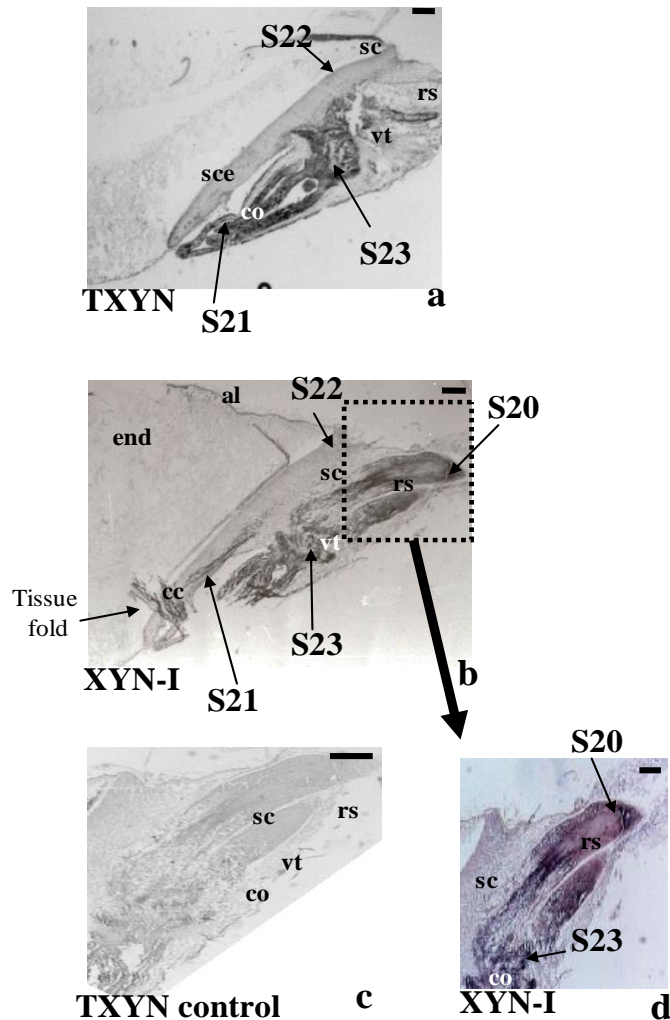
5.3.1.4: Modification stage M4 in Himalaya.

The main points of interest in Himalaya sections (Figure 5.7) for xylanase mRNA moving into Stage 4 was in the rootlets (H19), coleoptile and shoot primordium (Figure 5.7 a, b and d, H20) and ventral trace areas (Figure 5.7 a and b, H21), along with the scutellum epithelium area on the ventral side of the grain (Figure 5.7 a, H22). Expression of TXYN in the germ aleurone was also observed (Figure 5.7 c, H23) in areas where the layer still existed. Control sections were clear of staining (Figure 5.7 e).

5.3.1.5: Modification stage M4 and M5 in Sloop and M5 in Himalaya.

Signal on sections of Sloop grain at M4 (not shown) and M5 (Figure 5.9) were very similar. By the M5 stage (an average of approx 90 h from the start of steeping; Figure 5.8 showing Himalaya) the xylanase mRNA had decreased in the scutellum/embryo tissues and was mostly found at high levels throughout the aleurone layers (Figure 5.8 e, H24). A significant amount of mRNA as detected by both riboprobes was also still evident in the ventral trace (Figure 5.8 a and b, H25), embryonic seedling leaves (Figure 5.8 a and c, H26), rootlets (Figure 5.8 a, H27) and scutellum epithelium (Figure 5.8 a, H28). Control (sense riboprobes) treated sections were clear of staining (Figure 5.8 e). At these stages for Sloop, signal from both riboprobes was quite evident in the coleoptile (Figure 5.9 a, S24) and aleurone layers (Figure 5.9 a, S25) but very minimal in the scutellum and embryo. Although the preservation of the rootlets at this stage of germination is difficult, there appeared to be no xylanase mRNA in this tissue at M4 or M5. Control sections (Figure 5.9 b) were clear of staining.

For simplicity, a visual summary of the mRNA expression patterns observed for total xylanase and XYN-I using *in situ* hybridisation, on longitudinally cut barley grain sections, is presented in Figure 5.10.

5.3.2: Total xylanase enzyme levels.

Total xylanase enzyme activity was measured in extracts of grain samples taken at (32, 56 and 96 h) using the commercially available Megazyme assay. Increase in activity of xylanase was detected in Sloop extracts later in germination while levels in Himalaya appear constant from 32h onwards within the time frame of this study (Figure 5.11).

Figure 5.6: Messenger RNA for XYN-I and TXYN in Himalaya grain at the M3 stage in germination. *In situ* hybridisation on longitudinal sections of Himalaya at the M3 stage in germination with the following riboprobes. a) and b) – XYN-I anti-sense riboprobe. Control sections were treated with DIG labelled sense riboprobes corresponding to the anti-sense riboprobe TXYN (c). Bound riboprobes were detected with anti-DIG antibodies and NBT/BCIP staining. sc, scutellum; al, aleurone layer; vt, ventral trace; sce, scutellum epithelium; rs, rootlets; co, coleoptile and end, starchy endosperm. H16 – growing rootlets and shoots. H17 –scutellum epithelium, H18 – ventral trace region. Sacle bar represents 400 mm.
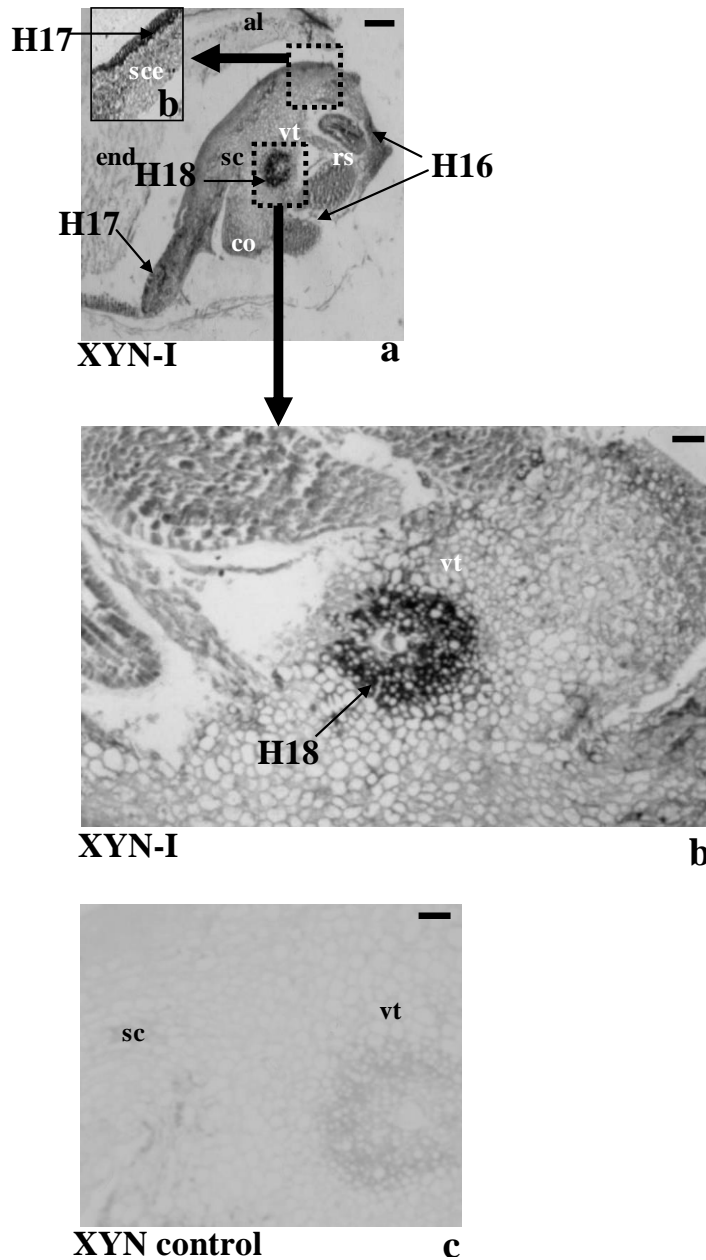
Figure 5.7: Messenger RNA for XYN-I and TXYN in Himalaya grain at the M4 stage in germination. *In situ* hybridisation on longitudinal sections of Himalaya at the M4 stage in germination with the following riboprobes. b), c) and e) – TXYN anti-sense riboprobe. a) and d) – XYN-I anti-sense riboprobe. Control sections were treated with DIG labelled sense riboprobes corresponding to the anti-sense riboprobes TXYN (e).Bound riboprobes were detected with anti-DIG antibodies and NBT/BCIP staining. sc, scutellum; al, aleurone layer; gal, germ aleurone; rs, rootlets; co, coleoptile and vt, ventral trace. H19 – growing rootlet, H20 – coleoptile, H21– ventral trace region
H22 – scutellum epithelium, H23 – germ aleurone. Scale bar represents 400 mm.
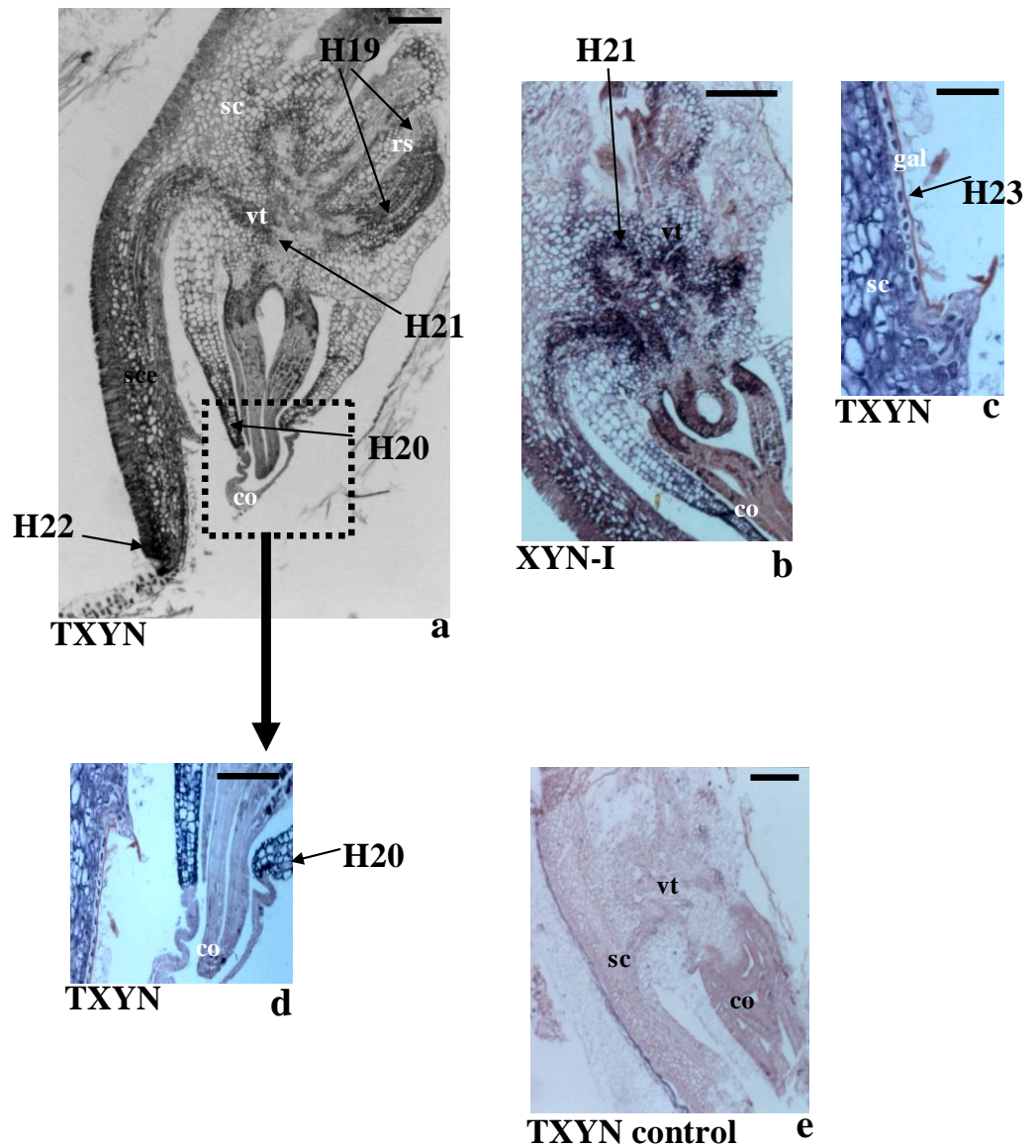
Figure 5.8: Messenger RNA for TXYN in Himalaya grian at the M5 stage in germination. *In situ* hybridisation on longitudinal sections of Himalaya at the M5 stage in germination with the following riboprobes. a), b), c) and e) – TXYN anti-sense riboprobe. Control sections were treated with DIG labelled sense riboprobes corresponding to the anti-sense riboprobe TXYN (d). Bound riboprobes were detected with anti-DIG antibodies and NBT/BCIP staining. sc, scutellum; al, aleurone layer; vt, ventral trace; sce, scutellum epithelium; rs, rootlets; co, coleoptile and end, starchy endosperm. H24 – aleruone tissue, H25 – ventral trace region, H26 – growing leaves, H27 – rootlets, H28 – scutellum epithelium. Scale bar represents 400 mm

Figure 5.9: Messenger RNA expression of XYN-I in Sloop grain at the M5 stage in germination. *In situ* hybridisation on longitudinal sections of Sloop at the M5 stage in germination with the following riboprobes. a) – XYN-I anti-sense riboprobe. Control sections were treated with DIG labelled sense riboprobes corresponding to the anti-sense riboprobe XYN-I (b). Bound riboprobes were detected with anti-DIG antibodies and NBT/BCIP staining. sc, scutellum; al, aleurone layer; co, coleoptile and end, starchy endosperm. S24 – Shoot growing between the aleurone and maternal tissue, S25 – aleruone tissue. Scale bar represents 400 mm.
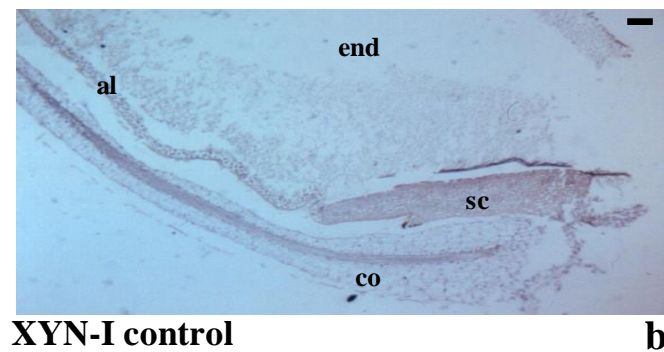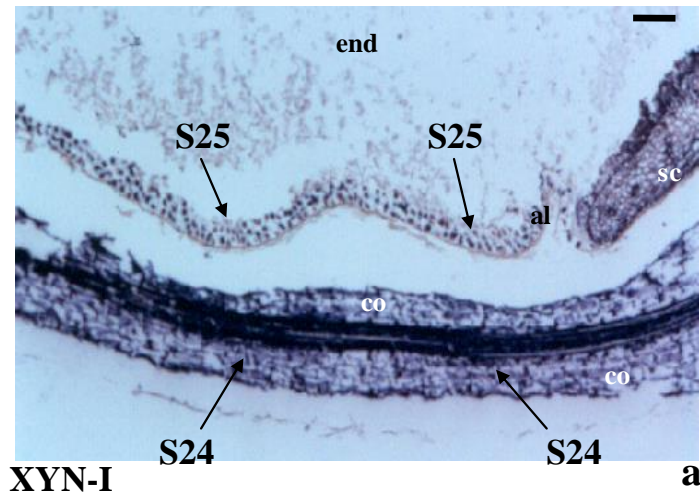
Figure 5.10: Hand drawings of the mRNA patterns observed for TXYN and XYN-I.

A: Sloop   M1   M3   M5

B: Himalaya   M1   M3   M5

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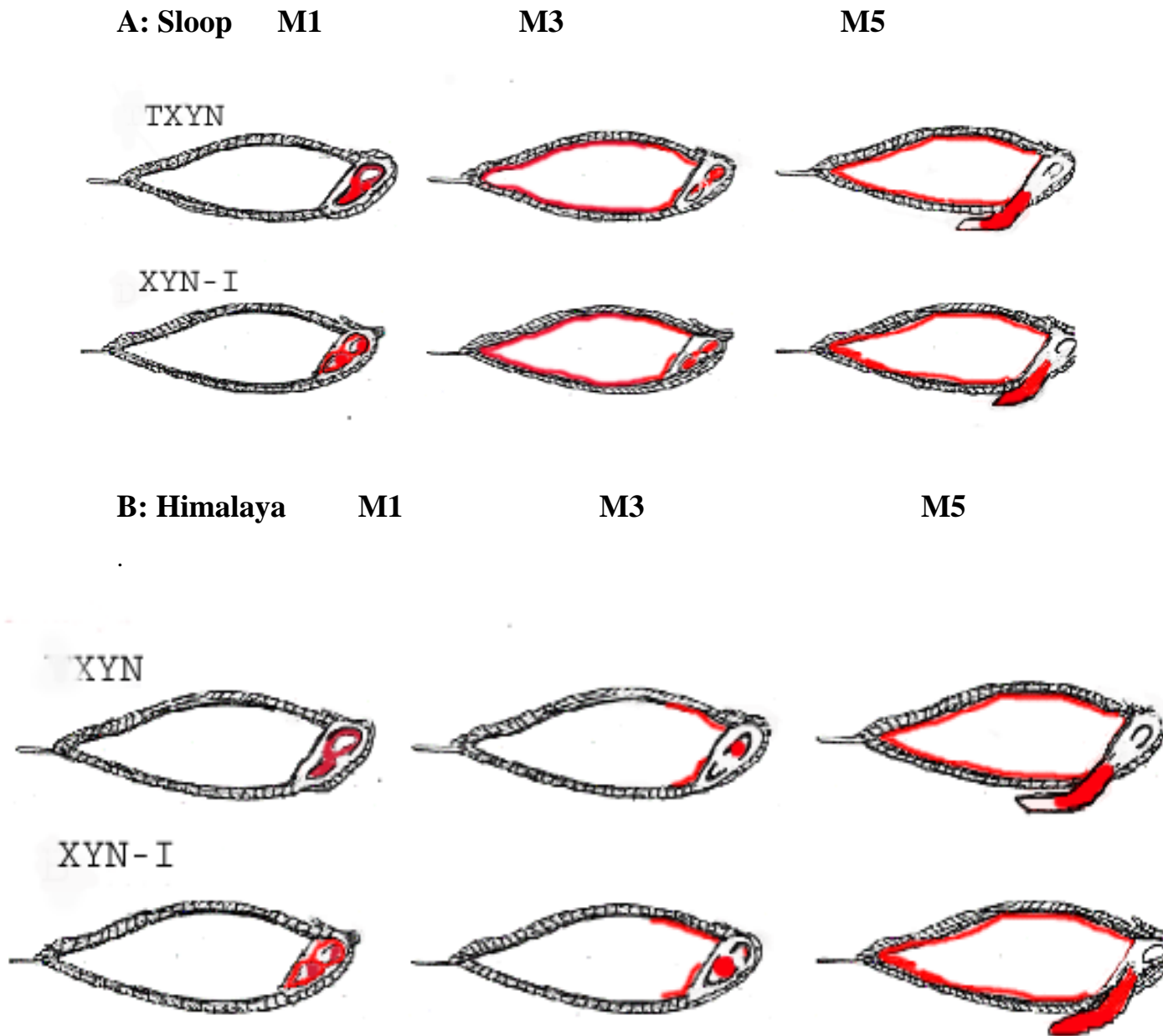
Hand drawn diagram showing staining is modified from Figure 2.1.   A: Sloop. B: Himalaya

Figure 5.11: Total (1→4)-β-xylanase β enzyme levels. Extracts of grain germinated and collected at the times shown (32, 56 and 96 h) were analysed to determine total active endxylanase enzyme levels. Values on the graph show absorbance (590 nm) and are expressed as specific activity per grain extract sample. H; himalaya, S; sloop,. h; hours
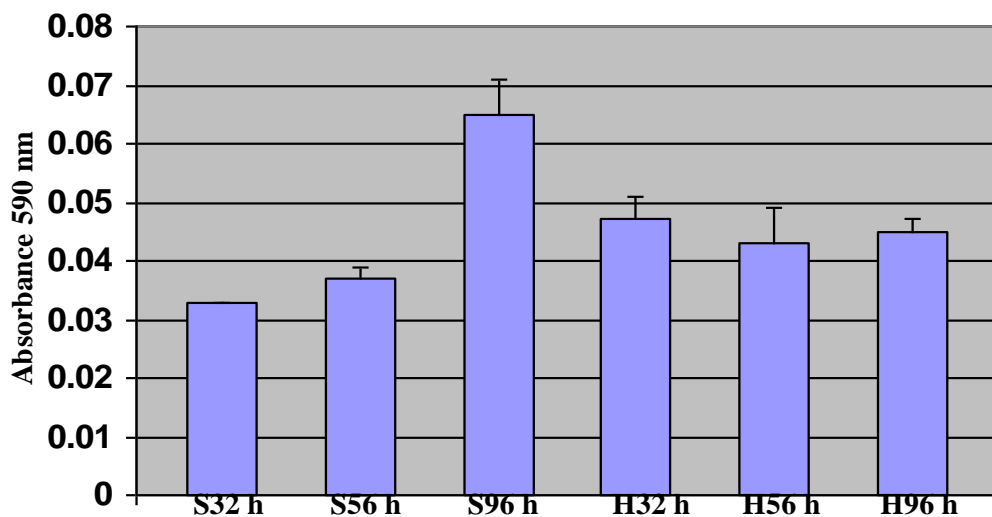
Name of grain sample	Absorbance 590nm	Xylanase, Somogyi milli-units/assay*
Sloop 32 h	0.032	**2**
Sloop 56 h	0.037	**2**
Sloop 96 h	0.065	**7**
Himalaya 32 h	0.047	**5**
Himalaya 56 h	0.042	**5**
Himalaya 96 h	0.043	**5**

*Activity from standard curve using *Trichoderma Longibrachiatum* xylanase (pI 9.0) and Xylazyme AX at pH 6.0 (Megazyme).

5.4: Discussion.

In this Chapter, the main focus was to directly document the mRNA patterns of endoxylanases during barley grain germination. This study has implications for the role that endoxylanase might play in arabinoxylan breakdown during barley grain germination, not just for the malting and brewing industries but also in the animal feed and food industries.

5.4.1: In situ hybridisation assessment of germinated barley grain for endoxylanase transcripts.

The appearance of (1→4)-β-xylanase mRNA transcripts using ISH was monitored in barley grain sampled throughout germination. It was found in the embryo early in germination followed by appearance in the aleurone layers of the grain as germination progressed in both varieties. A pattern was observed that seemed very similar to that seen for (1,3;1,4)-β-glucanase mRNA (Chapter 4), particularly that of the isoenzyme EI early in germination and the isoenzyme EII in later stages. This is not surprising, as arabinoxylan and β-glucan are major components of the aleurone and starchy endosperm cell walls, and their concomitant degradation is necessary for successful grain endosperm modification.

Others have suggested a similar profile for the glucanase and xylanase enzymes (Banik *et al.,* 1997), but is likely that distinctly different posttranslational modifications of each protein occurs, to render the enzymes active as suggested by Casper *et al.* (2001). This study may help explain why mRNA was observed in a range of tissues well before active enzyme was detected by the activity assay and as noted by past researchers (Figure 5. 12). This includes the presence of xylanase mRNA in the embryo early in germination and the previously unreported observation relating to xylanase mRNA in the aleurone and germ aleurone of the grain when compared to the expression of (1,3;1,4)-β-glucanase at the equivalent M3 stage (Figure 4.8). It is quite clear from the studies of Casper *et al.* (2001) that this enzyme may have a complicated post-translational activation system and/or may be under the influence of inhibitors. A monomeric barley xylanase inhibitor protein (HVXI, XIP-I or TAXI-I) has been previously identified (Gosaert *et al*., 2001), subsequently cloned by Elliott *et al.,* (2002), the X-ray crystallography structure deduced (Flatman *et al*., 2002) and then the specificity and mechanism of interaction reported by Payan *et al*. (2003). This inhibitor appears to be in two molecular forms and is homologous to *Triticum asetivum* L xylanase inhibitor I, an endoxylanase inhibitor found in wheat flour (Bonnin, *et al*.,

1999; Gosaert *et al*., 2001; Elliott *et al*., 2002; Gebruers *et al.,* 2002; Weng *et al.,* 2010). Subsequently the XIP and TAXI inhibitors have been identified in a range of cereals (Goesaert *et al*., 2000; 2003; Elliott *et al*., 2002). These inhibitors may play a role in the plant's defence system, as they show homology with other plant defence proteins (Elliott e*t al*, 2002). The xylanase inhibitors are predominantly located in the aleurone layer of wheat grain (Goesaert *et al.,* 2004) although Dornex (2009) noted that TAXI type inhibitors do not appear to inhibit plant type xylanases. Here xylanase mRNA as measured by ISH was found much earlier at 1 day after imbibition than that detected by Casper *et al*. (2001) at 4 days after imbibition.

The higher sensitivity of the ISH method used in this study could certainly account for the earlier detection when compared to the method which was used by Casper *et al*. (2001) based on protein detection using western blot analysis and RNA by Northern blots. An alternative explanation could be linked to the germination temperature used in this study, as highlighted by Slade *et al*. (1986), who demonstrated that xylanase was expressed earlier in grain germinated at 26^0C. The grain in this study was germinated at 22^0C, whilst that Casper *et al*. (2001) used was germinated at 15^0C.

5.4.1.1: Modification stage M1.

This study is the first to demonstrate that xylanase may play a role in the very early growth of the seedling, particularly in the rootlets and parenchyma tissue of the scutellum. Predominant expression of the xylanase-I isoenzyme is assumed at this stage, since a similar level of expression could be observed when using both the total XYN and XYN-I riboprobes. Expression in both barley varieties was quite obvious in the rootlet differentiation zone, coleorhiza, root cap, scutellum and the ventral trace region of the embryo tissue at an early stage. The root tissues are likely to be expanding quickly so the xylanase may be required to loosen cell wall components to allow growth.

As previously mentioned, a very similar expression profile was noted for both (1,3;1,4)-β-glucanase isoenzymes in Sloop grain sections, particularly at the tip of the embryo, near the acrospire, and the scutellum parenchyma around the rootlet (Figure 5.4). This matches the GFP activity noted for the AtXyn promoter fusion made by Suzuki *et al*. (2002), where primary expression was in the root and regions and coleorhiza (ventral trace area). Many reasons for glucanase expression in this area of the embryo early in germination are given in Chapter 4 (Section 4.4.2.1) and these could also be extended to explain the presence of xylanase mRNA, therefore assuming

that xylanase is part of this particular group of enzymes which play a distinct role early in germination. Although the presence of mRNA does not necessarily mean active protein and as xylanase protein expression was not studied due to the lack of availability of antibodies, a follow up study of grain early in germination would be worthwhile. The study by Kuntz and Bamforth (2007) is supportive, with xylanase and carboxypeptidase activity detected early in germination before glucanase expression. They attributed this finding to a steeping regime not dissimilar to the one presented here. The earlier expression of xylanase in the aleurone of Himalaya ahead of the other measured cytolytic enzymes is an interesting finding, as also reported by Kuntz and Bamforth (2007), who suggest a role for xylanase in surface micro-organism control.

5.4.1.2: Modification stage M3.

Similar patterns of expression were noted at M3 when both XYN-I and total XYN riboprobes were used for ISH. There was distinct XYN-I mRNA in the rootlets of both Sloop and Himalaya sections at M3, consistent with previous Northern blot analysis of xylanase mRNA (Banik *et al.,* 1996). This would imply a potential role for xylanase in combination with glucanase in early rootlet development, as discussed in Chapter 4.

Xylanase mRNA in the shoot was also noted at M3 which appears to be a novel finding. The presence of the 61 KDa inactive xylanase protein in roots and shoots of the early embryo was not investigated by Casper *et al*. (2001) but may be worth considering as the presence of message in grain at this stage is still much earlier when compared to the presence of active enzyme (Figure 5.12). This is particularly relevant in light of Van Campenhout *et al.* (2007) who indicated that the precursor form of X-I may indeed be active.

As with (1,3;1,4)-β-glucanase mRNA for isoenzyme EII (Chapter 4) the presence of xylanase mRNA was consistently seen at the ventral trace region of the scutellum/embryo, implicated as the site of GA_3 synthesis. The effect of GA_3 on xylanase mRNA was not assessed in this study, although it would be worthy of future investigation as levels of secreted xylanase isoenzyme I protein and mRNA are known to be enhanced by GA_3 (Banik *et al.,* 1996; Casper *et al.,* 2001).

5.4.1.3: Modification stages M4 and M5.

The aleurone layers appear to be another major site of endoxylanase mRNA later in germination. This is consistent with reports by several authors (Taiz and Honigham**,** 1976; Dashek and Chrispeels, 1977; Benjavongkvlchai and Spencer, 1989; Banik *et al.,*

1996; Figure 5.8e, H24). There is also an indication that mRNA staining was more predominant in the outer layer (lemma) at the seed coat interface than at the endosperm lumen, as reported to be the predominant location of wheat arabinoxylan using arabinoxylo-oligosaccharide antibodies by Guillon *et al.* (2004). Clarification is required to confirm whether the mRNA is translated to produce inactive protein, an active protein is secreted into the endosperm or an enzyme for the purpose of the degradation of the outer aleurone cell wall is produced as proposed by Gubler *et al*. (1987). Some believe that the xylanase may have a different role in germination and remains within the aleurone cells, bound to membranes, for an unknown function. Van Campenhout *et al.* (2007) showed that the precursor form of X-I is an endo-acting enzyme which can release xylose from polymeric xylan. The perceived release of xylanase into the endosperm has long been a debated topic, but unlike limit dextrinase (Chapter 6) the presence of a signal peptide suggests xylanase may be secreted. However, its' continued expression late into germination and apparent entrapment in the cytoplasm of aleurone cells and tissue is difficult to explain if this were the case. Further experiments could be designed to investigate this more closely, such as antibody specific immunostaining both at the light and electron microscope levels that may address some of these questions of location. In experiments not described in this thesis an attempt was made by Banik to obtain xylanase protein *in vitro* using the pET 14-b expression vector in *E. coli.* Small quantities of protein were obtained but were deposited as insoluble aggregates in inclusion bodies with no xylanase activity detectable (R. Burton, personal communication). It was not until the successful xylanase protein expression studies by Casper *et al*. (2001) that it became obvious that a full-length cDNA was necessary to ensure successful protein folding. The cDNA described by Banik *et al.* (1996) was in fact truncated at the 5' end by five amino acids. The use of specific xylanase antibodies such as described by Casper *et al.* (2001) would have been a valuable asset to this study.

Xylanase mRNA at this later stage of germination, in the scutellum, scutellum epithelium, ventral trace and rootlet areas was evident (Figure 5.7) for Sloop and (Figure 5.8) for Himalaya, particularly, as seen with glucanase, on the ventral side of the grain, parallel to coleoptile elongation.

The mRNA is present in the actively growing parts of the shoot apex (H26, Figure 5.8) and leaf primordia plus the coleoptile (H20, Figure 5.7). Work presented by Gibeaut *et al.* (2005) on barley coleoptile tissue has demonstrated that cell divisions were seen to

be greatest in the sub-epidermal layer, decreasing in the layers towards the inner epidermis. Gibeaut *et al.* (2005) also found that barley coleoptiles were in the most rapid phase of absolute elongation growth at day 3 after steeping. The role of xylanase in coleoptiles is unclear, but 4-day barley coleoptiles contain both 19% arabinoxylan and 19% (1,3;1,4)-β-glucan (Sakurai and Masuda, 1978), while older seedling leaves are reported to have 11% arabinoxylan and 16% β-glucan, (N. Sakurai, personal communication). A role for xylanase in the modification or degradation of coleoptile arabinoxylan may provide the growing embryo with an additional carbohydrate source late in the germination process; this may be particularly critical when poor germination conditions exist. Van Campenhout *et al.* (2005) also noted that the X-II isoenzyme appeared to predominantly be in the developing shoots and roots, particularly at the elongated stages, suggesting a role of the enzyme in the metabolism of vascular tissue. This is in contrast to other reports, which found up to 13% of xylanase activity in the roots and not in the shoots of growing seedlings (Slade, 1986; Benjavongkvlchai and Spencer, 1989; Banik *et al.,* 1996). This discrepancy could be due to the differences and accuracy between assays used to measure xylanase enzyme activity, whilst this study solely measures mRNA, which doesn't necessarily relate to comparable levels of protein expression. A follow up study would be to micro - dissect specific tissue from the grain, perhaps using a laser dissection microscope, to carry out a comparative enzyme and mRNA study.

At this stage, the xylanase mRNA in the root system of Sloop sections was also distinctive. It can be seen in Figure 5.9 that mRNA is spread over the whole developing coleoptile, including the central cylinder surrounded by the epidermis and cortex. The mRNA (Figure 5.9) is comparable to the activity noted by Suzuki *et al.* (2002) using ß-glucuronidase (GUS) activity in transgenic *A. thaliana* plants where it was fused to the AtXyn1 gene and expression was noted in the endodermis, pericycle, coleoptile and vascular bundles of the hypocotyl (primary root).

5.4.2: *Varietal differences for (1→4)-β–xylanase.*

The dynamics of xylanase-I mRNA patterns appear to be similar in the two varieties examined in this study, with only a slightly delayed appearance in sections of Himalaya grain (Figures 5.12 and 5.13). The exception to this is the predominant XYN-I mRNA in the aleurone noted early in germination for Sloop (Figure 5.3) and the obviously slower growth of the coleoptile in Himalaya grain at the M5 stage (Figure 5.8 b). Others have also found varietal differences relating to xylanases (Donrnez, 2009) and

even xylanase isoform difference between varieties (Sungurtas *et al.,* 2004). Many have attributed this variation to technical reasons arising from the various assays used to measure xylanase activity. Indeed, Megazyme at present offers at least three different xylanase enzyme measurement assays. The Megazyme Xylazyme AX tablets (dyed and cross linked wheat arabinoxylan) are reported to be accurate, reliable and have a sensitivity equivalent to viscometric assays, particularly when low levels of enzyme detection are required (McCleary, 1992). Although unpublished data reports that the kits detect xylanase in animal feed rather than malting barley.

5.4.3: *Comparison of the (1→4)-β–xylanase and (1,3;1,4)-β-glucanase in situ hybridisation.*

Similar staining patterns were seen between the two enzymes, particularly when comparing the EI glucanase isoenzyme to that of xylanase at all the modification stages in both varieties. The only observed exception was the xylanase pattern in Sloop at the M3 stage, which was more widespread through the aleurone layers. As highlighted in Chapter 4 when discussing the distribution of the glucanase mRNAs, a timing delay more than a location difference was observed between the two varieties.

5.5: Conclusions and further work.

The main objective of the work presented in Chapter 5 was to try and document the location and timing of xylanase mRNA appearance during barley germination, since the importance of xylanase to the malting and brewing industry makes it a worthwhile candidate for a broader study (Schwarz and Han, 1995). Work on this enzyme has been complicated by the fact that there are likely to be at least three isoenzymes, if not more, in barley.

a) It appears from these hybridization studies that xylanase isoenzyme I is likely to be the predominant isoenzyme from this family in these tissues, but until the other xylanase isoenzymes are characterized completely, this can only be a tentative conclusion.

b) From this study and that of Casper *et al*. (2001), it appears that xylanase is transcribed at high levels fairly early in germination in the scutellum, embryo tissue and in the aleurone layers but that there is a lag period until active enzyme appears.

It appears that xylanase may have a complicated enzyme activation mechanism involving either processing or the removal of inhibitors. This is not uncommon for

other barley enzymes present in the germinated grain, such as limit dextrinase (Chapter 6) and β amylase (Lauriere *et al*., 1986) and future work could focus on the use of specific antibodies to track the xylanase protein and ascertain whether inactive forms appear directly after mRNA synthesis which are then converted to active forms later.

Further work will be needed to complete the picture of mRNA appearance beyond 110 h from the start of steeping to determine if the mRNA is consistent with enzyme expression and is at a maximum very late in germination (164 h from the start of steeping), but maintaining intact grain samples will be technically challenging. A longer germination schedule goes beyond the feasible malting time presently set by the industry, but this may mean that maximal xylanase activity is never currently attained. The other enzymes worthy of examination in light of the work by Kuntz and Bamforth (2007) are carboxypeptidase, and α-4 arabinofuranosidase and β-d-xylopyranosidase which are also likely to participate in arabinoxylan breakdown (Taiz and Honigham, 1976; Dashek and Chrispeels, 1977) and which may very well show a similar or complementary expression profile to that of the xylanase presented here.

Figure 5.12: Summary of (1→4)-β-endo-xylanase expression in Himalaya grain

Tissue	M1	M1		M3	M3		M5	M5
	XYN-I	TXYN		XYN-I	TXYN		XYN-I	TXYN
Tip of scutellum (edge of the rootlet)	✓	✓		✓	✓			
Tip of scutellum (edge of the coleoptile)	✓	✓		✓	✓			
General scutellum tissue	✓	✓						
Scutellum epithelium				✓	✓		✓	✓
Rootlet tissue	✓	✓		✓	✓		✓	
Ventral trace region	✓	✓		✓	✓		✓	
Coleoptile							✓	✓
Shoot primordium							✓	✓
Aleurone	✓	✓		✓	✓		✓	✓

Figure 5.13: Summary of (1→4)-β-endo-xylanase expression in Sloop grain

Tissue	M1	M1		M3	M3		M5	M5
	XYN-I	TXYN		XYN-I	TXYN		XYN-I	TXYN
Tip of scutellum (edge of the rootlet)	✓	✓						
Tip of scutellum (edge of the coleoptile)								
General scutellum tissue	✓	✓						
Scutellum epithelium				✓	✓		✓	✓
Rootlet tissue				✓	✓			
Ventral trace region	✓	✓		✓	✓			
Coleoptile				✓	✓		✓	✓
Shoot primordium				✓	✓		✓	✓
Aleurone				✓	✓		✓	✓

Chapter 6

Identification and location of specific Limit Dextrinase and Limit Dextrinase Inhibitor mRNA in germinated *Hordeum vulgare* L grain

6.1: Introduction

Limit dextrinase (EC 3.2.1.41) is also known as amylopectin 6-glucoanohydrolase, debranching enzyme or pullulanase. To avoid confusion here limit dextrinase (LD) will be used in preference to other names.

Following the initial solubilization of starch granules by two α-amylases in the endosperm of germinating cereal grains, the subsequent hydrolysis to dextrins, oligosaccharides and glucose is achieved by the concerted action of α-amylase, β-amylase, α-glucosidase and LD (Manners, 1985; MacGregor, 1987). Both LD and α-glucosidase have the capacity to hydrolyse α-(1-6)-D-linkages in branched maltooligosaccharides, also referred to as limit dextrins (Lee *et al*., 1971). LD is the only diastase enzyme that cleaves the α-(1-6)-D-linkages to produce small fermentable sugars of value to the brewing industry (Moller, 2011; Enevoldsen and Schmidt, 1973).

The gene and full-length cDNA encoding LD (Burton *et al*., 1999; Kritsensen *et al*., 1999) have been described. The translation product from the LD cDNA contains a classical transit peptide that is unlikely to act as a signal peptide able to direct the LD polypeptide to the lumen of the endoplasmic reticulum (Burton *et al*., 1999). This raises a question concerning the mechanism of secretion from the aleurone cells and the subsequent involvement of LD in starch degradation in the starchy endosperm (Burton *et al*., 1999). Thus, the site of synthesis and secretion route of the LD protein in barley during germination is of particular interest, because the accepted view that LD is extensively involved in endosperm breakdown has been thrown into doubt. Studies highlighting aleurone cell death late in germination (Shahpiri *et al.,* 2014; Domínguez *et al*., 2004; Bethke *et al.,* 2004; Bethke and Jones, 2001; Fath *et al.,* 1999; 2001; 2002; Wang *et al*., 1996) may provide an alternative route for enzyme release into the endosperm, although this would occur at a stage where endosperm breakdown is already well advanced.

LD is thought to be either synthesized *de novo* during germination (Hardie, 1975; Longstaff and Bryce, 1991) or be laid down during development (Burton *et al.,* 1999). In either case, the rate of appearance of activity in germination does appear to be much slower than that of other hydrolytic enzymes (Fincher, 1989; MacGregor *et al*., 1994). Gibberellins (GA$_3$) have been reported to increase LD transcript levels during germination in the aleurone (Hardie, 1975; Palmer, 1973; Manners and Yellowlees, 1973; Lee and Pyler, 1984: Burton *et al.,* 1999) or increase secretion rates from isolated

aleurone layers (MacGregor *et al*., 1998). Burton et al. (1999) also indentified a GA response element in the promoter region of the LD gene. Involvement of the scutellum in LD secretion has not yet been verified (Fincher, 1989). According to the literature, LD appears to exist in a number of forms which have molecular weights of between 80 and 104.7 kDa's, a pI as measured by iso-electric focusing of 4.1 to 4.8, an optimal working temperature of 55^0C and optimal pH range of 4.5 to 5.5 (McCafferty et al., 2004; Lee and Pyler, 1984; MacGregor *et al*., 1987; Sissons *et al*., 1992; Kristensen *et al.,* 1999). These variations seem to be due to differences between cultivars used and variations in the methods used for measurement (Sissons, 1993; Schroeder and MacGregor, 1998; Longstaff and Bryce, 1991). The LD gene is present as a single highly-fragmented copy; it has 26 introns, in the barley genome (Burton *et al*., 1999; Kritsensen *et al*., 1999) associated with the short arm of chromosome one (Li *et al.,* 1999).

It has been reported that LD enzyme activity can be detected after 24 h of germination (Schroeder and MacGregor, 1998), increasing to reach a maximum after four (Manners and Yellowlees, 1973) five (Longstaff and Bryce, 1991; Kristensen, 1993; 1999) or 11 days of germination (Lee and Pyler, 1984; Pratt *et al*., 1981; Kneen and Spoerl, 1948). Others have also reported an increase in enzyme levels by 5 to 15 fold to then maintain an activity plateau until day 21 of germination (Kristensen *et al*., 1999). LD mRNA has been located in isolated aleurone layers and whole germinated grain by northern hybridization analysis, reaching high levels at day 4, to decrease around 7 to 8 days of germination (Burton *et al.,* 1999; Kristensen *et al*., 1999) and by more sensitive RT-PCR in young leaves, young roots and the scutellum in one day germinated grain (Burton *et al*., 1999).

Low molecular weight inhibitors (LDIN) appear to form soluble but inactive complexes with LD (Macri *et al*., 1993), and are believed to account for the bound inactive LD form (MacGregor *et al*., 1994). Two LDIN designated low pI (6.7, Mr 12, 928) and high pI (7.2, Mr 12,684 and 12, 744) have been isolated from barley (Macri *et al*., 1993; MacGregor *et al*., 1994). They were both found to have high sequence homology (98.5% identity) with a protein sequence encoded by an mRNA found in barley by Lazaro *et al*. (1988) and initially labelled as an α-amylase trypsin inhibitor (MacGregor *et al*., 2000). LDIN appear to be specific for LD, not inhibiting α-amylase, trypsin or fungal isoamylase (MacGregor *et al.,* 2000). They are encoded by a single gene, the difference in inhibitor molecular weights and pI values most likely due to post–

translational modification (MacGregor *et al.,* 2000). The LDIN isoforms have been found to differ by a glutathione residue bound to the free thiol group of the low pI form and cysteine of the high pI form (MacGregor et al., 2000). The majority of LDIN protein isolated has been from the endosperm of germinated grain (MacGregor *et al*., 1994), rather than from the aleurone or scutellum. The sequence has a classic signal peptide which implies that the protein is secreted.

Many theories have been proposed, but the most consistent amongst researchers has been that the increasing activity of LD as germination progresses is related more to a reduced inhibition than to increased translation efficiency of the LD mRNA, as shorter extraction times to release the inhibitor are consistently needed to yield maximum enzyme levels as germination progresses (Schroeder and MacGregor, 1998; Sissons, 1993; Laurier, 1985). In ungerminated barley grain almost all LD is present in an inactive "bound" form (Serre and Lauriere, 1989; Sissons, 1993; Schroeder and MacGregor, 1998). At day 3 of germination, 85 % of LD exists in a bound form, while at day 7 this is reduced to 35 % (Kristensen *et al*., 1999; Macgregor *et al*., 1994). Sometime between 5 and 10 days of germination the bound enzyme is rapidly converted into the free active enzyme, probably by proteolysis. This occurs *in vitro* specifically by the action of cysteine proteinase (Yamada, 1981; Sissons, 1993; Longstaff and Bryce, 1993). Thioredoxin has also been found to increase the activity of LD *in vitro*, by either inactivating a small disulfide inhibitor protein (Wong *et al.,* 1995), increasing the *de novo* synthesis (Hardie, 1975) or via another mechanism yet to be defined (Cho *et al*., 1999).

The presence of LDINs and therefore the complicated methods needed to measure LD enzyme levels in grain extracts means that an accurate study of enzyme activity has been difficult. To date, there is no evidence that the interaction between LD and LDIN even occurs *in vivo* with the possibility that it may only happen as an accidental association of the two proteins *in vitro* once the grain is broken open in the malting or mashing process. Although researchers have found that free LD activity is relatively heat labile at a mashing temperature of 65^0C, the bound and latent forms appear to be relatively heat stable (Sissons *et al.,* 1995, Stenholm *et al.,* 1997, Walker *et al.*, 2001), suggesting that the inhibitor may possibly offer protection against heat.

The existence of active LD enzyme has also been identified in small quantities in barley and rice both in the developing grain or mature plant, increasing rapidly at the onset of anthesis slowly reaching a maximum at 21-23 days post anthesis, and remaining

elevated through to maturity (Sissons, 1993; Burton *et al*., 1999; Nakamura *et al*., 1997). The publication of Stahl *et al.* (2004) using LDI antisense constructs in transgenic barley plants demonstrated a putative association between the presence of LD, LDI and starch synthesis in developing grain.

In this study, the distribution pattern of LD and LDIN mRNA was observed in cross sections of germinated barley grain, in order to gain a clearer picture about the sites and timing of both LD and LDIN mRNA transcription. *In situ* hybridization is an ideal procedure to do this, as it is not hampered by many of the problems previously outlined which occurs when the grain is destroyed and tissue contents become homogenized. As limited work has been done on examining the detailed location of LD mRNA in the germinating grain, this should complement information already gained from the previous chapters on other enzymes which are important for the successful transition of the barley grain to a growing seedling. Also, by examining serial sections for either LD or LDIN mRNA expression the association of these at the transcriptional level can be assessed.

6.2: Material and methods

6.2.1: Tissue preservation

The schedule of barley grain germination used in this study is outlined in section 2.2.3 of Chapter 2. All the results presented in this chapter are from grain sections fixed in 4 % paraformaldehyde and 0.25% glutaraldehyde, embedded in paraffin and sectioned with the microtome, as described in Chapter 2, sections 2.2.4; 2.2.5 and 2.2.6.

6.2.2: Modification pattern

Longitudinal sections of barley grain collected at set time points during germination were stained with the CW dye, as described in section 2.2.7 of Chapter 2. The degree of $(1\rightarrow3, 1\rightarrow4)$-$\beta$-glucan degradation as detected by the CW dye under UV determined the stage of grain modification.

From a total of 12 collection points over the germination schedule, grain sections were placed into one of five modification "M" stages called M1, M2 etc and assessed.

6.2.3: Riboprobe preparation

Single-stranded DIG labeled RNA (riboprobes) were used to detect the presence of specific mRNA in longitudinally cut, paraffin-embedded barley sections, as previously described in section 4.2.4 of Chapter 4. To prepare the LD DIG labeled riboprobes, cDNA encoding part of the internal coding region of LD was sub-cloned into pBluescript SK+, transformed into competent *Escherichia coli* XL-1 blue cells, and plasmid DNA was isolated as described in sections 3.2.6 and 3.2.7. For riboprobe production clones were linearised with the restriction enzymes *Sal* I (NEB) for anti-sense riboprobes made using T3 RNA polymerase or *Eco*RI (NEB) for sense riboprobes made using T7 RNA polymerase. The final LD DIG labeled riboprobes were 453 nucleotides in length.

The LDIN cDNA, encoding the entire protein was taken from a PCR fragment originally isolated from developing grain cDNA by R. Burton (School of Agriculture, Food and Wine, University of Adelaide, Waite Campus). This was originally identified by Lazaro *et al*. (1988) as an alpha amylase trypsin inhibitor, but later confirmed as LDIN by Macgregor *et al*. (2000).

The cDNA fragment was cloned into pBluescript SK+. For riboprobe production clones were linearised with the restriction enzymes *Xho*I (NEB) for sense riboprobes made using T3 RNA polymerase or *Spe*I (NEB) for anti-sense riboprobes made using

T7 RNA polymerase. The final LDIN DIG labeled riboprobes were 520 nucleotides in length.

All fragments subcloned into pBluescript SK+ vectors for use as templates for riboprobe transcription were sequenced to determine the insert orientation and to ensure nucleotide fidelity as described in section 4.2. All sequences were subsequently checked for cross hybridization using an advanced NCBI BLAST (version BLASTN 2.2.3) search (Altschul *et al*., 1996).

6.2.4: In situ hybridization assay on sectioned barley grain.

The *in situ* hybridization procedure developed as outlined in Chapter 3 was used with the LD and LDIN riboprobes. Images were routinely collected at 2.5 X, 10 X and 40 X magnifications, as described in section 2.5.6.

6.2.5: Total RNA isolation from whole barley grain.

As previously discussed in section 4.2.6 whole seedlings (grain, shoots and roots) were sampled and total RNA isolated at intervals throughout the germination procedure (32, 56 and 96 h from the start of steeping). A detailed procedure is outlined in section 4.2.6.

Figure 6.1a: Nucleotide sequencces of the cDNAs encoding the full length of the barley Limit dextrinase (LD) and Limit dextrinase Inhibitor (LDIN) with the primer locations indicated. LD: Begins at nucleotide 1337 (NCBI accession number; AF122049, Burton *et al*., 1999). LDIN: Begins at nucleotide 1 (NCBI accession number; X13443, Lazaro *et al*., 1988). Blue and green blocks indicate designed primers as per table 6.1.

Limit dextrinase nucleotide sequence

```
LD      1  atacaaaatg ccaatgccga tgcgaacgat gctgctccgc caccttagtc ccccctccgc
LD     61  cgtgcccaat ccccggcgct cctccgtctc ctcgccgcag cggataccgg tgagggcccg
LD    121  gccaccgccg ctgcattccg cccgcgccac cgcgctccgg gcccggcgga ggacgcccat
LD    181  ggcggtcggg gagaccggcg cctccgtctc cgccgccgag gccgaggccg aggccaccca
LD    241  ggcgttcatg ccggacgcca gggcgtactg ggtgacgagc gacctcatcg cctggaacgt
LD    301  cggcgagctg gaagcgcagt ccgtctgcct gtacgccagc agagccgccg cgatgagcct
LD    361  ctcgccgtcg aatggcggca tccaaggcta cgactccaag gttgagctgc aaccggagag
LD    421  cgccgggctc ccggaaaccg tgacccagaa gttccctttc atcagcagtt acagagcatt
LD    481  cagggtcccg agctctgtcg acgtcgccag ccttgtgaaa tgccaactgg tcgtcgcttc
LD    541  tttcggcgct gacgggaaac acgtagatgt tactggactg caattacccg gcgtgctgga
LD    601  tgatatgttc gcatacacgg gaccgctcgg tgcggttttc agcgaggact ctgtgagcct
LD    661  gcacctttgg gctcctacag cacagggcgt gagcgtgtgc ttctttgatg gtccagcagg
LD    721  ccctgcgcta gagacggtgc agctcaagga gtcaaatggt gtttggagtg tcactggacc
LD    781  aagagagtgg gaaaaccggt actatttgta tgaagtcgac gtgtatcatc caactaaggc
LD    841  gcaggttctg aaatgtttag ctggtgaccc ttatgctaga ggcctttctg caaatggagc
LD    901  gcgtacctgg ttggttgaca ttaacaatga gacattgaag ccggcttcct gggatgaatt
```

197

```
LD    961 ggctgatgag aagccaaaac ttgattcctt ctctgacata accatctatg aattgcacat

LD   1021 tcgtgatttt agcgcccacg atggcacagt ggacagtgac tctcgtggag catttcgtgc

LD   1081 atttgcatat caggcctcgg caggaatgga gcacctacgg aaattatctg atgctggttt

LD   1141 gactcatgtg catttgttgc caagctttca ttttgctggc gttgacgaca ttaagagcaa

LD   1201 ctggaaattt gtcgatgagt gtgaactagc aacattccct ccagggtcag atatgcaaca

LD   1261 agcagcagta gtagctattc aggaagagga cccttataat tgggggtata accctgtgct

LD   1321 ctggggggtt ccaaaaggaa gctatgcaag tgaccctgat ggcccgagtc gaattattga
```
                                      LD.1 →
```
LD   1381 atatcgtcag atggtccagg ccctcaatcg cataggtctt cgtgttgtca tggatgttgt

LD   1441 atacaatcat ctagactcaa gtggcccctg cggtatcagc tcagtgcttg acaagattgt

LD   1501 tcctgggtac tatgttagaa gggatactaa tggccagatt gagaacagtg cagctatgaa

LD   1561 caatacagca agtgagcatt tcatggttga taggttaatc gtggatgacc ttttgaactg

LD   1621 ggcagtaaac tacaaagttg acggggttcag atttgatctt atgggccata tcatgaaacg

LD   1681 cacaatgatg agagcaaaat ctgctcttca aagccttaca acagatgcac atggagttga

LD   1741 tggttcaaaa atatacttgt atggtgaagg atgggacttc gctgaagttgcacgcaatca
```
                                      ← LD.2
```
LD   1801 acgtggaata aatgggtccc agcttaatat gagtggaacg gggattggta gcttcaatga

LD   1861 tagaatccgg gatgctatta atgggggtaa tccctttggt aatccgctcc agcaaggctt

LD   1921 caatactggt ctgttcttag agccgaatgg gttttatcag ggcaatgaag cagataccag

LD   1981 gcgctcgctc gctacttatg ctgaccaaat acagattgga ctagctggta atctcaggga

LD   2041 ttatgtacta atatctcata ctggagaagc taagaaggga tcagaaattc acacttttga

LD   2101 tggattacca gtaggctata ctgcgtcccc aatagaaacg ataaactatg tttctgctca

LD   2161 tgacaatgag actctatttg atgttatcag tgtgaagacc ccaatgatcc tttcagttga

LD   2221 tgagagatgc aggataaatc atttggcctc cagcatgatg gcattatccc agggaatacc

LD   2281 cttcttccac gctggtgacg agatactaag atctaagtcc atcgaccgag attcatataa

LD   2341 ctctggtgat tggtttaaca agcttgattt tacctatgaa acaaacaatt ggggtgttgg

LD   2401 gcttcctcca agtgaaaaga acgaagataa ttggcccctg atgaaaccaa gattggaaaa

LD   2461 tccgtctttt aaacctgcaa aaggacacat tcttgctgcc ctagacagtt ttgttgacat

LD   2521 cttgaagatc agatactcat ctccactttt tcgtctcagt acagcaaatg acattaagca

LD   2581 aagggtacgc tttcacaaca cagggccctc cttagtccca ggtgttattg tcatgggcat

LD   2641 tgaagatgca cgaggtgaga gccccgagat ggctcaatta gacacgaact tctcttatgt

LD   2701 cgtaaccgtc ttcaatgtgt gtccgcacga agtgtccatg gatatccccg ctctcgcttc

LD   2761 gatggggttt gaactgcatc ctgtgcaggt gaattcatca gatactttgg tgaggaaatc

LD   2821 tgcgtacgag tccgcgacgt gcaggttcac cgtgcccgga agaaccgtgt cagtctttgt

LD   2881 cgaacctcgg tgttgatgcc tcctcggttt caacacgagg atctgttcta caagttgtcg

LD   2941 aagcaaccga agatctttct gtttttgtgt tgaagtaaat aataaatgaa gaataagaga

LD   3001 ggagaggcgc tgctgcctgc cagattacac tgctgaagga acaaacccaa gaataagaag

LD   3061 gttggcttat gtatatgtat agtgcccaag ctgcctagtc ctgttttttct ttcctaatcc

LD   3121 attttgattt tatatatcaa aaggaaaaca catatatcgt gttataagta ttgtataaca

LD   3181 tgtactccct ccaactgaaa atacttgtca taggaatgta tgtatctaga tgtattataa

LD   3241 ttctagatac atccatttt atacatttat gtgacaagta attccggacg gagggagtat

LD   3301 gtgttaaaca aatttcacaa aaatgtatgt gttaaacaag gatgcataat gattttgtac

LD   3361 tgtactctct ttttactatg tatattacat attatttttt accgaagaaa agaaagtaca

LD   3421 atttctcta
```

Limit dextrinase inhibitor nucleotide sequence

```
LDIN 1    gaaccaacga ccaataaact agtatcaaca atggcatccg accatcgtcg cttcgtcctc
LDIN 61   tccggcgccg tcttgctctc ggtcctcgcc gtcgccgccg ccaccctgga gagcgtcaag
LDIN 121  gacgagtgcc aaccaggggt ggacttcccg cataacccgt tagccacctg ccacacctac
LDIN 181  gtgataaaac gggtctgcgg ccgcggtccc agccggccca tgctggtgaa ggagcggtgc
LDIN 241  tgccgggagc tggcggccgt cccggatcac tgccggtgcg aggcgctgcg catcctcatg
LDIN 301  gacggggtgc gcacgccgga gggccgcgtg gttgagggac ggctcggtga caggcgtgac
LDIN 361  tgcccgaggg aggagcagag ggcgttcgcc gccacgcttg tcacggcggc ggagtgcaac
LDIN 421  ctatcgtccg tccaggagcc gggagtacgc ttggtgctac tggcagatgg atgacgatcg
LDIN 481  aaatgcgcca aggtaatgaa gcggagtact gtatacagaa taaaagtact cgagtgaaaa
LDIN 541  caaactcata aataaacctt gtgagatgta tgcgtatgat ctatggtgtg gacag
```

←LDIN.2

Figure 6.1b: Amino acid sequence of LD and LDIN.

Limit dextrinase protein sequence

```
LD   MPMPMRTMLLRHLSPPSAVPNPRRSSVSSPQRIPVRARPPPLHSARATALRARRRTPMAVGE
LD   TGASVSAAEAEAEATQAFMPDARAYWVTSDLIAWNVGELEAQSVCLYASRAAAMSLSPSNGG
LD   IQGYDSKVELQPESAGLPETVTQKFPFISSYRAFRVPSSVDVASLVKCQLVVASFGADGKHV
LD   DVTGLQLPGVLDDMFAYTGPLGAVFSEDSVSLHLWAPTAQGVSVCFFDGPAGPALETVQLKES
LD   NGVWSVTGPREWENRYYLYEVDVYHPTKAQVLKCLAGDPYARGLSANGARTWLVDINNETLKP
LD   ASWDELADEKPKLDSFSDITIYELHIRDFSAHDGTVDSDSRGAFRAFAYQASAGMEHLRKLSD
LD   AGLTHVHLLPSFHFAGVDDIKSNWKFVDECELATFPPGSDMQQAAVVAIQEEDPYNWGYNPVL
LD   WGVPKGSYASDPDGPSRIIEYRQMVQALNRIGLRVVMDVVYNHLDSSGPCGISSVLDKIVPGY
LD   YVRRDTNGQIENSAAMNNTASEHFMVDRLIVDDLLNWAVNYKVDGFRFDLMGHIMKRTMMRAK
LD   SALQSLTTDAHGVDGSKIYLYGEGWDFAEVARNQRGINGSQLNMSGTGIGSFNDRIRDAINGG
LD   NPFGNPLQQGFNTGLFLEPNGFYQGNEADTRRSLATYADQIQIGLAGNLRDYVLISHTGEAKK
LD   GSEIHTFDGLPVGYTASPIETINYVSAHDNETLFDVISVKTPMILSVDERCRINHLASSMMA
LD   LSQGIPFFHAGDEILRSKSIDRDSYNSGDWFNKLDFTYETNNWGVGLPPSEKNEDNWPLMKPR
LD   LENPSFKPAKGHILAALDSFVDILKIRYSSPLFRLSTANDIKQRVRFHNTGPSLVPGVIVMGI
LD   EDARGESPEMAQLDTNFSYVVTVFNVCPHEVSMDIPALASMGFELHPVQVNSSDTLVRKSAY
LD   ESATCRFTVPGRTVSVFVEPRC
```

Limit dextrinase inhibitor protein sequence

```
LDIN   MASDHRRFVLSGAVLLSVLAVAAATLESVKDECQPGVDFPHNPLATCHTYVIKRVCGGPSR
LDIN   PMLVKERCCRELAAVPDHCRCEALRILMDGVRTPEGRVVEGRLGDRRDCPREEQRAFAATL
LDIN   VTAAECNLSSVQEPGVRLVLLADG
```

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Table 6.1: Sequences of primers used in RT-PCR reactions. All primers manufactured by Geneworks, Adelaide, South Australia. *Include 1ul DMSO per PCR reaction;. Bp is base pairs. DG for developing grains. Tm, temperature for the PCR reactions.

Name of primer	5' nucleotide primer (5' to 3')	Name of primer	3' nucleotide primer (5'to 3')	Primer specific temperature for the PCR reaction	PCR product size
LDIN.1	GAACCAACGACCAATAAACTAG	LDIN.2	CTGTCCACACCATAGATCATACGC	$*51^0$C	595bp
LD.1	GGAAGCTATGCAAGTGAC	LD.2	CCCTGAAGCGACTTCAAC	52^0C	494bp
Actin Fw	GTCTTTCCCAGCATTGTAGG	Actin Rv	CGACACGGAGCTCATATAGAA	53^0C	453bp

6.2.6: Western blot analysis of LD and LDIN antibodies.

Sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis was performed followed by Western blot immunostaining to document specific LD and LDIN protein expression in the varieties selected for this study. The procedure was as outlined in Chapter four, section 4.2.8 using antibodies kindly provided by Sandy MacGregor (University of Manitoba, Winnipeg, Manitoba, Canada).

6.2.7: Complementary DNA synthesis and RT-PCR.

To complement the *in situ* hybridization findings, reverse transcription Polymerase Chain Reaction (RT-PCR) was employed to screen germinated barley grain total RNA samples from section 6.2.6 for specific LD and LDIN mRNA. Synthesis of cDNA from the germinated whole grain RNA samples was carried out using the Thermoscript RT-PCR system as outlined in section 4.2.7.

PCR reactions were performed to detect the presence of cDNAs encoding either LD or LDIN with the primers listed in Table 6.1a. The negative control included all reagents except the first strand cDNA population, while the positive control for these PCR reactions included cDNA made from RNA extracted from developing whole grain 9 days post anthesis supplied by R. Burton (School of Agriculture, Food and Wine, University of Adelaide, Waite Campus). PCR reactions consisted of the following reagents; cDNA (3 μL); 2.5 μL 10 X PCR buffer, 1.5 μL 25 mM $MgCl_2$; 1 μL 10 mM dNTP's; 0.5 μL each of 1-0.1 μg. $μL^{-1}$ primer; 1 μl Taq DNA polymerase; deoxyribonuclease free water to a final reaction volume of 25 μL. Reactions were performed in a Perkin-Elmer Cetus DNA Thermal Cycler as follows; 94°C, 2 min initial denaturation; 35 cycles of denaturation at 94^{0}C for 40 s annealing at primer specific temperature (Table 6.1) 40 s, extension at 72^{0}C, 1 min; followed by a 2 min final extension at 72°C. Amplified products were detected by electrophoresis at 100 V in 1.2% (w/v) agarose gel in 1 X TAE buffer containing 0.1 μg.mL^{-1} ethidium bromide (Sambrook *et al*., 1989).

6.2.8: Total LD enzyme analysis.

Total (bound and unbound) LD activity was measured in samples of whole grain for both varieties collected at 32, 56 and 96 h. LD activity was assayed by a modification of the method of Macleary (McCleary, 1992). Extracts (250 μL) prepared for SDS-PAGE Western bot analysis (section 4.2.9.1), once centrifuged, were added to 250 μL sodium acetate (100 mM, pH 5.0) containing 10 mM sodium azide; 50 mM calcium chloride ($CaCl_2$, Sigma), 25 mM dithiothreitol (DTT) and 0.5 mg/ml BSA, and

incubated for 5 min at 40^0C. A Limit Dextrixyme tablet (Megazyme, Deltagen, Australia) was added and incubated for a further 10 min at 40^0C. The assay was stopped by the addition of 5 mL 1 % Trizma base pH 8 (Sigma). The sample was vortexed, incubated at room temp for 5 min, vortexed again and filtered through Whatman 9 cm filter circles. The absorbance of the filtrate was measured at 590 nm against a reaction blank, and activity determined by reference to the standard curve supplied with the tablets to convert absorbance to milli-units per assay.   Values are expressed as Units/kg Malt.

LD activity units are defined as the amount of enzyme required to release one micromole of glucose reducing sugar equivalents per minute from azurine-cross linked pullulan under the defined assay conditions (McCleary, 1992). Duplicate analyses were performed on each extract.

6.2.9: Specificity analysis of the LD and LDIN riboprobes.

Southern blot analysis was used to confirm the specificity of the DIG labelled riboprobes. The procedure was essentially as described in detail in section 4.2.11. In this case, pBluescript SK+ clones containing the cDNA fragments of LD and LDIN were restriction cut with specific enzymes (*Sal*I/*Eco*RI or *Xho*I/*Spe* I respectively) as described in Chapter three (section 3.2.5) and aliquots of 20 ng run on a 1.2 % (w/v) agarose DNA grade gel in 1 X TAE buffer containing 0.1 μg.ml^{-1} ethidium bromide (Sambrook *et al*., 1989) to separate the specific fragments from the pBluescript SK+ vector. Ladder markers (10 μL, 1 kb) were loaded in the first well of the gels. DNA transfer to nitrocellulose, the hybridization procedure and Southern detection was as previously described for the probing of (1→3,1→4)-β-D-glucanase cDNA in section 4.2.11.

6.3: Results

The specific aims for Chapter 6 were to; 1) document the distribution of LD and LDIN mRNAs during barley germination; and 2) to relate transcript to detectable active enzyme which might indicate the role that LD plays in barley grain germination and its association with LDIN. The technique of *in situ* hybridization using non-radioactive riboprobes was employed to fulfill these aims.

6.3.1: Specificity analysis of the LD and LDIN riboprobes

The specificity of the riboprobes was tested by Southern blot analysis. The LD and LDIN riboprobes against cDNA sequences encoding the LD and LDIN on a Southern blot did not show any cross reactivity, as was the case with other cDNA sequences used as riboprobe transcripts in this study, such as the $(1\rightarrow3,1\rightarrow4)$-β-D-glucanase isoenzymes (data not shown). A small amount of cross reactivity was observed for both LD and LDIN anti-sense riboprobes against the pBluescript SK+ vector and endo-$(1\rightarrow4)$-β-xylanase for the LD riboprobes (data not shown).

6.3.2: Western blot analysis of LD and LDIN antibodies

It was found by Western blot immunostaining that the antibodies kindly provided by others for this study to document expression of specific LD and LDIN protein were non-specific (data not shown) and therefore were not suitable for use in the ISH assay on sectioned barley grain.

6.3.3: In situ hybridization assay on sectioned barley grain through germination for LD and LDIN mRNA expression

Grain sections collected through germination and categorized into distinct M stages as described in Chapter 2 were assessed for specific LD and LDIN mRNA. The results are expressed as a consistent pattern that was observed throughout a number of assays performed at each modification stage. Sense riboprobes as internal controls were routinely run in parallel with the anti-sense riboprobes.

The control staining generated by these sense riboprobes was assessed as minimal for all probes, although background staining was observed in the starchy endosperm and crushed cell layer of a number of tissue sections. This was seen with $(1\rightarrow3,1\rightarrow4)$-β-glucanase and endo-xylanase *in situ* hybridization results and therefore regarded as artifactual. Ungerminated grain of both varieties sampled immediately after the start of imbibition show similar LD or LDIN mRNA patterns as the control slides (data not shown).

6.3.3.1: Modification stage M1 for Sloop and Himalaya

The *in situ* hybridization results for the variety Sloop at the M1 stage (Figure 6.2 a and c) demonstrated that the mRNA for LD first appears in the scutellum, particularly the parenchyma of the scutellum (S26), the scutellar epithelium, and the root-cap (Figure 6.2 c, S27), along with site specific cellular staining in the coleorhiza and ventral trace region (procambial strands) of the embryo 16 h from the start of steeping (Figure 6.2 a S28). Control sections for both Sloop and Himalaya grain were clear of staining (Figure 6.2 b and d for Sloop). The presence of mRNA for LD was minimal in the Himalaya sections at this stage (Figure 6.2 e and f). Very little staining could be observed for LDIN at this M1 stage in either variety (Figure 6.3 a, b and c for Sloop, d for Himalaya) although a higher magnification of the coleorhizal parenchyma tissue surrounding the growing root in Sloop sections indicates some cytoplasmic staining (Figure 6.3 c S29). Control sections are clear of staining (Figure 6.3 e).

6.3.3.2: Modification stage M2 for Sloop and Himalaya

The M2 stage was not significantly different to the staining observed at the M1 stage. Aleurone mRNA for both LD and LDIN was not observed at either the M1 or M2 stage in both varieties. An interesting feature seen consistently only in Himalaya sections at the M2 stage for LDIN mRNA was the distinct staining seen at the tip of the growing coleoptile in the vascular bundles (Figure 6.4 a and b, H29) along with sparse staining in the ventral trace region and throughout the scutellum parenchyma (Figure 6.4 a and b, H30). Control sections were clear of staining (Figure 6.4 c).

6.3.3.3: Modification stage M3 for Sloop and Himalaya

Figure 6.5 shows the LD (a and b) and LDIN (c) mRNA in Sloop at the M3 stage. LD mRNA was observed in the growing rootlets possibly in the vascular tissue (Figure 6.5 a, S30) of the embryo along with slight staining at the coleoptile tip (Figure 6.5 a, S31). Higher magnification of the growing rootlet at this M3 point (Figure 6.5 b, S30) indicates specific LD mRNA in the cortex of the growing rootlet. Scutellum parenchyma or scutellum epithelium of the embryo tissue displayed very little LD or LDIN (Figure 6.5 a and c, S32). Very little LDIN inhibitor mRNA could be seen in sections from Sloop at this M3 stage and on further sections progressing into germination (Figure 6.5 c). Relevant control sections did not display staining at this stage for either LD or LDIN mRNA (Figure 6.5 d).

Figure 6.2: Messenger RNA for Limit Dextrinase in Himalaya and Sloop grain at the M1 stage in germination. *In situ* hybridisation on longitudinally cut sections of Sloop and Himalaya at the M1 stage in germination probed with the following: a), c) e) and f) – LD anti-sense riboprobe.Control sections were treated with DIG labelled sense riboprobes corresponding to the anti-sense riboprobe (b and d). a), b), c) and d) – Sloop sections e) and f) – Himalaya section. Bound riboprobes were detected with anti-DIG antibodies and NBT/BCIP staining. sc, scutellum; al, aleurone; rs, rootlets; vt, ventral trace region; co, coleoptile; em, embryo; sce, scutellum epithelium and end, starchy endosperm. Scale bar represents 400 mm. S26 – Scutellum parenchyma, S27 – Root cap and coleorhiza, S28 – Ventral trace
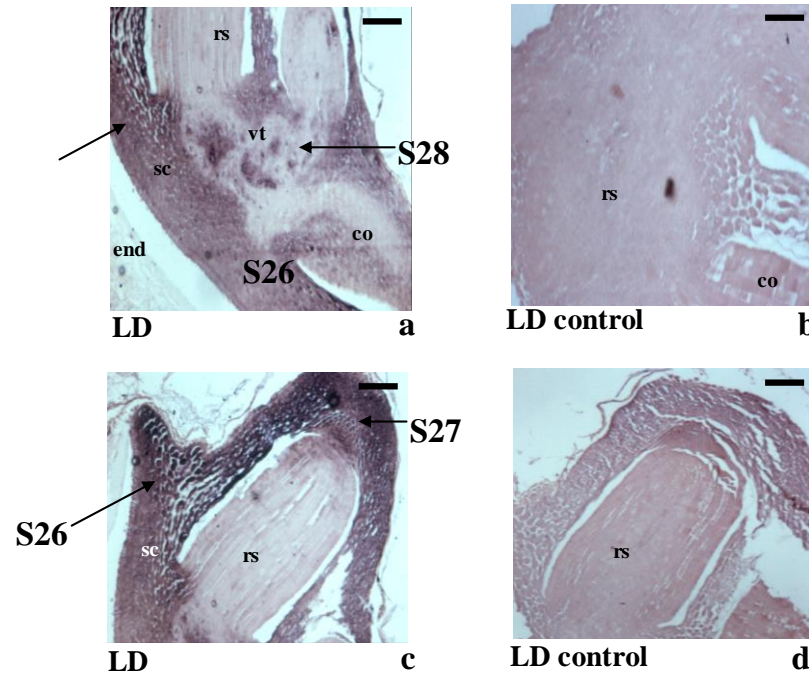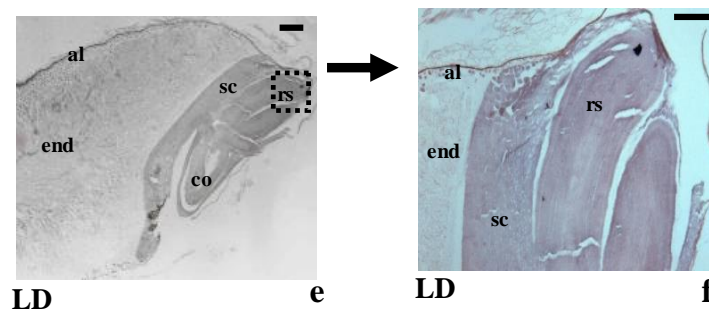
**Figure 6.3: Messenger RNA for Limit Dextrinase Inhibitor in Himalaya
and Sloop grain at the M1 stage in germination.** *In situ* hybridisation on longitudinally cut
sections of Sloop and Himalaya at the M1 stage in germination probed with the following:

a), b), c) and d) – LDIN anti-sense riboprobe. Control sections were treated with DIG labelled sense
riboprobes corresponding to the anti-sense riboprobe (e). a), b), and c) – Sloop sections

d) and e) – Himalaya sections. Bound riboprobes were detected with anti-DIG antibodies and NBT/BCIP
staining. sc, scutellum; al, aleurone; rs, rootlets; vt, ventral trace region; co, coleoptile; sce, scutellum
epithelium and end, starchy endosperm. Scale bar represents 400 mm.
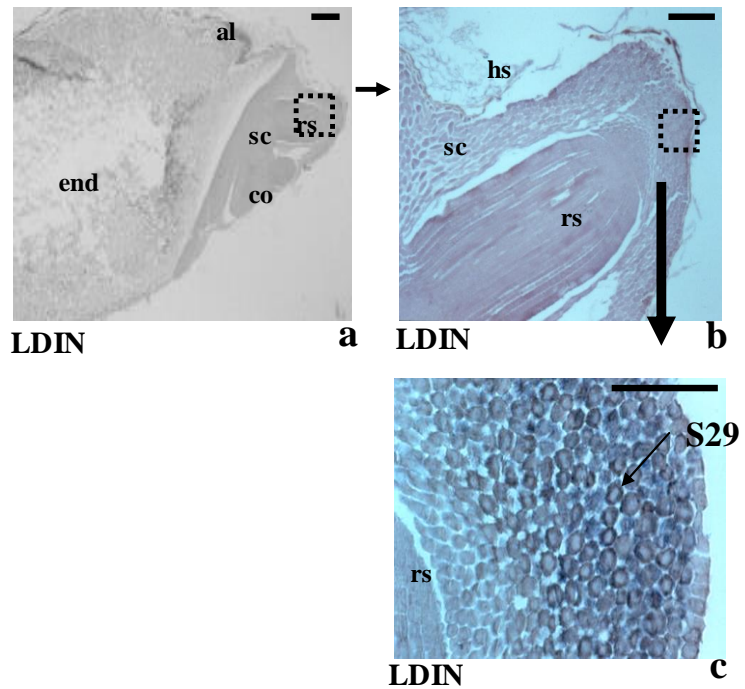
S29 - Coleorhiza
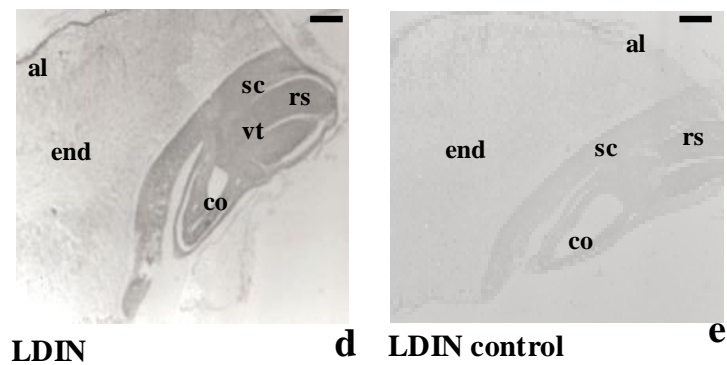
Figure 6.4: Messenger RNA for Limit Dextrinase Inhibitor in Himalaya grain at the M2 stage in germination. *In situ* hybridisation on longitudinally cut sections of Himalaya at the M2 stage in germination probed with the following:a) and b) – LDIN anti-sense riboprobe. Control sections were treated with DIG labelled sense riboprobes corresponding to the anti-sense riboprobe (c). Bound riboprobes were detected with anti-DIG antibodies and NBT/BCIP staining. sc, scutellum; al, aleurone; rs, rootlets; vt, ventral trace region; co, coleoptile; sce, scutellum epithelium and end, starchy endosperm. Scale bar represents 400 mm. H29 – Coleoptile, H30 – Scutellum Parenchyma
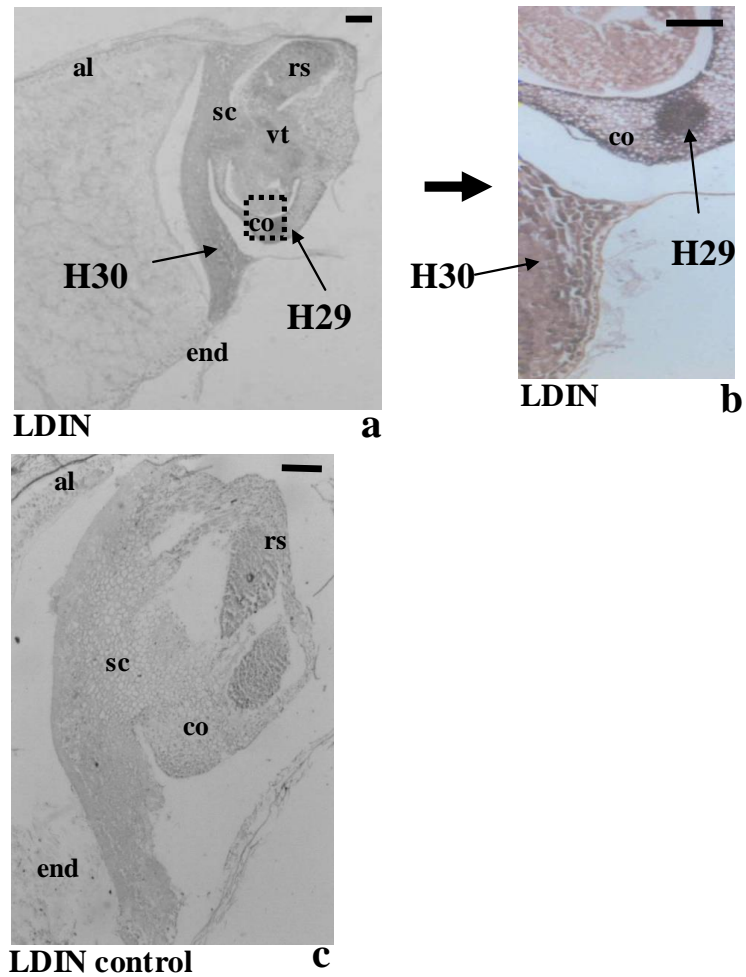
Figure 6.6 shows the mRNA pattern for both LD (a, b and c) and LDIN (d) in Himalaya sections at the M3 stage. A similar pattern was observed for both LD and LDIN mRNA, appearing to be specifically in the embryo, mainly in the coleorhiza, rootlets and coleoptile (Figure 6.6 a, b and c, H31 and H32), very little if any in the scutellum parenchyma and scutellum epithelium as was also seen with Sloop sections in the M3 stage. Relevant control sections did not display staining at this stage for either LD or LDIN mRNA (Figure 6.6 e). Also at this stage (M3) LD mRNA in all cells of the aleurone could be observed in both varieties (Figure 6.7 a, S33 representing Sloop sections) on both the ventral and dorsal side of the grain at the distal and proximal areas of the aleurone relative to the embryo. Nuclear sheaf cells lying in the ventral crease also displayed positive LD mRNA staining (data not shown).

The LD mRNA expression observed in the aleurone of Himalaya sections (Figure 6.7 b, H33) was not as strong as observed in Sloop sections (Figure 6.7 a S33). No visible staining was observed for the LDIN mRNA riboprobes in aleurone layers at this stage in either variety (data not shown) or control sections (Figure 6.7 c).

6.3.3.4: Modification stage M4 for Sloop and Himalaya

The LD mRNA in Sloop grain sections at the M4 stage displayed staining in the growing shoot of the grain in both the leaves and the cortex of the rootlet (Figure 6.8 a, b and d, S34), scutellum epithelium (data not shown) and quite distinctly in the aleurone layers (Figure 6.8 S35). These areas continue to be the main sites of expression in sections of Sloop going from the M4 into the M5 category. Low levels of LDIN mRNA in the scutellum could be seen above control staining in Sloop samples at M4 (Figure 6.8 c) along with now quite evident staining in the aleurone (Figure 6.8 e, S36) and developing root (Vascular tissue; Figure 6.8 e, S34) similar to that observed for LD mRNA (Figure 6.8 b).

Himalaya at M4 also displayed site location similarities between LD and LDIN mRNA expression (Figure 6.9, a for LD and c for LDIN), such as the scutellum epithelium (H34), parenchyma of the scutellum, growing coleoptile/shoot primordium (particularly the shoot apex and leaf area) and ventral trace (procambial strands) region (Figure 6.9 b and d, H35). Only LD mRNA can be detected in Himalaya aleurone layers and sheaf cells in the grain's crease at this stage (data not shown). LDIN mRNA was not detected (data not shown).

Figure 6.5: Messenger RNA for Limit Dextrinase and Limit Dextrinase Inhibitor in Sloop grain at the M3 stage in germination. *In situ* hybridisation on longitudinally cut sections of Sloop at the M3 stage in germination probed with the following: a) and b)– LD anti-sense riboprobe.c)– LDIN anti-sense riboprobe.Control sections were treated with DIG labelled sense riboprobe corresponding to the LDIN anti-sense riboprobe (d). Bound riboprobes were detected with anti-DIG antibodies and NBT/BCIP staining. sc, scutellum; rs, rootlets; co, coleoptile and end, starchy endosperm. Scale bar represents 400 mm. S30 – Root cortex, S31 – Coleoptile tip, S32 - Scutellum
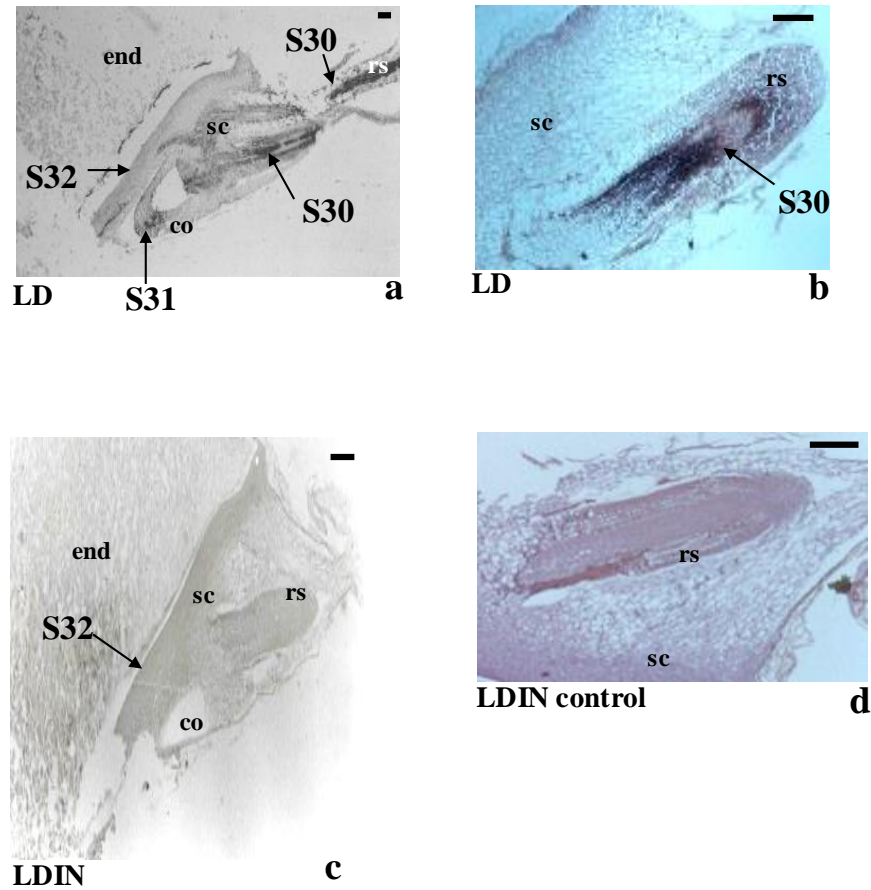
Figure 6.6: Messenger RNA expression of Limit Dextrinase and Limit Dextrinase Inhibitor in Himalaya grain at the M3 stage in germination. *In situ* hybridisation on longitudinally cut sections of Himalaya at the M3 stage in germination probed with the following:a), b) and c)– LD anti-sense riboprobe. d)– LDIN anti-sense riboprobe.Control sections were treated with DIG labelled sense riboprobes corresponding to the anti-sense riboprobe (e). Bound riboprobes were detected with anti-DIG antibodies and NBT/BCIP staining. sc, scutellum; rs, rootlets; co, coleoptile hs, husk; and end, starchy endosperm. Scale bar represents 400 mm. H31 – Rootlets, H32 – Coleoptile
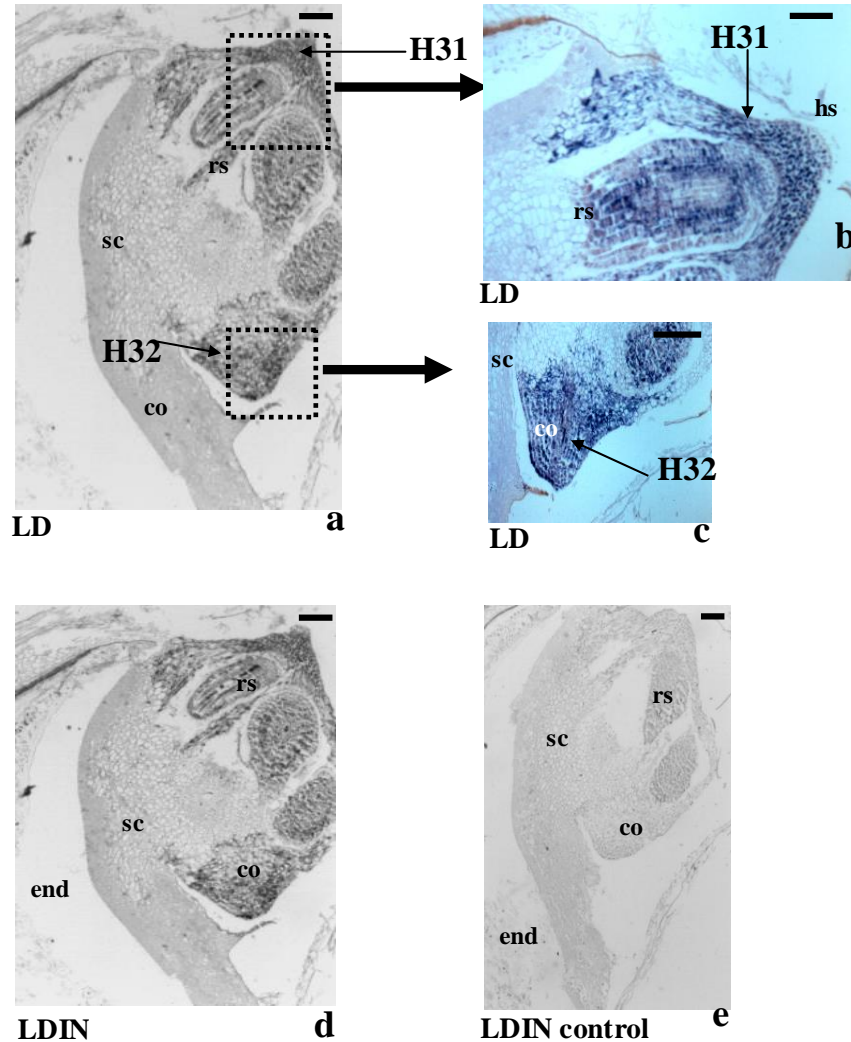
6.3.3 5: Modification stage M5 for Sloop and Himalaya

By the M5 stage (approx 90 h from the start of steeping; Figure 6.10 showing Himalaya) the majority of LD mRNA was concentrated in the expanding young leaves (Figure 6.10 a, H36) and the ventral trace region (H37) of the Himalaya sections and at high levels throughout the whole aleurone layer (data not shown) in both varieties. Low LDIN mRNA expression could be observed in the aleurone at this stage (data not shown), with no staining in the embryo or coleoptile tissue (Figure 6.10 b).

For simplicity a summary of the observed mRNA distribution pattern for LD and LDIN by *in situ* hybridization staining on longitudinally cut Sloop and Himalaya barley grain sections is presented in Figure 6.11.

6.3.4: Total RNA isolation and RT-PCR

At 32, 56 and 96 h into germination total RNA was isolated from whole grain samples and analysed for the presence of mRNA encoding the LD and limit dextrinase-inhibitor. cDNA encoding LD and LDIN was detected by RT-PCR (data not shown) for all the collection points, except for 96 h where the LD was undetectable. The absence of LD expression at 96 h is not consistent with data presented in the literature and the mRNA *in situ* hybridisation results presented here. It is considered to be a technical problem with the assay despite numerous attempts with changes in the PCR conditions. More intense LD bands in Sloop extracts at the early germination time point (32 h), compared to bands seen from Himalaya grain RNA extracts imply higher expression levels, but this assay method is only semi-quantative. LDIN product intensity increased from 56 to 96 h into germination, broadly indicative of increased levels.

A PCR fragment from LD in both varieties was also observed in developing grain extracts, consistent with reports in the literature on the presence of LD in this tissue (Burton *et al*. 1999; Sissons, 1993). LDIN PCR products were also generated from developing grain by RT-PCR (data not shown).

6.3.5: LD enzyme levels

LD enzyme activity was measured in extracts of grain samples taken at similar times to those used for the RT-PCR analysis (32, 56 and 96 h). A relationship between mRNA (transcription) and enzyme activity was assumed. Results in Figure 6.12 are consistent with increasing protein levels, as previously documented by others (Longstaff and Bryce, 1991; 1993). Total enzyme activity for LD increased as

germination proceeded in both varieties (Figures 6.12 and 6.13), although only Sloop grain extracts had detectable enzyme early in germination (32 h) complementing the observed *in situ* hybridization mRNA expression patterns (Figure 6.1), RT-PCR result (data not shown) and past literature. Activity in Himalaya extracts at the later stages of germination was comparable to Sloop (56 h) and significantly higher than Sloop at 96 h. A comparison of the LDIN protein levels would have been advantageous but the assay system is not feasible without large quantities of purified LDIN (MacGregor *et al*., 1994).

Figure 6.7: Messenger RNA for Limit Dextrinase in Sloop and Himalaya grain aleurone layers at the M3 stage in germination. *In situ* hybridisation on longitudinally cut sections of Sloop and Himalaya at the M3 stage in germination probed with the following: a) – LD anti-sense riboprobe (Himalaya). b) – LD anti-sense riboprobe (Sloop). Control sections were treated with DIG labelled sense riboprobes corresponding to the anti-sense riboprobe (c). Bound riboprobes were detected with anti-DIG antibodies and NBT/BCIP staining. al, aleurone and end, starchy endosperm.

Scale bar represents 400 mm. S33 – Aleurone cells, H33 – Aleurone cells
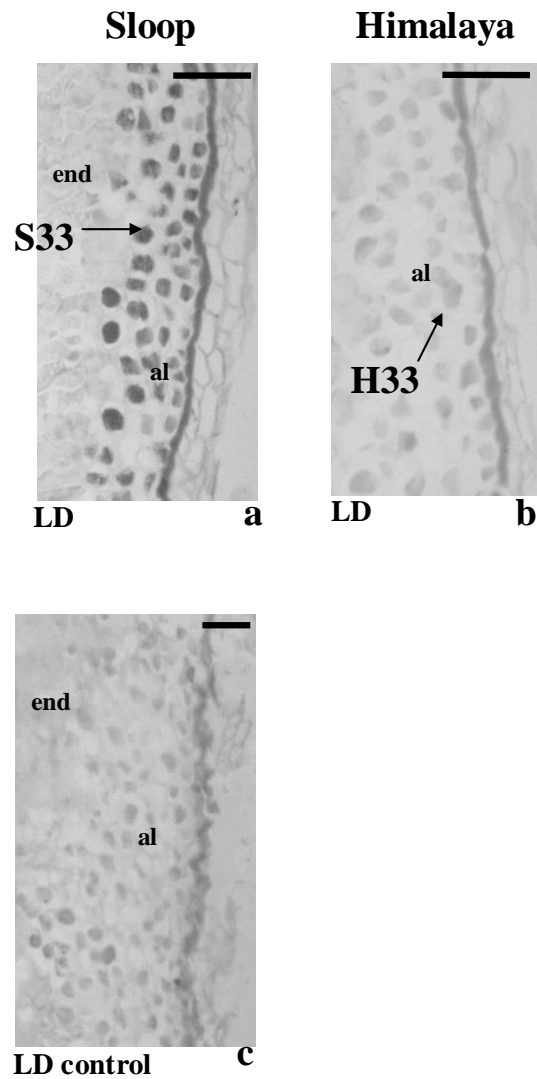
Figure 6.8: Messenger RNA for Limit Dextrinase and Limit Dextrinase Inhibitor in Sloop grain at the M4 stage in germination. *In situ* hybridisation on longitudinally cut sections of Sloop at the M4 stage in germination probed with the following: a), b) and d) – LD anti-sense riboprobe. c) and e) – LDIN anti-sense riboprobe.Control sections were treated with DIG labelled sense riboprobes corresponding to the anti-sense riboprobe (f). Bound riboprobes were detected with anti-DIG antibodies and NBT/BCIP staining. sc, scutellum; al, aleurone; rs, rootlets; vt, ventral trace region; co, coleoptile; em, embryo and end, starchy endosperm. Scale bar represents 400 mm. S34 – Vascular tissue, S35 – Aleruone cells, S36 – Young leaves

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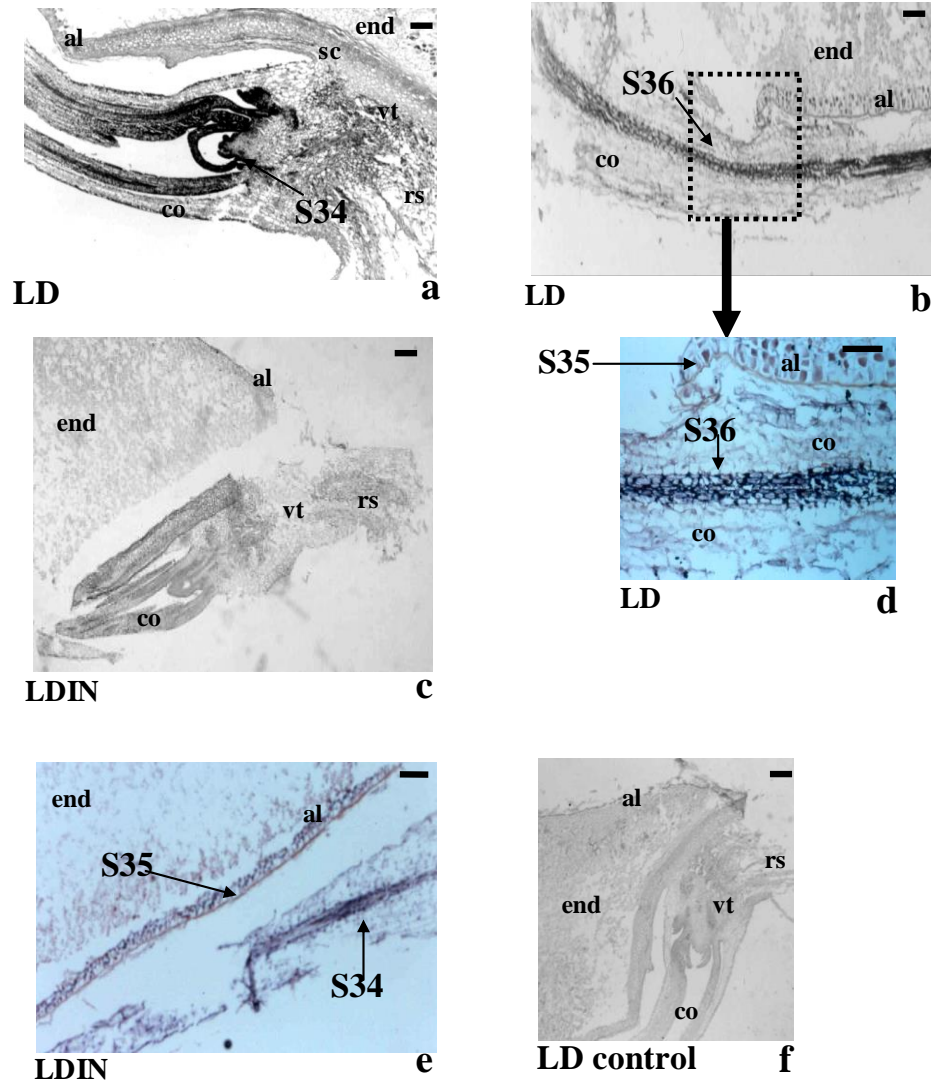
Figure 6.9: Messenger RNA for Limit Dextrinase and Limit Dextrinase Inhibitor in Himalaya grain at the M4 stage in germination. *In situ* hybridisation on longitudinally cut sections of Himalaya at the M4 stage in germination probed with the following: a) and b) – LD anti-sense riboprobe. c) and d) – LDIN anti-sense riboprobe.

Control sections were treated with DIG labelled sense riboprobes corresponding to the anti-sense riboprobe (e and f). Bound riboprobes were detected with anti-DIG antibodies and NBT/BCIP staining. sc, scutellum; al, aleurone; rs, rootlets; vt, ventral trace region; co, coleoptile and end, starchy endosperm. Scale bar represents 400 mm. H34 – Scutellum epithelium, H35 – Young leaves
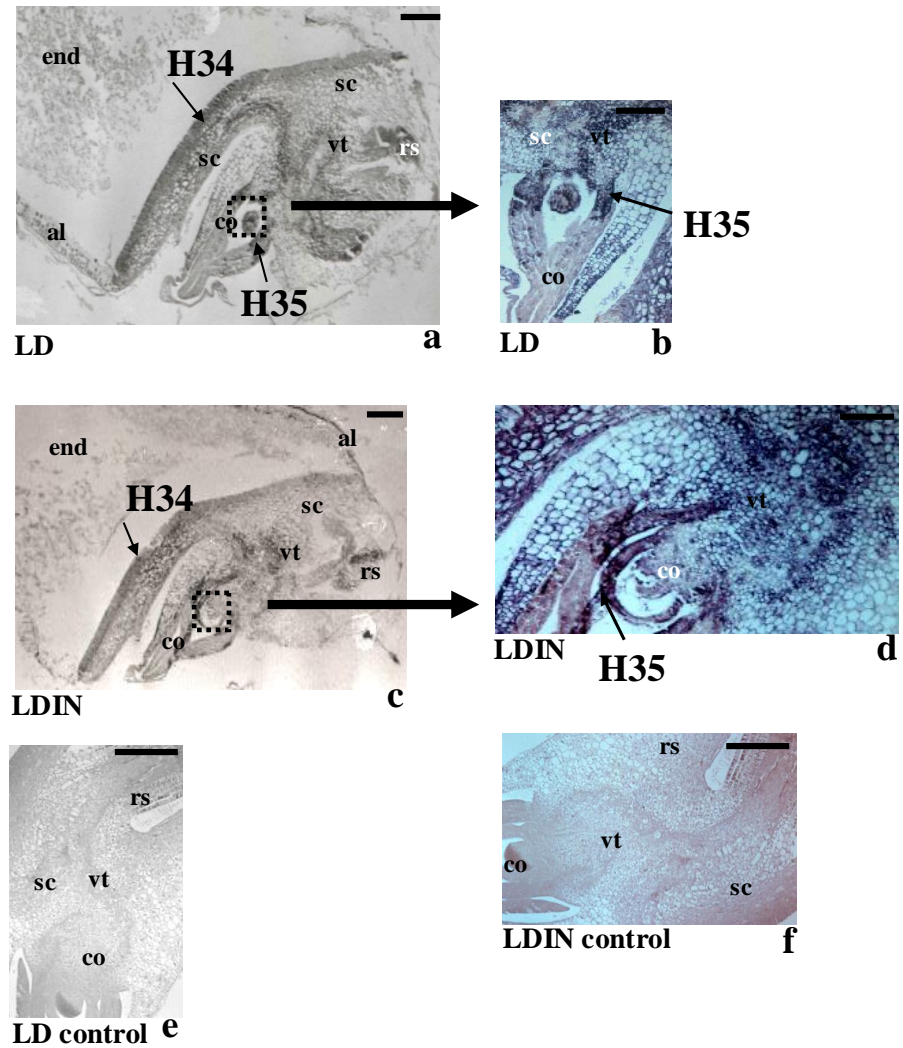
Figure 6.10: Messenger RNA for Limit Dextrinase and Limit Dextrinase

Inhibitor in Himalaya grain at the M5 stage in germination. *In situ* hybridisation on longitudinally cut sections of Himalaya at the M5 stage in germination probed with the following:

a)– LD anti-sense riboprobe. b)– LDIN anti-sense riboprobe. Bound riboprobes were detected with anti-DIG antibodies and NBT/BCIP staining. sc, scutellum; rs, rootlets; vt, ventral trace region and co, coleoptile. Control sections similar to those shown in Figure 6.9. Scale bar represents 400 mm

H36 – Ventral traceH37 – Young shoot leaves
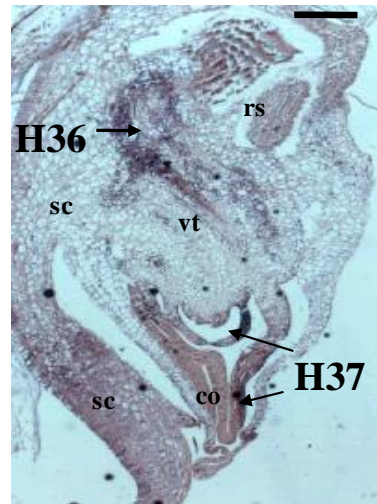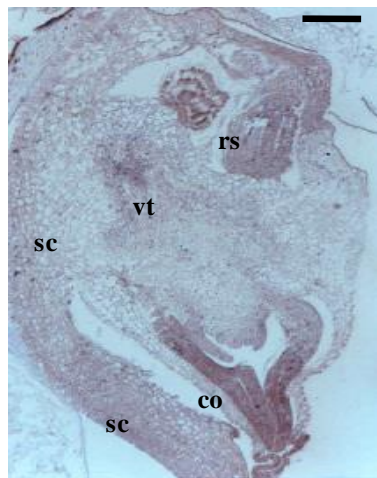
Figure 6.11: Hand drawings of compiled mRNA expression for LD and LDIN.

A (Sloop):   M1   M3   M5

B (Himalaya): M1   M3   M5
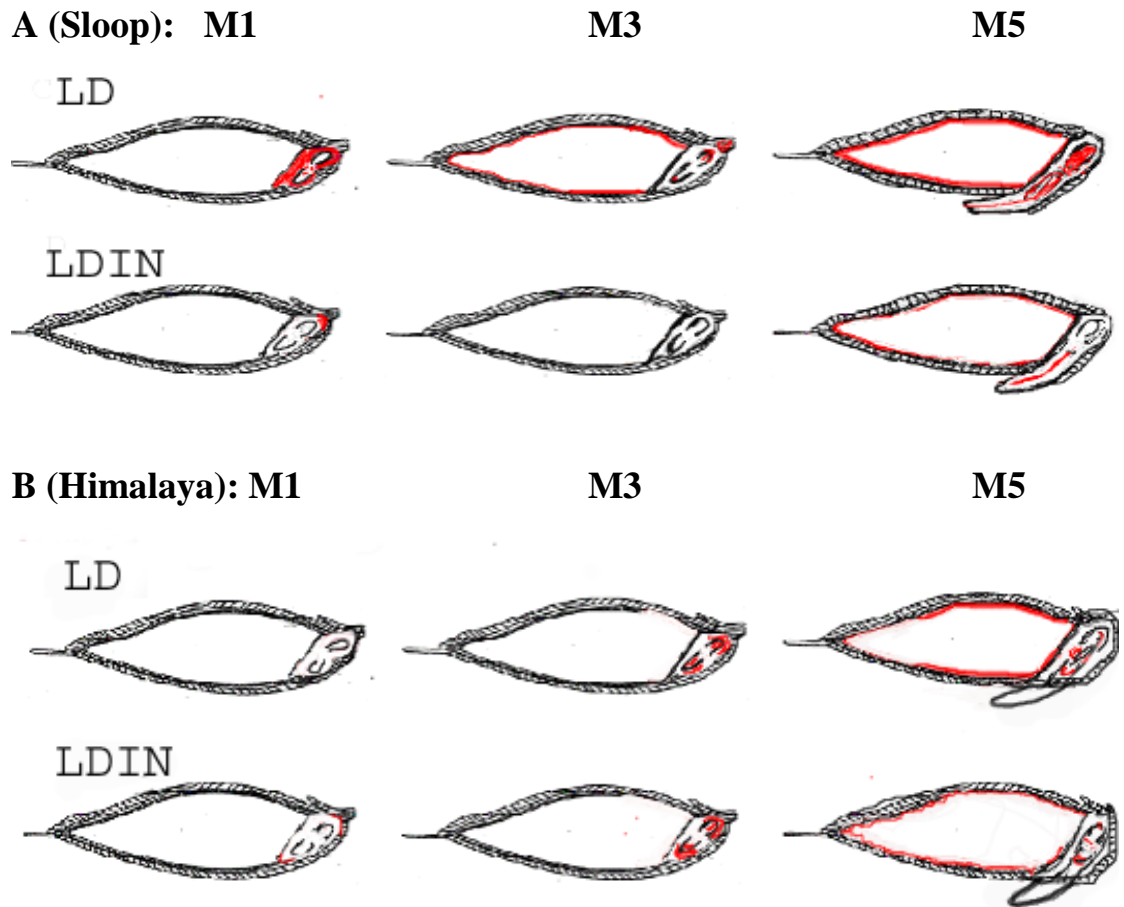
Figure 6.11: Hand drawn diagram showing site location of staining is taken from Figure 2.1. A: Sloop. B: Himalaya.

Figure 6.12: Total LD enzyme levels. Extracts of grain germinated and collected at the times shown (32, 56 and 96 h) were analysed by Megazyme Total LD enzyme assay to determine total active LD enzyme levels.
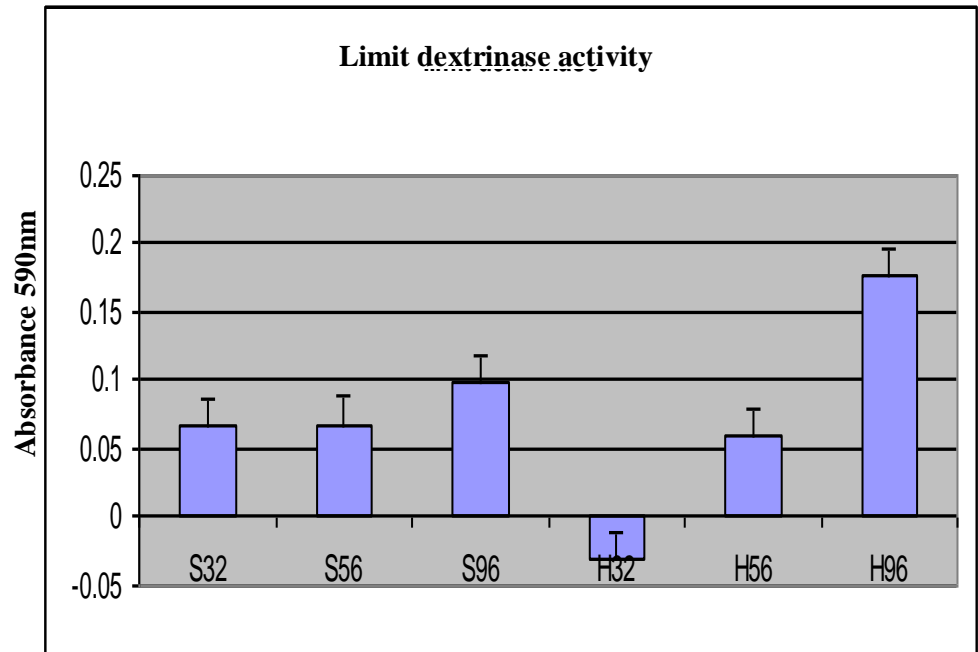
Name of grain sample	Absorbance	Milli-Units from standard curve*	Units/Kg malt [#]
Sloop 32 h	0.06	1.2	**38.4**
Sloop 56 h	0.07	1.4	**44.8**
Sloop 96 h	0.10	2	**64**
Himalaya 32 h	0	0	**0**
Himalaya 56 h	0.055	1.1	**35.2**
Himalaya 96 h	0.16	3.2	**102.4**

(# per assay, * supplied by Megazyme for the kit supplied substrate). Values are expressed according to McCleary (1992) and expressed as specific activity per grain extract sample. H, Himalaya; S, Sloop.

6.4: Discussion

The presence of LD mRNA in developing barley grain was found in this study, but was not detected in mature un-germinated grain (*in situ* hybridization assessment). The presence of total enzyme activity in un-germinated grain has been documented, but there is still a debate about whether this is likely to be due to residual protein laid down in the developing grain. As reported by Stahl *et al.* (2004) and observed in this study, LDIN mRNA was detected in developing grain by RT-PCR (data not shown). This is not an unexpected result as LDIN proteins are perceived to have an association with LD protein (Sissons, 1993) in developing grain and LD mRNA was also demonstrated in developing grain. Future studies would need to demonstrate that the detected mRNA indeed correlates to protein expression. Unfortunately no reliable antibodies were available for either LD or LDIN at the time this study was carried out.

6.4.1: Verification of riboprobe specificity

To ensure that the riboprobes were specific they were assessed by Southern blot analysis against cDNA fragments of LD, LDIN and endo-xylanase used in Chapter 5. Control (sense) riboprobes did not display any cross reactivity (data not shown).

A BLAST analysis (as per section 4.2.4) was also performed using the LDIN and LD cDNA sequences presented in section 4 2.4 which did not identify any similarities of significance.

6.4.2: In situ hybridization of germinated barley grain to determine LD and LDIN mRNA distribution

       6.4.2.1: Modification stage M1

The pattern of mRNA and protein expression seen in Figure 6.2 confirmed that the main site of LD early in germination is the scutellum, as noted by Burton *et al.* (1999) using RT-PCR, from immunological studies by Sissons (1991) and supported by enzyme secretion data (Longstaff and Bryce, 1991).

The mRNA pattern observed for LD early in germination was different to the classical scutellum epithelium expression seen for α-amylase, another starch degrading enzyme (Sugimoto *et al.*, 1998; Gonzalez *et al.*, 1998; Ranki, 1990; Gibbons, 1981). However, Van der Ma (2000) and Gibbons (1979) reported similar protein patterns for α-amylase across the entire scutellum at 24 h into germination, ascribing its presence to residual enzyme carried over from developing grain. Sugimoto *et al.* (1998), using *in situ* hybridization with a high pI α-amylase gene probe, has also

highlighted scutellum epithelium cell expression at this point in germination. Although not discussed by the authors their figures show a similar pattern of staining in the growing shoot, root tip and ventral trace region as presented here for LD mRNA. A possible role for LD at this point could be to act on the numerous starch granules seen in the parenchyma cells of the embryo; as illustrated on GMA embedded sections in Figure 2.2.4 and previously reported by Smart and O' Brien (1972) and Van der Meulen *et al.* (2000). Although the majority of the LD enzyme is believed to act on the large reservoir of starch in the endosperm; half the reported LD enzyme activity at this stage is reported to be in the embryo (Schroeder and MacGregor, 1998). A third possible function of LD in the embryo tissue at this stage could be in the turnover of starch that accumulates transiently in the amyloplasts in the parenchyma cells of the scutellum during grain germination (Smart and O'Brien, 1973). LDIN mRNA was detected, but at very minimal amounts at this early stage in germination. This could have several implications; for example, does this inhibitor once translated immediately bind to the LD enzyme at this early stage in germination as reported by others (Yamada 1981; MacGregor *et al*., 1994, Sissons, 1992; Longstaff and Bryce, 1991). Immunocytochemistry data with specific antibodies as presented in Chapter 4 with the (1,3;1,4)-β-glucanase isoenzymes would address this to some extent. Ideally, assessment of the bound (inactive) and unbound (active) LD enzyme levels would have been useful at this early stage in germination, along with the total activity as measured in Figure 6.13. Unpublished data from MacGregor *et al*. (personal communication) found LDIN protein predominantly in the starchy endosperm, most likely carried over from the developing grain. From a technical perspective this may be significant, because it may be possible to adjust mashing conditions to prevent the inhibitors from complexing with and inhibiting the LD. On the other hand, a totally different mechanism or inhibitor maybe responsible for the inactivation of LD at this early stage in germination.

6.4.2.2: Modification stage M2

The M2 stage (data not shown) was not significantly different to the mRNA or staining seen at the M1 stage, indicating that embryonic LD mRNA and the absence of LDIN mRNA was maintained until approx 32 h into germination. Aleurone mRNA for both genes was not observed at either the M1 or M2 stages in both varieties. An interesting feature of the M2 stage for LDIN mRNA in Himalaya sections was distinct staining seen at the vascular bundle of the growing coleoptile (Figure 6.4). The reasons for expression in this isolated area are unclear, but could imply the involvement of LDIN in a protective role.

6.4.2.3: Modification stage M3 and M4

For the first time in the germination schedule mRNA staining for both LD and LDIN can be seen in similar tissue locations, although this was only observed for Himalaya grain sections (Figure 6.6). Very little LDIN inhibitor mRNA could be seen in sections from Sloop at the M3 stage and on further sections progressing into germination (Figure 6.8). The location similarity is interesting but cannot answer questions about the role of the two proteins in the germination process.

The later appearance of LD mRNA as measured by *in situ* on both the Himalaya and Sloop sections and indicated by PCR expression, is in agreement with the sequential role that LD has in endosperm starch degradation following other hydrolytic enzymes (Schroeder and MacGregor, 1998). There appears to be a delayed production of LD mRNA in the scutellum epithelium during barley germination (Figure 6.8, M4 for Sloop and Figure 6.9, M4 for Himalaya) compared to $(1,3;1,4)$-β-glucanase mRNA (Figure 4.7, M3 for Sloop).

6.4.2.4: Modificationn stage M5

Consistent with data presented in past literature, such as enzyme expression studies (Schroeder and MacGregor, 1998), RT-PCR and Northern blot analysis (Burton *et al*., 1999; Kristensen *et al*., 1999) along with the profile of other enzymes involved in barley germination, the aleurone layers appear to be the main site of LD mRNA accumulation later in germination. To a lesser extent LDIN mRNA was also present in the aleurone layers at this later stage in germination and modification of the grain. It was found that all the cells in the rows of the aleurone (Figure 6.7) contain LD mRNA, which is consistent with transcript patterns of the other hydrolytic enzymes. The role that germ aleurone and sheaf cells apparently have in LD and LDIN protein expression may be just as important as the classical aleurone surrounding the endosperm. The ventral crease in which the sheaf cells lie is an idea channel for enzyme movement into the endosperm. LD mRNA appears over the whole aleurone in a short time, although if grain had been sampled at more points along the time course, a wave of accumulation may have been seen. The reason for this broad distrubution is unclear, particularly as it is different from the wave-like pattern noted for the $(1,3;1,4)$-β-glucanase isoenzymes in Chapter 4. One explanation could be the regulation of the promoter of the LD gene, *i.e*. through GA_3 stimulation, or the highly fragmented nature of the gene could possibly make regulation of LD more complex than other genes that are transcribed in aleurone layers of germinated barley (Burton *et al*., 1999; Kristensen *et al*., 1998; Fincher, 1989).

The presence of LD mRNA has been documented in growing young leaves and roots of barley (RT-PCR, Burton *et al*., 1999) and is supported by the *in situ* hybridisation assay presented here. The patterns of transcript in the actively growing parts of the shoot (shoot apex and leaf primordia, Figure 6.8) are very distinct in the shoot primordium surrounded by the coleoptile tissue. The extent of this should be investigated in light of the finding by Longstaff and Bryce (1991) where grain grown under germination conditions to encourage the development of roots and shoots (exposure to high levels of water), had higher levels of LD than under normal controlled malting conditions.

6.4.3: *Varietal and expression comparison between LD and LDIN*

The dynamics of LD mRNA accumulation appears to be very different between the two varieties, with a significant delay in sections of Himalaya grain compared to Sloop (Figures 6.11, 6.13 and 6.14), more so than can be accounted for by the obviously reduced growth of the coleoptile of Himalaya grain (Figures 6.6 and 6.10). The difference for LD is distinct between the two varieties as compared to the more subtle difference that was noted in the timing of $(1,3;1,4)$-β-glucanase mRNA apperance (Chapter 4) and in the endosperm modification pattern (Chapter 2) each variety displays. This could be explained by the more selective breeding pressure placed on grain with respect to $(1,3;1,4)$-β-glucanase isoenzyme EII and its role in malting versus the different function that LD is believes to have during grain germination. Other researches have previously noted a difference in LD and LDIN activity in different barley cultivars at the enzymic level (MacGregor *et al*., 1995; Longstaff and Bryce, 1993; Sisson, 1992; Lee and Pyler, 1984), which supports the results presented in this study.

As previously mentioned, unexpectedly the Himalaya grain sections appeared richer in LDIN mRNA during grain germination as compared to Sloop. This is possibly explained by the hulless nature of Himalaya leading to higher moisture content, as Longstaff and Bryce (1991) noted that LDIN expression was increased in grain grown in higher moisture. Similarly most of the past research has concentrated on malting varieties such as Harrington, and as a feed variety Himalaya appears to have a different profile.

Overall, detectable levels of LDIN mRNA as assessed by *in situ* hybridization and RT-PCR (not shown) indicated that message was more highly abundant later in germination, at a similar time and location to LD mRNA. If the *in situ* and RT-PCR

assessment had been taken beyond the 96 h format, a decrease in LDIN transcript would have been expected as reported by Macgregor (1995; 1994).

The timing of release of LD and LDIN proteins into the starchy endosperm during germination from the aleurone, and the association these two proteins has become a key topic of discussion. This stems from lack of evidence concerning the secretion of LD from isolated aleurone layers into the medium during *in vitro* studies (MacGregor *et al*., 1998) and the absence of a signal peptide sequence on the LD cDNA (Burton *et al.,* 1999; Kristensen *et al*., 1998; reviewed by MacGregor, 2004). Only a small fraction of total LD activity can be accounted for after 120 h of germination in the starchy endosperm, well into the time that the aleurone is actively accumulating LD mRNA (Figures 6.7 and 6.8).

The role of secretion (involving the transport of the enzymes into the ER and then across the plasma membrane) and release (diffusion of the enzyme through the aleurone cell wall) was not addressed in this study. But given that aleurone cells don't contain starch (MacGregor and Fincher, 1993), are nonphotosynthetic and that LD is found in the starchy endosperm (MacGregor *et al*., 1998), the presence of the transit peptide on the LD sequence is difficult to explain. Scutellum mRNA levels (Figures 6.6, 6.7, 6.10 and 6.11) do not appear to support a role for the scutellum as the sole source of the endosperm LD enzyme detected later in germination, although translation efficiencies were not measured. Researchers have reported evidence of autolysis or programmed cell death in barley aleurone following treatment with GA_3 (Kuo *et al*., 1996, Wang *et al*., 1996). The release of LD into the endosperm by this mechanism was dismissed by Burton *et al.* (1999) since unlike necrosis, the membranes of cells undergoing programmed cell death retain integrity and do not leak cellular contents. Indeed, the LD enzyme was not found to be secreted at an increasing volume during the stage when programmed cell death in aleurone layers was occurring (Schroeder and Macgregor, 1998) but further assessment of the paraffin grain sections used for the *in situ* studies could be carried out in parallel with a tunnel assay and immunostaining to link the presence of LD mRNA, protein and programmed cell death (Wang *et al*., 1996). Future immunostaining experiments both at the light, confocal or transmission electron microscopy level may also address some of these protein secretion questions.

It has also been proposed that the presence of a signal peptide sequence on the LDIN sequence (Lazaro *et al*., 1988) could be a means by which LD protein is escorted through the aleurone into the endosperm in a bound form.

Another theory that merits serious consideration is that the majority of LD activity seen during germination comes from protein that is laid down during development, persisting through drying and then becoming active as the grain hydrates to grow. As such the LDIN may complex with the LD protein in order to protect it during grain maturation and drying, and therefore opens the question of examining the appearance of both LD and LDIN mRNA in the developing grain, as performed by Burton *et al.*, (2002; 2006).

As with (1,3;1,4)-β-glucanase mRNA (Chapter 4), staining was consistently seen at the ventral trace region of the scutellum/embryo, where procambial stands which are channels that supply nutrients to the developing leaves are found (Bowes, 1996) and which are implicated as the site of GA_3 synthesis. The effect of GA_3 on LD and LDIN was not assessed in this study, but is worthy of future consideration as levels of secreted LD enzyme and mRNA are enhanced by GA_3 (Hardie, 1975; Manners and Yellowlees, 1973; Lee and Pyler, 1984; Schroeder and MacGregor, 1998; Burton *et al.,* 1999). Changes in accumulation around the ventral traces might be observed with GA_3 treatment of the germinated grain, and indeed influence expression in the growing rootlet and shoot near the ventral trace region.

6.4.4: Varietal comparisons between LD/LDIN, xylanase and the (1,3;1,4)-β-glucanase isoenzymes

The most prominent feature of the mRNA staining patterns observed for LD is the absence of staining in Himalaya grain sections at the early stages compared to that seen for either of the glucanase isoenzymes (Figure 4.18), and xylanase (Figure 5.17) particularly in the scutellum epithelium.

Common to all the mRNA measured was the widespread embryo scutellum staining at M1 and the expression in the growing rootlet and coleoptiles at the M3 stage. A different staining pattern was observed at both the M3 and M5 stage in the growing shootlets comparing the xylanase and LD probes. The distinct ventral trace staining observed for the glucanases and xylanase probes was absent from sections stained for LD. The LIDN probe was dissimilar to all the other probes at all stages in this study, indicative of a totally different expression pattern and function to the other tested enzymes.

6.4.5: Industry significance of LD/LDIN

Higher levels of LD can potentially result in a 2-4 % increase in fermentability (Evans *et al.,* 2005), of particular importance to those brewers who use rice adjuncts, thus

facilitating more efficient brewing (Kneen, 1984; Bryce *et al.,* 1995). A strong relationship has also been found between LD and hot water extract (HWE, Collins *et al.,* 2005), which suggests that LD is possibly the most limiting factor in the brewing process, in comparison to the other barley enzymes (Collins *et al*., 2005). Generally, barley breeders have traditionally not selected for LD in their programs, as it has only ever made up a small proportion of the diastase enzyme profile, but recent research combined with results presented here suggests that it may be unwise to ignore LD. Extra fermentable sugars in the form of limit dextrins as a result of higher LD activity can result in additional mouth feel (taste) in the brewed product (Ragot *et al.,* 1989). A market sector demanding lower carbohydrate or "lite" beers would also favor higher levels of LD activity.

The nature of the research presented in this Chapter in combination with the profile of the other enzymes presented in previous Chapters, broadens our understanding about the concerted action of these enzymes in the barley grain aiding both the barley breeders in the development of new cultivars, the maltster to optimize the malted grain, and the brewer in producing a final fermentable wort product. This knowledge enables breeders and maltsters to determine the appropriate areas to place selection and germination pressures.

6.5: Conclusions and further work

The main objective of this study was to document the location and timing of LD and LDIN mRNA appearance during barley germination. It was hoped that this would then answer questions relating to the association of LD and LDIN *in vivo* as well as confirming that peak LD expression occurs much later during germination than expected.

The main conclusions are that;

a) LD mRNA appears in the scutellum and other sites such as the embryo of the barley grain early in germination, in the aleurone at a later stage, but not in a wave-like fashion as noted for other enzymes in this study.

b) LDIN mRNA appears to be isolated to the growing tips of the roots and shoots in the early stages of germination, and follows a similar pattern of expression as LD in the later stages, but was stronger in Himalaya barley.

c) This presence of LD and LDIN mRNA at a similar location strengthens the concept of an association between the two translated proteins but it is hard to prove the formation of a complex. This similar location suggest that they are physically

near enough to interact but actual evidence of the direct interaction of LD and LDIN protein (MacGregor *et al.,* 2000) is required.

Further work will be needed to complete the picture of mRNA appearance beyond 110 h from the start of steeping to determine if the transcript is consistent with enzyme expression and is at a maximum very late in germination (164 h from the start of steeping), but maintaining intact grain samples will be technically challenging. The longer germination schedule also goes beyond the feasible malting time set by the industry. This has implications for current malting schedules used by the industry, as they may not be fully utilizing LD activity.

Ultimately this study should be co-ordinated with a follow up on LD and LDIN protein expression, as the presence of transcript does not account for translational efficiencies or other unknown inhibitors. Attempts were made during the course of this study to obtain specific antibodies against LD and LDIN proteins without success. The LD and LDIN antibodies showed considerable cross reactivity in Western blot analysis, which could be controlled with the use of appropriate dilutions but made them unusable for tissue immunostaining assessment (Sisson, 1992). Consideration was also given to heterologous expression of the LDIN protein for antibody production. Small quantities of protein were obtained (R. Burton, School of Agriculture, Food and Wine, Waite Campus, Adelaide University) but time constraints existed in obtaining antibodies. Caution needs to be exercised when examining LDIN at the antibody level due to the presence of LDIN inhibitor proteins (Macri *et al*., 1993).

The transgenic approach could also feature in future experimental studies; down-regulating the LDIN gene using RNAi technology and then assessing the effects could add to the definition of gene and protein function.

In addition to a role in starch hydrolysis in germinated grain, there is a suggestion that cereal debranching enzymes are involved in starch synthesis (Duffus and Cochrane, 1993). Both the LD enzyme (Sissons *et al.,* 1993; Kristensen *et al.,* 1993) and mRNA transcripts detected by RT-PCR (data not shown, Burton *et al.,* 1999; Stahl *et al.,* 2004) are present in the developing endosperm of immature grain. A future study using *in situ* hybridization should be directed towards monitoring LD and LDIN gene expression in the developing grain. Stahl *et al*. (2004) produced transgenic barley plants designed to down regulate LDIN by antisense, and showed pleiotropic effects

on other barley enzymes, such as α and β amylase, suggesting a link to these enzymes, starch synthesis and LD.

The debranching enzyme, isoamylase also mentioned by Burton *et al*. (2002) and described in barley and maize, maybe an interesting follow up candidate for the *in situ* hybridization assay in developing grain as it possibly also has a role in starch biosynthesis.

More recently it has also been suggested that starch hydrolysis and the enzymes involved difficult to determine because genes encoding enzymes involved come from a much larger and complex family than originally though (Mascher *et al*., 2017; Betts *et al*., 2017).

Figure 6.13: Summary of Limit Dextrinase and Limit Dextrinase Inhibitor mRNA in Himalaya grain

Tissue	M1	M1		M3	M3		M5	M5
	LD	LDIN		LD	LDIN		LD	LDIN
Tip of scutellum (edge of the rootlet)				X	X			
Tip of scutellum (edge of the coleoptile)	X			X	X			
General scutellum tissue								
Scutellum epithelium							X	X
Rootlet tissue				X	X		X	X
Ventral trace region							X	X
Coleoptile				X	X		X	X
Shoot primordium							X	X
Aleurone				X			X	X

Figure 6.14: Summary of Limit Dextrinase and Limit Dextrinase Inhibitor mRNA in Sloop grain

Tissue	M1	M1		M3	M3		M5	M5
	LD	LDIN		LD	LDIN		LD	LDIN
Tip of scutellum (edge of the rootlet)	X	X						
Tip of scutellum (edge of the coleoptile)	X							
General scutellum tissue	X							X
Scutellum epithelium							X	
Rootlet tissue	X			X			X	
Ventral trace region	X			X			X	
Coleoptile				X			X	
Shoot primordium							X	X
Aleurone				X			X	X

Chapter 7

Summary and future directions

A non-radioactive *in situ* hybridization technique for detecting specific mRNA in paraffin-embedded barley grain sections was developed to define spatial and temporal patterns for prominent barley enzymes; specifically (1,3;1,4)-β-glucanase isoenzymes EI and EII, (1→4)-β-endo-xylanase, limit dextrinase and limit dextrinase inhibitor. The coordinated expression of these enzymes is pivotal to successful cell wall degradation and maturation, which is fundamental for plant growth but also has significant industrial implications. Future improvements in the precision of malt analyses and in techniques of malting and brewing are more likely to come from experimental approaches such as presented here, than from any other avenue.

The main objective of this study was to establish if the transcripts of key enzymes involved in barley germination are synthesised in both a tissue specific manner and a fixed temporal sequence during grain germination. The general pattern of endosperm cell wall breakdown as assessed by CW fluorescent staining was consistent between the two varieties assessed in this study, except for a delayed, and subsequently slightly reduced, wave of emigrating degradation from the scutellum area of Himalaya grain, but the differences were not quantitated. Degradation of the endosperm areas close to aleurone layers in both varieties occurred at the later stages of germination. The progression of endosperm cell wall (1,3;1,4)-β-glucan modification presented in this study follows a reported pattern in general agreement with a number of other authors who described the breakdown as a front moving towards the distal end of the grain, extending parallel from the face of the scutellum. This therefore supports the "symmetrical" pattern of modification.

Some attempts were also made to relate the presence of the transcript to the presence of the protein and to try and elucidate if the expression of the enzymes is in parallel or sequential throughout the whole process. The role of individual isoenzymes was also addressed, as an ever increasing number of these are being identified in the functional genomics era.

The adaptation of the CW staining technique routinely used within malting laboratories is a novel approach to identifying the stages of modification. A modified method was necessary to preserve grain morphology and allow successful downstream ISH or immunostaining on the same grain blocks once the CW method had provided an accurate assessment of its modification stage.

In order to balance both morphological preservation with a functional assay such as the ISH, fixation combinations were tested on germinated barley grain and it was determined that the optimal preservation was obtained with a combination of glutaraldehyde at 0.25% and paraformaldehyde at 4%. As highlighted earlier, the direction and consistency of sectioning is critical, and indeed fundamental to the success of the whole procedure. The majority of work presented here used 7um longitudinal sections displayed at a pre-determined angle, allowing results to be compared across the whole study. But, as the endosperm is not structurally homogenous it would be beneficial to evaluate the whole kernel by varying the angle of sectioning. Also, to attempt a reconstruction of serial sections stained with CW, as seen with the work of Gibbons, (1980) and Suigimoto *et al*., (1998), would have been advantageous.

It took a methodical approach to develop a highly sensitive non-radioactive ISH assay compatible with germinated barely grain tissue. Options were considered from the earliest step of pre-fixation through to the sealing of the final product on the slide. As a result, a robust, reliable, and valuable ISH assay has been developed. The paraffin-embedded grain had many advantages, one in particular being the ability to yield sequential sections for analysis. The thermal instability of the grain, particularly the starchy endosperm, limited the incubation temperatures that could be used in the ISH assay and this warrants improvement to allow a wider range of probes to be tested. Improvements in the fixation process such as the use of n-heptane gas and/or vacuum fixation has been successfully attempted by other barley researchers and merit consideration for future studies to improve grain section stability on the slide. Other approaches could also be employed to improve sensitivity of the ISH by amplification of either the target nucleic acid sequence prior to ISH or in the method of signal detection after the hybridisation is completed (Qian and Lioyd, 2003).

The economic importance of any study on starchy endosperm degradation to the malting and brewing industry cannot be under-estimated. To understand particular enzyme profiles could possibly lead to a more rapid malting process, leading to less water usage, which is particularly advantageous in a time of enforced reduced water consumption. A greater understanding about the timing of enzyme activation in relation to endosperm mobilization may also lead to an enhanced understanding of industry problems such as black point on grain or pre-harvest sprouting and dormancy. These could be investigated using ISH to produce a diagnostic enzyme profile of the condition. The impact of seasonal variations on enzyme expression and the differences

between barley cultivars is also of key importance to the barley breeding and malting industries, and the CW staining procedure and ISH assay described here could be used as a screening tool to document modification patterns and link this to specific enzyme activity.

Critical to the whole germination and malting process is the association between water uptake and hydration of the grain. Optimal hydration is necessary for enzyme expression levels which are in turn necessary for the most favourable modification conditions. A study using ISH to examine enzyme expression and variable water uptake, monitored with techniques such as nuclear magnetic resonance (NMR) imaging should be considered (Kikuchi et al., 2006; Stark *et al.,* 2007). NMR allows the water distribution pattern within the grain to be examined without cutting or damaging the grain. This allows the grain to be subjected to subsequent tests or proceed with germination in an undamaged state. A recent study by Cu *et al*., (2016) adopted the calcofluor fluorescence screen presented in this study to show that the rate of water uptake is perhaps more genetically controlled than related to the thickness of the starchy endosperm cell walls.

Using the ISH technique to measure the effects of GA_3 is also worthy of future investigation, as mRNA and protein levels of many important enzymes are known to be enhanced or reduced by GA_3. This was not addressed in this study, but is a topic of interest to numerous current researchers and the malting and brewing industry.

The ISH technique developed here has been sensitive enough to discriminate between the EI and EII isoenzymes of (1,3;1,4)-β-glucanase providing the ability to document the presence of (1,3;1,4)-β-glucanase mRNA and protein in germinated grain tissue, confirming past studies and opening up the possibility to study the precise location of other isoenzymes in the future. This is particularly relevant to xylanase isoforms although at this stage final isoform numbers are unknown. It could also extend too many other barley enzyme families, such as others involved in starch biosynthesis or degradation. In fact the ISH has advantages over antibodies directed against isoenzymes and proteins as the ISH probes can potentially be more specific, particularly where isoenzyme similarities limits the ability of antibodies to distinguish between them.

There are still many unanswered questions arising from this study, for example the novel findings that xylanase mRNA is found in the coleoptile and that glucanases are present in the embryo tissue early in germination. The presence of the 61 KDa inactive

xylanase protein in roots and shoots of the early embryo was not investigated by Casper *et al.*, (2001) but is interesting. The presence of transcript in grain at this stage is still much earlier when compared to the presence of active enzyme, although Kuntz and Bamforth (2001, 2007) have reported earlier expression. But, one cannot dismiss the fact that although the presence of protein may reflect enzyme activities, this may not always be proportional to the apparent mRNA levels. The use of immunostaining with antibodies specific for key enzymes was attempted where possible in this study but this important approach needs more attention, particularly concerning xylanase. The pattern of mRNA in actively growing areas of the shoot apex and leaf primordia was interesting, also warranting future studies. For example the presence of the LD mRNA in the actively growing parts of the shoot is distinctively different when compared to the other enzymes studied here, appearing in the centre of the protective vascular tissue.

The ISH results presented here could also be used as a starting point to guide the use of laser capture micro dissection (LCM) (Emmert-Buck *et al.,* 1996; Espina *et al.,* 2007), particularly of areas that were of interest in this study, such as the embryo. LCM allows for visualisation and image capturing of tissue as it is micro dissected, critical to maintaining an accurate record of each dissection and later correlating histopathology with subsequent molecular results, such as real time PCR which subsequently provides a quantitative assessment of transcript levels. The accuracy of the laser beam is around 7.5 to 30 nm, allowing micro dissection of either groups of cells or single cells depending on the needs of the investigator (Nakazono *et al.,* 2003). Pin-pointing particular areas of the grain using ISH followed by LCM on serial sections could be a powerful technique.

The general phenomenon of programmed cell death in aleurone layers linked to secretion of particular enzymes could also be further assessed on the paraffin grain sections used for ISH. Assays such as the tunnel assay where DNA strand breaks characteristic of the late stages of apoptosis are detected enzymatically with labelled nucleotides (dUTP, biotin or fluorescein) could be performed. Results of this could be linked to the ISH or immunostaining assessment.

More work exploring very early plant growth could be combined with the ISH assay, targeting specific tissue areas, particularly the developing embryo to provide information on early seedling vigour. The ISH assay presented in this thesis is sensitive enough to be used on transgenic material, where genes can be either down regulated by dsRNAi approaches and/or over-expressed. Transgenes can be tagged with short non-

coding nucleic acid tags or antigenic regions such as HA, Myc or FLAG which can provide specificity for the transgenes as opposed to the endogenous transcripts or proteins (Jensen *et al.,* 1996; Stuart *et al.,* 2001; Jacob; Morosinotto *et al.,* 2006). The use of CRISPR-Cas9 technology could is also another option to target specific genes for use in the ISH assay.

The ISH method could also be used on developing grain to look at genes and proteins involved in key processes such as endosperm cell wall development, aleurone cell differentiation or transfer cell development. In this case it would be anticipated that such tissue would be easier to fix and section, although it would be necessary to develop an alternative, standard robust protocol. The fact that the current ISH process has been successfully developed for one of the most difficult plant tissues to fix, assess and section provides a true measure of its potential to provide valuable information about cellular processes in other related tissues.

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Appendix 1

As shown in appendix 1, Sloop is characterized by moderate levels of malt extract (HWE), low viscosity, high diastatic power (DP), high soluble nitrogen (Kolbach), very low malt wort β-glucan (H. Collins, The University of Adelaide, Waite Campus, personal communication), and good grain size. In comparison, hulless Himalaya has lower diastase, half the Kolbach, low wort β-glucan (Jorgensen and Aastrup, (1988) and four times the viscosity of Sloop. The malting quality of each variety was kindly assessed by the Waite Barley and Malt Quality Evaluation Laboratory, Waite campus, University of Adelaide (micro malted and tested by international standards in the Quality laboratory assessment program).

Variety	% HWE	Viscosity	Kolbach	β-glucan	Calculated hydration value at 48 h into germination	DP
Himalaya	67.5	8.4	22.8	4%[&]	84	327
Sloop	77.0	1.6	41.5	3.1%[*]	75	416

The malt samples were assessed using procedures routine in the Malt Quality Evaluation Laboratory, University of Adelaide (Waite Campus). The grain samples were malted in a Phoenix System micromalter at 15°C. The samples were steeped for 24 h using the schedule of wet:dry:wet, 7:10:5 h followed by germination for 94 h under controlled humidity and kilning for 19.5 h to a temperature of 80°C. For the PAHBAH procedure malted grain was ground to pass through a 0.5 mm screen and 250 mg is weighed into a 10 ml plastic centrifuge tube. Supernatant is collected from an extraction of the malt sample in 10 mls of 0.5 % NaCl at 25°C for 1 h. The values of key malting properties are given in the table. % HWE, hot water extract (IOB method, Industry of Brewing methods of analysis, 1997); Viscosity, the molecular weight of the extract determines the viscosity of the wort, measured under pressure (cP); Kolbach, ratio of wort soluble nitrogen over total grain nitrogen; *β-glucan value kindly provided by H.Collins, The University of Adelaide, Waite Campus; [&]β-glucan value from Jorgensen and Aastrup (1988); DP, PAHBAH Diastatic Power Assay, Inkerman PA *et al.* (1993). #Hydration assay on 30 grains per assessment at 48 h into germination according to Landau *et al.* (1995) and Cole *et al.* (1997). Hydration value is calculated by the equation (sum of the grains in a determined hydration category times score given to that category).