Isolation, Characterization and Differential Expression of Barley β-Glucan Exohydrolase Genes

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Abstract

Two barley β-glucan exohydrolases, designated isoenzyme ExoI and isoenzyme ExoII, have been purified previously from germinated seedlings. The primary aims of the work described in this thesis were to isolate and characterize the cDNAs that correspond to the two β-glucan exohydrolases. Two cDNAs were isolated from a barley germinated seedling cDNA library. Amino acid sequences deduced from the cDNAs were identical to NH_2-terminal sequence obtained for the purified enzymes.

Based on primary sequence analysis, the β-glucan exohydrolase enzymes are classified within the family 3 group of glycoside hydrolases. There are approximately 100 known members of the family 3, most of which are classified as β-glucosidases and originate from microorganisms. The only family 3 glycoside hydrolase for which a three-dimensional structure is available is the β-glucan exohydrolase isoenzyme ExoI from barley. Using the 3-dimensional structure determined for the barley β-glucan exohydrolase, selected members of the family 3 glycoside hydrolases belonging to several distinct phylogenetic clusters within the family were modelled, using homology modelling programs.

Southern hybridization analyses showed that the barley β-glucan exohydrolases are encoded by a family of approximately 8 genes, several of which were mapped to barley chromosomes 1H, 5H, and 7H. Expression patterns of two sub-sets of the genes were monitored using cDNAs encoding isoenzymes ExoI and ExoII. The genes of both sub-sets are transcribed in the scutella of germinated grain, in elongating coleoptiles, and in young roots and leaves. Only mRNA for β-glucan exohydrolase isoenzyme ExoI (or other members of its sub-family) could be detected in aleurone layers of germinated grain. These expression patterns are consistent with proposed roles for β-glucan exohydrolases during turnover of cell wall $(1\rightarrow3,1\rightarrow4)$-β-glucans in elongating coleoptiles and in young vegetative tissues.

Statement of Authorship

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

I consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

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Abbreviations

3D	Three-dimensional
ABA	Abscisic acid
Amp	Ampicillin
ATP	Adenosine triphosphate
βME	β-Mercaptoethanol
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CM	Carboxymethyl
CTAB	Cetyl-trimethylammonium bromide
CTP	Cytidine triphosphate
dATP	2'-Deoxyadenosine triphosphate
dCTP	2'-Deoxycytidine triphosphate
dGTP	2'-Deoxyguanosine triphosphate
dTTP	2'-Deoxythymidine triphosphate
dNTPs	2'-Deoxyribonucleotide triphosphates
DEAE	Diethylaminoethyl
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
DP	Degree of polymerization
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EST	Expressed sequence tag
EtBr	Ethidium bromide
ER	Endoplasmic reticulum
FUE	Far upstream element
GA	Gibberellic acid
HCA	Hydrophobic cluster analysis
IAA	Indole acetic acid
IPTG	Isopropyl-β-D-thiogalactopyranoside
LB	Luria-Bertani medium
mRNA	Messenger ribonucleic acid

MOPS	3-[N-Morpholino]propanesulfonic acid
NCF	Nitrocellulose filter
NUE	Near upstream element
nt	Nucleotide
4-NPG	4-Nitrophenyl β-D-glucopyranoside
Oligo(dT)	Oligodeoxythymidylic acid
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pfu	Plaque forming unit
PMSF	Phenylmethylsulphonyl fluoride
Poly(A)	Polyadenylic acid
PVP	Polyvinylpyrrolidone
RNA	Ribonucleic acid
RNase	Ribonuclease
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase polymerase chain reaction
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SSC	Sodium saline citrate
SSPE	Sodium saline phosphate ethylenediaminetetraacetic acid
TAE	Tris-acetate ethylenediaminetetraacetic acid
TE	Tris-HCl ethylenediaminetetraacetic acid
TLC	Thin layer chromatography
XET	Xyloglucan endotransglycosylase
XG	Xyloglucan
XGase	Xyloglucanase
X-gal	5-Bromo-4-chloro-3-indoyl-β-D-galactopyranoside

Amino acids are denoted by standard 1- or 3-letter abbreviations.

The composition of the solutions and media Denhardt's, LB, NZY, SM, SSC, SSPE, TAE, TE, and 2x YT are described in Appendix 1.

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Chapter 1

General Introduction

Barley (*Hordeum vulgare* L.) is a monocotyledonous plant that is classified in the Triticeae tribe of the family Poaceae in the order Poales. It is a commercially important cereal with uses ranging from animal feeds to human consumption. The work described in this thesis is part of a larger program in which enzymes involved in cell wall degradation in germinated barley grain are under examination. Particular attention has been directed towards the depolymerization of cell wall $(1\rightarrow3,1\rightarrow4)$-$\beta$-glucans, a process which requires the action of endo- and exohydrolases (Hrmova *et al.*, 1996). Here, the potential role of β-glucan exohydrolases in cell wall metabolism has been investigated.

Two β-glucan exohydrolases have been purified from 8-day germinated barley seedlings and are designated β-glucan exohydrolase ExoI and β-glucan exohydrolase isoenzyme ExoII (Hrmova *et al.*, 1996). These enzymes preferentially hydrolyze $(1\rightarrow3)$-β-glucosyl linkages as found in laminarin, but also hydrolyze $(1\rightarrow3,1\rightarrow4)$-$\beta$-glucans, $(1\rightarrow3,1\rightarrow6)$-$\beta$-glucans and many oligoglucosides and aryl β-glucosides (Hrmova *et al.*, 1996; Hrmova and Fincher, 1998). Their broad substrate specificity and the relative abundance of the β-glucan exohydrolase isoenzymes in young seedlings raise questions regarding their possible biological functions, which at this stage have not been defined unequivocally (Leah *et al.*, 1995; Hrmova *et al.*, 1996; Hrmova *et al.*, 1997; Kotake *et al.*, 1997; Hrmova and Fincher, 1998).

The young seedlings from which the β-glucan exohydrolases were originally purified (Hrmova *et al.*, 1996) consisted of several tissue types. Many hydrolytic enzymes involved in starchy endosperm mobilization following germination are synthesized in the aleurone or the scutellum of the grain, or may pre-exist in the starchy endosperm. Also present in the young seedlings were vegetative tissues such as the coleoptiles, young leaves and young roots. An understanding of the functional roles of β-glucan exohydrolases might be deduced from a detailed knowledge of their expression patterns in germinated barley grain and in young vegetative tissues; this was the focus and final objective of the experimental approach taken here. Before embarking on a description of the enzymes themselves, it is worth standing back and considering the biological context in which they function.

Information on $(1\rightarrow3,1\rightarrow4)$-$\beta$-glucan metabolism in germinated grain and in young seedlings is therefore provided in the following sections of this introductory chapter.

1.1 Barley Grain Morphology

The barley grain is comprised of two major components, the embryo and the endosperm (Fig. 1.1). These are surrounded by a testa, the remains of the periderm and pericarp, and an outer layer comprised of dead tissue formed by the fusion of the palea and lemma (Briggs, 1978).

Mature barley grains contain approximately 80% carbohydrate, 10% protein, 3% lipid and 2% minerals on a dry weight basis (Duffus and Cochrane, 1993). Of the carbohydrate, starch represents between 51 and 72% of the grain on a weight basis, depending on genetic and environmental factors. Polysaccharides of cell wall origin are also important and may constitute more than 10% of grain weight (Harris, 1962). Of the barley grain the $(1\rightarrow3,1\rightarrow4)$-$\beta$-glucan content is usually 3-6%, while arabinoxylan is present in similar amounts (Duffus and Cochrane, 1993).

At the basal end of the grain is the embryo, which is a living, diploid tissue derived from the fertilized ovum. It represents approximately 2.5% of the grain by weight and is comprised of approximately 34% protein, approximately 25% sugars (sucrose, 15%; raffinose, 5-10%), 14-17% lipids, 5-10% ash and some fructans (Briggs, 1978). During grain formation, the developing embryo accumulates sucrose, but only 20% of this is used for macromolecular synthesis in the grain. The remainder accumulates as a free pool (Duffus and Cochrane, 1993) which is readily available for the initial metabolic activities that follow germination (Briggs, 1992).

Structurally, the embryo is comprised of two major components, the embryonic axis and the scutellum. The embryonic axis has an apical meristem or plumule, sheathed by the coleoptile. At the other end is the radicle, which is surrounded by the coleorhiza (Briggs, 1978; Briggs, 1992). Following germination, the plumule gives rise to the young shoots and the radicle gives rise to young roots. The scutellum, from Latin 'a little shield', is a
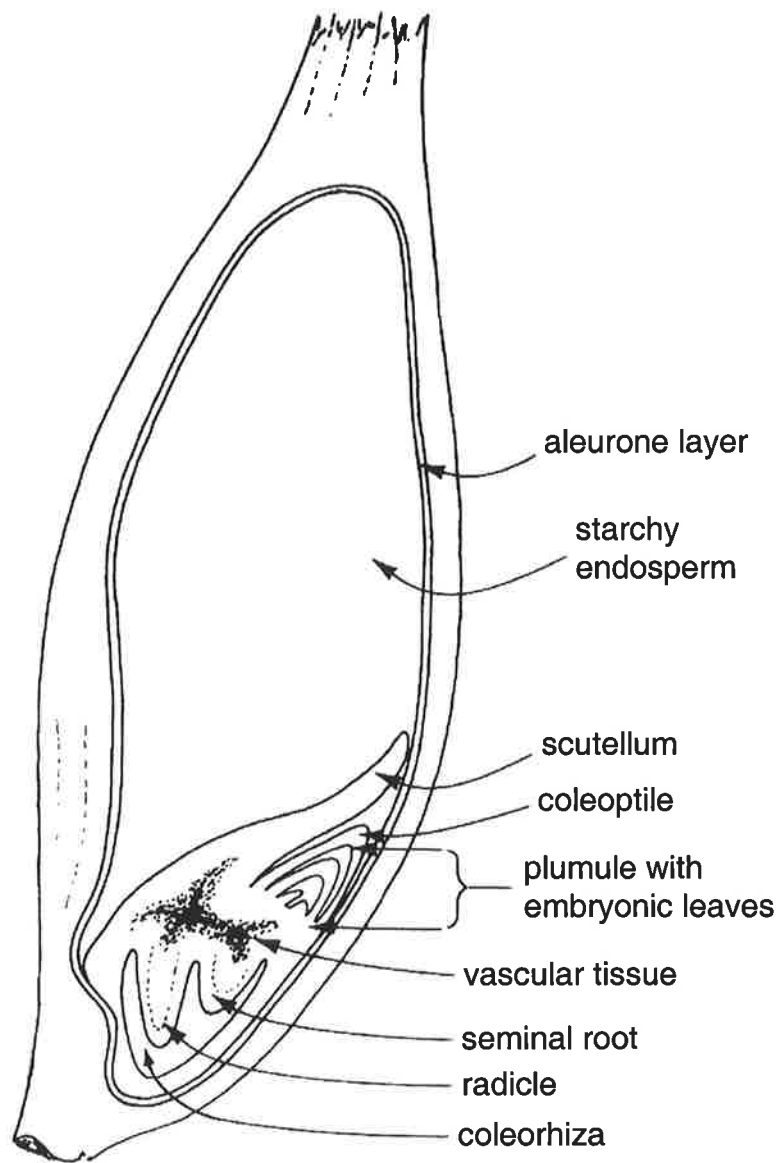
Figure 1.1 A schematic drawing of a barley grain
(adapted from Salisbury and Ross, 1978)

single modified cotyledon and forms a layer between the embryonic axis and the starchy endosperm. It consists mainly of thin-walled, essentially spherical parenchymatous cells but at the junction of the scutellum and the endosperm there is a monolayer of elongated, columnar epithelial cells that are approximately 4-5 times as long as they are wide (Nieuwdorp, 1963). This scutellar epithelium, which adjoins the storage tissues of the starchy endosperm, functions as a source of enzymes for the initial degradation of starchy endosperm reserves. In addition, the scutellar epithelium acts as an absorbing tissue for the uptake of products released by endosperm mobilization (Negbi, 1984).

The parenchymatous cells of the scutellum contain lipid reserves in small spheres that surround the numerous protein bodies and are present mainly near the nucleus and around the periphery of the cells (Smart and O'Brien, 1979a; Gram, 1982; Aisien et al., 1986). The protein bodies also contain inclusions of phytin, which acts as a source of phosphate during and following germination (Nieuwdorp, 1963; Swift and O'Brien, 1972a; Swift and O'Brien, 1972b; Smart and O'Brien, 1979a; Smart and O'Brien, 1979b; Smart and O'Brien, 1979c; Aisien et al., 1986; Stuart et al., 1986). The parenchyma cells of the scutellum contain a clearly visible nucleus, mitochondria, ribosomes and endoplasmic reticulum (ER) (Nieuwdorp and Buys, 1964; Swift and O'Brien, 1972a; Swift and O'Brien, 1972b). Small numbers of proamyloplasts which contain one or more starch granules are found (Gram, 1982). Prior to germination, starch is present only in the basal third of the scutellum (Smart and O'Brien, 1979a). The epithelial cells are similar in composition to the neighboring parenchymatous cells. In addition to the contents listed for the parenchyma cells, they show clearly visible middle lamella and plasmodesmata are present, but only in the lower regions where the epithelial cells bound parenchymatous scutellar cells (Nieuwdorp, 1963).

At the apical end of the barley grain is the other major structural component, the endosperm. This is the primary storage tissue of the grain and is composed of two morphologically and functionally distinct tissues: the aleurone and the starchy endosperm (Briggs, 1973). The aleurone layer is generally 3-4 cells thick in barley and surrounds the starchy endosperm, except where the endosperm adjoins the scutellum, and at the crease (Duffus and Cochrane, 1992). The aleurone cells are living and, once hydrated, they

respire and metabolize. The aleurone cells have thick cell walls which consist mainly of feruloylated and acetylated arabinoxylan, and $(1{\rightarrow}3,1{\rightarrow}4)$-$\beta$-glucans (Bacic and Stone, 1981a; Bacic and Stone, 1981b). These cells contain no starch but are rich in triacylglycerols (20%), protein (17-20%), minerals and some sugars (Briggs, 1992). The major function of the aleurone layer is to provide the biosynthetic machinery and biochemical reserves for hydrolytic enzyme production. The enzymes so produced are released from the aleurone for the mobilization of reserves in the starchy endosperm after germination.

The other component of the endosperm is the starchy endosperm, which constitutes approximately 75% of the grain by weight (Briggs, 1992) and contains the major reserves of the grain. Although the starchy endosperm cells of quiescent grain are non-living (Briggs, 1973; Briggs, 1978; Briggs, 1992), compacted remnants of nuclei, ribosomes, ER and RNA can be detected, particularly in the sub-aleurone regions (Bechtel and Pomeranz, 1981; McFadden *et al.*, 1988; Fincher, 1989). The starchy endosperm cell walls are thinner than those of the aleurone layer (Fincher and Stone, 1986), and the cells themselves are packed with starch granules embedded in a proteinaceous matrix (Pomeranz, 1972; Briggs, 1978; Bewley and Black, 1983).

Starch is the chief component of the grain (58-65%) and is mainly found in the starchy endosperm (Briggs, 1992). Lipids are a minor component of the starchy endosperm (2-3%) (MacGregor and Fincher, 1993) but polar lipids occur within the starch granules and may contribute to their structural characteristics (Morrison, 1988). Proteins constitute approximately 9% w/w of the starchy endosperm in barley (Briggs, 1992) and consist of approximately 30% hordein, 30% glutenin, and 10% globulin, with the remaining 30% made up mainly of albumins (Brandt, 1976).

1.2 Physiological Changes During and Following Germination

Germination is initiated by the uptake of water. This results in the dormant grain becoming vigorously metabolic and resuming protein and nucleic acid synthesis (Bewley and Black, 1994). Water enters through the micropylar region and penetrates to the rest of the grain (Briggs, 1978). Germination is considered to be complete when the coleorhiza emerges from the grain (Bewley and Black, 1994). At this point the grain has clearly defined root and shoot systems. Following germination, the coleoptile breaks through the testa and grows down the dorsal side of the grain (Briggs, 1978; Bewley and Black, 1994).

The reserves of the starchy endosperm are broken down by a battery of enzymes released from the scutellum and aleurone layer, and also by enzymes present in the starchy endosperm of the mature grain. Degradation products are translocated from the starchy endosperm through the scutellum, to support the growth of the embryo and the young seedling. In the ensuing sections the changes that occur within the scutellum, aleurone and starchy endosperm following the initiation of germination will be briefly considered.

1.2.1 Changes in the Scutellum

Following water uptake, large changes occur within the scutellar epithelium. Initially, protein bodies and associated phytin deposits are rapidly mobilized, the protein bodies become loose in structure, and vacuoles occupy an increasing proportion of cellular volume (Gram, 1982). The protein bodies have been identified as major sites for hydrolytic enzymes, which include endo- and exopeptidases (Mikola, 1987).

In addition, storage proteins are hydrolyzed to release amino acids that can subsequently be used for the synthesis of enzymes that are secreted into the starchy endosperm for the initial mobilization of grain reserves (Fincher, 1989). Lipid bodies are degraded more slowly (Fernandez et al., 1988). A number of morphological changes indicate that the change to a more active state occurs more rapidly in the epithelium (Gram, 1982), where rough ER appears and mitochondria, which may increase in number, exhibit distinct cristae (Nieuwdorp and Buys, 1964; Gram, 1982). The Golgi becomes more

apparent and well-defined in the epithelium, where the majority lie close to the plasma membrane (Nieuwdorp and Buys, 1964; Gram, 1982). This suggests that they are primarily involved in synthesizing hydrolytic enzymes to be secreted from the cells, for the mobilization of starchy endosperm reserves. The rapid degradation of scutellar reserves, together with the activation of the cellular protein synthesis machinery, correlate well with the scutellum's known role as an initial source of hydrolytic enzymes for hydrolysis of starchy endosperm reserves (Gibbons, 1981; Fincher, 1989).

Starch granules begin to accumulate in the scutellar cells shortly after water uptake (Nieuwdorp and Buys, 1964; Swift and O'Brien, 1972b; Aisien *et al.*, 1986). Synthesis of starch is so swift that it is probably made from pre-existing carbohydrate stores in the scutellum rather than from any products translocated from the starchy endosperm, because at this stage little degradation has occurred within the starchy endosperm (Swift and O'Brien, 1972b). The starch granules later disappear from the epithelial cells, but persist longer in the parenchymal cells (Swift and O'Brien, 1972b).

During germination, the scutellar epithelial cells undergo obvious morphological changes. They increase in length to almost twice that seen in ungerminated grain (Nieuwdorp and Buys, 1964; Swift and O'Brien, 1972b; Gram, 1982; Negbi, 1984), the middle lamellae of the anticlinal walls become loosened and split, and within 24 hours, cells begin to separate and become cylindrical (Swift and O'Brien, 1972b). The process of lateral disconnection of the epithelial cells takes approximately five days and produces a papillary-like surface with greatly increased surface area. This is consistent with the scutellum's function in the absorption of degradation products from the starchy endosperm (Gram, 1982; Fincher and Stone, 1993). Fine striations are visible within the cell walls and indicate the general direction of cellulose microfibrils (Nieuwdorp and Buys, 1964). The plasmodesmata that were visible in the ungerminated grain disappear and hence cannot be considered as either ducts for enzyme secretion or for the uptake of solutes (Nieuwdorp and Buys, 1964). Tangential fissures may occur locally within the epithelial cell walls (Nieuwdorp and Buys, 1964; Swift and O'Brien, 1972b).

The scutellum might also be the site of synthesis of the phytohormone gibberellic acid (GA), which diffuses to the aleurone and initiates the expression of specific genes (Fincher and Stone, 1993; Bewley and Black, 1994; Jacobsen *et al.*, 1995). However, the precise site(s) of GA synthesis have not been defined unequivocally.

1.2.2 Changes in the Aleurone Layer

While the scutellum is an initial source of certain secreted hydrolytic enzymes, it is clear that the aleurone is the major source of enzymes involved in degradation and mobilization of reserves from the starchy endosperm. Following germination of the barley grain, the aleurone layer secretes a broad range of hydrolytic enzymes involved in cell wall degradation and in the mobilization of carbohydrate, protein and nucleic acid reserves. Cell wall-degrading enzymes include $(1\rightarrow3,1\rightarrow4)$-$\beta$-glucanases (Stuart *et al.*, 1986; Slakeski and Fincher, 1992b), $(1\rightarrow4)$-β-xylan endohydrolases, α-L-arabinofuranosidases and β-xylosidases (Dashek and Chrispeels, 1977; Slade *et al.*, 1989; Banik *et al.*, 1996). Other major enzymes include α-amylase (Chrispeels and Varner, 1967; Filner and Varner, 1967), limit dextrinase (Hardie, 1975; Burton *et al.*, 1999), α-glucosidase (Hardie, 1975; Tibbot and Skadsen, 1996), peroxidase (Gubler and Ashford, 1983), endopeptidases (Koehler and Ho, 1988; Zhang and Jones, 1995; Cercós *et al.*, 1999), carboxypeptidases (Visuri *et al.*, 1969; Breddam *et al.*, 1983; Breddam, 1985; Breddam and Sørensen, 1987; Sørensen *et al.*, 1987), and nuclease I (Chrispeels and Varner, 1967).

Morphological changes observed in the aleurone layer following germination have been monitored using both light and electron microscopy (Van der Eb and Nieuwdorp, 1967; Obata, 1979; Gram, 1982), and can be attributed to the aleurone layer's role in synthesizing and secreting hydrolytic enzymes. Protein reserves within the aleurone grains begin to disappear, along with associated phytin and niacytin deposits (Van der Eb and Nieuwdorp, 1967; Gram, 1982). The protein reserves are likely to be used in enzyme synthesis as sources of free amino acids. Lipid bodies decrease in number, mitochondria become highly active (Van der Eb and Nieuwdorp, 1967; Gram, 1982) and Golgi bodies

become more abundant (Fernandez and Staehelin, 1985; Heupke and Robinson, 1985; Gubler *et al.*, 1986). ER appears and changes from single lamellae to well-defined stacks (Fincher and Stone, 1993). Later, the ER proliferates further and vesicles from the ER and Golgi apparatus increase in number.

1.2.3 Reserve Mobilization in the Starchy Endosperm

The starchy endosperm contains the majority of the reserves of the ungerminated grain. The enzymes that are required to degrade the stores of starch, reserve proteins and even residual DNA and RNA, are mostly synthesized in the scutellum and aleurone layers. However, some enzymes are already present within the starchy endosperm, having been synthesized there during grain maturation. Examples of pre-existing enzymes include β-amylase (Laurière *et al.*, 1986) and β-glucosidase (Simos *et al.*, 1994; Leah *et al.*, 1995).

Once the hydrolytic enzymes required for reserve mobilization have been synthesized in the scutellum and aleurone layers, they must make their way to the sites of reserve storage within the starchy endosperm. To do this they must cross the physical barriers presented by the scutellum or aleurone layer cell walls, and cell walls of the starchy endosperm. These cell walls are unlikely to be porous enough to allow passage of such large molecules (Carpita *et al.*, 1979; Tepfer and Taylor, 1981), and at least partial dissolution of the walls is almost certainly required to enable access of the reserve-mobilizing hydrolytic enzymes to their substrates within the starchy endosperm cells. Thus, degradation of cell walls is an important event that is necessary for efficient mobilization of the reserves within the starchy endosperm and, indeed, for the successful germination of the grain. The major enzymes involved in the breakdown of these cell walls and their modes of action are discussed in sections 1.3 and 1.4.

Starch

Starch granules are the major sites for polysaccharide storage within the barley grain (Mundy and Munck, 1985). There is a mixture of both large, lenticular granules (15-25 μm

in diameter) and more numerous but smaller, irregularly shaped granules (<10 μm) (MacGregor and Fincher, 1993). They contain about 25% amylose and 75% amylopectin and are degraded to glucose and small oligosaccharides by the combined actions of α-amylase, β-amylase, debranching enzymes and α-glucosidase.

Protein Reserves

The starchy endosperm contains about two-thirds of the total reserve protein of the mature barley grain (Mikola and Kolehmainen, 1972). In germinating barley, endo- and exopeptidases are responsible for the mobilization of these storage proteins. Levels of active peptidases are low in ungerminated grain and are thought to be those involved in the mobilization of aleurone and scutellar reserves. Following germination, peptidases that pre-exist in the starchy endosperm are activated and other peptidases are secreted from the scutellum and aleurone layer into the starchy endosperm (Mikola and Kolehmainen, 1972; Mikola, 1983; Mikola, 1987). Aminopeptidases are reported to be absent from the starchy endosperm but are present in the aleurone layer and scutellum (Mikola and Kolehmainen, 1972) and are probably not involved in hydrolysis of insoluble storage proteins within the starchy endosperm. Endopeptidases are important in this process, because they initially solubilize reserve proteins and provide shorter peptide substrates for carboxypeptidases (Jones and Poulle, 1988). Serine carboxypeptidases are the major exopeptidases involved in protein degradation within the starchy endosperm (Mikola, 1987).

In combination, the endopeptidases and the exo-acting serine carboxypeptidases hydrolyze the storage proteins of the starchy endosperm to free amino acids, and to di- and tri-peptides. These products are rapidly transported into the scutellum where the oligopeptides are converted to free amino acids (Sopanen, 1979; Higgins and Payne, 1981; Payne and Walker-Smith, 1987), which are transferred to the growing seedling or enter metabolic pathways within the scutellum (Mikola, 1987).

Nucleic Acids

Other hydrolytic enzymes are also released during germination in an effort to salvage the remnants of DNA and RNA which are present in significant amounts in the starchy endosperm (McFadden *et al.,* 1988). These enzymes include broad specificity endo-nucleases, phosphatases, nucleosidases, and phosphodiesterases (Chrispeels and Varner, 1967; Taiz and Starks, 1977; Lee and Pyler, 1985; Lee and Pyler, 1986).

1.2.4 Hormonal Regulation of Enzyme Synthesis

During and following germination, diffusible factors that stimulate target tissues elsewhere in the grain are released from the embryo. Of particular interest is the group of phytohormones called gibberellic acids (GAs). Over 70 forms of naturally-occurring GAs have been characterized from higher plants and they are known to produce a wide variety of effects in many tissues types and at many stages of development (Roberts and Hooley, 1988). Isolated barley embryos release gibberellins (Radley, 1967), and levels of secretion rise during germination (Brookes and Martin, 1975). In germinated barley grain, the major effect produced by GAs is the *de novo* synthesis of hydrolytic enzymes within the aleurone layer (Jones and Jacobsen, 1991). The production of α-amylase and other hydrolytic enzymes is dramatically increased when exogenous GA_3 is applied to isolated aleurone layers (Paleg, 1960; Yomo, 1960; Chrispeels and Varner, 1967; Jacobsen, 1983; Stuart *et al.,* 1986; Banik *et al.,* 1996). Furthermore, GA_3 is now implicated in the programmed cell death that eventually occurs in the aleurone of germinated grain (Bethke *et al.,* 1999). Although much work has been done on GA induction of enzyme synthesis in aleurone layers, very little has been done on the effects of GA on the synthesis and secretion of hydrolytic enzymes from the scutellum.

Another phytohormone of interest in barley is abscisic acid (ABA). ABA is an endogenous plant growth inhibitor that is linked to dormancy and abscission (Roberts and Hooley, 1988; Bewley and Black, 1994) and acts in two ways. Firstly, ABA suppresses expression of particular genes. In aleurone layers of germinated barley it suppresses some

GA-inducible genes involved in cell wall hydrolysis and reserve mobilization, including α-amylases (Jacobsen and Beach, 1985; Nolan and Ho, 1988), endopeptidases (Koehler and Ho, 1990), $(1\rightarrow3,1\rightarrow4)$-$\beta$-glucanases (Mundy and Fincher, 1986) and endoxylanases (Banik *et al.*, 1997). Direct suppression of genes may have evolved as a mechanism to prevent premature germination before the grain has finished filling, and to halt germination if environmental conditions become unsuitable (King, 1976; Green *et al.*, 1997).

Secondly, ABA is able to enhance the expression of other genes. Genes known as Late Embryogenesis Abundant (LEA) genes have been observed in a number of different species and are induced by ABA (Mundy and Chua, 1988; Harada *et al.*, 1989; Hatzopoulos *et al.*, 1990; Hughes and Galau, 1991; Morris *et al.*, 1991; Williams and Tsang, 1991). Another gene, encoding an inhibitor of endogenous α-amylase, is enhanced by ABA (Weselake *et al.*, 1985; Mundy and Rogers, 1986) and may play a role in preventing precocious germination.

During water stress, ABA levels in leaf tissue increase markedly and, to a lesser extent, levels rise in other tissues, including roots (Bradford and Hsiao, 1982). Under these conditions, ABA induces stomatal closure and inhibits shoot growth, both of which act to conserve water. Lowered cell turgor may be the trigger for ABA production (Bradford and Hsiao, 1982).

1.3 Cell Wall Composition and Degradation

In general, primary cell walls of higher plants consist of a framework of cellulose microfibrils interconnected by xyloglucan and/or glucuronoarabinoxylan chains to form an extensive network. In all flowering plants the cellulose microfibrils are made up of about three dozen linear chains of $(1\rightarrow4)$-β-glucan condensed to form a para-crystalline array (Carpita, 1996). Surrounding this framework is a matrix phase made up of various xyloglucans, glucuronoarabinoxylans, $(1\rightarrow3,1\rightarrow4)$-$\beta$-glucans and pectic polysaccharides (Table 1.1). Structural proteins may also play a role in strengthening the walls and are widely distributed as a second network within the matrix phase (Cassab and Varner, 1988; Albersheim *et al.*, 1994; Carpita, 1996). Enzymic proteins are present in cell walls, probably to allow growth and/or further modification of the cell walls, whether as part of normal function or in response to biotic or abiotic stresses (Fry, 1995).

In barley grain, the starchy endosperm cell walls contain approximately 75% $(1\rightarrow3,1\rightarrow4)$-$\beta$-glucan and 20% arabinoxylan with small amounts of cellulose and glucomannan (Fincher, 1975; Ballance and Manners, 1978). In aleurone cell walls, the proportions of the major components are approximately reversed, with 71% arabinoxylan and 26% $(1\rightarrow3,1\rightarrow4)$-$\beta$-glucan (Bacic and Stone, 1981a). Cellulose and glucomannan are also present in aleurone walls and, in contrast to starchy endosperm cell walls, approximately 1% $(1\rightarrow3)$-β-glucan is detectable. The low levels of cellulose present in both types of walls are consistent with the non-load bearing nature of the walls in grain and the need to degrade them rapidly after germination.

Both aleurone and starchy endosperm cell walls also contain ferulic acid (Fincher, 1976), which is covalently associated with arabinoxylans and causes autofluorescence under appropriate conditions. Aleurone walls contain much more ferulic acid than starchy endosperm cell walls (Fincher, 1976).

The composition of barley scutellar cell walls has not been determined, but both parenchyma and epithelial cell walls of maize scutellum contain arabinoxylans, and some $(1\rightarrow3,1\rightarrow4)$-$\beta$-glucan may be present (Seckinger *et al.*, 1960). The scutellar cell walls are

Table 1.1: Cell wall composition in barley*

Tissue	Major polysaccharide constituents		References
Aleurone (mature grain)	arabinoxylan	(71%)	Bacic and Stone (1981a,b)
	$(1\rightarrow3,1\rightarrow4)$-$\beta$-glucan	(26%)	
	cellulose	(2%)	
	glucomannan	(2%)	
	$(1\rightarrow3)$-β-glucan	(1%)	
Starchy endosperm (mature grain)	arabinoxylan	(20%)	Fincher (1975)
	$(1\rightarrow3,1\rightarrow4)$-$\beta$-glucan	(75%)	Ballance and Manners (1978)
	cellulose	(2%)	
	glucomannan	(2%)	
Coleoptiles (4 days)	arabinoxylan	(19%)	Sakurai and Masuda (1978)
	$(1\rightarrow3,1\rightarrow4)$-$\beta$-glucan	(19%)	
	cellulose	(55%)	
	pectin	(?)	
	xyloglucan	(?)	
Young Leaves (top 2 cm, dark grown)	arabinoxylan	(11%)	N. Sakurai
	$(1\rightarrow3,1\rightarrow4)$-$\beta$-glucan	(16%)	(personal communication
	cellulose	(63%)	cited in Fincher, 1992)
	pectin	(5%)	
	xyloglucan	(?)	
Stems (4th internode)	heteroxylan	(28%)	Kokubo et al., (1989)
	$(1\rightarrow3,1\rightarrow4)$-$\beta$-glucan	(5%)	
	cellulose	(65%)	

* Adapted from Fincher (1992)

intensely autofluorescent, mainly due to ferulic acid associated with arabinoxylans (Smart and O'Brien, 1979b; Smart and O'Brien, 1979c).

Despite the availability of detailed physical and chemical data relating to the composition of cereal grain cell walls, little is known about the actual physical structure of the matrix and how it interacts with the cellulosic microfibrillar network. Many models have been proposed that involve covalent and non-covalent linkage of the polysaccharide and protein constituents (Fincher and Stone, 1986; Albersheim *et al.*, 1994; Gibeaut and Carpita, 1994; Fry, 1995). It has been suggested that $(1\rightarrow3,1\rightarrow4)$-$\beta$-glucans combine with arabinoxylans to form a non-covalently-bound, three dimensional, gel-like matrix which would have the requisite properties for cell wall function (Fincher, 1975; Fincher and Stone, 1986). This type of structure would be functionally adapted to germinated grain, where hydrolytic enzymes can rapidly degrade the bulk of the wall to allow access of other enzymes to the reserves stored within the cell.

Arabinoxylans

Arabinoxylans or glucuronoarabinoxylans are the principal polymers that interlock cellulosic microfibrils in dividing cells of the Poaceae (Carpita and Gibeaut, 1993). In barley, arabinoxylans vary in chain length, degree of substitution and main chain substituents (Fincher and Stone, 1986; Viëtor *et al.*, 1992). They are particularly abundant in the starchy endosperm and aleurone cell walls (Table 1.1) and are also found in the husk (Aspinall and Ross, 1963). They consist of a linear chain of $(1\rightarrow4)$-β-linked xylanopyranosyl residues which may be substituted with single arabinosyl residues at the C(O)2 or C(O)3 positions (Fincher, 1975) and, less frequently, single glucuronosyl acid residues are found at the C(O)2 of xylosyl residues (Carpita and Gibeaut, 1993). The highly substituted arabinoxylans of barley endosperm contain mainly C(O)3 linked arabinosyl residues, but significant amounts of C(O)2- and doubly branched C(O)2- and C(O)3-linked arabinosyl residues are also found (Bacic and Stone, 1981a). No glucuronosyl acid residues are detected (Bacic and Stone, 1981a; Viëtor *et al.*, 1994). These substituents prevent extensive molecular aggregation and render the polysaccharides

soluble in water, but the extended conformation of the xylan backbone leads to asymmetry and causes them to produce solutions of high viscosity (Fincher and Stone, 1986).

Phenolic acids covalently bound to arabinoxylans have been detected in barley and constitute approximately 0.05% of walls of the starchy endosperm (Fincher, 1976; Viëtor *et al.,* 1992) and 1.2% of aleurone walls (Bacic and Stone, 1981a). The main types are *p–* coumaric acid, which is concentrated in the outer layers of the grain (Nordkvist *et al.,* 1984), and ferulic acid, which is ester linked to C(O)5 atoms of arabinofuranosyl substituents (Gubler *et al.,* 1985; Mueller-Harvey *et al.,* 1986). Neighboring feruloylated arabinoxylan chains may form cross-links through diferulate bridges (Geissman and Neukom, 1973; Markwalder and Neukom, 1976; Izydorczyk *et al.,* 1990). However, the extent of crosslinking has been questioned, because most phenolic acids are released by dilute alkali as ferulic acid rather than as diferulic acid (Fincher, 1976).

The degradation of arabinoxylan chains in endosperm cell walls during germination is achieved by a combination of endo- and exoxylanases, α-L-arabinofuranosidases and β-xylosidases (Preece and MacDougall, 1958; Taiz and Honigman, 1976). Xylan endohydrolases (EC 3.2.1.8) hydrolyze the $(1{\rightarrow}4)$-β-linkages of the xylan backbone in an essentially random fashion. Three endoxylanases have been isolated from germinated barley, but activity cannot be detected until 6-8 days after germination (Slade *et al.,* 1989). This suggests that degradation of arabinoxylans may not be essential in order for other hydrolytic enzymes, necessary for reserve mobilization in the starchy endosperm, to be released from the aleurone and scutellum. In general, endoxylanases only hydrolyze unsubstituted regions of the xylan chain, but an endoxylanase from *Bacillus subtilis* requiring a glucuronic acid substituent adjacent to the cleavage site has been reported (Nishitani and Nevins, 1991).

α-L-Arabinofuranosidases remove the arabinose substituents from arabinoxylans or released oligosaccharides, while β-xylosidases/β-xylan exohydrolases also degrade the small oligosaccharides released by endo-hydrolase activity. α-L-Arabinofuranosidase and β-xylosidase activities have been detected in germinated barley grain and in isolated

aleurone layers (Preece and MacDougall, 1958; Taiz and Honigman, 1976; Dashek and Chrispeels, 1977; Banik *et al.*, 1997).

(1→3,1→4)-β-Glucans

(1→3,1→4)-β-Glucans, consisting of a non-random combination of (1→3)- and (1→4)-β-linked glucosyl residues, have been found in cell wall preparations from graminaceous monocotyledons (Darvill *et al.*, 1980). They have been identified in several tissues in barley and constitute a major proportion of the starchy endosperm and aleurone cell walls (Table 1.1). Barley endosperm (1→3,1→4)-β-glucans are heterogeneous with respect to molecular size, solubility in aqueous solvents, and molecular structure (Bacic and Stone, 1981b; Woodward and Fincher, 1983; Woodward *et al.*, 1983), but generally consist of an unbranched linear chain of (1→3)- and (1→4)-β-linked glucosyl residues. They contain approximately 70% (1→4)- and 30% (1→3)-β-linked D-glucosyl residues (Parrish *et al.*, 1960; Woodward *et al.*, 1983). The distribution of the linkages is neither completely regular nor completely random (Staudte *et al.*, 1983; Edney *et al.*, 1991). Up to 90% of the polysaccharide consists of blocks of two or three adjacent (1→4)-β-linkages separated by single (1→3)-β-linkages (Woodward *et al.*, 1983) and can therefore be considered as a series of (1→3)-β-linked cellooligosaccharides, as follows:

non-red....G3G4G4G3G4G4G4G3G4G4G4G3G....red

cellotriose   cellotetraose   cellotetraose

where G represents a β-glucosyl residue and 3 or 4 represent the (1→3)- or (1→4)-glycosidic linkages. The "non-red" and "red" refer to the nonreducing and reducing termini of the polysaccharide, respectively. The remaining 10% of the polysaccharide is comprised of blocks of up to 14 adjacent (1→4)-β-linked glucosyl residues (Woodward *et al.*, 1983). Contiguous (1→3)-β-glucosyl residues are rare, if not totally absent, as determined by chemical analysis (Woodward *et al.*, 1983) and by computer modelling (Buliga *et al.*, 1986).

(1→3,1→4)-β-Glucan is present in significant amounts in other tissues in barley (Table 1.1). In the cell walls of young leaves it can represent up to 16% of the total carbohydrate (Fincher, 1992), but levels decrease during seedling development (Buchala and Wilkie, 1974). (1→3,1→4)-β-Glucan is also present in elongating coleoptiles where it constitutes approximately 19% by weight of 4-day-old seedlings, but again levels decrease during growth (Sakurai and Masuda, 1978).

1.4 (1→3,1→4)-β-Glucan Hydrolysis

As stated earlier, the main components of barley endosperm walls, and probably scutellar epithelial walls, are arabinoxylans and (1→3,1→4)-β-glucans. It is estimated that glucosyl residues in (1→3,1→4)-β-glucan may account for as much as 18% of the total glucose content of barley grain (Morrall and Briggs, 1978). The degradation of (1→3,1→4)-β-glucans following germination is therefore of great importance, not only in clearing the way for α-amylases, peptidases and nucleases from the aleurone and scutellum to reach their substrates in starchy endosperm cells, but also to recover the glucose component of wall (1→3,1→4)-β-glucan as an energy source for the developing seedling.

There are three types of β-glucan endohydrolases in germinated barley; (1→4)-β-glucan glucanohydrolase (cellulase; EC 3.2.1.4) (Hoy *et al.*, 1981; Manners *et al.*, 1982), (1→3)-β-glucan glucanohydrolase (EC 3.2.1.39) (Manners and Marshall, 1969; Bathgate *et al.*, 1974; Manners and Wilson, 1976; Høj *et al.*, 1989; Høj *et al.*, 1990; Hrmova and Fincher, 1993) and (1→3,1→4)-β-glucan 4-glucanohydrolase (EC 3.2.1.73) (Luchsinger *et al.*, 1960; Manners and Marshall, 1969; Manners and Wilson, 1976; Woodward and Fincher, 1982a; Woodward and Fincher, 1982b). However, (1→3)-β-glucanases are unable to hydrolyze the single (1→3)-β-linkage found in these polysaccharides (Høj and Fincher, 1995) and runs of contiguous (1→3)-β-linkages that would allow binding and hydrolysis by (1→3)-β-glucanases are not present (Luchsinger *et al.*, 1965; Woodward *et al.*, 1983; Hrmova *et al.*, 1995). Similarly, cellulases are relatively low in abundance in germinated barley grain, and it is therefore likely that (1→3,1→4)-β-glucanases are the major endohydrolases involved in the hydrolysis of cell wall (1→3,1→4)-β-glucans (Figure 1.2). A distinct type of (1→3,1→4)-β-glucan endohydrolase reported in maize is capable of hydrolyzing the (1→3,1→4)-β-glucan chain at regions of at least four contiguous (1→4)-β-linkages, to yield a collection of polymers with an approximate degree of polymerization (DP) between 60-100 (Huber and Nevins, 1981; Hatfield and Nevins, 1987; Thomas *et al.*, 2000). The enzyme is unable to hydrolyze these products to smaller

Hydrolysis of Endosperm Cell Wall (1→3, 1→4)-β-Glucans in Germinated Barley

Cell Wall

Endo-X (?)

(?) (1→3, 1→4)-β-Glucan Endohydrolases

(1→3,1→4)-β-Glucans

β-Glucan Exohydrolases

(1→3, 1→4)-β-Glucan Endohydrolases

Glucose and Tri- and Tetrasaccharides

β-Glucan Exohydrolases

β-Glucosidases

Glucose
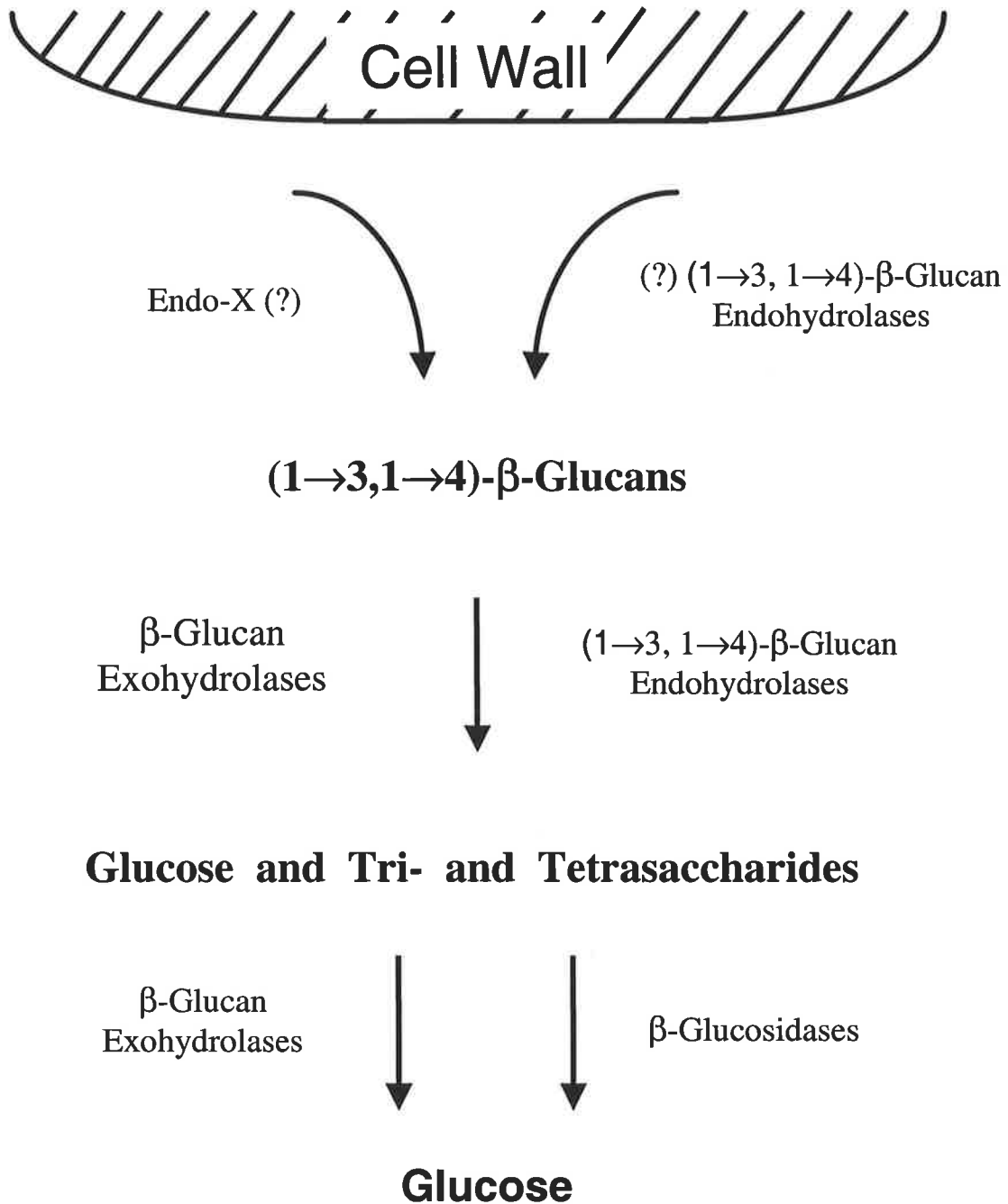
Figure 1.2 Hydrolysis of (1→3,1→4)-β-glucan in barley endosperm following germination. Unproven enzyme roles are indicated by question marks. Adapted from Hrmova and Fincher, 2001.

oligosaccharides. The presence of this type of (1→3,1→4)-β-glucan endohydrolase in barley has yet to be confirmed, but to avoid confusion with other endohydrolases, in this thesis it will be referred to as "Endo-X" (Figure 1.2).

Two (1→3,1→4)-β-glucan endohydrolases, designated isoenzymes EI and EII, have been purified from extracts of germinated barley (Woodward and Fincher, 1982a). The enzymes have identical substrate specificities. Both are capable of hydrolyzing (1→3,1→4)-β-glucan but show no activity on (1→3)-β-glucans or (1→4)-β-glucans (Woodward and Fincher, 1982b; Høj et al., 1989). The three-dimensional structure of (1→3,1→4)-β-glucanase isoenzyme EII, determined by X-ray crystallography, revealed a pronounced substrate-binding cleft that is able to accommodate 7-8 glucosyl residues (Varghese et al., 1994). The predicted catalytic amino acids lie within this cleft (Varghese et al., 1994).

Both genes (Litts et al., 1990; Slakeski et al., 1990) and their corresponding cDNAs (Fincher et al., 1986) have been isolated, and the deduced amino acid sequences of the two isoenzymes show 91% positional identity. The genes encoding (1→3,1→4)-β-glucanase isoenzymes EI and EII are located on the long arms of chromosomes 1H and 7H, respectively (Loi et al., 1988; MacLeod et al., 1991). Probes specific for each isoenzyme have shown that transcription of isoenzyme EII occurs mainly within the aleurone layer, with traces in the scutella (Slakeski and Fincher, 1992b). Northern hybridization analysis of RNA from GA$_3$-induced aleurone layers shows greatly enhanced levels of (1→3,1→4)-β-glucanase isoenzyme EII mRNA (Slakeski and Fincher, 1992a). Isoenzyme EI-specific probes also show that transcription of the isoenzyme EI gene occurs in the aleurone, but no GA$_3$ induction is apparent (Slakeski and Fincher, 1992a). Both isoenzymes are down-regulated by the addition of the phytohormone ABA (Slakeski and Fincher, 1992a). Isoenzyme EI mRNA is also detected in the scutellum of germinated grain, in young leaves, young roots, and in mature leaves infected with the powdery mildew fungus (*Erisiphe graminis*) (Slakeski, 1992). It is interesting to note that expression of either isoform in coleoptiles is not detected (Slakeski and Fincher, 1992a), although (1→3,1→4)-

β-glucan is present in these tissues and is actively degraded during coleoptile elongation (Sakurai and Masuda, 1978; Inouhe *et al.*, 1997).

The $(1\rightarrow3,1\rightarrow4)$-$\beta$-glucan endohydrolases specifically cleave the $(1\rightarrow4)$-β-linkage on the reducing end side of a $(1\rightarrow3)$-β-linkage, as follows:

$$\downarrow \qquad \downarrow \qquad \qquad \downarrow \qquad \qquad \downarrow$$

non-red....G4G3G4G4G3G4G4G4G3G4G4G4G3G4G4G....red

$$\Downarrow \qquad\qquad\qquad \Downarrow$$

$$\text{G4G3G}_{\mathbf{red}} \qquad\qquad \text{G4G4G3G}_{\mathbf{red}}$$
$$\text{Trisaccharide} \qquad\qquad \text{Tetrasaccharide}$$
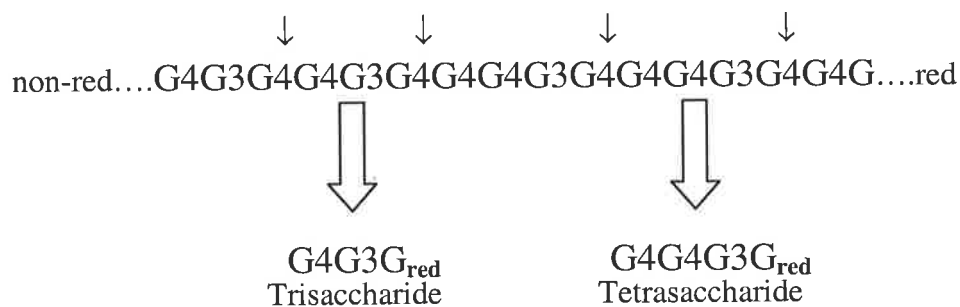
where $\downarrow$ indicates a potential hydrolysis site, '**red**' indicates the reducing end, '**G**' indicates a glucosyl residue, and '**3**' or '**4**' indicates the type of β-linkage. Thus, hydrolysis products of the endosperm cell wall $(1\rightarrow3,1\rightarrow4)$-$\beta$-glucan produced by $(1\rightarrow3,1\rightarrow4)$-$\beta$-glucan endohydrolases are largely in the form of tri- and tetrasaccharides (Parrish *et al.*, 1960; Anderson and Stone, 1975). Further depolymerization of these tri- and tetrasaccharides is necessary to release glucose for the developing seedling. Recent studies have suggested two possible candidate enzymes that may be involved in hydrolysis of the oligosaccharide breakdown products produced by $(1\rightarrow3,1\rightarrow4)$-$\beta$-glucan endohydrolase action.

The first candidate enzyme is β-glucosidase. At least two β-glucosidase isoenzymes have been purified from barley grain and characterized (Leah *et al.*, 1995; Hrmova *et al.*, 1996). Both the gene and a cDNA encoding one form of β-glucosidase have been isolated (Leah *et al.*, 1995). The β-glucosidases show no activity on $(1\rightarrow3,1\rightarrow4)$-$\beta$-glucan but degrade to glucose the tri- and tetrasaccharides produced when $(1\rightarrow3,1\rightarrow4)$-$\beta$-glucan is hydrolyzed by $(1\rightarrow3,1\rightarrow4)$-$\beta$-glucan endohydrolases (Leah *et al.*, 1995; Hrmova *et al.*, 1996). The β-glucosidases also hydrolyze cellodextrins and 4-nitrophenyl β-D-glucoside, and have been classified as $(1\rightarrow4)$-β-D-glucan glucohydrolases (EC 3.2.1.74) (Hrmova *et al.*, 1996). The β-glucosidases are synthesized in the starchy endosperm of developing

grain and their levels do not increase after germination (Simos *et al.,* 1994; Leah *et al.,* 1995).   Thus, their location and substrate specificity suggest that β-glucosidases are possible participants in the total hydrolysis of starchy endosperm cell wall (1→3,1→4)-β-glucan to glucose.

The second candidate for the hydrolysis of the tri- and tetrasaccharide products of (1→3,1→4)-β-glucan degradation are β-D-glucan exohydrolases, of which two isoenzymes have been isolated from germinated barley (Hrmova *et al.,* 1996; Kotake *et al.,* 1997). These enzymes remove glucosyl residues from the non-reducing termini of a range of substrates, including the (1→3)-β-glucan laminarin, (1→3,1→4)-β-glucan, a range of oligosaccharides with (1→2)-, (1→3)-, (1→4)- and (1→6)-β-glucosyl linkages, and the aryl β-glycosides 4-nitrophenyl β-D-glucoside and 2-nitrophenyl β-D-glucoside (Hrmova *et al.,* 1996; Hrmova and Fincher, 1998).   It is therefore possible that the β-glucan exohydrolases are also involved in the complete hydrolysis of the tri- and tetrasaccharide degradation products in germinated barley grain.

The isolation and characterization of cDNAs encoding these two barley β-glucan exohydrolases was one of the major objectives of this study.

1.5 Coleoptiles and Cell Extension

Another important tissue in barley that contains significant amounts of $(1\rightarrow3,1\rightarrow4)$-$\beta$-glucan is the coleoptile. The coleoptile is a protective sheath-like structure surrounding the plumule, or germinating shoot, of graminaceous species. It is basically a hollow cylinder comprised mainly of parenchymatous cells bounded by outer and inner epidermal cell layers (Figure 1.3). There are two vascular bundles which contain relatively more phloem than xylem, and which are bilaterally placed in the coleoptile. The thin-walled cells of the outer epidermis are elongated and contain one or a few rows of stomata which lie parallel to the vascular bundles and are more numerous at the tip of the coleoptile (Avery, 1930). Coleoptiles display both geo- and photo-tropism (Salisbury and Ross, 1985) and, because of their rapid growth following germination, coleoptiles have long been studied as a model for rapidly expanding or elongating tissues, and for examining the effects of auxin on plant growth.

Auxin is a plant growth regulator that induces rapid cell elongation in certain tissues (Roberts and Hooley, 1988). It is widely accepted that auxin stimulates elongation by increasing the mechanical extensibility of cell walls in a process known as cell wall loosening (Cleland, 1981; Taiz, 1984; Masuda and Yamamoto, 1985; Cosgrove, 1986; Masuda, 1990; Sakurai, 1991; Hoson, 1993; Cosgrove, 1999). During auxin-induced cell wall loosening, matrix phase polysaccharides are biochemically modified. In the Poaceae, this involves changes to wall $(1\rightarrow3,1\rightarrow4)$-$\beta$-glucans, arabinoxylans and xyloglucans (Masuda and Yamamoto, 1985; Masuda, 1990; Hoson, 1991; Sakurai, 1991; Hoson, 1993). In elongating barley coleoptiles, application of the auxin indole acetic acid (IAA) leads to a decrease of $(1\rightarrow3,1\rightarrow4)$-$\beta$-glucan content of walls over a six-hour period (Sakurai and Masuda, 1978). Decreases in $(1\rightarrow3,1\rightarrow4)$-$\beta$-glucan content are also induced by auxins in developing maize coleoptiles (Carpita and Kanabus, 1988; Inouhe and Nevins, 1991a). During normal maize coleoptile growth, $(1\rightarrow3,1\rightarrow4)$-$\beta$-glucan levels initially increase (Carpita, 1984), but subsequently decrease from 14% to 3% (w/w) between 5 d and 10 d after germination (Lutteneger and Nevins, 1985). When antibodies raised to $(1\rightarrow3,1\rightarrow4)$-
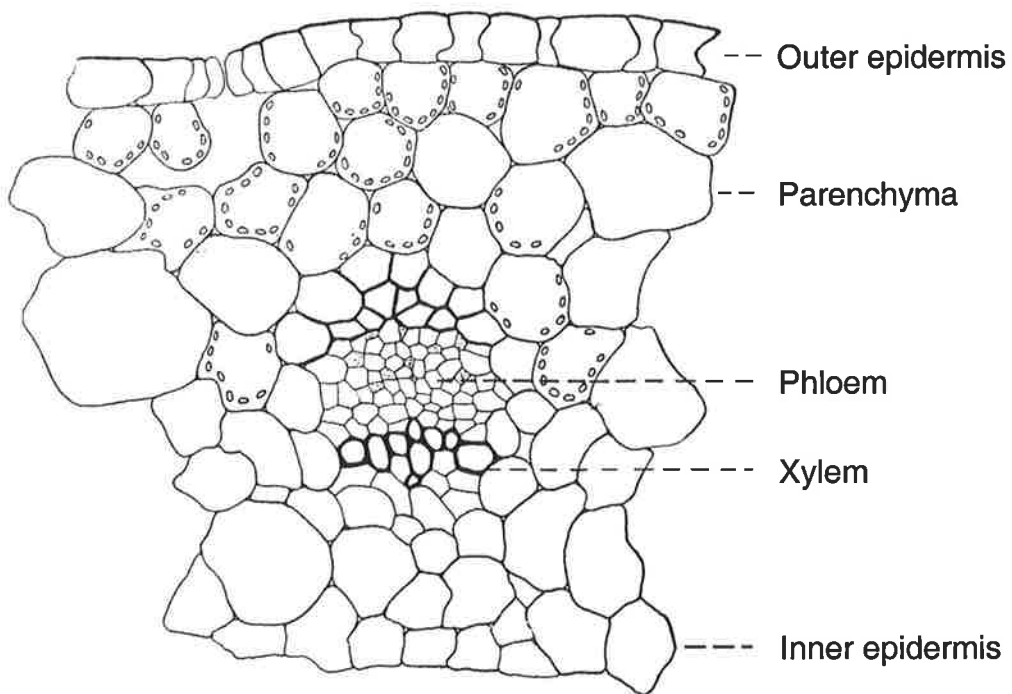
Figure 1.3 Transverse section of a small portion of a coleoptile including the vascular bundle and surrounding tissue. After Avery (1930).

β-glucan were applied to coleoptile segments, they inhibited auxin-induced elongation and wall loosening, as well as the autolytic decrease in glucose content (Hoson and Nevins, 1989a). This suggests that $(1{\rightarrow}3,1{\rightarrow}4)$-β-glucan hydrolysis is associated with the cell wall loosening that occurs during auxin-induced elongation. Further, $(1{\rightarrow}3,1{\rightarrow}4)$-β-glucan is enriched in the outer walls of the epidermal cells (Sauter and Kende, 1992; Miekle *et al.*, 1994), the area that has been implicated in control of elongation rates (Kutschera *et al.*, 1987; Kutschera and Kende, 1988).

As previously noted, no expression of $(1{\rightarrow}3,1{\rightarrow}4)$-β-glucanase isoenzymes EI or EII can be seen in barley coleoptiles (Slakeski and Fincher, 1992b), although an "Endo-X" type of endoglucanase has been detected in maize (Huber and Nevins, 1981; Hatfield and Nevins, 1987; Inouhe *et al.*, 1999). β-Glucan exohydrolases have also been detected in the coleoptiles of maize (Huber and Nevins, 1981; Inouhe *et al.*, 1999) and could, in conjunction with the maize endoglucanase, degrade the coleoptile $(1{\rightarrow}3,1{\rightarrow}4)$-β-glucan to glucose. Indeed, antibodies raised to each of these enzymes inhibit cell wall breakdown, wall loosening and auxin mediated elongation both *in vivo* and *in vitro*, indicating the involvement of both enzymes in the degradation of $(1{\rightarrow}3,1{\rightarrow}4)$-β-glucan in elongating maize coleoptiles (Hoson and Nevins, 1989b; Labrador and Nevins, 1989; Inouhe and Nevins, 1991b). In view of this information, and the fact that the two β-glucan exohydrolases recently isolated from barley by Hrmova *et al.* (1996) were from 8-day germinated barley seedlings that also included the coleoptiles, it became clear that a thorough investigation of the expression patterns of β-glucan exohydrolases in barley coleoptiles and other tissues was necessary to define the potential functions of the enzymes.

1.6 Objectives

The principal aim of this study was to isolate and characterize cDNAs and genes encoding barley β-glucan exohydrolases. The more specific aims of the project were;

- to screen cDNA libraries for near full-length cDNAs encoding β-glucan exohydrolase isoenzymes and to define the complete primary structures of the enzymes

- to use the primary structures to generate molecular models of similar enzymes from plants, fungi and bacteria, using the three-dimensional co-ordinates of barley β-glucan exohydrolase isoenzyme ExoI (Varghese *et al.*, 1999)

- to define the number and chromosomal location of barley β-glucan exohydrolase genes

- to define the physical and temporal expression patterns of the genes encoding β-glucan exohydrolase isoenzymes and the levels of the corresponding mRNAs.

The isolation and characterization of cDNAs encoding the two barley β-glucan exohydrolase isoenzymes (Hrmova *et al.*, 1996) are described in Chapter 2. Amino acid sequence alignments and hydrophobic cluster analyses showed that the enzymes can be classified as family 3 glycoside hydrolases (Henrissat, 1998). Comparisons of the amino acid sequences and 3D structures of the enzymes with other members of the family 3 group of glycoside hydrolases are described in Chapter 3.

The isolation and sequencing of several fragments of the barley β-glucan exohydrolase isoenzyme ExoI and isoenzyme ExoII genes are described in Chapter 4. Southern hybridization analyses were performed to define the number of β-glucan exohydrolase genes in the barley genome. To determine the chromosomal location of the β-glucan exohydrolase genes, wheat-barley addition lines were screened using the β-glucan exohydrolase cDNAs.

Finally, the sites of β-glucan exohydrolase gene expression, as measured by mRNA levels in Northern hybridization analyses, were investigated. Total RNA was isolated from germinated grain and young seedlings. The results of this work are presented in Chapter 5.

Chapter 2

Isolation and Characterization of Near-Full Length cDNAs Encoding Two Barley β-Glucan Exohydrolases

2.1 Introduction

Two β-glucan exohydrolases have been purified from barley seedlings and designated isoenzyme ExoI and isoenzyme ExoII (Hrmova *et al.,* 1996). The enzymes are members of the family 3 group of glycoside hydrolases (Henrissat, 1998). The primary objective of the work described in this Chapter was to isolate and characterize the cDNAs corresponding to these β-D-glucan exohydrolase isoenzymes. The isolated cDNAs would be useful for several reasons. The sequence of the cDNAs would enable us to deduce the complete primary structures of the isoenzymes, it would allow a comparison of translated protein sequences with those in databases, and it would be essential for molecular modelling of the enzymes. Molecular modelling would be useful, in turn, to compare members of the family 3 group of enzymes and to investigate their structures and molecular mechanisms of catalysis.

The cDNAs would also be useful to create probes for Southern and Northern hybridization analyses. The probes could be used to analyze the barley β-glucan exohydrolase gene family through Southern hybridization analyses, to study site-specific and temporal gene transcription patterns, and to monitor the effects of phytohormones on expression, using Northern hybridization techniques.

No cDNAs or genes corresponding to β-D-glucan exohydrolases of similar substrate specificity had been reported in the literature when this project commenced, and probes were therefore designed from amino acid sequence data obtained from the purified barley enzymes (Hrmova *et al.,* 1996). Initial NH_2-terminal amino acid sequencing of the β-glucan exohydrolase isoenzymes yielded the sequence of 52 amino acids for each isoenzyme (Hrmova *et al.,* 1996). This amino acid sequence information was sufficient to design a single degenerate oligonucleotide primer corresponding to a region of barley β-D-glucan exohydrolase isoenzyme ExoII. The degenerate oligonucleotide was radiolabelled and used to screen barley seedling cDNA libraries and a cDNA encoding β-glucan exohydrolase isoenzyme ExoII was subsequently isolated. However, a similar strategy for the isolation of a cDNA encoding isoenzyme ExoI was unsuccessful and the latter cDNA

was ultimately isolated when libraries were screened with a rice EST. In this Chapter the isolation and sequencing of near full-length cDNAs encoding barley β-glucan exohydrolase isoenzymes ExoI and ExoII are described.

2.2 Materials and Methods

2.2.1 Materials

A 5-day barley seedling library was obtained from Clontech Laboratories (Palo Alto, CA, USA). Restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase, dNTPs and *Taq* DNA polymerase were obtained from Promega (Madison, WI, USA) and New England Biolabs (Beverly, MA, USA). Tryptone, yeast extract and agar were purchased from Difco (Detroit, MI, USA). The Megaprime DNA labelling kit, autoradiographic film, nitrocellulose and nylon membranes, and $[\alpha^{-32}P]$-dCTP were from Amersham International Ltd. (Little Chalfont, Buckinghamshire, UK). The $[\alpha^{-35}S]$-dATP, $[\gamma^{-32}P]$-ATP, and BRESAclean DNA purification kit were from Bresatec (Adelaide, SA, Australia). Sephadex G-100, CM-Sepharose CL-6B and Blue Dextran 2000 were obtained from Pharmacia Biotech (Uppsala, Sweden). The manual DNA sequencing kits (Sequenase V2.0) were purchased from United States Biochemical Corporation (Cleveland, Ohio, USA). Agarose, ethidium bromide, ampicillin, maltose, herring sperm DNA, DTT, Ficoll, X-gal, IPTG, Orange G, PEG (6000), PVP, RNase and lysozyme were purchased from Sigma (St. Louis, MO, USA).

2.2.2 Bacterial growth and preparation of competent cells

For transformation of DNA into bacterial cells, *E. coli* strain DH5α [F⁻, φ80d*lac*ZΔM15, Δ(*lac*ZYA-*arg*F)U169, *deo*R, *rec*A1, *end*A1, *hsd*R17(r_k^-, m_k^+), *pho*A, *sup*E44, λ-, *thi*-1, *gyr*A96, *rel*A1] or strain XL1-Blue [*rec*A1, *end*A1, *gyr*A96, *thi*-1, *hsd*R17, *sup*E44, *rel*A1, *lac* (F' *pro*AB *lacI*^{q}ZΔM15 Tn10)] were grown in 50 ml LB medium at 37°C overnight. The overnight culture (5 ml) was used to inoculate 50 ml LB broth, which was subsequently incubated at 37°C with shaking until the optical density at 600 nm (OD_{600}) reached 0.6. Cells were harvested by centrifugation at 2000g for 15 min and resuspended in 20 ml ice-cold 50 mM $CaCl_2$. The suspension was incubated on ice for 30 min, centrifuged at 2000g for 15 min and resuspended in 5 ml ice-cold 50 mM $CaCl_2$. After incubating on ice for a further 4-24 h, the competent cells were transformed

immediately or stored in 50 μl aliquots at -80°C. For transformation, the competent cells (50 μl) were thawed on ice and approximately 100 ng plasmid DNA was added. The mixture was incubated on ice for 30 min and heat-shocked at 42°C for 90 sec. LB broth (1 ml) was added and the solution was incubated at 37°C for 1 h with gentle shaking. The bacterial cells were pelleted by centrifugation at 16,000 g for 2 min, resuspended in 200 μl LB broth and plated onto LB/Agar plates containing 100 μg/ml ampicillin.

XL1-Blue MRF' *E. coli* cells [Δ(*mcrA*)183 Δ(*mcrCB-hsdSMR*-mrr)173, *end*A1, *sup*E44, *thi*-1, *rec*A1, *gyr*A96, *rel*A1, *lac* (F' *proAB lacI^{q}Z*Δ*M15* Tn*10*)] to be used in plating out of cDNA libraries were grown overnight with shaking at 37°C in 50 ml LB broth containing 0.2% (w/v) maltose and 10 mM $MgSO_4$. Fresh LB broth (50 ml) containing 0.2% (w/v) maltose and 10 mM $MgSO_4$ was inoculated with 10 ml of the overnight culture and grown to an optical density (OD_{600}) of 0.5-0.7. Cells were harvested by centrifugation at 2000g for 10 min, resuspended in 10 ml 10 mM $MgSO_4$ and stored at 4°C for up to 2 days.

2.2.3 Screening of the cDNA libraries

The barley seedling cDNA library had been prepared from poly(A)$^+$ RNA from 5-day-old barley seedlings (*Hordeum vulgare* L., cv. Bomi) using both oligo(dT) and random primers. The cDNAs had been ligated into the λZAP II vector (Stratagene) using linker arms that contained an *Eco*RI restriction enzyme site at the 5'-end and an *Xho*I site at the 3'-end. Approximately 2-3x10^5 pfu of the library were plated out on 140 mm NZY plates using XL1-Blue *E.coli* cells that had been prepared for library plating. The plates were incubated for approximately 16 h at 37°C before chilling at 4°C for 2 h to prevent the nitrocellulose filters from sticking to the agar surfaces. Nitrocellulose filters were pre-wetted in 1% (w/v) NaCl solution, blotted dry and carefully placed onto the chilled agar surface for 2 min, or 4 min for the second of duplicate lifts. The transferred plaques were denatured with 0.5 M NaOH/1.5 M NaCl for 5 min, neutralized with 0.5 M Tris-HCl buffer, pH 7.5, containing 1.5 M NaCl, for 5 min, before rinsing in 2x SSC (Appendix I). The filters were baked at 80°C for 2 h (Sambrook *et al.*, 1989), prehybridized for 4 h in 6x

SSC, 1x Denhardt's solution, 0.1% (w/v) SDS and 100 µg/ml salmon sperm DNA, and hybridized in the same solution including either a labelled oligonucleotide at 37°C or a labelled cDNA fragment at 65°C for 16 h. After hybridization, oligonucleotide labelled filters were washed at 50°C with 2x SSC/0.1% SDS for 20 min, 1x SSC/0.1% SDS for 20 min, 0.5x SSC/0.1% SDS for 20 min, followed by 0.2x SSC/0.1% SDS for 20 min. The filters labelled with cDNA probes were washed at 60-65°C with 2x SSC/0.1% SDS for 20 min, 1x SSC/0.1% SDS for 20 min, 0.5x SSC/0.1% SDS for 20 min, followed by 0.2x SSC/0.1% SDS for 20 min. Filters were blotted dry and exposed against X-ray film in cassettes with intensifying screens for 2-20 h. Positive plaques were cored, placed in 500 µl SM buffer (Appendix I) containing 20 µl chloroform, vortexed and held at room temperature for at least 2 h before replating. Positive plaques were subjected to at least two rounds of screening, until only single, positive plaques could be detected on the plates.

2.2.4 Preparation of [³²P]-radiolabelled oligonucleotide and cDNA probes

For the preparation of [^{32}P]-radiolabelled oligonucleotide probes, 50 µl of a reaction mixture containing 100 ng oligonucleotide, 5 µl 10x T4 polynucleotide kinase buffer (700 mM Tris-HCl buffer, pH 7.6, containing 100 mM MgCl$_2$ and 50 mM DTT), 50 µCi [γ-^{32}P]-ATP and 2 units T4 polynucleotide kinase was incubated for 1 h at 37°C. The reaction was stopped by the addition of 1 µl 0.5 M EDTA. Labelled oligonucleotide was precipitated with 0.1 volumes of 3 M sodium acetate buffer, pH 5.2 and 2.5 volumes of ethanol overnight at –20°C. The precipitate was pelleted by centrifugation at 16,000 g for 30 min, washed with 70% ethanol, resuspended in 50 µl TE buffer, pH 8.0 (Appendix I), and added to the hybridization solution.

All cDNA probes were prepared with [α-^{32}P]-dCTP according to the protocol provided with the Megaprime labelling kit (Amersham). The cDNA fragment to be labelled (approximately 100 ng) was mixed with 5 µl random nonanucleotide primers and made up to 33 µl with sterile H$_2$O. The primer-cDNA mix was boiled for 5 min, cooled and pulsed in a centrifuge to collect all the liquid. After spinning, 10 µl labelling buffer, 5

µl 10 mCi/ml [α-^{32}P]-dCTP and 2 µl Klenow DNA polymerase (2 units) were added, and the mixture was incubated for 30 min at 37°C. The labelled DNA was separated from unincorporated nucleotides using a Sephadex G-100 column. A sterile Pasteur pipette was plugged with glass wool and pre-swollen Sephadex G-100 (equilibrated with TE buffer, pH 8.0) was added to a height of approximately 7 cm. The labelling reaction mixture was combined with 20 µl labelling dye containing 1.5% (w/v) Blue Dextran and 0.5% (w/v) Orange G in TE buffer, and the total mixture added to the column. Labelled DNA probe was collected as the blue dye eluted from the column. The purified DNA probe was boiled for 5 min and immediately cooled on ice, before addition to the hybridization solution.

2.2.5 Phagemid rescue of positive clones

Inserts from positively hybridizing plaques were isolated by placing the cored plaque in 500 µl SM buffer. To the SM buffer, 20 µl chloroform was added, and the mixture was vortexed and held for at least 2 h at room temperature. Phage stock (100 µl) was transferred to a 10 ml tube containing 200 µl XL1-Blue *E. coli* cells. ExAssist helper phage (1 µl) were added and the tube was incubated at 37°C for 15 min. Three millilitres of 2x YT media (Appendix I) was added and the tube incubated at 37°C with shaking for 2.5 h. An aliquot of 1 ml was transferred to an Eppendorf tube, heated at 70°C for 20 min and centrifuged at 6000g for 10 min. The supernatant was transferred to a sterile tube and stored at 4°C. The rescued phagemids were plated out by adding 50 µl of the supernatant to 200 µl SOLR *E. coli* cells [e14⁻(McrA-) Δ(*mcrCB-hsdSMR-mrr*)171 *sbcC recB recJ uvrC umuC::*Tn*5* (Kan^r) *lac gyrA96 relA1 thi-1 endA1* λ^R{F' *proAB laqI^{q}ZΔM15*}^{c} Su⁻] that had been grown to an OD$_{600}$ of 1.0. The mixture was incubated at 37°C for 15 min before plating onto LB plates containing 100 µg/ml ampicillin and incubating overnight at 37°C.

2.2.6 Southern blot analysis, restriction mapping and subcloning

Positively hybridizing clones rescued from λZAP into the pBluescript phagemid were excised using *Xho*I and *Eco*RI restriction enzymes. For further analyses and sequencing, excised inserts were digested with different restriction enzymes and subcloned into pBluescript SK(+). Fragment analysis was performed using 0.8-1% (w/v) agarose gels stained with approximately 0.5 µg/ml ethidium bromide. The DNA bands were excised from the gel and purified using BRESAclean DNA purification kits.

In preparation for sequence analysis, DNA fragments were subcloned into pBluescript SK(+) vector that had been digested with appropriate restriction enzymes and dephosphorylated (Sambrook *et al.*, 1989).

2.2.7 Nucleotide sequence analysis

Manual sequencing of denatured DNA was carried out in both directions for all nucleotide sequence determinations. Purified DNA was denatured in 2 M NaOH/1 mM EDTA for 30 min. The denatured DNA was recovered by centrifugation through custom-made CM-Sepharose CL-6B resin (Pharmacia) mini-spin columns. Sequencing reactions were performed using [α-^{35}S]-dATP and the Sequenase V2.0 kit (USBC), which incorporates the radiolabel in a technique based on the dideoxynucleotide chain termination method (Sanger *et al.*, 1977). Reaction products were separated on 45 cm 6% (w/v) polyacrylamide gels containing 46% w/v urea with 60 mA constant current at 55°C. Computer analyses were performed with the University of Wisconsin Genetics Computer Group software (Devereux *et al.*, 1984) in the ANGIS suite of programs developed in the Department of Electrical Engineering, University of Sydney, Australia.

2.3 Results

2.3.1 Isolation and characterization of a cDNA clone encoding barley β-D-glucan exohydrolase isoenzyme ExoII

NH$_2$-Terminal amino acid sequence data for both barley β-D-glucan exohydrolase isoenzyme ExoI and isoenzyme ExoII were reported by Hrmova *et al.* (1996). A single degenerate oligonucleotide of 23 nucleotides was designed from the sequence of β-glucan exohydrolase isoenzyme ExoII (Figure 2.1A). With the use of a single inosine (a neutral nucleotide that may pair with all four standard nucleotides), the primer was 64x degenerate (Figure 2.1B). This primer, designated **DPB**, was radiolabelled with [γ-^{32}P]-ATP and used to probe a barley 5-day-germinated seedling cDNA library. One faint, positively hybridizing plaque was detected. After a series of plaque purification steps to ensure homogeneity, phage DNA was purified, and the plasmid isolated by phagemid rescue. Restriction enzyme digestion with *Eco*RI and *Xho*I released a fragment of approximately 600 base pairs. Sequence analysis confirmed that the insert encoded the NH$_2$-terminal region of the β-glucan exohydrolase isoenzyme ExoII.

The 600 bp fragment was radiolabelled with [α-^{32}P]-dCTP and used to rescreen the 5-day seedling cDNA library. A positive clone with a cDNA insert of approximately 1600 bp was isolated. Digestion with *Eco*RI and *Xho*I released DNA fragments of approximately 600 and 1000 bp from the cDNA insert, the additional fragment resulting from an internal *Eco*RI site. These DNA fragments were separately subcloned into pBluescript plasmids and sequenced. The 600 bp fragment was identical in sequence to the previously isolated 600 bp cDNA clone. Further restriction analysis showed that the 1000 bp fragment was located 3' to the smaller fragment (Figure 2.2). The 1000 bp fragment was subsequently radiolabelled with [α-^{32}P]-dCTP and used to rescreen the 5-day seedling cDNA library. Several clones were isolated and rescued into pBluescript phagemid, the largest of which (clone 4) contained an insert of approximately 2700 bp (Figure 2.2).

Purified barley β-glucan exohydrolase isoenzymes ExoI and ExoII were isolated from 8-day germinated seedlings by Dr. Maria Hrmova. The purified isoenzyme ExoII had an

A.

Isoenzyme ExoI

```
D  Y  V  L  Y  K  D  A  T  K  P  V  E  D  R  V  A  D  L  L  -  R  M
2  2  4  6  2  2  2  4  4  2  4  4  2  2  6  4  4  2  6  6     6  1
                     ‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾
```

Isoenzyme ExoII

```
D  Y  L  K  Y  K  D  P  K  Q  P  L  G  V  R  I  K  D  L  L  -  -  M
2  2  6  2  2  2  2  4  2  2  4  6  4  4  6  3  2  2  6  6        1
         ‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾
```

B.

```
            A     T     K     P     V     E     D
                  A                 A
DPA   5' GC C ACI AA A CCI GT C GA A GA 3'
            G       G       G     G
            T                 T
```

C.

```
            K     Y     K     D     P     K     Q     P
            A     C     A     C           A     A
DPB   5' AA A TA C AA A GA C CCI AA A CA A CC 3'
            G     T     G     T           G     G
```
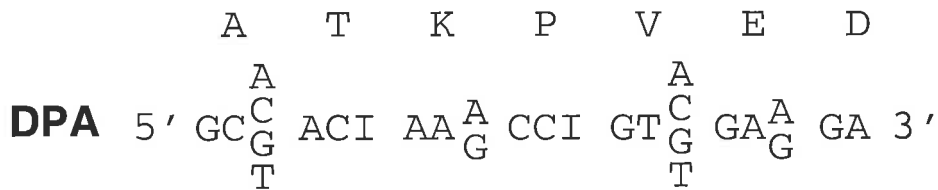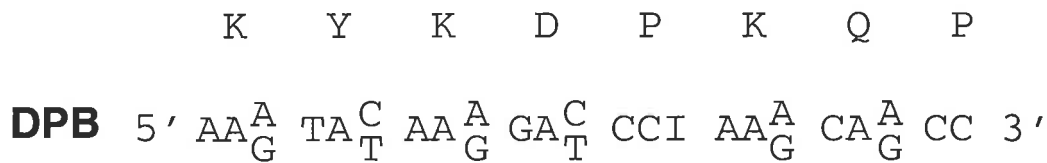
Figure 2.1 **A.** NH$_2$-Terminal amino acid sequence data of barley β-glucan exohydrolases ExoI and ExoII. Numbers below the line indicate the number of possible codons corresponding to each amino acid. Dashes indicate unassigned amino acids. **B.** The degenerate oligonucleotide primer **DPA** designed on the basis of barley β-glucan exohydrolase isoenzyme ExoI NH$_2$-terminal amino acid sequence. **C.** The degenerate oligonucleotide primer **DPB** designed on the basis of barley β-glucan exohydrolase isoenzyme ExoII NH$_2$-terminal amino acid sequence.

Figure 2.2 Barley β-glucan exohydrolase isoenzyme ExoII clones. Shown above the line are the NH$_2$-terminal amino acid sequence ▬, tryptic fragments ▬, signal peptide ▬, and translated coding region ▬. The direction and length of DNA sequencing experiments are shown below ➝. Restriction enzyme cut sites ▲, where applicable, are common to all clones except for *Eco*RI (in red) which is not present on the 1600bp clone.
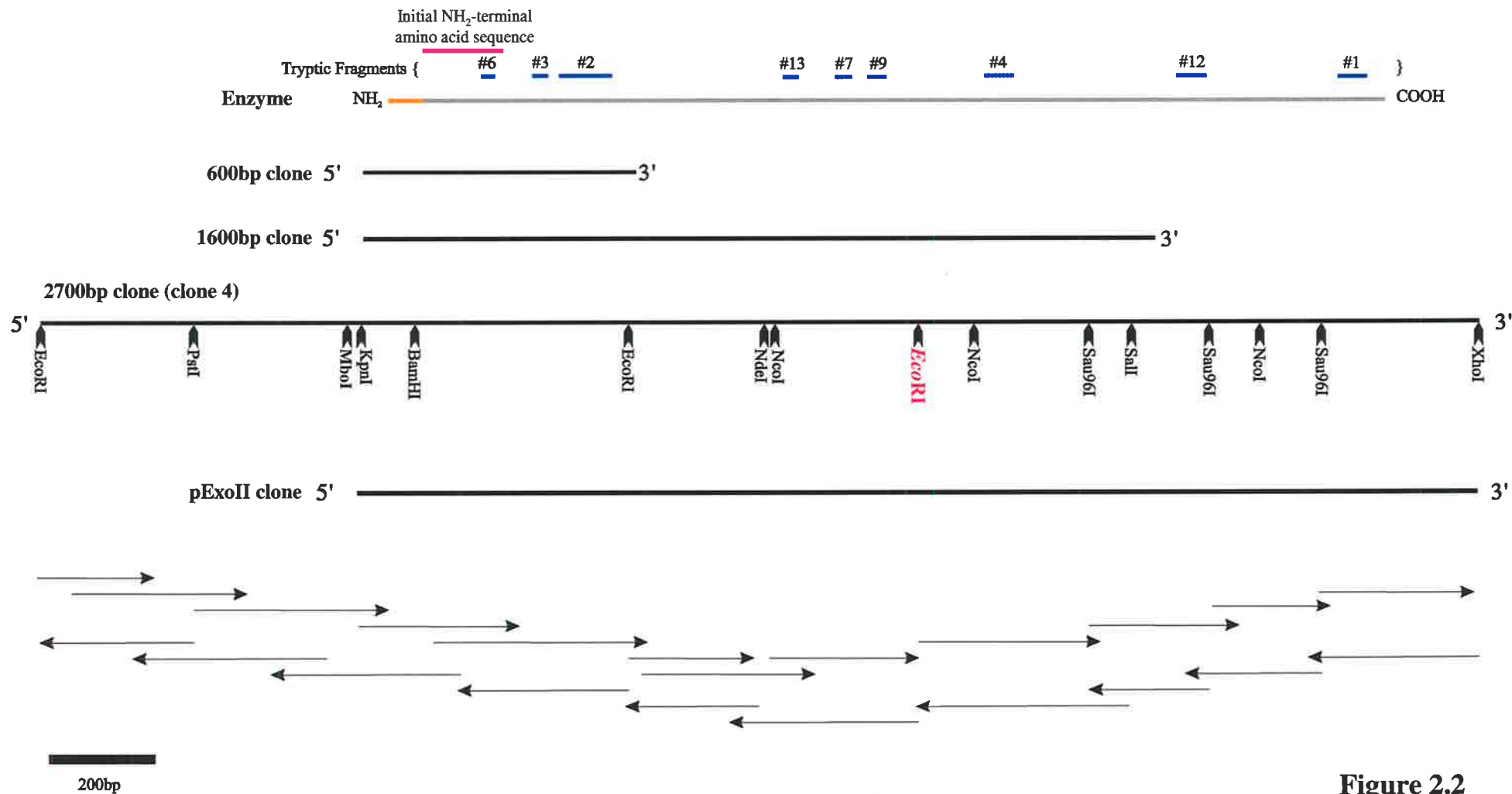
Figure 2.2

apparent molecular mass of approximately 71 kDa and NH_2-terminal amino acid sequencing resulted in the assignment of the first 52 amino acids (Hrmova *et al.*, 1996). This data, along with amino acid sequence information generated from several tryptic peptides of β-glucan exohydrolase isoenzyme ExoI (Dr Maria Hrmova, unpublished data), greatly assisted the cDNA sequencing experiments. In particular, the sequence of tryptic peptides provided regular checks that the reading frame of the nucleotide sequence was correct. From the apparent molecular mass of 71 kDa (Hrmova *et al.*, 1996), it was calculated that the protein contained around 600 amino acids and hence would be encoded by a cDNA of approximately 2-2.2 kb. Clone 4, isolated using the 1000bp fragment of the previous clone, was approximately 2700 bp and contained a poly(A)$^+$ tail of 30 bp. It was therefore presumed to be near full length.

However, sequence analysis revealed a cloning artefact at the 5'-end of the cDNA insert of clone 4. Comparison of the reverse translation of the 5' end of clone 4 and the SWISS-PROT protein database revealed a 97% sequence identity (100% sequence similarity) over 108 bp of clone 4 with a putative aldehyde dehydrogenase from *Pisum sativum* (Guerro *et al.*, 1990; accession number S25795). The dehydrogenase-like sequence was inserted in the reverse orientation, but it was difficult to assign the 5' boundaries of the two cDNAs within the insert. A point corresponding to an *Mbo*I restriction site GATC, was finally chosen as the 5' end of the β-glucan exohydrolase isoenzyme ExoII cDNA clone. Restriction enzyme analysis revealed a unique *Kpn*I site at 606 bp, immediately 3' to the *Mbo*I site, and by digesting with this enzyme a 2098 bp fragment was produced. The fragment was subcloned into the pBluescript plasmid, which was then designated pExoII.

The cDNA insert of pExoII was digested with various restriction enzymes, subcloned, and fully sequenced in both directions (Figure 2.2). Sequence comparison of the pExoII cDNA with the 1600 bp clone (Figure 2.2) used to rescreen the cDNA library showed an identical nucleotide sequence, with the exception of a single base, silent mutation in pExoII that resulted in the addition of an additional *Eco*RI restriction enzyme site.

The clone pExoII cDNA (Figure 2.3) contains an open reading frame which extends from nucleotides 26 to1897 and the amino acid sequence deduced from nucleotides 92-247 corresponds exactly with that determined directly from the 52 NH$_2$-terminal amino acids of β-glucan exohydrolase isoenzyme ExoII (Hrmova *et al.,* 1996).

The cDNA nucleotide sequence reveals the presence of a putative signal peptide of 22 amino acid residues encoded by bases 26-91, suggesting that the enzyme is secreted from cells in which it is expressed. The signal peptide is mainly composed of hydrophobic residues and follows the general pattern described by Watson (1984). It contains a charged amino acid within the first five residues (His5), approximately 10 non-polar residues in the central region, a helix-breaking residue within 4-8 amino acids of the cleavage site (Gly20) and an amino acid with a small, uncharged side chain (Ala22) at the COOH-terminus of the signal peptide. Overall codon usage within the signal peptide is balanced, with a 56% (G+C) content. However, at the wobble base position this shifted to an 86% (G+C) content. The significance of this is unknown, but codon usage will be considered further in the Discussion section of this chapter.

The region from the codon specifying the NH$_2$-terminal aspartic acid residue of the mature enzyme at nucleotide 92 to the stop codon at nucleotide 1898 encodes a polypeptide of 602 amino acid residues, with a calculated M_r of 65,102 and a calculated pI of 7.9. These values may be compared with an apparent molecular mass of 71 kDa and a pI of 8.0 for the purified enzyme itself (Hrmova *et al.,* 1996). The difference in apparent M_r calculated from the deduced amino acid sequence and that measured by SDS-PAGE may be accounted for by glycosylation of the mature enzyme, which carries four potential *N*-glycosylation sites (Figure 2.3). Overall, the nucleotide content of the coding region is balanced, with 54% (G+C); 65% of codons have (G+C) in the wobble base position.

The 3'-untranslated region of clone pExoII is 208 bp in length, from the stop codon TAG to a 30 bp poly(A)$^+$ tail. An near-upstream element (NUE) AATAAA consensus polyadenylation signal is present beginning 81 bp upstream from the poly(A)$^+$ tail. Far-upstream elements (FUE) that affect the overall processing efficiency at all polyadenylation sites (Rothnie, 1996) with sequence motifs such as CAYTG and YGTGTTYY (Joshi,

Figure 2.3 The complete nucleotide sequence and derived amino acid sequence of barley β-glucan exohydrolase isoenzyme ExoII. The putative signal peptide is <mark>highlighted</mark> and the mature NH$_2$-terminal aspartic acid residue is indicated with an arrow ↓, with the numbering of the amino acid residues starting at the Asp. Potential *N*-glycosylation sites are underlined. The catalytic nucleophile (D285)▲ and the catalytic acid (E491)▲ are indicated with arrows as shown (Varghese *et al.*, 1999). The stop codon is indicated by an asterisk and the potential polyadenylation signal is indicated by double underlining.

```
GATCGGTACCAGACACAGTGCCAAGATGGGGAACTTGCACAAGACCACCTTTGTTCTCCTCATGTTCTGCTTGGCGGCGTTGGGAAGCGC    90
                          M  G  N  L  H  K  T  T  F  V  L  L  M  F  C  L  A  A  L  G  S  A

  ↓
GGATTATCTCAAGTACAAGGATCCGAAGCAGCCTCTTGGCGTTCGCATCAAGGATCTGCTTGGTCGGATGACTCTCGCTGAAAAGATAGG    180
 D  Y  L  K  Y  K  D  P  K  Q  P  L  G  V  R  I  K  D  L  L  G  R  M  T  L  A  E  K  I  G     30

CCAGATGACCCAGATCGAGAGGGAGAATGCCACGGCAGAGGCCATGTCCAAGTACTTCATAGGTAGCGTACTGAGTGGTGGAGGCAGTGT    270
 Q  M  T  Q  I  E  R  E  N  A  T  A  E  A  M  S  K  Y  F  I  G  S  V  L  S  G  G  G  S  V     60

GCCTTCTCCTCAAGCATCCGCTGCGGCTTGGCAGTCGATGGTGAATGAAATGCAAAAGGGCGCCCTTTCTACCCGCCTAGGCATTCCGAT    360
 P  S  P  Q  A  S  A  A  A  W  Q  S  M  V  N  E  M  Q  K  G  A  L  S  T  R  L  G  I  P  M     90

GATCTACGGTATTGATGCTGTGCACGGTCACAACAACGTGTACAAAGCTACCATCTTCCCACATAATGTTGGCCTCGGTGCTACCAGGGA    450
 I  Y  G  I  D  A  V  H  G  H  N  N  V  Y  K  A  T  I  F  P  H  N  V  G  L  G  A  T  R  D    120

CCCTATGTTGGTAAAGAGGATAGGAGAAGCAACTGCTCTTGAAGTTAGAGCAACAGGAATTCCTTACGCTTTTGCTCCGTGTATTGCGGT    540
 P  M  L  V  K  R  I  G  E  A  T  A  L  E  V  R  A  T  G  I  P  Y  A  F  A  P  C  I  A  V    150

GTGTAGAGACCCAAGATGGGGACGCTGCTATGAAAGCTACAGCGAAGACCCAAAGGTTGTCCAGTCAATGACCACACTCATCTCTGGTCT    630
 C  R  D  P  R  W  G  R  C  Y  E  S  Y  S  E  D  P  K  V  V  Q  S  M  T  T  L  I  S  G  L    180

GCAAGGTGACGTTCCGGCTGGTTCTGAGGGAAGGCCATACGTTGGTGGAAGTAAGAAGGTTGCTGCATGCGCAAAGCACTATGTTGGTGA    720
 Q  G  D  V  P  A  G  S  E  G  R  P  Y  V  G  G  S  K  K  V  A  A  C  A  K  H  Y  V  G  D    210

TGGTGGTACGTTTATGGGGATCAACGAGAACGATACAATCATTGACGCCCATGGGCTGATGACTATCCATATGCCTGCTTATTACAATTC    810
 G  G  T  F  M  G  I  N  E  N  D  T  I  I  D  A  H  G  L  M  T  I  H  M  P  A  Y  Y  N  S    240

TATCATCAGAGGTGTCTCCACTGTTATGACCTCGTACTCTAGCTGGAACGGCAAGAAAATGCACGCCAACCATTTCCTTGTCACTGATTT    900
 I  I  R  G  V  S  T  V  M  T  S  Y  S  S  W  N  G  K  K  M  H  A  N  H  F  L  V  T  D  F    270

TCTGAAGAACAAGCTCAAATTTCGGGGTTTCGTGATTTCAGACTGGCAAGGCATTGATCGGATTACTAGCCCTCCAGGCGTGAACTATTC    990
 L  K  N  K  L  K  F  R  G  F  V  I  S  D  W  Q  G  I  D  R  I  T  S  P  P  G  V  N  Y  S    300
                                  ▲
TTATTCAGTTGAGGCTGGAGTTGGTGCCGGTATTGACATGATCATGGTTCCTTTTGCCTACACAGAATTCATTGATGATCTGACATACCA   1080
 Y  S  V  E  A  G  V  G  A  G  I  D  M  I  M  V  P  F  A  Y  T  E  F  I  D  D  L  T  Y  Q    330

AGTTAAGAACAACATCATCCCCATGAGCAGAATCAACGATGCTGTCTACAGGATTCTCAGGGTGAAGTTCACCATGGGTCTATTTGAGAG   1170
 V  K  N  N  I  I  P  M  S  R  I  N  D  A  V  Y  R  I  L  R  V  K  F  T  M  G  L  F  E  S    360

CCCCTATGCTGACCCAAGCCTCGTTGGTGAACTCGGGAAGCAGGAACACCGTGATCTTGCTCGTGAGGCCGTCAGGAAGTCATTGGTGTT   1260
 P  Y  A  D  P  S  L  V  G  E  L  G  K  Q  E  H  R  D  L  A  R  E  A  V  R  K  S  L  V  L    390

GCTGAAAAATGGAAAATCTGCCTCCACTCCATTGTTGCCTCTCCCAAAGAAGGCCGGTAAGATCCTCGTCGCTGGAAGCCACGCCGACGA   1350
 L  K  N  G  K  S  A  S  T  P  L  L  P  L  P  K  K  A  G  K  I  L  V  A  G  S  H  A  D  D    420

CTTGGGCAACCAGTGCGGAGGATGGACCATCACATGGCAAGGACAGACCGGCAACGACAAAACTGCCGGGACGACGATCCTTTCGGCGAT   1440
 L  G  N  Q  C  G  G  W  T  I  T  W  Q  G  Q  T  G  N  D  K  T  A  G  T  T  I  L  S  A  I    450

CAAGTCCACCGTCGACCCCAGCACGGAGGTGGTCTTCTCAGAGAATCCTGACAGCGCCGCCGTTGACAGCGGCAAGTACGACTACGCCAT   1530
 K  S  T  V  D  P  S  T  E  V  V  F  S  E  N  P  D  S  A  A  V  D  S  G  K  Y  D  Y  A  I    480

CGTGGTGGTCGGCGAGCCACCGTACGCTGAGACGTTCGGCGACAACCTGAACCTGACGATCCCTGCGCCCGGCCCCTCGGTGATCCAGAA   1620
 V  V  V  G  E  P  P  Y  A  E  T  F  G  D  N  L  N  L  T  I  P  A  P  G  P  S  V  I  Q  N    520
                         ▲
CGTGTGCAAGAGCGTCAGGTGCGTGGTGGTGCTCATCTCCGGCAGGCCGCTGGTGGTGGAGCCGTACATCAGCGCCATGGACGCGTTCGT   1710
 V  C  K  S  V  R  C  V  V  V  L  I  S  G  R  P  L  V  V  E  P  Y  I  S  A  M  D  A  F  V    540

CGCCGCGTGGCTGCCCGGCTCGGAGGGCCAGGGCGTGGCCGACGTGCTGTTCGGCGACTACGGGTTCTCCGGGAAGCTGGCGCGGACGTG   1800
 A  A  W  L  P  G  S  E  G  Q  G  V  A  D  V  L  F  G  D  Y  G  F  S  G  K  L  A  R  T  W    570

GTTCAAGTCGGCGGACCAGCTGCCGATGAACGTCGGCGACAAGCACTACGACCCGCTCTTCCCCTTCGGGTTCGGCCTCACCACCGAGGC   1890
 F  K  S  A  D  Q  L  P  M  N  V  G  D  K  H  Y  D  P  L  F  P  F  G  F  G  L  T  T  E  A    600

CAAGAAGTGAGTCGGGTGAGATGGGATGTGTCTCGATCGGTTGGGCTGTGACTGTGAGAGATGGGATACATACATACATTGCGTTTTGTG   1980
 K  K  *                                                                                     602

GTTGATTTGTTAGCAATAAAAGAGCGATGCAATCTTGCGGATGTTGTCGTGTTGGTTCTGTTGTGCAACAAAATCTAGCAATTGTGTTCT   2070

CTTGTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA                                                     2105
```

1987) are also present (CATTG, position 1967, and TGTGTTCT, position 2063, respectively).

Comparison of the nucleotide sequence with the DNA databases at the time of cloning revealed homology with several rice Expressed Sequence Tags (ESTs) and with a Sequence Tagged Site (STS) clone ABC254, from *Hordeum vulgare*. Clone ABC254, from Kadyrzhanova *et al.* (unpublished sequence data, GenBank accession number L43939), matched exactly with 164 bp of pExoII (1853-2016). This region is mainly within the 3'-untranslated region of the barley β-glucan exohydrolase isoenzyme ExoII clone. Further information regarding the STS clone ABC254 and its chromosomal location is discussed in Chapter 4.

2.3.2 Isolation and characterization of a cDNA clone encoding barley β-D-glucan exohydrolase isoenzyme ExoI

Following characterization of the barley β-glucan exohydrolase isoenzyme ExoII cDNA, efforts were made to isolate a cDNA corresponding to the other β-glucan exohydrolase that had been purified from seedlings, namely isoenzyme ExoI. Screening was performed on the barley 5-day germinated seedling cDNA library using a $[\alpha\text{-}^{32}P]$-dCTP labelled pExoII insert, that had been digested with *Kpn*I and *Pvu*I restriction enzymes to generate a 1916 bp fragment. Hybridization and washing were performed at 55°C and the filters were washed to a stringency of 0.5x SSC/0.1% SDS. Several positively hybridizing clones were isolated, but sequence analysis revealed only β-glucan exohydrolase isoenzyme ExoII cDNA inserts, or false positives. Several attempts were made to rescreen the barley 5-day germinated seedling cDNA library with probes made from different, shorter segments of the pExoII cDNA, to no avail.

Following an examination of the NH_2-terminal amino acid sequence obtained from purified barley β-glucan exohydrolase isoenzyme ExoI, a degenerate oligonucleotide of 20 bases was synthesized and designated **DPA** (Figure 2.1C). Because of the high level of degeneracy of this stretch of amino acid sequence, two inosines were used in the

degenerate oligonucleotide. Inosine forms stable base pairs with all four conventional bases and in most instances where there is three- or fourfold ambiguity at one position, inosine is preferable to a mixture of the conventional bases (Martin *et al.*, 1985). However, mismatched T:G pairs are somewhat more stable than I:G and thus may be preferable in certain circumstances (Sambrook *et al.*, 1989).

The degenerate oligonucleotide **DPA** was labelled with $[\gamma\text{-}^{32}P]$-ATP and used to probe the barley 5-day germinated seedling cDNA library. No positive clones were detected. A barley 12-day seedling cDNA library was also screened with this probe and subsequently with a $[\alpha\text{-}^{32}P]$-dCTP labelled pExoII insert probe, but again no positively hybridizing plaques were detected. An alternative approach to isolating the cDNA encoding β-glucan exohydrolase isoenzyme ExoI was therefore required.

When the 52 NH$_2$-terminal amino acid residues of barley β-glucan exohydrolase isoenzyme ExoI and isoenzyme ExoII are compared using the alignment tool Bestfit in the GCG suite of programs (Devereux *et al.*, 1984), they exhibited only 63% sequence identity (Hrmova *et al.*, 1996). The NH$_2$-terminal amino acid sequences of the barley β-glucan exohydrolase isoenzymes were compared to the EST databases, from which several homologous sequences were identified. Some EST sequences were similar to isoenzyme ExoI and others to isoenzyme ExoII. Although many sequences within the EST databank are truncated at the 5' end, one rice sequence was identified that was very similar to β-glucan exohydrolase isoenzyme ExoI and extended beyond the translated NH$_2$-terminus. This rice EST sequence (rics11657_1A: accession number D46775), which had been isolated from 8-day "green shoots" of rice, showed 80% sequence identity when translated and aligned against the first 52 amino acids of barley β-glucan exohydrolase isoenzyme ExoI. The cDNA of clone D46775 was subsequently provided by the MAFF DNA bank, National Institute of Agrobiological Resources at Tsukuba in Japan.

The cDNA of rice clone D46775 was digested with the *Eco*RI restriction enzyme, which released an insert of approximately 1600 bp. This DNA fragment, hereafter referred to as the 'rice EST Probe', was radiolabelled with $[\alpha\text{-}^{32}P]$-dCTP and used to screen the

barley 5-day germinated seedling cDNA library. Hybridization and washing steps were carried out at 60°C and a final wash stringency of 0.1x SSC, 0.1% SDS. Fourteen positively hybridizing plaques were observed, and were purified to homogeneity (Figure 2.4). Sequence analysis revealed that nine of these encoded part or all of the barley β-glucan exohydrolase isoenzyme ExoI.

Of the nine sequences that were isolated using the rice EST Probe, two were near-full length, spanning the entire coding region of barley β-glucan exohydrolase isoenzyme ExoI. The longer of these two clones, clone 8, was 2,265 bp in length, with a 81 bp 5'-untranslated region and a 294 bp 3'-untranslated region; the latter included a 13 bp poly(A)$^+$ tail. The shorter clone, clone 16, was 2,154 bp in length with a 19 bp 5'-untranslated region and a 245 bp 3'-untranslated region, including an 18 bp poly(A)$^+$ tail (Figure 2.5). Initial sequence data showed the two clones to be identical at both their 5' and 3' ends, with the exception of the different sites of the polyadenylation. The shorter clone, clone 16, was investigated first, and subsequent analyses are related to that sequence. Because the translation of the 5'-end of clone 16 showed that the amino acid sequence was identical to the 52 amino acids from the NH_2-terminus of barley β-glucan exohydrolase isoenzyme ExoI (Hrmova *et al.*, 1996), the clone was designated pExoI.

Restriction fragment analysis was carried out on the pExoI cDNA and the map is shown, together with the sequencing strategy, in Figure 2.6. The clone pExoI was digested with the 4-base cutters *Sau*3AI and *Taq*I before 'shot-gun' cloning into pBluescript. Sequence analyses of these inserts showed that some fragments were preferentially cloned and hence were sequenced several times, while other regions were never isolated. The "shot-gun" cloning resulted in approximately 80% of the pExoI cDNA being sequenced in both directions. For the remaining 20%, restriction enzyme fragments were isolated and subcloned. Some regions of pExoI proved difficult to sequence, because of several compressions that were hard to interpret. This was probably due to the very high G/C content of the cDNA. With the amino acid sequences of the tryptic fragments (Figure 2.5) that were previously isolated and sequenced by Dr. Hrmova, along with information provided by Dr. Varghese from X-ray crystallography data of barley β-glucan exohydrolase
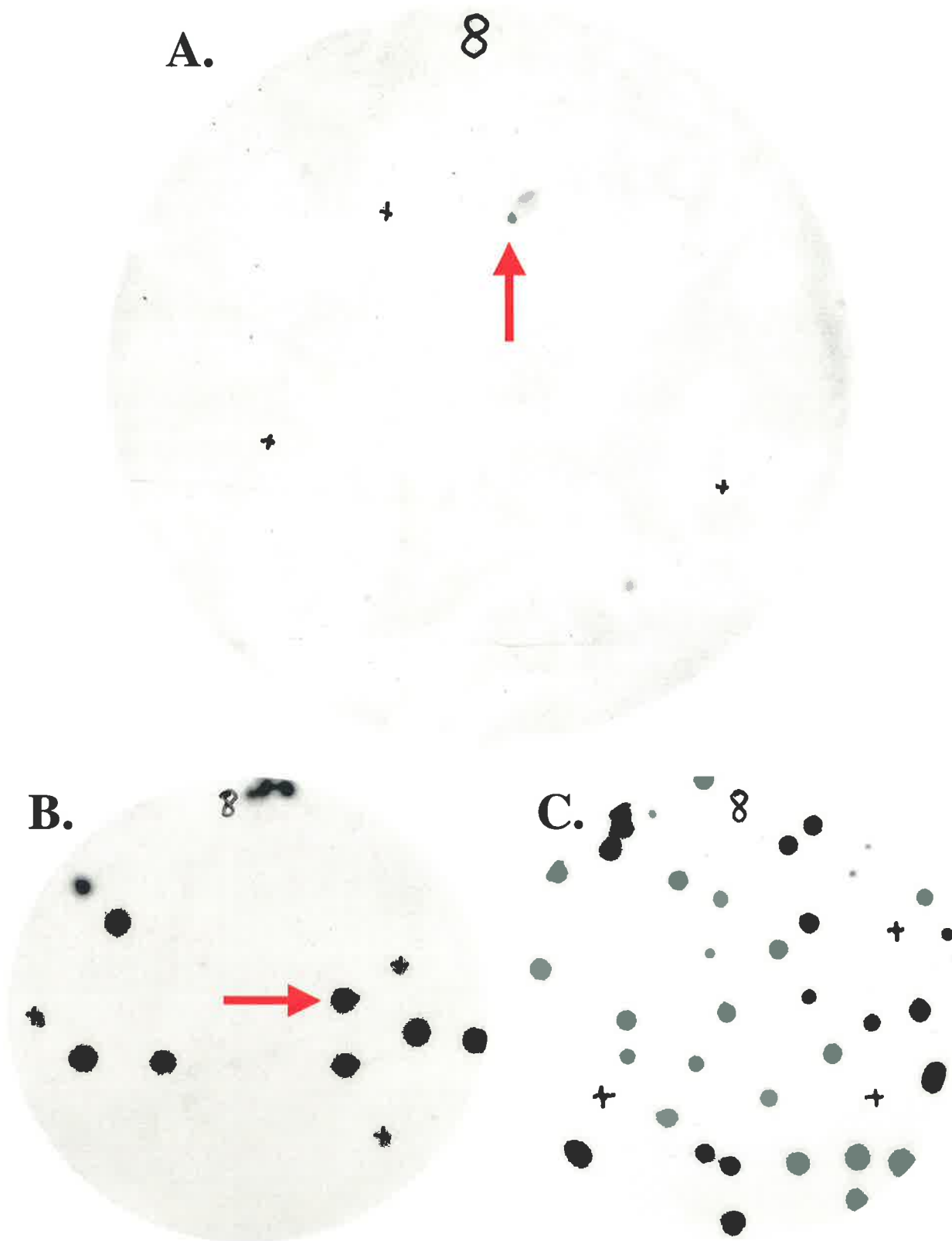
Figure 2.4 Positive plaques after screening the cDNA library with the rice EST Probe. Panel **A**, initial positive plaque after the 1st screen. Panel **B**, 2nd round screen with plug from 1st round plate 8. Panel **C**, homogeneous 'monoclones' after the 3rd screen. The red arrows indicates the plaques that were selected.

Figure 2.5 Complete nucleotide sequence and derived amino acid sequence of barley β-glucan exohydrolase isoenzyme ExoI. Arrows ➝ indicate the 5' and 3' boundaries of clone pExoI. The putative signal peptide is highlighted in orange and the mature NH₂-terminal aspartic acid residue is indicated with an arrow ↓, with the numbering of the amino acid residues starting at the Asp. The position of tryptic peptides is marked in blue. The catalytic nucleophile (D285) ▲ and the catalytic acid (E491) ▲ are indicated with arrows as shown (Varghese *et al.*, 1999). Potential *N*-glycosylation sites are underlined and the stop codon is indicated by an asterisk.

```
                                            ↓
CTCACGGCCCCCGCGGTGTTCGCCGCGCTCCTGCTCTTCTGGGCCGTGCTCGGCGGCACGGACGCCGACTACGTGCTCTACAAGGACGCC  180
 L  T  A  P  A  V  F  A  A  L  L  L  F  W  A  V  L  G  G  T  D  A  D  Y  V  L  Y  K  D  A     8

ACCAAGCCCGTGGAGGACCGCGTCGCCGACCTCCTGGGGAGGATGACGCTGGCGGAGAAGATCGGGCAGATGACCCAGATCGAGCGGCTC  270
 T  K  P  V  E  D  R  V  A  D  L  L  G  R  M  T  L  A  E  K  I  G  Q  M  T  Q  I  E  R      38

GTGGCCACGCCGGACGTGCTCCGGGACAACTTCATCGGCAGCCTGCTCAGCGGCGGCGGCAGCGTGCCGCGCAAAGGGGCCACGGCCAAG  360
 V  A  T  P  D  V  L  R  D  N  F  I  G  S  L  L  S  G  G  G  S  V  P  R  K  G  A  T  A  K   68

GAGTGGCAGGACATGGTGGACGGCTTCCAGAAGGCCTGCATGTCCACGCGGCTCGGCATCCCCATGATCTACGGCATCGACGCCGTCCAC  450
 E  W  Q  D  M  V  D  G  F  Q  K  A  C  M  S  T  R  L  G  I  P  M  I  Y  G  I  D  A  V  H   98

GGCCAGAACAACGTCTACGGCGCCACTATCTTCCCCCACAACGTCGGCCTCGGCGCCACCGGGACCCGTACCTCGTGAAGAGGATCGGC  540
 G  Q  N  N  V  Y  G  A  T  I  F  P  H  N  V  G  L  G  A  T  R  D  P  Y  L  V  K  R  I  G  128

GAGGCCACCGCGCTCGAAGTCAGAGCCACCGGCATCCAGTACGCCTTCGCGCCTTGCATCGCGGTGTGCAGAGATCCGAGATGGGGGCGG  630
 E  A  T  A  L  E  V  R  A  T  G  I  Q  Y  A  F  A  P  C  I  A  V  C  R  D  P  R  W  G  R  158

TGCTATGAGAGCTACAGCGAGGATCGCCGGATCGTGCAGTCCATGACGGAGCTCATCCCCGGCCTGCAGGGCGACGTCCCCAAGGACTTC  720
 C  Y  E  S  Y  S  E  D  R  R  I  V  Q  S  M  T  E  L  I  P  G  L  Q  G  D  V  P  K  D  F  188

ACCAGCGGCATGCCCTTCGTCGCCGGAAAGAACAAGGTGGCTGCATGCGCGAAGCATTTTGTGGGCGACGGCGGCACGGTGGACGGCATC  810
 T  S  G  M  P  F  V  A  G  K  N  K  V  A  A  C  A  K  H  F  V  G  D  G  G  T  V  D  G  I  218

AACGAGAACAACACCATCATCAACCGTGAGGGCCTGATGAACATCCACATGCCGGCGTACAAGAACGCCATGGACAAGGGGGTCTCCACC  900
 N  E  N̲  N̲  T̲  I  I  N  R  E  G  L  M  N  I  H  M  P  A  Y  K  N  A  M  D  K  G  V  S  T  248

GTCATGATCTCCTACTCCAGCTGGAACGGGGTCAAGATGCACGCCAACCAAGACCTCGTCACCGGATACCTCAAGGACACGCTCAAATTC  990
 V  M  I  S  Y  S  S  W  N  G  V  K  M  H  A  N  Q  D  L  V  T  G  Y  L  K  D  T  L  K  F  278

AAGGGCTTCGTGATCTCAGACTGGGAGGGCATTGACAGGATCACCACCCCTGCCGGATCTGACTACTCCTACTCGGTCAAGGCTTCCATT 1080
 K  G  F  V  I  S  D  W  E  G  I  D  R  I  T  T  P  A  G  S  D  Y  S  Y  S  V  K  A  S  I  308
                         ▲

CTTGCCGGCCTTGACATGATCATGGTGCCGAACAACTACCAGCAGTTCATCAGCATCCTGACCGGCCATGTCAACGGCGGCGTCATCCCC 1170
 L  A  G  L  D  M  I  M  V  P  N  N  Y  Q  Q  F  I  S  I  L  T  G  H  V  N  G  G  V  I  P  338

ATGAGCAGGATCGACGATGCCGTGACCCGGATCCTGCGGGTCAAGTTCACCATGGGTCTCTTCGAGAACCCCTACGCTGACCCTGCCATG 1260
 M  S  R  I  D  D  A  V  T  R  I  L  R  V  K  F  T  M  G  L  F  E  N  P  Y  A  D  P  A  M  368

GCCGAGCAGCTAGGAAAGCAGGAGCACAGGGATTTGGCGAGGGAGGCGGCGAGGAAGTCGCTGGTGCTGCTGAAGAACGGCAAGACTTCG 1350
 A  E  Q  L  G  K  Q  E  H  R  D  L  A  R  E  A  A  R  K  S  L  V  L  L  K  N  G  K  T  S  398

ACCGACGCGCCGCTTCTGCCGCTGCCCAAGAAGGCGCCCAAGATCCTGGTCGCCGGGAGCCACGCCGACAACCTGGGCTACCAGTGCGGC 1440
 T  D  A  P  L  L  P  L  P  K  K  A  P  K  I  L  V  A  G  S  H  A  D  N  L  G  Y  Q  C  G  428

GGCTGGACCATCGAGTGGCAGGGCGACACGGGCCGCACCACCGTGGGCACCACCATCCTGGAGGCGGTGAAGGCGGCCGTGGACCCGTCG 1530
 G  W  T  I  E  W  Q  G  D  T  G  R  T  T  V  G  T  T  I  L  E  A  V  K  A  A  V  D  P  S  458

ACGGTCGTGGTGTTCGCCGAGAACCCCGACGCGGAGTTCGTCAAGAGCGGCGGCTTCTCCTACGCCATCGTTGCCGTGGGCGAGCACCCG 1620
 T  V  V  V  F  A  E  N  P  D  A  E  F  V  K  S  G  G  F  S  Y  A  I  V  A  V  G  E  H  P  488

TACACGGAGACCAAGGGCGACAACCTGAACCTGACCATCCCGGAGCCCGGCCTGAGCACCGTGCAGGCGGTGTGCGGCGGCGTGCGGTGC 1710
 Y  T  E  T  K  G  D  N  L  N̲  L̲  T̲  I  P  E  P  G  L  S  T  V  Q  A  V  C  G  G  V  R  C  518
       ▲

GCGACGGTGCTCATCAGCGGGCGGCCCGTGGTGGTGCAGCCGCTGCTGGCCGCCTCCGACGCGCTGGTGGCGGCGTGGCTCCCCGGCTCG 1800
 A  T  V  L  I  S  G  R  P  V  V  V  Q  P  L  L  A  A  S  D  A  L  V  A  A  W  L  P  G  S  548

GAGGGGCAGGGAGTCACCGACGCGCTGTTCGGCGACTTCGGGTTCACCGGGAGGCTGCCGCGGACGTGGTTCAAGTCGGTGGACCAGCTG 1890
 E  G  Q  G  V  T  D  A  L  F  G  D  F  G  F  T  G  R  L  P  R  T  W  F  K  S  V  D  Q  L  578

CCGATGAACGTCGGCGACGCGCACTACGACCCGCTCTTCCGGCTCGGATACGGCCTCACCACCAACGCGACGAAGAAGTACTAGGTTATT 1980
 P  M  N  V  G  D  A  H  Y  D  P  L  F  R  L  G  Y  G  L  T  T  N̲  A̲  T̲  K  K  Y  *        605

ATACTAGACAGCATAATGGAGATAGGAAAACTGGGGACATGAACCGTAGGTTCTTGCTTCCCGCCATGGCTGCCGATCGATTTATTTCGC 2070

TCTCGGCCGATATTTCTTACTGTTGCTTACTACTGCTATTCTTTCTTACTGTTGCTGGTTGATCTGGTGTATTCAGTGAGATCTCACTCT 2160

GCAAGCAGTATATATCCAATTGACGAGCTATTTTATTCACTGAGGTGCTCGTGTTTTTACCTGTGCTTTGAATGTACAATCACTATCCTC 2250
             AAAAAAAAAAAAAAAAAA  3'    Clone 1
                   AAAAAAAAAAAAAAAAAA  3'    Clone pExoI                                 (2154)
                                    ▸
 TCAAAAAAAAAAAAAAAAAA  3'    Clone 8                                                      2269
```

Figure 2.6 Analysis of barley β-glucan exohydrolase isoenzyme ExoI clones. Shown above are the NH$_2$-terminal amino acid sequence ━ , tryptic fragments ━ , signal peptide ━ , and translated coding region ━ . The direction and length of DNA sequencing experiments are shown below ➤ . Restriction enzyme cut sites ⋀ are shown.
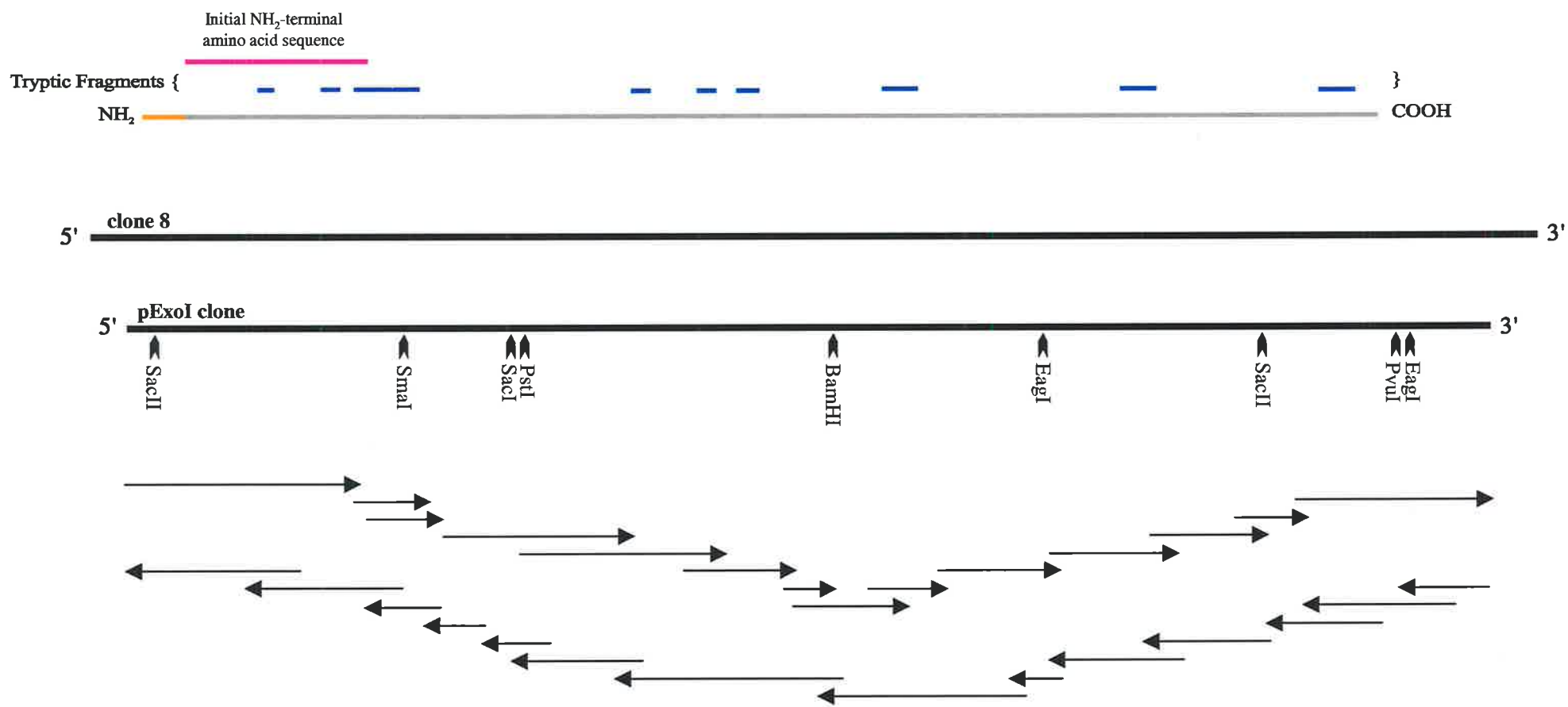
Figure 2.6

isoenzyme ExoI (Varghese *et al.,* 1999) and the use of specifically designed oligonucleotides, the complete nucleotide sequence for the full length pExoI cDNA was eventually defined (Figure 2.5).

The cDNA pExoI is 2154 bp in length and contains an open reading frame from nucleotides 20 to 1909. The amino acid sequence deduced from nucleotides 95-250 corresponds exactly to the first 52 amino acids determined by NH_2-terminal sequencing of barley β-glucan exohydrolase isoenzyme ExoI. Regions of the translated amino acid sequence also correspond exactly with sequences of eight tryptic peptides (Figure 2.5) generated by Dr. Hrmova (Harvey *et al.,* 2000). Within the sequence are three potential *N*-glycosylation sites. Analysis of the crystal structure of purified barley β-glucan exohydrolase isoenzyme ExoI has revealed that all three of these sites are glycosylated (Varghese *et al.,* 1999). No *O*-glycosylation sites were detected.

The region corresponding to the mature enzyme, nucleotides 95-1909, indicates that the mature protein has an M_r of 65,405 and a pI of 7.0. These values may be compared with the apparent molecular mass of 69 kDa deduced from SDS-PAGE and a pI of 7.8 from chromatofocusing (Hrmova *et al.,* 1996). The difference in apparent molecular mass may be accounted for by the glycosylation of the three sites on the mature enzyme, but the difference in pI is more difficult to explain. Possible explanations are mentioned in the Discussion section.

The 5'-untranslated region of clone pExoI is only 19 bases long and is unlikely to represent the full 5'-untranslated region of the mRNA. Clone 8, also produced by screening with the rice EST Probe, has an 81 bp 5'-untranslated region, but when this is compared to the 5'-end of the maize ExoII cDNA (an isoenzyme ExoI homologue) the first 47 bp show only a 30% match, whereas the remainder of the 5'-untranslated region shows 88% sequence identity. This suggests that one of these clones may be the result of either a cloning artefact or an mRNA processing error. At approximately the point where the sequences diverge, there is a 'cagG' sequence in isoenzyme ExoI clone 8; this might represent the right border of an intron (Watson *et al.,* 1987) that, due to faulty processing,

has not been removed. Thus, the precise sequence of the 5'-untranslated region of mRNA encoding barley β-glucan exohydrolase isoenzyme ExoI remains uncertain.

As with the sequence for barley β-glucan exohydrolase isoenzyme ExoII, the cDNA nucleotide sequence of the pExoI cDNA reveals the presence of a putative signal peptide of 25 amino acid residues, encoded by nucleotides 20-94. This again suggests that the enzyme is secreted from cells in which it is expressed. The putative signal peptide contains mainly hydrophobic residues, and there is no charged residue within the first five residues. There is, however, a hydrophilic threonine residue (Thr5), which may carry out a similar role. In other respects the signal peptide of pExoI is normal (Watson *et al.*, 1987), with a hydrophobic core, helix-breakers near the COOH-terminus (Gly21 and Gly22) and a small side-chain amino acid residue at the COOH-terminus (Ala25).

Beginning with the TAG stop codon of cDNA clone pExoI, there is a 245 bp 3'-untranslated region that includes an 18 bp poly(A)$^+$ tail. Two other cDNA clones isolated using the rice EST Probe contained alternative polyadenylation sites. Clone 1 contained a 17 bp poly(A)$^+$ tail that begins 9 bases upstream from that of the cDNA pExoI, and clone 8 contains a 3'untranslated region that is 49 bp longer than the cDNA pExoI, with a 17 bp poly(A)$^+$ tail. All 3'-untranslated sequences are identical where they overlap (Figure 2.5). Unlike the cDNA for barley β-glucan exohydrolase isoenzyme ExoII, the clones for isoenzyme ExoI contain no clearly identifiable NUE polyadenylation signal. This is perhaps not altogether surprising since less than 50% of reported plant mRNAs contain the AAUAAA consensus NUE sequence at their polyadenylation sites (Wu *et al.*, 1995).

Comparison of the nucleotide sequence of the cDNA encoding barley β-glucan exohydrolase isoenzyme ExoI (clone pExoI) with the sequence of the barley β-glucan exohydrolase isoenzyme ExoII cDNA (clone pExoII) shows 71% identity in the coding regions of the mature enzymes. Comparison of translated amino acid sequences of the same region reveals 73% identity and 83% similarity. When codon usage is analyzed, the cDNA clone for isoenzyme ExoII shows a relatively balanced usage with 65% of codons having G or C at the wobble base position. However, the cDNA for isoenzyme ExoI

exhibits a highly biased codon usage with 93% of codons having G or C at the wobble base position.

2.4 Discussion

Over the course of these studies, cDNAs that encode two β-glucan exohydrolase isoenzymes previously purified from barley seedlings have been isolated (Hrmova *et al.,* 1996). The 5-day barley seedling cDNA library was used because the purified barley β-glucan exohydrolase isoenzymes had been isolated from 8-day germinated barley seedlings. An oligonucleotide primer (**DPB**) designed from NH_2-terminal amino acid sequence was used initially to isolate a fragment encoding the 5'-end of the barley β-glucan exohydrolase isoenzyme ExoII cDNA from the cDNA library. Through a progression of re-probing experiments, a near-full length cDNA encoding isoenzyme ExoII was isolated. This cDNA clone, labelled pExoII, encodes a protein that has a calculated M_r and pI close to the values directly determined for the purified β-glucan exohydrolase isoenzyme ExoII. The difference between the calculated molecular mass and that determined for the purified enzyme may be accounted for by *N*-glycosylation, for which there are four potential sites (Figure 2.3).

Isolation of a cDNA encoding barley β-glucan exohydrolase isoenzyme ExoI initially proved difficult, because neither an isoenzyme ExoI-specific oligonucleotide (**DPA**) nor the β-glucan exohydrolase isoenzyme ExoII cDNA (pExoII) resulted in the identification of an isoenzyme ExoI cDNA. A cDNA encoding isoenzyme ExoI was eventually identified using a homologous rice EST (accession no. D46775) as a probe.

The calculated molecular mass for the protein encoded by pExoI is similar to that determined by SDS-PAGE for purified barley β-glucan exohydrolase isoenzyme ExoI, the difference may be accounted for by *N*-glycosylation. Three potential *N*-glycosylation sites are detected in the amino acid sequence (Figure 2.5) and recent X-ray crystallographic data has demonstrated that Asn221, Asn498 and Asn600 are indeed glycosylated (Varghese *et al.,* 1999). The difference in isoelectric points, where the pI measured for purified enzyme is approximately 0.8 units higher, is more difficult to explain. It is possible that the *N*-glycosylation may affect the pI by masking some surface charges, or some other post-

translational modification of the enzyme may have some effect. Further elucidation of the crystal structure may help clarify these possibilities.

Comparison between the two β-glucan exohydrolase cDNAs

When the two cDNAs encoding barley β-glucan exohydrolase isoenzyme ExoI and isoenzyme ExoII are compared, there is 73% sequence identity and 83% similarity at the amino acid level. This indicates that the cDNAs are products of two separate genes and, because barley is predominantly self-fertilizing and plants of established cultivars are essentially homozygous, they therefore represent true genetic isoenzymes. The 52 NH_2-terminal amino acid sequences of the two purified barley β-glucan exohydrolase isoenzymes match exactly with the deduced sequences from the isolated cDNAs. When the barley β-glucan exohydrolases were isolated from 8-day seedlings, two chromatographic peaks were seen for each isoform (Hrmova *et al.,* 1996). NH_2-Terminal sequencing showed that the sequences of the first 52 amino acids of each isoform within the pairs were identical, despite the differences in electrophoretic mobilities for each peak (Hrmova *et al.,* 1996). Because *N*-glycosylation is observed on all of the three possible sites of purified β-glucan exohydrolase isoenzyme ExoI (Varghese *et al.,* 1999), it is possible that minor differences in the amounts and compositions of carbohydrate linked to the isoenzymes are responsible for the observed electrophoretic differences of the isoforms.

At the DNA level, comparison of the cDNAs encoding barley β-glucan exohydrolase isoenzyme ExoI and ExoII show 71% identity over the sequence corresponding to the mature coding regions (Table 2.1). It might be expected that this level of sequence similarity would be sufficient to isolate the cDNA corresponding to one isoenzyme using the cDNA of the other as a probe, provided the hybridization was performed at low to moderate stringency. However, when the nucleotide composition is analyzed (Table 2.1), isoenzyme ExoI has a much higher (G+C) content overall (65% for the isoenzyme ExoI cDNA compared to 52% for the isoenzyme ExoII cDNA). The most surprising difference is found at the codon usage level, particularly with respect to the (G+C) content at the

DNA Coding Region % Identity	Barley β-Glucan Exohydrolase ExoI	Barley β-Glucan Exohydrolase ExoII	Nasturtium β-Glucosidase	Tobacco β-Glucan Exohydrolase	Maize β-Glucan Exohydrolase ExoII
Protein % Identity (left) / Protein % Similarity (right)					
Barley β-Glucan Exohydrolase ExoI — DNA	100	71	66	65	85
Barley β-Glucan Exohydrolase ExoI — Protein id / sim	100 / 100	73 / 83	71 / 83	72 / 84	83 / 91
Barley β-Glucan Exohydrolase ExoII — DNA		100	70	66	70
Barley β-Glucan Exohydrolase ExoII — Protein id / sim		100 / 100	77 / 86	76 / 85	72 / 83
Nasturtium β-Glucosidase (Acc. No. AJ006501) — DNA			100	73	66
Nasturtium β-Glucosidase (Acc. No. AJ006501) — Protein id / sim			100 / 100	81 / 88	71 / 84
Tobacco β-Glucan Exohydrolase (Acc. No. G3582436) — DNA				100	64
Tobacco β-Glucan Exohydrolase (Acc. No. G3582436) — Protein id / sim				100 / 100	72 / 85
Maizeβ-Glucan Exohydrolase ExoII (Acc. No. AF064707) — DNA					100
Maizeβ-Glucan Exohydrolase ExoII (Acc. No. AF064707) — Protein id / sim					100 / 100
Overall % (G+C) Content	65	52	47	45	67
% (G+C) in Wobble Base Position	93	65	46	41	94

Table 2.1 Comparison of DNA and translated amino acid sequences of five similar putative β-glucan exohydrolases. Numbers in upper, larger boxes are percentage identities of DNA sequences compared over the coding regions, while the lower, smaller boxes are percent identity (left) and percent similarity (right) for the amino acid sequences of translated coding regions of the mature proteins. The percentage (G+C) composition and percentage (G+C) in the wobble base position of codons for each enzyme are shown below.

wobble base position, where isoenzyme ExoI shows a biased codon usage (93% G+C) whereas isoenzyme ExoII is a more balanced 65% (G+C). It can be envisaged that probes made from cDNAs for one isoenzyme would have difficulty in binding to the DNA for the other isoenzyme where the sequence is different for every second or third codon, even when the amino acid sequences are relatively similar.

The high (G+C) bias in the wobble base position of codons in barley β-glucan exohydrolase isoenzyme ExoI may be related to the sites of transcription and is therefore of general interest. It would seem likely that some form of selective pressure has resulted in the shift of the isoenzyme ExoI gene from a relatively balanced codon usage to the extant form of the gene, in which more than 90% of codons end in G or C. Several cDNAs isolated from barley tissues, in particular aleurone cells following germination, have shown a similar bias in codon usage with a high (G+C) content at the wobble base position. These include cDNAs for (1→3,1→4)-β-glucan endohydrolases, (1→3)-β-glucan endohydrolases, (1→4)-β-xylan endohydrolases and other enzymes that are expressed at high levels in germinated cereal grains (Fincher and Stone, 1986; Fincher, 1989; Slakeski and Fincher, 1992a; Slakeski and Fincher, 1992b; Banik *et al.*, 1996). The genes corresponding to these cDNAs are all gibberellic acid-inducible and it has been suggested that the (G+C) bias may be related to their translational efficiency (Fincher, 1989). The rate-limiting step in the elongation cycle of protein synthesis is the stochastic search for the ternary aminoacyl-tRNA complex that corresponds to a particular codon, and differences in translation rates may be linked to the availability of specific tRNAs (Shewry and Miflin, 1985; Bulmer, 1987; Fincher, 1989). The difference in (G+C) usage at the wobble base position in the two barley β-glucan exohydrolase isoenzymes might be related to function, and to expression patterns of the two genes.

Comparison of the two β-glucan exohydrolase cDNAs to DNA and protein databases

When the cDNA sequences encoding barley β-glucan exohydrolase isoenzyme ExoI and isoenzyme ExoII are compared with sequences in the DNA and protein databases,

three sequences with reasonably high homologies are found. These sequences are the nasturtium (*Tropaeolum majus*) β-glucan exohydrolase (Crombie *et al.,* 1998; accession number AJ006501), a β-glucosidase from tobacco (*Nicotiana tabacum*; N. Koizumi, unpublished results, accession number G3582436) and a β-glucosidase from *maize (Zea mays*, Y.Y. Zhao and L. Bogorad, unpublished results, accession number AF064707). Comparisons of the nucleotide sequences and translated amino acid sequences of these three cDNAs and the sequences of both barley β-glucan exohydrolases are shown in Table 2.1. An alignment of the sequences using Pileup (Devereux *et al.,* 1984) was made and displayed using ALSCRIPT (Barton, 1993) (Figure 2.7). These data show that the maize cDNA encodes a close homologue of barley β-glucan exohydrolase isoenzyme ExoI.

The barley β-glucan exohydrolase isoenzyme ExoI and the maize β-glucan exohydrolase isoenzyme ExoII sequences have very similar codon usage patterns, especially in the wobble base position. The identity and similarity between the translated maize protein sequence and the translated barley β-glucan exohydrolase isoenzyme ExoI sequence are also high, with these two enzymes showing 91% sequence similarity (Table 2.1). The entry in the GenBank database relating to the maize sequence does not state the tissue or conditions from which the cDNA was isolated.

The two other comparable sequences found in the DNA databases are those of β-glucan exohydrolases from tobacco and nasturtium, both dicotyledonous species. The sequence from nasturtium is labelled as a β-glucosidase, but a more appropriate classification might be as a β-glucan exohydrolase (Hrmova *et al.,* 1996). Multiple sequence analysis (Figure 2.7) and representation within an unrooted radial phylogenetic tree (Figure 2.8) show the two dicotyledonous sequences to be more similar to barley β-glucan exohydrolase isoenzyme ExoII, than to isoenzyme ExoI. The (G+C) content and codon usage is essentially balanced within the two dicotyledonous exohydrolases.

When translated EST databases are searched with the barley β-glucan exohydrolase sequences, matches are found with several other dicotyledonous species including; cotton (*Gossypium hirsutum*, accession no. AI731185), soybean (*Glycine max*, accession no.

Figure 2.7 Multiple sequence alignment of five β-glucan exohydrolases from different plant species. Regions of dark green indicate identical amino acids, while yellow regions indicate homologous amino acids. The mature NH_2-terminus of barley β-glucan exohydrolase isoenzyme ExoI is indicated with a red arrowhead, the black arrowheads followed by **CHO** indicate the *N*-glycosylation sites of barley β-glucan exohydrolase isoenzyme ExoI and the hollow arrowheads indicate the catalytic amino acids that are conserved over all five sequences. Barley ExoII is *Hordeum vulgare* β-glucan exohydrolase isoenzyme ExoII (described in this thesis), Nasturtium is *Tropaeolum majus* β-glucosidase (Crombie *et al.*, 1998), Tobacco is *Nicotiana tabacum* β-glucan exohydrolase (Koizumi, unpublished, 1998), Maize ExoII is *Zea mays* β-glucan exohydrolase isoenzyme ExoII (Zhao and Bogorad, unpublished, 1999) and Barley ExoI is *Hordeum vulgare* β-glucan exohydrolase isoenzyme ExoI (described in this thesis).

Protein sequence alignment of Barley ExoII, Nasturtium, Tobacco, Maize ExoII, and Barley ExoI.

```
                1                                                    10              20
Barley ExoII  M G N L . . H . K T T F V L L M F C L A A L G S A D Y . . . . L K Y K D P K Q P L G V R I K D L L G R M T L A E K
Nasturtium    M G R F L L P . . I L G W F L L L S C L S A F T E A E Y . . . . M R Y K D P K K P L N V R I K D L M S R M T L A E K
Tobacco       M G R M S I P . M M G . F V V L C L W A V V A E G E Y . . . . M V K Y K D P K Q P V G A R I K D L M K R M T L E E K
Maize ExoII   M A L L T A P A V A A L L L L F W S S A Y G G D A Q G E G V P P P Y Q D A S K D V E V R V R D L L A R M T L A E K
Barley ExoI   M A L L T A P A V F A A L L L F W A V L G G T D A D Y . . . L Y K D A T K P V E D R V A D L L G R M T L A E K
                                                ▲NH2-Term.
```

```
      30              40              50              60              70              80              90
I G Q M T Q I E R E N A T A E A M S K Y F I G S V L S G G G S V P S P Q A S A A A W Q S M V N E M Q K G A L S T R L G I P M I
I G Q M T Q I E R K E A T P D V I S K Y F I G S V L S G G G S V P A P K A S P E A W V D L V N G M Q K A A L S T R L G I P M I
I G Q M T Q I E R K V A T A D V M K Q N F I G S V L S G G G S V P A P K A S A Q V W T N M V D E I Q K G S L S T R L G I P M I
V G Q M T Q I E R I V A S P Q A L R D Y Y I G S L L S G G G S V P R K Q A T A A E W V A M V S D F Q K A C L S T R L G I P M I
I G Q M T Q I E R L V A T P D V L R D N F I G S L L S G G G S V P R K G A T A K E W Q D M V D G F Q K A C M S T R L G I P M I
```

```
  100             110             120             130             140             150
Y G I D A V H G H N N V Y K A T I F P H N V G L G A T R D P M L V K R I G E A T A L E V R A T G I P Y A F A P C I A V C R D P
Y G I D A V H G H N N V Y N A T I F P H N V G L G V T R D P A L I K R I G E A T A L E C R A T G I P Y A F A P C I A V C R D P
Y G I D A V H G H N N V Y G A T I F P H N V G L G V T R D P D L V K R I G A A T A L E V R A T G I P Y A F A P C I A V C R N P
Y G I D A V H G H N N V Y G A T I F P H N V G L G A T R D P N L V K R I G A A T A L E V R A T G I Q Y A F A P C I A V C R D P
Y G I D A V H G Q N N V Y G A T I F P H N V G L G A T R D P Y L V K R I G E A T A L E V R A T G I Q Y A F A P C I A V C R D P
```

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      160             170             180             190             200             210
R W G R C Y E S Y S E D P K V V Q S M T T L I S G L Q G D V P A G S . E G R P Y V G G S K K V A A C A K H Y V G D G G T F M G
R W G R C Y E S Y S E D H T I V Q A M T E I I P G L Q G D V P P D V K K G V P F V G G K T K V A A C A K H F V G D G G T T K G
R W G R C Y E S Y S E D H R I V R S M T E I I P G L Q G D L P A K S K N G V P Y V G G K T K V A A C A K H F V G D G G T L H G
R W G R C Y E S Y S E D H R I V Q A M T E L I P G L Q G D V P Q N F T S G M P F A A G K D K V A A C A K H F V G D G G T Q N G
R W G R C Y E S Y S E D R R I V Q S M T E L I P G L Q G D V P K D F T S G M P F V A G K N K V A A C A K H F V G D G G T V D G
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  220             230             240             250             260             270             280
I N E N D T I I D A H G L M T H M P A Y Y N S I R G V S T V M T S Y S S W N G K K M H A N H F L V T D F L K N K L K F R G
I D E N N T V I D S R G L F S H M P A Y H D S I K K G V A T V M V S Y S S W N G L R M H A N R D L V T G Y L K N K L K F R G
V D E S N T V I S S N S L F S H M P A Y Y D S L R K G V A T V M V S Y S S W N G R K M H A N R D L V T G F L K D K L K F R G
I N E N N T I I D R Q G L I S H M P A Y L D A L R K G F S T V M I S Y S S W N G L K M H A N H N L I T G F L K D R L N F Q G
I N E N N T I I N R E G L M N I H M P A Y K N A M D K G V S T V M I S Y S S W N G V K M H A N Q D L V T G F L K D T L K F K G
      ▲CHO
```

```
  290             300             310             320             330             340
F V I S D W Q G I D R I T S P P G V N Y S Y S V E A G V G A G I D M I M V P F A Y T E F I D D L T Y Q V K N N I P M S R I N
F V I S D W E G I D R I T D P P G R N Y S Y S V E A G V G A G I D M I M V P E D N F T K F L N E L T S Q V K K N I P M S R I D
F V I S D W Q G I D R I T D P P H A N Y S Y S V Q A S I L A G L D M I M V P N N Y Q N F I T I L T G H V N S G L P M S R I D
F T I S D W E G I D R V T S P P G A N Y S Y S V Q A S I L A G L D M I M V P N K Y Q Q F I S I L T G H V N G G V P M S R I D
F V I S D W E G I D R I T T P A G S D Y S Y S V K A S I L A G L D M I M V P N K Y Q Q F I S I L T G H V N G G V P M S R I D
  △Catalytic
```

```
D A V Y R I L R V K F T M G L F E S P Y A D P S L V G E L G K Q E H R D L A R E A V R K S L V L L K N G K S A . S T P L L P L
D A V K R I L R V K F T M G L F E S P L A D Y S L A N Q L G S Q E H R D L A R E A V R K S L V L L K N G E S A . D K P F V P L
D A V K R I L R V K F V M G L F E N P M S D P S L A N Q L G S Q E H R E L A R E A V R K S L V L L K N G K T P . S Q P L L P L
D A V T R I L R V K F T M G L F E N P M P D P S L A D Q L G K Q E H R D L A R E A V R K S L V L L K N G K P G . D A P L L P L
D A V T R I L R V K F T M G L F E N P Y A D P A M A E Q L G K Q E H R D L A R E A A R K S L V L L K N G K T S T D A P L L P L
      350             360             370             380             390             400
```

```
P K K A G K I L V A G S H A D D L G N Q C G W T I T W Q G Q T G N D K T A G T T I L S A I K S T V D P S T E V V F S E N P D
P K N A K K I L V A G S H A D N L G R Q C G G W T I E W Q G V N G N D L T T G T T I L N A I K K T V D P T T Q V I Y N E N P D
P K K A P K I L V A G T H A D N L G Y Q C G G W T I E W Q G D V G R T . T I G T T I L N T D A V K K T V A D P S T Q V V Y Q Q N P D
P K K A A R I L V A G S H A D N L G Y Y Q C G G W T I E W Q G D A G R T . T V G T T T L E A V K A V D P S T V V V F A E S P D
P K K A P K I L V A G S H A D N L G Y Q C G G W T I E W Q G D G R T . T V G T T T L E A V K A V D P S T V V V F A E N P D
      410             420             430             440             450             460
```

```
S A A V D S G K Y D Y A I V V G E P P Y A E T F G D N L N L T I P A P G P S V I Q N V C K S V R C V V V L I S G R P L V V E
S N Y V K T N S F D Y A I V V G E V P Y A E M Q G D S F N L T I P E P G P T T I S S V C G A V K C V V V V I S G R P V V L Q
A N E F V R S N K F S Y A I V V G E V P Y A E M F G D S N L T I A E P G P S T I S V N C G S V K C V V V V V S G R P V V L E
A A E F V R S G G F S Y A I V A V G E H P Y T E T K G D S M N L T I P A P D P G P S T V Q T V C A A V R C V T L I S G R P V V Q
A A E F V K S G G F S Y A I V A V G E H P Y T E T K G D N L N L T I P E P G L S T V Q A V C G G V R C A T V L I S G R P V V Q
      470             480             490             500             510             520             530
                                        Catalytic △       ▲CHO
```

```
P Y I S A M D A F V A A W L P G S E G Q G V D V L F G D Y G F S G K L A R T W F K S A D Q L P M N V G D K H Y D P L F P F G
P Y V S Y M D A L V A A W L P G T E G Q G V T D V L F G D Y G F T G K L A R T W F K T V D Q L P M N V G D K H Y D P L F P F G
P Y V S K M D A L V A A W L P G T E G Q G V A D A L F G D Y G F T G K L A R T W F K R V D Q L P M N F D D A H V D P L F P F G
P F L G A M D A V V A A W L P G T E G Q G V T D V L F G D Y G F T G K L P R T W F R S V D Q L P M N Y G D A H Y D P L F R L G
P L L A A S D A L V A A W L P G S E G Q G V T D A L F G D F G F T G R L P R T W F K S V D Q L P M N V G D A H Y D P L F R L G
      540             550             560             570             580             590
```

```
F G L T T E A K K . R T E F I G L I F G D L E M F S R Y Y V E G C K D G V
F G L T T K P S N R T E F I G L I F G D L E M F S R Y Y V E G C K D G V
F G L T T K P V K G Y . . . . . . . . . . . . . . . . . . . . . . . . . .
F G L T T Q G . K M Y Q N . . . . . . . . . . . . . . . . . . . . . . . .
Y G L T T N A T K K Y . . . . . . . . . . . . . . . . . . . . . . . . . .
      600
      ▲CHO
```

Nicotiana tabacum

Tropaeolum majus

Hordeum vulgare
Isoenzyme ExoII

Hordeum vulgare
Isoenzyme ExoI

Zea mays
Isoenzyme ExoII

0.1

Figure 2.8 Unrooted radial phylogenetic tree showing relationships between five plant β-glucan exohydrolases.

AI460825), *Arabidopsis* (*Arabidopsis thaliana*, accession no. ATTS1387), tomato (*Lycopersicon esculentum*, accession nos. AI489350, AI489610) and aspen (*Populus tremula* x *Populus tremuloides*, accession no. AI165893). Assuming that these matches indicate the presence of β-glucan exohydrolases within these plants, such a wide range of species seems to suggest a general role for these enzymes in plant function. All of the dicotyledonous species sequences show relatively balanced (G+C) contents and codon usage.

EST Database searches also give a plethora of matches with rice (*Oryza sativa*) sequences as well as matches with maize (*Zea mays*) EST sequences. The presence of these homologous sequences is not surprising given the evolutionary relatedness of the monocotyledonous species. Matches are found with ESTs from various tissues, including: rice panicle, rice seedling root, rice immature leaf, rice shoot, rice etiolated shoot, maize ear and maize leaf primordia. As more EST database projects are established around the world, the number of matches found to barley β-glucan exohydrolase sequences would be expected to increase rapidly. The use of the information derived from these matches is limited however, since the length of EST sequences is generally only a few hundred bases, and most tend to be short at the 5'-end. These matches are useful in providing information as to where these sequences are being expressed, and perhaps at what times during the life cycle of the plant. More about this issue is discussed in Chapter 5.

2.5 Conclusion

In the work described in this Chapter, two cDNAs encoding β-glucan exohydrolases were isolated. Their translated sequences matched exactly to NH$_2$-terminal sequence data of the two previously purified β-glucan exohydrolase isoenzymes (Hrmova *et al.,* 1996). It was demonstrated by sequence comparisons that there are a number of plant genes that are highly similar to the barley β-glucan exohydrolase isoenzyme cDNAs. The determination of the complete amino acid sequences for the barley enzymes (Figures 2.3 and 2.5) allows the enzymes to be classified in the family 3 group of glycoside hydrolases. In Chapter 3 the basis for this classification is described in detail, together with an extensive analysis of family 3 glycoside hydrolases that has been made possible by the availability of the amino acid sequence (Figure 2.5) and 3D structure of the β-glucan exohydrolase isoenzyme ExoI which was determined by X-ray crystallography.

Chapter 3

Molecular Modelling of Family 3 Glycoside Hydrolases

3.1 Introduction

In Chapter 2 of this thesis the isolation and characterisation of two barley β-glucan exohydrolases was described. Sequence comparisons at both DNA and protein level showed that similar glycoside hydrolases occur widely amongst plants, bacteria and fungi. Many of these glycoside hydrolases are able to hydrolyse several substrates and some exhibit a very broad specificity. For example, the barley β-glucan exohydrolases described in this thesis are able to hydrolyse synthetic aryl β-glycosides such as 4-nitrophenyl β-D-glucoside, and might therefore be classified as a β-glucosidases in the EC 3.2.1.21 group (Hrmova *et al.*, 1996). Nevertheless, the preferred substrates for the barley β-glucan exohydrolase are (1→3)-β-glucans, from which the enzyme releases non-reducing terminal β-glucosyl residue (Hrmova and Fincher, 1998); this specificity would place the enzyme in the EC 3.2.1.58 class.

While substrate specificity will remain a cornerstone of enzyme classification, additional criteria are clearly needed to identify evolutionary and functional relationships between individual glycoside hydrolases. Structural information is increasingly used to discriminate between enzymes or, conversely, to classify them into related groups. Thus, glycoside hydrolase classifications based on linear, one-dimensional (1D) amino acid sequences were initially proposed to take into account primary protein structures (Henrissat, 1991) and these were subsequently supplemented with hydrophobic cluster analysis (HCA) (Gaboriaud *et al.*, 1987). The HCA analyses provide 2D structural information and are particularly useful where amino acid sequence identities between proteins are low (Callebaut *et al.*, 1997). The 1D and 2D structural information has been applied to the glycoside hydrolases, which fall into more than 80 different families under this classification scheme (Henrissat and Davies, 1997; Henrissat, 1998).

The identification of glycoside hydrolases through 3D structural models and their classification into family groups rely on the availability of at least one experimentally-determined 3D structure per family. Until recently, no 3D structures were available for family 3 glycoside hydrolases, which currently include enzymes that are variously

designated as β-glucosidases, *N*-acetyl β-glucosaminidases and β-xylosidases. However, the structure of a family 3 β-glucan exohydrolase from barley has now been solved to 2.2 Å resolution by X-ray crystallography (Hrmova *et al.,* 1998b; Varghese *et al.,* 1999). Solution of this structure relied heavily on the primary amino acid sequence deduced from the pExoI cDNA described in Chapter 2 (Figure 2.5). This, in turn, allowed comparative protein modelling to examine domain structures within the family 3 group of glycoside hydrolases, to define the disposition of catalytic amino acids and structures of active sites, and to determine local variations in structures across the family.

3.2 Materials and Methods

3.2.1 Evolutionary analysis

A total of 99 members of the family 3 group of glycoside hydrolases, as classified on the Carbohydrate-Active enZYmes (CAZY) server (http://afmb.cnrs-mrs.fr/~pedro; as at June 1, 2000) (Coutinho and Henrissat, 1999), were aligned using the PileUp program. An unrooted, radial phylogenetic tree was constructed using the EprotPars program of the University of Wisconsin Genetics Computers Group (GCG) package (Devereux *et al.*, 1984).

The accession numbers at the GenBank/EMBL and SWISS-PROT databases of the 99 family 3 glycoside hydrolases are as follows: *Acetobacter xylinus* AB010645 (a), *Acetobacter xylinus* AB015802 (b), *Agrobacterium tumefaciens* M59852, *Ajellomyces capsulatus* U20346, *Alteromonas sp.* D17399, *Arabidopsis thaliana* AL353995 (a), AL133292 [ORF F13I12.100] (b), AL133292 [ORF F13I12.50] (c), AL133292 [ORF F13I12.60] (d), AL133292 [ORF F13I12.90] (e), AL353994 (f), AL162651 (g), AC009243 (h), *Aspergillus aculeatus* D64088, *Aspergillus kawachi* AB003470, *Aspergillus nidulans (Emericella nidulans)* Y13568, *Aspergillus niger* Z84377, *Aspergillus oryzae* AB009972, *Aspergillus wentii* P29090, *Azospirillum irakense* AF090429 β-glucosidase (SalA) (a), *Azospirillum irakense* AF090429 β-glucosidase (SalB) (b), *Bacillus sp.* AB009411, *Bacillus subtilis* L19954, *Bacteroides fragilis* AF006658, *Borrelia burgdorferi* AE001115 (a), *Borrelia burgdorferi* AE001163 (b), *Botryotinia fuckeliana* AJ130890, *Butyrivibrio fibrisolvens* M31120, *Cellulomonas biazotea* AF005277, *Cellvibrio gilvus* D14068, *Chryseobacterium meningosepticum* AF015915, *Clostridium stercorarium* Z94045, *Clostridium thermocellum* X15644, *Coccidioides immitis* U87805 (a), *Coccidioides immitis* AF022893 (b), *Cochliobolus heterostrophus* AF027687, *Debaryomyces hansenii* AJ223815, *Deinococcus radiodurans* AE001979, *Dictyostelium discoideum* L21014, *Erwinia chrysanthemi* U08606, *Escherichia coli* U15049 (a), *Escherichia coli* AE000211 (b), *Gaeumannomyces graminis* U35463 (a), *Gaeumannomyces graminis* U17568 (b), *Glycine max* AF000378, Haemophilus *influenza* U32777, *Hordeum vulgare* β-glucan

exohydrolase isoenzyme Exo1 AF102868, *Hordeum vulgare* β-glucan exohydrolase isoenzyme Exo2 U46003, *Kluyveromyces fragilis* X05918, *Listeria monocytogenes* U78883, *Microbispora bispora* L06134, *Mycobacterium tuberculosis* AL021929 (a), Z97050 (b), *Neisseria meningitidis* AE002408 (a), *Neisseria meningitides* AL162754 (b), *Neurospora crassa* AL355929, *Nicotiana tabacum* AB017502, *Phanerochaete* avenaria AJ276675, Phanerochaete *chrysosporium* AF036872, *Pichia anomala (Candida pelliculosa)* X02903, *Prevotella ruminicola* U35425, *Pseudomonas aeruginosa* U56077, *Pseudomonas fluorescens* X65527, *Ruminococcus albus* U92808 (a), *Ruminococcus albus* X15415 (b), *Ruminococcus flavefaciens AJ132472, Saccharomycopsis fibuligera* M22475 (a), M22476 (b), *Saccharopolyspora erythraea* Y14327, *Salmonella typhimurium* D86507, *Schizophyllum commune* M27313, *Schizosaccharomyces pombe* AL355920, *Septoria lycopersici* U24701, *Streptomyces antibioticus* AF055579, *Streptomyces coelicolor* AL121596 (a), AL136519 (b), AL023702 (c), AL352956 (d), AL117385 (e), AL355913 (f), AL031013 (g), *Streptomyces lividans* AF043654, *Streptomyces thermoviolaceus* AB008771, *Streptomyces venezuelae* AF079762, *Synechocystis sp.* D90914, *Thermoanaerobacter brockii* Z56279, *Thermoanaerobacter ethanolicus* AF135015, *Thermotoga maritima* AE001690 (a), AE001694 (b), AE001748 (c), *Thermotoga neapolitana* Z77856 (a), Y17983 (b), U58632 (c), *Trichoderma reesei (Hypocrea jecorina)* U09580 (a), Z69257 (b), *Tropaeolum majus* AJ006501, unidentified bacterium U12011, *Vibrio furnissii* U52818 and *Zea mays* AF064707. Lower case letters after accession numbers indicate individual members when multiple entries of a species are listed.

3.2.2 Analysis of domain arrangements

Analyses of all the family 3 glycoside hydrolases were effected through the ProDom 99.1 server (http://www.toulouse.inra.fr/multalin.html) (Corpet *et al.,* 1998), using the sequence of barley β-glucan exohydrolase isoenzyme ExoII. The automated program compares the non-fragmentary sequences with the PSI-BLAST tool (Sonnhammer and Kahn, 1994), taken from the SWISS-PROT database, and the resultant families are further

processed with the MKDOM program (Altschul *et al.*, 1997). The original output is filtered to yield non-redundant similarities.

3.2.3 Protein structure modelling

Comparative (homology) protein modelling was performed using the Modeller version 4.0 program (Sali and Blundell, 1993), based on satisfaction of spatial restraints and statistical analysis of the relationships between pairs of homologous structures in order to produce the protein fold. The CHARM 22 energy function was implemented during energy minimization to produce an objective function. The resultant model was refined by employing a conjugate gradient and molecular dynamics with simulated annealing (Sali and Blundell, 1993). This experimental approach requires the identification of a known three-dimensional (3D) structure (template) related to the target sequences of family 3 glycoside hydrolases and uses the coordinates of the template protein as a basis for further modelling. The template in this case was the barley β-glucan exohydrolase isoenzyme ExoI (Varghese *et al.*, 1999). The second step in the process of modelling is the alignment of the template with the target sequence. This was done with the Bestfit program, using the implemented gap penalty function and the local homology algorithm of Smith and Waterman (Smith and Waterman, 1981) of the University of Wisconsin Genetics Computer Group (GCG) package (Devereux *et al.*, 1984), with all other parameters unchanged from the default settings. During the sequence alignments, no correlations for loops connecting secondary structural elements were introduced. In some instances the Swiss-Pdb Viewer's alignment tool (Guex and Peitsch, 1997) was used and the resultant alignments were found to be similar. In the case of the *Aspergillus nidulans* β-xylosidase (Perez-Gonzales *et al.*, 1998), the alignment with the template sequence was also effected with the ClustalW algorithm (Thompson *et al.*, 1994) (http://www2.ebi.ac.uk/clustalw), using the Blosum matrix. In this particular alignment, the first 50 NH_2-terminal residues of the β-xylosidase were deleted from the sequence; the amino acid sequence identity of the first 50 residues was 14% with respect to the barley enzyme. Finally, 15 protein models, based on the

template-target alignments, were constructed on a Silicon Graphics model O2 computer, running IRIX 6.4.

3.2.4 Evaluation of models

The stereochemical quality of models was evaluated using PROCHECK (Laskowski et al., 1993). Overall G-factors, which are measures of normality of main chain bond lengths and bond angles obtained from the small molecule library of Engh and Huber (1991), were calculated from PROCHECK. The rms (root mean square) deviation values in the C^α positions between barley β-glucan exohydrolase isoenzyme ExoI and the modelled structures of family 3 glycoside hydrolases were determined with the program "O" (Jones et al., 1991).

3.3 Results

3.3.1 Phylogenetic tree

The amino acid sequences of 99 members of the family 3 group of glycoside hydrolases were aligned and an unrooted, radial phylogenetic tree was constructed (Figure 3.1). Amino acid sequence identities between the barley β-glucan exohydrolase isoenzyme ExoI and other family 3 members vary from 19% to 83%, although it must be emphasized that not all sequences are full-length. Approximately 65% of the family members are 20-30% identical with the barley enzyme and about 21% fall in the 30-40% identity range.

For subsequent analysis and modelling, the phylogenetic tree (Figure 3.1) was divided into six major branches of sequences and 14 members were modelled (Table 3.1). The majority of higher plant enzymes are closely related and are located in a cluster on one branch of the tree. Members of this group exhibited amino acid sequence identities of between 57% and 83%, compared with the sequence of barley β-glucan exohydrolase isoenzyme ExoI. Most of the other major branches of the tree included representatives from bacteria, yeast and fungi (Figure 3.1). To obtain good alignments for the bacterial β-glucosidases from *Butyrivibrio fibrisolvens* (Lin *et al.*, 1990) and *Ruminococcus albus* (K. Ohmiya, unpublished results, GenBank/EMBL accession number X15415) it was necessary to change the order of the NH_2-terminal and COOH-terminal domains, because in these two enzymes there is a reversal of the two domains compared with their order in the barley β-glucan exohydrolase isoenzyme Exo1 (see section 3.3.2 below). However, another β-glucosidase from *Ruminococcus albus* has its two domains arranged in the same order as the barley enzyme (Figure 3.1).

3.3.2 Domain structures

The arrangements of protein domains in selected enzymes from the family 3 glycoside hydrolases were further investigated using the ProDom server (Corpet *et al.*, 1998). The enzymes were selected so that each of the major branches on the phylogenetic

Figure 3.1: The sequences were aligned using the PileUp program and the tree was constructed with the EprotPars program of the University of Wisconsin GCG package (Devereux *et al.*, 1984). The 99 sequences (for accession numbers see Experimental Procedures) are subdivided into 6 major branches, or clusters. The representatives that have been modeled are shown in bold typeface. To obtain good alignments for the bacterial β-glucosidases from *Butyrivibrio fibrisolvens* and *Ruminococcus albus*, it was necessary to change the order of the NH_2-terminal and COOH-terminal domains. Lower case letters after accession numbers indicate individual members when multiple entries of a species are listed. All family 3 glycoside hydrolases listed in the databases on June 1, 2000 are included in this Figure, which is available on the University of Adelaide's web site at http://planta.waite.adelaide.edu.au/labs/gbf/modeling.htm.
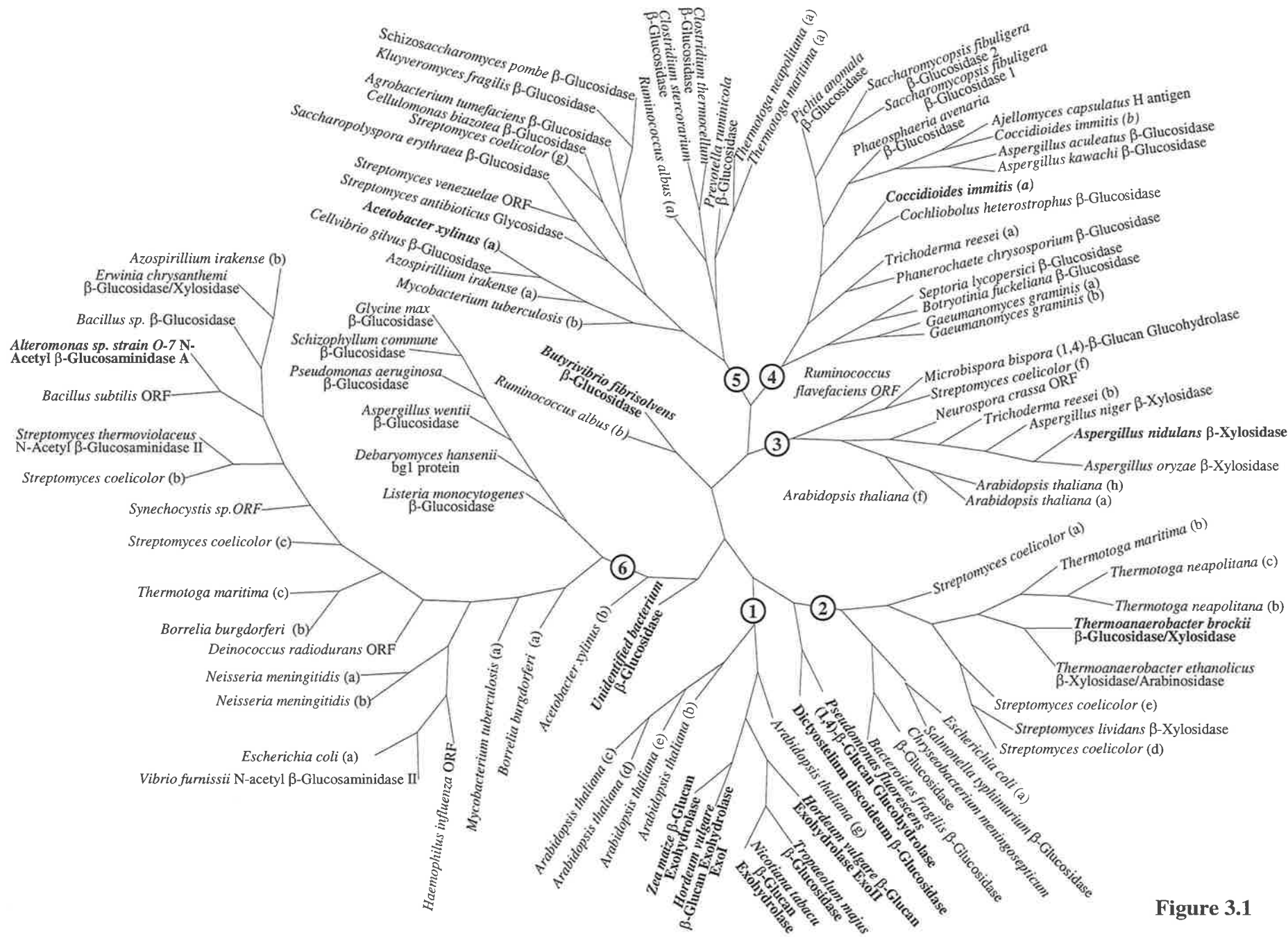
Figure 3.1

Table 3.1 Protein sequence identities and similarities, rms deviation values, overall G-factors and Ramachandran plot statistics of the template barley β-glucan exohydrolase isoenzyme ExoI and target sequences selected from family 3 glycoside hydrolases.

Entry[a]	Identity % Bestfit[b]	Similarity % Bestfit[b]	rms[c] Å (aa)	G-factor PROCHECK[d]	Ramachandran values[e] % Allowed	Disallowed
Barley Exo1	100	100	0	-0.28	99.8	0.2
Barley Exo2	72	82	0.50 (596)	-0.15	99.4	0.6
Maize	83	91	0.25 (598)	-0.04	99.6	0.4
Nasturtium	70	82	0.47 (599)	-0.18	99.4	0.6
Tobacco	71	84	0.53 (601)	-0.08	99.4	0.6
Acetobacter	26	49	1.10 (495)	-0.64	95.8	4.2
Alteromonas	22	48	0.96 (502)	-0.46	96.2	3.8
Aspergillus	29	51	1.02 (527)	-0.56	95.3	4.7
Butyrivibrio $(\alpha/\beta)_8$	25	49	0.20 (278)	-0.52	97.1	2.9
Butyrivibrio $(\alpha/\beta)_6$	22	50	1.39 (166)	-0.74	94.6	5.4
Coccidioides	28	51	1.42 (495)	-0.75	96.4	3.6
Dictyostelium	33	55	0.91 (577)	-0.51	97.6	2.4
Pseudomonas	45	64	0.64 (579)	-0.27	98.6	1.4
Thermoanaerobacter	33	56	0.76 (510)	-0.42	96.1	3.9
Unidentified bacterium	36	60	0.96 (320)	-0.25	98.3	1.7
Barley β-glucosidase (family 1)	19	44	1.83 (317)	-1.05	94.6	5.4

[a] Representatives were chosen from different branches on the unrooted phylogenetic radial tree.

[b] Using Bestfit program of the University of Wisconsin GCG package (Devereux *et al.*, 1984).

[c] Rms deviations of the C^α backbone were determined for the Modeler pdb files using program "O" (Jones *et al.*, 1991). Numbers in brackets (aa) indicate the number of amino acid residues aligned to generate the rms deviations.

[d] Overall G-factors (Engh and Huber, 1991) of models calculated by PROCHECK (Laskowski *et al.* 1993).

[e] Ramachandran plot statistics excluding Gly and Pro residues.

tree was represented and include β-glucosidases from fungal and bacterial sources, β-glucan exohydrolases from bacteria and higher plants, a bacterial N-acetyl β-glucosaminidase, a β-glucosidase from *Dictyostelium discoideum*, and a β-glucosidase from a thermophilic bacterium. Some of the selected enzymes are shown in bold type in Figure 3.1, and possible domain arrangements assigned by the ProDom program are represented diagrammatically in Figure 3.2.

The barley β-glucan exohydrolases are known to have an NH_2-terminal domain (domain 1) that adopts a $(\alpha/\beta)_8$ TIM barrel fold and is connected *via* a 16-amino acid linker to a second domain, which forms an $(\alpha/\beta)_6$ sandwich (domain 2); a 42 amino acid antiparallel loop is located at the COOH-terminus (Varghese *et al.*, 1999). The ProDom program detected the two major domains in all the sequences analysed and indicated that in each enzyme their lengths were similar (Figure 3.2). The $(\alpha/\beta)_8$ barrel domain is usually separated from the $(\alpha/\beta)_6$ sandwich domain by a 'linker' region of variable length and other possible domains of unknown structure are also predicted by the program. As mentioned above, in the bacterial β-glucosidases from *Butyrivibrio* and *Ruminococcus* domains 1 and 2 are reversed in order, compared with their arrangements in the other enzymes (Figure 3.2). Because the ProDom program is based on sequence alignments that indicate likely domain boundaries, rather than on 3D structural information, the data presented in Figure 3.2 must be interpreted with some caution. Nevertheless, the program does predict that almost all the family 3 glycoside hydrolases examined have the two major protein domains, that both of these are similar in length to the equivalent domains in the barley enzyme, and that in two bacterial enzymes domain 2 precedes domain 1 (Figure 3.2). In some instances, such as the *Alteromonas* N-acetyl β-glucosaminidase, the program could not detect the presence of domain 2 in the sequence. This could be related to the absence of a clear signature that is required by the program to delineate the domain boundary, rather than the absence of blocks of amino acid residues equivalent to those in domain 2 of the barley β-glucan exohydrolase (see Figure 3.5).

Figure 3.2: Schematic display of domain arrangements of selected members of family 3 glycoside hydrolases. Entries available through the SWISS-PROT database were analysed with the automated ProDom server using the BLAST, MKDOM and MultAlin programs (Corpet *et al.*, 1998). Note that 2 members of the group (*Butyrivibrio* and *Ruminococcus*) show reverse orientation of domains 1 and 2, in comparison with the barley β-glucan exohydrolase isoenzyme ExoII. The amino acid sequence of barley isoenzyme ExoII has 73% identity with that of barley β-glucan exohydrolase isoenzyme ExoI.

Barley ExoII

Acetobacter

Alteromonas

Aspergillus

Coccidioides

Dictyostelium

Pseudomonas

Thermoanaerobacter

Butyrivibrio

Ruminococcus

Domain 1 - $(\alpha/\beta)_8$ barrel

Putative linker region

Domain 2 - $(\alpha/\beta)_6$ sandwich

Other ProDom domains (unknown structures)

100 a.a.
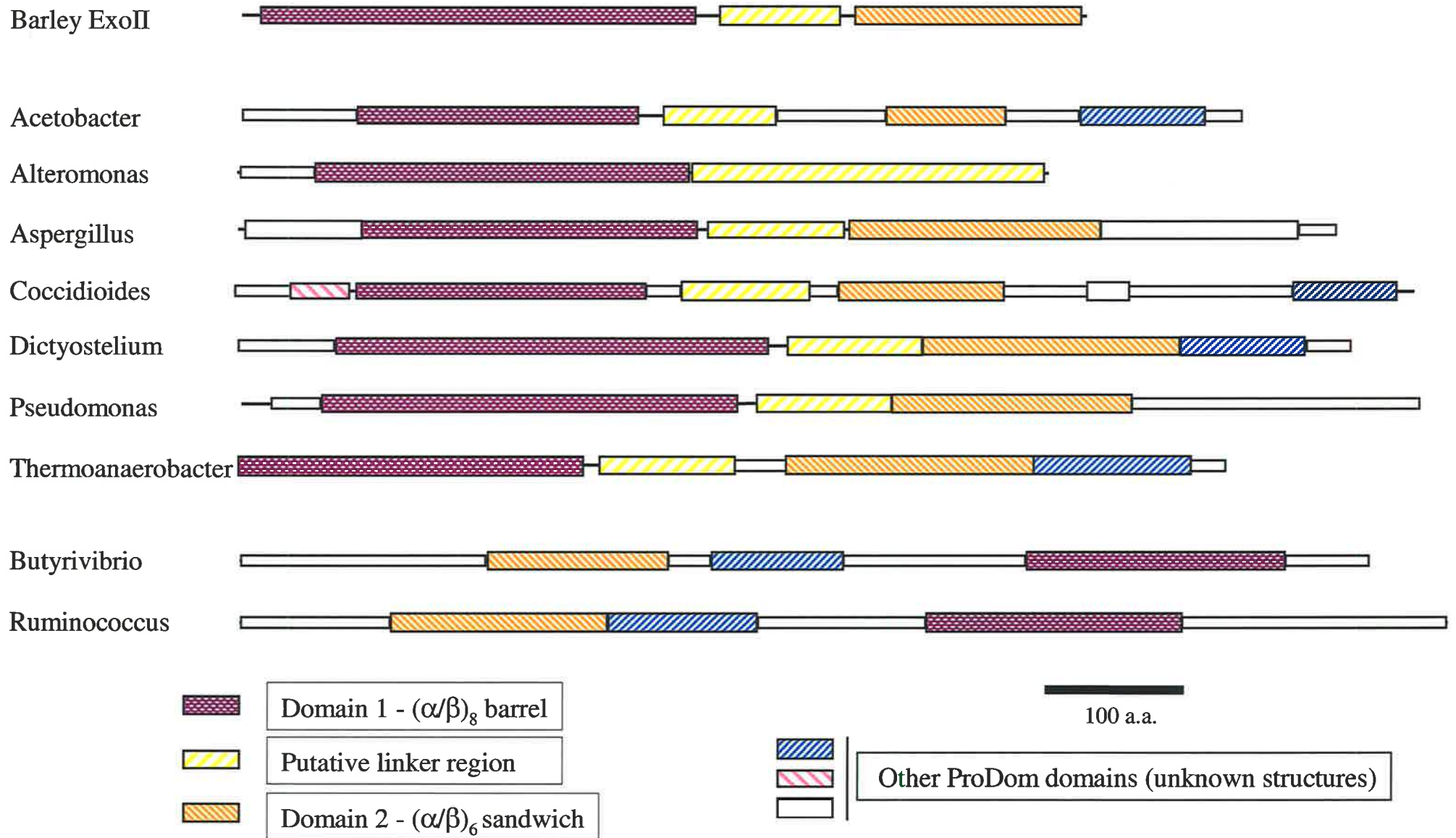
Figure 3.2

3.3.3 Protein modelling

A total of 14 models were built from the amino acid sequences of family 3 glycoside hydrolases, using the Modeller program (Sali and Blundell, 1993). With the exception of some *Arabidopsis* members, the known plant enzymes were modelled, together with the selection of fungal and bacterial enzymes that was indicated in Figure 3.1 and that was analysed for domain arrangement (Figure 3.2).

The amino acid sequence similarities of the selected enzymes, the rms deviation values for modelled C^{α} chains, the PROCHECK program G-factors, which measure how reliable the models are, and the percentage of 'allowed' amino acid residues from Ramachandran plots (Laskowski *et al.*, 1993) are compared in Table 3.1. Amino acid sequence identities of the modelled enzymes, compared with barley β-glucan exohydrolase isoenzyme ExoI, range from about 20% to more than 80% (Table 3.1). The plant enzymes share the highest degree of amino acid sequence identity, at 72-83% identity, but the amino acid sequences of the other selected enzymes are only 22% to 45% identical when aligned with the barley enzyme (Table 3.1). The C^{α} backbones of both barley β-glucan exohydrolase isoenzymes are compared in Figure 3.3.

The rms deviations in C^{α} positions between the modelled enzymes and the barley β-glucan exohydrolase template range from 0.25 Å to more than 1.4 Å, over 500-600 amino acid residues (Table 3.1). These values may be compared with an rms deviation of 0.65 Å in C^{α} positions for two very closely-related family 17 β-glucan endohydrolases from barley (Varghese *et al.*, 1994). The predicted 3D structure of the maize β-glucan exohydrolase is more similar to barley β-glucan exohydrolase isoenzyme ExoI than is the other barley enzyme, isoenzyme ExoII (Table 3.1). A model of the negative control enzyme, the family 1 β-glucosidase from barley (Hrmova *et al.*, 1998a), shows that the sequence of this β-glucosidase can be 'forced' onto the β-glucan exohydrolase structure, but that the rms deviation is more than 1.8 Å (Table 3.1).

The values for rms deviations in C^{α} positions between the models and the template β-glucan exohydrolase isoenzyme ExoI are also reflected in the overall G-factors and

Figure 3.3 Superimposition of modelled barley β-glucan exohydrolase isoenzyme ExoII (green) onto the structure of barley β-glucan exohydrolase isoenzyme ExoI (yellow). The structure of β-glucan exohydrolase isoenzyme ExoI was determined experimentally by X-ray crystallography. The two structure are almost perfectly aligned with only minor local variation due mainly to sequence insertions and deletions.

The values for rms deviations in C^{α} positions between the models and the template β-glucan exohydrolase isoenzyme ExoI are also reflected in the overall G-factors and Ramachandran values (Table 3.1). The G-factors are a measure of model reliability, in which more positive values generally indicate better reliability; values of less than -1.0 indicate an unreliable model (Engh and Huber, 1991; Jones *et al.,* 1991). Similarly, Ramachandran plot statistics which place 95-97% or more of the amino acid residues in 'allowed' positions are considered reliable in modelling experiments, and this indicates how well the structures fit the expected main chain length and torsion angle distribution (Ramachandran *et al.,* 1963; Morris *et al.,* 1992; Laskowski *et al.,* 1993; Kleywegt and Jones, 1996). Higher numbers of residues in the disallowed region reflect a distorted geometry or steric clashes in the models, because there are higher proportions of residues falling outside the limits of main chain bond length and torsion angles of the small molecule library (Engh and Huber, 1991).

Table 3.1 shows that correlations between sequence similarity, rms deviation of C^{α} chains, G-factors or Ramachandran values could be detected, and some general trends were evident (Table 3.1). The higher plant enzyme models were the most reliable and it can be concluded that all of these modelled enzymes are likely to adopt very similar protein folds (Table 3.1). This is consistent with their position on a single branch of the phylogenetic tree (Figure 3.1). However, some of the *Arabidopsis* entries, specifically those designated "β-xylosidase-like", are positioned on other branches of the phylogenetic tree (Figure 3.1). Enzymes that are relatively close to the higher plant enzymes on the phylogenetic tree, in particular the β-glucosidases from *Dictyostelium* (Bush *et al.,* 1994), *Thermoanaerobacter* (Breves *et al.,* 1997) and an unidentified bacterium (Healy *et al.,* 1995), and the (1→4)-β-glucan glucohydrolase from *Pseudomonas* (Rixon *et al.,* 1992), also appear to be most similar in 3D structure with the barley β-glucan exohydrolase (Table 3.1, Figure 3.1). Conversely, the *Alteromonas* N-acetyl β-glucosaminidase (Tsujibo *et al.,* 1994) and the *Coccidioides* β-glucosidase (JJ Yu, PW Thomas, K Seshan, GT Cole; unpublished results in GenBank/EMBL entry U87805) are located relatively further from the barley enzyme on the phylogenetic tree (Figure 3.1) and their 3D structures are less similar to the barley

enzyme, as indicated by the lower overall G-factors and Ramachandran plot values (Table 3.1).

Despite these differences, it remains likely that the family 3 glycoside hydrolases consist of an $(\alpha/\beta)_8$ TIM barrel domain and an $(\alpha/\beta)_6$ sandwich domain.

To illustrate the range of molecular conformations in the family 3 group, the barley β-glucan exohydrolase isoenzyme ExoI structure is compared with models of the closely-related maize β-glucan exohydrolase and the more distantly related N-acetyl β-glucosaminidase from the marine bacterium *Alteromonas* (Tsujibo *et al.,* 1994) in Figure 3.4. For comparative purposes, the amino acid sequence alignments of the same three enzymes are shown in Figure 3.5, together with the secondary structural elements of the barley β-glucan exohydrolase isoenzyme ExoI.

3.3.4 Inserted and deleted blocks of amino acids

The most noteworthy differences between the plant and the bacterial enzymes are the insertions and deletions of blocks of up to 13 amino acid residues, and the observation that about half of the COOH-terminal loop is missing in the *Alteromonas* enzyme (Figure 3.5). The inserted or deleted groups of amino acid residues are generally located in surface loop regions of the protein fold, or near the ends of α-helices (Figure 3.5). Furthermore, there appear to be more surface insertions and deletions in the first 300-350 amino acid residues, which constitute domain 1 of the enzymes (Figures 3.4 and 3.5). Similar effects are observed when the model of the $(1 \rightarrow 4)$-β-glucan glucohydrolase from *Pseudomonas* (Rixon *et al.,* 1992) is compared with the 3D structure of the barley β-glucan exohydrolase (Figure 3.6), where insertions are shown in red and the positions of deletions are indicated in blue.

Figure 3.4: Stereoview ribbon representation of barley β-glucan exohydrolase isoenzyme ExoI and modeled structures of *Zea mays* β-glucan exohydrolase and *Alteromonas sp.* N-acetyl β-D-glucosaminidase A. Domain 1, domain 2 and the linker of each enzyme are coloured in magenta, cyan and yellow, respectively. The catalytic nucleophiles and catalytic acids are shown in red and blue, respectively. No other catalytic acid is required in the *Alteromonas* enzyme. The Figure was generated with MOLSCRIPT (Kraulis, 1991).

Hordeum ExoI

Zea mays

Alteromonas sp.
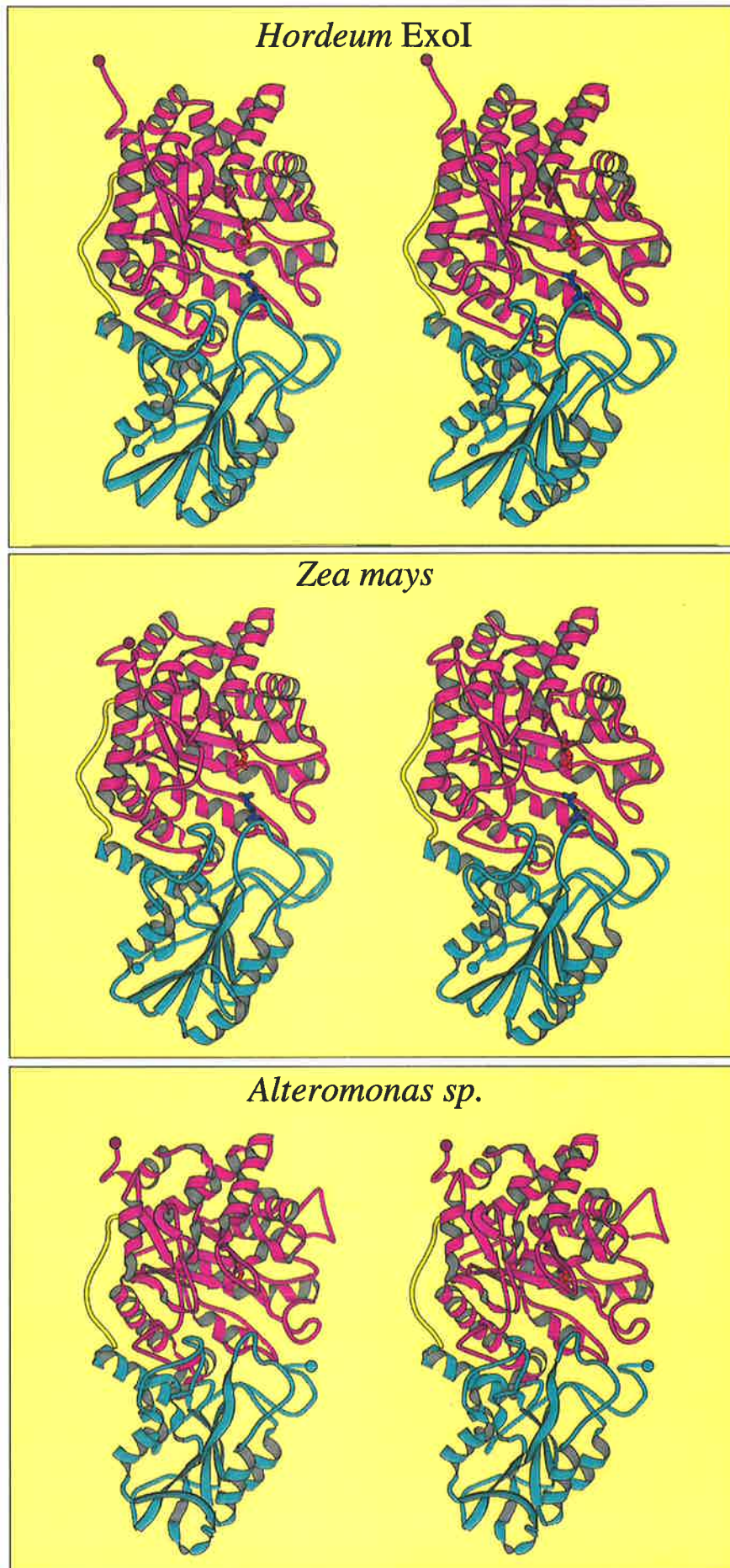
Figure 3.4

Figure 3.5: Amino acid sequence alignments of family 3 glycoside hydrolases. The amino acid sequence of barley β-glucan exohydrolase isoenzyme ExoI (AJ Harvey, M Hrmova and GB Fincher, unpublished data, GenBank/EMBL accession number AF102868) is aligned with related enzymes from maize (YY Zhao and L Bogorad 1999, unpublished results, GenBank accession number AF064707) and *Alteromonas sp.* (Tsujibo *et al.*, 1994). The sequences were aligned using the PileUp program. Green areas highlight identical residues and yellow areas represent regions of high conservation. Open arrowheads point to the catalytic residues and black arrowheads to the *N*-glycosylation sites. Numbers below the sequences indicate amino acid residues of the mature barley β-glucan exohydrolase isoenzyme ExoI. Secondary structural elements of domain 1 of β-glucan exohydrolase isoenzyme ExoI are shown as purple cylinders (α-helices) and purple horizontal arrows (β-strands), connected by purple lines (coils). Cyan cylinders (α-helices) and cyan horizontal arrows (β-strands), connected by cyan lines (coils), indicate secondary structural elements of the domain 2 $(\alpha/\beta)_6$ sandwich. Yellow and red lines correspond to the linker and the COOH-terminal antiparallel loop, respectively. The Figure was prepared using ALSCRIPT (Barton, 1993).

Protein sequence alignment of Barley ExoI, Maize ExoII, and Alteromonas.

```
               1                                                          60
Barley ExoI    D Y V L Y K D A I K P V E D R V A D L L G R M T L A E K I G Q M T Q I E R L V A T P D V L R D N F I G S L L S G G G S V
Maize ExoII    V P P P Y Y Q D A S K D V E V R V R D L L A R M T L A E K V G Q M T Q I E R I V A S P Q A L R D Y Y I G S L L S G G G S V F
Alteromonas    . . . . Y Y C G E S K K P S G D C R A A M T . . T L P P E L S E L . . I S R Y D I G G A I L . . . . . . . . . . . . . . F
                                10              20            30              40            50              60
                          A-2              A-1       a                A              b
```

```
              61                                                         120
Barley         P R K G A T A K E W Q D M V D G F Q K A C M S T R L G I P M I Y G I D A V H G . . . Q N N V Y G A T I F P H N V G L G A T . . . R D
Maize          P R K Q A T A A E W V A M V S D F Q K A C L S T R L G I P M I Y G I D A V H G . . . H N N V Y G A T I F P H N V G L G A T . . . R D
Alteromonas    A E N V Q N T A Q I I S L T N A L Q S A A Q Q S K S Q L P L F I A I D Q E G G R V A R I N R E Q A T S F T G N M S I G A T Y P K Q G
                              70            80            90              100             110              120
                          B                  c                      C1
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             121                                                         180
Barley         P Y L V K R I G E A T A L E V R A T G I Q Y A F A P C I A V C R D P R W G R C . Y E S Y S E D R R I V Q S M T E L I P G L Q G D V P
Maize          P N L V K R I G A A T A L E V R A T G I Q Y A F A P C I A V C R D P R W G R C . Y E S Y S E D H R I V Q A M T E L I P G L Q G D V P
Alteromonas    D I Y A T K V A S A A G K E L N S L G I N V N F A P T V D V N S N P N N P V I N V R S F S E N P T V V T K L L . . . . . . . . . . . .
                              130             140           150              160             170              180
                          C2                 d                            D
```

```
             181                                                         240
Barley         K D F T S G M P F V A G K N K V A A C A K H F V G D G G T V D G . . . . . . . N E N N T I I N R E G L M N I H M P A Y K N A M D K
Maize          Q N F T S G M P F A A G K D K V A A C A K H F V G D G G T Q N G . . . . . . . N E N N T I I D R Q G L L S I H M P A Y L D A L R K
Alteromonas    . G L A Q V K A F E E A . . G V L S A L K H F P G H G D T H V D S H T G L P R V D H D R D K I N Q Q D L L P F . . A E I I K A S P P
                              190             200           210              220             230              240
                                        e                      ▲CHO            E1                E2
```

```
             241                                                         290
Barley         G . V S T V M I S V S S W N G V K M H A N Q . . . . . . . . . . . . D L V T G Y L K D T L K F K G F V I S D W E G I D R I T T P A G
Maize          G . F S T V M I S V S S W N G L K M H A N H . . . . . . . . . . . . N L I T G F L K D R L N F Q G F T I S D W E G I D R V T S P P G
Alteromonas    G M I M T A H I Q Y P A L D N S K V V N S Q G E S M I R P A T M S Y Q I M T Q L L R H E L G Y Q G G V T V T D A L D M A G I . . . . .
                              250             260                          270             280              290
                          f                                            F                g      △Catalytic    G1
```

```
             291                                                         350
Barley         S D Y S Y S V K A S I L . . . A G L D M I M V P . . . . . . . . . N K Y Q Q F I S I L T G H V N G G V I P M S R I D D A V T R I L R
Maize          A N Y S Y S V Q A S I L . . . A G L D M I M V P . . . . . . . . . N N Y Q N F I T I L T G H V N S G L I P M S R I D D A V T R I L R
Alteromonas    S D F Y N V Y D A T I E T F N A A G V D I A L M P I A I R N R A D I K R F E E Q Y M A Q L A D A L E T N K L N Q E Q L S S S M A R I A K
                              300                 310                      320             330              340             350
                          G2                  h                            H1                            H2
```

```
             351                                                         410
Barley         V K F T M G L F E N P Y A D P A M A E Q L G K Q E H R D L A R E A A R K S L V L L K N G K T S T D A P L L P L P K K A P K I . L V A
Maize          V K F T M G L F E N P M P D P S L A D Q L G K Q E H R D L A R E A V R K S L V L L K N G K P G . D A P L L P L P K K A A R I . L V A
Alteromonas    L K T K L P Q S S A S L A I A N . . S T L G N P S H R R L E A E L A L A A I T E V K N D . . . . G V L P L R D N A Q V H L I M
                              360             370           380              390             400              410
                          I                                                i                                  j
```

```
             411                                                         480
Barley         G S H A D N L G Y Q C G G W T I E W Q G D T G R T T V G T T I L E A V K A A V D P S T V V V F A E N P D A E F V K S G G F S Y A I V
Maize          G S H A D N L G Y Q C G G W T I E W Q G D T G R T T V G T T V L D A V K A A V D P S T E V V F A E S P D A E F V R S G G F S Y A I V
Alteromonas    P D R Q K C F A L E Q A L Q T Y S K N S . . . L T L S C T S L Q A Y D P D I A H D A I . . . . . . K Q A D M I I A A H A S P P Q S
                              420             430           440              450             460              470             480
                          J1                                            J2                k                  K                  l
```

```
             481                                                         540
Barley         A V G E H P Y T E T K . . . G D N L N L T I P E P G L S T V Q A V C G G V R C A T V L I S G R . P V V V Q P L L A A S D A L V A A W
Maize          A V G E H P Y T E T K . . . G D S M N L T I P D P G P S T V Q T V C A A V R C V T V L I S G R . P V V I Q P L F G A M D A V V A A W
Alteromonas    A V E I G G M D D V K K L R E H G V A R N V Q P A A L K A L L Q Y G Q Q Q G K K Q L F I S L R A P Y E I S T F G P L S N A V L A S Y
                              490                 500           510          520              530              540
                          △Catalytic                   ▲CHO         L                m              M                n
```

```
             541                                                         600
Barley         L P G S E G Q G V T D A L F G D F G F T G R L P R T W F K S V D Q L P M N V G D A H Y D P L F R L G Y G L T T N A T K K Y . .
Maize          L P G T E G Q G V T D V L F G D F G F T G K L P R T W F R S V D Q L P M N Y G D A H Y D P L F P L G F G L T T Q G . K M Y Q N
Alteromonas    A Y N V D V N H D K K V A G P A Y T A L A K V I L G I A K A E G S L P V T V N H . . . . . . . . . . . . . . . . . . . . . . . .
                              550             560           570              580             590              600
                          N
```

Figure 3.6: Stereoview ribbon representation of modelled structure of *Pseudomonas fluorescens* (1→4)-β-glucan glucohydrolase, showing positions of amino acid deletions and insertions. The *Pseudomonas* enzyme structure (Rixon *et al.*, 1992) was modelled using the barley β-glucan exohydrolase isoenzyme ExoI as a template. Domains 1 and 2 are coloured in white and grey, respectively. Residues that are inserted in the *Pseudomonas* enzyme but are absent from the barley enzyme are coloured red, and amino acid residues flanking deletions are in blue. Deletions and insertions are generally located on surface loops. The Figure was generated using the MOLSCRIPT program (Kraulis, P., 1991).

3.3.5 Domain reversal in two bacterial enzymes

As noted in Figure 3.2, the two domains in the β-glucosidases from *Butyrivibrio* and *Ruminococcus* are arranged in the reverse sequence, compared with all the other members of the family. Thus, the $(\alpha/\beta)_6$ sandwich domain precedes the $(\alpha/\beta)_8$ barrel in these two enzymes. Although the reliability of the $(\alpha/\beta)_6$ sandwich domain of the *Butyrivibrio* enzyme model is relatively poor, the other domain models well (Table 3.1). It was possible to generate a composite model, in which partial models of the two domains could be arranged so that the overall shape of the enzyme was similar to that of the barley β-glucan exohydrolase (Figure 3.7). It is not clear exactly how the two domains are connected, but there are 96 amino acid residues between the COOH-terminal end of the $(\alpha/\beta)_6$ sandwich domain and the NH$_2$-terminus of the $(\alpha/\beta)_8$ barrel domain; this would be easily long enough to connect the two domains or even to insert a new subdomain into the vacant gap (Figure 3.7). However, the absence of an appropriate template that corresponded to this 96-residue sequence precluded further modelling of regions between the two domains.

3.3.6 Catalytic amino acids

Careful examination of the 3D structure (Varghese *et al.*, 1999), together with studies of the effects of active site-specific inhibitors (M Hrmova, JN Varghese, H Driguez and GB Fincher, unpublished), indicate that the catalytic nucleophile and the catalytic acid of barley β-glucan exohydrolase isoenzyme ExoI are D285 and E491, respectively. Sequence alignments indicated that the position of the catalytic nucleophile (D285 or its equivalent) is absolutely conserved in all family 3 glycoside hydrolases. In the plant enzymes the catalytic nucleophile is located in a highly conserved GFVIS<u>D</u>W sequence, and is surrounded by a landscape of amino acid residues with similar characteristics in other family 3 enzymes (Table 3.2). Molecular modelling and HCA procedures indicate that the catalytic nucleophile is always found near the COOH-terminus of β-strand 'g' in domain 1 (Figure 3.5), and that this β-strand is always positioned in the shallow active site pocket that is located at the interface of the two domains (Varghese *et al.*, 1999).

Figure 3.7: Ribbon representation of barley β-glucan exohydrolase isoenzyme ExoI (left), and domains 1 and domains 2 of the *Butyrivibrio fibrisolvens* β-glucosidase (right). Domain 1, domain 2 and the linker region of each enzyme are coloured in magenta, cyan and yellow, respectively, and the domain arrangement of the two enzymes are compared below the structures, using the same colour code. The $(\alpha/\beta)_8$ barrel and $(\alpha/\beta)_6$ sandwich of the *Butyrivibrio* enzyme represent partial folds. The catalytic nucleophiles (D285 in the barley enzyme and D769 in the *Butyrivibrio* enzyme) (Varghese *et al.*, 1999; Lin *et al.*, 1990) and catalytic acid (E491 in the barley enzyme) are shown in red and blue, respectively. The dotted yellow line represents the linker between the NH_2-terminus of the $(\alpha/\beta)_8$ barrel and COOH-terminus of the $(\alpha/\beta)_6$ sandwich of the composite *Butyrivibrio* model. The Figure was generated with MOLSCRIPT (Kraulis, 1991).

Barley β-Glucan Exohydrolase

Butyrivibrio fibrisolvens β-Glucosidase

Domain 1
$(\alpha/\beta)_8$ barrel

$(\alpha/\beta)_8$ barrel

Linker

Linker

Domain 2
$(\alpha/\beta)_6$ sandwich

$(\alpha/\beta)_6$ sandwich

$(\alpha/\beta)_8$ barrel   linker $(\alpha/\beta)_6$ sandwich

Barley

Butyrivibrio

$(\alpha/\beta)_6$ sandwich

$(\alpha/\beta)_8$ barrel
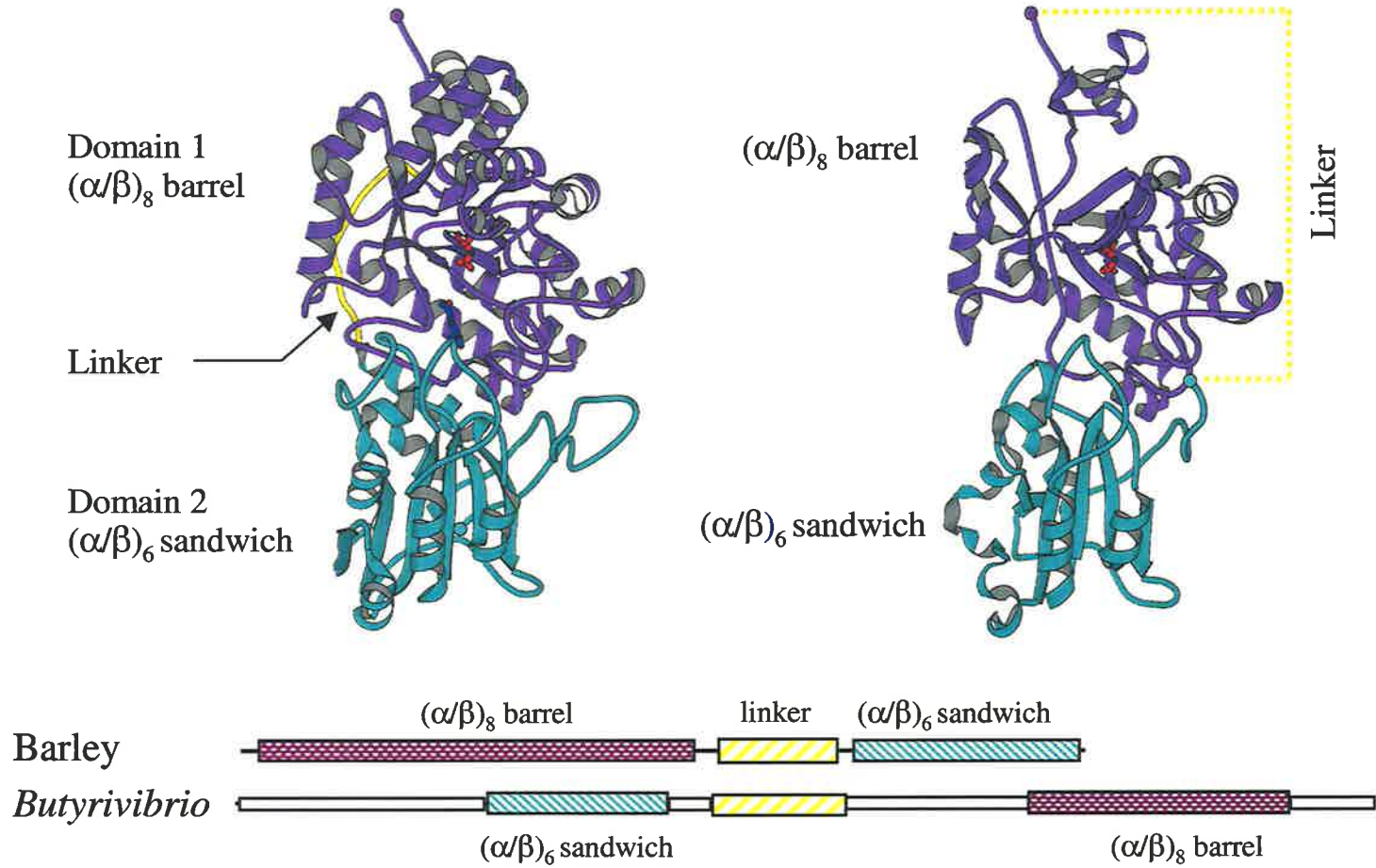
Figure 3.7

Table 3.2 A comparison of conserved sequence motifs of family 3 glycoside hydrolases.

Entry[a]	205-209[b]	280-286[c]	429-435[d]	488-492[e]	589-598[f]
Barley Exo1	AKHFV	GFVISDW	GWTIEWQ	PYTET	PLFRLGYGLT
Barley Exo2	AKHYV	GFVISDW	GWTITWQ	PYAET	PLFPFGFGLT
Maize	AKHFV	GFTISDW	GWTIEWQ	PYTET	PLFRLGYGLT
Nasturtium	AKHFV	GFVISDW	GWTIEWQ	PYAEM	PLFPFGFGLT
Tobacco	AKHFV	GFVISDW	GWTIEWQ	PYAEM	PLFPFGFGIT
Acetobacter	LKHYA	GFVMSDW			PLYPFGYGLT
Alteromonas	LKHFP	GVTVTDA			
Aspergillus	AKHYA	GYVSGDC			PVYEFGHGLF
Butyrivibrio $(\alpha/\beta)_8$	PKHFA	GFVVTDY			
Butyrivibrio $(\alpha/\beta)_6$					YPFGYGLS
Coccidioides	AKHLV	GFVMTDW			PRYHFGYGLS
Dictyostelium	AKHYF	GVAVTDW	GWSVHWQ	PEAET	PLFQFGDGLS
Pseudomonas	AKHFI	GLVVGDW	GWSVSWQ	PYAEM	PLFPYGYGLS
Thermoanaerobacter	GKHFV	GIVVSDY			PLYPFGYGLS
Unidentified bacterium	AKHYI	GFVVTDW			

[a] Representatives were chosen from different branches on the unrooted phylogenetic radial tree. Identical residues are enclosed in boxes.

[b] Conserved sequence containing a putative carbohydrate-binding site (Varghese _et al._, 1999).

[c] Conserved sequence containing the catalytic nucleophile (Varghese _et al._, 1999) (▼).

[d] Conserved sequence joining helices J1 and J2 of $(\alpha/\beta)_6$ sandwich (portion of a Rossmann-like fold; Varghese _et al._, 1999).

[e] Conserved sequence containing the catalytic acid (Varghese _et al._, 1999) (◆).

[f] Conserved COOH-terminal antiparallel loop sequence (Varghese _et al._, 1999).

The equivalent amino acid residue to the putative catalytic acid of the barley enzyme, E491, is highly conserved in the plant enzymes but is sometimes difficult to detect in more distantly related members of the family (Table 3.2). The catalytic acid in barley is positioned on the $(\alpha/\beta)_6$ sandwich domain in a loop region that varies across the family (Figures 3.4 and 3.7; Table 3.2). A potentially equivalent acidic amino acid residue is often detected on that loop of domain 2, but models suggest that it is located further from the catalytic nucleophile as the particular enzyme diverges from the barley enzyme. Another example is the cell-wall associated β-xylosidase from *Aspergillus nidulans* (Perez-Gonzales *et al.,* 1998). This enzyme was chosen to show that distantly related (see Figure 3.1) enzymes with different substrate specificities still retain the same catalytic nucleophile. The C^{α} chain of the *Aspergillus* enzyme is superimposed on the C^{α} chain of the barley enzyme in Figure 3.8A, the equivalent amino acid, E475, is positioned on the loop that joins β-strand 'I' and α-helix 'L', but is predicted to be 30 Å from the catalytic nucleophile, D273. There are two other acidic residues on this loop of domain 2 in the *Aspergillus* β-xylosidase (D478, E480). The closest to the catalytic nucleophile is D478, but this is still 15 Å away from the nucleophile (Figure 3.8B). A more likely catalytic acid in the *Aspergillus* enzyme is E196, which is located on domain 1 about 6.5 Å from the catalytic nucleophile D273. The E196 residue is similar in both sequence and 3D position to E220 of the barley enzyme; this residue is highly conserved in the plant enzymes (Figures 3.4 and 3.7). As shown in Figure 3.8B, the distance between D285 and E220 in the barley enzyme is 6.4 Å.

Figure 3.8: Stereoview of superimposed ribbon representations of loops positioned in the catalytic region of barley β-glucan exohydrolase isoenzyme ExoI and the modeled structure of *Aspergillus nidulans* β-xylosidase. Overall, the coils and labels showing the particular amino acid residues are shown in cyan for the *Aspergillus* enzyme (Perez-Gonzales *et al.*, 1998) and, for the barley enzyme, coils are shown in white and labels are in black. The sidechains of catalytic nucleophiles (D285 in barley and D273 in *Aspergillus*) are on domain 1 and are shown in red; they are located in similar positions in the 3D structures of the two enzymes. The sidechain of the putative catalytic acid of the barley enzyme (E491) is on domain 2 and is in blue. The sidechain of the putative catalytic acid of the barley enzyme (E491) is 6.4 Å from the D285 nucleophile. In the *Aspergillus* enzyme, the amino acid residue that corresponds to the catalytic acid on domain 2 of the barley enzyme is D478 and is shown in CPK colours. This residue is about 15 Å from the catalytic nucleophile D273; this distance is larger than usual for retaining glycoside hydrolases and, as a result, D478 might not be the actual catalytic acid. A more likely catalytic acid residue in the *Aspergillus* enzyme is E196 from domain 1, which is coloured blue. The corresponding amino acid residue on domain 1 of the barley enzyme is E220, shown in orange. The distance between D273 and E196 is around 6.5 Å. Glucose (Glc) trapped in the active site of the barley enzyme is shown in CPK colours. The Figure was generated with MOLSCRIPT (Kraulis, P., 1991).

Hordeum ExoI / *Aspergillus*
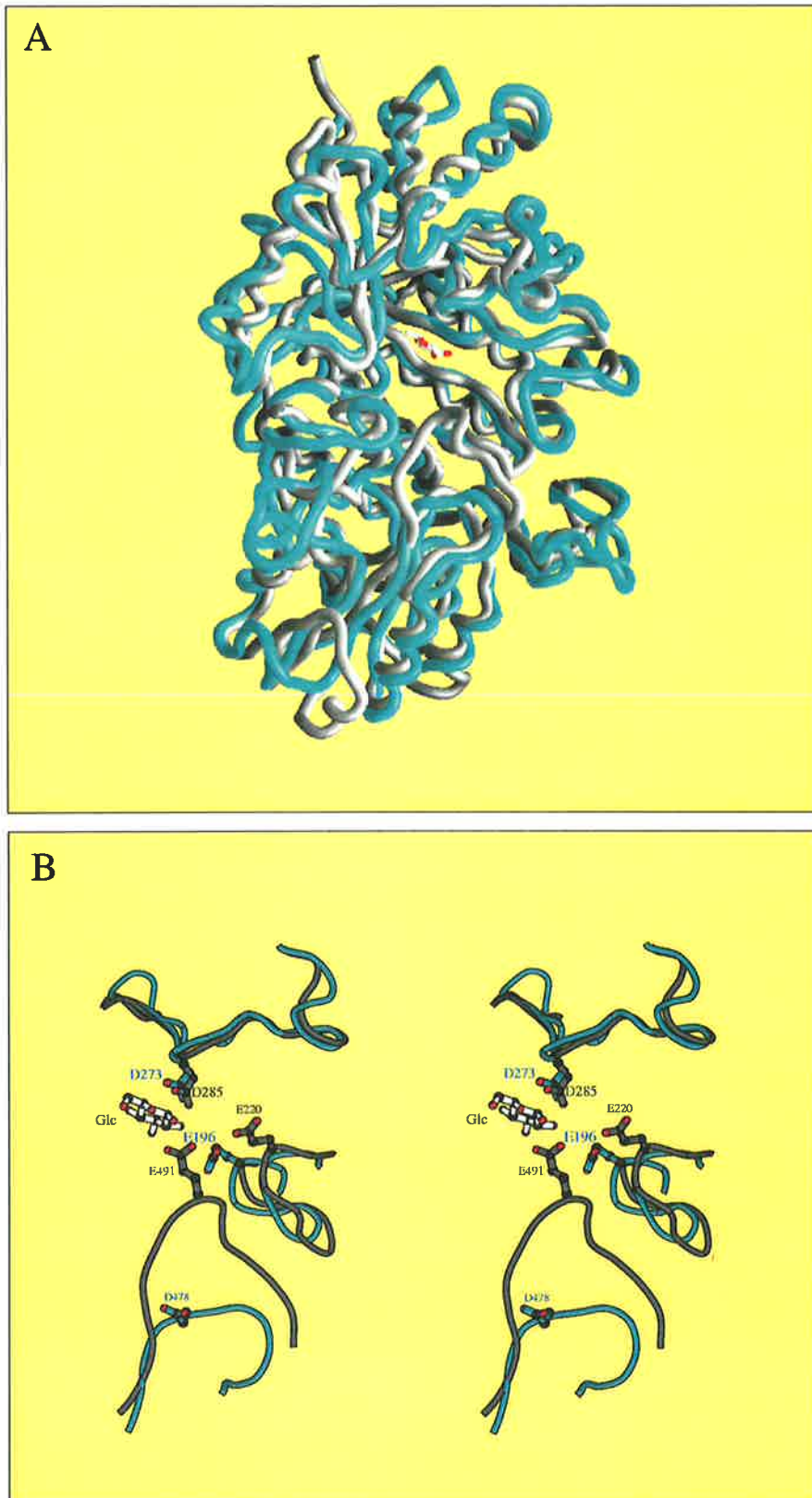
Figure 3.8

3.4 Discussion

The three-dimensional structure of a broad specificity β-glucan exohydrolase from barley (Varghese *et al.,* 1999) has been used to examine the structures of family 3 glycoside hydrolases, which include enzymes from higher plants, *Dictyostelium*, fungi and bacteria (Figure 3.1). Although amino acid sequence similarities between family members are often less than 30% (Table 3.1), it is possible through molecular modelling to show that most of the 64 family members consist of an NH_2-terminal domain that adopts a $(\alpha/\beta)_8$ barrel fold (Figures 3.4, 3.6 and 3.7). The NH_2-terminal domain is attached to the second domain *via* a linker region which is 14-41 amino acids in length. Domain 2 adopts an $(\alpha/\beta)_6$ sandwich conformation (Figures 3.4, 3.6 and 3.7), and is followed by a long antiparallel loop at the COOH-terminus of the enzymes, which is also conserved across most of the family (Table 3.2). In two bacterial enzymes, from *Butyrivibrio fibrisolvens* and *Ruminococcus albus*, in which the order of the two domains is reversed, it is still possible to build models that are similar in fold to the barley β-glucan exohydrolase (Figure 3.7). Heterologous expression of the *Butyrivibrio* gene (*bglA*) enzyme in *E. coli* confirmed that the domains were indeed reversed and that the expressed enzyme was catalytically active (Lin *et al.,* 1990). The reversal of domain sequence suggests that recombination or other shuffling machinery may have caused 'domain' rearrangements in the genes that encode the two enzymes (Stemmer, 1994). It is noteworthy that β-glucosidases from *Ruminococcus albus* can have either the 'normal' or 'reversed' arrangement of domains 1 and 2 (Figure 3.1).

The modelling programs also allowed the relative dispositions of the catalytic nucleophiles and catalytic acids of the enzymes to be examined. The crystal structure of barley β-glucan exohydrolase isoenzyme ExoI indicates that the catalytic nucleophile, Asp285, and the catalytic acid, Glu491, are situated approximately 6.5 Å apart, and each are 3.0 to 4.5 Å from the anomeric C(1) atom of the bound glucose (Varghese *et al.,* 1999). These distances are typical of those found in other retaining glycoside hydrolases (McCarter and Withers, 1994; Davies and Henrissat, 1995), and are similar in all the higher

plant enzymes in family 3. Varghese *et al.* (1999) noted that the catalytic nucleophile and the catalytic acid of the barley enzyme are located on different domains, and suggested that the linker region between the two domains could act as a molecular hinge that would enable the second domain, which carries the catalytic acid, to move away from, or closer to, the active site on domain 1; this could regulate enzyme activity.

While the catalytic nucleophile is absolutely conserved across the family 3 glycoside hydrolases, it has become apparent here that the catalytic acid is not highly conserved, especially in the distantly related members of the family (Table 3.2). Nevertheless, there appear to be other acidic amino acids in the active site region, such as Glu196 on domain 1 of the *Aspergillus* β-xylosidase (Figure 3.8), and these might be recruited to act as catalytic acids in some members of the family. Furthermore, it is possible that the distantly related *N*-acetyl β-glucosaminidases in family 3 require only one acidic catalytic residue in the active site. This is found in bacterial *N*-acetyl β-glucosaminidases belonging to family 20, where the *N*-acetoamido group on C-2 of the *N*-acetyl β-D-glucosaminyl residue of the substrate itself acts as the catalytic nucleophile (Tews *et al.*, 1996; Drouillard *et al.*, 1997).

The active site of the barley β-glucan exohydrolase consists of a shallow pocket that has the appearance of a coin slot and is located near the interface of domains 1 and 2 (Varghese *et al.*, 1999). The pocket is about 13 Å deep and would accommodate only two glycosyl residues of bound substrate; the remainder of the substrate would project away from the surface of the enzyme. The active site geometry can be reconciled with the broad substrate specificity of the barley β-glucan exohydrolases (Hrmova and Fincher, 1998), because the shallow active site pocket would allow the enzyme to bind to the non-reducing termini of a wide range of substrates and the overall shape of the substrate would not be a key determinant for the formation of the enzyme-substrate complex. Accordingly, substrates with a range of linkage types might be accommodated in the active site and this, in turn, would explain the broad substrate specificity of the enzyme (Hrmova and Fincher, 1998). The interactions between substrate and enzyme of this active site pocket must also be responsible for the higher activity of the barley β-glucan exohydrolases towards (1→3)- and (1→3,1→4)-β-glucans than towards 4-NPG. Further studies are currently be carried

out to ascertain which amino acid residues are involved and how the interactions are mediated.

On the basis of the geometry of the active site pocket of the barley β-glucan exohydrolase, one might predict that many of the other family 3 glycoside hydrolases will also have broad substrate specificities. Relatively few of the enzymes have been purified and characterized in detail. Many are simply deduced from open reading frames in cloned genes or cDNAs. Those that have been partially purified have been tested against a narrow selection of potential substrates, and many are classified as β-glucosidases because they can hydrolyse synthetic aryl β-glucosides such as 4-nitrophenyl β-D-glucoside. It should be noted that β-glucosidases which hydrolyse this substrate also fall into the family 1 group of glycoside hydrolases (Henrissat and Davies, 1997). Family 1 'β-glucosidases' do have an active site pocket on their surfaces but, in contrast to the shallow pocket found in family 3 enzymes, the family 1 enzymes have a much deeper pocket which is more like a dead-end tunnel, and can accommodate about six glucosyl residues of the substrate (Barrett *et al.*, 1995; Hrmova *et al.*, 1998a). The deep, narrow tunnel of the family 1 enzymes would undoubtedly place much greater constraints on the conformation of substrates that could fit into the active site; relatively straight $(1{\rightarrow}4)$-β-linked oligoglucoside substrates are required (Hrmova *et al.*, 1998a). Thus, while family 1 and family 3 glycoside hydrolases will catalyse the hydrolysis of *p*-nitrophenyl β-D-glucoside, in most cases their actual substrate specificities have not been rigorously evaluated. There are likely to be fundamental differences in both the breadth of their specificities and in the length of substrates that are hydrolysed.

These considerations of substrate specificity raise several issues regarding functions of family 3 glycoside hydrolases, which have been identified in a wide range of plants and microorganisms, but not in animals. This suggests that they might have a fundamental function, which could be linked to plant cell wall degradation. Family 3 glycoside hydrolases have two domains; the active site is located on domain 1 but catalytic amino acids might also be located on domain 2 (Figure 3.8). Could there be another function for the second domain? Hrmova and Fincher (1998) presented kinetic evidence for positive

cooperativity of binding for barley (1→3,1→4)-β-glucan by the barley β-glucan exohydrolases, and Varghese *et al.* (1999) suggested that this might be explained by a second binding site for the (1→3,1→4)-β-glucan substrate on domain 2. A Rossmann-like fold that lies at the ends of β strands 'j' and 'l' and on the loop between helices J1 and J2 of the second domain is conserved in the plant enzymes and in some bacterial enzymes (Table 3.2). This fold is similar to conformations found in carbohydrate-binding regions of lectins and other proteins (Brändén, 1980).

However, (1→3,1→4)-β-glucans are not found in dicotyledonous plants, although family 3 glycoside hydrolases with two domains have been detected in the dicotyledons tobacco (N. Koizumi, unpublished results, GenBank accession number AB017502) and nasturtium (Crombie *et al.*, 1998). Perhaps the second domains of these enzymes bind xyloglucans, which are polysaccharides analogous to the (1→3,1→4)-β-glucans in location, and possibly function, in cell walls of dicotyledons.

Observations of this kind provide circumstantial evidence for a role of domain 2 in attaching the enzyme to insoluble substrates or to cell walls, in a fashion similar to that observed for the carbohydrate-binding domains of cellobiohydrolases (Gilkes *et al.*, 1991; Teeri *et al.*, 1998), chitinases (Watanabe *et al.*, 1994), glucoamylases (Sigurskjøld *et al.*, 1998) and related polysaccharide hydrolases. The plant enzymes might function in wall loosening during elongation of young vegetative tissues (Sakurai and Masuda, 1978; Cosgrove, 1999), while the microbial family 3 glycoside hydrolases could be participants in the enzymic degradation of cell walls during pathogenesis or during the normal autotrophic utilization of plant residues. In any case, detailed analyses of substrate specificities and the precise definition of kinetic properties of other family 3 glycoside hydrolases, in particularly the more distantly related enzymes from microbial sources, will be necessary before we can confidently assign functions to this widely distributed group of enzymes.

Chapter 4

Isolation of Gene Fragments and Chromosomal Location of Barley β-Glucan Exohydrolases

4.1 Introduction

The cDNAs encoding barley β-glucan exohydrolases isoenzyme ExoI and isoenzyme ExoII were isolated and characterized as described in Chapter 2. The relationships between these sequences and similar sequences from other organisms were examined in Chapter 3 and selected structures were modelled.

In this Chapter, experiments aimed at defining the number of genes encoding barley β-glucan exohydrolases and their chromosomal locations are described. As has been previously noted, two isoforms of β-glucan exohydrolase have been purified from germinated barley seedlings (Hrmova *et al.*, 1996) indicating that there must be at least two genes. A barley β-glucan exohydrolase has also been isolated from cell wall-bound fractions of barley coleoptiles (Kotake *et al.*, 1997). The latter enzyme apparently has a different substrate specificity compared with the isoenzyme ExoII isolated by Hrmova *et al.*, (1996) although experimental designs were different as were some of the substrates. For example the (1→3)-β-glucan used was laminarin from *Eisenia arborea* rather than from *Laminaria digitata*, used by Hrmova *et al.* (1996). These substrates contain different levels of branching which would have an effect on hydrolytic rates. NH$_2$-Terminal amino acid sequencing showed that the enzyme purified by Kotake *et al.* (1997) has an identical sequence, at least over the first thirty amino acids, to the mature form of the barley β-glucan exohydrolase isoenzyme ExoII. Nevertheless, it is unclear whether the enzyme isolated by Kotake *et al.* (1997) is actually isoenzyme ExoII, or a closely related protein encoded by a different gene. Similarly, two distinct forms of (1→3)-β-glucan exohydrolase have been described in maize (YY Zhao and L Bogorad 1999, unpublished results, GenBank accession number AF064707; Kim *et al*, 2000). Thus, the work described in this Chapter was undertaken to determine the number of genes encoding barley β-glucan exohydrolases both by the isolation and characterization of genomic DNA clones and through Southern hybridization analysis.

It was also of interest to define the chromosomal location of these genes. The location of the genes might provide information on potential linkages to other genes of

666666interest. The cDNAs encoding the barley β-glucan exohydrolase isoenzymes (Chapter 2) were used in Southern hybridization analyses to probe wheat-barley addition line DNA for the determination of chromosomal location of the genes.

4.2 Materials and Methods

4.2.1 Materials

The barley genomic library was prepared by Mr. Ronald Osmond (Department of Plant Science, University of Adelaide, South Australia) from partially digested DNA from 7-day-old seedlings of *Hordeum vulgare* (cv. Galleon) cloned into the *Eco*RI restriction enzyme site of the bacteriophage vector λDASH II (Stratagene, La Jolla, CA, USA). Bacterial cells *E. coli* XL1-Blue MRA (P2) [Δ*(mcrA)183* Δ*(mcrCB-hsdSMR-mrr)173*, *end*A1, *sup*E44, *thi*-1, *gyr*A96, *rel*A1, *lac*^{c}, P2 lysogen] and the plasmid vector pBluescript SK(+) were purchased from Stratagene. Restriction enzymes, T4 DNA ligase, T4 polynucleotide kinase, dNTPs and *Taq* polymerase were obtained from Promega (Madison, WI, USA) and New England Biolabs (Beverly, MA, USA). Tryptone, yeast extract and agar were purchased from Difco (Detroit, MI, USA). The Wettex sponge was from Johnson & Son (Auckland, New Zealand). 3MM Chromatography paper and DEAE-cellulose DE52 were from Whatman International (Maidstone, England). The Megaprime DNA labelling kit, autoradiographic film, nitrocellulose and nylon membranes and [α-^{32}P]-dCTP were from Amersham International Ltd. (Little Chalfont, Buckinghamshire, UK). [α-^{35}S]-dATP and [α-^{33}P]-dATP, and BRESAclean DNA purification kit were from Bresatec (Adelaide, SA, Australia). Sephadex G-100, CM-Sepharose CL-6B and Blue Dextran 2000 were obtained from Pharmacia Biotech (Uppsala, Sweden). The manual DNA sequencing kits (Sequenase V2.0) were purchased from United States Biochemical Corporation (Cleveland, Ohio, USA). Agarose, ethidium bromide, ampicillin, maltose, herring sperm DNA, DTT, Ficoll, X-gal, IPTG, Orange G, PEG (8000), PVP, RNase and lysozyme were purchased from Sigma (St. Louis, MO, USA). Miracloth was from Calbiochem (La Jolla, CA, USA).

4.2.2 Screening the barley genomic library

The genomic library was plated out on lawns of *E. coli* XL1-Blue (P2) cells and allowed to grow overnight at 37°C.   Membrane filter plaque lifts were performed essentially as described in section 2.2.3.   Positively hybridizing plaques were rescreened until it was confirmed that they were monoclonal (section 2.2.3).   Individual positive plaques were removed from the plate using a 1 ml Gilson pipette tip cut for the purpose, resuspended in 500 µl SM buffer containing 20 µl chloroform, and the phage stock stored at 4°C.

DNA for probes were from barley β-glucan exohydrolase isoenzyme cDNA clones pExoI and pExoII digested with *Kpn*I and *Pvu*I.   Thus, the complete cDNAs were used. Probes were labelled using [α-^{32}P]-dCTP as described previously (section 2.2.3).

4.2.3 Preparation of λ phage DNA

Positive clone phage stock (200 µl) was added to 400 µl of overnight cultured *E. coli* XL1-Blue (P2) cells and incubated at 37°C for 15 min to allow infection to occur.   The mixture was transferred to a 100 ml conical flask containing 50 ml LB medium.   The flask was incubated at 37°C with shaking for approximately 8 h until the bacterial cells flocculated.   The culture was centrifuged at 12,000 g for 10 min to pellet the cell debris. The supernatant was transferred to a fresh centrifuge tube and 20 µl DNase and RNase (10 mg/ml in 10 mM Tris-HCl buffer, pH 7.5, containing 15 mM NaCl) was added before incubation at 37°C for 30 min.   The solution was adjusted with PEG 8000 and NaCl to final concentrations of 10% (w/v) and 1 M, respectively.   The solution was stored at 4°C overnight.   The phage particles were pelleted by centrifugation at 12,000 g for 20 min at 4°C.   The supernatant was discarded and the tube inverted on filter paper for 15 min to drain off residual PEG.   The drained pellet was resuspended in 700 µl LB, transferred to a 1.5 ml Eppendorf tube and 700 µl DEAE-cellulose DE-52 (equilibrated with LB) was added to remove bacterial proteins and DNA.   The tube was thoroughly mixed by inversion and centrifuged at 16,000 g for 5 min at room temperature.   The supernatant was transferred to a fresh Eppendorf tube and the process repeated.   Following centrifugation,

the supernatant was transferred to another Eppendorf tube and mixed with 15 μl Proteinase K (0.1 mg/ml) and 35 μl 10% (w/v) SDS. The tube was incubated at room temperature for 5 min to lyse the phage particles. Next, 130 μl 3 M potassium acetate buffer, pH 4.8, was added and the solution incubated for 20 min at 88°C and subsequently chilled on ice for 10 min. The tube was centrifuged at 16,000 g to pellet phage debris. The supernatant was transferred to a fresh tube and phage DNA was precipitated by the addition of an equal volume of isopropanol. Following incubation at -20°C for 1 h, the precipitate was pelleted by centrifugation, washed with 70% ethanol, dried and resuspended in 100 μl TE buffer.

4.2.4 Restriction digestion, Southern hybridization and subcloning

Purified phage DNA from positively hybridizing plaques was digested with appropriate restriction enzymes and separated by electrophoresis through a 1% (w/v) agarose gel. The DNA was transferred to Hybond-N$^+$ nylon membrane (Amersham) (see section 2.2.6) and barley β-glucan exohydrolase gene fragments were identified by hybridization with either β-glucan exohydrolase isoenzyme ExoI or ExoII cDNA probes radiolabelled with [α-^{32}P]-dCTP.

Fragments that hybridized strongly with the barley β-glucan exohydrolase probes were excised from the agarose gel and extracted using BRESAclean DNA purification kit (Bresatec) following the manufacturer's instructions. The fragments were ligated into pBluescript (SK$^+$) that had been cut with the appropriate restriction enzyme and dephosphorylated with alkaline phosphatase. Further restriction analyses, separation through a 1% agarose gel and Southern hybridization confirmed insertion of the correct fragments.

4.2.5 Preparation of genomic DNA for Southern hybridization analyses

Pieces of young barley leaf tissue (0.5-0.75 g) were ground to a powder in liquid nitrogen using a mortar and pestle. The frozen, powdered tissue was transferred to a 30 ml centrifuge tube and resuspended in 15 ml extraction buffer (100 mM Tris-HCl buffer, pH

8.0, containing 50 mM EDTA, 500 mM NaCl and 10 mM β-mercaptoethanol). The solution was mixed thoroughly with 1 ml 20% (w/v) SDS, and incubated at 65°C for 10 min. Proteins and polysaccharides were precipitated by the addition 5 ml 5 M potassium acetate, the tube was vigorously shaken and incubated at 0°C on ice for 20 min. The proteins and polysaccharides were pelleted by centrifugation at 25,000 g for 15 min. The supernatant was filtered through Miracloth (Calbiochem, USA) into a fresh centrifuge tube containing 10 ml isopropanol. The solution was mixed and incubated at -20°C for 30 min. The DNA was pelleted by centrifugation at 20,000 g for 15 min. The supernatant was decanted and the pellet allowed to dry by inverting the tube on filter paper. The pellet was redissolved in 0.7 ml 50 mM Tris-HCl buffer, pH 8.0, containing 10mM EDTA, and transferred to an Eppendorf tube. The tube was spun at 16,000 g for 10 min to remove insoluble debris. The supernatant was transferred to a fresh Eppendorf tube and mixed with 75 μl 3 M sodium acetate and 500 μl isopropanol. The DNA clot was pelleted in a microfuge for 30 sec and the supernatant was removed. The pellet was washed with 80% (v/v) ethanol, dried and redissolved in 100 μl 10 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA.

The genomic DNA was digested with the restriction enzymes *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hind*III and *Pst*I. Duplicate tubes were prepared so that parallel hybridization with either barley β-glucan exohydrolase isoenzyme cDNAs could be performed. Approximately 10-12 μg DNA was added to 6 μl SDB buffer (10x SDB: 330 mM Tris-HCl buffer, pH 7.8, containing 650 mM potassium acetate, 100 mM magnesium acetate, 40 mM spermidine and 50 mM dithiothreitol), 2 μl restriction enzyme was added and the volume was made up to 30 μl with Milli-Q H₂O. The solutions were incubated at 37°C for 4 h and duplicate preparations separated on a single 1.2% (w/v) agarose gel at approximately 35V (31mA) overnight. The gel was stained with 1 μg/ml ethidium bromide for 10 min and destained with several changes of sterile H₂O for 1 h before being photographed on an UV-lightbox.

The DNA was transferred to Hybond-N⁺ (Amersham) using 0.4 M NaOH as a transfer solution. A piece of Wettex sponge (Johnson & Son) the size of the agarose gel

was placed in the bottom of a shallow tray containing a small amount of the 0.4 M NaOH. Onto this was carefully laid a piece of 3MM Whatman paper (Whatman International) followed by a screen cut from an overhead transparency sheet so as to have a hole just smaller that the agarose gel. The gel was placed upside down on top of the screen followed by a piece of Hybond-N$^+$ (Amersham) that had been presoaked in sterile H$_2$O. Above the nylon filter were placed two layers of 3MM Whatman paper followed by a 6 cm stack of paper towelling cut to size. Care was taken during the addition of the Whatman paper layers, the agarose gel and the nylon filter to ensure that no air bubbles were trapped between the layers. The 0.4 M NaOH solution in the tray was topped up and maintained just below the level of the plastic screen and the blot allowed to transfer for 6-20 h. After transfer the blot was disassembled and the upper right-hand corner of the nylon membrane was clipped as a reference marker. The membrane was rinsed for 2 min in 2x SSC and blotted dry on 3MM Whatman paper.

Barley β-glucan exohydrolase gene fragments were identified by hybridization with either β-glucan exohydrolase isoenzyme ExoI or ExoII cDNA probes radiolabelled with [α-^{32}P]-dCTP, at 65°C overnight. Membranes were washed in 2x SSC, 0.1% SDS for 20 min once and twice in 0.1x SSC, 0.1% SDS in 65°C for 20 min. Labelled filters were exposed using X-ray film or using a PhosphorImager screen (Molecular Dynamics), where the image was analyzed using ImageQuaNT software (Molecular Dynamics).

4.2.6 Chromosomal location of barley β-glucan exohydrolase genes

Wheat-barley addition lines described by Islam *et al.* (1981) were kindly provided by Drs. AKRM Islam and KW Shepherd. The chromosome 1H addition line is a double monosomic addition containing both the 1H and 6H barley chromosomes, while all other addition lines contain only the wheat chromosomes and the individual barley chromosome. The wheat (*Triticum aestivum* cv. Chinese Spring) and barley (*Hordeum vulgare* cv. Betzes) parental line DNA was also provided by Drs. AKRM Islam and KW Shepherd. Restriction enzyme digestion and Southern hybridization analyses were performed as described in section 4.2.5. The DNA was provided at approximately 0.5 mg/ml and

approximately 8 μg was digested with *Bam*HI for each lane on the gel.   Southern hybridization analyses were performed with a membrane probed first with barley β-glucan exohydrolase isoenzyme ExoI cDNA probe, stripped and reprobed with β-glucan exohydrolase isoenzyme ExoII cDNA probe.

4.3 Results

### *4.3.1 Isolation of gene fragments encoding barley β-glucan exohydrolase isoenzymes*

Approximately 1×10^6 plaques of the barley (*Hordeum vulgare* cv. Galleon) genomic library were screened using the barley β-glucan exohydrolase isoenzyme ExoII cDNA probe. DNA was isolated from purified phage from 8 positive plaques, and after digestion with *Eco*RI, Southern hybridization analysis showed that all but one (λ8) of the clones contained DNA fragments that hybridized with the isoenzyme ExoII cDNA probe (Figure 4.1). Several hybridizing DNA fragments were subcloned into pBluescript (SK⁺) for further analyses. An approximately 4 kb DNA fragment was isolated from genomic clone λ6 and subcloned. The resulting plasmid was designated λ6A.

Sequence analysis using primers from both ends of clone λ6A revealed no similarity to either barley β-glucan exohydrolase isoenzyme cDNA sequences. Comparison to DNA databases also failed to provide any matches. Further restriction analysis (Figure 4.2) showed a strongly-hybridizing DNA fragment of approximately 800 bp released by digestion with restriction enzyme *Pst*I. This DNA fragment was excised from the agarose gel and subcloned into pBluescript (SK⁺) that had been digested with *Pst*I. Sequence analysis of this new plasmid, designated Exo2λ6, showed the fragment to be almost identical to the central part (~1000bp from 5' end) of the barley β-glucan exohydrolase isoenzyme ExoII cDNA; only three mismatches were found over two hundred and forty-nine nucleotides of overlapping sequence (Figure 4.3). The sequence also contained an intron of unknown length toward its 3'-end.

A further two DNA fragments were subcloned from different λ clones. An approximately 4 kb fragment was subcloned from genomic clone λ4 into pBluescript (SK⁺) and designated Exo2λ4. An approximately 1.2 kb fragment from genomic clone λ5 was subcloned and designated Exo2λ5. These clones were sequenced at either end and compared to barley β-glucan exohydrolase isoenzyme ExoII cDNA sequence (see Figure
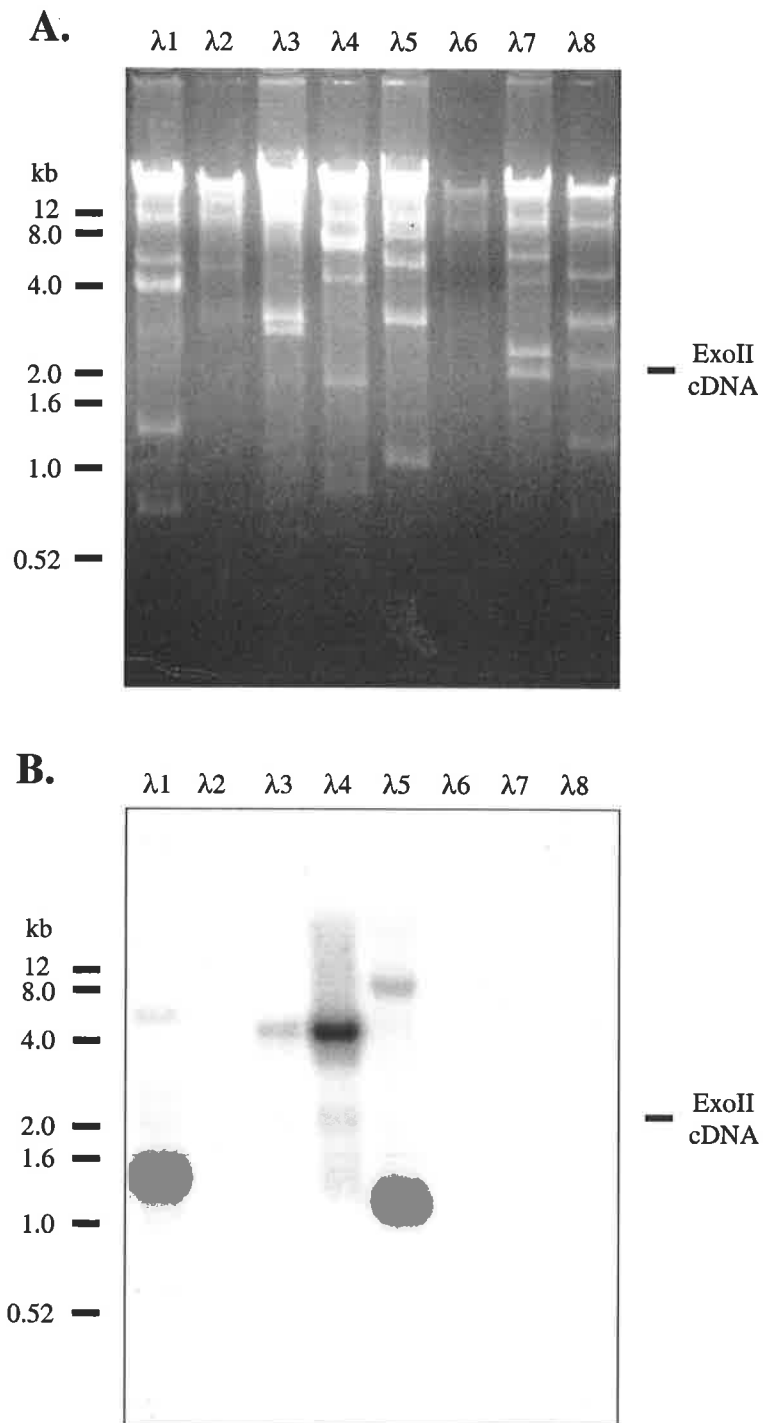
Figure 4.1 Southern hybridization analysis of phage DNA from barley (cv. Galleon) genomic clones digested with *Bam*HI restriction enzyme. **A.** EtBr-stained agarose gel **B.** Southern blot analysis using barley β-glucan exohydrolase isoenzyme ExoII cDNA probe. DNA size markers are shown on the left, with the size of isoenzyme ExoII cDNA on the right.
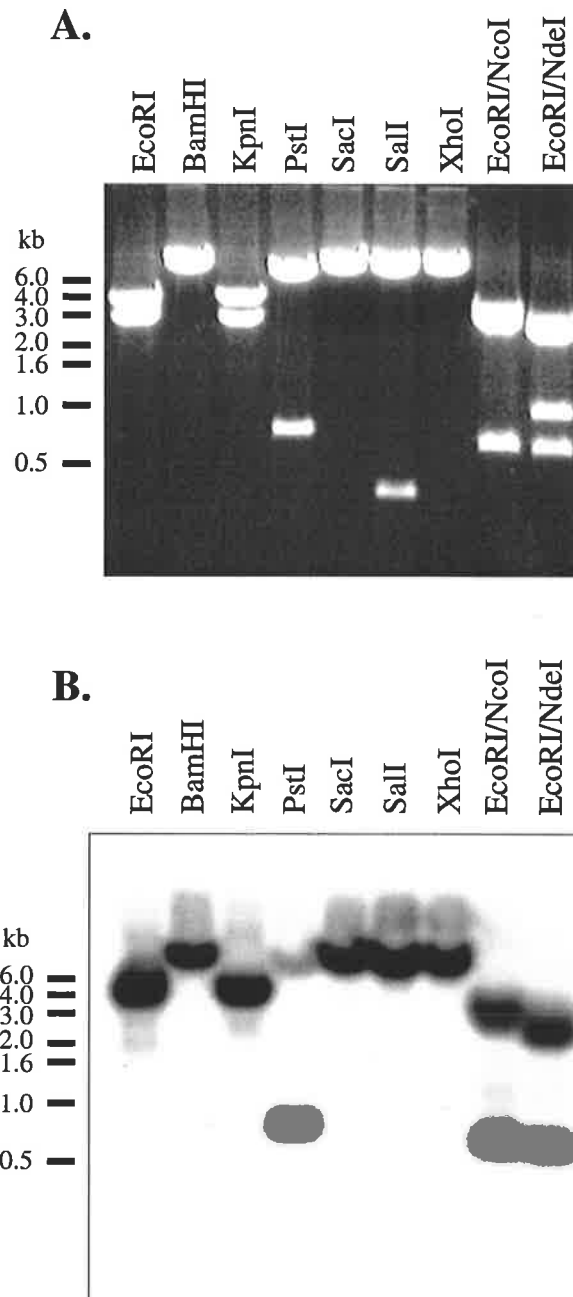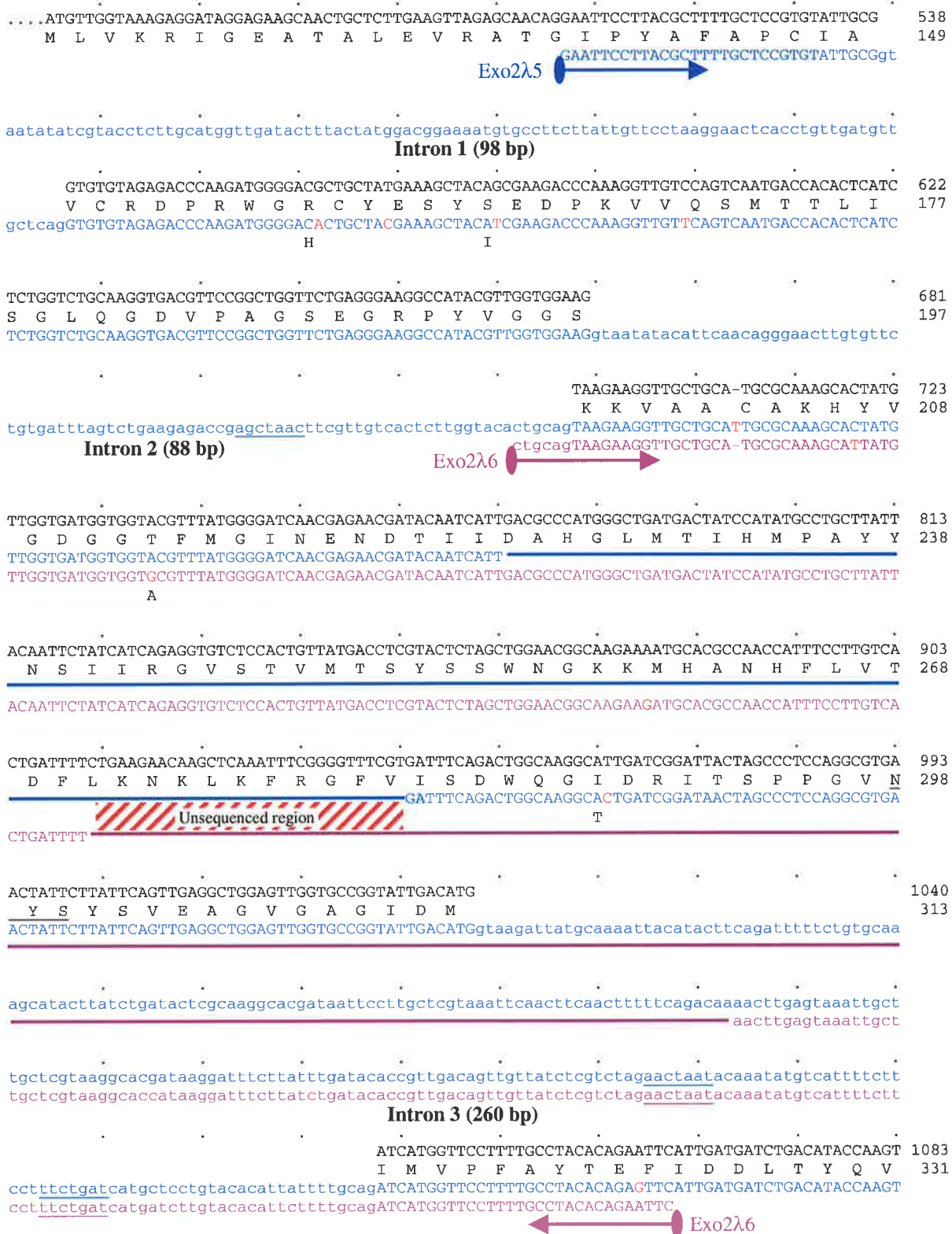
Figure 4.2 Southern hybridization analysis of plasmid DNA from barley (cv. Galleon) genomic subcloned λ6A in pBluescript digested with various restriction enzymes. Panel **A**. EtBr-stained agarose gel. Panel **B**. Southern blot analysis using barley β-glucan exohydrolase isoenzyme ExoII cDNA probe. DNA size markers are shown on the left.

Figure 4.3 Sequence data comparing barley β-glucan exohydrolase isoenzyme ExoII cDNA sequence to gene fragments isolated using the same cDNA as a probe. Numbers on the right refer to cDNA base pairs and mature amino acid sequence. Bases in red indicate sequence differences and changed amino acids are indicated below sequence where changes are non-silent. Ends of gene fragments are indicated with coloured arrows e.g.⟜▶. Thin coloured lines indicate internal unsequenced regions and the red striped box indicates the region unsequenced by any genomic clone. Blue sequence and lines indicate clone Exo2λ5, purple indicate Exo2λ6, and green indicate clone Exo2λ4. Lower case bases indicate intron regions of gene fragments. The underlined regions within the intron indicate putative branch points.

See Figure 4.4 for graphical representation of barley β-glucan exohydrolase isoenzyme ExoII genomic clones.

Figure 4.3 Barley β-Glucan Exohydrolase Isoenzyme ExoII cDNA/genes

```
....ATGTTGGTAAAGAGGATAGGAGAAGCAACTGCTCTTGAAGTTAGAGCAACAGGAATTCCTTACGCTTTTGCTCCGTGTATTGCG      538
    M  L  V  K  R  I  G  E  A  T  A  L  E  V  R  A  T  G  I  P  Y  A  F  A  P  C  I  A       149
                                                       GAATTCCTTACGCTTTTGCTCCGTGTATTGCGgt
              Exo2λ5

aatatatcgtacctcttgcatggttgatactttactatggacggaaaatgtgccttcttattgttcctaaggaactcacctgttgatgtt
                              Intron 1 (98 bp)

    GTGTGTAGAGACCCAAGATGGGGACGCTGCTATGAAAGCTACAGCGAAGACCCAAAGGTTGTCCAGTCAATGACCACACTCATC      622
    V  C  R  D  P  R  W  G  R  C  Y  E  S  Y  S  E  D  P  K  V  V  Q  S  M  T  T  L  I       177
gctcagGTGTGTAGAGACCCAAGATGGGGACACTGCTACGAAAGCTACATCGAAGACCCAAAGGTTGTTCAGTCAATGACCACACTCATC
                                   H              I

TCTGGTCTGCAAGGTGACGTTCCGGCTGGTTCTGAGGGAAGGCCATACGTTGGTGGAAG                                  681
S  G  L  Q  G  D  V  P  A  G  S  E  G  R  P  Y  V  G  G  S                                  197
TCTGGTCTGCAAGGTGACGTTCCGGCTGGTTCTGAGGGAAGGCCATACGTTGGTGGAAGgtaatatacattcaacagggaacttgtgttc

                                          TAAGAAGGTTGCTGCA-TGCGCAAAGCACTATG      723
                                          K  K  V  A  A     C  A  K  H  Y  V      208
tgtgatttagtctgaagagaccgagctaacttcgttgtcactcttggtacactgcagTAAGAAGGTTGCTGCATTGCGCAAAGCACTATG
      Intron 2 (88 bp)                                     ctgcagTAAGAAGGTTGCTGCA-TGCGCAAAGCATTATG
                                       Exo2λ6

TTGGTGATGGTGGTACGTTTATGGGGATCAACGAGAACGATACAATCATTGACGCCCATGGGCTGATGACTATCCATATGCCTGCTTATT   813
 G  D  G  G  T  F  M  G  I  N  E  N  D  T  I  I  D  A  H  G  L  M  T  I  H  M  P  A  Y  Y   238
TTGGTGATGGTGGTACGTTTATGGGGATCAACGAGAACGATACAATCATT
TTGGTGATGGTGGTGCGTTTATGGGGATCAACGAGAACGATACAATCATTGACGCCCATGGGCTGATGACTATCCATATGCCTGCTTATT
          A

ACAATTCTATCATCAGAGGTGTCTCCACTGTTATGACCTCGTACTCTAGCTGGAACGGCAAGAAAATGCACGCCAACCATTTCCTTGTCA   903
 N  S  I  I  R  G  V  S  T  V  M  T  S  Y  S  S  W  N  G  K  K  M  H  A  N  H  F  L  V  T   268

ACAATTCTATCATCAGAGGTGTCTCCACTGTTATGACCTCGTACTCTAGCTGGAACGGCAAGAAGATGCACGCCAACCATTTCCTTGTCA

CTGATTTTCTGAAGAACAAGCTCAAATTTCGGGGTTTCGTGATTTCAGACTGGCAAGGCATTGATCGGATTACTAGCCCTCCAGGCGTGA   993
 D  F  L  K  N  K  L  K  F  R  G  F  V  I  S  D  W  Q  G  I  D  R  I  T  S  P  P  G  V  N   298
                                           GATTTCAGACTGGCAAGGCACTGATCGGATAACTAGCCCTCCAGGCGTGA
         ///////// Unsequenced region /////////                T
CTGATTTT

ACTATTCTTATTCAGTTGAGGCTGGAGTTGGTGCCGGTATTGACATG                                             1040
 Y  S  Y  S  V  E  A  G  V  G  A  G  I  D  M                                              313
ACTATTCTTATTCAGTTGAGGCTGGAGTTGGTGCCGGTATTGACATGgtaagattatgcaaaattacatacttcagatttttctgtgcaa

agcatacttatctgatactcgcaaggcacgataattccttgctcgtaaattcaacttcaacttttttcagacaaaacttgagtaaattgct
                                                                          aacttgagtaaattgct

tgctcgtaaggcacgataaggatttcttatttgatacaccgttgacagttgttatctcgtctagaactaatacaaatatgtcattttctt
tgctcgtaaggcaccataaggatttcttatctgatacaccgttgacagttgttatctcgtctagaactaatacaaatatgtcattttctt
                              Intron 3 (260 bp)

                    ATCATGGTTCCTTTTGCCTACACAGAATTCATTGATGATCTGACATACCAAGT 1083
                     I  M  V  P  F  A  Y  T  E  F  I  D  D  L  T  Y  Q  V   331
cctttctgatcatgctcctgtacacattattttgcagATCATGGTTCCTTTTGCCTACACAGAGTTCATTGATGATCTGACATACCAAGT
cctttctgatcatgatcttgtacacattcttttgcagATCATGGTTCCTTTTGCCTACACAGAATTC
                                      ATCATGGTTCCTTTTGCCTACACAGAATTC     Exo2λ6
```

```
          .         .         .         .         .         .         .
TAAGAACAACATCATCCCCATGAGCAGAATCAACGATGCTGTCTACAGGATTCTCAGGGTGAAGTTCACCATGGGTCTATTTGAGAGCCC  1173
 K  N  N  I  I  P  M  S  R  I  N  D  A  V  Y  R  I  L  R  V  K  F  T  M  G  L  F  E  S  P   360
TAAGAACAACATCATCCCCATGAGCAGAATCAACGATGCTGTCTACAGATTCTTCAGGGTGAAGTTCACCATGGGTCTATTTGAGAACCC
                                                 F  F                                    N

          .         .         .         .         .         .         .
CTATGCTGACCCAAGCCTCGTTGGTGAACTCGGGAAGCAG                                                    1213
 Y  A  D  P  S  L  V  G  E  L  G  K  Q                                                       373
CTATGCTGACCCAAGCCTCGTTCATGAACTCGGGAAGCATgttagttctcaacaccacattttctttctatcacactccatttgtcaaga
                      H              H
        CCAAGCCTCGCCGGTGAACTTGGCAGCCAAgtcggttctgaacaactcaatttcatttctctcacacttcatccatcttga
Exo2λ4  ────────────────►  A              S                                    Intron 4 (90 bp)

          .         .                             .         .         .         .
           ◄──────────── Exo2λ5      GAACACCGTGATCTTGCTCGTGAGGCCGTCAGGAAGTCATTGGTGTTGCT  1263
tccatgaattc                          E  H  R  D  L  A  R  E  A  V  R  K  S  L  V  L  L   390
ttctactatatttagagatgtatcctcttctacctggtagGAACACCGCGAAGTTGCCCGTGAAGCTGTCAGGAAATCCTTGGTGTTGCT
                                             A  E  V

          .         .         .         .         .         .         .
GAAAAATGGAAAATCTGCCTCCACTCCATTGTTGCCTCTCCCAAAGAAGGCCGGTAAGATCCTCGTCGCTGGAAGCCACGCCGACGACTT  1353
 K  N  G  K  S  A  S  T  P  L  L  P  L  P  K  K  A  G  K  I  L  V  A  G  S  H  A  D  D  L   420
GAAGAACGGAAAATCTTCCTACGCTCCATTGCTGCCTCTCCCGAAGAAGGCCGGTAAGATCCTCGTCGCCGGGAGCCACGCCGACAACCT
            S  Y  A                                                               N

          .         .         .         .         .         .         .
GGGCAACCAGTGCGGAGGATGGACCATCACATGGCAAGGACAGACCGGCAACGACAAAACTGCCG                           1418
 G  N  Q  C  G  G  W  T  I  T  W  Q  G  Q  T  G  N  D  K  T  A                              442
GGGCAACCAGTGTGGAGGGTGGACGATCACATGGCAAGGAGGGCCTGGCAATAACAACACTGCAGgtaaatgctcgtccgttcctcgttg
                                   G  P         N  N

          .         .         .         .         .         .         .
atacttgattgcttacacgagtgtactcgatgctcgacagattactggatgcaggacacgatcg───────────◄──┤~4kb├──  Exo2λ4
       Intron 5 (89+ bp)
```

4.3). The relative size and position of these clones compared with β-glucan exohydrolase isoenzyme ExoII cDNA are shown in Figure 4.4.

The approximately 1 x 10^6 plaques of the barley (*Hordeum vulgare* cv. Galleon) genomic library were rescreened using the barley β-glucan exohydrolase isoenzyme ExoI cDNA probe, and several positive plaques were detected. λDNA was isolated for three positive clones and digested with different restriction enzymes. The DNA fragments were separated on a 1% (w/v) agarose gel, blotted onto Hybond-N$^+$ (Amersham) and hybridized with the barley β-glucan exohydrolase isoenzyme ExoI cDNA probe (Figure 4.5). The DNA fragments apparent at approximately 2.6 kb in clones λ1B and λ10B digested with *Bam*HI were separately cloned into pBluescript (SK$^+$), designated ExoIλ1 and ExoIλ3, and sequenced at both ends. It was determined that, over the regions sequenced, both inserts were exactly the same. Comparison of ExoIλ3 and barley β-glucan exohydrolase isoenzyme ExoI cDNA sequence showed that ExoIλ3 corresponded to a region near the 5'-end of isoenzyme ExoI cDNA (Figure 4.6). Over a region of two hundred and sixty seven base pairs where the sequences correspond to each other, there are five base mismatches that caused three changes in the translated amino acid sequence.

Thus, gene fragments encoding both β-glucan exohydrolase isoenzymes were isolated and partially sequenced. The genes were not completely sequenced because of time constraints and because they were highly fragmented with numerous intervening introns. Attention was focussed instead on the mapping and expression experiments.

4.3.2 Southern hybridization analyses of genomic DNA

DNA from young leaves of barley (*Hordeum vulgare* cv. Schooner) was digested with restriction enzymes *Bam*HI, *Dra*I, *Eco*RI, *Eco*RV, *Hind*III and *Pst*I before separation of fragments on an agarose gel and Southern hybridization. Duplicate filters were probed with barley β-glucan exohydrolase isoenzyme ExoI and isoenzyme ExoII cDNA probes (Figure 4.7). The isoenzyme ExoI cDNA probe hybridized with approximately four DNA fragments in each lane. One DNA fragment usually appeared stronger than the others. The

Figure 4.4 Graphical representation of gene fragments isolated using the barley β-glucan exohydrolase isoenzyme ExoII cDNA as a probe. Upper line indicates size of encoded protein with signal peptide indicated in orange. The pExoII cDNA clone depicts the barley β-glucan exohydrolase isoenzyme ExoII cDNA, with restriction sites marked by ↑. Names of gene fragments are shown above appropriate clones. Thick coloured lines indicate regions of genomic clones. Blue bars ▬▬ correspond to exons of clone Exo2λ5, purple bars ▬▬ corresponds to exons of clone Exo2λ6, and green bars ▬▬ corresponds to exons of clone Exo2λ4. Red regions ▬▬ correspond to introns. Sizes of introns are indicated on the lower left. Dashed lines e.g. ▯▯▯▯ indicate unsequenced regions.

A.
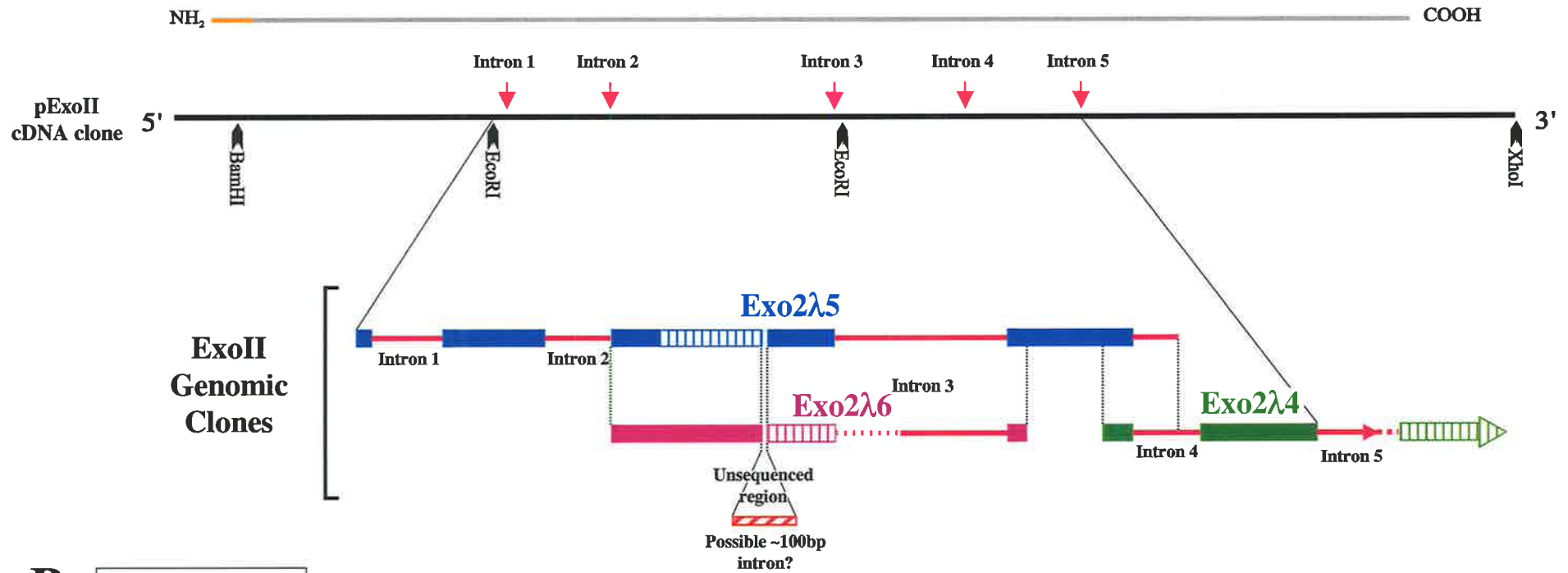
B.

Intron 1	98bp
Intron 2	88bp
Intron 3	260bp
Intron 4	90bp
Intron 5	89⁺bp

Figure 4.4
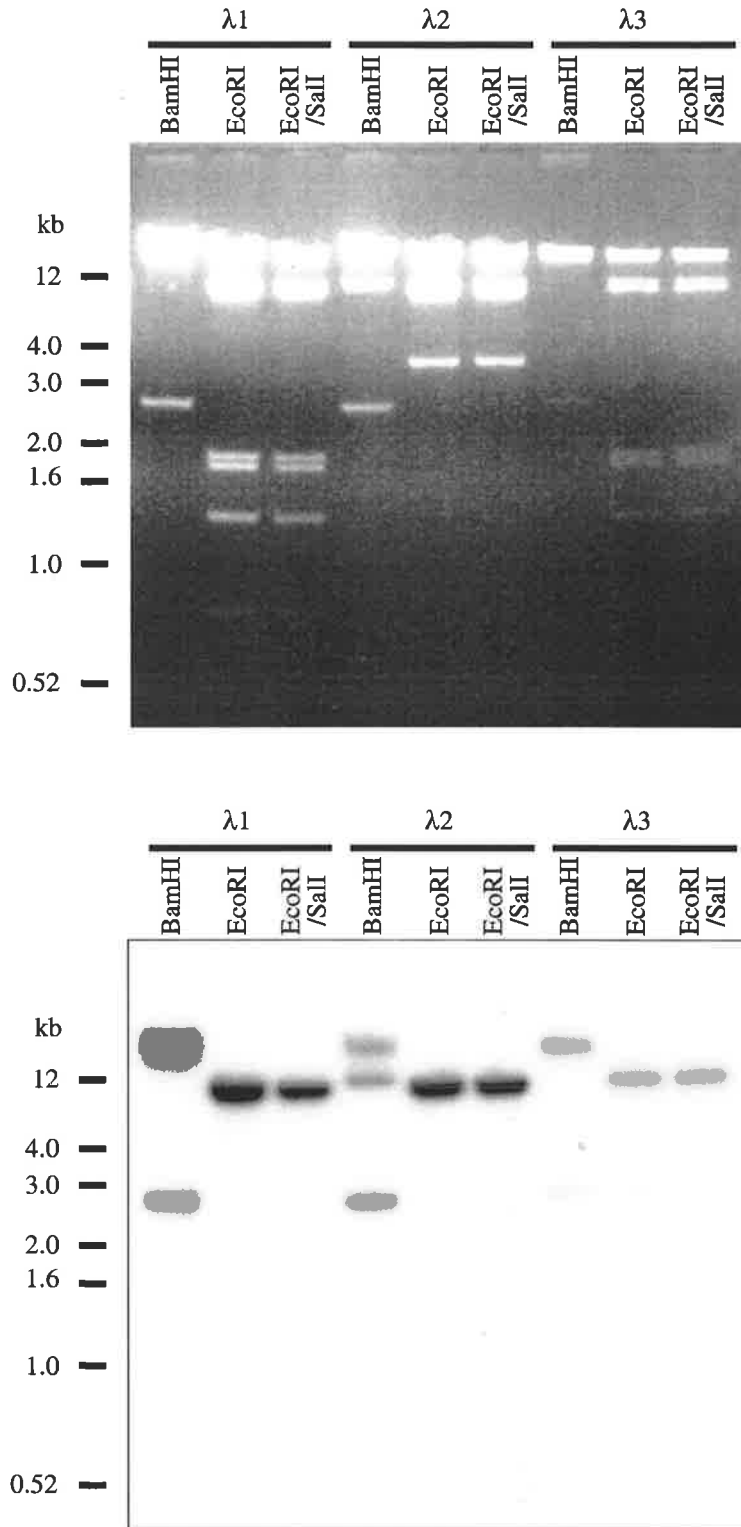
Figure 4.5 Phage DNA from barley (cv. Galleon) genomic clones λ1, λ2 and λ3 digested with shown restriction enzyme. **A.** EtBr-stained agarose gel **B.** Southern hybridization analysis using barley β-glucan exohydrolase isoenzyme ExoI cDNA probe. DNA size markers are shown on the left.

Panel A

```
TGTCAACGGCGGCGTCATCCCCATGAGCAGGATCGACGATGCCGTGACCCGGATCCTGCGGGTCAAGTTCACCATGGGTCTCTTCGAGAA 1238
 V   N   G   G   V   I   P   M   S   R   I   D   D   A   V   T   R   I   L   R   V   K   F   T   M   G   L   F   E   N  361
                                                              CTGCGGGTCAAGTTCACCATGGGTCTCTTCGAGAA
                                          Exo1λ3 ▬▬▬▶
```

```
CCCCTACGCTGACCCTGCCATGGCCGAGCAGCTAGGAAAGCAG                                                 1281
 P   Y   A   D   P   A   M   A   E   Q   L   G   K   Q                                       375
CCCCTACGCTGACCCTGCCATGGCCGAGCAGCTAGGAAAGCAGgtactgaacaagctctgatgctgaatttggttggcatggtgattctg
```

```
                            GAGCACAGGGATTTGGCGAGGGAGGCGGCGAGGAAGTCGCTGGTGCTGC 1330
                             E   H   R   D   L   A   R   E   A   A   R   K   S   L   V   L   L  392
aactctgaaagtctgaccttcctactcgatctggaatgcagGACCACAAGGATTTGGCGAGGGAGGCGGCGAGGAAGTCGCTGGTGCTGC
                             D       K
```

```
TGAAGAACGGCAAGACTTCGACCGACGCGCCGCTTCTGCCGCTGCCCAAGAAGGCGCCCAAGATCCTGGTCGCCGGGAGCCACGCCGACA 1420
 K   N   G   K   T   S   T   D   A   P   L   L   P   L   P   K   K   A   P   K   I   L   V   A   G   S   H   A   D   N  422
TGAAGAACGGTAAGACTTCGACCGACGCGCCGCTTCTGCCGCTGCCCAAGAAGGCGGCCAAGATCCTGGTCGCCGGGAGCCACGCCGACA
                                                                   A
```

```
ACCTGGGCTACCAGTGCGGCGGCTGGACCATCGAGTGGCAGGGCGACACGGGCCGCACCACCGTGGGCACCACCATCCTGGAGGCGGTGA 1510
 L   G   Y   Q   C   G   G   W   T   I   E   W   Q   G   D   T   G   R   T   T   V   G   T   T   I   L   E   A   V   K  428
ACCTGGGCTACCAGTGCGGCGGCTGGACCATCGAGTGGCAGGGAGACACGG ▬▬▬▬▬▬▬▬◀═ ~2.2kb ═▶● Exo1λ3
```

Panel B
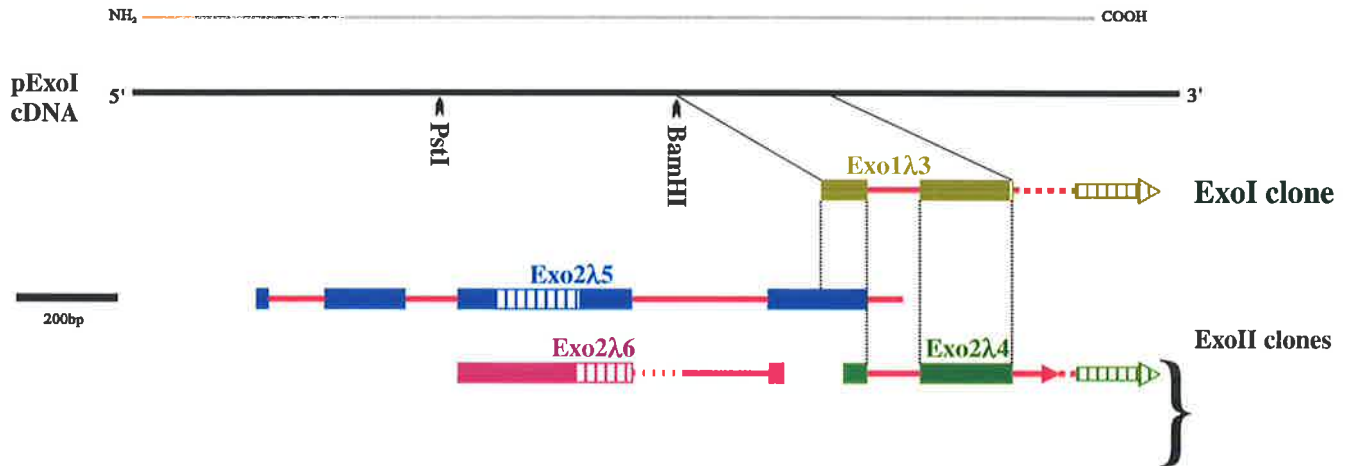
Figure 4.6 Sequence comparison of barley β-glucan exohydrolase isoenzyme ExoI cDNA and Exo1λ3 gene fragment isolated using the same cDNA as a probe. Panel **A**. Numbers on right refer to cDNA base pairs and mature amino acid sequence. Bases in red indicate sequence differences and amino acids substitutions are indicated below sequence where applicable. Ends of the gene fragment are indicated with arrows ●▬▶ . Lower case bases indicate intron region of gene fragment. The underlined region within the intron is the putative branch point. Panel **B**. Representation of the size and position of all of the β-glucan exohydrolase genomic clones, with the isoenzyme ExoI genomic clone Exo1λ3 shown in khaki. Red regions ▬ correspond to introns. Dashed lines e.g. ▭▭▭ indicate unsequenced regions.
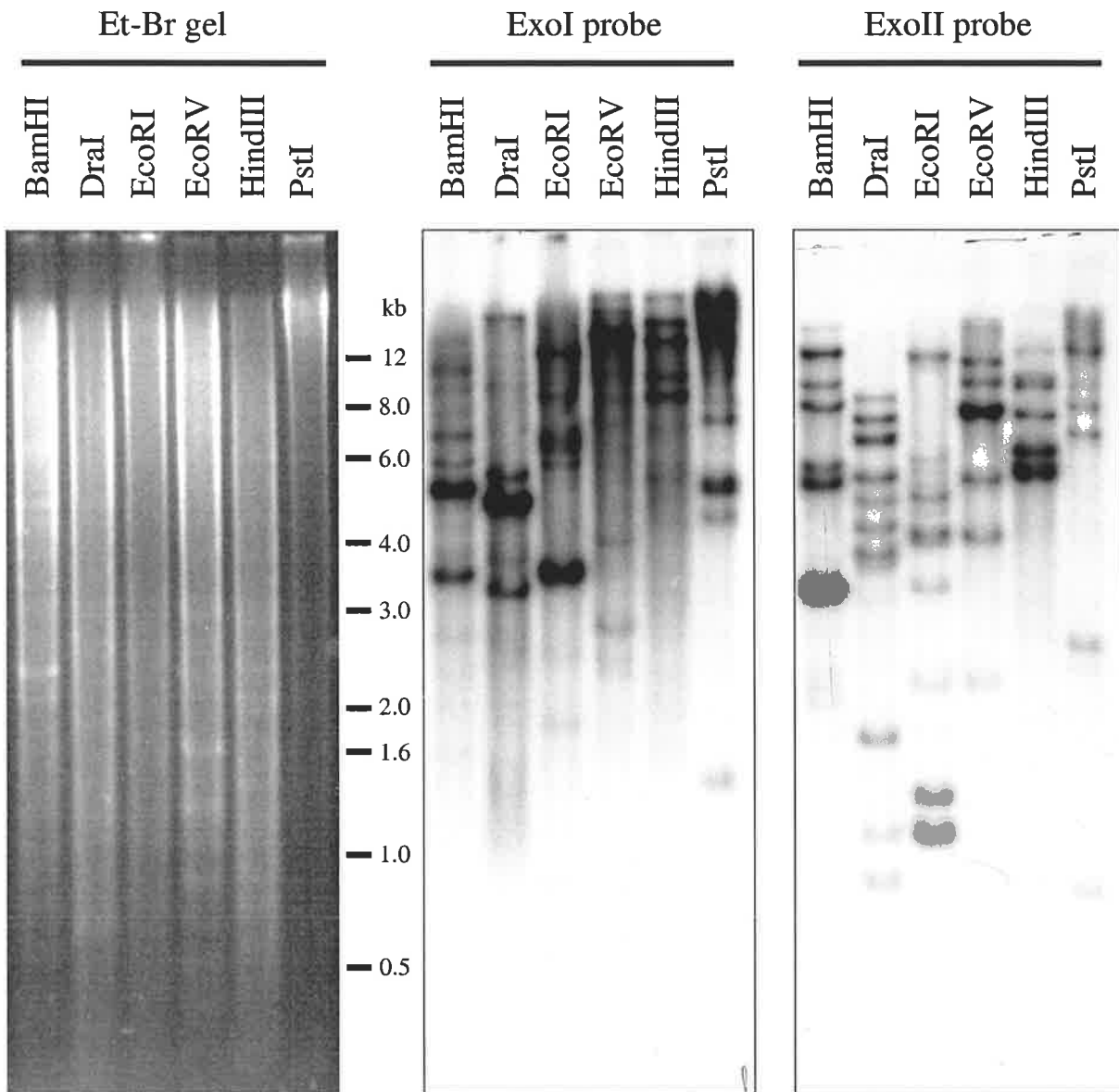
Figure 4.7 Southern hybridization analyses of barley (*Hordeum vulgare* cv. Schooner) genomic DNA digested with various restriction enzymes and probed with barley β-glucan exohydrolase isoenzyme ExoI and isoenzyme ExoII cDNA probes. The Et-Br stained agarose gel is shown on the left.

isoenzyme ExoII cDNA probe hybridized to four to ten DNA fragments, again with one DNA fragment appearing stronger than the others.

4.3.3 Chromosomal location using Southern hybridization analyses of addition-line DNA

Southern hybridization analyses of genomic DNA for parental lines of wheat (*Triticum aestivum* cv. Chinese Spring), barley (*Hordeum vulgare* cv. Betzes) and wheat-barley addition lines were performed first with the barley β-glucan exohydrolase isoenzyme ExoI cDNA probe. The membrane was stripped and reprobed with the β-glucan exohydrolase isoenzyme ExoII cDNA probe (Figure 4.8). For the β-glucan exohydrolase isoenzyme ExoI cDNA probe there are three or four DNA fragments that hybridize with a reasonably intense signal in the barley parental line cv. Betzes (Figure 4.8). The probe also binds to homologous gene DNA fragments in wheat, but three DNA fragments corresponding to DNA fragments in the barley parent of approximately 3.4, 5.0, and 6.0 kb are seen in wheat addition line 5H. No DNA fragments corresponding to other barley parental DNA fragments can be discerned in the addition line digests.

For the β-glucan exohydrolase isoenzyme ExoII cDNA probe there are approximately four DNA fragments that hybridize strongly in the barley parental line. It can be clearly seen that the DNA fragment of approximately 3.5 kb corresponds to a DNA fragment in addition line 1H/6H. No DNA fragment is present in 6H at this position so it can be presumed that the DNA fragment is indeed present on chromosome 1H of barley. The next largest DNA fragment in the barley parent, at approximately 5.2 kb, corresponds to a DNA fragment in addition line 7H. It is not clear where the DNA fragment of the barley parent, of approximately 8 kb, sits within the addition lines. It may be present in the addition line 1H (Figure 4.8C). The largest strongly hybridizing DNA fragment of over 12 kb, seen in the barley parental line, appears to correspond to a DNA fragment in wheat-barley addition line 5H, although this assignment is debatable (Figure 4.8C).

Further Southern blots were performed using parental and addition line DNA digested separately with *Dra*I, *Eco*RI and *Hind*III restriction enzymes. The results from

Figure 4.8 Genomic DNA from wheat parent (Chinese-Spring), barley parent (Betzes) and wheat-barley addition lines (1H-7H) digested with *Bam*HI restriction enzyme. Panel **A**. EtBr-stained agarose gel. Panel **B**. Southern hybridization filter probed with barley β-glucan exohydrolase isoenzyme ExoI cDNA probe. Panel **C**. Stripped filter probed with barley β-glucan exohydrolase isoenzyme ExoII cDNA probe. Red arrowheads show extra hybridizing bands in addition-lines, indicating presence of genes on these chromosomes.

A.

Chinese-Spring, Betzes, 1H/6H, 2H, 3H, 4H, 5H, 6H, 7H, Betzes

kb
12
8.0
6.0
4.0
3.0
2.0
1.6
1.0
0.5

B.

Chinese-Spring, Betzes, 1H/6H, 2H, 3H, 4H, 5H, 6H, 7H, Betzes

ExoI cDNA probe

C.

Chinese-Spring, Betzes, 1H/6H, 2H, 3H, 4H, 5H, 6H, 7H, Betzes
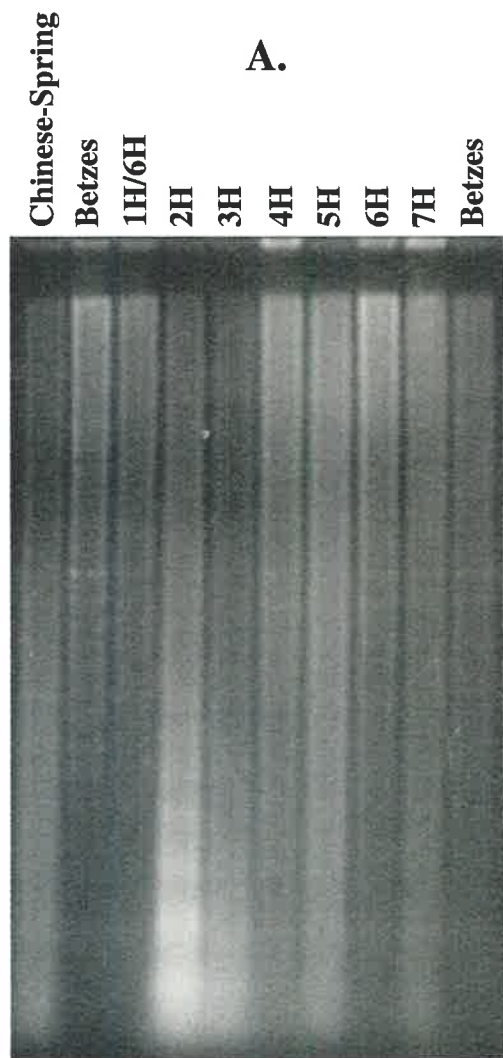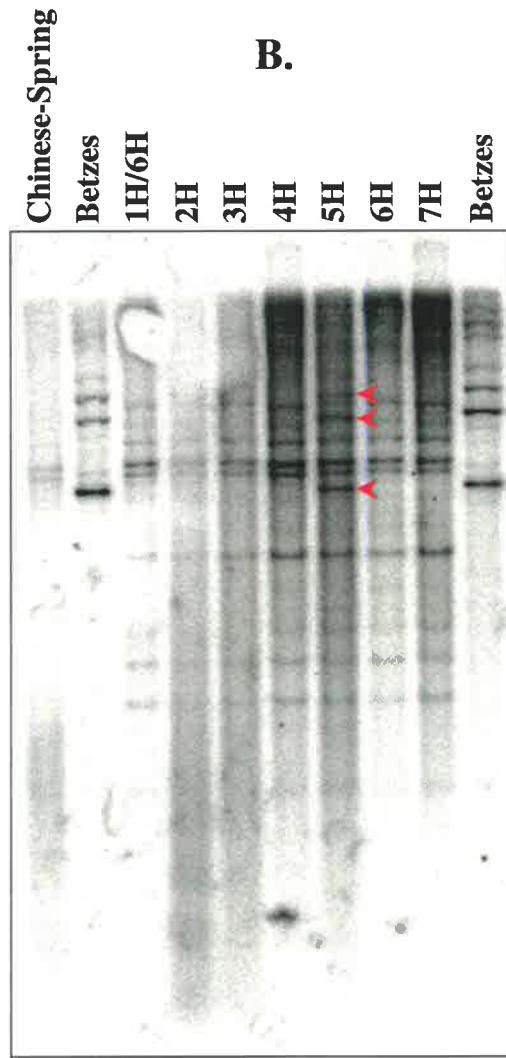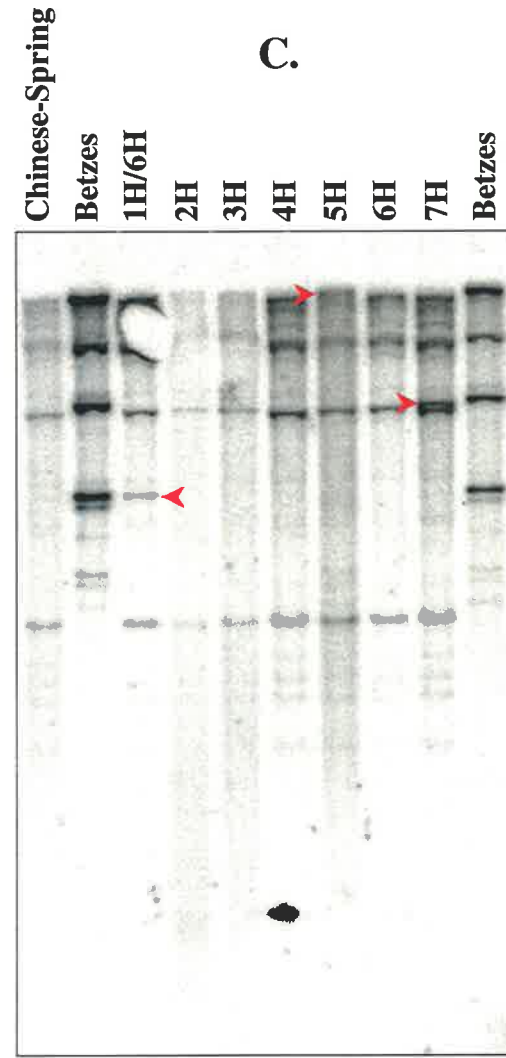
ExoII cDNA probe

Figure 4.8

probing these filters with barley β-glucan exohydrolase isoenzyme ExoI (Figure 4.9) and isoenzyme ExoII probes confirmed the results seen in Figure 4.8.

Figure 4.9 Genomic DNA from wheat parent (Chinese-Spring), barley parent (Betzes) and wheat-barley addition lines (1H-7H) digested with *Bam*HI and DraI restriction enzymes. Southern hybridization filter was probed with barley β-glucan exohydrolase isoenzyme ExoI cDNA probe. Red arrowheads show extra hybridizing bands in addition-lines, indicating presence of genes on these chromosomes.

C.S. Betzes 1H/6H 2H 3H 4H 5H 6H 7H   C.S. Betzes 1H/6H 2H 3H 4H 5H 6H 7H
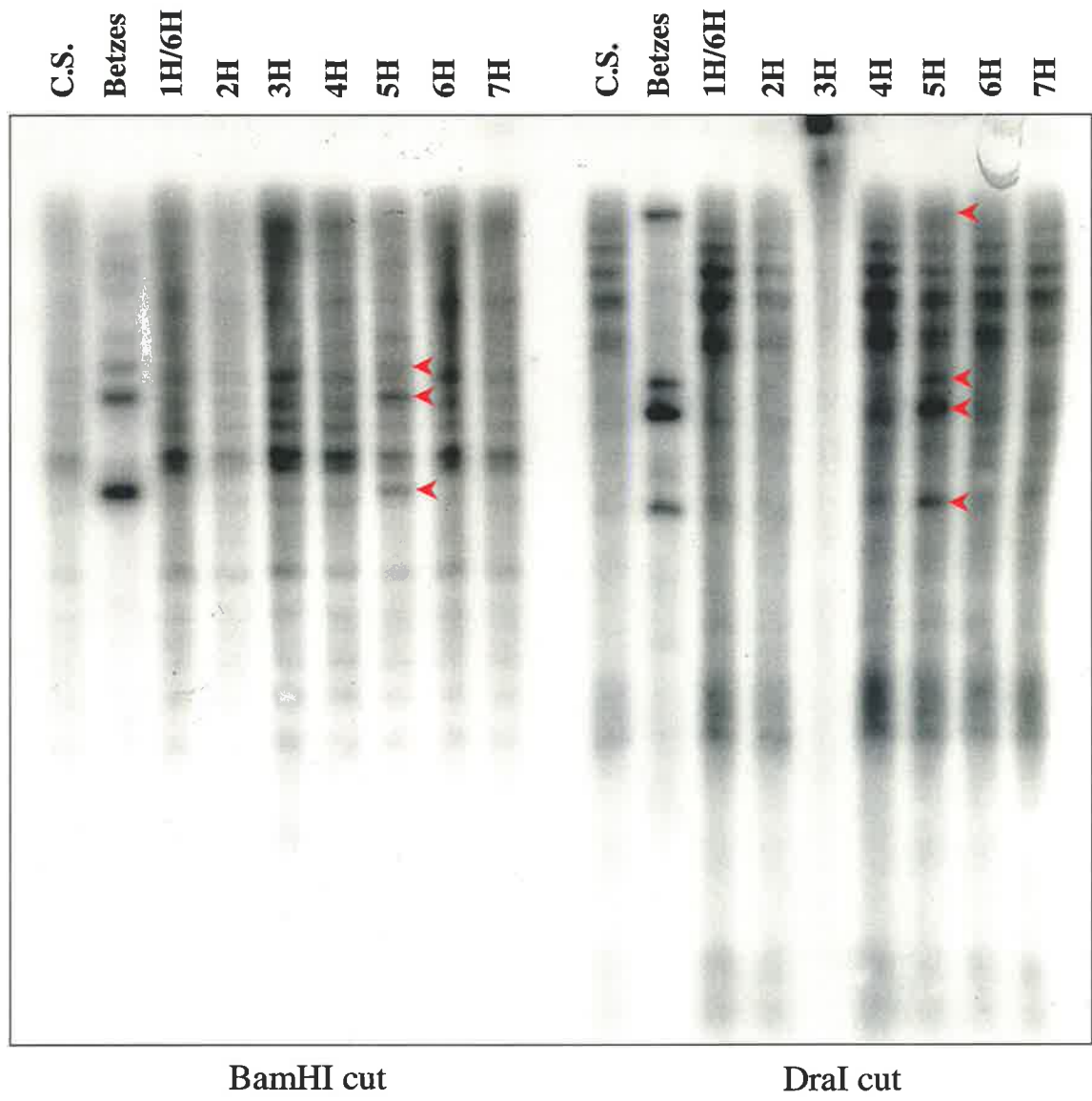
ExoI probe

BamHI cut   DraI cut   **Figure 4.9**

4.4 Discussion

In this study one gene fragment was isolated from a barley (cv. Galleon) genomic library using the barley β-glucan exohydrolase isoenzyme ExoI cDNA probe and three overlapping gene fragments were isolated using the β-glucan exohydrolase isoenzyme ExoII cDNA probe. Comparison of the sequence of these fragments with the appropriate β-glucan exohydrolase isoenzyme cDNA sequences showed the fragments to be highly similar at both the DNA and deduced amino acid sequence level (Figures 4.3 and 4.6). Thus, there is one gene similar to isoenzyme ExoI and three distinct genes that are similar to isoenzyme ExoII.

Barley β-glucan exohydrolase isoenzyme ExoI genomic clone

The genomic clone ExoIλ3, isolated using the barley β-glucan exohydrolase isoenzyme ExoI cDNA probe, is approximately 2.2 kb long. Over the region that has been sequenced, there are 258 bp in exons with 98% identity and only five base pair mismatches compared with the isoenzyme ExoI cDNA sequence. These differences may be due to varietal differences, because the genomic library was made using DNA from the barley cultivar Galleon, while the isoenzyme ExoI cDNA was isolated from a seedling cDNA library from the barley cultivar Bomi. Three of the mismatches cause amino acid substitutions, namely E376D, R378K and P411A. All three of the amino acids that are changed are on the surface of β-glucan exohydrolase isoenzyme ExoI, with their R-groups directed into the solvent (Varghese *et al.*, 1999). This would provide sufficient space for the substitutions, with minimal to nil structural disruptions.

Clone ExoIλ3 also has an 88 bp intron which contains the normal, simple plant intron/exon junction signals of 'GT' at the 5' boundary and 'AG' at the 3' boundary (Breathnach and Chambon, 1981). According to Brown (1988), the consensus sequence for the 5' boundary of an intron is 'AG↓gtaag', where bases in lower case indicate intron sequence, and the 3' boundary of an intron is 'tttgcag↓'. These may be compared with the

intron in clone ExoIλ3, which has a 5' junction of 'AG↓gtact' and a 3' boundary of 'aatgcag↓'. In Table 4.1, a closer examination is made of intron 5' and 3' splice junction regions and also of putative branch points within the introns. Introns are removed during RNA processing as lariat RNAs, where the 5' end of the intron forms a 5'-2' phosphodiester bond with the 2'-OH of an adenosine residue (branch point) lying between 18 and 40 nucleotides from the 3' splice site (Brown, 1986). In yeast the branch point consensus 'TACTAAC' is highly conserved (Teem *et al.,* 1984), while in plants and animals this sequence is less highly conserved, with a consensus of 'CT^A/$_G$A^C/$_T$' (Brown, 1986). The clone ExoIλ3 intron contains the sequence 'CTGAC' which begins 31 bp from the 3' splice junction point. This sequence complies with the consensus and is positioned within the expected region (Brown, 1986).

The clone ExoIλ3 intron has a (G+C) content of just 45%. The sequenced regions of exons in clone ExoIλ3 have a (G+C) content of 62.5% and the same regions of barley β-glucan exohydrolase isoenzyme ExoI cDNA have a (G+C) content of 64.4%, very close to the 65.3% (G+C) for the entire coding region. Plant introns generally show a higher 'A+T' than 'G+C' content (Litts *et al.,* 1990; Slakeski *et al.,* 1990; Wolf, 1991; Wang *et al.,* 1992; Xu *et al.,* 1992; Banik *et al.,* 1997). Effective splicing of adjacent introns is reported to require 'A+T'-rich regions within the introns, near the intron boundaries (Goodall and Filipowicz, 1989).

The length of the intron in ExoIλ3 is relatively short, but larger than the minimum functional length of 70-73 nucleotides (Goodall and Filipowicz, 1990). It has been reported that most introns within plant genes are between 100 and 2000 bp (Brown, 1986). The intron also contains stop codons in all three reading frames, which again is typical of plant introns.

Barley β-glucan exohydrolase isoenzyme ExoII genomic clones

Three overlapping clones were isolated from a barley genomic library using the barley β-glucan exohydrolase isoenzyme ExoII cDNA as a probe. These clones have been

Table 4.1 Sequence motifs in the introns of gene fragments isolated using barley β-glucan exohydrolase isoenzyme cDNA probes. The letters n, r, and y denote nucleotide, purine and pyrimidine respectively. The down arrow ↓ indicates the intron slice site. The position numbers in square brackets [] are in relation to the appropriate β-glucan exohydrolase isoenzyme cDNA sequence (see Figures 4.3 and 4.6).

Motif	Region (Ref.)	Sequence	Position
5' Junction	Plant Consensus[1]	$\frac{C}{A}$AG↓gtaagt	
	Monocot Consensus[2]	$\begin{smallmatrix}A\\C\\G\end{smallmatrix}$AG↓gta$\frac{a}{t}g\frac{ttt}{cac}$	
	Dicot Consensus[2]	$\frac{A}{T}$G↓gtaag$\frac{tatt}{ataa}$	
	ExoI Intron	CAG↓gtactga	[1279]
	ExoII Intron 1	GCG↓gtaatat	[536]
	ExoII Intron 2	AAG↓gtaatat	[679]
	ExoII Intron 3	ATG↓gtaagat	[1038]
	ExoII Intron 4	CAT↓gttagtt	[1211]
	ExoII Intron 5	CAG↓gtaaatg	[1416]
3' Junction	Plant Consensus[1]	tt$\frac{a}{t}$tt$\frac{tttt}{rrrr}$tgcag↓G	
	Monocot Consensus[2]	$\frac{tttttttt}{ccgacacg}$nntncag↓	
	Dicot Consensus[2]	$\frac{tttttttatt}{aanaaaataa}t\frac{gc}{at}$ag↓	
	ExoI Intron	cgatctggaatgcag↓G	+88bp
	ExoII Intron 1	gttgatgttgctcag↓G	+98bp
	ExoII Intron 2	cttggtacactgcag↓T	+88bp
	ExoII Intron 3	cacattcttttgcag↓A	+260bp
	ExoII Intron 4	tcttctacctggtag↓G	+90bp
	ExoII Intron 5	Unsequenced	
Putative Branch Points	Yeast Consensus[3,4]	tactaac	18-40bp 5' to the 3' splice site
	Plant/Animal Consensus[1]	ctray	
	ExoI Intron	gtctgac	-31 bp
	ExoII Intron 1	aactcac / acctgtt	-24/-19 bp
	ExoII Intron 2	agctaac	-34 bp
	ExoII Intron 3	ttctgat	-34 bp
	ExoII Intron 4	tactata	-37 bp
	ExoII Intron 5	Unknown	

References: (1) Brown (1986); (2) Hanley and Shuler (1988); (3) Langford and Gallwitz (1983); (4) Pikielny *et al.* (1983).

designated Exo2λ4, Exo2λ5 and Exo2λ6. The clones cover a region corresponding to approximately 890 bp on the isoenzyme ExoII cDNA (Figure 4.4), but are believed to represent 3 different genes.

Of the three clones isolated using the β-glucan exohydrolase isoenzyme ExoII cDNA as a probe, Exo2λ6 has the highest similarity to the isoenzyme ExoII cDNA. It differs in only three bases over a sequenced region of 250 bp (99% identity), of which only one causes an amino acid change, namely T213A. Clone Exo2λ5 is also highly similar, with only ten base differences over 538 bases of exon when compared with the isoenzyme ExoII cDNA (98% identity). There are seven amino acid changes from the isoenzyme ExoII sequence.

Other than the above noted changes between Exo2λ5 and β-glucan exohydrolase isoenzyme ExoII cDNA sequence, there is an insertion of a deoxythymidine after base 707, relative to isoenzyme ExoII cDNA. This insertion, which was confirmed by several sequencing runs in both directions, creates a frame-shift for protein translation that is not recovered or amended elsewhere. Although the insertion may potentially have resulted from a cloning/replication artefact, there are other possibilities which will be discussed shortly.

Clone Exo2λ4 is approximately 4 kb in length (Figure 4.1), from which only 414 bp were sequenced at the 5'-end. Of this sequence, 235 bp aligned with the β-glucan exohydrolase isoenzyme ExoII cDNA and clearly represent exons, and 179 bp are inserted as introns. Across the exons there were thirty-seven base changes when compared with the β-glucan exohydrolase isoenzyme ExoII cDNA (84% identity). These changes result in thirteen amino acid differences. The number of base changes and amino acid differences suggest that the Exo2λ4 gene encodes part of a different β-glucan exohydrolase isoenzyme. When the exon regions are compared with the isoenzyme ExoI cDNA, there is only 69% identity at the DNA level.

There have been other putative isoforms of barley β-glucan exohydrolase purified, which may be encoded by the gene corresponding to clone Exo2λ4 (Kotake *et al.,* 1997).

NH$_2$-Terminal sequencing of one isoform (Kotake *et al.,* 1997) showed that the first thirty amino acids were exactly the same as β-glucan exohydrolase isoenzyme ExoII purified by Dr M. Hrmova (Hrmova *et al.,* 1996) and it is possible that, due to differing isolation procedures, that it is also isoenzyme ExoII. In further work Kotake (2000) purified another β-glucan exohydrolase isoenzyme to near homogeneity and labelled it isoenzyme ExoIII. This enzyme has the same substrate specificity, but has a different NH$_2$-terminal amino acid sequence. Using this information to create degenerate oligonucleotide primers, Kotake (2000) was able to clone partial length cDNAs encoding two new β-glucan exohydrolases by using RT-PCR techniques. It was reported that the deduced amino acid sequences of these clones were similar to β-glucan exohydrolase isoenzyme ExoII (Kotake, 2000). This supports the evidence shown in Southern hybridization analyses in this Chapter, where isoenzyme ExoII hybridizes to at least 4 fragments (Figure 4.7).

β-Glucan exohydrolase isoenzyme ExoII genomic clone introns

Within the DNA sequence of the three genomic clones isolated using the β-glucan exohydrolase isoenzyme ExoII cDNA, are five introns of varying length. For convenience, the introns were numbered relative to their position on β-glucan exohydrolase isoenzyme ExoII cDNA beginning at the 5'-end. On the whole, the introns conform to the expected criteria for 5' and 3' junctions and splice sites. This information is summarised in Table 4.1. They also all contain elevated percentages of 'A+T' base-pairs relative to the coding regions

Regions flanking β-glucan exohydrolase genomic clones

As noted earlier, clone Exo2λ5 contains a one base insertion at nucleotide 707, numbering relative to isoenzyme ExoII cDNA sequence. If present in the gene, this would disrupt expression of a functional gene enzyme, since the new reading frame would contain a stop codon 21 bases after the insertion.

The subcloned fragments of Exo2λ4 and Exo1λ3 are reasonably large and contain flanking sequence 3' to the β-glucan exohydrolase isoenzyme gene sequence. Sequence comparisons between these 3' ends and DNA databases show high levels of homology to copia-like retrotransposons. The 3' sequence of Exo2λ4 is 83% identical to a *Hordeum vulgare* insertion sequence in a copia-like retroelement BARE-1, named gypsy-type retrotransposon BARE-100 DNA, solo-LTR (E. Ohtsubo, 1999; GenBank accession number AB014756; unpublished results). The Exo2λ4 3' sequence also matches with a region within the right LTR (Long Terminal Repeats) of BARE-1 (Manninen and Schulman, *1993*). The 3' sequence obtained from Exo1λ3 is 95% identical to regions within both LTRs of BARE-1 (Manninen and Schulman, 1993).

The 3' sequence of clone Exo2λ4 matches at a lower degree of homology (approximately 70%) with a region of DNA from chromosome 4H of *Hordeum vulgare*.

The presence of the LTRs sequences of BARE-1 suggests that the genes for Exo2λ4 and Exo1λ3 are flanked, at least on one end, by elements of retrotransposons. Retrotransposons are mobile regions of DNA that are copied by reverse transcription of RNA and the DNA copies are subsequently inserted elsewhere in the genome (Watson *et al.,* 1987). Errors in processing during retrotransposon duplication may introduce insertions, deletions or base changes. It is difficult to predict what effects (if any) the retrotransposons have had on the β-glucan exohydrolase isoenzyme genes. If copies are improperly made, as seems to be the case with Exo2λ5, the result is a pseudo-gene, i.e. a gene that is similar in structure to the normal genes but is non-functional. This obviously creates problems in determining the number of "active" β-glucan exohydrolase genes.

If some of the barley β-glucan exohydrolase genes are indeed placed within retrotransposons or even tightly linked with retrotransposons, this may result in the creation of multiple copies of the genes within the barley genome. Depending on how many generations ago the retrotransposons moved around the genome, natural mutation rates might have introduced a number of base changes. This suggests a possible explanation for the small difference between the sequences of clones Exo2λ5 and Exo2λ6, assuming the

transposition was relatively recent. Transposition and mutation may also be the reason for differences between the Exo2λ5, Exo2λ6 and β-glucan exohydrolase isoenzyme ExoII cDNA as well as between Exo1λ3 and β-glucan exohydrolase isoenzyme ExoI cDNA. Since at least two of these gene fragments are apparently associated with retrotransposons, a confirmation of transposition events would be a larger than expected number of genes detected by Southern hybridization.

Southern hybridization analyses

Initial Southern hybridizations were carried out by creating a filter of restriction enzyme-digested genomic DNA, probing it with one β-glucan exohydrolase probe, stripping and reprobing with the other β-glucan exohydrolase probe. Questions were raised as to whether the filter had been effectively stripped, due to the multiplicity of DNA fragments observed, particularly when probing with the barley β-glucan exohydrolase isoenzyme ExoII cDNA probe. To alleviate this problem, duplicate membranes were probed/washed at exactly the same time under exactly the same conditions (Figure 4.7). It has been deduced from careful examination of these filters and others produced through the course of study (e.g. Figure 4.8), that the two β-glucan exohydrolase isoenzyme cDNA probes do not cross-hybridize to any significant extent under the hybridization conditions used (Appendix II).

It is evident that β-glucan exohydrolase isoenzyme ExoI cDNA probe generally binds to one major DNA fragment and three or four minor DNA fragments. It must be noted that restriction enzyme *Pst*I only partially digested the DNA, as indicated by the large, diffuse DNA band in the upper regions of that lane (Figure 4.7). The duplicate filter probed with the β-glucan exohydrolase isoenzyme ExoII cDNA probe produced a similar result, with one major DNA fragment and four or more minor DNA fragments recognised by the probe.

The possibility must be considered that multiple DNA fragments may be produced by internal restriction enzyme cleavage sites within the genes, producing two of more DNA fragments of varying intensity on the Southern. The presence of many introns, as shown by

the β-glucan exohydrolase genomic clones, increases this likelihood. Restriction enzyme maps of the barley β-glucan exohydrolase isoenzyme cDNAs (Figures 2.2 and 2.6) show both sequences contain a single *Bam*HI restriction enzyme site. Isoenzyme ExoI cDNA also contains a single *Pst*I site, while isoenzyme ExoII cDNA contains two *Eco*RI sites. It can be seen that the β-glucan exohydrolase isoenzyme ExoII probe produces smaller hybridizing DNA fragments when the DNA is digested with *Eco*RI, as would be expected from the restriction enzyme sites within the gene/s. Neither β-glucan exohydrolase isoenzyme cDNA contains *Dra*I, *Eco*RV or *Hin*dIII restriction sites. It could therefore be expected that these lanes on Southern hybridizations would show fewer hybridizing DNA fragments. However, examination of the Southern hybridizations (Figure 4.7) shows that the β-glucan exohydrolase isoenzyme ExoI cDNA probe shows a similar number of hybridizing DNA fragments for each restriction enzyme, while β-glucan exohydrolase isoenzyme ExoII cDNA probe shows a greater number of hybridizing DNA fragments in the *Dra*I restricted lane.

The restriction enzyme *Dra*I cuts at the palindromic restriction site 5′...TTT↓AAA...3′. Since it contains only adenosine and thymidine residues, it would be expected to cut more frequently in the (A+T) 'rich' non-coding regions such as in introns. Due to the relatively large number of introns observed in the genomic clones isolated using the barley β-glucan exohydrolase isoenzyme cDNA probes, the presence of *Dra*I recognition sites within the whole genes seems likely. On inspection of the *Dra*I digested lanes on the Southern (Figure 4.7), the Southern hybridized with β-glucan exohydrolase isoenzyme ExoII cDNA does indeed produce many DNA fragments, more so than in lanes digested with other restriction enzymes.

The information gathered from these Southern hybridizations on the number of functional β-glucan exohydrolase genes remains inconclusive. The activities of retrotransposons may not only have created multiple copies of genes but may have led to, directly or indirectly, the creation of pseudogenes. It seems likely however, that there are

more than the two functional genes that encode for β-glucan exohydrolase isoenzyme Exo I and isoenzyme Exo II.

Chromosomal location using wheat/barley addition lines

Genomic DNA was obtained from parental lines of wheat (*Triticum aestivum* cv. Chinese Spring), barley (*Hordeum vulgare* cv. Betzes) and seven wheat-barley addition lines. The addition lines contain a normal complement of Chinese Spring wheat chromosomes, together with an additional barley cv. Betzes chromosome or chromosome arm. The wheat lines carrying individual barley chromosomes 2H-7H are all fertile and reasonably stable. Wheat lines carrying barley chromosome 1H on its own are self-sterile, and chromosome 6H is therefore included in this addition line for stability (Islam *et al.*, 1981).

A wheat/barley addition line filter, created using the restriction enzyme *Bam*HI, was sequentially probed with both barley β-glucan exohydrolase isoenzyme cDNAs (Figure 4.8). Despite the undigested DNA in the 3H lane, all the DNA fragments present in the barley parent digested with *Dra*I are present on chromosome 5H.

Panel C on Figure 4.8 shows the wheat/barley addition line membrane re-probed with isoenzyme ExoII cDNA. In this hybridization there are four DNA fragments hybridizing with approximately equal intensity in the barley parental lanes. Three of these DNA fragments correlate to DNA fragments on chromosomes 1H/6H, 5H and 7H. Since the DNA fragment seen on 1H/6H is not observed on chromosome 6H, it is concluded that the DNA fragment does indeed belong to chromosome 1H. The second largest positively hybridizing DNA fragment of approximately 9 kb may correlate to a fragment from chromosome 1H, but due to technical difficulties encountered in making the Southern hybridization filter, it is impossible to positively assign this DNA fragment.

Both isoenzyme ExoI and isoenzyme ExoII cDNA probes showed DNA fragments on chromosome 5H. However, the position of the DNA fragment on chromosome 5H in panel

C of Figure 4.8 produced with the isoenzyme ExoII cDNA probe is much larger than those shown with isoenzyme ExoI cDNA probe, indicating that it is most likely a different gene.

4.5 Conclusions

Four gene fragments were isolated from a barley genomic library. One was similar to β-glucan exohydrolase isoenzyme ExoI cDNA and three were similar to the cDNA of isoenzyme ExoII. Sequence analysis showed the fragments represented separate genes, and demonstrated that there are at least four β-glucan exohydrolase genes, at least in the barley cultivar Galleon. Southern hybridization analyses showed that there are likely to be even more β-glucan exohydrolase genes, with at least four restriction fragments hybridizing to each β-glucan exohydrolase isoenzyme cDNA probe. This is in agreement with the work of Kotake (2000) who indicated the presence of five transcribed β-glucan exohydrolase sequences.

Probing DNA digests from the wheat/barley addition lines confirmed that there are several genes that hybridize to the barley β-glucan exohydrolase isoenzyme ExoI and isoenzyme ExoII cDNA probes, and that these genes map to chromosomes 1H, 5H and 7H. Future approaches to resolve which genes encode which isoenzymes will need to use gene specific probes or PCR approaches, although this will be difficult to achieve without first obtaining all members of the gene family. Screening genomic DNA libraries demonstrated that there are a number of similar sequences to be found, and future efforts must be initially directed toward isolating and characterizing these clones.

Chapter 5

Developmental Regulation of Barley β-Glucan Exohydrolases

5.1 Introduction

Two cDNAs encoding barley β-glucan exohydrolase isoenzymes ExoI and ExoII have been isolated and characterized (Chapter 2). Analysis of Southern hybridizations of genomic DNA from barley and wheat-barley addition lines (Chapter 4) revealed that each of the two cDNAs binds to approximately four different genes, or pseudogenes. The two β-glucan exohydrolase isoenzyme cDNA sequences are only moderately homologous (72% sequence identity) across the coding region and, as discussed in Chapter 2, codon usage differs dramatically between the genes encoding β-glucan exohydrolases isoenzyme ExoI and isoenzyme ExoII. This results in cDNAs that show very little cross-hybridization under stringent hybridization conditions (Appendix II). Thus, the two cDNAs hybridize to two subsets of genes within the overall β-glucan exohydrolase gene family, and can be used to track the spatial and temporal regulation of the two subsets of β-glucan exohydrolase genes.

Previously, β-glucan exohydrolase enzymes have been isolated from young seedlings of barley (Hrmova *et al.*, 1996; Kotake *et al.*, 1997), maize coleoptile cell walls (Inouhe *et al.*, 1999; Kim *et al.*, 2000), nasturtium cotyledons (Crombie *et al.*, 1998) and cell walls of cultured *Acacia* cells (Lienart *et al.*, 1980). It seems a reasonable assumption that barley β-glucan exohydrolase isoenzymes are expressed in coleoptiles, and possibly within other elongating tissues. Evidence has linked β-glucan exohydrolase and $(1 \rightarrow 3, 1 \rightarrow 4)$-β-glucan endohydrolase expression in elongating maize coleoptiles (Inouhe and Nevins, 1997b; Inouhe *et al.*, 1999), suggesting that expression sites of $(1 \rightarrow 3, 1 \rightarrow 4)$-β-glucan endohydrolases in barley might reflect sites of β-glucan exohydrolase synthesis. The differential expression of two barley $(1 \rightarrow 3, 1 \rightarrow 4)$-β-glucan endohydrolase genes in various tissues has been described (Slakeski *et al.*, 1992b). The $(1 \rightarrow 3, 1 \rightarrow 4)$-β-glucan endohydrolase isoenzyme EII gene is expressed mainly in the aleurone of germinated grain, while the $(1 \rightarrow 3, 1 \rightarrow 4)$-β-glucan endohydrolase isoenzyme EI is more widely transcribed, with mRNA being detected in the scutella of germinated grain, in young leaves and in young roots (Slakeski *et al.*, 1990; Slakeski, 1992; Slakeski and Fincher, 1992b).

However, transcripts of $(1{\rightarrow}3,1{\rightarrow}4)$-$\beta$-glucan endohydrolase genes are not detected in 3-6 day barley coleoptiles (Slakeski and Fincher, 1992b).

Studies in this Chapter were focussed on detecting β-glucan exohydrolase isoenzyme mRNA in various tissues from young seedlings. Experiments are described in which cDNAs are used as probes in Northern hybridization analyses for β-glucan exohydrolase mRNA in scutella, aleurone, coleoptiles, and young roots and young leaves. In an effort to correlate enzyme and mRNA levels, the tissue locations of the barley β-glucan exohydrolase isoenzymes themselves were determined using standard protein purification techniques.

5.2 Materials and Methods

5.2.1 Materials

Barley grain (cv. Bomi) was kindly provided by Professor A. Barr, Department of Plant Science, University of Adelaide. Sodium hypochlorite as 'Milton' solution, equivalent to 1% (w/v) available chlorine, was from Proctor & Gamble (Parramatta, Australia). Nystatin, neomycin sulphate, chloramphenicol, penicillin-G, phenylmethylsulphonyl fluoride (PMSF), diethyl pyrocarbonate (DEPC), 4-nitrophenyl β-D-glucopyranoside (4-NPG), $(1\rightarrow3)$-β-glucan (laminarin from *Laminaria digitata*), and sodium azide were from Sigma Chemical Company (St. Louis, MO, USA). Trizol was from Gibco BRL (Gaithersburg, MD, USA). HETS transfer buffer was from Biotex Laboratories (Houston, TX, USA). Barley $(1\rightarrow3,1\rightarrow4)$-β-glucan was from Megazyme International (Wicklow, Ireland).

5.2.2 Growth of tissues

Barley grain (*Hordeum vulgare* cv. Bomi) was surface-sterilized with 1% (v/v) sodium hypochlorite for 20 min, rinsed thoroughly with sterile H_2O, and soaked for 4 h in sterile H_2O containing 100 U/ml nystatin, 100 μg/ml neomycin, 100 U/ml penicillin G and 10 μg/ml chloramphenicol. Grains were subsequently germinated on moist filter paper in the dark for up to 6 days at 25°C. Scutella were isolated from germinated grain, taking particular care to exclude embryo and aleurone tissues. Similarly, care was taken to exclude any leaf material from excised coleoptiles. For the preparation of vegetative tissues, surface-sterilized grain was sown in a moist vermiculite/soil mixture and grown at 18°C in a natural day/night cycle (14 h light, 10 h dark) for up to 8 days. Total RNA was isolated from 1 d scutella, 5 d coleoptile, 5 d leaf and 5 d root tissue. Total RNA from 48 h GA_3-treated aleurone layers was kindly provided by Mr. Robert Lee, Department of Plant Science, University of Adelaide.

5.2.3 RNA isolation

Plant material was ground to a fine powder under liquid N_2. Powdered tissue (100 mg) was suspended in 1 ml Trizol solution (Gibco BRL) (Chomczynski and Sacchi, 1987) in a 2 ml plastic tube. After the addition of 0.2 ml chloroform, phases were separated by centrifugation at 12,000 g. Total RNA was recovered from the aqueous phase by the addition of 0.5 ml isopropanol and recovered by centrifugation at 12,000 g. The RNA pellet was washed with 75% ethanol (v/v), centrifuged at 7,500 g, and dried at room temperature. Samples were resuspended in 40 µl DEPC-treated H_2O and frozen at -80°C.

5.2.4 Northern hybridization analyses

Total RNA samples (10 µg) were separated in 1.2% (w/v) agarose gels containing 3 ml 37% (v/v) formaldehyde per 100 ml. Concentrations of RNA samples were determined by absorbance at 260 nm and solutions containing 10 µg were dried in a Speed Vac (Savant). Samples were resuspended in 2 µl 10x MOPS/EDTA buffer, pH 7.0 (Appendix III), and 13.5 µl of a solution of formamide/formaldehyde/H_2O (3.5/10/3.5 by volume). Dissolved samples were incubated at 68°C for 10 min and cooled on ice. Approximately 2 µl RNA loading buffer (Appendix III) was added and the samples were loaded onto a gel that had been pre-run at 60 V for 30 min. RNA size markers were also loaded. Gels were run at 60 V for 30 min, and subsequently at 100 V for approximately 2 h. RNA was transferred to Hybond N^+ nylon membranes (Amersham) as described for DNA transfers (Chapter 4, section 4.2.5), except that HETS transfer buffer (Biotex Laboratories) was used instead of 0.4 M NaOH. Membranes were cross-linked using an UV-Stratalinker (Stratagene) with a total energy of 70,000 kJ/cm². Membranes were probed with cDNAs labelled to at least 10^9 dpm/µg with {α-^{32}P]-dCTP and random sequence nonanucleotides (Feinberg and Vogelstein, 1983) as described in Section 2.2.4. Membranes were pre-hybridized in a solution containing 3 ml 50 x Denhardt's solution, 5 ml 20 x SSPE, 1 ml 10% (w/v) SDS, 2 ml 10 mg/ml carrier DNA, and 9 ml formamide at 42°C for 24h. The pre-hybridization solution was removed and replaced with 2 ml 50 x Denhardt's solution, 5 ml 20 x SSPE, 1 ml 10% (w/v) SDS, 1 ml 10 mg/ml carrier DNA, 9 ml formamide, and 2

ml 25% (w/v) dextran sulphate. The labelled probe was heated at 100°C for 5 min, chilled on ice and added to the hybridization solution. Hybridization was carried out at 42°C for 24 to 48h. Membranes were washed and exposed in a PhosphorImager cassette essentially as described in section 4.2.5. Where possible duplicate membranes were made to facilitate the use of the two barley β-glucan exohydrolase isoenzyme cDNA probes under identical conditions. Photographs were taken of ethidium bromide-stained RNA gels so that loadings could be compared. Following hybridization and washing, signal strength was quantitated by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

5.2.5 Protein extraction

Barley grain (cv. Clipper, 1 kg dry weight) was germinated for 3 or 8 days as described in Section 5.2.2. No microbial contamination was observed during this period. Grains germinated for 3 days were homogenized in an Omni-Mixer (Sorvall, USA) for 3 x 1 min on ice, with 1 min cooling periods, in approximately 2 vol 100 mM sodium acetate buffer, pH 5, containing 10 mM EDTA, 10 mM sodium azide, 3 mM 2-mercaptoethanol and 3 mM phenylmethylsulphonyl fluoride (the extraction buffer). For 8-day seedlings, the coleoptiles, leaves and roots were removed from the grain and independently homogenized for 3 x 1 min as described above. Roots were homogenized for 5x 1 min in the presence of 1g acid-washed sand per 20 g wet weight roots. The homogenates were stirred at 4°C for 120 min to extract enzymes. Insoluble material from the mixtures was removed by centrifugation and by filtration through Miracloth.

Buffer-Soluble Fraction. The soluble extracts were fractionated by precipitation with 85% saturated ammonium sulphate for 2 h at 4°C. Ammonium sulphate precipitates were collected by centrifugation, redissolved in extraction buffer and stored at -20°C prior to further enzyme fractionation.

LiCl-Soluble Fraction. Insoluble material recovered by centrifugation for the initial tissue homogenates was washed once with 50 mM sodium acetate buffer, pH 5.25, containing 50 mM NaCl, once with 10 mM sodium acetate buffer, pH 5.25, containing 5 mM NaCl, once with cold acetone (-20°C) and finally with 50 mM NaCl (Inouhe *et al.,*

1999).   Pellets were recovered by centrifugation and cell-wall bound enzymes were extracted by resuspending the pellets in 50 mM sodium acetate buffer, pH 5.25, containing 3.5 M LiCl and 10 mM NaCl.   The mixtures were incubated with stirring for 24 h at 4°C. The suspensions were centrifuged, pellets were resuspended in the same buffer and incubated with stirring for another 2 h at 4°C.   Following centrifugation, supernatants were stored at -20°C prior to further enzyme fractionation.

CM-Sepharose Chromatography.   To evaluate the levels of β-glucan exohydrolase isoenzymes ExoI and ExoII in various tissues, extracts from 3-day seedlings and from 8-day leaves, coleoptiles, roots and grain were dialyzed in 20 mM sodium acetate buffer, pH 4.9, containing 3 mM 2-mercaptoethanol.   The dialyzed extracts were separated on a column of CM-Sepharose (2.5 x 10 cm), equilibrated in 50 mM sodium acetate buffer, pH 4.9.   After unbound proteins were eluted, a linear gradient (60 ml) of 0-0.5 M NaCl in the same buffer was applied to the column at a linear flow rate of 76 cm.h^{-1}.   Fractions (1-1.5 ml) were tested for activity against (1→3)-β-glucan (laminarin from *Laminaria digitata*, Sigma), barley (1→3,1→4)-β-glucan, and 4-nitrophenyl β-D-glucopyranoside (4-NPG).

Enzyme Analyses.   Enzyme activities against laminarin, barley (1→3,1→4)-β-glucan and 4-NPG were measured as follows.   (1→3)- and (1→3,1→4)-β-Glucanase activities were determined reductometrically by monitoring the increase in reducing sugars (Nelson, 1944; Somogyi, 1952) released in a 0.2% (w/v) solution of laminarin (Sigma) or barley (1→3,1→4)-β-glucan (Megazyme) in 0.1 M sodium acetate buffer, pH 5.0, at 37°C.   One unit of activity is defined as the amount of enzyme required to release 1 μmol of glucose equivalents per min.   Activity on 4-NPG was determined spectrophotometrically at 410nm on a 0.04% (w/v) 4-NPG solution in 50 mM sodium acetate buffer, pH 5.0, at 37°C.   The reaction was stopped by adding 2 vols of 4% (w/v) Na_2CO_3 (Biely *et al.*, 1980). One unit of activity is defined as the amount of enzyme required to release 1 μmol of 4-nitrophenol from 4-NPG per min.   SDS-PAGE was from the procedure of Laemmli (1970) as modified by Hrmova and Fincher (1993).   Protein content was measured by the Coomassie Brilliant Blue reagent (Bradford, 1976), using bovine serum albumin (BSA) as standard.

A summary of the purification procedures is shown in Scheme 5.1.

Germinated Grain (8 days, 21±2° C)

Tissue isolation

Grain   Coleoptiles   Leaves   Roots

Homogenization
Centrifugation (5,000g, 30 min, 4° C)

Pellet (insoluble fraction)

Buffer washes, pH 5.0
Acetone wash (-20° C)
LiCl extraction

Supernatant (buffer soluble fraction)

$(NH_4)_2SO_4$ precipitation

CM-Sepharose (pH 4.9)

Unbound fraction   Bound fraction

Elution with linear
0-0.5 M NaCl gradient

Peak 1   Peak 2

Isoenzyme ExoI   Isoenzyme ExoII

Scheme 5.1 Summary of procedures used to partially purify β-glucan exohydrolase isoenzymes ExoI and ExoII from young barley tissues.

5.3 Results

5.3.1 Tissue-specific transcription of barley β-glucan exohydrolase genes

To study the expression sites of the two β-glucan exohydrolase gene families within barley tissues, total RNA was prepared for Northern hybridization analyses. The Trizol method from Gibco BRL (Chomczynski and Sacchi, 1987)was found to be the most effective for extracting RNA from most barley tissues. Initial Northern hybridization analyses using isoenzyme specific oligonucleotides from both coding and 3'-untranslated regions produced only weak hybridization signals, so further experiments were carried out using the two β-glucan exohydrolase isoenzyme cDNAs as probes. Initial experiments also showed that signal levels produced by barley β-glucan exohydrolase isoenzyme cDNA probe hybridizations on X-ray film were very faint. In subsequent experiments the higher sensitivity PhosphorImager screens and associated equipment from Molecular Dynamics (Sunnyvale, CA, USA) were used.

When total RNA preparations from tissues of young barley seedlings were subjected to Northern hybridization analyses, mRNA transcripts encoding both β-glucan exohydrolases were most abundant in the scutellum and in coleoptiles, but lower levels of mRNA were also detected in young leaves and roots (Figure 5.1). There were no major differences in hybridization intensities between isoenzyme ExoI and isoenzyme ExoII probes in RNA extracts from these tissues, although the isoenzyme ExoII mRNA appeared to be slightly more abundant than isoenzyme ExoI mRNA transcripts in young leaves (Figure 5.1b). In aleurone layers, isoenzyme ExoI mRNA could be detected in low abundance, but isoenzyme ExoII mRNA was not seen.

5.3.2 Developmental regulation of barley β-glucan exohydrolase isoenzymes

In view of the relatively high abundance of both groups of transcripts in scutella and coleoptiles, the development of β-glucan exohydrolase mRNA levels was monitored in these tissues over several days. Scutella were dissected from grain 1-3 days after the
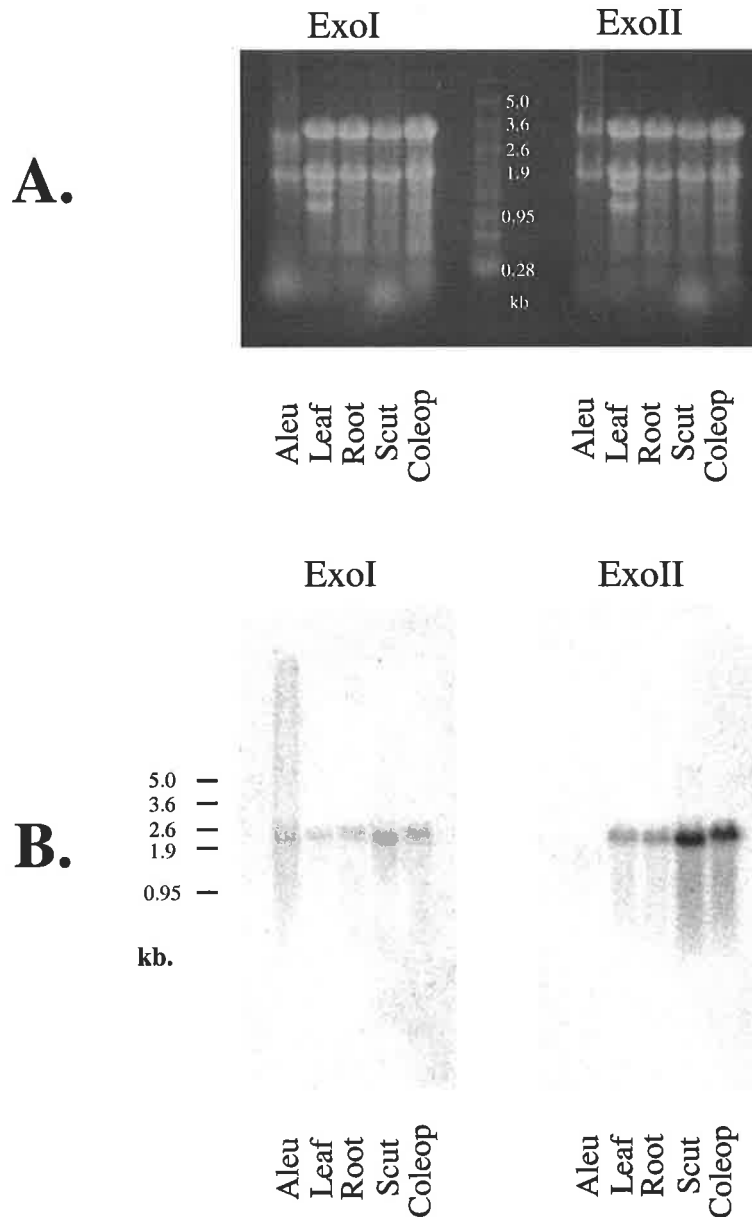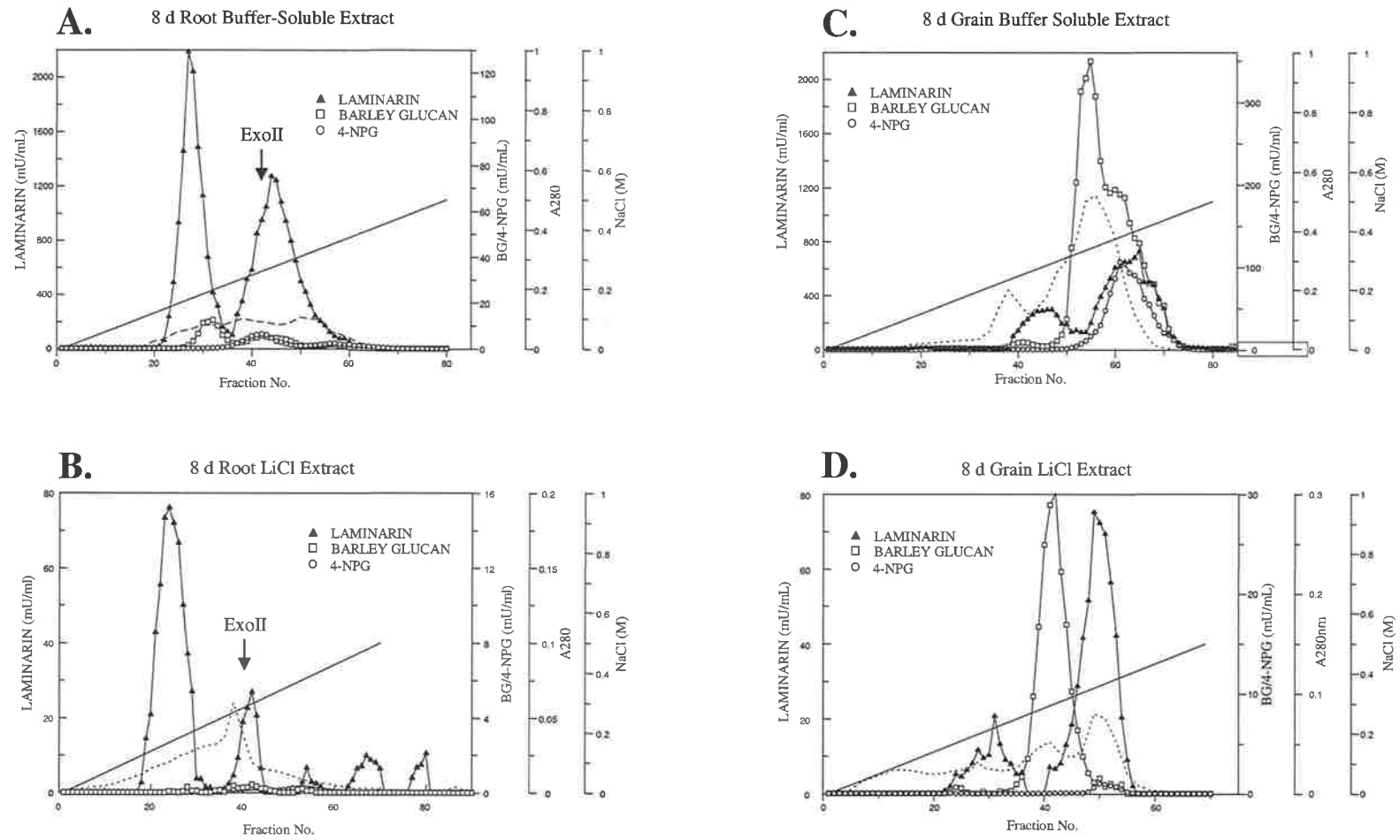
Figure 5.1 Northern hybridization analyses of total RNA preparations (10μg/lane) probed with cDNAs encoding either the barley β-glucan exohydrolase isoenzyme ExoI or isoenzyme ExoII. Panel **A**:. Total RNA preparations separated on a denaturing agarose gel and stained with EtBr. The gel was blotted onto Hybond N+ and the filter was cut in half before probing with separate probes. Gels were stained with EtBr to compare RNA loadings. Panel **B**: Total RNA preparations from isolated aleurone layers after 48 h treatment with GA$_3$ (Aleu), from 5 d leaves, 5 d roots, 1 d scutella (Scut), and from 5 d coleoptiles (Coleop).

initiation of germination, and coleoptiles were harvested 4-6 days after the initiation of germination. In scutella, mRNA detected by both probes reached a maximum at 1-2 days, and thereafter declined (Figure 5.2a). In coleoptiles, isoenzyme ExoI transcript levels appear to increase from 4-6 days, but isoenzyme ExoII mRNA appeared to decrease in abundance over this period (Figure 5.2b).

5.3.3 Expression of barley β-glucan exohydrolase isoenzymes in young vegetative tissues

In attempts to relate apparent mRNA abundance to enzyme activity, extracts of various barley tissues were fractionated on CM-Sepharose (Scheme 1) under conditions used in the initial purification steps of the two barley β-glucan exohydrolases (Hrmova *et al.*, 1996; Hrmova and Fincher, 1998). The CM-Sepharose fractionation is the first step in the purification process where the two isoenzymes ExoI and ExoII are separated (Hrmova and Fincher, 1998). In addition, enzymes were extracted from tissue homogenates using both dilute aqueous buffer and 3.5 M LiCl (Scheme 1). The LiCl extraction procedure has been widely used to extract β-glucan exohydrolases from maize and barley coleoptiles (Huber and Nevins, 1980; Huber and Nevins, 1981; Kotake *et al.*, 1997; Inouhe *et al.*, 1999).

The β-glucan exohydrolases could be detected in the CM-Sepharose eluate by their unique ability to hydrolyze each of the three substrates: 4-NPG, laminarin and barley $(1{\rightarrow}3,1{\rightarrow}4)$-β-glucan (Hrmova and Fincher, 1998). Other enzymes capable of hydrolyzing one of these substrates will undoubtedly be present in the extracts, including barley $(1{\rightarrow}3,1{\rightarrow}4)$-β-glucanases, $(1{\rightarrow}3)$-β-glucanases and β-glucosidases (Woodward and Fincher, 1982b; Hrmova and Fincher, 1993; Leah *et al.*, 1995). However, neither the barley $(1{\rightarrow}3,1{\rightarrow}4)$-β-glucan endohydrolases nor the barley $(1{\rightarrow}3)$-β-glucan endohydrolases can hydrolyze 4-NPG, and barley β-glucosidases are unable to hydrolyze the two polymeric substrates (Hrmova and Fincher, 1998). The barley β-glucan exohydrolases isoenzyme ExoI and isoenzyme ExoII could be distinguished by their characteristic elution volumes on the CM-Sepharose column (Hrmova and Fincher, 1998),

A.   Scutellum Development

B.   Coleoptile Development
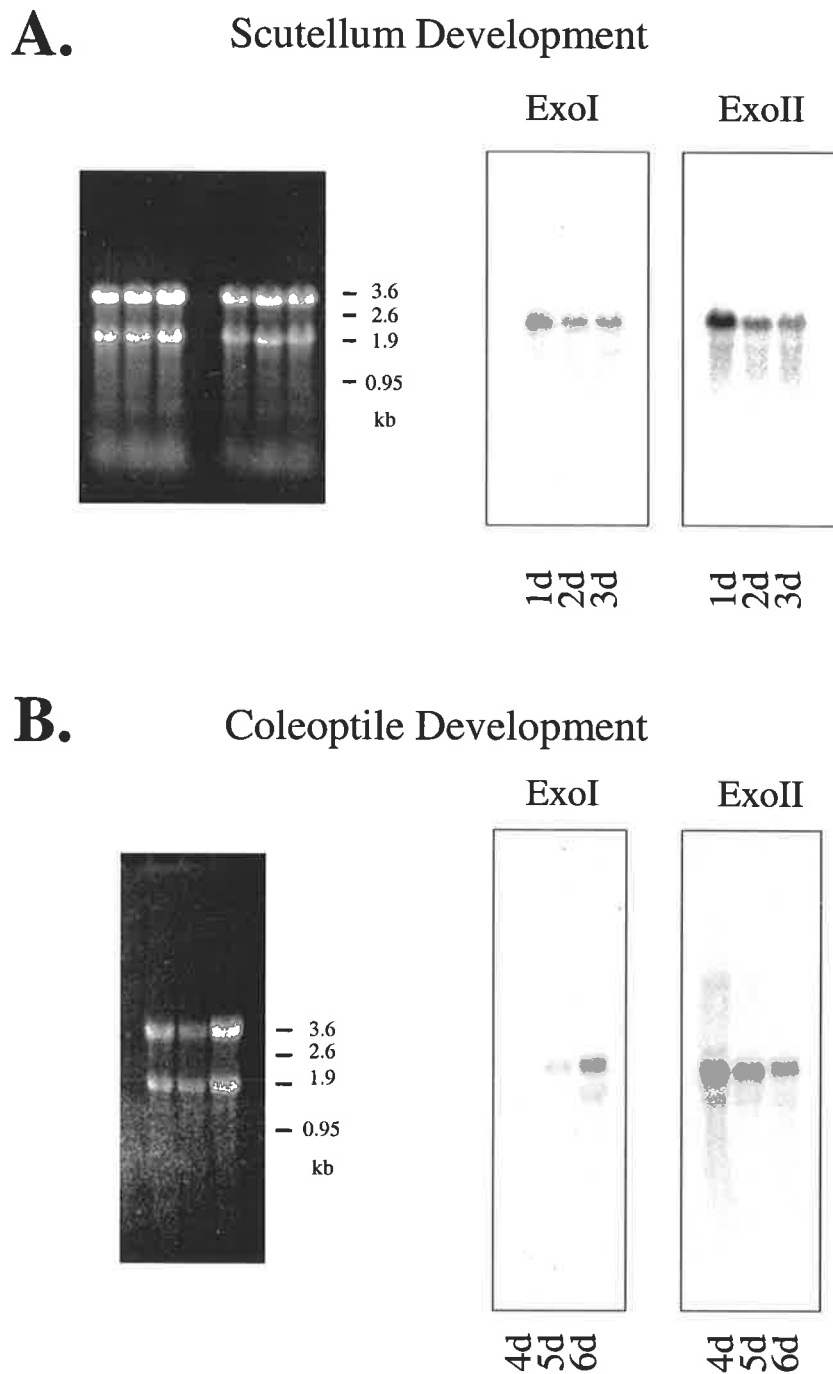
Figure 5.2 Northern hybridization analyses of total RNA preparations (10µg/lane) probed with cDNAs encoding either the barley β-glucan exohydrolase isoenzyme ExoI or isoenzyme ExoII. Panel **A**: Total RNA preparations from scutella isolated after 1, 2, or 3 days after germination, separated, hybridized and probed. Panel **B**: Total RNA preparations from coleoptiles isolated after 4, 5, or 6 days after germination, separated, hybridized and probed. In both **A** & **B** the left hand panels show gels stained with EtBr to ensure approximately equal loading of RNA had occurred.

Figure 5.3 Ion-exchange chromatography of buffer-soluble and LiCl extracts from 8 d coleoptile (A and B) and 8 d leaf (C and D). Dialysed tissue extracts were applied to the CM-Sepharose column and, after removing unbound proteins, retained proteins were eluted with a linear gradient of 0-0.5 M NaCl (—). Fractions (1ml) were assayed for activity against laminarin (▲), barley (1→3,1→4)-β-glucan (□), 4-NPG (○) and protein (----). Note that no activity was detected in the 8d coleoptile LiCl extracts for any of the substrates.

Figure 5.3

A. 8 d Coleoptile Buffer-Soluble Extract

B. 8 day Coleoptile LiCl Extract

C. 8 d Leaf Buffer-Soluble Extract

D. 8 day Leaf LiCl Extract
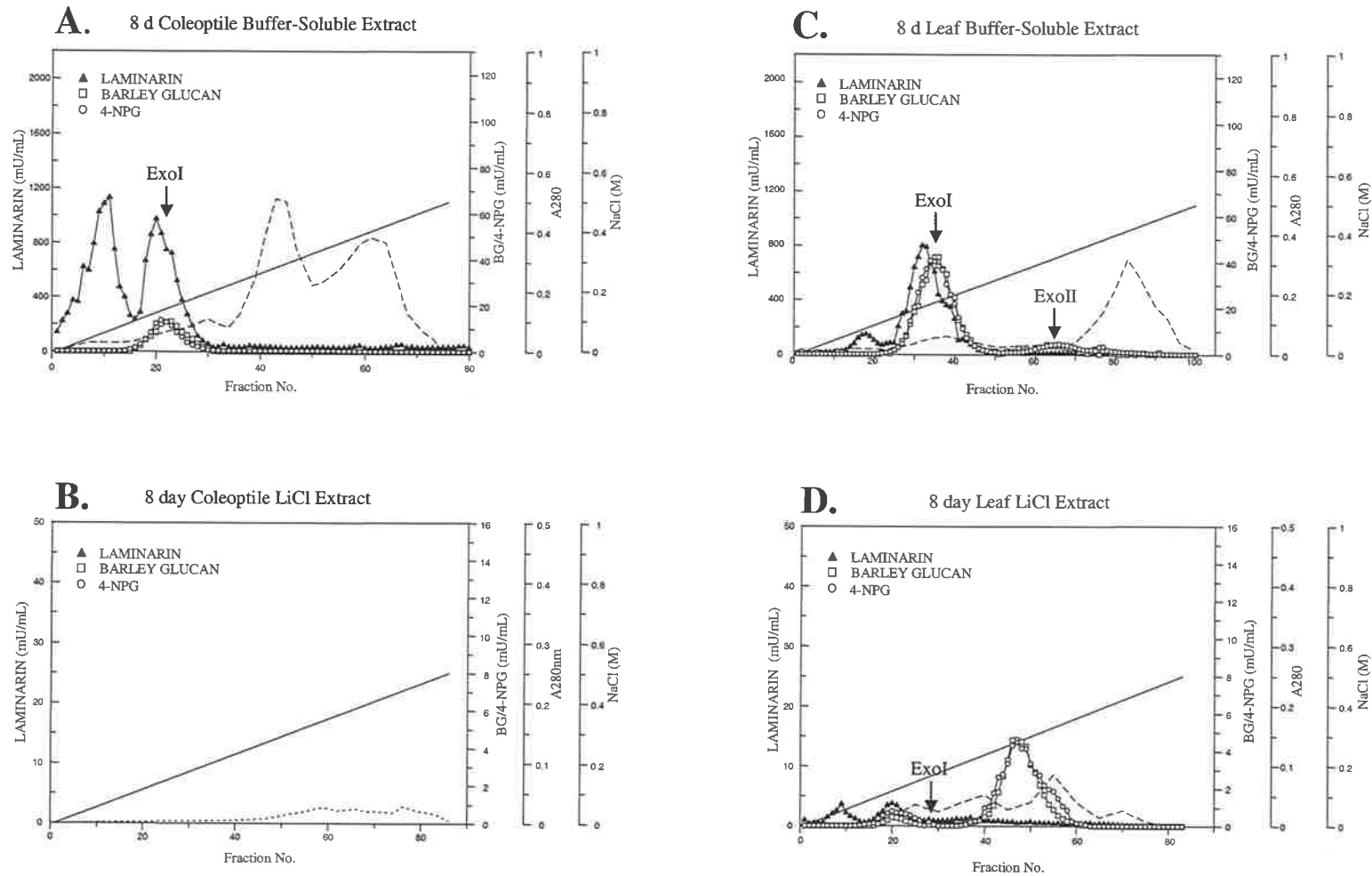
Figure 5.4 Ion-exchange chromatography of buffer-soluble and LiCl extracts from 8 d root (A and B) and 8 d grain (C and D). Dialysed tissue extracts were applied to the CM-Sepharose column and, after removing unbound proteins, retained proteins were eluted with a linear gradient of 0-0.5 M NaCl (—). Fractions (1ml) were assayed for activity against laminarin (▲), barley (1→3,1→4)-β-glucan (□), 4-NPG (○) and protein (----).

A. 8 d Root Buffer-Soluble Extract

▲ LAMINARIN
□ BARLEY GLUCAN
○ 4-NPG

ExoII

B. 8 d Root LiCl Extract

▲ LAMINARIN
□ BARLEY GLUCAN
○ 4-NPG

ExoII

C. 8 d Grain Buffer Soluble Extract

▲ LAMINARIN
□ BARLEY GLUCAN
○ 4-NPG

D. 8 d Grain LiCl Extract

▲ LAMINARIN
□ BARLEY GLUCAN
○ 4-NPG
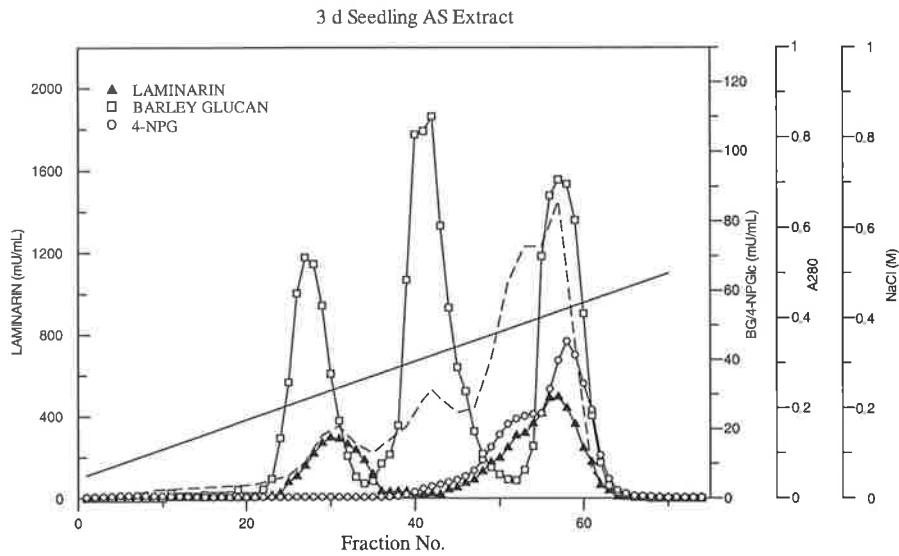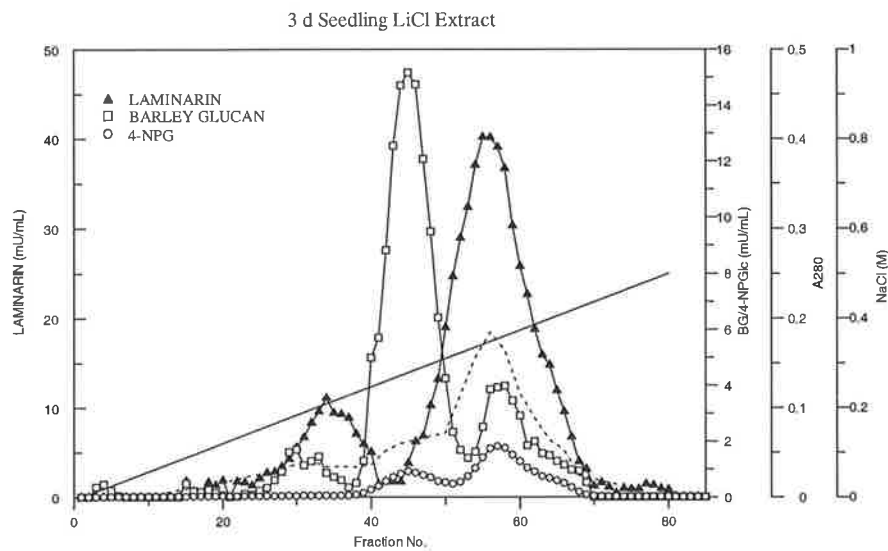
Figure 5.4

Figure 5.5 Ion-exchange chromatography of buffer-soluble extract (A) and LiCl extracts (B) from 3 d seedlings. Dialyzed tissue extracts were applied to the CM-Sepharose column and, after removing unbound proteins, retained proteins were eluted with a linear gradient of 0-0.5 M NaCl (—). Fractions (1ml) were assayed for activity against laminarin (▲), barley (1→3,1→4)-β-glucan (□), 4-NPG (○) and protein (----).

Table 5.1 Yields of enzyme activities and mRNA levels of β-glucan exohydrolase isoenzymes ExoI and ExoII in buffer-soluble and LiCl fractions of 8 d germinated root, leaf and coleoptile extract fractions and 3 d germinated seedling extracts.

		Enzyme activity[1] (mU)		mRNA[2]	
		ExoI	ExoII	ExoI	ExoII
8d Root	Buffer-soluble[3]	0	84	++[5]	++[5]
	LiCl-fraction[4]	0	3		
8d Leaf	Buffer-soluble[3]	682	91	+[5]	++[5]
	LiCl-fraction[4]	5	0		
8d Coleoptile	Buffer-soluble[3]	148	0	++[5]	+++[5]
	LiCl-fraction[4]	0	0		

3d Grain	Buffer-soluble[3]	0	46	+++[6]	++++[6]
	LiCl-fraction[4]	0	17		

[1] Enzyme activity determined against 4-NPG and expressed in mU per integrated area of the peak.

[2] mRNA levels determined from Northern hybridization analysis; ++++ relatively strong signal, +++ intermediate signal , ++ relatively weak signal, + trace (see Figures 5.1 and 5.2).

[3] Activity determined in the buffer-soluble fraction after ammonium sulphate precipitation.

[4] Activity determined in the insoluble fraction after LiCl extraction.

[5] Determined for 6 d leaf, coleoptile and root.

[6] Determined for 1 d scutella.

where isoenzyme ExoI (pI 7.8) is eluted earlier than isoenzyme ExoII (pI 8.0).   CM-Sepharose elution profiles are shown in Figures 5.3, 5.4, and 5.5.   No β-glucan exohydrolase activity was detected in the CM-Sepharose unbound fraction.

Semi-quantitative estimates of isoenzyme ExoI and isoenzyme ExoII activities were obtained by measuring areas of peaks eluted from the CM-Sepharose column, and are presented in Table 5.1.   It must be emphasized that activities were measured in partially purified mixtures and that the values presented in Table 5.1 should be considered as estimates.   Standard errors are likely to be in the vicinity of ±10%.   It should be noted that no β-glucan exohydrolase activity was detected in the CM-Sepharose unbound fraction.

Despite these interpretative constraints, it is clear that activity is highest in extracts from young leaves and coleoptiles, while activity in young roots and germinated grain is lower (Table 5.1).   β-Glucan exohydrolase isoenzyme ExoI is abundant in leaves and coleoptiles, but is not detected in roots or grain.   Isoenzyme ExoII is relatively abundant in roots, leaves and grain, but is not detected in coleoptiles.

Another noteworthy observation is that most activity can be extracted from tissues with 100 mM sodium acetate buffer, pH 5.0 if homogenates are left at 4°C for 2h, and that the addition of 3.5 M LiCl is generally not necessary for enzyme extraction (Table 5.1). Some isoenzyme ExoII was detected in LiCl extracts of germinated grain (Table 5.1).

When enzyme activity is compared with the apparent abundance of mRNA transcripts (Table 5.1), no clear relationships between mRNA levels and activity are evident.   Indeed, mRNA encoding both β-glucan exohydrolase isoenzymes was detected in all tissues tested here, and levels of mRNAs for isoenzymes ExoI and ExoII were similar in these tissues (Table 5.1).   In contrast, large differences in activity attributable to isoenzymes ExoI and ExoII were apparent (Table 5.1).   It might be argued that the mRNAs detected by the probes correspond to barley β-glucan exohydrolase genes other than those encoding isoenzymes ExoI and ExoII, but no other fractions containing β-glucan exohydrolase activity were detected in any column eluates.

5.4 Discussion

Because the barley β-glucan exohydrolase cDNAs that encode isoenzymes ExoI and ExoII are only about 70% identical at the nucleotide level, they could be used as probes in Northern analyses to separately monitor transcriptional activity of the two genes, provided hybridizations were performed at sufficiently high stringency (Figure 5.1). However, before it could be claimed that the cDNAs represented gene-specific probes, genomic Southern hybridization analyses were required to determine the number of β-glucan exohydrolase genes in barley. These analyses, described in Chapter 4, showed that a subset of as few as 2-3 genomic DNA fragments hybridized with the isoenzyme ExoI probe and another subset of as few as 4 different fragments hybridized with the ExoII probe (Chapter 4; Figure 4.7). Some of the multiple fragments could have originated through restriction enzyme hydrolysis of internal sites not present in the cDNAs, but contained within introns of the genes themselves. In extensive studies of β-glucan exohydrolases in tissues of young barley seedlings, Hrmova and co-workers have never detected more than two isoforms (Hrmova *et al.,* 1996; Hrmova and Fincher, 1998), but additional isoforms of the enzyme have been shown to be present (Kotake, 2000). In any case, it is concluded here that the β-glucan exohydrolase isoenzyme ExoI and ExoII cDNAs can be used as subfamily-specific probes to examine transcriptional patterns of the two subsets of genes by Northern hybridization analysis, at least in tissues of young seedlings.

Overall, there were no major differences in levels of mRNA transcripts for the isoenzyme ExoI and ExoII gene subfamilies in the tissues examined (Figure 5.1). Transcript levels detected with both probes were highest in the scutellum of 1-2 day-germinated grain and in 5-day-old coleoptiles (Figure 5.1B). Kim *et al.* (2000) isolated a β-glucan exohydrolase from shoots of maize and used tissue printing and polyclonal antibodies for the β-glucan exohydrolase to show that the expression occurs in the basal regions of the coleoptile and in the growing region of the mesocotyl.

In Chapter 2 the G/C contents of the two cDNAs encoding barley β-glucan exohydrolase isoenzymes ExoI and ExoII were compared, and a dramatic difference in codon usage was shown. It would seem likely that some form of selective pressure has

resulted in the shift of the isoenzyme ExoI gene from a relatively balanced codon usage to the extant form of the gene, in which more than 90% of codons end in G or C. Isoenzyme ExoI mRNA is detected in aleurone layers, but isoenzyme ExoII mRNA is not (Figure 5.1B). Whether or not the bias in codon usage results in alterations in translational efficiency of the mRNA cannot be determined without a robust procedure for quantitating individual isoenzyme levels in extracts of individual tissues. However, the expression of the isoenzyme ExoI gene in the aleurone layer of germinated grain can be reconciled with earlier observations that many genes expressed in aleurone layers in response to GA_3 are characterized by a codon usage biased towards G+C in the wobble base position (Fincher, 1989).

It has been consistently noted that more isoenzyme ExoI can be purified from young barley seedlings than isoenzyme ExoII (Hrmova *et al.*, 1996). Although it is difficult to assign β-glucan exohydrolase activity in tissue extracts to specific isoenzymes, particularly in the presence of a broad range of $(1\rightarrow3)$- and $(1\rightarrow3,1\rightarrow4)$-β-glucan endohydrolases, careful selection of substrates, coupled with the differential elution of the enzymes on CM-Sepharose, has allowed the relative activities of isoenzyme ExoI and isoenzyme ExoII to be estimated (Figures 5.3-5.5, Table 5.1).

These activity measurements showed that isoenzyme ExoI is abundant in leaves and coleoptiles, but could not be detected in roots or grain. In contrast, isoenzyme ExoII activity is found in roots, grain and leaves, but not in coleoptiles (Table 5.1). The fact that mRNA levels for the two enzymes are approximately equal in these tissues (Figure 5.1B; Table 5.1) serves to emphasise again that the relative abundance of mRNA transcripts estimated from Northern hybridization analyses does not necessarily correspond with activities of the enzymes themselves (Table 5.1). It is quite possible that the dramatic differences in codon usage between the isoenzyme ExoI and ExoII mRNAs could account for these discrepancies through markedly different translational efficiencies of the two mRNAs (Fincher, 1989). Similarly, the presence of specific activators (Inouhe and Nevins, 1997a) or inhibitors of the enzymes would also result in an apparent discrepancy between

mRNA levels and enzyme activity. Specific monoclonal antibodies coupled with a precise immunological assay could be useful in further addressing these questions.

An observation that emerged during the isolation of barley β-glucan exohydrolases was that the majority of enzyme activity could be extracted with dilute aqueous buffers, and that 3.5 M LiCl was therefore not required to extract the enzymes from seedling cell walls (Table 5.1). In order to ensure sufficient buffering capacity to retain total enzyme activity in the crude homogenates, we used 100mM sodium acetate buffer. This use of a relatively dilute aqueous buffer can be contrasted to the routine use of buffers containing high salt concentrations to extract this class of enzymes from cereal coleoptile or seedling cell walls (Labrador and Nevins, 1989; Kotake *et al.,* 1997; Kim *et al.,* 2000). Kim *et al.* (2000) reported the isolation of a plasma membrane-bound β-glucan exohydrolase from maize shoots, and attributed the binding to the plasma membrane to a hydrophobic region in the $(\alpha/\beta)_6$-sandwich domain of the enzyme. However, the same hydrophobic region is present in both barley β-glucan exohydrolases. Indeed the barley β-glucan exohydrolase isoenzyme ExoI exhibits an even greater level of hydrophobicity in this area, but can still be extracted with dilute aqueous buffers. Thus it seems unlikely that the β-glucan exohydrolases are plasma membrane-bound, but as extracellular enzymes they could be physically occluded into the cell wall.

The final consideration here is to question whether the tissue locations of β-glucan exohydrolase gene transcription cast any further light on possible functions for the enzymes. We believe that the results presented here, through a process of elimination, do provide important clues on the likely functions of the enzymes. Both barley β-glucan exohydrolase isoenzymes have signal peptides typical of the type required for targeting of nascent enzyme to the endoplasmic reticulum and thence to the extracellular space (Chapter 2, Figures 2.3 and 2.5). Evidence to support a role for the β-glucan exohydrolases in the degradation of (1→3,1→4)-β-glucans in cell walls during starchy endosperm mobilization is not strong, and the low levels of β-glucan exohydrolase mRNA in the aleurone layers of germinated grain would also argue against such a role for the enzyme in germinated grain. Indeed, it has been suggested by various groups that the complete

hydrolysis to glucose of (1→3,1→4)-β-glucooligosaccharides released from wall (1→3,1→4)-β-glucans by endohydrolase action is probably mediated by the β-glucosidase/(1→4-β-glucan glucohydrolase enzymes found in the starchy endosperm of germinated cereal grains (Leah *et al.,* 1995; Hrmova and Fincher, 1998; Hrmova *et al.,* 1998a). It is possible that the β-glucan exohydrolases located in the scutellum are involved in the hydrolysis of (1→3,1→4)-β-glucooligosaccharides that may have been transported there from the starchy endosperm. However, there is no evidence to date for the transport of (1→3,1→4)-β-glucooligosaccharides.

The β-glucan exohydrolases are more likely to function in (1→3,1→4)-β-glucan (or xyloglucan in dicotyledons) turnover or modification in walls of elongating cells, or possibly in senescing tissues. The relative abundance of both enzyme activity and mRNA in elongating coleoptiles would be consistent with such a role (Figure 5.1). Similarly, the observed elongation of scutellar epithelial cells in germinated grain (Nieuwdorp and Buys, 1964) might involve (1→3,1→4)-β-glucan turnover and could explain the transcription of β-glucan exohydrolase genes in that tissue (Figure 5.1). Furthermore, it is not clear how an exohydrolase that hydrolyses the non-reducing terminal glycosidic linkage of its substrates could loosen the non-covalent cross-links between matrix phase wall polysaccharides and cellulose microfibrils. Intuitively, an endohydrolase would appear to be better equipped to play such a role in wall loosening, although both classes of enzymes might be required (Hrmova and Fincher, 1998; Inouhe and Nevins, 1998; Varghese *et al.,* 1999). Perhaps the β-glucan exohydrolases, through their capacity to catalyze glycosyl transfer reactions (Hrmova and Fincher, 1998), could be involved in the "re-construction" of cell wall polysaccharides in much the same way as other cell wall enzymes are believed to alter xyloglucan structure in walls of expanding vegetative tissues (Fry, 1995; Cosgrove, 1999). Comparison of growth kinetic data with the results of Northern analyses using tissues in different developmental stages from scutella and coleoptiles may also be of use in determining whether the β-glucan exohydrolases function in cell elongation, but the required growth kinetics have not yet been done.

Dicotyledons contain no $(1\rightarrow3,1\rightarrow4)$-$\beta$-glucan, yet β-glucan exohydrolase enzymes have been detected in soybean (*Glycine max*) cultures (Cline and Albersheim, 1981), *Acacia verek* cells (Lienart et al., 1986) and nasturtium (*Tropaeolum majus*) (Crombie *et al.*, 1998). Crombie *et al.* (1998) found that a β-glucan exohydrolase from nasturtium can hydrolyse oligoglucosides of the type released from wall xyloglucans by endohydrolases, provided the non-reducing glucosyl residue of the oligosaccharide is not substituted. Xyloglucans have been shown to be present in elongating coleoptiles of maize and barley (Carpita and Gibeaut, 1993; D.M.Gibeaut, pers. comm.), and in the starchy endosperm of rice (Shibuya and Misaki, 1978). Thus, the β-glucan exohydrolases can hydrolyze polymeric $(1\rightarrow3,1\rightarrow4)$-$\beta$-glucans, or oligosaccharides released by endohydrolases from both $(1\rightarrow3,1\rightarrow4)$-$\beta$-glucans and xyloglucans (Hrmova and Fincher, 2000).

In the absence of unequivocal evidence that the β-glucan exohydrolases participate in wall loosening and cell elongation (Cosgrove, 1999), are there other possible functions that might be performed by these enzymes? It has been shown that they participate in the hydrolysis of oligoxyloglucosides during xyloglucan degradation or turnover in dicotyledons (Crombie *et al.*, 1998), so do they actually participate in this role in monocotyledons? Their broad substrate specificity might mean that they are involved in a more general strategy to recover glucose from a range of different classes of polysaccharides or oligosaccharides. They could contribute to the conversion of xyloglucans, glucomannans and $(1\rightarrow3,1\rightarrow4)$-$\beta$-glucans to their constituent monosaccharides.

Another possibility is that the β-glucan exohydrolases are expressed pre-emptively to counter potential pathogen attack in young tissues that are particularly vulnerable to fungal infection. Initial experiments have indicated an up-regulation of β-glucan exohydrolase isoenzyme ExoI gene transcription in barley leaves upon infection with the causal agent of barley scald, *Rhynchosporium secalis* (A.J.Harvey and G.B.Fincher, unpublished data). The enzymes hydrolyze the $(1\rightarrow3)$-β-glucans and $(1\rightarrow3,1\rightarrow6)$-$\beta$-glucans that are found in cell walls of many fungi (Hrmova and Fincher, 1998). They might act in concert with $(1\rightarrow3)$-β-glucan endohydrolases to degrade walls of invading fungi.

At this stage, accumulated evidence favours a role in wall turnover in elongating tissues as the most likely function of the β-glucan exohydrolases in higher plants.

Chapter 6

Summary and Future Directions

6.1 Summary of Work Described Here

The primary aim of the work described in this thesis was to isolate and characterize the cDNAs that correspond to the two β-glucan exohydrolases that have previously been purified from young seedlings of barley (Hrmova *et al.*, 1996; Kotake *et al.*, 1997).

Using a variety of molecular techniques, two cDNAs encoding β-glucan exohydrolases were isolated and characterised, as described in Chapter 2. Amino acid sequences deduced from the cDNAs matched exactly to NH_2-terminal sequence data of the two previously purified β-glucan exohydrolase isoenzymes (Hrmova *et al.*, 1996), and the cDNAs were therefore designated as the corresponding cDNAs of β-glucan exohydrolase isoenzyme ExoI and isoenzyme ExoII. The two β-glucan exohydrolase cDNA sequences were only 71% identical and also revealed differences in their (G+C) content and codon usage. Sequence comparisons of the isolated cDNAs with GenBank and EMBL databases at both the DNA and translated protein level showed that similar glycoside hydrolases occur widely amongst plants, bacteria and fungi.

The identification of glycoside hydrolases through 3D structural models and their classification into family groups rely on the availability of at least one experimentally-determined 3D structure per family. The structure of a family 3 β-glucan exohydrolase from barley, which was one of the isoenzymes studied here, has recently been solved to 2.2 Å resolution by X-ray crystallography (Hrmova *et al.*, 1998; Varghese *et al.*, 1999). Solution of this structure relied on the primary amino acid sequence deduced from the pExoI cDNA described in Chapter 2. This, in turn, allowed the use of comparative protein modelling to examine domain structures within the family 3 group of glycoside hydrolases, to define the disposition of catalytic amino acids and structures of active sites, and to determine local variations in structures across the family (Chapter 3).

Before determining the spatial and temporal expression patterns of genes encoding β-glucan exohydrolases, the number of genes encoding barley β-glucan exohydrolases needed to be defined. In Chapter 4, work undertaken to determine the number of genes encoding

barley β-glucan exohydrolases, both by the isolation and characterization of genomic DNA clones and through Southern hybridization analysis, was described.

It was also of interest to define the chromosomal location of these genes. The location of the genes might provide information on potential linkages to other genes of interest in cell wall metabolism, barley productivity, or quality. The cDNAs encoding the barley β-glucan exohydrolase isoenzymes were used in Southern hybridization analyses to probe wheat-barley addition line DNA for the determination of chromosomal location of the genes. The results of these studies revealed that each barley β-glucan exohydrolase cDNAs bound to approximately 4 DNA fragments, indicating the presence of two distinct sub-families of genes (or pseudo-genes) positioned on at least 3 chromosomes.

Previous studies on β-glucan exohydrolases showed that the enzymes are expressed in coleoptiles, and possibly within other elongating tissues (Inouhe and Nevins, 1997; Inouhe *et al.*, 1999). Studies described in Chapter 5 were focussed on detecting β-glucan exohydrolase isoenzyme mRNA in various tissues from young seedlings. Experiments in which β-glucan exohydrolase cDNAs were used to probe Northern hybridization membranes showed the presence of β-glucan exohydrolase mRNA in scutella, aleurone, coleoptiles, and young roots and leaves. Overall, there were no major differences in levels of mRNA transcripts for the isoenzyme ExoI and ExoII gene subfamilies in the tissues examined. Transcript levels detected with both probes were highest in the scutellum of 1-2 day-germinated grain and in 5-day-old coleoptiles. Only β-glucan exohydrolase isoenzyme ExoI mRNA was detected in extracts of aleurone layers from germinated grain. In an effort to correlate enzyme and mRNA levels, tissue locations of the barley β-glucan exohydrolase isoenzymes themselves were determined using standard protein purification techniques. These studies indicated that there were no clear correlations between the levels of β-glucan exohydrolase enzymes and β-glucan exohydrolase mRNAs, although the lack of gene-specific probes limited results to broad conclusions only.

6.2 Future Directions

Perhaps the most important outstanding question regarding the barley β-glucan exohydrolases relates to their true biological function(s). Background information is still incomplete. Thus, detailed information on the size of the gene family, expression patterns of specific genes, and the actual, *in vivo* substrates(s) of the enzymes is not yet available. Work by Dr. T. Kotake (2000) has shown that there might be more β-glucan exohydrolases in barley seedlings than the two discussed in this thesis. Kotake (2000) was able to purify a third β-glucan exohydrolase from barley seedlings which he designated isoenzyme ExoIII, and he also isolated two partial β-glucan exohydrolase cDNAs than encode putative β-glucan exohydrolases isoenzyme ExoIV and isoenzyme ExoV (Kotake, 2000). This is in agreement with the results of Southern hybridization experiments described in Chapter 4 of this thesis, where the β-glucan exohydrolase isoenzyme ExoI and isoenzyme ExoII cDNA probes bound to a total of approximately 8 DNA fragments. This suggests that there are more β-glucan exohydrolases in barley that have yet to be purified. Complete analysis of the barley β-glucan exohydrolase family would require that all genes be isolated and characterised.

Once all the β-glucan exohydrolase genes have been isolated and characterised, gene-specific probes could be designed. These gene-specific probes could be used to define barley chromosomes on which the β-glucan exohydrolase genes are located. Barley doubled haploid mapping populations could subsequently be used to determine their chromosome map locations (Langridge *et al.*, 1995). The knowledge of the location of the β-glucan exohydrolase genes in the barley genome may be useful for future barley breeding programs where inclusion or exclusion of specific β-glucan exohydrolase genes may be required.

Gene-specific probes would also be useful for the full analysis of barley β-glucan exohydrolase transcription patterns. Gene specific probes may possibly be designed from 3'-untranlated regions, due to the lower level of homology in these regions of mRNAs

from members of the same gene family. Preliminary work for this thesis that attempted to use PCR amplified 3'-untranlated regions as probes to determine mRNA levels failed to detect any β-glucan exohydrolase transcripts in RNA preparations from a range of tissues. This is most likely due to the level of β-glucan exohydrolase transcript being too low. Future attempts at determining β-glucan exohydrolase transcript levels using 3'-untranslated regions of β-glucan exohydrolase cDNAs as probes will require a different methodology than the standard $[\alpha-^{32}P]$-dCTP labelled DNA techniques used in this thesis. Preliminary work using an $[\alpha-^{32}P]$-UTP labelled transcribed RNA probe instead of a DNA probe, gave promising early results (data not shown). This system has the advantage that only complementary strand RNA is transcribed and labelled. This contrasts with the standard random-primed probe synthesis kits, where both strands are labelled. Also, RNA:RNA binding is used in place of the less efficient DNA:RNA hybridization. However, time constraints in the present study precluded further development of the procedures to monitor the expression patterns of all the barley β-glucan exohydrolase genes.

Once a more efficient and precise detection system for specific β-glucan exohydrolase isoenzyme mRNAs had been determined, spatial and temporal studies on β-glucan exohydrolase gene transcription could be examined more comprehensively. Studies could then be carried out on the effect of plant growth regulators, such as auxin, on β-glucan exohydrolase transcript levels. As previously noted, auxin stimulates elongation by increasing the mechanical extensibility of cell walls in a process known as cell wall loosening. In the Poaceae, this involves changes to wall (1→3,1→4)-β-glucans, arabinoxylans and xyloglucans (Masuda and Yamamoto, 1985; Masuda, 1990; Hoson, 1991; Sakurai, 1991; Hoson, 1993). In elongating barley coleoptiles, application of the auxin analogue IAA leads to a decrease of (1→3,1→4)-β-glucan content of walls over a six-hour period (Sakurai and Masuda, 1978). Preliminary studies using barley β-glucan exohydrolase isoenzyme ExoI and isoenzyme ExoII cDNAs as probes have shown that IAA has little or no effect on β-glucan exohydrolase transcript levels in barley coleoptiles

(A.J.Harvey and G.B.Fincher, unpublished data). This is surprising, because β-glucan exohydrolases have been linked to cell elongation (Huber and Nevins, 1981). However, work by Inouhe *et al.* (1999) has also shown that β-glucan exohydrolase enzyme levels in coleoptiles of maize are unaffected by the addition of IAA, even though auxin-induced elongation is evident. Analyses of the effects of other plant growth regulators such as GA_3, ABA, cytokinins, and various hormone combinations may also be of interest.

Further studies need to be carried out on the ability of β-glucan exohydrolases to hydrolyse a variety of different substrates. Hrmova and Fincher (1998) have shown that β-glucan exohydrolases are able to hydrolyse $(1→3)$-β-glucan from laminarin and $(1→3, 1→4)$-β-glucan from barley. Crombie *et al.* (1998) noted that a β-glucan exohydrolase (designated a β-glucosidase) from nasturtium can hydrolyse oligoglucosides of the type released from wall xyloglucans by endohydrolases, provided the non-reducing glucosyl residue of the oligosaccharide is not substituted. Due to the high level of amino acid sequence identity between the barley and nasturtium enzymes (71-77%), it would be expected that the barley enzyme could likewise hydrolyse xyloglucan oligosaccharides but this needs to be tested. Another potential substrate that needs to be tested is glucomannan, where $(1→4)$-β-linked glucosyl and mannosyl units are arranged essentially at random in an unbranched backbone (Piro *et al.*, 1993).

Once the background information of the type described above is available, emphasis could be moved to the functional analysis of individual enzymes. The cloning of two barley β-glucan exohydrolase cDNAs described in this thesis has enabled the possibility of producing the β-glucan exohydrolase isoenzymes through heterologous expression in foreign hosts such as bacteria and yeast. This provides an opportunity to extend our understanding of many aspects of the structure and function of β-glucan exohydrolases. To begin with, expressing only the first domain of the protein, the $(\alpha/\beta)_8$-barrel, would be useful to determine if this domain alone is catalytically competent (Varghese *et al.*, 1998). The function of the second domain, the $(\alpha/\beta)_6$-sandwich, can also be investigated by expressing it separately. The $(\alpha/\beta)_6$-sandwich is unlikely to be catalytically active on its

own, however binding studies with oligo- or polysaccharides would be of interest because this domain might function to attach the enzyme to cell walls (Hrmova and Fincher, 1998). Mutation of the putative carbohydrate-binding region on domain 2 to see whether it binds $(1 \rightarrow 3)$-β-glucan, $(1 \rightarrow 3, 1 \rightarrow 4)$-β-glucan, and/or xyloglucan oligosaccharides may also be useful. Deletion of the long, conserved COOH-terminal loop could test its significance with respect to catalytic rates, substrate binding, protein folding, and thermostability.

Further studies using point mutations of the β-glucan exohydrolases could be carried out in order to study substrate binding and catalytic activity. For example, if the catalytic nucleophile D285 was mutated to N285 there would be minimal structural changes, but would all catalytic activity be lost as expected? Likewise, if the putative catalytic acid E491 were altered to Q491, what would happen to catalytic activity? The two other acidic amino acids in the active site, E220 and D95, could be changed to neutral amino acids and the rate of reaction checked again. There are a number of changes that could be effected with these amino acids that may alter catalytic activity and give us a greater insight to the mechanism of action of the β-glucan exohydrolases. If the two 'coin-slot' trytophan amino acids at the mouth of the active site, W286 and W434 (Varghese *et al.*, 1999), which appear to guide the substrate into the active site, were changed to amino acids with short, non-aromatic sidechains, how would this affect substrate binding and catalytic activity? Would this also change the substrate specificity of the β-glucan exohydrolases? Would it be possible to engineer an enzyme with an altered substrate specificity? Numerous other single and multiple amino acid mutations are possible in order to gain further knowledge on the binding, mode of action, and catalytic activity of β-glucan exohydrolases in heterologous expression systems.

Recently, rapid advances have been made in the transformation of 'foreign' genes into plants using a variety of methods, either to knock out the expression of a gene ("loss of function") or to insert a gene that was not present before ("gain of function"). One such method is virus-induced gene silencing (Burton *et al.*, 2000) which uses antisense technology to knock out translation of specific genes. If the β-glucan exohydrolase cDNAs were inserted into barley plants via a virus-induced gene silencing or *Agrobacterium-*

mediated infection, gene knockout may give morphological changes that could help identify or at least suggest the roles of β-glucan exohydrolases. Transformed plants could also be challenged with infectious agents to see if pathogenesis-related responses have been altered.

Appendices

Appendix I : Solutions and Media

50x Denhardt's solution

1% (w/v) Ficoll, 1% (w/v) polyvinylpyrrolidone, 1% (w/v) BSA

20x SSC

3 M NaCl, 0.3 M sodium citrate (pH 7.0)

20x SSPE

3 M NaCl, 0.2 M NaH_2PO_4, 20 mM EDTA

SM

50 mM Tris-HCl buffer, pH 7.5, containing 10 mM NaCl, 8 mM $MgSO_4$, 0.01% gelatin

10x TAE

0.4 M Tris-acetate buffer, pH 8.0, containing 10 mM EDTA

10x TE

0.1 M Tris-HCl buffer, pH 8.0, containing 10 mM EDTA

LB media

1% (w/v) Bacto-tryptone, 0.5% (w/v) Bacto-yeast extract, 0.5% (w/v) NaCl

NZY media

0.5% (w/v) Bacto-yeast extract, 0.2% (w/v) $MgSO_4.7H_2O$, 0.5% (w/v) NaCl, 1% (w/v) NZ amine (casein hydrolysate)

2 x YT media

1.6% (w/v) Bacto-tryptone, 1% (w/v) Bacto-yeast extract, 0.5% (w/v) NaCl

Appendix II : Cross-Reactivity Southern Hybridization Analysis

To test for cross-reactivity between the two barley β-glucan exohydrolase cDNAs, a Southern hybridization Hybond-N+ nylon membrane was prepared containing plasmid DNA from both cDNA constructs. The membrane was probed with [α-^{32}P]-radiolabelled β-glucan exohydrolase isoenzyme ExoI cDNA following the methods described in Chapter 2, sections 2.2.3 and 2.2.4. The membrane was washed in 2x SSC, 0.1% SDS for 20 min once and twice in 0.1x SSC, 0.1% SDS in 65°C for 20 min. It was then exposed to X-ray film.

ExoII   ExoI

A. B. C.   D. E.
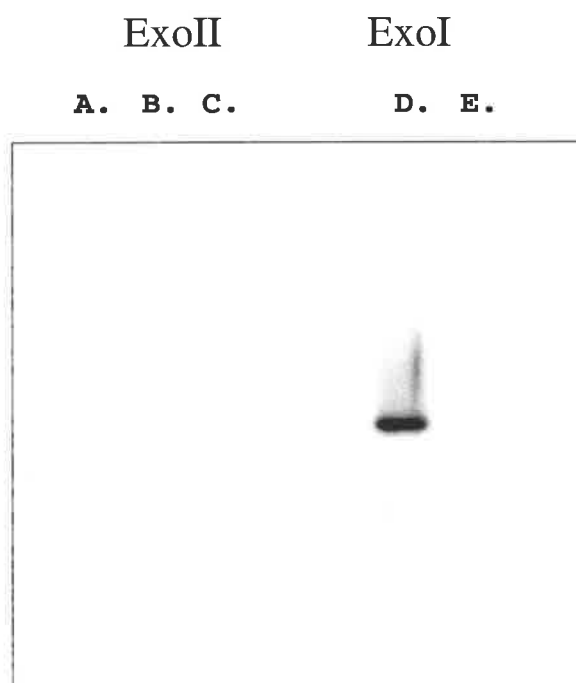
A. = 1μg pExoII undigested
B. = 1μg pExoII digested with KpnI and PvuI
C. = 1ng pExoII undigested
D. = 1μg pExoI undigested
E. = 1ng pExoI undigested

Appendix III : RNA Isolation and Analysis Associated Solutions

10x MOPS/EDTA Buffer

0.5 M MOPS buffer, pH 7.0, containing 10 mM EDTA

RNA Buffer A

294 µl 10x MOPS/EDTA buffer, pH 7.0, and 706 µl DEPC-treated H_2O

RNA Gel Loading Buffer

322 µl RNA Buffer A, 5 mg xylene cyanol, 5 mg bromocresol green, 178 µl 37% formaldehyde, 500 µl formamide, and 400 mg sucrose

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