



**STRUCTURE, HORMONAL REGULATION AND  
CHROMOSOMAL LOCATION OF GENES  
ENCODING BARLEY (1→4)- $\beta$ -XYLAN  
ENDOXYDROLASES**

by

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## STATEMENT OF AUTHORSHIP

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## Publications

The following publications have arisen from work described in this thesis.

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## ABBREVIATIONS

A	Absorbance
aa	Amino acid
ABA	Abscisic acid
ABRE	Abscisic acid response element
Amp	Ampicillin
AMV	Avian myeloblastosis virus
ATP	Adenosine triphosphate
bp	Base pair
BSA	Bovine serum albumin
°C	Degrees Celsius
cDNA	Complementary DNA
Ci	Curie
cpm	Counts per minute
CTAB	Cetyl-trimethylammonium bromide
CTP	Cytidine triphosphate
dATP	2'-Deoxyadenosine triphosphate
dCTP	2'-Deoxycytidine triphosphate
dGTP	2'-Deoxyguanosine triphosphate
DEAE	Diethylaminoethyl
DEPC	Diethyl pyrocarbonate
Dimethyl POPOP	1,4-Bis [2(4-methyl-5-phenyl-oxazoly)] benzene
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTPs	Deoxyribonucleotide triphosphates
DP	Degree of polymerization
DTT	Dithiothreitol

dTTP	2'-Deoxythymidine triphosphate
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
ER	Endoplasmic reticulum
g	Gram
GA	Gibberellic acid
GARC	Gibberellic acid response complex
GARE	Gibberellic acid response element
h	Hour
HCA	Hydrophobic cluster analyses
IPTG	Isopropylthio- $\beta$ -D-galactoside
kb	Kilobases
kDa	Kilodalton
LB medium	Luria-Bertani medium
M	Molar
mA	Milliampere
mg	Milligram
$\mu$ g	Microgram
min	Minute
ml	Milliliter
mRNA	Messenger ribonucleic acid
$\mu$ M	Micromolar
M-MLV	Moloney murine leukaemia virus
MOPS	3-[N-Morpholino]propane-sulfonic acid
$M_r$	Molecular mass
NaPPi	Sodium pyrophosphate
NCF	Nitrocellulose filter
nm	Nanometer

nt	Nucleotide
4-NP	4-Nitrophenol
NTES	Sodium chloride Tris ethylenediaminetetraacetic acid Sodium dodecyl sulphate
Oligo(dT)	Oligodeoxythymidylic acid
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
Pers. comm.	Personal communication
pET	Plasmid for expression by T7 polymerase
pfu	Plaque forming unit
pI	Isoelectric point
PMSF	Phenyl methyl sulphonyl fluoride
Poly(A)	Polyadenylic acid
pp	Pages
PPO	2,5-Diphenyloxazole
PVP	Polyvinylpyrrolidone
RACE	Rapid amplification of complementary DNA ends
RFLP	Restriction-fragment-length polymorphism
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
RT	Reverse transcriptase
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SSC	Sodium saline citrate
SSPE	Sodium saline phosphate EDTA
TAE	Tris-acetate EDTA
TBE	Tris-borate EDTA
TCA	Trichloroacetic acid

TE	Tris EDTA
TLC	Thin layer chromatography
TSP	Transcription start point
UV	Ultraviolet
Vol	Volume
v/v	Volume/volume
w/v	Weight/volume
X-gal	5-Bromo-4-chloro-3-indoyl- $\beta$ -D-galactoside
(1 $\rightarrow$ 4)- $\beta$ -xylanase	(1 $\rightarrow$ 4)- $\beta$ -xylan endohydrolase

## ABSTRACT

Arabinoxylans and (1→3,1→4)-β-D-glucans represent up to 95% of barley endosperm cell walls by weight. After germination, cell walls constitute a physical barrier that limits the access of hydrolytic enzymes secreted from the aleurone layer or the scutellum to their substrates within cells of the starchy endosperm. Degradation of cell wall arabinoxylans to their constituent monosaccharides in germinated barley is mediated, in part at least, by the action of (1→4)-β-xylan endohydrolases (EC 3.2.1.8). In the present study, a cDNA of 1.3 kb in length was amplified from poly(A)<sup>+</sup>-RNA isolated from gibberellic acid-treated barley aleurone layers using 3' RACE-PCR (Rapid Amplification Complementary DNA Ends-polymerase chain reaction). The NH<sub>2</sub>-terminal amino acid sequence deduced from the cDNA showed almost complete identity with the NH<sub>2</sub>-terminal sequence previously determined from purified barley (1→4)-β-xylanase itself.

The PCR product was used to isolate two cDNAs encoding different (1→4)-β-xylanases from cDNA libraries constructed from gibberellic acid-treated barley aleurone layers and 5-day old seedlings. The (1→4)-β-xylanase isoenzyme X-I cDNA is a near full-length cDNA which encodes a polypeptide containing 427 amino acid residues; this includes a putative signal peptide of 32 amino acids. The deduced polypeptide sequence of the mature enzyme has two putative glycosylation sites and contains 395 amino acid residues. It has a calculated M<sub>r</sub> of approximately 44,600, an isoelectric point of 6.1, and is likely to adopt an (α/β)<sub>8</sub>-barrel conformation. The cDNA for isoenzyme X-II is truncated at its 3' end and shows approximately 13% divergence in its deduced amino acid sequence compared with isoenzyme X-I. The cDNAs show a sequence identity of 91% at the nucleotide level. This indicates that the (1→4)-β-xylanase cDNAs are derived from two separate genes. Both enzymes exhibit sequence and structural similarities with microbial xylanases.

In attempts to obtain large quantities of pure (1→4)-β-xylanase protein, the near full-length cDNA for isoenzyme X-I was expressed in the heterologous host *E. coli* using

the plasmid pET 14-b expression vector. The protein was expressed at high levels, but was deposited in insoluble aggregates. No xylanase activity could be detected, even after the partial solubilization of the inclusion bodies.

Examination of the expression patterns of the (1→4)-β-xylanase genes in barley was based on measuring levels of (1→4)-β-xylanase mRNA by Northern blot analyses. These analyses showed that expression of the (1→4)-β-xylanase genes in germinated barley grain appeared to be confined largely to the aleurone layer. No mRNA transcripts could be detected in either young or mature vegetative tissues. Treatment of aleurone layers with gibberellic acid, abscisic acid or both, indicated that gibberellic acid induces both transcriptional activity of the gene and the secretion of active enzyme from the layers. Abscisic acid abolishes the gibberellic acid induction of (1→4)-β-xylanase gene expression.

A gene encoding (1→4)-β-xylan endohydrolase isoenzyme X-I was isolated from a barley genomic library and the nucleotide sequence of a 2704 bp fragment defined. The gene contains a single intron of 91 bp in the coding region of the mature enzyme, but additional introns might be present in the 5'-untranslated region. Promoter sequences and *cis*-acting elements previously identified as components of gibberellic acid response complexes in other plant genes were detected within 200 bp of the putative transcription start site. The *cis*-acting elements include a pyrimidine box CTCTTTCC, together with TAACGAC and TATCCAT boxes. The hormonal responses described above were consistent with the presence of this gibberellic acid response complex.

Genomic Southern blots indicated that the barley (1→4)-β-xylanase enzymes are encoded by a family of at least 3 separate genes. Analysis of six barley cultivars demonstrated that polymorphism exists between the cultivars, and this has been used in a separate study to incorporate the genes into high density genetic maps. A genomic blot of wheat-barley addition lines revealed that the three genes encoding barley (1→4)-β-xylan endohydrolase isoenzymes are all located on barley chromosome 7 (5H).

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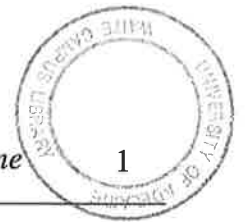
## APPENDICES

**Appendix 1: Solutions**

**Appendix 2: Nutrient media**

# **CHAPTER I**

## **GENERAL INTRODUCTION**



Barley (*Hordeum vulgare* L.) is a commercially important cereal that has been used for thousands of years for the production of alcoholic beverages, for human consumption and as an important constituent of animal feed. It is a monocotyledonous plant which is classified in the Triticeae tribe of the family Poaceae in the order Poales (Bothmer *et al.*, 1991). One of the distinguishing features of the Poales, even within the Monocotyledonae, is the unusual composition of their cell walls (Carpita, 1996). Although cell walls only account for about 5% by weight of barley grains, constituent polysaccharides of the walls exert a disproportionately large influence during the commercial utilization of barley grain, particularly in the brewing, distilling and stockfeed industries (Fincher, 1992). As a result, there has been considerable research attention directed towards the analysis of cell walls in barley grain and towards hydrolytic enzymes that depolymerize the major polysaccharide components of the walls in the germinated grain. The most important enzymes in wall degradation in germinated grains are probably the (1→3,1→4)-β-glucan endohydrolases and the family of enzymes that hydrolyse arabinoxylans. While the (1→3,1→4)-β-glucanases have been characterized in detail and their genes have been cloned (Høj and Fincher, 1995), much less information is available on the enzymes which hydrolyse the arabinoxylans. The isolation and characterization of the gene(s) and corresponding cDNAs encoding barley (1→4)-β-D-xylan endohydrolases were the major objectives of this study.

In this Chapter the anatomy, morphology and composition of the barley grain are reviewed, together with the structural and physiological changes which accompany reserve mobilization after germination. In addition, the role of arabinoxylans in the cell wall and the characteristics of enzymes responsible for arabinoxylan degradation are outlined. Finally, the specific objectives of the work described in this thesis are listed.

## 1.1 Barley grain anatomy and composition

The barley grain consists of two major structural components, the embryo and the endosperm (Figure 1.1). These grain components are surrounded by a testa, the remnants of the pericarp and the fused, dead tissue of the palea and lemma. The latter are referred to collectively as the husk. Harvest-ripe, mature barley grains contain approximately 80% carbohydrate, 10% protein, 3% lipid, and 2% minerals (Duffus and Cochrane, 1993). Oligosaccharides, mainly raffinose and low molecular weight fructans, represent about 2% of the dry weight, as does sucrose.

At the basal end of the grain is the embryo, which contains living tissues and is diploid in nature. The embryo consists of an apical meristem (plumule), sheathed by the coleoptile, and the primary rootlet (radicle), which is protected by the coleorhiza (Briggs, 1978, 1992). Following germination, the plumule and radicle give rise to the young shoots and roots, respectively. The embryo represents only about 2.5% of grain weight. It is rich in protein (34%), sugars (sucrose, 15%; raffinose, 5-10%), some fructans, lipids (14-17%) and ash (5-10%). The embryo contains more free sugar than the endosperm and is therefore well-supplied with a readily available source of energy to support its initial metabolic activities during germination and growth (Briggs, 1992).

An important component of the embryo is the scutellum, a shield-shaped tissue which is situated between the embryonic axis and the endosperm. The scutellum is made up mainly of thin-walled, cuboid parenchymatous cells. However, at the interface with the endosperm it has a single-celled layer of palisade-type columnar epithelium cells (Briggs, 1992). The scutellar epithelium cells contain a nucleus, mitochondria, ribosomes and endoplasmic reticulum (ER) (Nieuwdorp and Buys, 1964; Swift and O'Brien, 1972 a, b) and are packed with phytin-containing protein bodies which serve as a source of amino acids and phosphate during and following germination (Swift and O'Brien, 1972 a, b; Smart and O'Brien, 1979 a, b, c; Nieuwdorp, 1963; Aisien *et al.*, 1986).

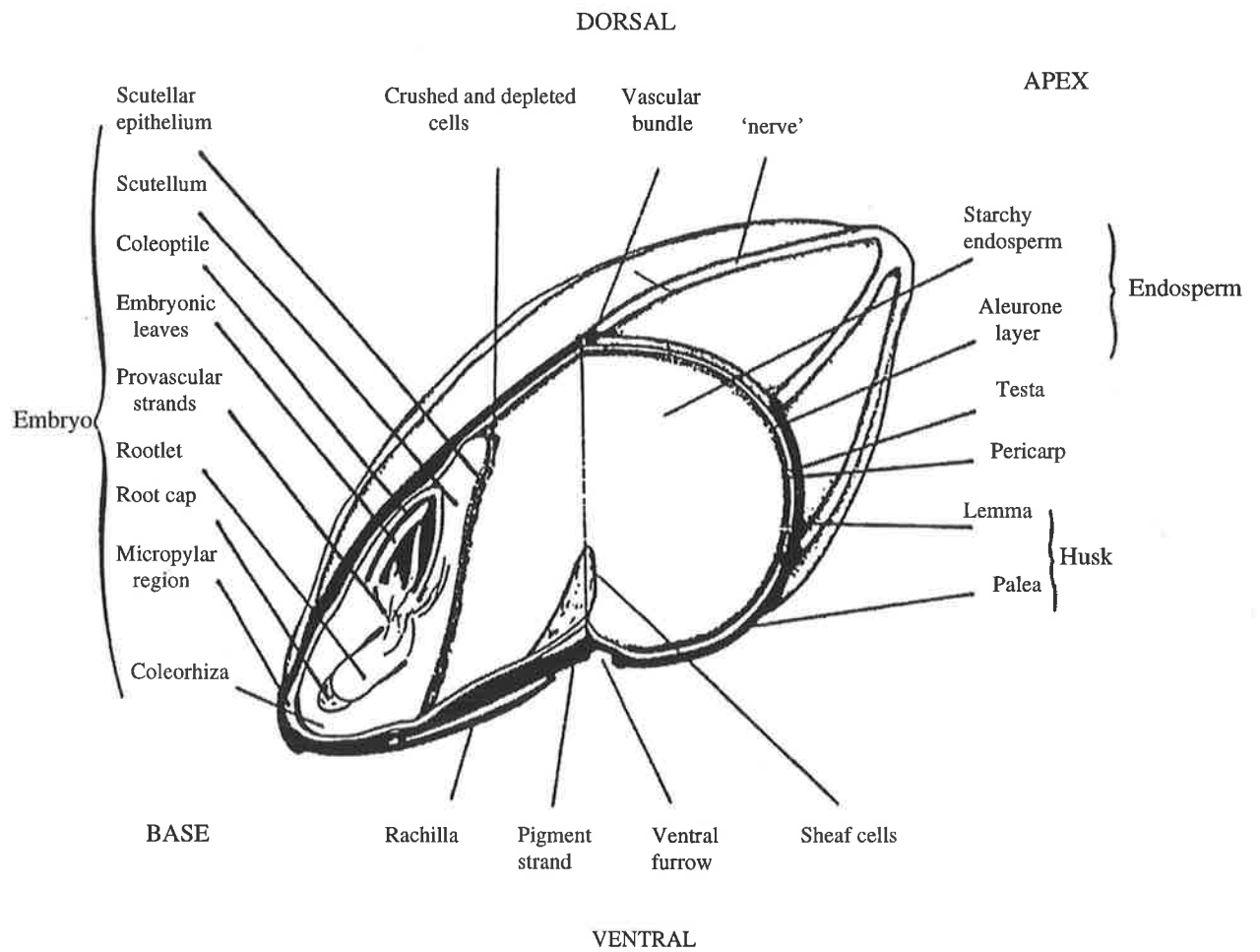


Figure 1.1 A schematic drawing of a barley grain (redrawn from Briggs, 1978)



The other major component of the barley grain is the endosperm. This tissue is the primary site of nutrient storage in the grain and consists of two morphologically and functionally distinct components; the aleurone layer and the starchy endosperm. The starchy endosperm is surrounded by the aleurone layer, except in the region of the scutellum. The aleurone consists of a thin layer of living cells 50-110  $\mu\text{m}$  in diameter; the cells are roughly cuboid and are separated by thick cell walls in which there are numerous plasmodesmata. The tissue contains no starch but is rich in protein (17-20%), triacylglycerols (20%), minerals and sugars (including sucrose, raffinose and probably stachyose, verbascose and fructans) (Briggs, 1992). Mature aleurone layer cells contain nuclei, mitochondria and endoplasmic reticulum (ER), and are packed with specialized protein bodies called aleurone grains. The aleurone grains contain proteins which are relatively rich in basic amino acids. Embedded in the aleurone grains are deposits of both phytin and niacytin (Bacic and Stone, 1981 a; Fulcher *et al.*, 1977). Thus, the aleurone layer serves as a store of amino acids, phosphate, niacin, sugars and minerals which are subsequently mobilized for the generation of the enzymes necessary for endosperm mobilization. In addition, the aleurone has the cellular machinery necessary for protein synthesis and secretion (Fincher, 1989).

The non-living starchy endosperm is the main storage tissue in the mature barley grain and constitutes over 75% of the grain by weight (Briggs, 1992). The starchy endosperm consists of thin-walled cells which are tightly packed with starch granules embedded in a proteinaceous matrix (Bewley and Black, 1983; Briggs, 1978). Starch, which itself constitutes 58-65% grain dry weight, is almost exclusively located in starchy endosperm cells (Briggs, 1992). Polar lipids occur within starch granules and appear to contribute to their structural characteristics (Morrison, 1988). In addition, the starchy endosperm contains approximately 9% (w/w) protein (Briggs, 1992). Proteins in the starchy endosperm of harvest-ripe barley consist of approximately 30% hordein, 30%

glutenin and 10% globulin. The remaining 10% is composed of albumins and free amino acids (Brandt, 1976).

## 1.2 Composition and organization of cell walls

Primary cell walls in higher plants are polysaccharide-rich extracellular structures which are generally composed of cellulosic fibres embedded in a matrix of non-cellulosic polysaccharides and proteins. The cellulose is organized into bundles of approximately three dozen linear chains of (1→4)- $\beta$ -glucan; these aggregates constitute the cylindrical microfibrils that can be observed under the electron microscope (Darvill *et al.*, 1980; Carpita, 1996). The cellulosic microfibrils in most higher plant cell walls are closely associated with adherent xyloglucan and/or glucuronoarabinoxylan chains and form an extensive, reinforcing network throughout the wall (Albersheim *et al.*, 1994). The matrix phase of the cell wall consists variously of glucuronoarabinoxylan, xyloglucans, (1→3,1→4)- $\beta$ -glucans and pectic polysaccharides (Fincher and Stone, 1986; Bacic *et al.*, 1988; Albersheim *et al.*, 1994; Carpita, 1996). Structural protein, including hydroxyproline-, threonine-hydroxyproline- and glycine-rich glycoproteins, are widely distributed constituents of the matrix phase and might provide a second network to strengthen or fortify fully elongated or differentiated cells (Albersheim *et al.*, 1994; Cassab and Varner, 1988; Carpita, 1996). Walls also contain enzymic proteins that modify cell wall components during normal growth and development or in the face of biotic and abiotic stress (Fry, 1995).

As mentioned at the beginning of this Chapter, cell walls of the Poales have several important features that distinguish them from those of the Dicotyledonae and other Monocotyledonae. These differences include the relative abundance of glucuronoarabinoxylans and the exclusive occurrence of the (1→3,1→4)- $\beta$ -glucans in the Poales (Bacic *et al.*, 1988; Carpita, 1996). Xyloglucans are much less abundant and

important differences are observed in the fine structure of the pectic polysaccharides (Carpita, 1996). Furthermore, primary cell walls of the Poales are enriched in ferulate and *p*-coumarate esters which are attached to the C(O)5 atom of arabinosyl residues in glucuronoarabinoxylans (Rudall and Caddick, 1994).

Walls of barley endosperm cells, including those of both the aleurone and the starchy endosperm, exhibit specialized compositions that are clearly related to their function in the grain. Levels of cellulose are low, as might be expected in non load-bearing primary walls that must be rapidly degraded in the germinated grain. Endosperm walls are composed predominantly of (1→3,1→4)-β-glucans and arabinoxylans; the latter are associated with esterified ferulic acid and some *p*-coumaric acid, but are low in glucuronic acid residues (Fincher, 1975, 1976).

Although detailed chemical and physical data are available on the (1→3,1→4)-β-glucan and arabinoxylan fractions of cereal grain cell walls, the molecular organization and interactions of these components in the wall matrix and at the matrix-microfibril interface have not been clearly defined (Woodward *et al.*, 1983). Several complex models involving both covalent and non-covalent cross-linking of the polysaccharide and protein constituents of cell walls have been proposed (Fincher and Stone, 1986; Fry, 1995; Albersheim *et al.*, 1994; Gibeaut and Carpita, 1994; Carpita, 1996), together with the suggestion that (1→3,1→4)-β-glucans and arabinoxylans form a non-covalent, three-dimensional gel network with all the properties of porosity, elasticity and mechanical strength required for cell wall function (Fincher and Stone, 1986).

The arabinoxylans and (1→3,1→4)-β-glucans together account for up to 95% of polysaccharides in cell walls of the aleurone layer and starchy endosperm of the mature barley grain. The walls also contain small amounts of cellulose and glucomannan, but pectin, xyloglucan, lignin and hydroxyproline-rich glycoproteins have not been reported. A substantial proportion of the protein present in cell wall preparations is believed to be of cytoplasmic origin (Fincher, 1975; Bacic and Stone, 1981 b). The compositions of cell

walls from the aleurone layer and starchy endosperm of mature barley grain, and from vegetative tissues are compared in Table 1.1. The striking difference between walls in the endosperm is that those of the starchy endosperm cells contain approximately 70% (1→3,1→4)-β-glucan and 20% arabinoxylan, whereas the aleurone layer cell walls contain approximately 26% (1→3,1→4)-β-glucan and 67% arabinoxylan (Fincher, 1975; Bacic and Stone, 1981 b).

In contrast to the walls of the endosperm, walls of vegetative tissues of young barley seedlings are comprised of 55-65% cellulose and may contain some lignin, in keeping with the requirement of the load-bearing cell walls in these tissues to provide greater structural rigidity than is needed in the starchy endosperm of the germinated grain (Bacic *et al.*, 1988; Fincher, 1992) (Table 1.1). In addition to (1→3,1→4)-β-glucan and arabinoxylan, which constitute only 30-40% of these walls, pectic polysaccharides and xyloglucan may also be present (Fincher, 1992).

*(1→3,1→4)-β-Glucan structure:* The (1→3,1→4)-β-glucan component of barley endosperm cell walls consists of linear chains of glucopyranosyl residues polymerized through (1→3)- and (1→4)-β-linkages. These glucan molecules are heterogeneous with respect to size, solubility and molecular structure (Bacic and Stone, 1981 b; Woodward and Fincher, 1983). They are unbranched polymers containing approximately 70% (1→4)- and 30% (1→3)-linked β-glucosyl residues arranged predominantly in blocks of two or three (1→4)-β-linkages separated by single (1→3)-linkages (Parrish *et al.*, 1960; Woodward and Fincher, 1983). In addition, blocks of up to 10-12 contiguous (1→4)-β-glucosyl residues are found and account for approximately 10% by weight of the polysaccharide (Woodward and Fincher, 1983). The major structural features of (1→3,1→4)-β-glucan molecules found in cell walls can therefore be represented as follows:

Table 1.2 Characteristics of xylan endohydrolases purified from different microorganisms

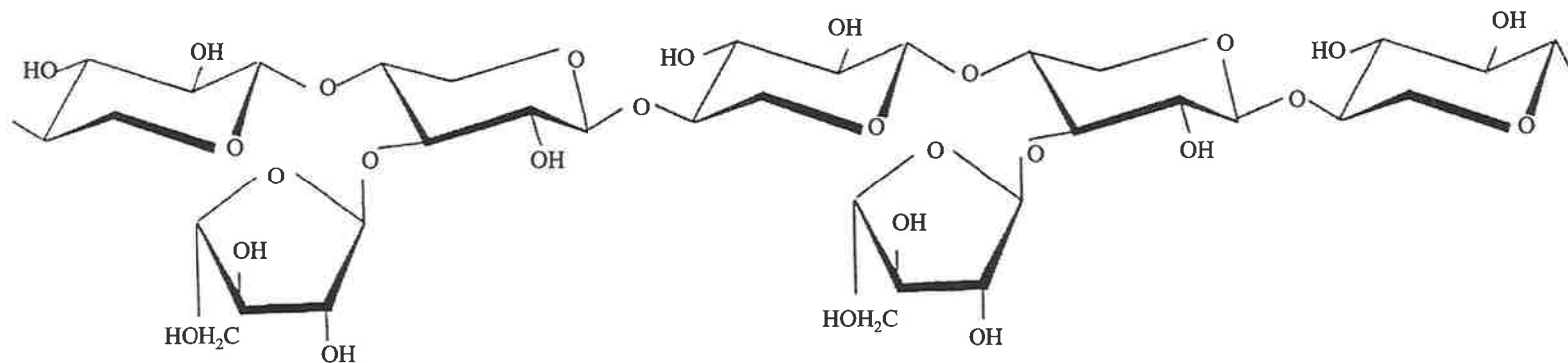
Source	Molecular weight (kDa)	Optimum pH	Isoelectric point (pI)	References
<i>Fibrobactor succinogenes</i> S85	53.7 66.0	7.0 6.3	8.9 8.0	Matte and Forsberg (1992)
<i>Penicillium Chrysogenum</i>	35.0	6.0	4.2	Haas <i>et al.</i> (1992)
<i>Aspergillus nidulans</i>	34.0	6.0	3.4	Fernández-Espinar <i>et al.</i> (1994)
<i>Piromyces</i>	12.5	6.0	9.1	Teunissen (1993)
<i>Actinomycete microtetraspora flexuosa</i>	26.3 16.8	8.4 9.45	6.0	Berens <i>et al.</i> (1996)
<i>Bacillus sp strain bp-23</i>	32.0	5.5	9.3	Blanco <i>et al.</i> (1995)
<i>Aspergillus nidulans</i>	22.0	5.5	6.4	Fernández-Espinar (1993)
<i>Talaromyces emersonii</i>	74.85 54.2	4.2 3.5	5.3 4.2	Tuohy <i>et al.</i> (1993)

non-red-----G4G3G4G4G3G4G4G4G3G4G4G3G----- red.

In the above diagram,  $\beta$ -glucopyranosyl residues are represented as G, the numbers 3 and 4 denote (1 $\rightarrow$ 3)- and (1 $\rightarrow$ 4)- $\beta$ -linkages respectively, and "non-red" and "red" denote the non-reducing and reducing termini of the polysaccharide chain.

*Arabinoxylan structure:* Arabinoxylans consist predominantly of the two pentoses, arabinose and xylose, and are commonly referred to as pentosans (Figure 1.2). However, cereal arabinoxylans sometimes contain hexoses and hexuronic acids as minor constituents and if this is the case they are more precisely referred to as heteroxylans or glucuronoarabinoxylans (Fincher and Stone, 1986). The arabinoxylans in the starchy endosperm cell walls of barley contain linear chains of (1 $\rightarrow$ 4)- $\beta$ -xylanopyranosyl residues, with one or two attached  $\alpha$ -L-arabinofuranose residues (Fincher and Stone, 1986; Viëtor *et al.*, 1993; MacGregor and Fincher, 1993; Cleemput *et al.*, 1995). The main chain of the arabinoxylan is analogous to that of cellulose, but is composed of (1 $\rightarrow$ 4)-linked- $\beta$ -xylosyl instead of  $\beta$ -glucosyl residues. Substituents consist mainly of  $\alpha$ -L-arabinosyl residues linked to the C(O)3 position of xylosyl residues; some arabinosyl residues are linked to the C(O)2 position and some xylosyl residues are substituted with arabinosyl residues at both C(O)2 and C(O)3.  $\alpha$ -D-Glucuronopyranosyl residues and their 4-methyl ethers constitute up to 4% by weight of the arabinoxylan from the husk of barley grains and are linked to the C(O)2 position of xylosyl residues. However, substituents other than arabinose have not been detected in the arabinoxylan in walls of barley endosperm cells (Aspinall and Ross, 1963; Fincher, 1975; Ballance *et al.*, 1986).

Phenolic acids such as ferulic acid and *p*-coumaric acid are also associated with arabinoxylans, where they are usually esterified to C(O)5 of arabinosyl residues. The phenolic acids constitute approximately 0.05% of starchy endosperm walls in barley grain and 1.2% of barley aleurone walls (Bacic and Stone, 1981 a; Fincher, 1976; Gubler *et al.*, 1985; Mueller-Harvey *et al.*, 1986; Viëtor *et al.*, 1993).



**Figure 1.2 Structure of cereal arabinoxylans.** Molecules consist of a linear (1→4)-β-xylosyl backbone which is substituted at the O-2 and/or O-3 positions of xylosyl residues with α-arabinofuranosyl residues. Ferulic acid residues are sometimes esterified to the O-5 of arabinofuranosyl residues (MacGregor and Fincher, 1993)

The degree of arabinosyl substitution in cell wall arabinoxylans in plants depends on the tissue location and the source of the polysaccharides (Carpita, 1996). Similarly, arabinoxylans in barley vary widely with respect to their xylose to arabinose ratios. For example, ratios of approximately 9:1 are observed in heteroxylans from husks (Aspinall and Ferrier, 1957), whereas in arabinoxylan preparations from the starchy endosperm or aleurone layer walls, ratios of 1:1 to 2.3:1 are normally found (Fincher, 1975; McNeil *et al.*, 1975; Bacic and Stone, 1981 b; Ballance and Manners, 1978; Ballance *et al.*, 1986).

*Organization of polysaccharides within the walls:* McNeil *et al.* (1975) have attempted to describe the interactions between arabinoxylans and cellulose within walls of the barley aleurone layer using *in vitro* cellulose-binding experiments. Assuming that arabinoxylans bind non-covalently to cellulose *in vivo*, it appears likely that any binding would be controlled by the degree of arabinosyl substitution. The results of the cellulose-binding experiments demonstrate that arabinoxylans require regions of at least four contiguous, unbranched (1→4)-β-xylosyl residues for binding to occur (McNeil *et al.*, 1975). Thus, the unsubstituted regions of the arabinoxylan may be able to form non-covalent associations with the cellulosic chain, and the interaction is likely to be stabilized by the formation of extensive hydrogen bonding of the type found in cellulosic microfibrils. The regions of arabinoxylan/cellulose association have been referred to as “junction zones” (Rees and Welsh, 1977; Dea, 1979). It seems likely that arabinoxylans could bind not only to cellulose, but also to (1→3,1→4)-β-glucans and other arabinoxylan molecules, to form a two component gel that would constitute the matrix phase of the primary cell wall (Fincher and Stone, 1986). It has been postulated that a decrease in the number of arabinosyl substituents in cell-wall arabinoxylans would facilitate the formation of junction zones (Fincher and Stone, 1986) and that the strength and porosity of the cell wall matrix could therefore depend on the number and arrangement of arabinosyl substituents along the xylan backbone.



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Although the binding of arabinoxylans in barley endosperm cell walls probably relies mainly on non-covalent interactions, covalent cross-linking of wall polysaccharides is also possible (Fincher, 1986; Fry and Miller, 1989; Carpita, 1996). It has been suggested that ferulic acid substituents are involved in the formation of firm gels by oxidative dimerization of ferulic acid residues and the attendant cross-linkage of arabinoxylan chains (Izydorczyk *et al.*, 1990; Geissman and Neukom, 1973). The ferulic acid residues detected in barley endosperm cell walls might therefore act as a covalent cross-linking agent between arabinoxylan chains or in polysaccharide/protein interactions in the cell wall matrix (Markwalder and Neukom, 1976; Fincher and Stone, 1986; Izydorczyk *et al.*, 1990). However, the existence of such diferulic acid cross-links in plant cell walls has not always been rigorously demonstrated.

### **1.3 Physiological changes following germination of the barley grain**

Germination of viable, non-dormant barley grain is initiated by the uptake of water, which mostly enters through the micropylar region and subsequently penetrates to the interior of the grain (Briggs, 1992). When water is taken up, the grain swells as the embryo and endosperm become turgid. The first visible sign of the completion of germination is the emergence of the coleorhiza from the base of the grain. The coleoptile then breaks through the testa and grows up the dorsal side of the grain (Briggs, 1978; Bewley and Black, 1994). Following germination, the reserves of the starchy endosperm are mobilized and their breakdown products are translocated to support the growth and development of the embryo and young seedling. The mobilization process is effected by a battery of enzymes, many of which are secreted into the starchy endosperm by the aleurone layer and the scutellar epithelium. Some enzymes pre-exist in the starchy endosperm of the mature grain and are released or activated after germination (Fincher, 1989). In the sections below, the changes observed in the aleurone layer and scutellum of

germinated grain are related to functions of these tissues in starchy endosperm mobilization, and regulation of enzyme synthesis in aleurone layers and scutellar cells is briefly reviewed.

### ***1.3.1 Changes in the scutellum***

Following germination, dramatic ultrastructural changes occur in the scutellum. The scutellar epithelial cells elongate to approximately twice their original length (Smart and O'Brien, 1979 a). The outer wall of the scutellar epithelium is digested and a relatively thin inner wall layer remains. This results in the formation of extended, "papillae-like" cells with a greatly enhanced surface area for absorption of starchy endosperm degradation products. The protein bodies and associated phytin inclusions of the scutellar epithelium dissolve and appear to be converted to large vacuoles (Gram, 1982), whilst lipid bodies gradually disappear. Mitochondria become active, and well-defined ER and Golgi bodies appear (Nieuwdorp and Buys, 1964; Swift and O'Brien, 1972 a, b; Gram, 1982).

While it is generally accepted that the aleurone layer is the major source of hydrolytic enzymes following grain germination, the scutellum is also thought to contribute to the secretion of enzymes into the starchy endosperm, particularly in the early stages of endosperm mobilization (Fincher and Stone, 1986). Thus, the scutellum serves several functions after germination. One major function is believed to be the synthesis and secretion of the phytohormone gibberellic acid (GA). This hormone diffuses to the aleurone layer, where it initiates the expression of specific genes (Fincher and Stone, 1993; Bewley and Black, 1994; Jacobsen *et al.*, 1995).

A second function of the scutellum, as indicated above, is to synthesize and secrete hydrolytic enzymes. These enzymes participate in the early stage of the depolymerization of starchy endosperm reserves. Starch and cell wall degradation in germinated barley initially occurs adjacent to the scutellar epithelium (Briggs, 1972; Gibbons, 1979, 1980,

1981; Okamoto *et al.*, 1980; Gram, 1982; Stuart *et al.*, 1986 a; McFadden *et al.*, 1988; Bewley and Black, 1994). Compared with the aleurone layer, the scutellum synthesizes a relatively large amount of (1→3,1→4)-β-glucanase isoenzyme EI (Stuart *et al.*, 1986 a; Slakeski and Fincher, 1992 a) and carboxypeptidase I (Mundy *et al.*, 1985; Ranki *et al.*, 1990), but relatively small amounts (5-10% of total) α-amylase (Mundy *et al.*, 1985; Stuart *et al.*, 1986 a; Ranki, 1990; Jacobsen *et al.*, 1995). The scutellum produces α-amylase for 1-2 days, whereas aleurone layer production of α-amylase peaks after 3-4 days (Jacobsen *et al.*, 1995).

The third role of the scutellum during reserve mobilization is to absorb degradation products from the starchy endosperm and translocate them to the growing embryo, and this function is clearly facilitated by the increase in surface area of scutellar epithelium cells described above.

### ***1.3.2 Changes in the aleurone layer***

The aleurone layer is probably the most important source of hydrolytic enzymes for starchy endosperm degradation. Several research groups have shown that isolated aleurone layers secrete α-amylase (Chrispeels and Varner, 1967; Filner and Varner, 1967), (1→3,1→4)-β-glucanase (Stuart *et al.*, 1986 a; Slakeski and Fincher, 1992 a), xylan endohydrolase, α-arabinofuranosidase, β-xylosidase (Dashek and Chrispeels, 1977; Slade *et al.*, 1989), limit dextrinase (Hardie, 1975; X. Zhang, R. Burton and G. B. Fincher, unpublished data), α-glucosidase (Hardie, 1975; Tibbot and Skadsen, 1996), peroxidase (Gubler and Ashford, 1983), (1→3)-β-glucanases (Taiz and Jones, 1970) and nuclease I (Chrispeels and Varner, 1967). Thus, the aleurone layer secretes a battery of enzymes that is capable of depolymerizing starch, protein, cell wall polysaccharides and residual nucleic acids that are stored in the starchy endosperm.

Cells of the aleurone layer exhibit ultrastructural changes in keeping with their primary role of synthesizing and secreting hydrolytic enzymes. Within the aleurone layer cells, the protein bodies and their inclusions of phytin and niacytin gradually disappear, and the protein bodies are transformed into vacuoles which occupy a large proportion of the cell (Van Der Eb and Nieuwdorp, 1967). Furthermore, there is a proliferation of ER, lipid bodies diminish in number, mitochondria become highly active (Van Der Eb and Nieuwdorp, 1967; Gram, 1982) and Golgi bodies are more abundant (Fernandez and Staehelin, 1985; Heupke and Robinson, 1985; Gubler *et al.*, 1986). It is clear, therefore, that reserve proteins, lipids, niacytin and phytin are mobilized during the generation of the protein-synthesizing machinery that becomes very active after germination.

### ***1.3.3 Reserve mobilization in the starchy endosperm***

The scutellum and aleurone layer both participate in endosperm mobilization and dissolution patterns reflect a temporally and spatially coordinated secretion of hydrolytic enzymes from these specialized tissues that surround the starchy endosperm. Enzymes secreted from the scutellar epithelium initially degrade the starchy endosperm adjacent to the scutellum. However, after about 1 day the aleurone layer becomes the principal source of hydrolytic enzymes (Gibbons, 1981; McFadden *et al.*, 1988). As a result, barley endosperm dissolution begins in the region immediately adjacent to the scutellum and progresses towards the distal end of the grain as a front that is approximately parallel to the face of the scutellar epithelium (Briggs and MacDonald, 1983; Briggs, 1992; Fincher and Stone, 1993; Bewley and Black, 1994).

Three major phases involved in the mobilization of the starchy endosperm reserves can be identified:

- degradation of the aleurone layer and starchy endosperm cell walls;
- depolymerization of proteins, starch and other storage reserves;

- translocation of the degradation products to the growing embryo.

*Degradation of cell walls:* Because hydrolytic enzymes are secreted, in large part, from tissues which surround the starchy endosperm, and because the cell walls of the endosperm are not likely to be sufficiently porous to allow free diffusion of enzymes of the size found in germinated grain (Carpita *et al.*, 1979; Tepfer and Taylor, 1981; Fincher, 1992), the barrier presented by the cell walls must first be removed so that the starch- and protein-degrading enzymes can reach their target substrates within the starchy endosperm cells. Thus, cell wall degradation is an important event which is essential for the efficient mobilization of starchy endosperm reserves. The mode of action of the major enzymes involved in cell wall breakdown are described in detail in section 1.4.

*Starch degradation:* Starch is degraded to glucose and small oligosaccharides by the combined action of  $\alpha$ -amylases,  $\beta$ -amylases, limit dextrinases and  $\alpha$ -glucosidases.  $\alpha$ -Amylases (EC 3.2.1.1) are endohydrolases that cleave internal (1 $\rightarrow$ 4)- $\alpha$ -glucosyl linkages of amylose or amylopectin in an essentially random fashion. The  $\alpha$ -amylases synthesized in germinated barley grain are Ca<sup>2+</sup>-dependent.  $\alpha$ -Amylases are classified into two groups on the basis of their isoelectric points; low pI isoforms (AMY1, pI 4.5 to 5.0) and high pI isoforms (AMY2, pI 5.9 to 6.4) (Callis and Ho, 1983). A third group, AMY3 (pI 6.5), is comprised of high pI isoforms complexed with an  $\alpha$ -amylase inhibitor (MacGregor and MacGregor, 1987). Immunocytochemical studies show that the initial production of  $\alpha$ -amylase occurs in the region of the scutellum (Briggs and MacDonald, 1983; Gibbons, 1981), but represents a small proportion of the total  $\alpha$ -amylase in the grain. Most  $\alpha$ -amylase is synthesized in the aleurone layer (Ranki, 1990; Chandler and Mosleth, 1990; MacGregor *et al.*, 1984). MacGregor and Marchylo (1986) have suggested that low-pI  $\alpha$ -amylases are predominant in barley embryos.

$\beta$ -Amylases (EC 3.2.1.2) are exohydrolases that cleave the penultimate (1 $\rightarrow$ 4)- $\alpha$ -linkage from the non-reducing end of (1 $\rightarrow$ 4)- $\alpha$ -glucans to release maltose. In contrast to  $\alpha$ -amylase,  $\beta$ -amylase is preformed in mature, quiescent barley grains and little or none is synthesized following germination. Instead, the  $\beta$ -amylases are released from the bound state, probably through the action of peptidases (Sopanen and Lauriere, 1989; Guerin *et al.*, 1991).  $\beta$ -Amylases are unable to hydrolyze intact starch granules. However, they act synergistically with  $\alpha$ -amylases to degrade the granules following the secretion of the endohydrolases from the scutellum or aleurone layer (Maeda *et al.*, 1978; Sissons *et al.*, 1992).

Limit dextrinases (EC 3.2.1.41) are endohydrolases which hydrolyze branch point (1 $\rightarrow$ 6)- $\alpha$ -linkages in amylopectin or in branched (1 $\rightarrow$ 4,1 $\rightarrow$ 6)- $\alpha$ -oligoglucosides. Limit dextrinases are detected in low amounts in mature dry grains of barley (Manners and Hardie, 1977), and increase following germination (Lee and Pyler, 1984). Although purified limit dextrinase has no action on starch granules, it enhances the rate of granule dissolution when added to a mixture of  $\alpha$ - and  $\beta$ -amylases (Maeda *et al.*, 1978; Sissons *et al.*, 1992).

$\alpha$ -Glucosidases (EC 3.2.1.20) are exohydrolases which liberate glucose from polymeric  $\alpha$ -glucans and  $\alpha$ -oligoglucosides. Barley  $\alpha$ -glucosidases have been reported to initiate the hydrolysis of native starch granules *in vitro* at a rate comparable to  $\alpha$ -amylase (Sun and Henson, 1990, 1991). Barley  $\alpha$ -glucosidase is present during early grain development and decreases during late maturation (MacGregor and Lenoir, 1987; Stark and Yin, 1987). Very low amounts are present in the aleurone layer, pericarp and embryo of ungerminated grain (Jorgensen, 1965; MacGregor and Lenoir, 1987; Watson and Novellie, 1974). After germination, activity increases about six-fold (Jorgensen, 1965) and this new activity is found in the scutellum, aleurone layer, starchy endosperm and embryo (MacGregor and Lenoir, 1987; Clutterbuck and Briggs, 1973; Hardie, 1975; Jorgensen, 1965; Stark and Yin 1987). Thus, the concerted action of  $\alpha$ -amylases,  $\beta$ -

amylases, limit dextrinases, and  $\alpha$ -glucosidases allows the complete depolymerization of both amylopectin and amylose to glucose.

*Protein degradation:* The starchy endosperm contains about two thirds of the total protein reserves of the barley grain (Mikola and Kolehmainen, 1972). Levels of active peptidases in the resting grain are low, and are thought to result mainly from the activity of the enzymes necessary for the mobilization of the aleurone layer or scutellar reserves (Fincher, 1989). In germinated grain, several endo- and exo-peptidases are responsible for the mobilization of the storage proteins. The peptidases either pre-exist in the starchy endosperm and are activated after germination or are secreted by the aleurone layer and scutellum during and following grain germination (Mikola and Kolehmainen, 1972; Mikola, 1983, 1987). The endopeptidases are important in the initial solubilization of reserve protein and provide shorter peptide substrates for carboxypeptidases (Jones and Poulle, 1988). Cysteine endopeptidases are the most abundant endopeptidases, accounting for approximately 90% of the proteolytic activity in the germinated grain. Some cysteine endopeptidases are secreted from isolated aleurone layers (Hammerton and Ho, 1986). Serine carboxypeptidases are the major exo-peptidases involved in protein degradation in the starchy endosperm (Mikola, 1987), and five serine carboxypeptidases (EC 3.4.16.1) with different substrate specificities have been identified in germinated barley (Mikola, 1983). Final products of the action of endo- and exo-peptidases include free amino acids and small peptides of two or three amino acid residues. These di- and tri-peptides are rapidly transported into the scutellum and converted to free amino acids (Sopanen, 1979; Higgins and Payne, 1981; Payne and Walker-Smith, 1987).

Along with the starch- and protein-degrading enzymes, other hydrolytic enzymes such as phosphatases, nucleosidases, nucleases and phosphodiesterases are also released to salvage the constituents of residual DNA and RNA in the starchy endosperm (Chrispeels

and Varner, 1967; Taiz and Starks, 1977; Lee and Pyler, 1985, 1986, McFadden *et al.*, 1988).

#### ***1.3.4 Hormonal regulation of enzyme synthesis***

During and following germination, gibberellins (GA) are synthesized in the embryo and diffuse to the aleurone layer, where they trigger the synthesis and secretion of several hydrolytic enzymes (Paleg, 1960; Yomo, 1960; Chrispeels and Varner, 1967; Jacobsen, 1983). GA<sub>3</sub> is widely accepted to be the principal hormone involved in the initiation of hydrolase synthesis. It is released from isolated barley embryos (Radley, 1967) and is capable of stimulating the production of hydrolytic enzymes in aleurone layers isolated from barley and from other cereal grains (Paleg, 1960; Yomo, 1960; Chrispeels and Varner, 1967). There are high levels of specific GAs present in developing grain and these may therefore have an additional role to play in grain development. The GA content of the barley grain decreases during maturation and dry grain contains very low amounts of GAs. The GA content of the grain rises again during and following germination, GA<sub>1</sub> being the predominant type in mature barley; low amounts of other GAs (GA<sub>3</sub>, GA<sub>8</sub>, GA<sub>17</sub>, GA<sub>19</sub>, GA<sub>20</sub>, GA<sub>29</sub>, GA<sub>34</sub> and GA<sub>48</sub>) are also present in ungerminated grain (Gaskin *et al.*, 1984; Croker *et al.*, 1990; Jacobsen *et al.*, 1995). After germination, GA<sub>3</sub> predominates. It is not clear whether specific GAs are synthesized in different tissues of the grain, or whether target cells are differentially sensitive to the different forms of GA (Fincher, 1989; Fincher and Stone, 1993). Similarly the exact site and mechanism of synthesis of the hormones have not been fully determined. Several researchers have suggested that the embryonic axis (MacLeod and Palmer, 1967; Grosselindemann *et al.*, 1991), the scutellum (Radley, 1967; Lenton *et al.*, 1994) or the aleurone layer itself (Atzorn and Weiler, 1983; Lenton *et al.*, 1994) are possible sites of GA synthesis.



The major effect of GA in the grain is the induction of *de novo* synthesis and secretion of hydrolytic enzymes in the aleurone layer (Jones and Jacobsen, 1991). Mundy and Munck (1985) showed that if the embryo is removed from barley endosperm earlier than 24 h after the start of imbibition there is no increase in GA in the endosperm and very little  $\alpha$ -amylase is produced. This result indicates that GA from the embryo stimulates the production of  $\alpha$ -amylase. As mentioned earlier, application of GA<sub>3</sub> to isolated barley aleurone layer cells induces the secretion of a range of hydrolases, including  $\alpha$ -amylase (Chrispeels and Varner, 1967; Filner and Varner, 1967; Nicholls *et al.*, 1986), endopeptidases (Jacobsen and Varner, 1967; Hammerton and Ho, 1986; Koehler and Ho, 1990); ribonucleases (Chrispeels and Varner, 1967; Bennett and Chrispeels, 1972), (1 $\rightarrow$ 3,1 $\rightarrow$ 4)- $\beta$ -D-glucanases (Mundy and Fincher, 1986; Stuart *et al.*, 1986 a; Slakeski and Fincher, 1992 b), limit dextrinase (Hardie, 1975; X. Zhang, R. Burton and G. B. Fincher, unpublished data),  $\alpha$ -glucosidase (Clutterbuck and Briggs, 1973; Hardie, 1975), peroxidase (Gubler and Ashford, 1983), acid phosphatase (Ashford and Jacobsen, 1974; Jones and Jacobsen, 1991), protease (Jacobsen and Varner, 1967) and arabinoxylan-degrading enzymes (Taiz and Honigman, 1976; Slade *et al.*, 1989).

Little is known about the precise molecular mechanisms of the action of GA (Jones, 1985), although it is clear that the hormone's action is reflected in altered transcriptional activity of specific genes (Jacobsen and Beach, 1985, Fincher, 1989). Studies on the regulation of gene expression, using isolated barley aleurone layers as a model system, have revealed great variations in the response of different genes to GA<sub>3</sub>. The induction patterns of the mRNA for  $\alpha$ -amylase (Chandler *et al.*, 1984; Rogers, 1985), (1 $\rightarrow$ 3,1 $\rightarrow$ 4)- $\beta$ -glucanase (Mundy and Fincher, 1986) and a putative thiol protease (Rogers *et al.*, 1985) indicate that GA<sub>3</sub> exerts its control at the level of transcription (Jacobsen and Beach, 1985). The accumulation of low-pI mRNA increases approximately 20-fold after GA<sub>3</sub> treatment, in contrast to high-pI  $\alpha$ -amylase mRNA, which increases by at least 100-fold (Rogers, 1985; Bewley and Black, 1994). In addition, several groups have demonstrated

an approximately ten-fold stimulation of transcription of  $\alpha$ -amylase genes using nuclei isolated from GA<sub>3</sub>-responsive barley aleurone layer protoplasts (Gubler and Jacobsen, 1992; Sutcliff *et al.*, 1993; Gubler *et al.*, 1995; Jacobsen and Beach, 1985). Although many studies have been focussed on the hormonal regulation of total  $\alpha$ -amylase mRNA levels, less attention has been directed to examining the regulation of individual  $\alpha$ -amylase mRNAs of the  $\alpha$ -amylase gene family (Higgins *et al.*, 1976; Jacobsen and Beach, 1985; Muthukrishnan *et al.*, 1984; Zwar and Hooley, 1986).

Very little information is available concerning the effects of GA on the scutellum during the synthesis and secretion of hydrolytic enzymes from this tissue. Levels of  $\alpha$ -amylase secreted from excised barley scutella do not increase in the presence of exogenous GA<sub>3</sub> (MacGregor and Marchylo, 1986). However, the effects of exogenous GA<sub>3</sub> may be masked by the presence of the endogenous hormone in the excised scutella or, alternatively, the scutellar cells may respond to other GAs (Fincher, 1989).

Several groups (Chrispeels and Varner, 1967; Jones and Jacobsen, 1983, 1991; Stuart *et al.*, 1986 a; Jones *et al.*, 1987) have shown that the addition of Ca<sup>2+</sup> to isolated barley aleurone layers stimulates the secretion of  $\alpha$ -amylases, (1→3,1→4)- $\beta$ -glucanases, (1→3)- $\beta$ -glucanases, endopeptidases, nucleases and acid phosphatases. The Ca<sup>2+</sup> is generally assumed to exert its influence at the intracellular transport level, although the mechanism of its action remains unclear and it may also be involved in signal transduction associated with hormonal responses (Varner and Mense, 1972; Moll and Jones, 1982; Bush *et al.*, 1989; Jones and Bush, 1991; Drozdowicz and Jones, 1995).

Abscisic acid (ABA) is another important phytohormone that may play a central role in the process of grain maturation (King, 1976) by inducing the expression of "ABA-specific" genes (Lin and Ho, 1986; Mundy and Fincher, 1986; Jacobsen and Chandler, 1987; Williamson and Quatrano, 1988; Clarke *et al.*, 1991). These genes include those encoding  $\alpha$ -amylase inhibitors (Mundy, 1984; Weselake *et al.*, 1985; Mundy and Rogers, 1986), protease inhibitors (Svensson *et al.*, 1986) and various heat-stable proteins of

unknown function (Jacobsen and Shaw, 1989). ABA also suppresses the expression of genes which respond to GA<sub>3</sub> (Fincher, 1989; Jones and Jacobsen, 1991). It reverses GA<sub>3</sub>-promoted changes in protein synthesis and transcription in the barley aleurone layer, including that of (1→3,1→4)-β-glucanases (Mundy and Fincher, 1986), α-amylases (Jacobsen and Beach, 1985; Nolan and Ho, 1988; Jacobsen *et al.*, 1995; Bewley and Black, 1994) and endopeptidases (Koehler and Ho, 1990).

## 1.4 Cell wall degradation

### 1.4.1 Degradation of starchy endosperm and aleurone layer cell walls

Hydrolytic enzymes secreted from the aleurone layer or from the scutellum must cross the walls of the cells from which they are secreted, as well as walls of the starchy endosperm itself. During reserve mobilization, the outer layer of the aleurone layer cell wall is completely degraded while the inner layer remains resistant to digestion, presumably to provide continued structural support for the protoplast (Taiz and Jones, 1970, 1973; Bacic and Stone, 1981 a, b; Gubler *et al.*, 1987; Fincher, 1992). The residual, resistant inner wall layer appears to be sufficiently porous to allow secreted enzymes to cross into the starchy endosperm. Thus, immunological studies have revealed that α-amylase from GA<sub>3</sub>-treated barley aleurone layers diffuses through the inner layer of aleurone walls, but secretion of the enzyme into the starchy endosperm requires the formation of pronounced channels in the outer wall layer of the aleurone (Gubler *et al.*, 1987).

In the case of the scutellum, partial dissolution of the outer wall layers of the scutellar epithelium separates the cells into cylindrical papillae at the endosperm/embryo interface. As mentioned in section 1.3.1, this process results in an increase in surface area of the epithelial cells and may be important for the translocation of degradation products of

the starchy endosperm reserves from the endosperm to the embryo (Nieuwdorp and Buys 1964). In any case, changes in the scutellar epithelial and aleurone cell walls in germinated grain are similar in that an inner wall layer remains apparently intact after dissolution of an outer wall layer (Fincher, 1989; Bacic and Stone, 1981 a).

Following the release of cell wall-degrading enzymes from the aleurone layer and scutellum, cell walls of the starchy endosperm are degraded in an apparently biphasic process. Initially, most of the walls are degraded but some remnants can be detected; these remnants gradually disappear as a second front of enzymes diffuses across the starchy endosperm (Selvig *et al.*, 1986; Fincher, 1989). The major polysaccharide constituents of both aleurone layer and starchy endosperm cell walls are (1→3,1→4)-β-glucans and the arabinoxylans (Table 1.1). The enzymology of (1→3,1→4)-β-glucan depolymerization has been studied in detail for a number of years and is briefly summarized in section 1.4.2 below. In contrast to the large body of information available on (1→3,1→4)-β-glucan hydrolysis, relatively little is known about the enzymes required for arabinoxylan degradation. Similarly, the spatial and temporal regulation of genes encoding the enzymes that participate in arabinoxylan hydrolysis has not been described. Available information on arabinoxylan depolymerization is presented in section 1.4.3 below.

#### 1.4.2 Hydrolysis of (1→3,1→4)-β-glucan

It might be expected that (1→3,1→4)-β-glucans could be hydrolysed by the following three classes of β-glucan endohydrolases, all of which have been detected in germinated barley: (1→4)-β-glucan glucanohydrolase (cellulase, EC 3.2.1.4, Hoy *et al.*, 1981; Manners *et al.*, 1982), (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, 1982 a, b), and (1→3)-β-glucan glucanohydrolase (EC 3.2.1.39, Manners and Marshall, 1969; Manners and Wilson, 1976; Bathgate *et al.*, 1974; Høj *et al.*,

1988, 1989; Hrmova and Fincher, 1993). In quantitative terms, the (1→3,1→4)-β-glucanases are probably the most important, because (1→3)-β-glucanases are unable to hydrolyse the single, isolated (1→3)-β-linkages found in these polysaccharides (Høj and Fincher, 1995) and because cellulases are relatively low in abundance (Hoy *et al.*, 1981). The (1→3,1→4)-β-glucanases specifically cleave (1→4)-β-linkages of (1→3,1→4)-β-glucan, only where the corresponding glucosyl residue is substituted at the C(O)3 position, as follows:



Two (1→3,1→4)-β-glucanases, designated isoenzymes EI and EII, have been purified from germinated barley and characterized (Woodward and Fincher, 1982 a). Isoenzyme EI is predominantly synthesized in the scutellum, while isoenzyme EII is found exclusively in the aleurone layer (Stuart *et al.*, 1986 b). Neither of the barley (1→3,1→4)-β-glucanases can hydrolyze (1→3)-β-glucans or (1→4)-β-glucans (Woodward and Fincher, 1982 b; Høj *et al.*, 1989).

The major products of cell wall (1→3,1→4)-β-glucans released by these (1→3,1→4)-β-glucan endohydrolases are the trisaccharide 3-*O*-β-cellobiosyl D-glucose (G4G3G<sub>red</sub>) and the tetrasaccharide 3-*O*-β-celotriosyl D-glucose (G4G4G3G<sub>red</sub>) (Woodward and Fincher, 1982 b). Although the cell wall (1→3,1→4)-β-glucans will be solubilized after hydrolysis with the endohydrolase, the oligosaccharide products represent a valuable source of glucose for the developing seedling. Indeed, Morall and Briggs (1978) have calculated that cell wall (1→3,1→4)-β-glucans account for up to 18.5% of total glucosyl residues in the barley grain.

Enzymes capable of converting the (1→3,1→4)-β-oligosaccharides to glucose include β-glucan exohydrolases and β-glucosidases, both of which have been detected in homogenates of germinated grain (Preece and Hoggan, 1956; Manners and Marshall,

1969; Leah *et al.*, 1995; Hrmova *et al.*, 1996). Hrmova *et al.* (1996) have suggested that  $\beta$ -glucan exohydrolases are the most important enzymes in this process, because they rapidly release glucose from the non-reducing terminus of (1 $\rightarrow$ 3)-, (1 $\rightarrow$ 3,1 $\rightarrow$ 4)-, (1 $\rightarrow$ 4)- $\beta$ -glucans and derived oligosaccharides, while the  $\beta$ -glucosidases hydrolyse (1 $\rightarrow$ 3)- $\beta$ -glucosyl linkages very slowly.

### 1.4.3 Arabinoxylan hydrolysis

#### 1.4.3.1 Degradation of arabinoxylan

The depolymerization of barley endosperm cell wall arabinoxylans during starchy endosperm mobilization is probably achieved by the combined action of endo- and exoxylanases,  $\alpha$ -arabinofuranosidases and  $\beta$ -xylosidases (Figure 1.3; Preece and MacDougall, 1958; Taiz and Honigman, 1976).

*Endohydrolases:* Xylan endohydrolases (EC 3.2.1.8) catalyse the hydrolysis of internal linkages in (1 $\rightarrow$ 4)- $\beta$ -xylans in an essentially random fashion (Figure 1.3) and the final products of hydrolysis are generally xylooligosaccharides with a degree of polymerization (DP) of two or three (Preece and MacDougall, 1958; Dekker, 1989; Dekker and Richards, 1976). The enzymes may act on xylooligosaccharides as short as xylotriose, but at much lower rates than they act on substrates with a higher DP (Reilly, 1981). Endoxylanases may be distinguished from one another by the substrates upon which they act and by the final products of such reactions. For example, Nishitani and Nevins (1991) have described a sequence-dependent endoxylanase that requires a glucuronic acid substituent adjacent to the cleaved (1 $\rightarrow$ 4)- $\beta$ -xylosyl linkage.

In general however, polymeric xylans are cleaved only at unsubstituted regions and yield a mixture of unsubstituted xylooligosaccharides, together with short-and



longer-chain substituted xylooligosaccharides. Thus, degradation products arising from the early hydrolysis of arabinoxylan by endoxylanases are xylooligosaccharides of mixed constitution, usually containing arabinose and/or other substituents such as glucuronic acid (or its 4-O-methyl derivative). The DPs of these early hydrolysis products vary, but are normally greater than 4. As hydrolysis proceeds, xylose, xylobiose and xylotriose also accumulate. Some highly purified microbial endoxylanases show transglycosylation activity (Biely *et al.*, 1981). Thus, these microbial xylanases hydrolyse the main chain of xylan, but are also capable of synthesizing oligosaccharides from high concentrations of the low molecular weight hydrolysis products.

The extent of hydrolysis of arabinoxylans by the combined action of mainchain- and substituent-cleaving enzymes might exceed the sum of hydrolysis by each enzyme acting alone. A clear-cut example of such a synergy is that occurring between endoxylanase,  $\beta$ -xylosidase and arabinoxylan arabinofuranohydrolase in the hydrolysis of wheat arabinoxylans (Kormelink and Voragen, 1992). Puls and Poutanen (1989) have shown that the xylanolytic enzymes from *Trichoderma reesei* act synergistically on a number of different xylans. Hydrolysis starts by the action of endoxylanases, which decrease the average DP of the substrate. The side-group-cleaving enzymes have their highest activity towards short, substituted xylooligosaccharides, and hydrolysis is therefore completed by the synergistic action of both endo- and exohydrolases.

*$\beta$ -Xylosidases and xylan exohydrolases:* Xylan exohydrolases and  $\beta$ -xylosidases, both classified as members of the (EC 3.2.1.37) group, are often difficult to distinguish, as indeed are exo-glycanases and glycosidases generally (Hrmova *et al.*, 1996). While xylan exohydrolases normally release mono- or disaccharides from the non-reducing ends of polysaccharide chains,  $\beta$ -xylosidases are often identified on the basis of their ability to hydrolyse aryl and alkyl  $\beta$ -xylosides. The  $\beta$ -xylosidases generally hydrolyse polymeric xylans slowly, if at all (Reilly, 1981). Nevertheless,  $\beta$ -xylosidases can generally hydrolyse



short-chain xylooligosaccharides, liberating xylose from the non-reducing end, and the substrate affinity of these enzymes usually decreases with increasing DP of the substrate. Thus, xylosidases can act synergistically with endoxylanases (and perhaps other enzymes) to accomplish extensive hydrolysis of polymeric heteroxylan substrates. Most  $\beta$ -xylosidases cannot hydrolyse substituted xylooligosaccharides until the substituents are removed by relevant ancillary enzymes (Puls, 1992; Coughlam and Hazelwood, 1993; Herrmann *et al.*, 1996).

*Arabinofuranosidases:*  $\alpha$ -L-Arabinofuranosidases (EC 3.2.1.55) can hydrolyse  $\alpha$ -L-arabinofuranosyl residues from  $\alpha$ -L-arabinofuranosides, arabinoxylans and arabinogalactans (Figure 1.3). Kaji (1984) subdivided  $\alpha$ -L-arabinofuranosidases into two groups: an *Aspergillus niger*-type  $\alpha$ -L-arabinofuranosidase and a *Streptomyces purpurascens*-type  $\alpha$ -L-arabinofuranosidase. The former group hydrolyses substrates of low molecular weight, including L-arabinooligosaccharides, polymeric arabinans, arabinoxylans and arabinogalactans. The latter group can act only on aryl or alkyl  $\alpha$ -L-arabinosides, and on  $\alpha$ -L-arabinooligosaccharides of low molecular weight. Kormelink *et al.*, (1991) purified a (1 $\rightarrow$ 4)- $\beta$ -D-arabinoxylan arabinofuranohydrolase (AXH) from *Aspergillus awamori* which is highly specific for arabinoxylan, but is only capable of hydrolysing non-reducing terminal  $\alpha$ -L-arabinofuranose residues from singly-substituted xylopyranosyl residues of the arabinoxylan chain. Unlike other  $\alpha$ -L-arabinofuranosidases, AXH does not show any activity towards *p*-nitrophenyl  $\alpha$ -L-arabinofuranoside, arabinans or arabinogalactan.

#### 1.4.3.2 Plant xylanases

A very large number of microbial endo- and exo-(1 $\rightarrow$ 4)- $\beta$ -xylanases have been purified and characterized (Fernández-Espinar *et al.*, 1993, 1994; Blanco *et al.*, 1995;

Berens *et al.*, 1996), and these have attracted considerable attention because of their potential to hydrolyse heteroxylans during the industrial conversion of plant residues to fermentable sugars. Some properties of microbial  $\beta$ -xylan endohydrolases are listed in Table 1.2. These enzymes vary widely in their isoelectric points, pH optima and molecular weights.

In contrast, information on plant xylan hydrolases is very limited. Lienart *et al.* (1985) partially purified an endoxylanase from cultured cells of *Acacia verec*; the enzyme had an apparent molecular weight of 55,000 and an isoelectric point of 5.7. The presence of xylan endo- and exohydrolases in secretions from isolated barley aleurone layers has been reported, but neither group of enzymes has been purified or characterized (Taiz and Honigman, 1976; Dashek and Chrispeels, 1977). Three (1 $\rightarrow$ 4)- $\beta$ -xylan endohydrolases (EC 3.2.1.8) with an approximate molecular weight of 41,000 have been purified from extracts of 5-day-germinated barley grain (Slade *et al.*, 1989). All three isoenzymes have identical NH<sub>2</sub>-terminal amino acid sequences, they have pI values of approximately 5 and, although their genetic origins are not entirely clear, the authors concluded that they are the products of separate genes (Slade *et al.*, 1989). These three isoenzymes can be clearly distinguished from a xylanase of M<sub>r</sub> 34,000 and pI 4.6 that has been purified from the medium around GA<sub>3</sub>-treated aleurone layers (Benjavongkulchai and Spencer, 1986). The relationship of the endo-xylanases purified by Benjavongkulchai and Spencer (1986) and Slade *et al.* (1989) has not been satisfactorily explained. It seems unlikely that different endo-xylanases from barley aleurone layers would have such widely divergent molecular weights. Nevertheless, NH<sub>2</sub>-terminal amino acid sequences of the three enzymes studied by Slade *et al.* (1989) were used in the present work to generate oligonucleotides for the isolation of cDNAs encoding barley (1 $\rightarrow$ 4)- $\beta$ -xylan endohydrolases.

Table 1.2 Characteristics of xylan endohydrolases purified from different microorganisms

Source	Molecular weight (kDa)	Optimum pH	Isoelectric point (pI)	References
<i>Fibrobactor succinogenes</i>	53.7	7.0	8.9	Matte and Forsberg (1992)
S85	66.0	6.3	8.0	
<i>Penicillium Chrysogenum</i>	35.0	6.0	4.2	Haas <i>et al.</i> (1992)
<i>Aspergillus nidulans</i>	34.0	6.0	3.4	Fernández-Espinar <i>et al.</i> (1994)
<i>Piromyces</i>	12.5	6.0	9.1	Teunissen (1993)
<i>Actinomycete microtetraspora flexuosa</i>	26.3	8.4	6.0	Berens <i>et al.</i> (1996)
	16.8	9.45		
<i>Bacillus sp strain bp-23</i>	32.0	5.5	9.3	Blanco <i>et al.</i> (1995)
<i>Aspergillus nidulans</i>	22.0	5.5	6.4	Fernández-Espinar (1993)
<i>Talaromyces emersonii</i>	74.85	4.2	5.3	Tuohy <i>et al.</i> (1993)
	54.2	3.5	4.2	

## 1.5 Regulation of genes encoding hydrolytic enzymes

During and following germination, specific genes are differentially expressed or repressed in the various tissues within the endosperm. The high level of spatial and temporal coordination of gene expression is demonstrated by the transcriptional activity of barley (1→3,1→4)- $\beta$ -glucanases (Fincher, 1989; Fincher and Stone, 1993). McFadden *et al.* (1988) have shown by *in situ* hybridization that (1→3,1→4)- $\beta$ -glucanase mRNA accumulates initially in the scutellum. Subsequently, levels decrease in the scutellum, but increase strongly in the aleurone layer. Transcription of the genes in the aleurone occurs near the embryo initially, but proceeds towards the distal end of the grain. Although tissue-specific sequential regulation of gene expression is not well understood, it is clear that the regulation of many genes in the aleurone layer is mediated at the transcriptional level (Fincher, 1989; Fincher and Stone, 1993). Furthermore, transcriptional regulation often involves the phytohormones GA and ABA. Indeed, barley aleurone layers have been extensively used as a convenient model system for the study of molecular mechanisms involved in gibberellin-regulated gene expression (Fincher, 1989; Hooley, 1994).

Most progress has been made in understanding the regulation by GA of  $\alpha$ -amylase production in germinated barley. A large number of cereal  $\alpha$ -amylase genes have now been cloned and this has enabled the analysis of promoter regions involved in the control of transcription. In particular, *cis*-acting elements that confer GA regulation, tissue-specificity and high levels of expression have been identified. Sequence analysis of the promoters of both the low- and high-pI barley  $\alpha$ -amylase genes has revealed that three conserved regions within the proximal 200-300 bp upstream of the transcription start site are responsible for GA-induced transcription (Huttly and Baulcombe, 1989; Huttly and Phillips, 1995; Jacobsen and Close, 1991; Kim *et al.*, 1992; Lanahan *et al.*, 1992; Jacobsen *et al.*, 1995). These sequences include the pyrimidine box (C/TCTTTTC/T),

and the TAACAA/GA and TATCCAC/T boxes, which are highly conserved among the promoters of GA<sub>3</sub>-responsive  $\alpha$ -amylase genes of wheat, barley and rice (Huang *et al.*, 1990; Gubler and Jacobsen, 1992). Analysis of a high-pI barley  $\alpha$ -amylase promoter (AMY6-4) indicates that the TAACAAA box represents a particularly important GA-response element (GARE) (Skriver *et al.*, 1991). Removal of the sequence TAACAAA by site-directed mutagenesis abolishes the GA<sub>3</sub> response of the barley  $\alpha$ -amylase gene promoter (Gubler and Jacobsen, 1992; Jacobsen *et al.*, 1995). Mutagenesis of the TATCCAC box results in a decrease of GA<sub>3</sub>-regulated expression. These results indicate that both boxes are absolutely required for the GA<sub>3</sub>-induction of genes and form a complex gibberellic acid response complex (GARC) (Skriver *et al.*, 1991). The TAACAAA box also appears to be linked with ABA induced reductions in the transcriptional activity of barley  $\alpha$ -amylase genes (Gubler and Jacobsen, 1992).

The expression of (1→3,1→4)- $\beta$ -glucanase genes is induced by GA and is controlled at the transcriptional level. The barley (1→3,1→4)- $\beta$ -glucanase isoenzyme EII gene promoter carries a motif, TAACAAC, at position -169; this sequence is similar to the TAACAAA component of the GARE of the barley  $\alpha$ -amylase gene (Skriver *et al.*, 1991). The sequence in the corresponding position of the gene encoding isoenzyme EI is CAACAAC and similar motifs (TAAGAAA, CAACAAA) are also present in the isoenzyme EI promoter region at -151 bp. Specific cDNA probes encoding (1→3,1→4)- $\beta$ -glucanase isoenzymes EI and EII are available to discriminate between the expression of the specific genes (Slakeski *et al.*, 1990; Wolf, 1992). Amounts of isoenzyme EII mRNA are strongly enhanced after treatment of aleurone layers with GA<sub>3</sub>. However, no induction of isoenzyme EI gene transcription was detected (Slakeski and Fincher, 1992 b; Wolf, 1992). Isoenzyme EI mRNA levels were relatively high in untreated control aleurone layers (Slakeski and Fincher, 1992 b). Wolf (1992) also reported that the promoter of the gene encoding isoenzyme EI is not affected by GA<sub>3</sub> in barley aleurone protoplasts and it is likely that specific deviations from the sequence TAACAAA disturb

the binding of a putative *trans*-acting element. These experiments clearly demonstrate that the genes encoding the two isoenzymes EI and EII are subject to tissue-specific and hormonal regulation, and that significant differences exist in their regulation.

To date, there are no published reports describing the cloning of cDNAs or genes encoding (1→4)-β-xylanases from any plants. Thus, there is no information on the structure of plant xylanase genes or on factors that regulate the transcriptional activity of these genes.

### 1.6 Aims of the present study

The principal aim of the present study was to isolate and characterize cDNAs and genes encoding barley (1→4)-β-xylan endohydrolases. The more specific aims of the project were;

- to generate cDNA libraries from GA<sub>3</sub>-treated barley aleurone layers
- to screen the cDNA libraries for near full-length cDNAs for (1→4)-β-endohydrolases and to sequence these cDNAs
- to construct vectors for the heterologous expression of (1→4)-β-xylan endohydrolases in *E. coli*
- to use the cDNAs in Northern blot analyses to define the sites of transcription of barley (1→4)-β-xylan endohydrolase genes and to examine the effect of phytohormones on the mRNA levels
- to isolate and characterize barley (1→4)-β-xylan endohydrolase genes
- to define the number and location of (1→4)-β-xylan endohydrolases genes in the barley genome.

Three barley (1→4)-β-xylan endohydrolases have been purified previously and the sequences of 30 amino acid residues at the NH<sub>2</sub>-terminal end of the enzymes were defined (Slade *et al.*, 1989). This information has now been used to design

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oligonucleotide primers for the amplification by the polymerase chain reaction (PCR) of a (1→4)- $\beta$ -xylan endohydrolase cDNA. The synthesis, amplification, cloning and characterization of the cDNA clone were performed by reverse transcriptase-rapid amplification complementary DNA ends (RT-RACE PCR) and are described in Chapter 2. The isolation and characterization of a full-length cDNA for (1→4)- $\beta$ -endohydrolase isoenzyme X-I and a partial cDNA for isoenzyme X-II are presented in Chapter 3, together with properties of the enzymes deduced from their primary structures.

The heterologous expression of the (1→4)- $\beta$ -xylan endohydrolase isoenzyme X-I cDNA and characterization of the expressed protein are described in Chapter 4.

Investigations were carried out on the sites of xylanase gene expression, as measured by mRNA levels in Northern analyses. Total RNA was isolated from tissues of germinated grain and young seedlings. The effects of various plant growth regulators on the transcription of barley (1→4)- $\beta$ -xylan endohydrolase isoenzyme genes were also investigated. The results of this work are presented in Chapter 5.

In Chapter 6, the use of the cDNA to screen a barley genomic library for the isolation of a genomic clone encoding the barley (1→4)- $\beta$ -xylan endohydrolase is described. Nucleotide sequence analysis of the genomic clone revealed several important characteristics of the gene. Finally, Southern blot analyses were used to determine the number of (1→4)- $\beta$ -xylan endohydrolase genes present in the barley genome, using the (1→4)- $\beta$ -xylan endohydrolase isoenzyme X-I cDNA as a probe. In addition, the chromosomal locations of the (1→4)- $\beta$ -xylan endohydrolase genes were determined.

## **CHAPTER 2**

**ISOLATION OF A cDNA ENCODING BARLEY**

**(1→4)- $\beta$ -XYLAN ENDOHYDROLASE BY**

**RACE-PCR**



## 2.1 INTRODUCTION

As stated in section 1.6, the primary objective of the work described in this thesis was to isolate and characterize cDNAs encoding barley (1→4)- $\beta$ -xylan endohydrolases (EC 3.2.1.8). The cloning experiments were to rely completely on amino acid sequence information obtained from the purified enzymes (Slade *et al.*, 1989). The available sequence was restricted to the 30 NH<sub>2</sub>-terminal amino acids (Figure 2.1). Because the xylanase enzymes purified by Slade *et al.* (1989) were obtained in very low yield, there was insufficient material for tryptic digestion and for the subsequent determination of internal amino acid sequences (Slade *et al.*, 1989). Nevertheless, two regions within the 30 NH<sub>2</sub>-terminal amino acids were suitable for the generation of oligonucleotide probes or primers (Figure 2.1)

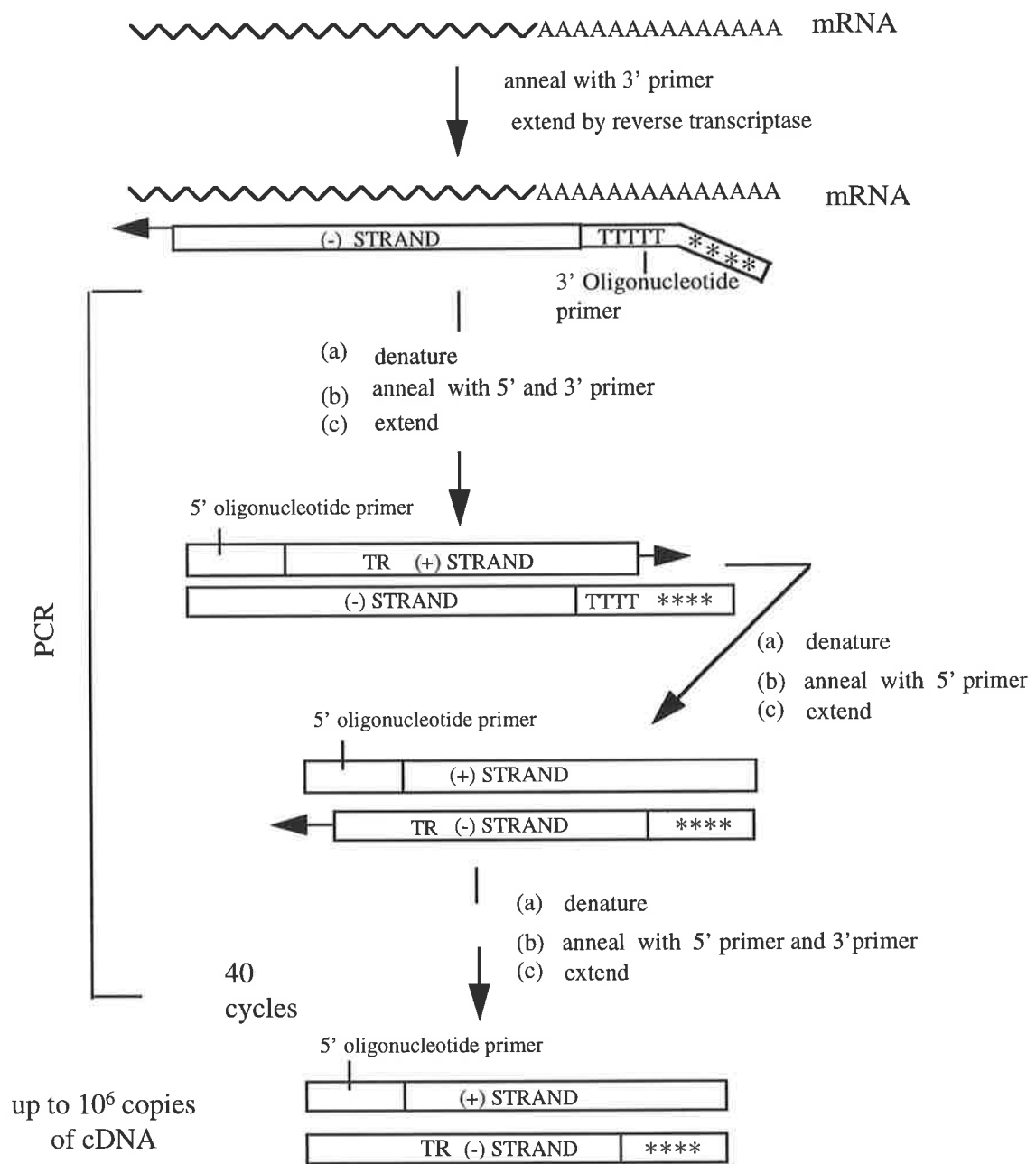
With regard to the appropriate tissue from which the poly (A)<sup>+</sup>-RNA should be isolated, published data indicated that GA<sub>3</sub>-treated aleurone layers were likely to be a suitable source of RNA for cDNA synthesis. Thus, Taiz and Honigman (1976), Dashek and Chrispeels (1977), Benjavongkulchai and Spencer (1986) and Slade *et al.* (1989) all showed that xylan endohydrolases were secreted from isolated barley aleurone layers following treatment with GA<sub>3</sub>.

Based on this information, two approaches were used to isolate barley (1→4)- $\beta$ -xylan endohydrolase cDNA(s) from aleurone layer poly(A)<sup>+</sup>-RNA. Preliminary attempts involved screening a cDNA library prepared from aleurone layer poly(A)<sup>+</sup>-RNA using degenerate oligonucleotides as probes (Figure 2.1). The two degenerate oligonucleotides were designated Xy1 and Xy2 and corresponded to the amino acid sequences VYPVDHKA and KDKTDKA, respectively, from the NH<sub>2</sub>-terminal amino acid sequence. The degenerate oligonucleotides were used as probes to screen the aleurone cDNA library, according to Doan and Fincher (1988). However, no positive clones were detected and the procedures are therefore not described in detail here.



The other approach for cloning barley (1→4)-β-xylanase cDNA(s) was based on the protocol developed by Frohman *et al.* (1988), which has been designated "Rapid Amplification of cDNA Ends" (RACE). The RACE protocol employs the reverse transcription of poly (A)<sup>+</sup>-RNA using an oligo-dT adaptor-primer to generate single-stranded cDNA. The single-stranded cDNA is subsequently amplified in the polymerase chain reaction (PCR), using the adaptor-primer in conjunction with another 5' primer. This particular RACE procedure, which is referred to as 3'-RACE, therefore generates cDNAs by using PCR to amplify copies of the region between an appropriate position in the corresponding mRNA transcript (in this case the region between the NH<sub>2</sub>-terminus of the mature enzyme and the 3' end of the mRNA (Figure 2.2, Frohman and Martin, 1989; Frohman, 1993; Frohman *et al.*, 1988).

RACE-PCR techniques are advantageous for several reasons. Firstly, it is possible to amplify a cDNA fragment from an oligonucleotide primer which is designed from a relatively short amino acid sequence. Secondly, it is much simpler, faster and cheaper, and requires very small amounts of template compared to normal cDNA library preparation and screening. Thirdly, this technique provides a method to obtain 5' and 3' ends which might be missing from partial cDNAs isolated from libraries by conventional methods. Furthermore, it is possible to use RACE-PCR to create overlapping 5' and 3' cDNA ends that can later be ligated together to form a full-length cDNA. RACE-PCR has also been used as a highly sensitive method for directly detecting and amplifying specific RNA molecules in small samples of plant or animal tissues (Frech and Peterhans, 1994). The disadvantage of RACE-PCR is that it often results in non-specific PCR products; these can be minimized by gradually raising the annealing temperature until non-specific products are no longer observed (Frohman, 1994). An additional disadvantage is that a high error rate is associated with the PCR conditions in which efficient RACE amplification takes place, and a high number of clones must therefore be sequenced to identify those without point mutations (Frohman, 1994).



**Figure 2.2 Diagrammatic representation of the 3'-RACE protocol.** The \*\*\*\* sequence represents an introduced restriction enzyme site. Oligonucleotides: TTTT(dT)17-adaptor primer, \*\*\*\* adaptor. TR (+) and (-) strands designated as truncated and which are shorter than original (+) and (-) strands. Rectangles with an arrow show the DNA strand being actively synthesized (redrawn from Frohman *et al.*, 1988).

In the work described in this Chapter, 3'-RACE-PCR was used to synthesize, amplify and isolate putative barley (1→4)- $\beta$ -xylanase cDNAs.

## 2.2 MATERIALS AND METHODS

### 2.2.1 Materials

Barley (*Hordeum vulgare* L., cv. Himalaya) was obtained from the Victorian Institute of Dryland Agriculture (VIDA), Horsham, Victoria, Australia. Plasmid pBluescript II (SK<sup>+</sup>) and *E. coli* strain XL1-Blue were obtained from Stratagene (La Jolla, CA, USA). Avian Myeloblastosis Virus Reverse Transcriptase (AMV-RT), T<sub>4</sub> DNA ligase, *Taq* polymerase and dNTPs were obtained from Promega Corporation (Madison, WI, USA). Restriction enzymes were purchased from Promega Corporation or New England Biolabs (Beverly, MA, USA). Tryptone, yeast extract and agar were purchased from Difco (Detroit, MI, USA). The Megaprime cDNA labelling kit, Hybond-N<sup>+</sup> membranes, autoradiographic film and [<sup>35</sup>S]-methionine were from Amersham International Ltd. (UK). The IPTG, X-Gal and herring sperm DNA were from Boehringer Mannheim GmbH (Sandhofer Strasse, Mannheim, Germany). The radionucleotides [ $\gamma$ -<sup>32</sup>P]ATP and [ $\alpha$ -<sup>35</sup>S]dATP were obtained from Bresatec (Adelaide, SA). Agarose, ampicillin, maltose, DTT, SDS, PVP, Ficoll, RNase and lysozyme were from Sigma Chemical Company (St. Louis, MO, USA). The FastTrack mRNA isolation kit was purchased from *Invitrogen* (San Diego, CA, USA). The DNA Sequencing kit Version 2.0 was purchased from United States Biochemical Corporation (USBC; Cleveland, Ohio, USA).

## 2.2.2 Isolation of poly (A)<sup>+</sup>-RNA from barley aleurone layers

### 2.2.2.1 Preparation of tissues

Aleurone layers were isolated essentially as described by Chrispeels and Varner (1967). The proximal end of each barley grain, which contained the embryo, was excised and discarded. The embryoless half grains, which contained the aleurone layer and starchy endosperm tissues, were surface sterilized for 20 min with 1.5% (v/v) sodium hypochlorite. The half grains were rinsed twice with sterile distilled water, soaked in 0.1 M HCl for 20 min and further rinsed with six washes of sterile distilled water. Sterilized grain sections were incubated on sterile filter paper in Petri dishes for 3 days in the dark at room temperature, with the crease side of the grain facing downwards. Starchy endosperm tissue was removed and 100 aleurone layers were incubated with shaking in 50 ml conical flasks at 25°C in 5 ml 10 mM CaCl<sub>2</sub> (containing 10 µg/ml chloramphenicol, 100 µg/ml neomycin, 100 units/ml nystatin, and 5 µM GA<sub>3</sub>) for 44 h. A 10 mM GA<sub>3</sub> stock solution was prepared in ethanol. The aleurone layers were rinsed in DEPC-treated water and stored in liquid N<sub>2</sub>.

Careful surface sterilization was important for the removal of commensal microorganisms that inhabit the surface of the grain (Hoy *et al.*, 1981), especially when prolonged incubation of grain or grain sections was necessary. As a further precaution, samples of the surface-sterilized half-grains were incubated for 44 h on malt extract agar plates (pH 5.5) to detect fungal growth and on nutrient agar media (pH 7.4) to detect bacterial growth (Slade, 1990). The absence of microbial growth on these media indicated that the surface sterilization procedure had been successful.

### 2.2.2.2 Isolation of total RNA

Total RNA was isolated from aleurone layers essentially as described by Chandler and Jacobsen (1991). Approximately 350 frozen aleurone layers were ground to a fine powder in liquid N<sub>2</sub> with a mortar and pestle. Extraction buffer (10 ml/100 layers) consisting of 0.1 M sodium glycinate buffer, pH 9.5, (containing 10 mM EDTA, 100 mM NaCl, 1% w/v SDS, 1% w/v sodium deoxycholate, 10% w/v insoluble PVP) was added to the powdered aleurone layers, which were subsequently homogenized at 0°C in an Omnimixer for 2 min at 55,000 rpm. The homogenate was centrifuged for 20 min at 10,000 rpm in a Sorvall SS-34 rotor and the supernatant was transferred to a tube containing an equal volume of phenol:chloroform (1:1 by vol). Phenol was pre-equilibrated with 10 mM Tris-HCl buffer, pH 7.5 (containing 0.1M NaCl, 1 mM EDTA, 0.1% w/v SDS).

The mixture was centrifuged for 10 min to separate the organic and aqueous phases. During the phenol:chloroform extraction, RNA remains in the aqueous phase, but denatured proteins are found at the interface. The aqueous phase was re-extracted at least twice with phenol:chloroform until no more proteinaceous material could be seen at the interface. The aqueous phase was precipitated with 0.1 vol 3 M sodium acetate buffer, pH 5.2 and 2.5 vol absolute ethanol at -20°C for 16 h. Following centrifugation at 10,000 rpm for 15-20 min, the RNA pellet was dissolved in 3 ml 0.5 M sodium acetate buffer, pH 5.8. After stirring on a vortex mixer, 400 µl 1% (w/v) cetyltrimethylammonium bromide (CTAB) was added, and the mixture was incubated on ice for 1 h. After centrifugation at 13,200 rpm for 20 min, the supernatant was discarded and the pellet was washed once with cold 0.1 M sodium acetate buffer, pH 6.0, once with 70% ethanol and centrifuged again. The pellet was dissolved in 600 µl NTES 10 mM Tris-HCl buffer, pH 7.5 (containing 0.1M NaCl, 1 mM EDTA, 0.1% w/v SDS), and transferred to a microfuge tube containing 500 µl phenol. The tube was vortexed and



centrifuged for 5 min at 13,000 rpm. The nucleic acids contained in the upper phase were precipitated by the addition of 0.1 vol 3 M sodium acetate buffer, pH 6.0 and 2.5 vol ethanol for 30 min at -80°C. After centrifugation for 15 min at 13,000 rpm, the pellet was dissolved in 100 µl 1% DEPC-treated water and 1 ml 3 M sodium acetate buffer, pH 6.0. The tube was incubated on ice for 2 h. Following centrifugation for 15 min at 13,000 rpm, the pellet was dissolved in sterile water and reprecipitated with 0.1 vol 3 M sodium acetate buffer pH 5.8 and 2.5 vol ethanol at -20°C for 16 h. After centrifugation for 15 min the final pellet was dissolved in 80 µl 10 mM Tris-HCl buffer, pH 7.5 (containing 0.7 M NaCl, 1 mM EDTA and 0.2% lauryl sarcosine) and stored at -80°C.

#### 2.2.2.3 Isolation of poly (A)<sup>+</sup>-RNA

The total RNA isolated from GA<sub>3</sub>-treated barley aleurone layers was used for preparation of poly (A)<sup>+</sup>-RNA using the *Invitrogen* FastTract mRNA isolation kit (version 3.2) as recommended by the manufacturers, with minor modifications. Total RNA (80 µl) was heated at 65°C for 5 min, immediately chilled on ice, and diluted to 3 ml with 10 mM Tris-HCl buffer pH 7.5, containing 0.7 M NaCl, 1 mM EDTA and 0.2% lauryl sarcosine in a 50 ml Lux tube. One oligo (dT)-cellulose tablet was added to the 3 ml RNA and the tube was sealed. After allowing 2 min for the tablet to swell, 10 ml "binding buffer" (composition not specified by the manufacturers) was added. After complete dispersion of the tablet, the tube was shaken gently at room temperature for 1 h. The oligo dT-cellulose was collected by centrifugation for 8 min at 4,000 x g, washed in 20 ml "binding buffer" and collected by centrifugation. The washing was repeated using 10 ml "binding buffer", and the resultant pellet was resuspended in 10 ml "low-salt-wash buffer". The washing process was repeated twice until the buffer was no longer cloudy. Following the final wash, the oligo dT-cellulose was resuspended in 0.8

ml "low-salt-wash buffer". The sample was pipetted into a spin-column (supplied in the Fast Track mRNA isolation kit, *Invitrogen*), centrifuged at room temperature for 10 sec at 5,000 x g, and the liquid discarded. The process was repeated at least 3 times until the  $A_{260}$  of the flow-through was less than 0.05. The spin-column was placed in a microfuge tube, 200  $\mu$ l "elution buffer" was added and mixed into the cellulose bed with a sterile pipette tip, and the column recentrifuged. The eluent was collected in a microfuge tube, and a further 200  $\mu$ l "elution buffer" was added, mixed and spun through the column. The final eluents were combined to give a total volume of approximately 400  $\mu$ l. The eluted poly (A)<sup>+</sup>-RNA was precipitated with 1.5 vol 2 M sodium acetate buffer, pH 5.2 and 2.5 vol ethanol and placed at -80°C for approximately 15 min. The tube was centrifuged at 12,000g for 15 min and the pellet washed with 70% ethanol. The washed pellet was dried under vacuum and dissolved in 20  $\mu$ l DEPC-treated sterile distilled water. The yield and purity of the resultant poly (A)<sup>+</sup>-RNA was determined from the ratio of  $A_{260}/A_{280}$ , and by *in vitro* translation (Sambrook *et al.*, 1989).

#### 2.2.2.4 *In vitro* translation of poly (A)<sup>+</sup>-RNA

Poly (A)<sup>+</sup>-RNA (0.5  $\mu$ g) isolated from GA<sub>3</sub>-treated aleurone layers was incubated with 10  $\mu$ l rabbit reticulocyte lysate containing 1  $\mu$ l 1200 Ci/mmol [<sup>35</sup>S]-methionine and 1  $\mu$ l amino acid mixture (Amersham) at 30°C for 1 h. The translated products (2  $\mu$ l) were spotted onto 3 MM filter paper and boiled in 10% (w/v) TCA for 10 min. The TCA was cooled by adding ice and the filter was sequentially rinsed with water, absolute ethanol and acetone. The filter was air-dried and transferred to a scintillation vial containing 10 ml scintillation fluid (75% v/v Triton-X 114, 0.3% w/v PPO and 0.2% w/v dimethyl POPOP). Incorporation of [<sup>35</sup>S]-methionine into TCA-insoluble products was measured in a Rackbeta liquid scintillation spectrometer (Pharmacia-LKB, Sweden). In addition, *in vitro* translation products were analysed by

electrophoresis using 12.5% (w/v) SDS-polyacrylamide gels. Gels were dried and exposed against X-ray Hyperfilm (Amersham) using an intensifying screen for 2 h at -80°C.

### **2.2.3 Amplification of the 3' end of the (1→4)-β-xylanase cDNA by 3'-RACE PCR**

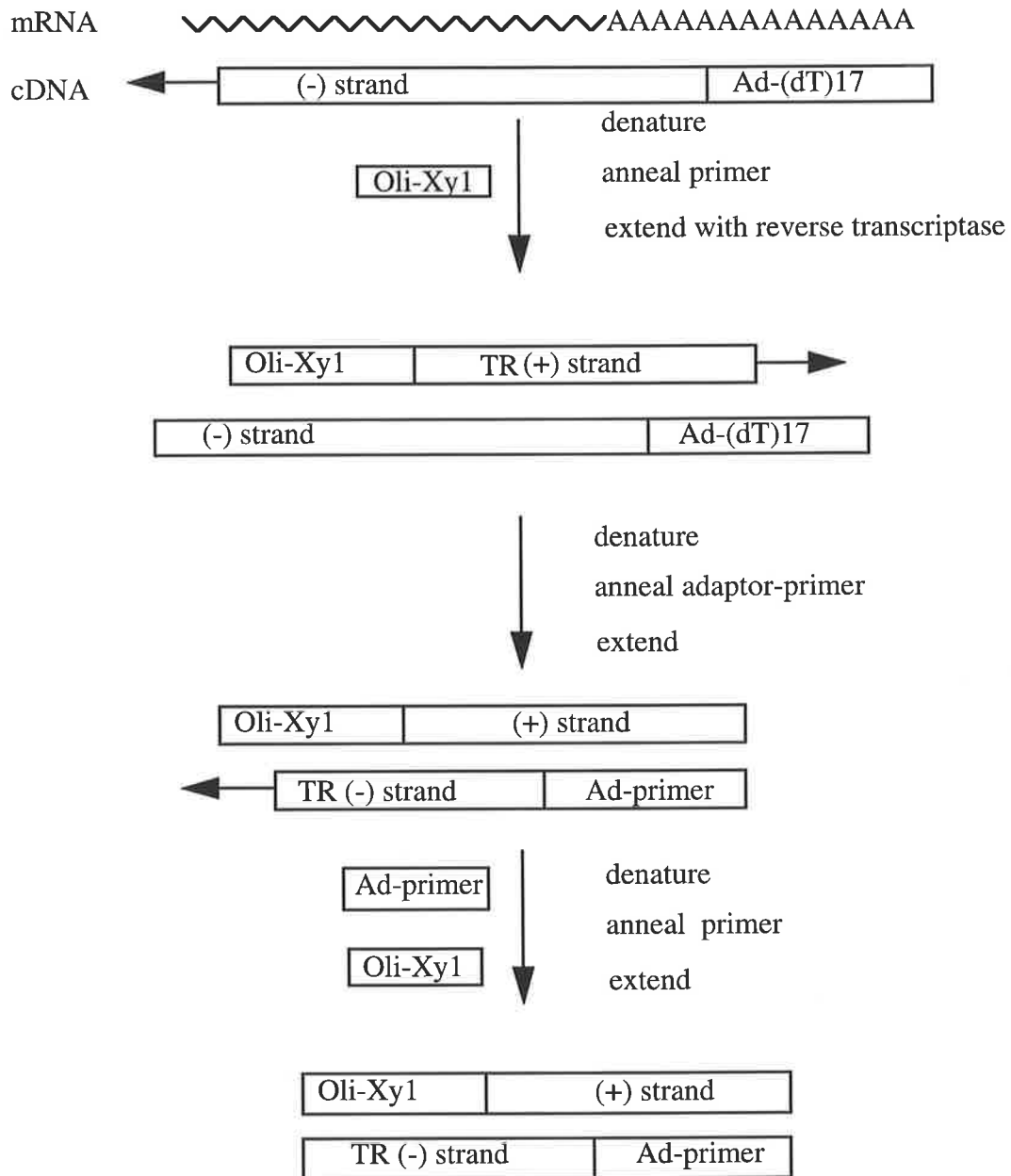
Reverse transcription was used to generate single stranded cDNA, which was subsequently used as a template in the PCR reaction. First strand cDNA was synthesized from poly (A)<sup>+</sup>-RNA by reverse transcription using a 35-base oligonucleotide containing 17 dT residues, as follows;

5' GACTCGAGTCGACATCGATTTTTTTTTTTTTTTTTTTT 3'.

The oligo-dT tail annealed to the poly(A) tail of the mRNA. Extension of first strand cDNAs from the 3' ends of the mRNA back to the region of known sequence was accomplished using an adaptor primer of sequence

5' GACTCGAGTCGACATCG 3'

which annealed to the 3' end of the coding strand of the double stranded cDNA (Figure 2.3). A xylanase-specific amplification primer (Xy1) was annealed to the first strand (-) of the cDNA produced by reverse transcription of the RNA and extended to generate the complementary (+) DNA strand. The resulting double-stranded DNA was amplified by PCR using a 5' Xy1 oligonucleotide and a 3' primer containing the adaptor sequence but no oligo(dT) tail (Figure 2.3). To avoid any non-specific binding during the second round of PCR, the amplification was performed at an elevated temperature (45°C). Details of the PCR procedures are described below.



**Figure 2.3** Diagrammatic representation of the 3'-RACE protocol for cDNA synthesis and amplification of xylanase cDNA using Xy1 xylanase-specific oligonucleotide primer. Ad-(dT) and Ad-primer represent adaptor-primers with 17-dT residues and without dT residues, respectively. Rectangles with an arrow represent DNA strands being actively synthesized and the (-) or (+) strand is designated as truncated (TR) when it is shorter than original (-) or (+) strands.

### 2.2.3.1 First strand cDNA synthesis and PCR

First strand cDNA synthesis was performed using 1 µg GA<sub>3</sub>-treated aleurone layer poly (A)<sup>+</sup>-RNA in a reaction mixture of 50 mM Tris-HCl buffer, pH 8.3, containing 25 mM KCl, 10 mM MgCl<sub>2</sub>, 4 mM DTT, 1 mM NaPPi (sodium pyrophosphate), 1 mM dNTPs, 1 U ribonuclease inhibitor, 21 U AMV reverse transcriptase (Promega) and 0.5 µg dT-17 anchor-primer (Frohman *et al.*, 1988). The anchor region contains *Xho*I, *Sal*I, and *Cla*I recognition sites. The reaction mixture (20 µl) was incubated at 42°C for 1 h and diluted with water to 500 µl. A 23-mer degenerate primer (Xy1) corresponding to the eight NH<sub>2</sub>-terminal amino acid residues (YYPVDHKA) of the previously purified (1→4)-β-D-xylan endohydrolase protein from barley (Slade *et al.*, 1989) was synthesized on an Applied Biosystems DNA synthesizer. Inosine was incorporated at wobble base positions in which more than two bases could be selected. The cDNA reaction mixture (10 µl), 25 pmol Xy1 primer and 25 pmol of the anchor-primer (minus dT tail) were mixed in a 50 µl PCR cocktail containing 1x*Taq* buffer (Promega), dNTPs at individual concentrations of 1.5 mM, 10% (v/v) DMSO and 2.5 Units *Taq* polymerase in a 0.5 ml microcentrifuge tube. The mixture was overlaid with 40 µl mineral oil and the PCR reaction was performed in a Perkin Elmer Cetus DNA Thermal Cycler. Thirty cycles of amplification were carried out using the following step cycle programme: 94°C for 2 min, 37°C for 1 min 30 sec, 72°C for 2 min. After 30 cycles, the mixture was heated at 72°C for 10 min, and held at 4°C.

The amplified product was analysed by electrophoresis on 1% (w/v) agarose gels and located by ethidium bromide staining (Sambrook *et al.*, 1989). The appropriate DNA band was excised from the gel and extracted from the agarose slice by electroelution. The excised agarose slice was transferred to a sterile dialysis tube containing 1 ml 1x TAE buffer and placed in an electrophoresis tank with 0.5 x TAE for 15 min at 100 V. The DNA was eluted from the agarose gel into the buffer inside the

dialysis tube, transferred into a microfuge tube, and extracted successively with phenol:chloroform and chloroform alone to remove protein contaminants. The supernatant was precipitated with 0.1 vol 3 M sodium acetate buffer, pH 5.2 and 2.5 vol ethanol. The DNA was pelleted by centrifugation at 13,200 rpm in a microcentrifuge for 15 min, washed with 70% ethanol, dried under vacuum and dissolved in 50 µl TE. The purified DNA (1 µl) was used as a template for a second round of PCR amplification, utilizing the same primer and conditions outlined above, with the exception that the annealing step temperature was changed to 45°C for 1 min.

The reamplified DNA was again extracted from agarose gels by electroelution (Sambrook *et al.*, 1989) and precipitated as described above. Purified DNA was cloned into the pBluescript SK (+) T-vector for analysis.

#### 2.2.3.2 Construction of the T-vector

To prepare the T-vector, pBluescript SK(+) plasmid was digested with the *EcoRV* restriction enzyme and incubated with *Taq* polymerase (1 U/mg plasmid) as recommended by the manufacturer (Promega), in the presence of 2 mM dTTP for 2 h at 70°C (Marchuk *et al.*, 1991). This resulted in the addition of a single thymidine at the 3' end of each fragment. The DNA was extracted with phenol:chloroform and precipitated with 1 vol 3M sodium acetate buffer, pH 5.2 and 2.5 vol ethanol. The DNA was collected by centrifugation at 13,200 rpm in a microfuge for 15 min. The pellet was washed with 70% ethanol, dried and dissolved in 50 µl sterile distilled water.

#### 2.2.3.3. Transformation and plasmid DNA preparation

*Preparation of competent cells:* Purified PCR-amplified DNA was ligated into the T-vector for 16 h at 14°C using T4 DNA ligase. The recombinant plasmid was

transformed into competent XL1-Blue cells, which were prepared as follows: XLI-Blue cells were grown overnight at 37°C with shaking at 250 rpm in Miller's LB broth containing 15 µg/ml of tetracycline. One ml of the overnight culture was aseptically transferred to a 500 ml Erlenmeyer flask containing 100 ml Miller's LB broth and 15 µg/ml tetracycline, and the flask was incubated at 37°C with shaking at 250 rpm until the A<sub>600</sub> was 0.5. Cells were transferred to 50 ml polypropylene centrifuge tubes and centrifuged at 3,000 rpm for 15 min at 4°C. The supernatant was discarded, the cells were resuspended in one-third of the original volume of "competency buffer I" (containing 100 mM KCl, 30 mM potassium acetate, 60 mM CaCl<sub>2</sub> and 0.15% (v/v) glycerol, pH 5.8; U.S.E. mutagenesis kit, manufacturer instructions, Pharmacia Biotech) and incubated on ice for 1 h. The cells were centrifuged at 3000 rpm for 15 min at 4°C and the supernatant was again discarded. The final cell pellet was resuspended in 1/25th of the original volume of "competency buffer II" (containing 10 mM MOPS, 10 mM KCl, 75 mM CaCl<sub>2</sub> and 0.15% (v/v) glycerol, pH 6.8; U.S.E. mutagenesis kit, manufacturer instructions, Pharmacia Biotech) and left on ice for 15 min. The competent cells were quick-frozen using liquid N<sub>2</sub> and stored in 100 µl aliquots at -80°C.

*Transformation:* Frozen competent cells (100 µl) were thawed on ice and mixed gently to assure that cells were evenly suspended. Cells were transferred to pre-chilled microcentrifuge tubes and the ligation mix (10 µl, see section 2.2.3.2) was gently mixed with the competent cell suspension. Tubes were incubated on ice for 30 min, at 42° for 90 sec, and incubated again on ice for 2 min. One ml LB medium was added to all tubes, which were incubated for 90 min at 37°C with shaking at 180 rpm. The tubes were centrifuged and all but approximately 100 µl of the supernatant was removed. The cell pellet was resuspended in the remaining 100 µl supernatant and spread onto LB plates containing 100 µg/ml ampicillin, 40 µg/ml X-gal and 12.5 µg/ml IPTG. The plates were incubated at 37°C for approximately 16 h. White colonies were picked and transferred

to LB media containing 100 µg/ml ampicillin using sterile tooth picks. The cultures were shaken at 250 rpm overnight at 37°C.

*Plasmid DNA preparation* : Cells were pelleted by centrifugation at 3000 rpm for 15 min at 4°C and the supernatant was discarded. Pellets were resuspended in 50 mM Tris-HCl buffer, pH 8.0, (containing 8% w/v sucrose, 0.1% (v/v) Triton X-100 and 50 mg/ml lysozyme) and incubated at room temperature for 15 min. The lysed cells were heated at 100°C for 1 min and pelleted by centrifugation at 13,000 rpm for 15 min. The resultant pellet was removed from the microfuge tube using a sterile toothpick and the supernatant was incubated with 5 µl 10 mg/ml RNase A at 68°C for 10 min. The supernatant was chilled on ice and 60 µl 5% (w/v) CTAB was added to precipitate plasmid DNA. The precipitate was pelleted by centrifugation for 10 min at 13,200 rpm, dissolved in 300 µl 1.2 M NaCl, and precipitated with 2.5 vol ethanol. The mixture was centrifuged for 10 min at 13,200 rpm and the DNA pellet was washed with 70% ethanol and resuspended in TE buffer.

To check the insert size, 1µg plasmid DNA was digested with *EcoRI* and *XhoI* restriction endonucleases. The DNA sample was mixed with 5 x DNA loading buffer (0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol, 40% w/v sucrose) and separated by 1% (w/v) agarose gel electrophoresis as described by Sambrook *et al.* (1989). A 1 kb ladder (Gibco BRL) was used as a DNA standard. DNA sequencing and Southern blot analysis were used where appropriate to ensure the plasmid contained the correct insert.

#### 2.2.3.4 Preparation of [<sup>32</sup>P]-radiolabelled oligonucleotide probes

For the preparation of oligonucleotide probes, 50 µl of a reaction mixture containing oligonucleotide (100 ng), 5 µl 10xT4 polynucleotide kinase in 700 mM Tris-



HCl buffer, pH 7.6, (containing 100 mM MgCl<sub>2</sub>, 50 mM DTT), 50 mCi [ $\gamma$ -<sup>32</sup>P]ATP and 2 units T4 polynucleotide kinase was incubated for 1 h at 37°C. The reaction was stopped by adding 1  $\mu$ l 0.5 mM EDTA. Labelled oligonucleotide was precipitated with 0.1 vol 3 M sodium acetate buffer, pH 5.2 and 2.5 vol ethanol overnight at -20°C. The precipitate was pelleted by centrifugation at 13,200 rpm for 30 min, washed with 70% ethanol, resuspended in 50  $\mu$ l TE buffer pH 8.0, and added to the hybridization solution.

#### 2.2.3.5 Southern blot analysis

Southern blot analysis was used to confirm that plasmid preparations carried cDNAs encoding (1 $\rightarrow$ 4)- $\beta$ -xylanase. The gel containing digested DNA was soaked in 200 ml denaturing solution (1.5 M NaCl and 0.5 M NaOH) for 30 min. The denaturing solution was decanted and the gel was neutralised with 200 ml 0.5 M Tris-HCl buffer, pH 7.0 (containing 1.5 M NaCl, 1 mM EDTA,) for 30 min. A piece of clean sponge was soaked in 20 x SSC and placed in a plastic tray, and two sheets of Whatman 3MM filter paper were placed individually on top of the sponge. The edges of the filter paper were sealed with a cellulose acetate-sheet frame and the gel was placed upside-down on the paper, without trapping air bubbles. Hybond N<sup>+</sup> membrane was placed on top of the gel and any air bubbles were removed by rolling a glass pipette over the surface of the membrane. Two additional sheets of Whatman 3 MM filter paper were soaked in 20 x SSC, placed on top of the membrane and overlaid by a stack of dry paper towels. A glass plate was placed on top of the stack and the tray was filled with 20 x SSC until the sponge was covered. DNA was allowed to transfer overnight. The membrane containing the transferred DNA was fixed by placing it for 20 min on a piece of 3 MM filter paper soaked with 0.4 M NaOH. The membrane was then ready for Southern hybridization.

The membrane was prehybridized for 4 h with 6 x SSC, 1 x Denhardt's solution (Denhardt, 1966), 1% (w/v) SDS and 100 µg/ml salmon sperm DNA, and hybridized with the same solution containing the labelled oligonucleotide probe at 37°C for 16 h. After hybridization, the filter was washed with 2 x SSC/0.1% SDS for 30 min, followed by 1 x SSC/0.1% SDS for 30 min. The membrane was dried and exposed against X-ray Hyperfilm (Amersham) for 2 h.

#### 2.2.3.6 Nucleotide sequence analysis

Double-stranded DNA inserts contained within purified plasmid DNA were sequenced in both directions using DNA sequencing kits (Sequenase version 2.0 DNA Sequencing Kit, USBC, USA) according to the manufacturer's instructions. The sequencing kit was based on the dideoxynucleotide chain termination method (Sanger *et al.*, 1977), and [ $\alpha$ -<sup>35</sup>S]dATP was used as the radioactive label. DNA templates (approximately 4 µg per reaction) were denatured in 2M NaOH/1mM EDTA for 30 min and the denatured DNA was recovered using custom-made Sepharose 6BL (Pharmacia) resin-spin columns. Denatured DNA was used as a template for the sequencing reaction and the reaction products were separated on 6% (w/v) polyacrylamide gels (containing 46% w/v urea) by electrophoresis at 60 mA constant current at 55°C. Computer analyses were performed using the University of Wisconsin Genetics Computer Group package (Devereux *et al.*, 1984) in the ANGIS suite of programs developed in the Department of Electrical Engineering, University of Sydney, Australia, and the SeqEd™ version 1.0.3 (Applied Biosystems Inc.) programs.

## 2.3 RESULTS AND DISCUSSION

### 2.3.1 Isolation of poly (A)<sup>+</sup>-RNA

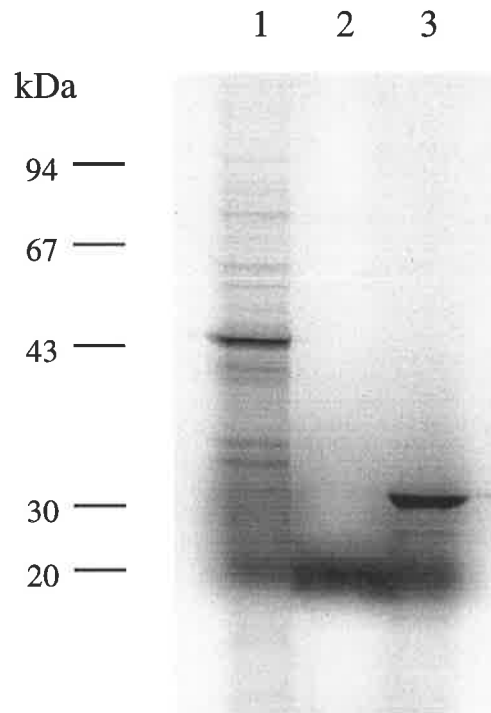
Approximately 435 µg total RNA was isolated from about 1000 GA<sub>3</sub>-treated aleurone layers. Approximately 20 µg poly (A)<sup>+</sup>-RNA was recovered from the total RNA preparation. The purity of the isolated poly(A)<sup>+</sup>-RNA was analysed spectrophotometrically. An A<sub>260/280</sub> ratio of 2.0 indicated that the RNA preparation was of high purity (Sambrook *et al.*, 1989). To further examine the integrity of the poly(A)<sup>+</sup>-RNA, a sample was translated *in vitro* and products examined by SDS-PAGE and autoradiography. The *in vitro* translation experiment showed that the poly (A)<sup>+</sup>-RNA was indeed translatable. Compared to the elution buffer control, the incorporation of [<sup>35</sup>S]-methionine into TCA-insoluble products was 7-fold higher for aleurone layer poly(A)<sup>+</sup>-RNA and 8-fold higher for the positive control luciferase RNA provided by the manufacturer (Table 2.1). The size distribution of the translation products is shown in Figure 2.4 and it is clear that the mRNA specified polypeptide products of up to 70 kDa in size. This indicated that the mRNA was essentially intact and that the isolated poly (A)<sup>+</sup>-RNA was suitable for first strand cDNA synthesis.

### 2.3.2 Amplification and cloning of a (1→4)-β-xylanase cDNA

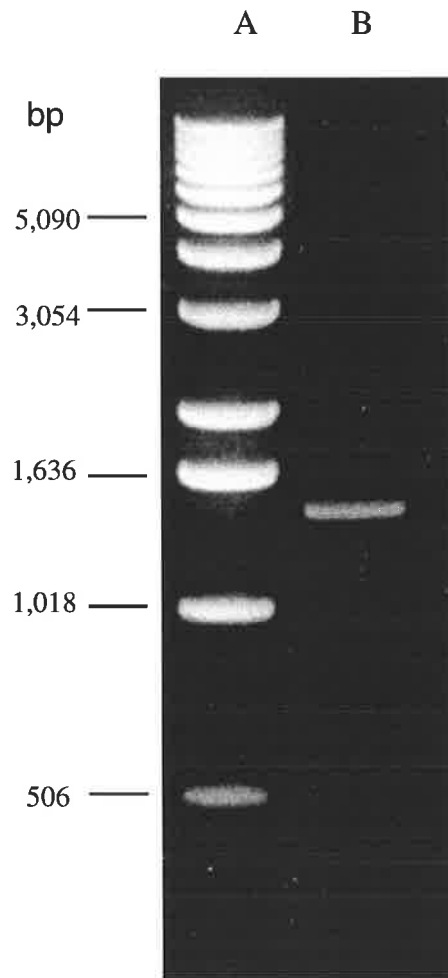
A cDNA of 1.35 kb was amplified (Figure 2.5). The expected size of the xylanase cDNA could be estimated from the molecular weight (approximately 41,000) of the purified protein (Slade *et al.*, 1989). A protein of M<sub>r</sub> 41,000 would be expected to contain approximately 400 amino acids, which would be encoded by an mRNA of approximately 1200 bp. Addition of a 3' untranslated region, which might be from 150-300 bp in length, would mean the predicted size of the PCR product would be in the

Table 2.1 *In vitro* translation of isolated poly(A)<sup>+</sup>-RNA

Sample	TCA-insoluble material (cpm)
Elution buffer	3581
Luciferase Control RNA	28234
Aleurone Poly (A) <sup>+</sup> - RNA	26323



**Figure 2.4** *In vitro* translation products of poly(A)<sup>+</sup>-RNA from GA<sub>3</sub>-treated aleurone layers. Lanes 1, 2 and 3 represent the *in vitro* translation products from aleurone poly (A)<sup>+</sup>-RNA, wheat germ extract control (without added RNA) and luciferase RNA, respectively. The sizes of proteins are as indicated. The strong band of M<sub>r</sub> approximately 45,000 kDa in lane 1 is presumably α-amylase precursor.



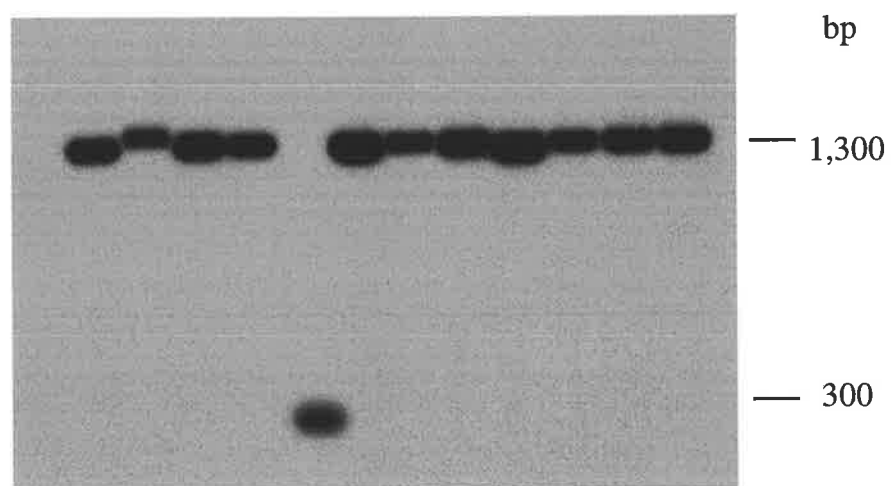
**Figure 2.5** Agarose gel electrophoresis of cDNA products of 3' RACE-PCR from poly(A)<sup>+</sup>-RNA of GA<sub>3</sub>-treated barley aleurone layers. A. 1kb ladder DNA markers; B. PCR product of approximately 1.3 kb.

range 1350-1500 bp, as observed (Figure 2.5). Final amplification products were cloned into dT-tailed, *EcoRV*-cleaved pBluescript vector (T-vector). Plasmid DNA was prepared from 12 randomly selected recombinant transformants and digested with *EcoRI* and *XhoI* restriction enzymes. To confirm the identity of the positive transformants another oligonucleotide primer (Xy2), which corresponds to the NH<sub>2</sub>-terminal amino acid sequence (KDKTDKA) of the xylanase enzyme, was used as a probe for Southern blot analysis. Among the recombinants analysed, 11 produced very strong hybridization signals and had cDNA inserts of identical sizes. One clone showed hybridization of the probe to a shorter fragment (Figure 2.6); this clone was not studied further.

### 2.3.3 Characterization of the cDNA clone

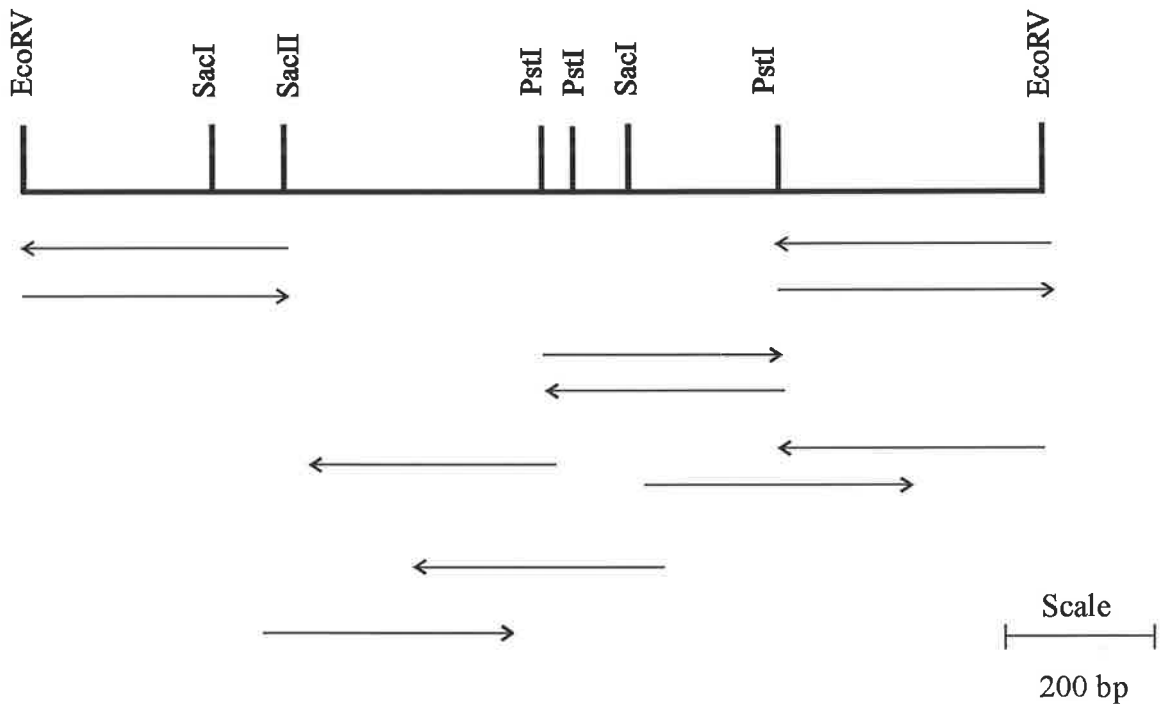
The 1.35 kb cDNA generated by PCR was designated pMXI. The detailed restriction map of pMXI and the strategy used for nucleotide sequence analysis are shown in Figure 2.7. Figure 2.8 shows the complete nucleotide sequence and reveals that the cDNA is 1334 bp in length and has an open reading frame that encodes 395 amino acid residues. The 5' end of this cDNA product contains a sequence which corresponds exactly to the sequence of the first 30 amino acids at the NH<sub>2</sub>-terminal end of the purified barley (1→4)-β-D-xylanase (Slade *et al.*, 1989), except that deduced amino acid residue number 11 was arginine in the PCR-generated cDNA and lysine in the purified enzyme.

The translation stop signal (TGA) is located at nucleotides 1186-1188. The sequence contains 116 bp of 3' untranslated region and a 30 bp polyadenylic acid tail, which starts at nucleotide 1305 (Figure 2.8). Four independent clones were sequenced at their 5' and 3' ends and although all four cDNAs were identical at the 5' ends, they differed at their 3' ends (Figure 2.9).



**Figure 2.6** Southern blot analysis of 12 individual PCR-generated cDNA clones using the Xy2 oligonucleotide as a probe. The cDNA inserts were excised from the recombinant pBluescript SK+ plasmids with *EcoRI/HindIII*, separated on 1% agarose gels and blotted onto nylon membrane prior to probing with the Xy2 oligonucleotide. DNA fragment lengths are as indicated on the right.





**Figure 2.7 Restriction map and sequencing strategy for the cDNA clone pMX1.**

The arrows indicate the direction and length of individual sequence analyses.

No consensus polyadenylation signal (AATAAA) was observed. However, a putative polyadenylation signal GATAAT is found 61 bp upstream from the poly(A) tail (Figure 2.8). This may represent the polyadenylation signal, in view of the variability of such signals in plant mRNAs (Lycett *et al.*, 1983; Fray *et al.*, 1994; Li and Hunt, 1995). The distance between this putative polyadenylation signal and the actual site of polyadenylation showed considerable variation among the four clones (Figure 2.9).

Because the 5' primer used in the RACE-PCR reaction corresponded to NH<sub>2</sub>-terminal sequence of the mature protein, no Met start codon was found and the clone was clearly not full-length at its 5' end. In other words, the experimental design was such that the PCR products corresponding to the cDNA would not be full-length because sequences representing the signal peptide and 5' untranslated regions would be missing.

In conclusion, the RACE-PCR protocol proved effective for the isolation of a xylanase cDNA. The pMX1 cDNA described in this Chapter was not full-length but it was long enough to use as a probe to screen cDNA libraries to obtain full-length cDNAs encoding barley (1→4)-β-xylan endohydrolases. The use of the pMX1 cDNA to isolate full-length cDNAs from aleurone libraries is described in the next Chapter.

GTGTATCCGGTGGACCACAAAGCGCGCTTCAGGCAGCTCAAGGACAAGACTGACAAGGCG	60
<b>V Y P V D H K A R F R Q L K D K T D K A</b>	20
CGCAAGAGGGACGTGATTTCTCAAGCTGGGCACGCCGGCGGAGCGGGAGCGGGCGCGCG	120
<b>R K R D V I L K L G T P A G A G A G A A</b>	40
GCGTCCGTGGCGTGGTGCAGTTGGACAACGCCTTCCCCTTCGGGACATGCATCAACACG	180
A S V R V V Q L D N A F P F G T C I N T	60
TCCGTCAATCCAGAAGCGGCCCTTCTCGACTTCTTACCAACCACCTTCGACTGGGCCGTC	240
S V I Q K P A F L D F F T N H F D W A V	80
TTCGAGAACGAGCTCAAGTGGTACCACACGGAGGTGCAGCAGGGCCAGCTCAACTACGCC	300
F E N E L K W Y H T E V Q Q G Q L N Y A	100
GACCCGACGCGCTGCTCGCGTTCGCGACCGCTGGCAAGACCGTCCGCGGCCACTGTC	360
D A D A L L A F C D R L G K T V R G H C	120
GTCTTCTGGTCCGTGGACGGCGACGTGCAGCAGTGGTCAAGAACCTCAACAAGACCAG	420
V F W S V D G D V Q Q W V K N L N K D Q	140
CTCAGGTCTCCATGCAGAGCCGCTCGAGGGCCTCGTCTCCCGCTACGCCGCGAGTTTC	480
L R S S M Q S R L E G L V S R Y A G R F	160
AAGCACTACGACGTCAACAACGAGATGCTGCACGGCCGCTTCTTCCGGGACCGCCTCGGC	540
K H Y D V N N E M L H G R F F R D R L G	180
GACGAGGACGTCCCGCGTACATGTTCAAGGAGGTGGCGCGGCTGGACCCGGAGCCCGTG	600
D E D V P A Y M F K E V A R L D P E P V	200
CTCTTCGTCACGACTACAACGTGGAGTGGCGCAACGACCCCAACGCGACGCCGAGAAG	660
L F V N D Y N V E C G N D P N A T P E K	220
TACCCGAGCAGGTCCGATGGCTGCAGAGTGCAGCGCGGTGGTGCAGCGCATCGGGCTG	720
Y A E Q V A W L Q S C G A V V R G I G L	240
CAGGGCCAGTGC AAAACCCGGTCGGGAGGT CATCTGCGCCGCGCTCGACAGGCTCGCC	780
Q G H V Q N P V G E V I C A A L D R L A	260
AAGACGGGGTGCATCTGGTTCACCGAGCTCGACGTGCCGGAGTACGACGTGGGCTC	840
K T G V P I W F T E L D V P E Y D V G L	280
CGGCCAAGGACCTGGAGTGGTGTCCGGGAGGCGTACGCGCACCCGGCCGTGGAGGGC	900
R A K D L E V V L R E A Y A H P A V E G	300
ATCGTGTCTGGGGCTTCAATGCAGGCACAATGTGGGCCAGAACGCTTGGCTCGTCGAC	960
I V F W G F M Q G T M W R Q N A W L V D	320
<b>GCCGATGGCACCGTCAACGAGGCGGGCCAGATGTTCTGAATCTGCAGAAGGAGTGGAA</b>	1020
<b>A D G T V N E A G Q M F L N L Q K E W K</b>	340
ACGACGCGCGGGGAACTTCGACGGCGACGGAACTTCAAGTTCAGGGGCTTCTACGGC	1080
T D A R G N F D G D G N F K F R G F Y G	360
AGATACGTCGTGGAGTTACGACGGCGAAGCGGAAGCAGATGCTCAAGACCTTCACGGTG	1140
R Y V V E V T T A K R K Q M L K T F T V	380
GAGAAAGGGGACAACACACCTGTCGTCGTGGATTGGCTGACGCTGACGGTGAATCTAT	1200
E K G D N T P V V V D L A D A *	395
CTAAGAACTATTTATTTATACCTATCTAATTACATGCAACACGTC AAGGGATAATGGT	1260
TGTATAATTTTACATTTCTAAGGTAACGGGTATTGTATTTGTAAAAAAAAAAAAAAAA	1320
AAAAAAAAAAAAA	1334

**Figure 2.8 Complete nucleotide sequence and deduced amino acid sequence of cDNA pMX1.** The 30 NH<sub>2</sub>-terminal amino acid residues from the purified protein (Slade *et al.*, 1989) are shown in bold. A putative polyadenylation signal is underlined. The stop codon is marked by an asterisk.



## **CHAPTER 3**

# **ISOLATION AND CHARACTERIZATION OF A FULL-LENGTH cDNA ENCODING BARLEY (1→4)- $\beta$ -XYLAN ENDOHYDROLASE**

### 3.1 INTRODUCTION

In contrast to microbial xylanases, for which primary structures of bacterial and fungal xylanases have been deduced from the nucleotide sequences of cloned cDNAs and genes (Shareck *et al.*, 1991; Sakka *et al.*, 1993; Gibbs *et al.*, 1995), there are no published reports describing the cloning of plant xylanase cDNAs or genes, and the primary structures of these plant enzymes have remained undefined. Although the barley xylanase cDNA isolated by RACE-PCR and described in Chapter 2 was not full-length, the deduced amino acid sequence could be used to predict the complete primary structure of the mature enzyme. However, the RACE-PCR product was truncated at its 5' end and the position of the initiating Met codon and the sequence of the 5' untranslated region could therefore not be defined, nor could the presence or absence of a signal peptide be determined. In addition, it was possible that cDNAs encoding more than one xylanase isoform might be present in the available cDNA libraries and that these might provide information on the barley xylanase gene family.

In attempts to isolate full-length cDNAs, the RACE-PCR product was therefore used as a probe to screen libraries prepared from RNA of GA<sub>3</sub>-treated barley aleurone layers and 12 day-old barley seedlings. The structures of cDNAs encoding two distinct xylanase isoenzymes are described in this Chapter and the characteristics of the proteins have been analysed in detail. Amino acid sequences are aligned with those of selected microbial xylanases.

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## 3.2 MATERIALS AND METHODS

### 3.2.1 *Materials*

The cDNA synthesis, cloning and packaging kits were purchased from Stratagene (La Jolla, CA, USA). A cDNA library prepared from barley aleurone layers (cv. Himalaya) which had been treated for 18 h with GA<sub>3</sub> was generously provided by Drs. Frank Gubler and Jake Jacobsen, CSIRO Division of Plant Industry, Canberra. A barley seedling library was purchased from Clontech Laboratories Inc. (Palo Alto, CA, USA). Restriction enzymes and T4 ligase were obtained from Promega (Madison, WI, USA) and New England Biolabs (Beverly, MA, USA). Sephadex G-100 was obtained from Pharmacia Biotech (Uppsala, Sweden). X-Gal, IPTG, DEPC and gibberellic acid were purchased from Sigma. The multiprime labelling kit, autoradiographic film and nylon membrane were from Amersham International. [ $\alpha$ -<sup>32</sup>P]dCTP and [ $\gamma$ -<sup>32</sup>P]ATP were from Bresatec or Amersham International. The DNA sequencing kits (Sequenase version 2.0) were purchased from United States Biochemical Corporation.

### 3.2.2 *Construction of a cDNA library*

#### 3.2.2.1 *cDNA synthesis*

An outline of the cDNA synthesis technique is shown in Figure 3.1. Double-stranded cDNA was synthesized using a  $\lambda$ ZAP-cDNA synthesis kit (Uni-ZAP XR, Stratagene) according to the manufacturer's instructions. Five micrograms of poly (A)<sup>+</sup>-RNA were used for first strand cDNA synthesis. Moloney-Murine Leukemia Virus Reverse Transcriptase (M-MuLV-RT) was used instead of Avian Myeloblastosis Virus Reverse Transcriptase (AMV-RT). The nucleotide mixture for the first strand synthesis

contained 10 mM dATP, dGTP, dTTP and the analog 5-methyl dCTP. Thus, the first strand cDNA had a methyl group on each cytosine base to protect the cDNA from restriction enzymes in subsequent cloning steps. The second-strand cDNA was synthesized from the first strand using second strand reaction buffer (1 x) containing 10 mM dNTPs, 1 mM DTT, 4.5 U RNase H and 7 U DNA polymerase I. During second-strand synthesis RNase H nicks the mRNA which is bound to the first strand cDNA and produces fragments which serve as primers for DNA polymerase I. The dNTP mixture was supplemented with dCTP to reduce the incorporation of 5-methyl dCTP in the second strand.

*Analysis of first strand and second strand cDNA products:* First- and second-strand cDNA synthesis products were analyzed by alkaline agarose gel electrophoresis. A sample containing 10,000-30,000 cpm of radioactivity (approximately 5  $\mu$ l) was taken from the cDNA synthesis mix and placed in a microcentrifuge tube with 20  $\mu$ l carrier DNA solution (100  $\mu$ g/ml salmon sperm DNA). Approximately 10  $\mu$ l 1 M NaOH was added and the mixture was incubated for 30 min at 46°C. The tube was spun briefly to return the mixture to the bottom of the tube and 10  $\mu$ l 1 M HCl was added. Finally, 10  $\mu$ l 1 M Tris-HCl buffer, pH 8.0 was added. The resulting mixture was extracted with an equal vol of phenol/chloroform (1:1, v/v) and the aqueous phase was removed and re-extracted with phenol:chloroform, followed by chloroform alone. The aqueous phase was precipitated with an equal volume of 4 M ammonium acetate and two volumes of cold (-20°C) ethanol. The tube was chilled for 15 min at -80°C, warmed to room temperature with shaking to dissolve the unreacted dNTPs which were precipitated during chilling, and spun for 10 min at 13,200 rpm. The pellet was washed once with 50  $\mu$ l 2 M ammonium acetate and 100  $\mu$ l cold ethanol. The resulting pellet was washed with 200  $\mu$ l cold ethanol and, following centrifugation, the supernatant was removed. The cDNA pellet was dried, resuspended in TE buffer, pH 8.0 and separated on a 1.2%



(w/v) alkaline agarose gel overnight at 20 mA to analyse the size distribution of the product. The gel was dried, covered with Glad Wrap plastic film and examined by exposing the gel against X-ray Hyperfilm at -70°C with an intensifying screen for 1 day.

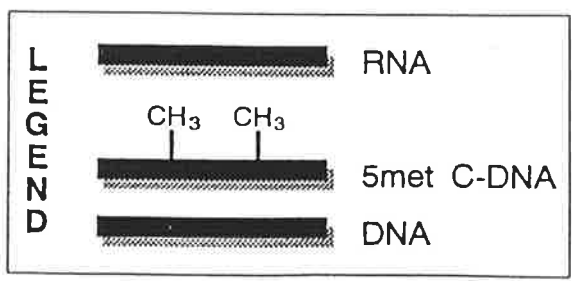
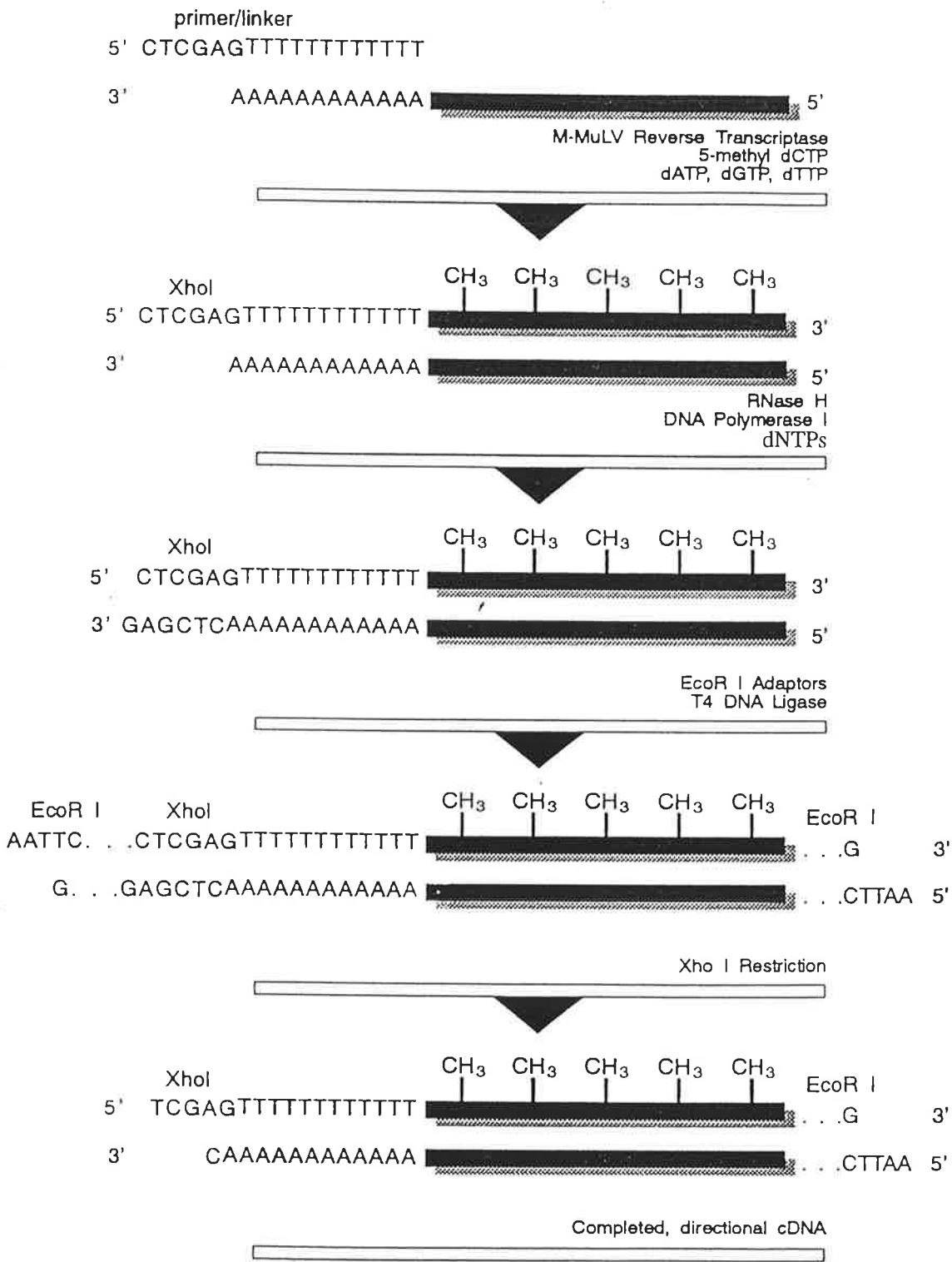
### 3.2.2.2 Cloning in the $\lambda$ ZAP II vector

The uneven ends of the double-stranded cDNA preparation were filled using T4 DNA polymerase and *Eco*R1 adaptors were ligated to the blunt ends, according to the manufacturer's instructions. The adapted cDNA ends were digested with *Xho*I (Figure 3.1). As a result the cDNA contained an *Eco*RI site at its 5' end and a *Xho*I site at its 3' end, to allow directional cloning into the *Eco*RI/*Xho*I-digested Uni-ZAP II vector. After ligation, the vector containing the cDNA inserts was packaged into  $\lambda$ ZAP using a Gigapack<sup>(R)</sup> Gold packaging kit, according to the manufacturer's instructions (Stratagene). The packaged recombinant phage particles were infected into *E. coli* SURE cells [e14<sup>-</sup>(*mcr*A),  $\Delta$  (*mcr*CB-*hsd* SMR-*mrr*) 171, *sbc*C, *rec*B, *rec*J, *umu*C :: Tn5(Kan<sup>r</sup>), *uvr*C, *sup*E44, *lac*, *gyr*A96, *rel*A1, *thi*-1, *end*A1, {F'*pro*AB, *lac*I<sup>q</sup>Z  $\Delta$  M15} Tn10(*tet*<sup>r</sup>)]. *E. coli* SURE is a *rec*A<sup>-</sup>, *mcr*A<sup>-</sup>, *mcr*CB, and *mrr*<sup>-</sup> strain which does not degrade methylated DNA.

### 3.2.2.3 Analysis of the cDNA library

To check that insert sizes of cDNAs were satisfactory prior to screening the library, random clones were isolated and insert sizes examined. The cDNA library was plated out using the SURE strain of *E. coli*. The number of plaque forming units (pfu) was determined and twelve single plaques were picked at random. The inserts were rescued from  $\lambda$ ZAPII by *in vivo* excision into the pBluescript plasmid according to the supplier's instructions. Each plaque was picked from the agar plate and transferred to 500  $\mu$ l SM

**Figure 3.1 Strategy for cDNA synthesis (Diagram from Stratagene product literature).** The first strand cDNA is synthesized using reverse transcriptase with the oligo (dT)/*Xho*I linker-primer in the presence of 5-methyl dCTP, dATP, dGTP and dTTP. The second-strand cDNA is synthesized from the first-strand cDNA using DNA polymerase I, RNaseH and dNTPs. The resulting double-stranded cDNA is blunt-ended with T4 DNA polymerase, ligated to *Eco*RI adaptors and finally the cDNA is digested with *Xho*I.



buffer. Chloroform (20 $\mu$ l) was added and the tube vortexed to release the phage particles into the SM buffer, which was incubated for 2 h at room temperature. Phage stock (100  $\mu$ l) was incubated at 37°C for 15 min with 200  $\mu$ l X11-Blue cells [recA1, endA1, gyrA96, thi-1, hsdR17, SupE44, relA1, lac, (F', ProAB, lacI<sup>q</sup>, Z $\Delta$ M15, Tn10tet<sup>r</sup>)] and 1 $\mu$ l ExAssist helper phage (1x10<sup>6</sup> pfu/ml) in a 10 ml tube. Following incubation, 3 ml of 2 x YT media was added and incubation was continued for 2.5 h at 37°C with shaking. The cell suspension (1ml) was transferred into a sterile microfuge tube and heated at 70°C for 20 min. Following centrifugation at 6,000 x g for 10 min the supernatant was transferred into a fresh microfuge tube and stored at 4°C. To plate the rescued phagemid, 200  $\mu$ l *E. coli* SOLR {e<sup>14-</sup> (mcrA),  $\Delta$ (mcrCB-hsdSMR-mrr) 171, sbcC, recB, recJ, umuC::Tn5(kan<sup>r</sup>), uvrC, lac, gyrA96, relA1, thi-1, endA1,  $\lambda^R$ , [F' proAB, lacI<sup>q</sup>Z $\Delta$ M15] Su<sup>-</sup>} cells (A<sub>600</sub> approximately 1.0) were incubated with 50  $\mu$ l rescued phagemid stock for 15 min at 37°C. The mixture was plated on LB plates containing 100  $\mu$ g/ml ampicillin and incubated at 37°C overnight.

Plasmid DNA was prepared from overnight cultures by the alkaline-lysis method (Sambrook *et al.*, 1989). The cDNA inserts in pBluescript plasmid were excised with *Xho*I and *Eco*RI restriction enzymes and separated by electrophoresis using a 1% (w/v) agarose gel.

### 3.2.3 Preparation of [<sup>32</sup>P]-radiolabelled cDNA probes

The cDNA clone pMX1 was labelled with [ $\alpha$ -<sup>32</sup>P]-dCTP according to the protocol provided with the Megaprime labelling kit (Amersham). The DNA fragment (approximately 100 ng) was mixed with 5  $\mu$ l random nonanucleotide primers and made up to a volume total of 50  $\mu$ l with sterile milliQ H<sub>2</sub>O. The primer-DNA mix was boiled for 5 min and immediately transferred to ice. After cooling it was mixed with 10  $\mu$ l labelling buffer, 5  $\mu$ l [ $\alpha$ -<sup>32</sup>P]-dCTP and 2  $\mu$ l Klenow DNA polymerase enzyme, and

incubated for 20 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 was added to a height of 7 cm. The column was equilibrated with 1 x TE buffer pH 8.0. To the labelling reaction mixture was added 20 µl 1% (w/v) Blue Dextran and 1% (w/v) Orange G in TE buffer and, following equilibration, the reaction mixture was added slowly to the column. Labelled probe was collected as the blue dye eluted from the column. The purified probe was boiled for 5 min and immediately cooled on ice before being added to the hybridization solution.

#### **3.2.4 Screening of the cDNA libraries**

The recombinant plaques were grown overnight at 39°C on a lawn of the Sure strain of *E. coli* on NZY broth plates. To prevent agar sticking to the nitrocellulose filters, the plates were chilled to 4°C for 2 h. Nitrocellulose filters were placed on the chilled agar surface for 2 min. The transferred plaques were denatured with 0.5M NaOH/1.5 M NaCl and neutralized with 0.5 M Tris-HCl buffer pH 7.5/1.5M NaCl for 5 min each. The filter was baked at 80°C for 2 h (Sambrook *et al.*, 1989), prehybridized for 4 h and hybridized for 16 h at 65°C in 6 x SSC [1 x SSC is 150mM NaCl, 15mM sodium citrate buffer, pH (7.0)], 1x Denhardt's solution [0.02% (w/v) BSA, 0.02% (w/v) Ficoll and 0.02% (w/v) PVP] (Denhardt, 1966), 1% w/v SDS, 100 µg/ml denatured herring sperm DNA and the [ $\alpha$ -<sup>32</sup>P]-labelled pMX1 cDNA probe. The filters were washed at 65°C in 2 x SSC/0.1% (w/v) SDS for 20 min, followed by 1 x SSC/0.1% (w/v) SDS for 20 min, 0.5 x SSC/0.1% (w/v) SDS for 20 min and 0.1 x SSC/0.1% (w/v) SDS for 20 min. Finally, the filters were washed with 0.1 x SSC/0.1 (w/v) SDS at 70°C for 30 min. The nitrocellulose filters were dried and exposed against X-ray Hyperfilm (Amersham International, UK) using an intensifying screen at -80°C for 3 h. The positive clones were subjected to three rounds of plaque purification.

### 3.2.5 Southern blot analysis, restriction mapping and subcloning

The inserts were rescued from  $\lambda$ ZAP into the pBluescript plasmid and cDNAs were excised with *Xho*I and *Eco*RI restriction enzymes. The inserts were further digested with other restriction enzymes for subcloning into pBluescript. Resulting fragments were fractionated on a 1% (w/v) agarose gel. The DNA was transferred to a nylon membrane using the procedure described in Section 2.2.3.5. Hybridization was carried out with [ $\alpha$ - $^{32}$ P] dATP-labelled pMX1 cDNA as the probe (section 3.2.4).

Bands were excised from the gel and purified using Gene Clean (Bresatec). For complete nucleotide sequence analysis the purified DNA fragments were subcloned into pBluescript II SK(+) which had been digested with appropriate restriction enzymes and dephosphorylated (Sambrook *et al.*, 1989).

### 3.2.6 Comparisons of three-dimensional structures of (1 $\rightarrow$ 4)- $\beta$ -xylanase by computer analysis

Coordinates for the three-dimensional structures of a bifunctional xylanase/exo- $\beta$ -glucanase from *Cellulomonas fimi* (2exo) (White *et al.*, 1994), xylanase A from *Streptomyces lividans* (1xas, Ca only) (Derewenda *et al.*, 1994) and xylanase A from *Pseudomonas fluorescens* (1xys, Ca only) (Harris *et al.*, 1994) were taken from the Protein Data Bank (Bernstein *et al.*, 1977). Coordinates for xylanase Z from *Clostridium thermocellum* (1xyz) (Dominguez *et al.*, 1995) were kindly provided by the authors. Structures were superimposed using the automated procedure in the program O (Jones *et al.*, 1991). Structurally conserved segments were defined as those that were superimposable in all pairwise comparisons. These comparisons were kindly undertaken by Dr. Tom Garrett, Biomolecular Research Institute, Parkville, Victoria 3052.

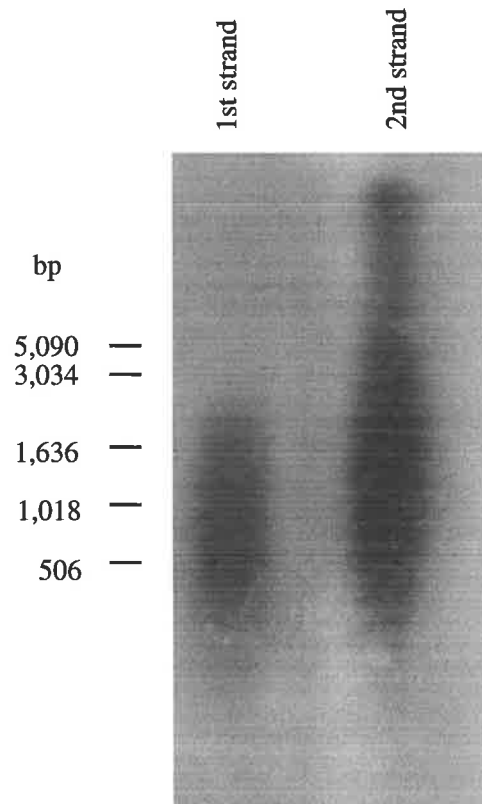
### 3.3 RESULTS

#### 3.3.1 Construction of the cDNA library

An alkaline agarose gel of the first strand and double-stranded cDNA showed a smear of cDNAs ranging from 500 to more than 3000 bp (Figure 3.2). The cDNA library produced from the double-stranded cDNA contained approximately  $1.2 \times 10^6$  pfu; these were generated from 5  $\mu$ g poly(A)<sup>+</sup>-RNA. Twelve plaques were selected at random and pBluescript plasmid containing cDNA inserts were excised from the  $\lambda$ ZAPII vector. The cDNA inserts were excised from pBluescript by *Xho*I and *Eco*RI digestion. Their sizes ranged from 500-2500bp (Figure 3.3). Because the molecular weight of the purified xylanase protein from germinated barley grain extracts is 41,000 (Slade *et al.*, 1989) and the expected size of a full-length xylanase cDNA (including both 5' and 3' untranslated regions) is therefore 1450-1600bp, the presence of cDNA inserts of up to 2500 bp indicated that the cDNA library was satisfactory and justified subsequent screening for full-length xylanase cDNAs.

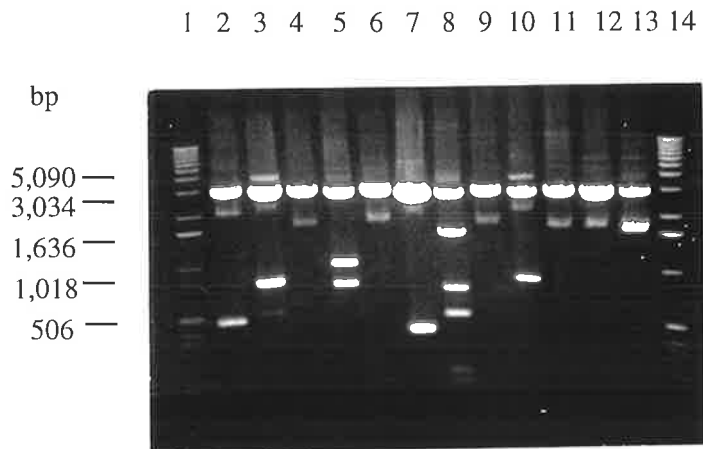
#### 3.3.2 Isolation of cDNAs encoding barley (1 $\rightarrow$ 4)- $\beta$ -xylan endohydrolase

Three cDNA libraries were screened in attempts to obtain full-length xylanase cDNAs. Approximately  $1 \times 10^5$  pfu of the GA<sub>3</sub>-treated aleurone cDNA library generated here were screened and thirty strongly positive clones were obtained and purified (Figure 3.4). The sizes of the cDNA inserts were checked by restriction digestion with *Xho*I and *Eco*RI and analysed by Southern blotting using the 5' oligonucleotide Xy2 as a probe (section 2.2.3.5). With this probe none of the clones gave a positive signal. Among the plaques, detected using the pMX1 cDNA, the largest cDNA insert obtained was 900 bp and this clone was named pMX1 (0.9A) (Figure 3.5).



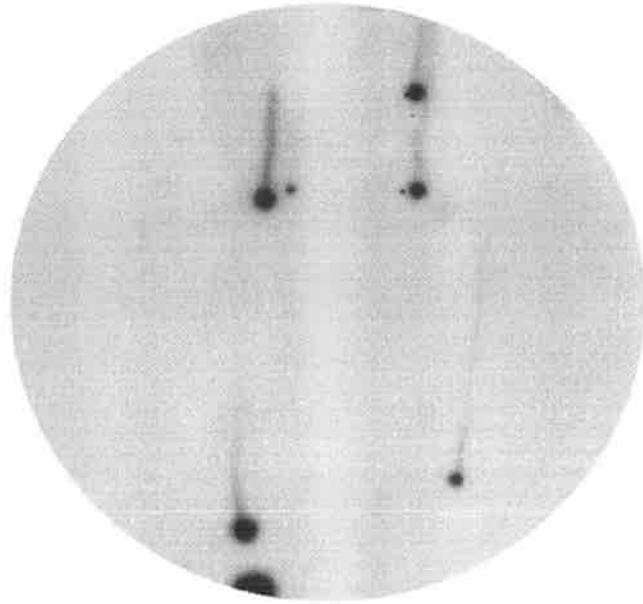
**Figure 3.2** Alkaline agarose gel electrophoresis of the first-strand and double-stranded cDNAs synthesized using poly(A)<sup>+</sup>-RNA from GA<sub>3</sub>-treated barley aleurone layers. DNA size markers are shown on the left.



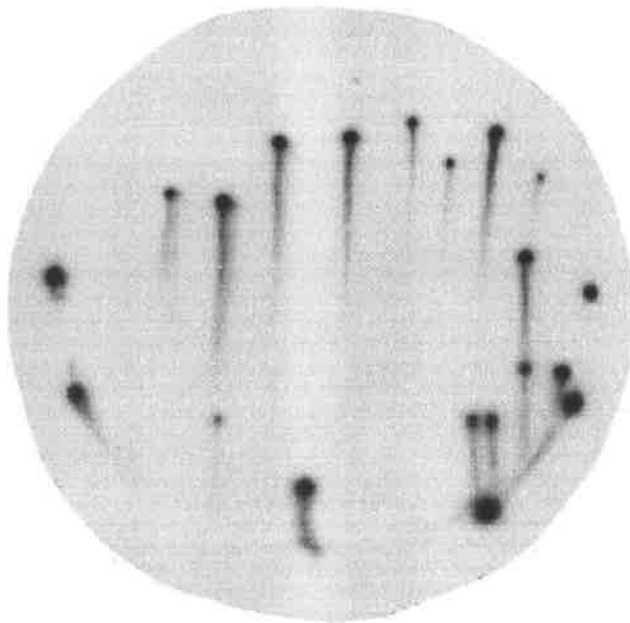


**Figure 3.3** Agarose gel electrophoresis showing restriction digests (*Eco*RI and *Xho*I) of the 12 random clones from the GA<sub>3</sub>-treated barley aleurone cDNA library. Lanes 1 and 14, 1 kb DNA size markers; lanes 2-13, inserts from twelve different clones.

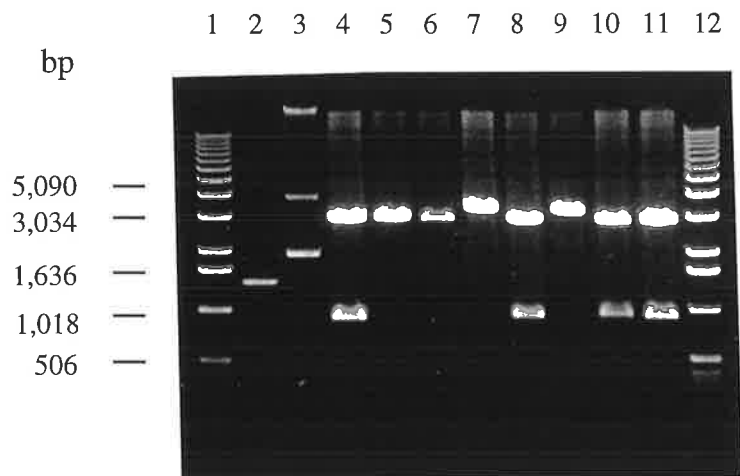
**A**



**B**



**Figure 3.4** Positive plaques after screening the cDNA library with the pMX1 cDNA probe. Panel A, positive plaques after the 2nd screen. Panel B, a single “monoclonal” after the 3rd screen.



**Figure 3.5** Restriction analysis of positive cDNA clones isolated from the GA<sub>3</sub>-treated barley aleurone layer cDNA library. Lanes 1 and 12, 1 kb ladder DNA markers; lane 2, pMX1 PCR cDNA insert; lane 3, an undigested positive cDNA clone, lanes 4-11, different positive clones after excision of cDNA inserts with *EcoRI/XhoI*.

A second cDNA library ( $1.2 \times 10^5$  pfu) prepared by Drs. Gubler and Jacobsen from 18 h GA<sub>3</sub>-treated aleurone layer poly (A)<sup>+</sup>-RNA was screened. Eighteen positives were obtained from the library. Insert sizes were determined by restriction digestion of the rescued clones (Figure 3.6 A) and the presence of xylanase sequences confirmed by Southern blot analysis with the 685 bp *Pst*I fragment of the pMX1 clone (Figure 3.6 B, lane 19). Among the 18 clones, the biggest insert size was 1560 bp and this clone was named pMX1(1.6A).

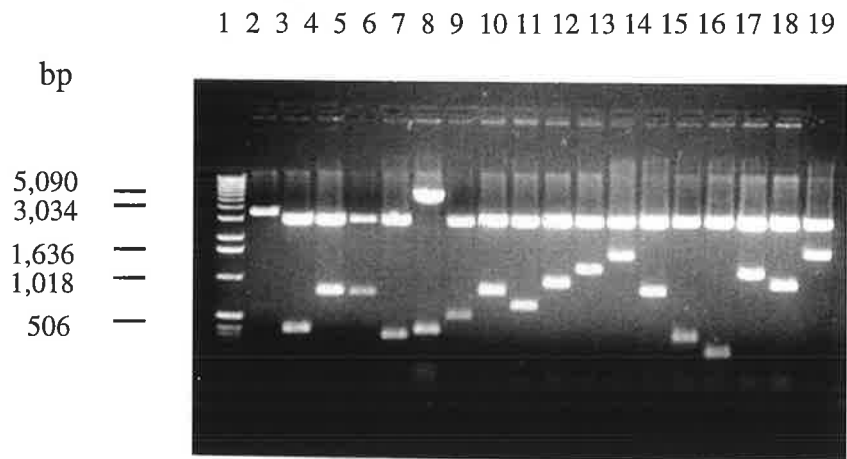
The third library ( $1.5 \times 10^5$  pfu) prepared in  $\lambda$ gt11 from 12-day-germinated barley seedling RNA was also screened but only one positive clone was obtained. The size of its cDNA insert was 2.7 kb which included 1.8 kb and 0.9 kb *Eco*RI fragments (Figure 3.7). After subcloning, the plasmid was digested with *Eco*RI (Figure 3.8A) and Southern blot analysis showed that the pMX1 cDNA probe bound only to the 1.8 kb *Eco*RI fragment (Figure 3.8 B). No hybridization was obtained with the 0.9 kb fragment. The 1.8 kb cDNA fragment was recloned and named pMX2(1.1S); the (1.1S) designation was given because it was likely that the first 700 bp at the 5' end of this cDNA resulted from a cloning artifact (section 3.4).

In summary, the following cDNAs were isolated and characterized: a 900 bp cDNA clone pMX1(0.9A) from the aleurone layer cDNA library generated in the present work; a near full-length, 1560 bp cDNA clone pMX1(1.6A) from the aleurone layer cDNA library provided by Drs. Gubler and Jacobsen; and a 1140 bp cDNA clone pMX2(1.1S) from the CLONTECH seedling library.

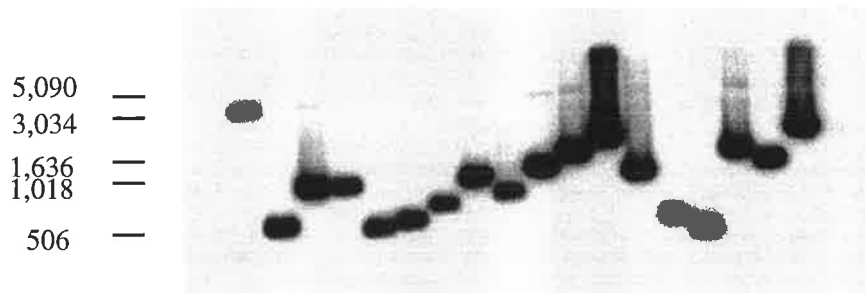
### 3.3.3 Characterization of cDNAs

Of the three cDNAs, pMX1(0.9A) had a poly(A) tail and appeared to be missing about 600 bp from its 5' end. The pMX1(1.6A) clone was nearly full-length but did not contain a poly (A) tail, while the 1140 bp pMX2(1.1S) was almost complete at its 5' end

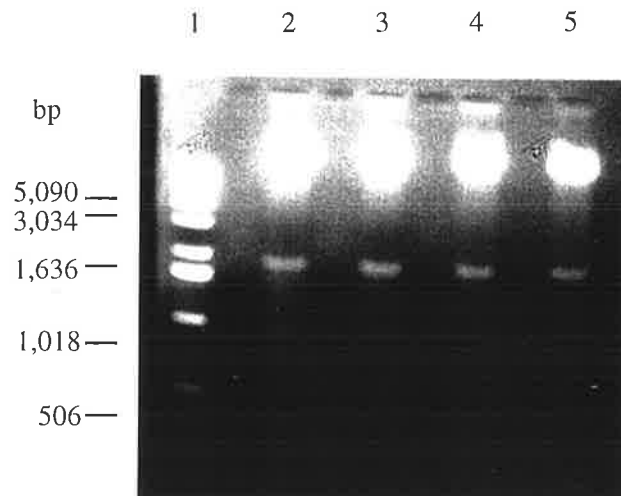
**A**



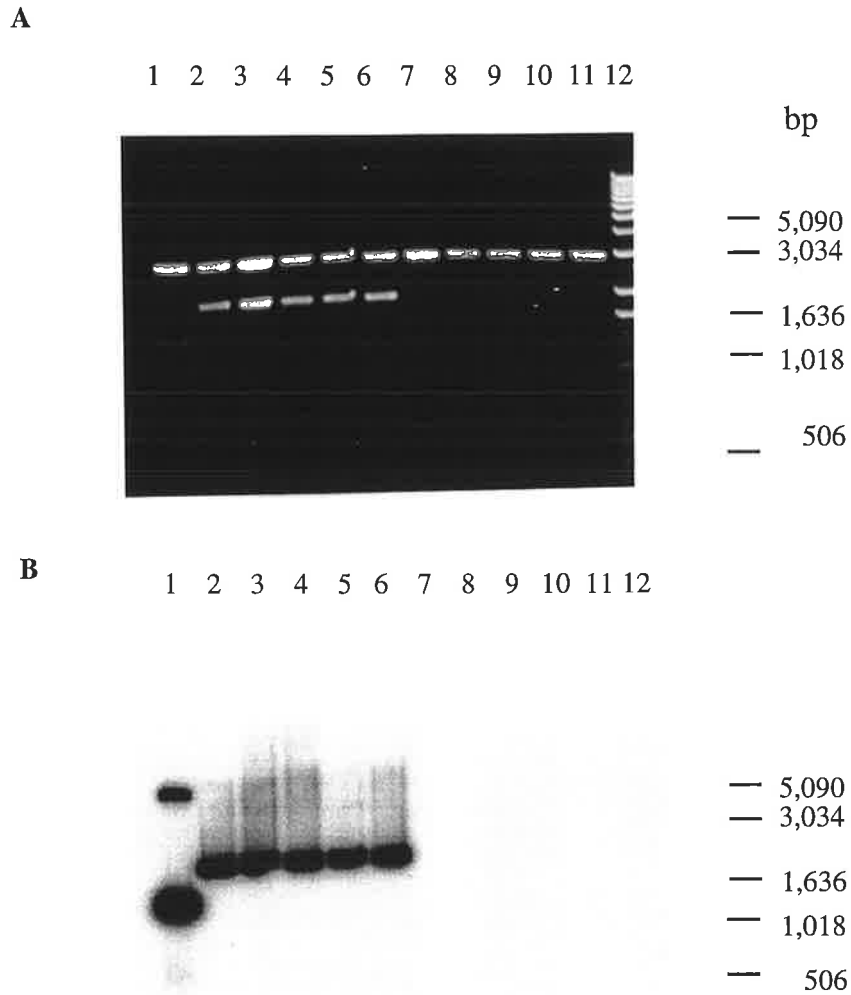
**B**



**Figure 3.6** *EcoRI* restriction digest of positive clones isolated from the GA<sub>3</sub>-treated barley aleurone layer cDNA library. Panel A, ethidium bromide-stained agarose gel showing different positive clones prior to transfer to a nylon membrane. Panel B, Southern blot analysis of the positive clones using the 685 bp *PstI* fragment of the pMXI cDNA as a probe.

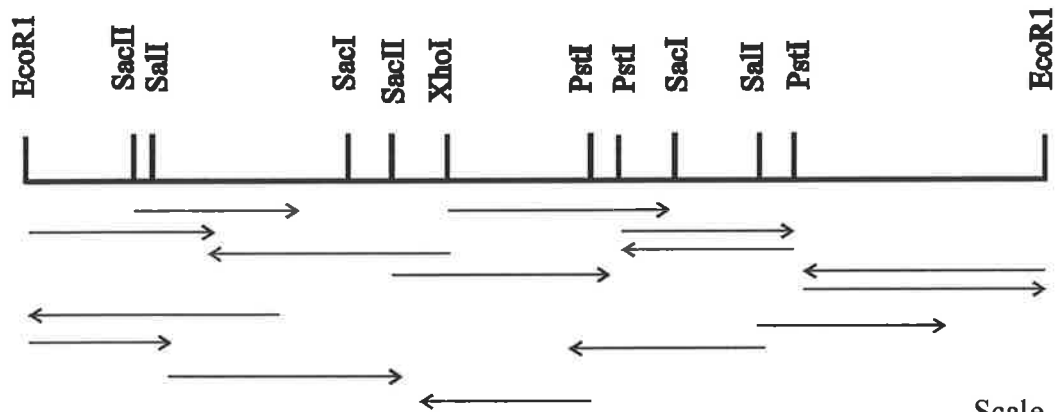


**Figure 3.7** Agarose gel electrophoresis of the positive clone [pMX2(1.1S)] isolated from a 12-day seedling library following restriction digestion with *EcoRI*. Lane 1, 1 kb DNA ladder; lanes 2-5, four individual transformants of the positive clone showing the 1.8 kb and 0.9 kb cDNA fragments.

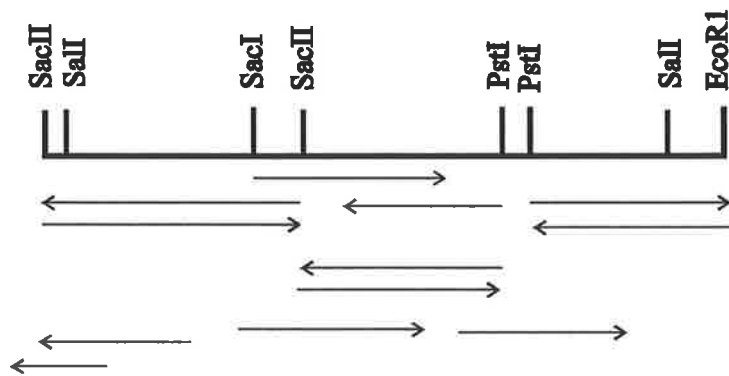


**Figure 3.8** Subcloned 1.8 kb and 0.9 kb fragments from the pMX2(1.1S) cDNA clone isolated from a 12-day-germinated barley seedling library. Panel A, ethidium bromide-stained agarose gel of plasmid DNA digested with *EcoRI* prior to transfer onto a nylon membrane for blotting. Lane 1, pMX1 digested with *Bam*HI and *Hind*III; lanes 2-6, 1.8 kb inserts; lanes 7-11, 0.9 kb inserts; lane 12, 1 kb DNA markers. Panel B, Southern blot analysis of digested DNA following transfer to a nylon membrane using pMX1 cDNA as a probe.

A



B



Scale  
200 bp

**Figure 3.9** Restriction map of barley (1→4)-β-xylanase cDNAs. A. near full-length cDNA pMX1(1.6A) from aleurone layers. B. cDNA clone pMX2(1.1S) from young seedlings. The arrows indicate the direction and length of nucleotide sequences determined during sequence analysis.



**Figure 3.10** The complete nucleotide sequence and derived amino acid sequence of barley (1→4)-β-xylanase isoenzyme X-I. The signal peptide is shown in bold and the NH<sub>2</sub>-terminal valine residue of the mature enzyme is indicated with an arrow, with the numbering of amino acid residues starting at the Val. Potential N-glycosylation sites are indicated by dashed underlining, the likely catalytic nucleophile (Glu 168) and catalytic acid (Glu 270) by double underlining, and the potential polyadenylation signals are underlined with solid lines. The translation stop codon is marked with an asterisk, and the position where the poly(A) tail for the pMX1 clone begins is shown by a leaf symbol.



**Figure 3.11 Partial nucleotide sequence and deduced amino acid sequence of (1→4)-β-xylanase isoenzyme X-II.** The signal peptide is in bold and the NH<sub>2</sub>-terminal valine residue of the mature enzyme is indicated with an arrow. Potential N-glycosylation sites are indicated by dashed underlining, and a putative catalytic nucleophile (Glu 163) and catalytic acid (Glu 265) by double underlining.



but was missing about 400 bp from its 3' end. As mentioned above, the pMX2 (1.1S) had a very long (700 bp) 5' untranslated region, part of which was probably a cloning artifact. Therefore, the 1.14 kb region of pMX2(1.1S) was compared with the pMX1(1.6A) clone.

The nucleotide sequences of the PCR-generated cDNA pMX1(1.3) and the aleurone layer library cDNAs pMX1(0.9A) and pMX1(1.6A) were identical where they overlapped, except for a few base mismatches in the PCR-generated cDNA. Only one amino acid change was evident. The (1→4)-β-D-xylan endohydrolase encoded by these cDNAs was designated isoenzyme X-I. The nucleotide sequence of the seedling library cDNA pMX2(1.1S) showed a 91% sequence identity with the other cDNAs, but the amino acid sequence deduced from it differed significantly from the (1→4)-β-xylanase isoenzyme X-I sequence. The (1→4)-β-xylanase encoded by the seedling library cDNA pMX2(1.1S) was therefore designated isoenzyme X-II.

The restriction maps and nucleotide sequencing strategies for the full-length aleurone layer cDNA pMX1(1.6A) and the seedling library cDNA pMX2(1.1S) are compared in Figure 3.9. The complete nucleotide sequences of pMX1(1.6A) and pMX2 (1.1S), together with deduced amino acid sequences, are shown in Figures 3.10 and 3.11, respectively. The pMX1 (1.6A) cDNA has a 5' untranslated region of 136 bp, which is followed by a putative Met initiation codon. This codon is the first in an open reading frame that encodes 427 amino acid residues. The deduced sequence includes a putative signal peptide of 32 amino acid residues, followed by the NH<sub>2</sub>-terminal amino acid sequence of the mature enzyme (Slade *et al.*, 1989) (Figure 3.10). There is an additional in-frame Met codon much closer to the NH<sub>2</sub>-terminal Val residue (Figure 3.10). The translation start point indicated in Figure 3.10 was chosen because the nucleotide sequence in this region more closely matched the consensus sequence for translation start points in plant genes (Joshi, 1987 a). The pMX2(1.1S) also encodes a signal peptide of 32 amino acids, but only 345 amino acids of the mature protein sequence. The signal

peptides encoded by pMX2(1.1S) and pMX1(1.6A) have only two differences in amino acids.

In the region of the pMX1(1.6A) cDNA that encodes the mature (1→4)-β-xylanase there is an overall (G+C) content of 66% and this is attributable in large part to an extreme bias towards the use of G and C in the wobble base position of codons. Of the 395 codons in the region encoding the mature enzyme, only 17 have A or T in the wobble base position (Figure 3.10).

The translation stop codon beginning at nucleotide 1418 represents the beginning of a 195 bp 3' untranslated region and a 18 bp polyadenylate tail (Figure 3.10). The poly(A) tail therefore begins at a different position than that observed for the PCR-generated cDNA (Figure 2.8 cf 3.10). This indicates that although the two mRNAs originated from a single gene, alternative polyadenylation sites are used during 3' processing of the primary transcript in the aleurone layer cells. No AATAAA consensus polyadenylation signals are observed in the 3' untranslated region of the barley (1→4)-β-xylanase cDNA, although related sequences such as GATAAT (Joshi, 1987 b) are present (Figure 3.10).

#### **3.3.4 Properties of the encoded (1→4)-β-xylanase**

The mature enzyme encoded by the pMX1(1.6A) nucleotide sequence shown in Figure 3.10 contains 395 amino acid residues. It has a calculated molecular weight of 44,623 and two potential N-glycosylation sites (Figure 3.10). The isoelectric point calculated from the deduced amino acid sequence is 6.1.

The complete amino acid sequence of barley (1→4)-β-xylanase isoenzyme X-I is aligned with the partial sequence of isoenzyme X-II in Figure 3.12. Although the pMX2(1.1S) cDNA is truncated at its 3' end, the alignment shows clear differences between the sequences of the two putative isoenzymes (Figure 3.12). Thus, 50 changes

**Figure 3.12** Alignment of the complete amino acid sequence of barley (1→4)- $\beta$ -xylanase isoenzyme X-I, deduced from the nucleotide sequence of the near full-length cDNA pMX1(1.6A), with the partial amino acid sequence of barley (1→4)- $\beta$ -xylanase isoenzyme X-II deduced from the pMX2(1.1S) cDNA, which was truncated at its 3' end. The arrow indicates the NH<sub>2</sub>-terminal Val residue in the mature enzyme (amino acid number 1), dashes show the region of the isoenzyme X-I sequence which is absent from the isoenzyme X-II sequence, dots indicate the identical residues and doubly underlined Glu residues are the putative catalytic residues.



MGAFRLRTEPRSAAVYVHGAPAGVDVKVMDLRVYPVDHKARFRQLKDKTDKARKRDVILK	28	X-I
.....S.....Q.....FHA.R...T.....V..	28	X-II
LGTPAGAGAGAAASVRVVQLDNAFPFGTCINTSVIQKPAFLDFFTNHFDWAVFENELKWY	88	X-I
..-----A.TG..R.....S.....T...N...V.....L.....	83	X-II
HTEVQOGQLNYADADALLAFCDRLGKTVRGHCVFWSVDGDVQQWVKNLNKDQLRSSMQSR	148	X-I
...A.....D.....RA.....T.....DR.....AV...	143	X-II
LEGLVSRYAGRFKHYDVN <u>NE</u> MHLHGRFFRDRLGDEDVPAYMFKEVARLDPEPVLVNDYINV	208	X-I
IQ.....P.....AA.....	203	X-II
ECGNDPNATPEKYAEQVAWLQSCGAVVRGIGLQGHVQNPVGEVICAALDRLAKTGVPTWF	268	X-I
.....D.....IS.....G.....A...V..	263	X-II
<u>TE</u> LDVPEYDVGLRAKDLEVVLRAYAHPAVEGIVFWGFMQGTMRQNAWLVDADGTVNEA	328	X-I
.....C.A.....Q.....I...K...KD.....	323	X-II
GQMFLNLQKEWKT DARGNFDGDGNFKFRGFYGRYVVEVTTAKRKQMLKTF <sup>T</sup> VEKGDNTPV	388	X-I
...LM..H.....V.NA	345	X-II
VVDLADA	395	X-I



are apparent in the 383 amino acid residues that can be aligned and isoenzyme X-I has an extra group of 5 contiguous amino acids inserted near its NH<sub>2</sub>-terminus (Figure 3.12).

### 3.3.5 *Fold recognition*

Dr. T.P.J. Garrett used the program THREADER (Jones *et al.*, 1992) to see if the barley xylanase sequence was consistent with a known protein structure. The standard dictionary of 254 structures was modified by the addition of a xylanase/exo- $\beta$ -glucanase, 2exo. Three structures gave significant Z-scores ( $<-3.0$ ), the best two being  $\alpha/\beta$  barrels. Scores were 2exo (-3.79), 1nar, narbonin (-3.46) and 1cse, subtilisin (-3.30). The top two structures stood out more clearly for the filtered weighted Z-scores, which were -3.01, -3.16 and -2.51, respectively (Dr. T.P.J. Garrett, personal communication).

### 3.3.6 *Comparisons of sequences and three-dimensional structures.*

Although there are no sequences for plant xylanases in the DNA and protein databases, the primary structures of many xylanases of microbial origin have been determined. Furthermore, when searching the SWISSPROT database for similar sequences it was the microbial sequences that were most similar to the barley  $\beta$ -xylan endohydrolase. Overall positional identities were about 20%, but were as high as 28% for xylanase A from *Streptomyces lividans* (XYLA\_STRLI). Selected bacterial and fungal xylanase sequences have been aligned with the amino acid sequence of the barley (1 $\rightarrow$ 4)- $\beta$ -xylan endohydrolase isoenzyme X-I in Figure 3.13. This shows that blocks of identical or highly conserved residues are distributed throughout the microbial sequences and that many of these residues are also conserved in the barley enzyme.

The three-dimensional conformations for four microbial xylanases have been determined by x-ray crystallography (Derewenda *et al.*, 1994; Harris *et al.*, 1994;

**Figure 3.13 Amino acid sequence alignment of the barley (1→4)-β-xylanase isoenzyme X-I (hv) with selected microbial (1→4)-β-xylanases from *Penicillium chrysogenum* (pc), *Aspergillus niger* (an), *Streptomyces lividans* (sl) and *Bacillus stearothermophilus* (bs). The alignments were performed using the PRETTYBOX program.**

```

pc . . . . . M I P N I T Q L K K T A A L V . . M L F A G Q A L S G P V E S R Q A S E S I D A K F K A H . . G K K Y . . . . . L 47
an . . . . . . . . . . M V O I K A A A L A . . M L F A S H V L S E P I E P R Q A S V S I D S K F K A H . . G K K Y . . . . . L 43
sl M G S Y A L P R S G V R R S I R V L L A A L V V G V L G T A T A L I A P P G A H A A E S T L G A A . . . . . F 57
bs- . . . . . M R N V V R K P L T I G L A L T L L L P M G M T A T S A K N A D S Y A K K P H I S A L N A P Q L D Q R Y K N E F T I 58
hv . . V Y P V D H K A R F R Q L K D K T D K A R K R D V I L K L G T P A G A G A G A A A S V R V V Q L D N A . . . . . F P F 54
Consensus - - - - - R - - - - - A A L - - - - - L - - - - - A L S - P - - - - - A S - - - - - Y - - - - - 65

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pc G N I A D Q G T L N G N P K T P A I I K A N F G Q L S P E N S M K W D A T E P S Q G Q F S F A G S D Y F V E F A E T N G K L I R G 112
an G N I G D Q Y T L T K N S K T P A V I K A D F G A L T P E N S M K W D A T E P S R G Q F S F S G S D Y L V N F A Q S N N K L I R G 108
sl G T A I A S G R L S D S T Y T . S I A G R E F N M V T A E N E M K I D A T E P Q R G Q F N F S S A D R V Y N F A V Q N G K K I R G 121
bs- G A A V E P Y Q L . Q N E K D V Q M L K R H F N S I V A E N V M K P I S I Q P E E G K F N F E Q A D R I V K F A K A N G M D I R F 122
hv G T C I N T . S V I Q K P A F L D F F T N H F D W A V F E N E M K W Y H T E V Q Q G Q F N Y A D A D A L L A F C D R L G K T I R G 118
Consensus G - - - - - L - - - - - N - K T - - - - - K - - - - - F - - - - - E N - - - - - M K W D A T E P - - - - - G Q F N F - - - - - A D - - - - - V - F A - - - - - N G - - - - - K - I R G 130

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pc H T L V W . . H S Q L P S W . . . . . V S S I T D K T T L T D V M K N H I T T V M K Q Y K G K L Y A W D V V 159
an H T L V W . . H S Q L P S W . . . . . V Q A I T D K N T L I E V M K N H I T T V M Q H Y K G K I Y A W D V V 155
sl H T L A W . . H S Q Q P G W . . . . . M Q S L S G R . P L R Q A M I D H I N G V M A H Y K G K I V Q W D V V 167
bs- H T L V W . . H S Q V P P Q W F F L D K E G K P M V N E T D P L K R K E T H I K T V E R Y K D D I K Y W D V V 185
hv H C V F W S V D G D V Q Q W . . . . . V K N L . N K D Q L R S S M Q S R L E G L V S R Y A C R F K H W D V N 166
Consensus H T L V W - - H S Q - - P - W - - - - - V - - - - - K - - - - - L - - - - - M - - - - - H I - T - - - - - V M - - - - - Y K G K I - - - - - W D V V 195

```

```

pc N E I F E E D . . G T L R D S V F S R V L G E D F V R I A F . E T A R E . A D P E A K L Y I N D Y N L D S A T S A K L Q G . . M V 218
an N E I F N E D . . G A L R D S V F Y K V I G D D Y V I R I A F . E T A R A . A D P N A K L Y I N D Y N L D S A S Y P K L A G . . M V 214
sl N E A F A D G S S G A R R D S N L Q R S . G N D W I E V A F . R T A R A . A D P S A K L C Y N D Y N V E N W T W A K T Q A . . M Y 227
bs- N E V V G D D . . G K L R N S P W Y Q I A G I D Y I K V A F . Q A A R K Y G G D N I K L Y M N D Y N T E V E . . P K R T A . . Y 243
hv N E M H G R F . . F R D . . . R L G D E D V P A Y M F K E V A R . . L D P E P V L F V N D Y N V E C G N D P N A T P E K Y A 222
Consensus N E - F - - D - - G - - L R D S - - - - - R - - - - - G - D - - - - - A F - - - - - E T A R - - - - - A D P - - - - - A K L Y - N D Y N - - - - - E - - - - - P K - - - - - M - - - - - 260

```

```

pc S H V K K W I A A G V P I D G I G S Q T H L G A G A . . G A A A S G A L N A L A S A G T E E V A V T E L D I . . . . . A G A T S T 276
an S H V K K W I E A G I P I D G I G S Q T H L S A G G . . G A G I S G A L N A L A G A G T K E I A V T E L D I . . . . . A G A S S T 272
sl N M V R D F K Q R G V P I D C G F Q S H F N S G S P Y N S N F R T T L Q N A A L G V . D V A I T E L D I . . . . . Q G A P A S 286
bs- N L V K Q L K E E G V P I D G I G H Q S H I Q I G W P S E A E I E K T I N M A A L G L D N . Q I T E L D V S M Y G W P P R A Y P 307
hv E Q V A W L Q S C G A V V R G I G L Q G H V Q . . N P V G E V I C A A L D R L A K T G V P T W . F T E L D V P E Y D . V C L R A K 283
Consensus - - V K - - - - - G - - - - - V P I D G I G - Q - - - - - H - - - - - G - P - G A - - - - - I - - - - - A L N - L A - - - - - G - - - - - A - T - - - - - E L D I - - - - - G A - - - - - 325

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pc D Y V D V V N A . C L D Q P . . . . . K C V G I T V W G V A D P . . . . . D S W . . . . . 305
an D Y V E V V E A . C L D Q P . . . . . K C I G I T V W G V A D P . . . . . D S W . . . . . 301
sl T Y A N V T N D . C L A V S . . . . . R C L G I T V W G V R D S . . . . . D S W . . . . . 315
bs- T Y D A P K Q K F L D Q A A R Y D R L F K L Y E K L S D K I S N V T F W G I A D N H T W L D S R A D V Y Y D A N G N V V V D P N 372
hv D L E V V L R E A A H P A . . . . . V E G I V F W G F M Q G T M W . . . R Q N A W L V D A D G T V N E A G 329
Consensus D Y - - - - - V - - - - - C - - - - - L D Q - - - - - K - - - - - C - G I T V W G V A - - - - - D - - - - - D S W - - - - - - - - - - - 390

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pc . . . . . R A D E S P L L F D A S Y N P K . E A Y . N . . V S Q L L . . . . . 330
an . . . . . R S S S T P L L F D S N Y N P K . P A Y T A . . . . . A N A L . . . . . 327
sl . . . . . R S E Q T P L L F N N D G S K K . A A Y T A . . . . . V L D A L . . . . . 341
bs- A P Y A K V E K G K G K D A E V F G P D Y K V K . P A Y W A . . . . . I D H K . . . . . 407
hv Q M F L N L Q K E W K T D A R G N F D G D G N F K F R G Y G R Y V V E V T T A K R K Q M L K T F T V E K G D N T P V V V D L A D 394
Consensus - - - - - R - - - - - P L L F D - - - - - D Y N - K - - - - - A Y - - - - - A - - - - - V - - - - - L - - - - - - - - - - - 455

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Törrönen *et al.*, 1994; White *et al.*, 1994). The structures have been superimposed and their alignment with the barley enzyme is shown in Figure 3.14, along with the major secondary structural elements. Of the 34 amino acids which are totally conserved, 23 form a cluster around the putative catalytic glutamate residues (Figure 3.15). Residue Tyr206 in the barley enzyme (Tyr171 in 2exo) also lies in this patch and, although not completely conserved, is an aromatic residue in all cases.

**Figure 3.14 Structural homology of xylanases.** The structurally superimposable segments of the four xylanases are shown in uppercase letters. The remaining sequence is shown in lowercase. The top line is the bifunctional xylanase/exoglucanase (2exo) from *Cellulomonas fimi*; the second line is xylanase A from *Streptomyces lividans*; the third line is xylanase A from *Pseudomonas fluorescens*; the fourth line is xylanase Z from *Clostridium thermocellum*; and the fifth line is (1→4)-β-xylanase from *Hordeum vulgare*. Secondary structural elements for 2exo are given above the sequences (→ for β-sheet, H for helix, elements of the α/β barrel are numbered). The sequence for the barley xylanase was aligned by similarity with the other sequences. Because there is little similarity at the COOH terminus, the alignment in that region may be unreliable.

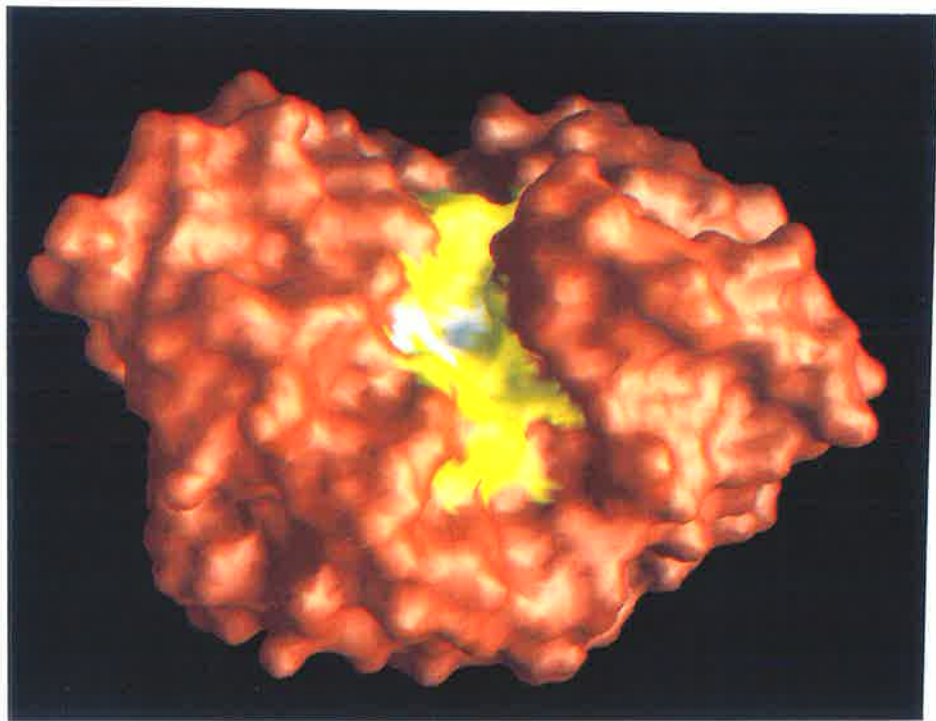
$\alpha 0$	$\beta 1$	$\alpha 1$	$\beta 2$	$\alpha 2$	$\beta 3$	
HHHHHHH	----->	HHHHHHHHHH	---->	HHHH	HHHHHHHHHHHHHH	----->
atTLKEaadgaGRDFGFALDp	-----nrls	EAQYKAIADSEFNLVVAENAMKWDATE	EpsqnSFSFGAGDRVAS	YAADTGKELYGHTLVWHS		86
LGAAaaq-sGRYFGTAIAS	-----grls	DSTYTSIAGREFNMVTAENEMKIDATE	EpqrgQFNFSADR	VYNWAVQNGKQVRGHTLAWHS		87
GLASla---DFPIGVAVAasggnadift	SSARQNIVRAEFNQITAENIMKMSYMYsgs	-NFSFTNSDRLVSWAAQNGQTVHGHALVWHP				85
nALRDyaearGIKIGTCVNypf	---ynns	DPTYNSILQREFSMVVCENEMKFDALQ	prqnvFDfSKGDQLLAF	ERNGMQMRGHTLIWHN		602
aSVRVvqldnAFPFGTCINT	-----sviq	KPAFLDFFTNHFDWAVFENELKWYHTE	vqqgQLNYADADALLAFCDRLGKT	VRGHCVFWSV		125
	+	+	** *	+	+ ** *	

$\alpha 3$	$\beta 4$	$\alpha 4$	
HHHH	HHHHHHHHHHHHHHHHHH	----->	HHHHHH
---qlpdwakln--gs	AFESAMVNHVTKVADHFEGKVASWDVVNEAFADgd	-----grrqdSAFQqkl	-NGYIETAFRAARAADP
---qqpgwmqsls--gr	PLRQAMIDHINGVMAHYKGIKIVQWDVVNEAFADgss	-----garrdSNLQrs	--GNDWIEVAFRTARAADP
syqlpnwasdsn---	aNFRQDFARHIDTVAAHFAGQVKS	WDVVNEALFDsaddpdgrgsangyrq	-SVFYrqfgGPEYIDEAFRRARAADP
--qnpswltngnwnrd	SLLAVMKNHITVMTHYKGI	VEWDVANECMDDs	-----gnglrs-SIWRnvi
gdvqgwkln--kd	QLRSSMQSRLEGLVSR	YAGRFKHYDVNNEMLHGr	-----FFRdrlgDEDVPAYMFKEVARLDP
	+	+ ** *	+ ** *

$\beta 5$	$\alpha 5$	$\beta 6$	$\alpha 6$	$\beta 7$	
----->	HHHHHHHHHHHHHHHH	---->	HHHHHHHHH	----->	
TAKLCINDYNVEGINA--	kSNSLYDLVKDFKARGVPLDCVGFQSHL	Ivg--qvpg-dFRQNLQRFADLg	--VDVRITELDIRMrtps	d-	243
SAKLCYNDYNVENWTwa--	kTQAMYNMVRDFKQRGVPIDCVGFQSHFN	sgs-pyns-nFR TTLQNF	AAALg--VDVAITELDIQGa	-----	242
TAELYNDNFNTEENGA--	kTTALVNLVQRLLNNGVPIDGVGFQMHVM	nd--ypsi	anIRQAMQKIVALsptL	KIKITELDVRLnpydg	257
DALLFYNDYNIEDLGP---	kSNAVFNMIKSMKERGV	PIDGVGFQCHFIngmspeyl	asIDQNIKRYAEI	g--VIVSFTEIDIRIpsenp	765
EPVLFVNDYNVECGNDp	natPEKYAEQVAWLQSCGAVVRGIGLQGHV	Qn--pvge-vICAALDRLAKTg	--VPTWFTELDVPEy	-----	276
	+	+ ** *	+ ** *		

$\alpha 7$	$\beta 8$	$\alpha 8$	
HHHHHHHHHHHHHHHHHH	----->	HHHH	HHHHHHHHH
-----atkl	atQAADYKKVVQACMQVT	---rcqGVTVWGITDKYSWVpdvf	pgeGAALVWDASYAKKPAYAAVMEAF
-----PASTYANVTNDCLAVS	---rcLGITVWGV	RSDSWRs	-----eQTPLLFNNDGSKKAAYTAVLDA
nssndytnrndcavscagldr	QKARYKEIVQAYLEV	VppgrrgGITVWGIADPDSWLythqnl	pdWPLLFNDNLQPKPAYQGVVEAlS
-----atafqv	QANNYKELMKICLANP	---ncnTFVMWGF	TDKYTWIpgt fpgyGNPLIYDSNYPKPAYNAIKEAlm
-----dvgl	RAKDLEVVLR	EAYHP---aveGIVFWGFMQGT	MWRq   gdNTPVVVDLADA
		** *	*

NAWLVDADGTVNEAGQMFLNLQKEWKT DARGNF DGDGNFKFRGFYGRYVVEVTTAKRKQMLKTFTVEK



**Figure 3.15** Conserved active sites in plant and microbial xylanases. The molecular surface of the bifunctional xylanase/exoglucanase (exo) is shown in blue with the conserved patch (yellow; residues marked with an asterisk in Figure 3.14) which surrounds the catalytic glutamate residues (Glu168 and Glu270; white). The highly conserved Tyr171 is included in the conserved patch.

### 3.4 DISCUSSION

An oligonucleotide primer corresponding to an NH<sub>2</sub>-terminal amino acid sequence of barley (1→4)-β-xylan endohydrolases (Slade *et al.*, 1989) was used in 3' RACE PCR experiments to generate a cDNA from poly(A)<sup>+</sup>-RNA prepared from GA<sub>3</sub>-treated barley aleurone layers (Chapter 2). The PCR product was subsequently used to screen a near full-length, 1560 bp cDNA [pMX1(1.6A)] from a barley aleurone layer cDNA library. Nucleotide sequence analysis (Figure 3.10) of the cDNA confirmed that it encoded a barley (1→4)-β-xylanase, which is now designated isoenzyme X-I. The deduced NH<sub>2</sub>-terminal amino acid sequence is almost identical to that determined directly from three separate endoxylanase enzymes by Slade *et al.* (1989). A second cDNA [pMX2(1.1S)] from a young seedling library was shown to encode a related yet distinct enzyme, designated (1→4)-β-xylan endohydrolases isoenzyme X-II.

Although the cDNA encoding isoenzyme X-II is not full-length at its 3' end, it is long enough to allow the alignment of nearly 400 amino acids (Figure 3.12). The alignment shows a sequence identity value of approximately 87% at the amino acid level; this may be compared with a value of 91% identity at the nucleotide level. These results indicate that the xylanase cDNAs are derived from two separate genes and, because barley is predominantly self-fertilizing and plants of established cultivars are essentially homozygous, the products of the genes represent true genetic isoenzymes. The three barley (1→4)-β-xylanase isoforms purified by Slade *et al.* (1989) had identical NH<sub>2</sub>-terminal amino acid sequences, whereas the isoenzymes X-I and X-II examined here exhibit significant differences in their NH<sub>2</sub>-terminal sequences (Figure 3.12). It may now be concluded, therefore, that the three isoforms described by Slade *et al.* (1989) probably originated by differences in post-translational modification of a single gene product. The presence of two potential N-glycosylation sites (Figure 3.10) provides



scope for heterogeneity in attached carbohydrate, which might be reflected in variations in chromatographic behaviour during the purification process.

The cDNA sequences revealed some information about the barley (1→4)- $\beta$ -xylanase gene structure. In particular, there is an extreme (G+C) bias in the wobble base position of codons (Figure 3.10). Similar strong biases towards the use of G and C in the third position of codons have been observed in genes encoding barley (1→3,1→4)- $\beta$ -glucanases, (1→3)- $\beta$ -glucanases and other enzymes that are expressed at high levels in germinated cereal grains (Slakeski and Fincher 1992 a, b; Fincher 1989; Fincher *et al.*, 1986). It should be noted, however, that a (G+C) bias in the wobble base position is not found in all genes expressed in germinated grains; a carboxypeptidase and a  $\beta$ -glucan exohydrolase from germinated barley both have a balanced codon usage (Doan and Fincher 1988; Hrmova *et al.*, 1996). There is also evidence for alternative polyadenylation sites during 3' processing of the primary transcript in the aleurone layers of germinated barley (Figure 3.10). Tissue-specific 3' processing of the pre-mRNA for barley (1→3)- $\beta$ -glucanase isoenzyme GV has been described (Xu *et al.*, 1994), although the functional significance of the alternative polyadenylation sites is not yet clear.

A putative signal peptide of 32 amino acid residues (Figure 3.10) also suggests that the mature enzyme is secreted from the aleurone cells. This putative signal peptide has charged residues near its NH<sub>2</sub>-terminus, it has a relatively short hydrophobic core and becomes more hydrophilic towards the NH<sub>2</sub>-terminal residue of the mature enzyme (Figure 3.10). These characteristics are typical of plant signal peptides that target nascent polypeptides to the endoplasmic reticulum (Watson, 1984) for eventual secretion from the cell.

Nucleotide sequence analysis of the barley (1→4)- $\beta$ -xylanase cDNA enabled the complete primary sequence of 395 amino acids in the mature enzyme to be deduced (Figure 3.11). The (1→4)- $\beta$ -xylanase isoenzyme X-I has a calculated molecular weight of mature enzyme of 44,623 and a pI of 6.1. These values can be compared with

apparent molecular weight values of 41,000 and pIs of 5.2 directly determined for the three (1→4)- $\beta$ -xylan endohydrolases that have been purified previously from extracts of germinated barley grain (Slade *et al.*, 1989). The discrepancies between values deduced from the cDNA and those directly measured from the purified enzyme are somewhat larger than expected. Whether the highly charged nature of the enzyme, which has 108 charged acidic and basic residues, together with associated carbohydrate, causes aberrant behaviour during SDS-PAGE or isoelectric focusing is not yet known. Another possibility is that the mature enzyme purified from germinated grain was subject to limited degradation by carboxypeptidases present in the grain. This would create carboxy-terminal heterogeneity with respect to both length and hence in pI of the various truncated forms. This phenomenon of carboxy terminal processing (or degradation) has been reported for the  $\alpha$ -amylase of germinated barley (Lundgard and Svensson 1987).

Alignment of the barley (1→4)- $\beta$ -xylanase sequence with microbial endoxylanases reveals the presence of several highly conserved blocks containing 2-6 amino acid residues (Figure 3.13). Microbial xylan endohydrolases have been classified into two distinct families on the basis of amino acid sequence alignments and hydrophobic cluster analyses (HCA) (Wong *et al.*, 1988; Gilkes *et al.*, 1991; Henrissat 1991; Henrissat and Bairoch, 1993) as follows;

a) a low molecular weight group of basic xylanases which have  $M_r$  values of approximately 22 kDa or less and isoelectric points (pI) of approximately 8.3-10.0. This group is a member of the G group of  $\beta$ -glycanases (Henrissat and Bairoch, 1993), which is well-characterized in terms of three dimensional structures. The G family of xylanases consists of enzymes containing a single-domain  $\beta$ -sheet protein (Wakarchuk, 1994; Arase *et al.*, 1993; Campbell *et al.*, 1993; Törrönen *et al.*, 1993).

b) a high molecular weight group of acidic xylanases, which have  $M_r$  values of approximately 43 kDa and above, and pI values in the range 3.6-4.5. These xylanases

have been classified in the F family of  $\beta$ -glycanases (Henrissat and Bairoch, 1993), which are generally eight-fold  $\alpha/\beta$  barrels (Harris *et al.*, 1994) (Table 3.1).

Despite this classification, the well-characterized microbial (1 $\rightarrow$ 4)- $\beta$ -xylan endohydrolases do not always fit obviously into one group or another and care must be exercised in oversimplifying xylanase classification. Exceptions to the two groups can be seen amongst the enzymes listed in Table 1.2 (Chapter 1).

The similarities of the barley (1 $\rightarrow$ 4)- $\beta$ -xylanases with conserved sequences in F family microbial xylanases for which crystal structures are defined suggest that the two groups of enzymes have similar protein folds. The microbial xylanases, from the F family group of endo- $\beta$ -glycanases are all ( $\alpha/\beta$ )<sub>8</sub> barrel structures (Davies and Henrissat 1995; Derewenda *et al.*, 1994; Harris *et al.*, 1994; White *et al.*, 1994). Although the overall amino acid sequence identities between the barley  $\beta$ -xylan endohydrolase and the microbial xylanases are not particularly high, it appears that the plant enzyme will prove to be quite similar to F family microbial xylanases both in overall three-dimensional structure and in the location of catalytic sites (Figure 3.15). It is noteworthy that barley (1 $\rightarrow$ 3,1 $\rightarrow$ 4)- $\beta$ -glucanase also adopts an ( $\alpha/\beta$ )<sub>8</sub> barrel fold (Varghese *et al.*, 1994).

The catalytic domains of several hundred glycosyl hydrolases and related enzymes can be classified into a relatively small number of distinct families, based on similarities in amino acid sequences (Henrissat and Bairoch, 1993). The barley (1 $\rightarrow$ 4)- $\beta$ -xylanase sequence deduced here (Figure 3.10) indicates that the enzyme falls into family F (Henrissat and Bairoch, 1993). Based on this classification, the catalytic acid residue of the barley (1 $\rightarrow$ 4)- $\beta$ -xylanase isoenzyme X-I would be expected to be Glu 168 and the catalytic nucleophile Glu270; the anomeric configuration of the xylosyl residues would be expected to be retained during hydrolysis (Henrissat and Bairoch, 1993; Chen *et al.*, 1995; Hrmova *et al.*, 1996). The potential participation of these residues in the catalytic mechanism can now be confirmed, using chemical modifications and suicide inhibitors of the type used by Chen *et al.* (1993) to define the catalytic amino acids in barley

Table 3.1 Properties of a selection of basic and acidic xylanases from microorganisms

Species	Molecular weight (kDa)	Isoelectric point (pI)	Xylanase family	References
<i>Aspergillus niger</i> 11	50.0	4-4.5	F	John <i>et al.</i> (1979)
<i>Clostridium stercorarium</i>	44.0	4.5	F	Bérenger <i>et al.</i> (1985)
	62.0	4.4	F	
	72.0	4.4	F	
<i>Bacillus</i> sp W2	50.0	3.7	F	Okazaki <i>et al.</i> (1985)
	22.5	8.3	G	
<i>Streptomyces lividans</i>	43.0	5.2	F	Morosoli <i>et al.</i> (1986)
	31	8.4	G	Kluepfel <i>et al.</i> (1990)
	22	10.25	G	Kluepfel <i>et al.</i> (1992)
<i>Trichoderma harzianum</i> E58	20.0, [8-11.5]	9.4	G	Tan <i>et al.</i> (1985)
<i>Trichoderma viride</i> (Onozuka)	17.8	9.2	G	Sinner and Dietrichs (1975, 1976)
	53.0	5.3	F	
	57.0	4.4	F	
<i>Cryptococcus albidus</i>	48.0	5.3	F	Morosoli <i>et al.</i> (1986)

(1→3)-β- and (1→3,(1→4)-β-glucanases. However, these experiments would rely on the availability of reasonable quantities of highly purified, stable enzyme, but problems encountered by Slade *et al.* (1989) with enzyme instability would probably preclude this approach. An alternative approach would be to express the cDNA in a heterologous system to generate sufficient enzyme for a thorough characterization. Attempts to achieve high-level expression of the barley xylanase isoenzyme X-I cDNA in *E. coli* are described in the next Chapter.

## **CHAPTER 4**

# **HETEROLOGOUS EXPRESSION OF THE cDNA ENCODING (1→4)- $\beta$ -XYLAN ENDOXYDROLASE ISOENZYME X-I**

## 4.1 Introduction

Slade *et al.* (1989) encountered serious difficulties during the purification of barley (1→4)- $\beta$ -xylanases. Dramatic losses of activity were evident during the purification processes, and it proved very difficult to isolate the (1→4)- $\beta$ -xylanases in large quantities. Nevertheless, they managed to purify microgram quantities of three (1→4)- $\beta$ -xylanase isoenzymes from germinated grains. Purification factors were close to 1800-fold, but less than 2% of activity detected in initial extracts was recovered in the purified enzyme preparations. The first 30 NH<sub>2</sub>-terminal amino acids were identical in all three isoforms, although the enzymes showed different chromatographic mobility when analysed by isoelectric focusing. As a result of the low yields, tryptic fragments could not be generated for additional sequence information (Slade *et al.*, 1989). It was therefore not possible to conclude whether the three isoforms were products of separate genes or were derived from a single gene product by post-translational modification.

Because these difficulties in isolating large quantities of pure active barley xylanase precluded detailed kinetic and enzyme analyses, an attempt was made here to express the xylanase cDNAs described in Chapter 3 in *E. coli*.

Several heterologous hosts for the expression of foreign genes have been developed, including mammalian cells, insect cells, fungal cells, bacterial cells and transgenic plant or animal cells. The choice of expression system for a given gene depends upon the likely properties of the encoded protein, since some enzymes require post-translational modifications to become active, whereas others may require special processing if they are to be used as diagnostic reagents (Yarranton and Mountain, 1994).

Many eukaryotic genes have been characterized by heterologous expression in bacteria (Gold, 1990; Henner 1990, Xu *et al.*, 1994). *E. coli* is the most widely-used and convenient host for heterologous production of foreign proteins in large quantities. The advantages of this system include the availability of suitable expression vectors, and

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the speed and adaptability of the system. Although *E. coli* is generally considered to be the heterologous system of choice, it does present some disadvantages and limitations. While significant progress has been made in the design of vectors for expression of foreign genes in *E. coli*, high-level expression is still not routine. Unlike eukaryotic cells, bacterial cells do not generally perform post-translational modifications. When foreign proteins are "overexpressed" intracellularly they sometimes aggregate or precipitate to form insoluble inclusion bodies (Marston, 1986; Schein, 1989; Kohno *et al.*, 1990). Furthermore, proteins produced in such systems are often inactive because eukaryotic polypeptide chains do not routinely adopt their native conformation in prokaryotic cells. Another problem related to the overexpression of recombinant DNA in *E. coli* is that the foreign protein synthesized is sometimes detrimental or toxic to the host cell (Schertler, 1992).

Yeast can be used as an alternative eukaryotic host for the heterologous expression of foreign proteins. Unlike bacteria, yeast possesses a complex cell biology more typical of multicellular organisms and can mediate the secretion and post-translational modification of many eukaryotic proteins. The main advantage of the yeast system is that it allows the production of proteins in a soluble and active form, and purification of expressed proteins is often simplified. Yeast expression systems have some disadvantages which include the accumulation of inactive expressed proteins in insoluble inclusion bodies (Kikuchi and Ikehara, 1994). One of the major disadvantages of the yeast expression system is the low transcription rate of foreign genes, which can result in relatively low yields of the expressed protein (Romanos *et al.*, 1992). This is not always the case however, and high levels of expression have been obtained for hepatitis B core antigen (40% of total cell protein, Kniskern *et al.*, 1986) and human superoxide dismutase (30-70% of total cell protein, Hallewell *et al.*, 1987). In comparison with the bacterial systems, yeast expression systems are technically more complicated and time consuming, and the transformation frequency is often low. In addition, expression levels



generally depend on such conditions as the composition of the culture medium, temperature and pH, and sometimes on the toxicity or secretory characteristics of the products.

Another commonly used heterologous eukaryotic expression system is the insect cell/baculovirus system. This system allows the expression of foreign genes to exceptionally high levels while also allowing for the post-translational modification of expressed proteins. The insect cell/baculovirus system has been used for the expression of genes from viruses (Vlak *et al.*, 1990; Sanchez-Martinez and Pellett, 1991), bacteria (Pennock *et al.*, 1984), animals (Atkinson *et al.*, 1990; Lithgow *et al.*, 1991) and plants (Bustos *et al.*, 1988; Nagai *et al.*, 1992; Doan and Fincher, 1992; Kamp *et al.*, 1992; Korth and Levings, 1993), and utilizes cultured *Spodoptera frugiperda* (sf9) cells and a vector which contains a strong viral polyhedrin promoter to direct transcription of cloned genes. However, the cell cultures and viral constructs are very difficult to manipulate and the system is very labour intensive (Summers and Smith, 1987). Cloning of foreign genes into the baculovirus genome requires the preliminary cloning of the foreign gene into a transfer plasmid vector and subsequent transfer of the gene into the baculovirus genome by homologous recombination (Summers and Smith, 1987). A final problem associated with the baculovirus system is the fact that some foreign proteins are rapidly degraded either during, or shortly after, synthesis. Others are lost during cell breakage and subsequent purification. In fact, proteolysis represents one of the most significant barriers to heterologous gene expression in any organism (Marston, 1986; Gold, 1990). Despite the general practical difficulties associated with the insect cell/baculovirus system, there have been a number of recent improvements in the system which are discussed in a review by Sridhar *et al.* (1994). These improvements mainly focus on improving the efficiency of producing recombinant baculoviruses by giving them a selective advantage over their wildtype counterparts (Kitts and Possee, 1993).

After analysing the various characteristics of the available heterologous expression systems, it was decided to attempt to express the barley (1→4)-β-xylanase cDNAs in *E. coli*. This decision was based on the ease of handling the *E. coli* system, the recent availability of vectors and host strains that would facilitate expression and purification of expressed proteins and, in particular, on the successful expression of barley (1→3,1→4)- and (1→3)-β-glucanases in *E. coli*. (Xu *et al.*, 1994; Chen *et al.*, 1995). It was also deemed important that large quantities of the barley (1→4)-β-xylanases be produced, since (1→4)-β-xylanases have some potential industrial applications, and an important criterion for industrial implementation is the ability to produce highly active enzyme in bulk quantities. Thus, xylanases have potential applications in the biodegradation of lignocellulosic biomass to fuels and chemicals, improvement of rumen digestion (Wong *et al.*, 1988), bleaching of kraft pulps (Koponen, 1991; Kovasin and Tikka, 1992; Lavielle *et al.*, 1992; Nissen *et al.*, 1992; Buchert *et al.*, 1994) and in the improvement of fibre properties (Mora *et al.*, 1986). Xylanases also have widespread application in the production of chicken feed supplements and bread improver mixtures (McCleary, 1992; Nissen *et al.*, 1992) and in the malting and brewing industries (Dekker, 1979).

Two cDNAs for barley isoenzymes X-I and X-II were isolated as described in Chapter 3. The isolated (1→4)-β-xylanase isoenzyme X-I cDNA was full-length whereas the isoenzyme X-II cDNA was truncated at its 3' end (Figure 3.10 and Figure 3.11). An obvious pre-requisite for the efficient production of active protein for functional analysis is the availability of a full-length cDNA. The construction of an appropriate expression vector and expression of the full-length (1→4)-β-xylanase isoenzyme X-1 cDNA in *E. coli* is described in this Chapter. Attempts to purify the expressed enzyme by affinity chromatography are discussed.

The pET plasmid vector was chosen for the heterologous expression studies. The pET (plasmid for Expression by T7 RNA polymerase) vector system is a powerful system for the cloning and expression of recombinant proteins in *E. coli* (Resenberg *et*

*al.*, 1987; Studier *et al.*, 1990). Expression is driven from the T7 RNA polymerase promoter which is inducible by IPTG and, downstream from the cloned DNA fragment, the vector has stop codons in all 3 reading frames to terminate translation. The pET-14b vector includes a sequence at the 5' end of the foreign gene which encodes 6 consecutive histidine residues. The resulting protein therefore consists of the foreign protein of interest fused at the NH<sub>2</sub>-terminus to a 6-residue histidine leader sequence. The poly-histidine sequence (His-tag) has a high and specific affinity for a commercially-available nickel-resin. This characteristic facilitates the purification of the expressed recombinant protein, in a single step, by affinity chromatography.

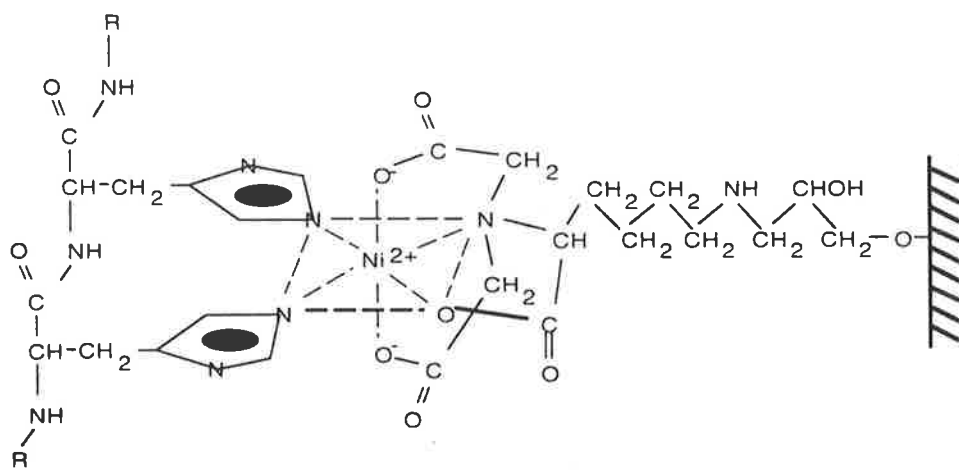
The poly-histidine segment can be fused to the NH<sub>2</sub>- or COOH-terminus of the expressed protein. The NH<sub>2</sub>-terminal fusion system has an advantage over the COOH-terminal fusion system in that NH<sub>2</sub>-terminal sequencing of the gene product can be easily performed. Furthermore, NH<sub>2</sub>-terminal fusion requires only that a short segment of 5' nucleotide sequence is known. Also, NH<sub>2</sub>-terminus-fused proteins are often expressed 2-4 times more efficiently than proteins tagged at their COOH-terminus (The QIAexpressionist, product literature, Qiagen). One potential disadvantage of the poly-histidine tagging can arise when the 3-dimensional folding of the expressed protein positions the tag at the interior of the protein. In such a case, the tag can cause unfavourable biochemical interactions during folding and loss of enzyme activity, it may be sterically occluded so that affinity chromatography is no longer possible and its position can inhibit the efficient cleavage of the tag following purification.

For expression of the barley (1→4)-β-xylanase cDNA, the expression construct was transformed into *E. coli* strain BL21 (DE3) pLysS. This strain has the potential advantage that, as a B strain, it is deficient in the *Ion* protease and lacks the *omPT* outer membrane protease that can degrade proteins following cell lysis (Grodberg and Dunn, 1988). The host genome of *E. coli* BL21 (DE3) contains a T7 RNA polymerase gene under the control of the *lac* operon, and thus expression of the polymerase can be

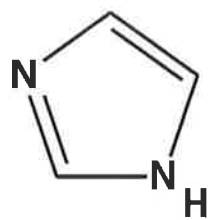
induced by IPTG. A second plasmid, designated pLysS, is also present in the expression host cells to confer resistance to chloramphenicol; it contains a gene encoding for bacterial lysozyme under the control of the *tet* promoter. Because lysozyme is a natural inhibitor of T7 RNA polymerase, its presence reduces background expression prior to induction. In addition to being an inhibitor of T7 RNA polymerase, lysozyme also has the capacity to hydrolyse the peptidoglycan layer of the *E. coli* extracellular envelope (Inouye *et al.*, 1973). Following expression of the lysozyme gene from plasmid pLysS, lysozyme is located in the cytoplasm and therefore does not have access to the carbohydrates on the internal layers of the bacterial extracellular envelope (Inouye *et al.*, 1973). However, rupture of the envelope and cell membrane by the freeze/thaw method releases the lysozyme and facilitates complete lysis.

After the pET vector containing the (1→4)-β-xylanase foreign cDNA is transformed into *E. coli* BL21(DE3), expression of the foreign gene is induced in the presence of plasmid pLysS by the addition of IPTG to the growth medium. Ni-NTA (Nickel-nitro-tri-acetic acid) spin columns are used to purify the His-tagged protein from the bacterial cell lysate following induction of expression. The Ni-NTA resin is composed of a high surface concentration of NTA ligand attached to Sepharose CL-6B. The NTA ligand has four of the six ligand binding sites in the coordination sphere of the Ni<sup>2+</sup> ion, leaving two sites free to interact with the 6 X His-tag (Figure 4.1). The high affinity of the Ni-NTA resin for His-tagged proteins is due to the specificity of the interactions between histidine residues and the immobilized Ni<sup>2+</sup> ions on the NTA resin. Imidazole is used to competitively elute the histidine-tagged protein from the binding sites on the resin, since imidazole is a structural analogue of histidine (Figure 4.2).

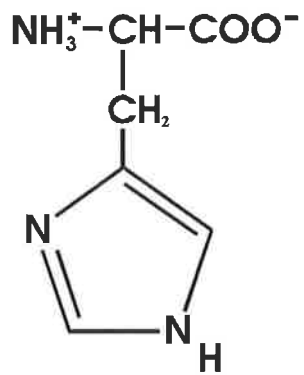
In this Chapter, experiments designed to express barley (1→4)-β-xylanase isoenzyme X-I cDNA in *E. coli* lysates are described. Although the protein was expressed at relatively high levels, it was mostly deposited in inclusion bodies and attempts to isolate active (1→4)-β-xylanase were unsuccessful.



**Figure 4.1 Interaction between histidine residues and the Ni-NTA resin**  
 (from The QIAexpressionist, product literature, Qiagen).



**Imidazole**



**Histidine**

**Figure 4.2 Chemical structures of imidazole and histidine.**

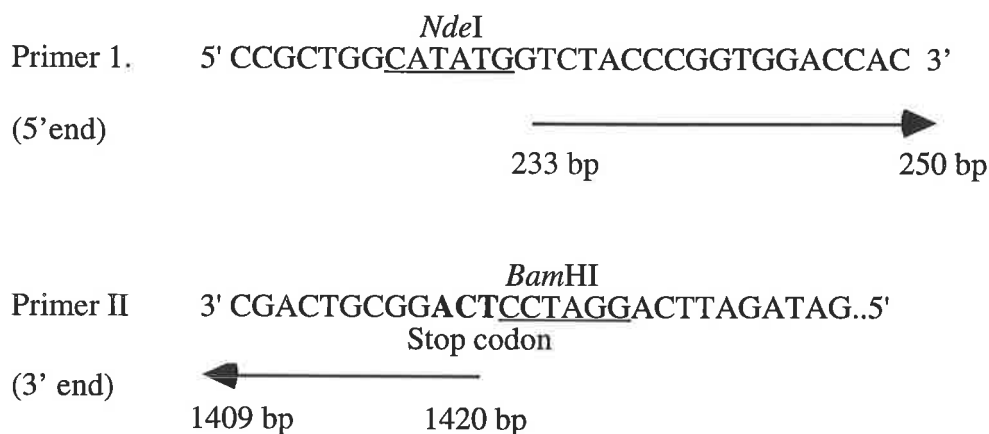
## 4.2 MATERIALS AND METHODS

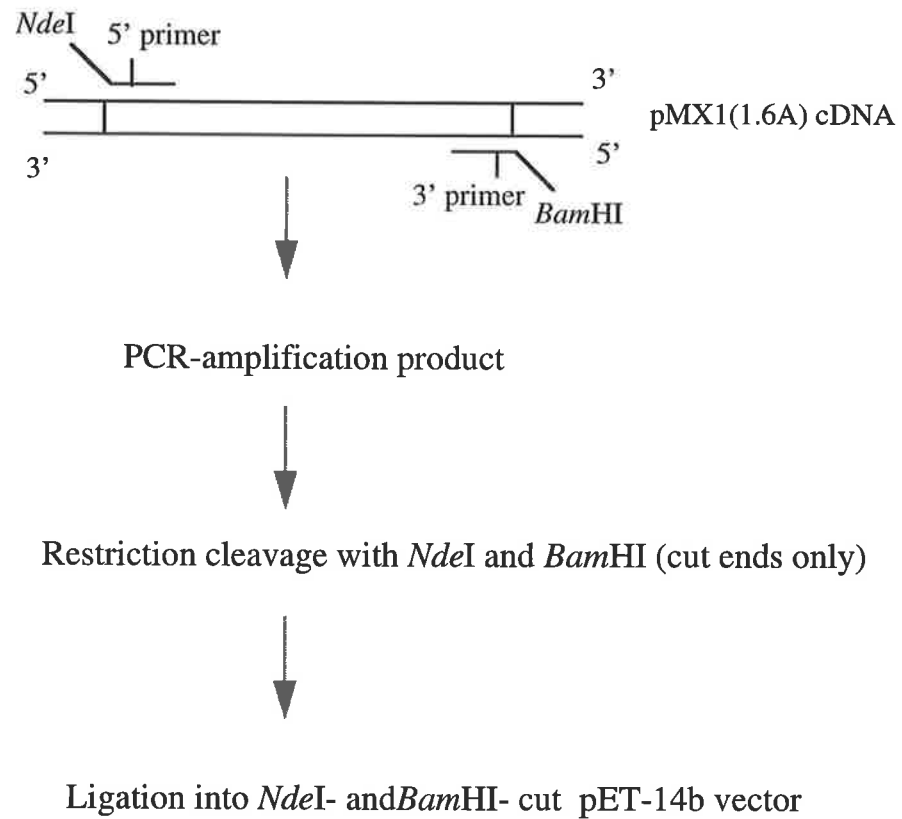
### 4.2.1 Materials

The pET-14b vector and *E. coli* cells HMS174 and BL21 were obtained from Novagene, USA. *E. coli* X11-Blue was from Stratagene. Ni-NTA spin columns and imidazole were from QIAGEN Inc (Chatsworth, CA, USA). Oligonucleotides were synthesized using an Applied Biosystems DNA Synthesizer. Oat-spelt xylan, betaine, sorbitol, PMSF, SDS, IPTG, bacto-tryptone, guanidine-HCl and urea were purchased from Sigma.

### 4.2.2 Construction of the (1→4)- $\beta$ -xylanase isoenzyme X-I expression plasmid

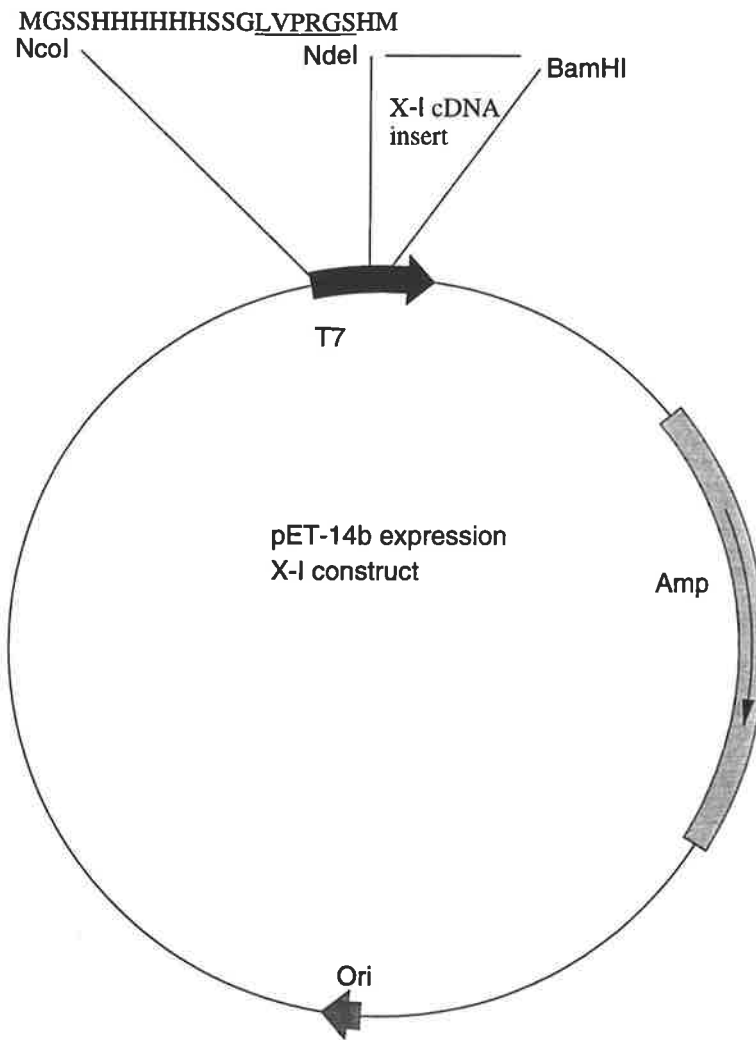
*Construction of expression plasmid:* The coding region of the cDNA encoding barley (1→4)- $\beta$ -xylanase isoenzyme X-I was synthesized by PCR amplification using pMXI (1.6A) cDNA as a template. The ends of the coding region were modified during the PCR to create suitable restriction sites, in the correct reading frame, to enable the region to be subcloned into the expression vector pET-14b (Figures 4.3 and 4.4). Two oligonucleotide primers were designed to include convenient restriction enzyme sites.





**Figure 4.3** A diagrammatic representation of the preparation of (1→4)- $\beta$ -xylanase isoenzyme X-I cDNA for subcloning into the pET-14b expression vector. The PCR product was digested with *NdeI* and *BamHI* restriction enzymes and ligated into the corresponding enzyme sites in the pET-14b expression plasmid.





**Figure 4.4** Design and construction of the pET-14b-X-I cDNA expression vector. The barley (1→4)-β-xylanase isoenzyme X-I cDNA was inserted between the *NdeI* and *BamHI* sites of the pET-14b vector. Histidine residues are denoted by H and the specific thrombin cleavage site is underlined. The cDNA insert is represented by a solid line. The translation start codon is located within the *NcoI* site of the pET-14b vector.

Primers I and II were used to amplify the entire coding region of the cDNA, including a stop codon adjacent to the *Bam*HI site of primer II. Primer I contained a 5'-overhang with an *Nde*I site and the cDNA sequence corresponding to the region 233-250 bp (Figure 3.10, Chapter 3). Primer II contained a 5'-overhang, with a *Bam*HI site and part of the cDNA coding region corresponding to base pairs 1409-1420 (Figure 3.10, Chapter 3). The PCR was performed using Vent polymerase for 30 cycles. Before adding the polymerase, the PCR cocktail, including template and primer, was preheated to 95°C for 5 min. Thereafter the following cycle was used : 94°C, 2 min; 50°C, 1 min; 72°C, 3 min; with a final 10-min extension at 72°C after the 30 cycles were completed. The PCR-amplified fragments were purified by Bresa-Clean according to the manufacturer's instructions (Bresatec). The purified fragment was digested with *Nde*I and *Bam*HI for 2 h at 37°C. Digested products were further purified by extracting with phenol:chloroform and chloroform. The aqueous, upper phase was precipitated with 0.1 vol 3M sodium acetate buffer, pH 5.2 and 2.5 vol ethanol. The resulting pellet was washed with 70 % ethanol, dried and dissolved in 20 µl water. The PCR-amplified fragment was now ready to ligate into the pET-14b expression vector.

*pET-14b vector*: For vector preparation, the pET-14 b vector was digested with *Nde*I and *Bam*HI restriction enzymes for 4 h at 37°C. Following digestion, the vector DNA was separated in a 1% (w/v) agarose gel. The digested vector band was excised and the vector DNA was purified by Bresa-Clean (Bresatec). The vector DNA and cDNA fragments were ligated for 16 h at 14°C, using conditions described in section 2.2.3.3, to construct the expression plasmid.

*Bacterial strains*: Expression constructs were transformed into *E. coli* strain X11-Blue, as described in Section 2.2.3.2 to maintain the construct. For induction of expression, the *E. coli* strain BL21 (DE3) containing pLysS was used.

### **4.2.3 Induction of expression**

The following protocol was used for induction of protein expression; 3 ml rich media [containing 1.2% (w/v) bacto-tryptone, 2.4% (w/v) bacto-yeast, 0.4% (v/v) glycerol, 17 mM K<sub>2</sub>PO<sub>4</sub>, 72 mM K<sub>2</sub>HPO<sub>4</sub>] was added to a lawn of transformed cells grown overnight at 37°C. The cells were resuspended in this medium and 100 µl of the cell suspension was incubated in 50 ml rich media in a 250 ml Erlenmeyer conical flask containing 100 µg/ml ampicillin and 25 µg/ml chloramphenicol at 37°C with shaking until the A<sub>600</sub> reached 0.6. One ml of culture was centrifuged, the pellet was resuspended as an uninduced sample control and stored at -20°C until further analysis. IPTG (0.5 mM) was added to the remaining culture and incubation was continued at 23°C for 3 h. The flask was placed on ice for 5 min and the cells harvested by centrifugation at 3,000 rpm for 20 min at 4°C. The supernatant was removed and the cell pellets were stored at -70°C.

To study the time course of (1→4)-β-xylanase gene expression after induction with IPTG, 1 ml aliquots were taken at 1 h intervals for up to 5 h. The cells were collected by centrifugation and stored at -20°C for further analysis. Frozen cell pellets were thawed and resuspended in 100 µl SDS sample loading buffer prior to analysis by SDS-PAGE .

### **4.2.4 Solubilization of inclusion bodies and protein refolding**

Stored cell pellets were thawed for 15 min on ice and resuspended in 1ml 50 mM sodium phosphate buffer, pH 7.8, containing 300 mM NaCl, 20 mM imidazole and 1 mM PMSF. The suspension was incubated on ice for 30 min, cells were lysed by sonication for 30 sec and the resulting lysate was centrifuged at 13,200 rpm for 20 min at 4°C. The supernatant was retained for analysis by SDS-PAGE and for purification of the expressed (1→4)-β-xylanase under native conditions.

The cell pellet, which contained insoluble inclusion bodies, was resuspended in 6 M guanidine-HCl or 8 M urea containing 0.1 M sodium phosphate buffer, pH 7.8 and 0.01 M Tris-HCl buffer, pH 8.0, respectively. The cells were gently stirred on a rocking platform in the denaturing buffer for 1 h at room temperature. The lysate was centrifuged for 15 min at 13,200 rpm and the supernatant containing the solubilized, denatured proteins was retained. The denatured proteins were analysed by SDS-PAGE and used for the subsequent purification of the expressed (1→4)- $\beta$ -xylanase on Ni-NTA column.

#### ***4.2.5 Purification of the overexpressed protein***

To purify the tagged (1→4)- $\beta$ -xylanase from the soluble cell lysate prepared under non-denaturing conditions, a Ni-NTA spin column was pre-equilibrated with 600  $\mu$ l 50 mM sodium phosphate buffer pH 8.0, containing 300 mM NaCl and 20 mM imidazole. The column was centrifuged for 2 min at 2,000 rpm at room temperature. Following equilibration, 600  $\mu$ l cell lysate was added to the column, which was recentrifuged for 2 min at 2,000 rpm. The flow-through was collected for SDS-PAGE analysis to check the efficiency of binding. The column was washed twice with the same buffer, and once with the same buffer containing 40 mM imidazole. Bound protein was eluted twice with 100  $\mu$ l buffer containing 250 mM imidazole.

To purify the protein that had been re-solubilized from inclusion bodies, the Ni-NTA column was pre-equilibrated with 600  $\mu$ l sodium phosphate buffer pH 7.8 (containing 6 M urea, 0.01 M Tris-HCl, pH 8.0, 20 mM imidazole) and centrifuged for 2 min at 2,000 rpm. Solubilized cell lysate (600  $\mu$ l) was added onto a pre-equilibrated column, centrifuged for 2 minutes at 2,000 rpm, and the flow-through was retained for SDS-PAGE analysis to check the efficiency of binding. The column was washed twice with the same buffer, and twice with the same buffer adjusted to pH 6.3. Next, the

column was washed twice with the same buffer (pH 6.3) containing 40 mM imidazole and bound protein was finally eluted with 200  $\mu$ l buffer containing 250 mM imidazole. For SDS-PAGE analysis, 10-20  $\mu$ l eluted protein was resuspended in SDS-loading buffer.

#### 4.2.6 Renaturation of the protein

Attempts were made to refold the purified, denatured proteins by gradual dilution and removal of the denaturing agents by dialysis (Stern *et al.*, 1993; Saavedra-Alanis *et al.*, 1994). Purified protein was transferred to dialysis tubing and dialysis was carried out by step-wise dilution of denaturants using concentrations in the range 8 M $\rightarrow$ 0.2 M urea or 6 M $\rightarrow$ 0.2 M guanidine-HCl and glycerol concentrations in the range 20% (v/v) to (v/v) 5%. The dialysis buffer also contained 0.02 M Tris-HCl buffer, pH 7.4 with 1 mM PMSF, 300 mM NaCl and 5 mM 2-mercaptoethanol. Each dialysis step was carried out for 3 h with slow stirring at 4°C. The final dialysis was performed in 0.02 M Tris-HCl buffer, pH 7.4 containing 300 mM NaCl and 1 mM PMSF. The extent of enzyme refolding was determined by measuring enzyme activity.

#### 4.2.7 (1 $\rightarrow$ 4)- $\beta$ -Xylanase activity

Enzyme activity was measured reductometrically using the Somogyi-Nelson procedure (1952). High background levels of reducing sugars in some substrates can limit the usefulness of the Somogyi Nelson procedure, but this problem was not encountered with the substrates used here. However, the substrates sometimes contained traces of soluble starch. Oat-spelt xylan was therefore treated with  $\alpha$ - and  $\beta$ -amylase to remove any contaminating starch before using it as a substrate for the reductometric assay for xylanase activity. One gram of substrate was dissolved in 60 ml water and

boiled for 15 min. The dissolved substrate was centrifuged briefly, the supernatant was transferred to a fresh tube and the solution was adjusted to a final volume of 100 ml in 50 mM sodium acetate buffer, pH 5.5. The  $\alpha$ - and  $\beta$ -amylase (20 units) were added to the substrate and incubated at 37°C overnight. The substrate was heated for 30 min to inactivate the amylases and centrifuged. Finally the supernatant was dialysed against 50 mM sodium acetate buffer pH 5.5.

It was very important to treat the xylan with the amylases before the colorimetric determination of xylanase activity because soluble starch present in the substrate created problems with the specificity of the assay. In experiments not described in this thesis an attempt was made to purify (1 $\rightarrow$ 4)- $\beta$ -xylanase from extracts of germinated barley grain using untreated xylan from larchwood (Sigma) as a substrate. Two putative (1 $\rightarrow$ 4)- $\beta$ -xylanases were purified. However, the substrate contained a small but significant amount of starch and other non-cellulosic polysaccharides and when the two pure proteins were sequenced they were shown to be a  $\beta$ -amylase and a  $\beta$ -glucosidase.

For the oat-spelt-xylan-based enzyme assays, the enzyme activity was measured using 400  $\mu$ l of a 3% (w/v) oat-spelt xylan substrate in 50 mM sodium acetate buffer, pH 5.5, mixed with 100  $\mu$ l soluble fraction and incubated for up to 4 h at 37°C. The reaction was stopped by the addition of 500  $\mu$ l copper reagent (Somogyi solution) and the reaction mixture boiled for 10 min. After cooling in cold water, 500  $\mu$ l arsenomolybdate solution was added to develop the blue complex. The absorbance was measured at 660 nm against substrate and enzyme blanks. A standard curve of 0-150  $\mu$ g/ml xylose was constructed and enzyme activity was expressed as  $\mu$ g xylose equivalents released per min.

#### **4.2.8 SDS-PAGE**

Protein samples were analysed by SDS-PAGE essentially as described by Laemmli (1970). Samples were mixed with equal volumes of 2 x SDS-PAGE sample buffer, boiled for 5 min, loaded onto a polyacrylamide gel (12%, w/v) and separated on gels for 3 h at a constant current of 20 mA. Gels were stained with Coomassie Brilliant Blue R-250 prepared in 20% (v/v) ethanol and 7% (v/v) glacial acetic acid, at 60°C for 30 min. The gels were destained with 20% (v/v) ethanol and 7% (v/v) glacial acetic acid at 60°C for 30 min. Molecular weight markers used were phosphorylase b ( $M_r$  95,000); BSA ( $M_r$  68,000); ovalbumin ( $M_r$  43,000); carbonic anhydrase ( $M_r$  30,000); trypsin inhibitor ( $M_r$  20,000);  $\alpha$ -lactalbumin ( $M_r$  14,400) (Electrophoretic Calibration Kit, Pharmacia LKB, Biotechnology Inc, Uppsala, Sweden).

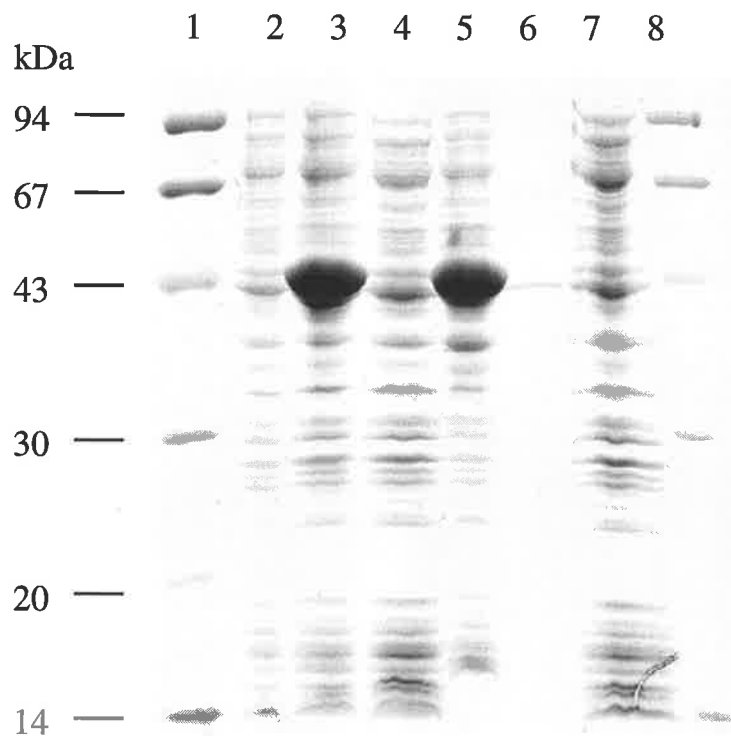
## 4.3 RESULTS AND DISCUSSION

### 4.3.1 Construction of expression plasmid

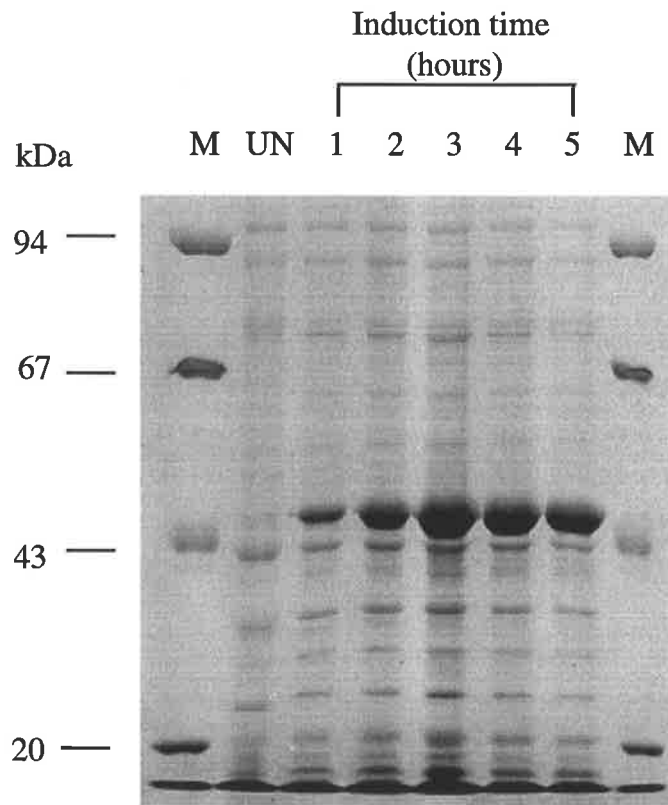
The coding region of the isolated barley (1→4)- $\beta$ -xylanase isoenzyme X-I was modified during PCR amplification to introduce *Nde*I and *Bam*HI restriction sites at the 5' and 3' ends of the insert, respectively. The *Nde*I and *Bam*HI sites were used for directional cloning to create an in-frame orientation with respect to the histidine tag in the pET-14b vector. The coding sequence containing *Nde*I and *Bam*HI ends was subsequently inserted into the *Nde*I and *Bam*HI sites of vector pET-14b to create a translation fusion, designated pET-His-X-I (Figure 4.4). The expression vector contained a T7 promoter, a ribosome binding sequence, a translation start codon (ATG), a 6 x Histidine tag, a thrombin cleavage site, the cDNA insert and a T7 terminator sequence. The in-frame insertion of the cDNA into pET-14b vector was confirmed by sequencing both the 5' and 3' ends (data not shown).

For expression of the (1→4)- $\beta$ -xylanase clone, the construct was transformed into bacterial strain *E. coli* BL21 (DE3) containing the pLysS plasmid. Freshly transformed cells were used for induction. Expression was induced by the addition of IPTG and, following expression, the cells were pelleted, lysed and the (1→4)- $\beta$ -xylanase fusion protein was purified on the Ni-NTA column. Induced and uninduced fractions were analysed by SDS-PAGE, which revealed that the major induced protein had an apparent  $M_r$  ranging from approximately 43,000 (Figure 4.5) to approximately 45,000 (Figure 4.6). This can be compared with a calculated  $M_r$  of 44,600 for the (1→4)- $\beta$ -xylanase translation product and suggests that no reading frame errors were introduced during the PCR. Assuming that 43-45 kDa protein represents expressed (1→4)- $\beta$ -xylanase protein, the (1→4)- $\beta$ -xylanase protein was located predominantly in the pellet of the cell lysate. A small amount of expressed (1→4)- $\beta$ -xylanase protein was seen in the soluble fraction





**Figure 4.5** The heterologous expression and purification of (1→4)- $\beta$ -xylanase isoenzyme X-I. SDS-PAGE analysis of proteins in the *E. coli* lysate after induction with IPTG. Lanes 1 and 8, protein molecular mass (kDa) standard markers; lane 2, cell lysate before induction; lane 3, cell lysate after induction; lane 4; protein released from cells after sonication; lane 5, protein in insoluble pellet; lane 6, bound soluble protein eluted from the Ni-NTA column with 100 mM sodium phosphate buffer, pH 7.9 containing 250 mM imidazole; lane 7, unbound soluble protein wash through the column.



**Figure 4.6** SDS-PAGE analysis of barley (1→4)- $\beta$ -xylanase isoenzyme X-I following expression in *E. coli* for varying times. M, molecular weight protein markers (kDa). UN, cell lysate before induction; lanes 1-5, cell lysate after induction with IPTG for different times (h).

of the cell lysate. (Figure 4.5, lane 4). When the soluble fraction of the cell lysate was purified through the Ni-NTA column, a weak band corresponding to the expressed protein was observed (Figure 4.5, lane 7). However, when the soluble extracts and Ni-NTA column eluants were checked for (1→4)- $\beta$ -xylanase activity using oat-spelt xylan as a substrate, no activity could be detected.

#### 4.3.2 Variation to culture conditions

Preliminary experiments were conducted using two different growth temperatures (23°C and 37°C) following induction. Several research groups have shown that when *E. coli* cultures are grown at temperatures lower than 30°C, most of the proteins were expressed in an active and soluble form, while proteins from cultures grown at 37°C are often insoluble (Schein and Noteborn, 1988; Haase-Pettingell and King, 1988; Piatak *et al.*, 1988; R. Stewart and G.B. Fincher, unpublished data). However, in this case the lower temperature did not have any effect on the expression of the (1→4)- $\beta$ -xylanase protein in a soluble form.

To monitor the expression of the (1→4)- $\beta$ -xylanase cDNA a time course experiment was conducted. Cells were induced for 1, 2, 3, 4 or 5 h with 0.5 mM IPTG at 23°C. SDS-PAGE analysis revealed that a 3 h induction period gave the highest levels of expression (Figure 4.6), but again no (1→4)- $\beta$ -xylanase activity was detected in the soluble fraction of the cell lysate (data not shown).

Several other experiments were conducted in an attempt to express the (1→4)- $\beta$ -xylanase protein in an active, soluble form (data not shown). By employing enriched growth media, Moore *et al.* (1993) found a deaminase could be induced in a soluble active form to a level of at least 20% of cellular protein. In the present work, cells were therefore induced in two different media, FTB and LB, but the composition of the growth medium appeared to have little effect on (1→4)- $\beta$ -xylanase protein solubility or

activity. The host strain was also changed to *E. coli* HMS174 in the expectation that this strain might tolerate overexpressed protein (pET system product literature, Novagen, Madison, WI). However, no soluble (1→4)-β-xylanase protein was obtained. A further set of experiments was carried out to examine the effect of cell density and growth times on protein solubility. Cells were induced with IPTG when their A<sub>600</sub> reached 1.0 and expression was continued for 60 or 90 min following induction. However, increasing the cell density or decreasing expression times proved unsuccessful in increasing (1→4)-β-xylanase solubility or activity.

Blackwell and Horgan (1991) have reported that inducing *E. coli* cells under osmotic stress in the presence of sorbitol and glycyl betaine can significantly enhance the production of soluble, active protein. In a final attempt to obtain soluble (1→4)-β-xylanase enzyme, cells were grown and induced in LB medium containing 660 mM sorbitol and 2.5 mM glycyl-betaine at 23°C but again no soluble (1→4)-β-xylanase was produced.

#### **4.3.3 Solubilization of inclusion bodies and renaturation**

As detailed in the previous sections, expression of the barley (1→4)-β-xylanase cDNA was performed under a variety of conditions including different media compositions, a range of pHs, different temperatures, three host cell lines, high and low cell densities, and a range of IPTG concentrations. In every case a strongly-induced protein of the correct molecular weight could be seen in the cell lysates but it became apparent that the expressed enzyme was always deposited in an inactive, insoluble 'inclusion body' complex in the *E. coli* cells. Several research groups have reported that high levels of expression often lead to the formation of inclusion bodies (Arango *et al.*, 1992; Landman *et al.*, 1992; Oono *et al.*, 1992; Rinas and Bailey, 1992; Schulze *et al.*, 1994; Sirawaraporn *et al.*, 1993). Schein (1989) suggested that inclusion bodies arise by

inappropriate aggregation of partially folded or incorrectly folded intermediates. The production of proteins as insoluble inclusion bodies sometimes allows an easier purification, but when active enzyme is required, correctly folded protein is clearly essential.

Our attempts to refold denatured protein from the inclusion bodies were also unsuccessful. Solubilization of the insoluble protein aggregates was achieved with strong denaturing agents. In the presence of 6 M guanidine-HCl and 8 M urea as strong chaotropic agents, proteases are also denatured and the procedure can therefore be performed at room temperature without any deleterious effects on protein integrity. In an attempt to refold the solubilized protein, 6 M guanidine-HCl was gradually removed by dialysis, but the protein re-precipitated during this process. This result was in agreement with the report of Hartley and Kane (1988) who also found that solubilized protein re-precipitated during dialysis, although a number of proteins have been successfully returned to their native or active state after renaturation of inclusion bodies using the step dialysis procedure (Arango *et al.*, 1992; Narciandi *et al.*, 1993; Brown *et al.*, 1993; Cox and Johnson, 1993; Friedhoff *et al.*, 1994). Another disadvantage of using guanidine-HCl is that it often precipitates in the presence of SDS, thereby rendering subsequent analysis by SDS-PAGE difficult.

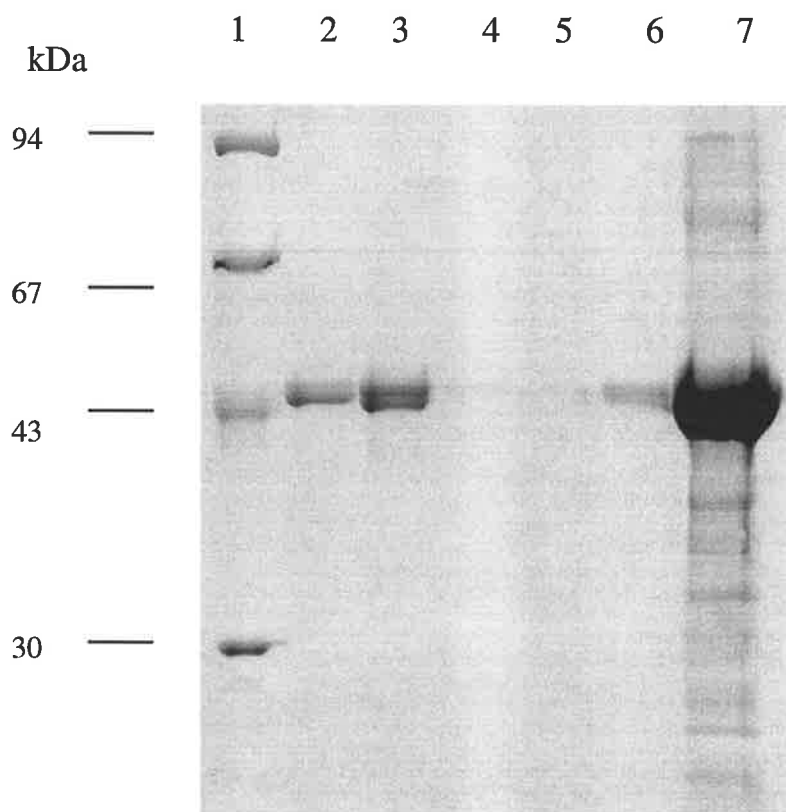
In a second attempt to renature the expressed protein, 8 M urea was used to solubilize the protein aggregates, and the solubilized (1→4)-β-xylanase was purified using a Ni-NTA column. Again refolding of the purified, denatured protein was attempted by dialysis against an appropriate buffer containing decreasing concentrations of urea. In this case expressed protein did not precipitate during dialysis. Following the final dialysis step into a urea-free buffer, the protein remained in solution but when enzyme activity was assayed against 0.3% (w/v) oat-spelt xylan none could be detected. This suggested that the protein had not folded correctly during this treatment, although it had remained in solution. Soluble protein was analysed by SDS-PAGE both prior to and

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following dialysis (Figure 4.7). Most of the expressed protein was not bound to the Ni-NTA column. Bound and eluted protein had no (1→4)- $\beta$ -xylanase activity.

Although no further attempts were made to express barley (1→4)- $\beta$ -xylanase in an active form, there are other strategies which might be pursued in the future. There are now several commercially-available *E.coli* host strains which have been designed specifically to enhance the solubility of foreign proteins and to keep the proteins folded (The QIAexpressionist, product literature, Qiagen). A new gene expression system based on the use of the *E. coli* thioredoxin pTrxFus vector (LaVallie *et al.*, 1993) has proven to be successful in overcoming problems of protein insolubility. Furthermore, it has been shown that co-expressing foreign proteins with molecular chaperones or the addition of molecular chaperones such as GroEL or GroES during protein folding can lead to significant increases in protein solubility (Buchner *et al.*, 1991; Hendrick and Hartl, 1993; Todd *et al.*, 1994; Ellis, 1994; Rudolph and Lilie, 1996).

Finally, if active barley (1→4)- $\beta$ -xylanase cannot be successfully expressed in *E.coli*, perhaps the use of eukaryotic heterologous systems such as those based on insect cell/baculovirus or yeast protocols should be investigated. Due to time constraints it was not possible to try these other expression systems in the present study. Doan *et al.* (1993) successfully expressed barley (1→3,1→4)- $\beta$ -glucanase in a baculovirus system, but this enzyme can also be expressed in an active form in *E. coli* (R. J. Stewart and G. B. Fincher, pers. commun.).



**Figure 4.7 SDS-PAGE analysis of expressed (1→4)- $\beta$ -xylanase isoenzyme X- I protein purified under denaturing conditions.** Lane 1, Molecular weight protein standards (kDa); lanes 2 and 3, bound protein (20  $\mu$ l) eluted with 100 mM sodium phosphate buffer, pH 7.9 containing 300 mM imidazole from Ni-NTA column following and prior to dialysis; lane 4, column eluant following washing with 100 mM sodium phosphate and 10 mM Tris-HCl buffer, pH 6.3 containing 40 mM imidazole and 8 M urea; lane 5, column eluant following washing with 100 mM sodium phosphate and 10 mM Tris-HCl buffer, pH 7.8 containing 8 M urea; lane 6, column eluant following washing with 100 mM sodium phosphate buffer, pH 7.8 containing 8 M urea; lane 7, unbound protein fraction.

## **CHAPTER 5**

# **DEVELOPMENTAL REGULATION OF (1→4)- $\beta$ -D-XYLANASE GENE EXPRESSION**



## 5.1 INTRODUCTION

Two cDNAs encoding barley (1→4)- $\beta$ -xylanase isoenzymes X-I and X-II have been isolated and characterized as described in Chapters 2 and 3. The cDNA for isoenzyme X-I was full-length, while the cDNA for isoenzyme X-II was truncated at its 3' end. These cDNAs were therefore available as probes to examine the spatial, temporal and hormonal regulation of xylanase gene expression in various tissues of barley. In this Chapter experiments are described in which the full-length cDNA encoding (1→4)- $\beta$ -xylanase isoenzyme X-I was used to probe Northern blots for xylanase mRNA in extracts of vegetative tissues of mature plants, in tissues from the germinated grain, and in tissues from young seedlings. The cDNAs encoding isoenzyme X-I and X-II were very similar in sequence (91% identity) and the 3' untranslated region of the isoenzyme X-II cDNA, which might have shown sequence divergence from the same region of the isoenzyme X-I cDNA, was not available for use as a probe. As a result, it was not possible to design isoenzyme-specific oligonucleotide or cDNA probes to study the developmental regulation of the specific, individual genes coding for (1→4)- $\beta$ -xylanase isoenzymes X-I and X-II. Rather, the probe detects both mRNA species and possibly mRNAs from other (1→4)- $\beta$ -xylanase genes.

In initial work described in this Chapter, the expression of xylanase genes, as measured by mRNA transcripts in Northern blot analyses, was detected only in aleurone layers. Barley aleurone layers have been used extensively as a convenient model system to examine the hormonal control of gene expression in plants (Taiz and Honigman, 1976; Dashek and Chrispeel, 1977; Ho *et al.*, 1987; Jacobsen and Chandler, 1987; Fincher, 1989; Jones and Jacobsen, 1991; Fincher, 1992; Jacobsen *et al.*, 1995). Aleurone layers were therefore treated with the phytohormones GA<sub>3</sub> and ABA to determine whether or not transcription was mediated by these hormones. Concurrently, the activities of various other enzymes that would be involved in arabinoxylan depolymerization were

measured in the medium surrounding isolated aleurone layers. Furthermore, the levels of expression of (1→4)- $\beta$ -xylanase in isolated aleurone layers were compared with those of the (1→3,1→4)- $\beta$ -glucanase; the latter enzyme also plays a key role in cell wall degradation.

## 5.2 MATERIALS AND METHODS

### 5.2.1 Materials

Wheat flour arabinoxylan and barley (1→3,1→4)- $\beta$ -glucan were obtained from Megazyme (Warriewood, NSW, Australia). 4-Nitrophenyl- $\alpha$ -L-arabinofuranoside (4-NPA), 4-nitrophenyl- $\beta$ -D-xyloside (4-NPX), MOPS, DEPC, 2-mercaptoethanol, orcinol, guanidine thiocyanate, gibberellic acid (GA), abscisic acid (ABA) and low melting-point agarose were obtained from Sigma Chemical Co. Hybond-N<sup>+</sup> autoradiographic film was purchased from Amersham. Radioactively-labelled [ $\alpha$ -<sup>32</sup>P]-dCTP was from Bresatec. Thin layer chromatography (TLC) plates Kieselgel 60 were from Merck (Germany), Ostwald viscometers were from Cannon (USA), and RNA markers were purchased from Promega.

*Plant Material:* Barley (*Hordeum vulgare* L. cv. Richard) was obtained from Ms. Amanda Kerry, Department of Plant Science, University of Adelaide, South Australia. Grains were surface sterilized in 1% (v/v) sodium hypochlorite for 10 min, rinsed thoroughly with sterile distilled water and sown directly into moist vermiculite. Barley grains were germinated at 24°C under ambient conditions of light and temperature. Leaves were harvested from 5, 7, 9, 12, 17, 35 and 50 day-old seedlings, and roots were harvested from 5, 7, 9 and 12 day-old seedlings. Scutella were excised from grains 24 h after soaking in water. For the 5-day germinated grain sample, roots and shoots were first removed from the grain. All samples were washed with DEPC-treated sterile water, frozen in liquid N<sub>2</sub> and stored at -80°C prior to RNA extraction.

*Hormone treatment of seedlings:* Five-day germinated seedlings were sprayed with GA<sub>3</sub> (5  $\mu$ M) or GA<sub>3</sub>+ABA (5  $\mu$ M and 25  $\mu$ M, respectively, in 20 mM potassium citrate

buffer, pH 6.0) until leaves were completely wet. Potassium citrate buffer (20 mM, pH 6.0) was sprayed onto the seedlings as a negative control. Sampling was commenced 12 h after spraying the seedlings with the hormone solution. Leaves and roots were removed and washed with sterile DEPC water, frozen in liquid N<sub>2</sub> and stored at -80°C.

### 5.2.2 Isolation and hormonal treatment of aleurone layers

The hull-less barley variety Richard was used throughout this work, mainly because the absence of a husk facilitates the extraction of RNA. Grains were surface sterilized in 1% (v/v) sodium hypochlorite for 10 min and rinsed thoroughly with water. It was particularly important to ensure that the grain was thoroughly surface-sterilized because of the extended incubation periods that were necessary for monitoring (1→4)- $\beta$ -xylanase secretion (Slade *et al.*, 1989) and because any fungal contaminants might also be expected to produce (1→4)- $\beta$ -xylanases (Peltonen, 1995).

Barley aleurone layers were prepared essentially as described in section 2.2.2.1. Isolated layers (100) were incubated in 5 ml 10 mM CaCl<sub>2</sub>, 20 mM sodium acetate, pH 5.5 (containing 100 units/ml nystatin, 100  $\mu$ g/ml neomycin and 10  $\mu$ g/ml chloramphenicol) and gently shaken for up to 72 h in the dark at 22°C in the presence or absence of 5  $\mu$ M gibberellic acid (GA<sub>3</sub>), 25  $\mu$ M abscisic acid (ABA) or both. Layers were rinsed with sterile 10 mM CaCl<sub>2</sub>, frozen in liquid N<sub>2</sub> and stored at -80°C prior to RNA extraction. Aliquots of the media around the aleurone layers were centrifuged to remove cellular debris, dialyzed and concentrated in a Microcon 10 ultrafilter (Amicon Inc, Beverly, MA, USA) in 50 mM sodium acetate buffer, pH 5.5 containing 3 mM 2-mercaptoethanol and 5 mM sodium azide. These samples were used to assay enzyme activities.

### 5.2.3 Enzyme assays

*(1→4)-β-Xylanase and (1→3,1→4)-β-glucanase*: Because reducing sugar assays have several limitations, especially where background levels of sugars are high (as discussed in section 4.2.7), (1→4)-β-xylanase and (1→3,1→4)-β-glucanase secreted into the incubation media were measured viscometrically. The assays involved the use of highly viscous polysaccharide substrates and a reduction in the viscosity of the particular substrate was used as a sensitive indicator of enzyme activity. Thus, (1→4)-β-xylanase and (1→3,1→4)-β-glucanase activities were measured viscometrically using as substrates 0.3% (w/v) wheat flour arabinoxylan or 0.3% (w/v) barley (1→3,1→4)-β-glucan, respectively (Woodward and Fincher, 1982 a; Slade *et al.*, 1989). To dissolve the arabinoxylan substrate, 1 g polysaccharide was soaked in a beaker with 6 ml of 95% (v/v) ethanol and 80 ml of water was added. The beaker was placed on a magnetic stirrer hot plate and heated to 100°C with vigorous stirring until the substrate was completely dissolved (manufacturer's instructions, Megazyme). The solution was adjusted to a volume of 100 ml with water and stored in a sealed glass container. Sodium azide was added to 5 mM to inhibit microbial growth. (1→3,1→4)-β-Glucan substrate was prepared essentially as described by Woodward and Fincher (1982 a).

Buffers contained 50 mM sodium acetate buffer, 400 µg/ml BSA, 5 mM sodium azide and 5 mM 2-mercaptoethanol and were adjusted to pH 5.5 for the (1→4)-β-xylanase assay or pH 5.0 for the (1→3,1→4)-β-glucanase assay. For each assay 50 µl of an appropriately diluted sample was added to 550 µl substrate that had been equilibrated at 40°C for at least 3 h. Flow rates were recorded over 30 min. One unit of enzyme activity is defined as the change in the reciprocal of specific viscosity ( $1/\eta_{sp}$ ) per min (Woodward and Fincher, 1982 a). The equation used for calculating activity units was as follows:

$$\Delta 1/\eta_{sp} = 1/\eta_{sp_{t_5}} - 1/\eta_{sp_{t_0}} = 1/(t_5/t_b - 1) - 1/(t_0/t_b - 1)$$

where  $t_b$  is the flow time (sec) of the buffer alone;  $t_0$  is the flow time of the substrate before enzyme was added;  $t_5$  is the flow time 5 min after the addition of enzyme solution to the substrate (Woodward and Fincher, 1982 a).

*Glycosidases*:  $\alpha$ -L-Arabinofuranosidase and  $\beta$ -D-xylopyranosidase activities were determined colorimetrically on 4-nitrophenyl- $\alpha$ -arabinofuranoside (4-NPA) or 4-nitrophenyl- $\beta$ -xylopyranoside (4-NPX). Substrate solutions (0.5 mg/ml; 450  $\mu$ l) in 50 mM sodium acetate buffer, pH 5.5 were incubated with 50  $\mu$ l dilute enzyme extract at 40°C for 30 min. The reaction was stopped with 500  $\mu$ l 4% (w/v) sodium carbonate solution (Biely *et al.*, 1980) and the absorbance of liberated 4-nitrophenol (4-NP) was measured at 410 nm. One unit of activity is expressed as the amount of enzyme required to release 1  $\mu$ mol 4-NP per min.

#### 5.2.4 Thin layer chromatography

For analysis of the products released from the substrate during hydrolysis, the media (20  $\mu$ l) surrounding aleurone layers was added to 100  $\mu$ l 0.5% (w/v) arabinoxylan substrate and incubated at 37°C for up to 24 h. Samples (15  $\mu$ l) were taken after 0.25 h, 0.5 h, 2 h, 6 h, and 24 h incubation, and the enzyme was heat-inactivated at 100°C for 3 min. Substrate and enzyme controls were also treated in the same manner. All samples were concentrated to a final volume of 5  $\mu$ l under vacuum, and spotted onto thin layer chromatography (TLC) plates. Hydrolysis products were separated twice in ethyl acetate:acetic acid:water (3:2:1, v/v). The TLC plates were air-dried and sugar products were detected by spraying with the orcinol reagent (5 ml H<sub>2</sub>SO<sub>4</sub>, 45 ml ethanol, 0.5 g orcinol in a total volume of 50 ml) and followed by heating at 110°C for 5 min.

### 5.2.5 RNA isolation

Three methods were used to isolate total RNA from different tissues. A modified acid guanidine thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987) was used to isolate RNA from barley scutella. Scutellar tissue (1 g) was ground to a fine powder in liquid N<sub>2</sub> using a mortar and pestle. Ground samples were transferred to 50 ml sterile plastic tubes and 10 ml 4 M guanidine thiocyanate, containing 25 mM sodium citrate buffer, pH 7.0, 0.5% (w/v) sarkosyl and 0.1M 2-mercaptoethanol was added. The samples were thoroughly vortexed and the homogenate was transferred to a 30 ml Corex tube and centrifuged to remove cellular debris for 20 min at 12,000 rpm using an SS34 rotor at 4°C. The supernatant was transferred to a fresh Corex tube and extracted with an equal volume of acid phenol:chloroform. The phenol had been equilibrated with 50 mM sodium acetate buffer, pH 4.0. The resulting mixture was centrifuged at 12,000 rpm at 4°C and the extraction was repeated until no protein was visible at the interface. The final aqueous phase containing the RNA was precipitated with 1 ml isopropanol at -20°C overnight. The precipitate was pelleted by centrifugation for 20 min at 12,000 rpm at 4°C. The resulting pellet was dissolved in 1 ml extraction buffer, transferred to a 1.5 ml plastic tube and re-extracted twice with phenol:chloroform. The supernatant was precipitated with an equal volume of 4 M LiCl at -20°C overnight. Following centrifugation at 13,200 rpm at 4°C for 20 min, the resulting pellet was dissolved in TE buffer, pH 8.0. Samples were vortexed until the pellet was dissolved, and RNA was precipitated with 0.1 vol ethanol for 1 h at -20°C. The RNA was pelleted by centrifugation for 20 min at 4°C and washed with 70% ethanol, dried under vacuum, dissolved in TE buffer, pH 8.0 and stored at -80°C.

In the second method, total RNA was isolated from barley leaves and roots using a modification of the method described by Verwoerd *et al.* (1989). Leaf pieces (1 gm) were ground to a fine powder in liquid N<sub>2</sub> using a mortar and pestle, and the powder was

transferred to 2 ml plastic tubes and placed on ice. Extraction buffer (1 ml) containing phenol (the phenol had been equilibrated with TE buffer, pH 8.0), 0.1 M LiCl, 0.1 M Tris-HCl pH 8.0, 10 mM EDTA and 1% (w/v) SDS was heated to 85°C and added to the ground powder. The powder and buffer mixture was vortexed, 500 µl chloroform:isoamyl alcohol (24:1, v/v) was added and the tubes were vortexed for 30 sec. Following centrifugation for 5 min at 13,200 rpm, the aqueous phase was removed and mixed with 1 vol 4 M LiCl. Following centrifugation, RNA was precipitated with 0.1 vol 3 M sodium acetate buffer, pH 5.2 and 2 vol ethanol, at -20°C. After centrifugation, the pellets were washed with 70% ethanol, dried under vacuum and redissolved in RNase-free, DEPC-treated sterile water.

The third method involved extraction with sodium glycinate buffer (Chandler and Jacobsen, 1991) used to isolate total RNA from aleurone layers, as described in section 2.2.2.2.

### 5.2.6 Northern blot analysis

Total RNA was denatured, fractionated in a formaldehyde gel and transferred to a nylon membrane essentially as described by Sambrook *et al.* (1989). Total RNA (10 µg) was mixed with sample buffer, pH 7.0, [2 µl 10 x MOPS/EDTA buffer (1 x MOPS/EDTA consisted of 5 mM MOPS and 1 mM EDTA, pH 7.0,) 10 µl formamide, 3.5 µl 37% (v/v) formaldehyde in a 20 µl reaction mixture]. RNA was gently mixed with the buffer and heated to 70°C for 15 min. After heating, the sample was immediately placed on ice and 2 µl sample loading dye was added. The RNA was separated electrophoretically in a 1.2% (w/v) agarose gel (100 ml) containing 10 ml 10 x MOPS/EDTA buffer consisted of 0.5 M MOPS and 0.01 M EDTA, pH 7.0, and 1.5 ml 37% (v/v) formaldehyde. The gel was pre-run for 30 min at 50 V. The denatured RNA sample was loaded and run for 2-3 h at 70 V. Standard RNA markers were loaded with



the RNA samples to estimate the size of the mRNA transcripts from the Northern blot analyses. The RNA markers were also denatured prior to loading.

Following electrophoresis, the gel was thoroughly washed with water and stained in 0.1 mg/ml ethidium bromide for 10 min. The gel was soaked in water for 30 min prior to being photographed on an ultraviolet (UV) illuminator. The gel was soaked in 20 x SSC for 20 min and the RNA was transferred overnight to a Hybond-N<sup>+</sup> membrane, as described in section 2.2.3.5. Following transfer, the filter was rinsed with 4 x SSC, blot-dried, baked at 80°C for 1 h under vacuum, and fixed under short-wave ultraviolet illumination for 7 min.

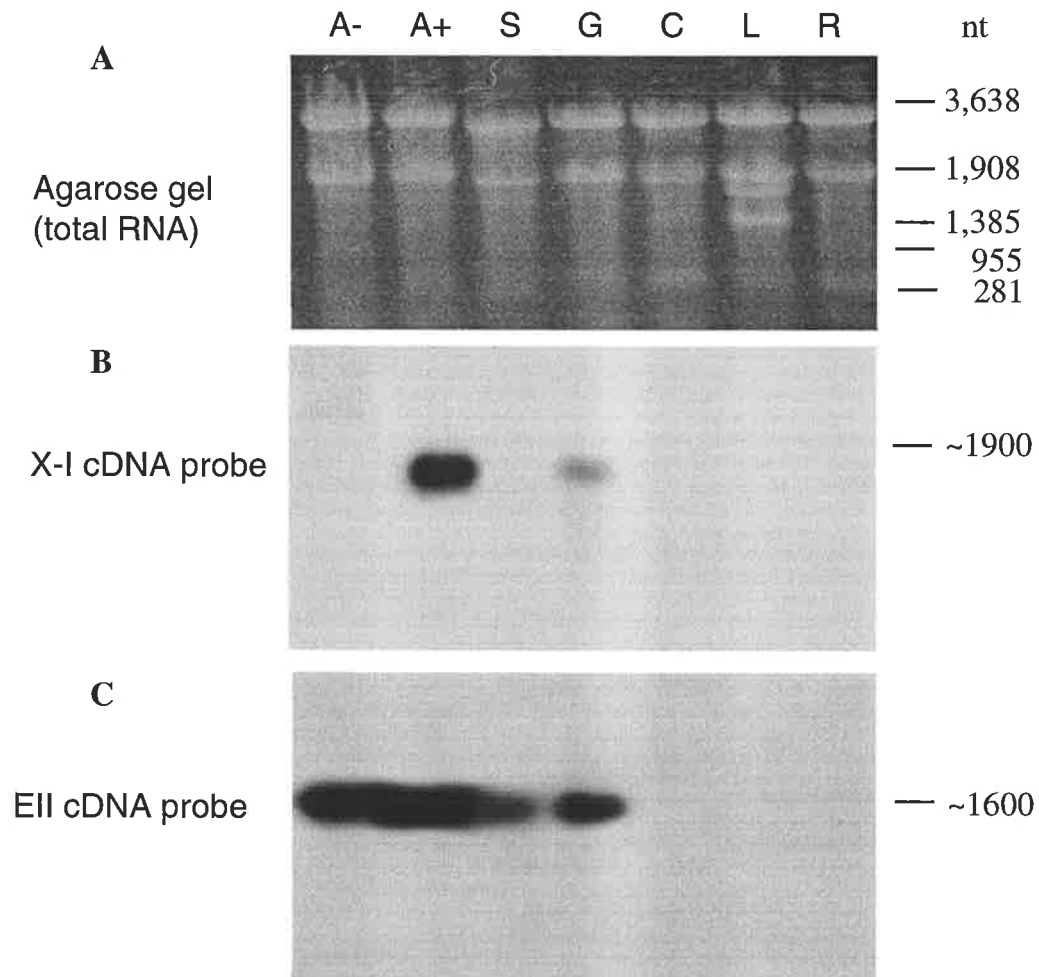
The membrane was soaked in 5 x SSC for 1 min and rolled into a hybridization bottle. Prehybridization solution [3 ml 50 x Denhardt's reagent, 5 ml 20 x SSPE, 1 ml 10% (w/v) SDS, 2 ml salmon sperm DNA (10 mg/ml) and 9 ml formamide] was added to the hybridization bottle. The membrane was prehybridized for at least 6 h at 42°C. The prehybridization solution was removed and hybridization solution [2 ml 50 x Denhardt's reagent, 5 ml 20 x SSPE, 1 ml 10% (w/v) SDS, 1 ml (10 mg/ml) salmon sperm DNA, 9 ml of formamide], containing 1 ml of the cDNA probe was added in a total volume of 20 ml. Following hybridization for at least 24 h at 42°C, the membrane was washed with 2 x SSC/0.1% (w/v) SDS at 42°C for 20 min, 1 x SSC/0.1% (w/v) SDS at 42°C for 20 min, 0.5 x SSC/0.1% (w/v) SDS at 42°C for 20 min, and 0.1 x SSC/0.1% (w/v) SDS at 42°C for 20 min. The membrane was finally washed with 0.1 x SSC/0.1% (w/v) SDS at 55°C for 20 min and blot-dried. Filters were subjected to phosphorimage analysis (Molecular Dynamics) for the quantitation of signal strength.

## 5.3 RESULTS

### 5.3.1 Tissue location of (1→4)- $\beta$ -xylan endohydrolase mRNAs

To study the expression sites of the barley (1→4)- $\beta$ -xylanase genes, total RNA was prepared from different tissues including 5-day germinated grain, leaves, roots, coleoptiles, scutella from 1-day germinated grain, and aleurone layers treated with or without GA<sub>3</sub>. Northern blot analysis was performed using equal amounts of total RNA (10  $\mu$ g) isolated from the various tissues, with the isoenzyme X-I cDNA as a probe. A strong signal corresponding to a mRNA transcript size of 1.9 kb was observed in total RNA isolated from GA<sub>3</sub>-treated barley aleurone layers. A weaker hybridization signal corresponding to a transcript of the same size was also detected in RNA preparations from 5-day germinated whole grain (Figure 5.1 B). No hybridization signal was observed in root, leaf, scutellum or coleoptile RNA probed with the barley xylanase isoenzyme X-I cDNA. Photographs of the ethidium-bromide stained RNA gel (before transfer of the RNA to the Hybond N+ membrane) under an ultraviolet illuminator revealed that the RNA loadings from different tissues were essentially equal (Figure 5.1 A). From the resultant Northern blot analysis it is clear that xylanase gene expression is tissue-specific, and that the genes are expressed predominantly in GA<sub>3</sub>-treated aleurone layers.

No (1→4)- $\beta$ -xylanase mRNA was detected in 5 day-old leaves (Figure 5.1 B). However, high levels of (1→3,1→4)- $\beta$ -glucanase isoenzyme EI have been shown in young leaves, but only after 8-10 days (Slakeski and Fincher, 1992 b). These transcripts were also present in RNA from young roots. To determine whether (1→4)- $\beta$ -xylanase gene expression might follow a similar pattern, additional Northern analyses of the RNA extracted from leaves up to 50 days after germination and from roots up to 12 days were performed. The results showed that no xylanase transcripts were detected during the first



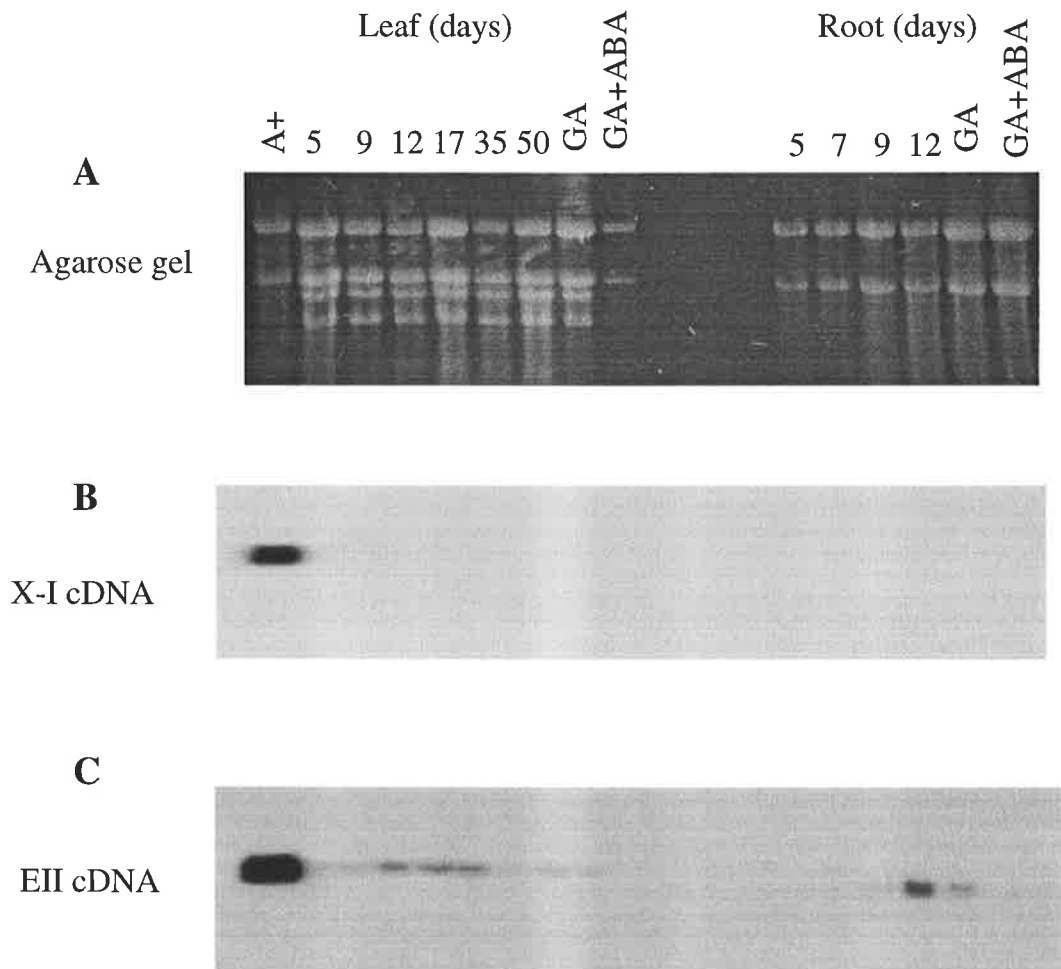
**Figure 5.1 Northern blot analysis of total RNA preparations from different tissues of barley.** A. Formaldehyde agarose gel of total RNA after staining with EtBr but before transfer to the nylon membrane. In each case 10  $\mu$ g RNA from aleurone layers with or without GA<sub>3</sub> (A+ and A-), scutellum (S), grain (G), coleoptile (C), leaf (L) and root (R) was loaded onto the gel. B. Membrane probed with (1 $\rightarrow$ 4)- $\beta$ -xyylanase isoenzyme X-I cDNA. C. The same membrane stripped probed with (1 $\rightarrow$ 3,1 $\rightarrow$ 4)- $\beta$ -glucanase isoenzyme EII cDNA. RNA size markers are shown on the right.

50 days of leaf development or during the first 12 days of root development (Figure 5.2 B). On the same Northern blot, an equal amount of total RNA from GA<sub>3</sub>-treated aleurone layers was used as a positive control (Figure 5.2 C). Ethidium bromide-stained, UV-irradiated gels showed well-defined ribosomal RNA bands and indicated that approximately equal amounts of the total RNA samples were loaded (Figure 5.2 A).

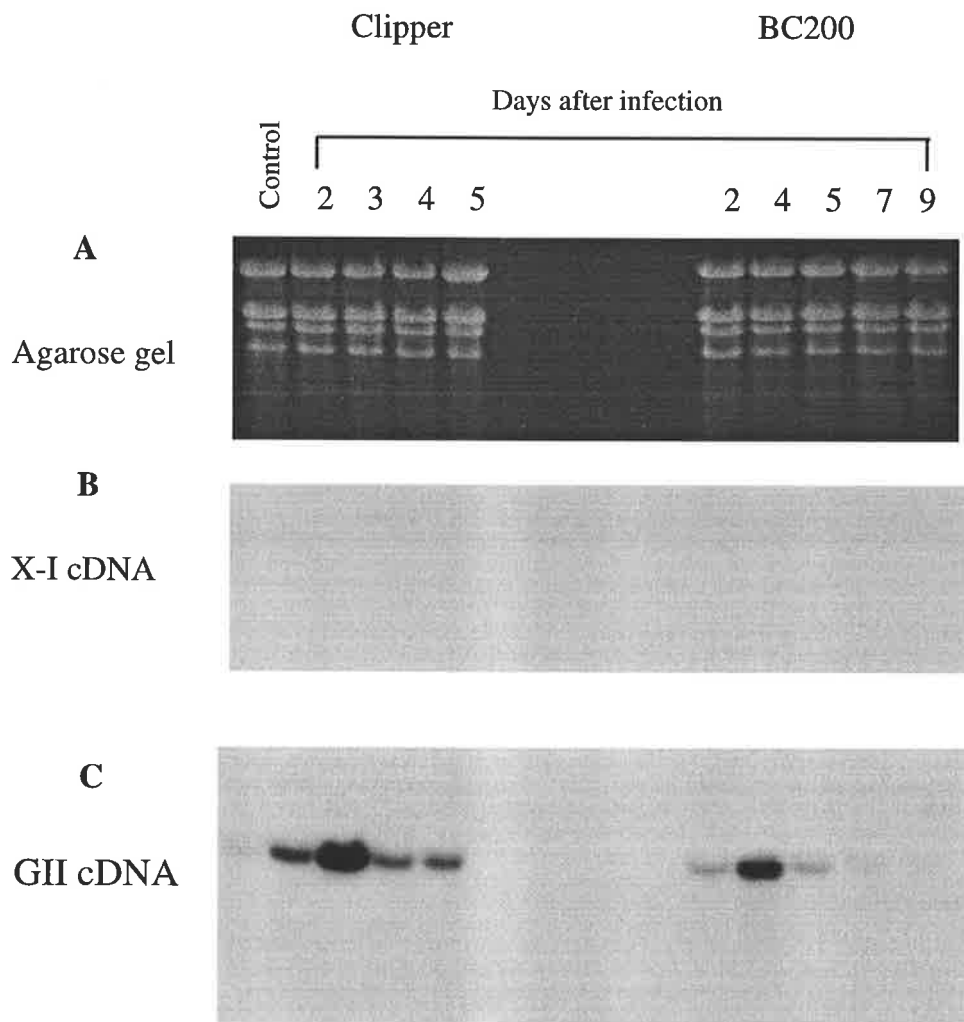
Slakeski (1992) observed that the (1→3,1→4)-β-glucanase isoenzyme EI gene was expressed following fungal infection of mature barley leaves. To determine whether or not (1→4)-β-xylanase gene expression is induced by pathogen attack, total RNA was isolated from young leaves of near-isogenic lines of barley which were either resistant or susceptible to the scald fungus *Rhynchosporium secalis*. The two barley lines used were Clipper, which is susceptible to the scald fungus, and a backcross line of Clipper, BC-200, which is resistant to the scald fungus (Brown *et al.*, 1988). The *Rhynchosporium secalis* isolate used was R144. Total RNA from infected leaves for both the susceptible and resistant varieties was kindly provided by Dr. Samuel Roulin, Department of Plant Science, University of Adelaide. When total RNA from healthy and infected leaves was subjected to Northern blot analysis, no xylanase mRNA transcripts were detected (Figure 5.3 B). For a positive controls, the filter was probed with a cDNA encoding barley (1→3)-β-glucanase isoenzyme GII (Figure 5.3 C).

### ***5.3.2 Effects of phytohormones on secretion of (1→4)-β-xylan endohydrolases from barley aleurone layers***

Northern blot analyses (section 5.3.1) suggested that transcription of (1→4)-β-xylanase genes is restricted to the aleurone layer of germinated grain; no xylanase transcripts could be detected in the scutellum or in young vegetative tissues. Experiments described here on the regulation of barley (1→4)-β-xylanase gene expression were therefore focussed on isolated aleurone layers.



**Figure 5.2 Northern blot analysis of RNA from different developmental stages of root and leaf.** In each case 10  $\mu\text{g}$  total RNA was loaded onto a 1.2% (w/v) agarose gel. A. The RNA gel after Ethidium bromide staining, confirming that approximately equal amounts of RNA were loaded; Only 5  $\mu\text{g}$  of  $\text{GA}_3$ +ABA-treated leaf RNA was loaded. B. Filter probed with (1 $\rightarrow$ 4)- $\beta$ -xylanase isoenzyme X-I cDNA. C. The same filter stripped and probed with (1 $\rightarrow$ 3,1 $\rightarrow$ 4)- $\beta$ -glucanase isoenzyme EII cDNA.

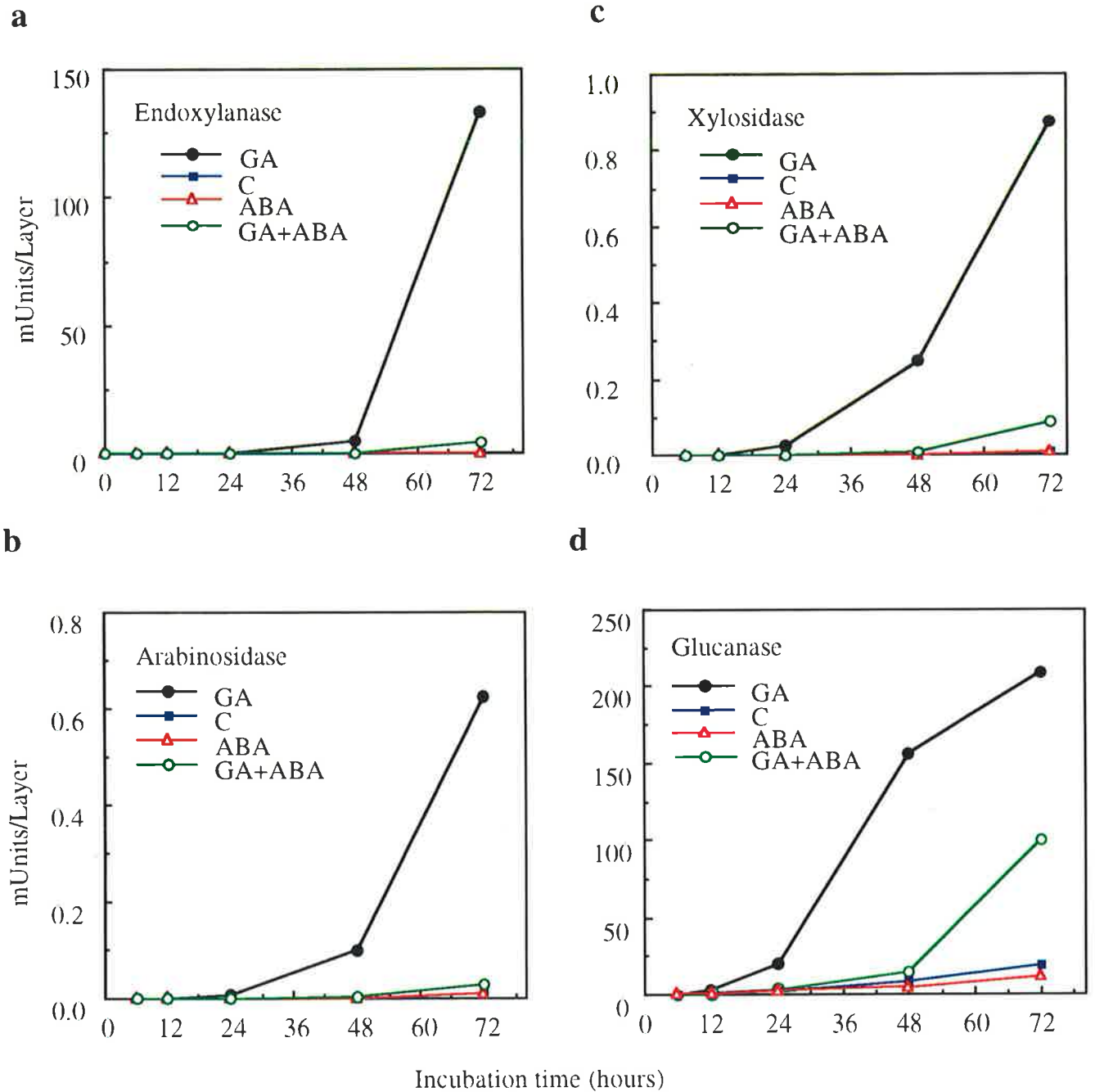


**Fig 5.3 Northern blot analysis comparing mRNA transcripts after infection of barley leaf with *Rhynchosporium secalis*, A.** EtBr-stained agarose gel showing approximately equal RNA loadings. **B.** Membrane probed with (1→4)- $\beta$ -xylanase isoenzyme X-I cDNA. **C.** Same membrane probed with (1→3)- $\beta$ -glucanase isoenzyme GII cDNA.

When barley aleurone layers were treated with GA<sub>3</sub>, (1→4)-β-xylanase secretion into the surrounding medium could be detected after 48 h but these levels had increased dramatically by 72 h (Figure 5.4 a). No enzymic activity was found in the medium surrounding untreated aleurone layers, and the addition of ABA abolished the GA<sub>3</sub> induction of (1→4)-β-xylanase secretion (Figure 5.4 a). Similar patterns of secretion were observed for α-L-arabinofuranosidase and β-D-xylopyranosidase; other enzymes that are likely to participate in arabinoxylan hydrolysis (Figures 5.4 b and 5.4 c). However, compared with (1→4)-β-xylanase secretion, relatively high levels of the α-L-arabinofuranosidase and β-D-xylopyranosidase were detected after 48 h (Figures 5.4 a, 5.4 b and 5.4 c; Taiz and Honigman, 1976; Dashek and Chrispeels, 1977). Similarly, (1→3,1→4)-β-glucanase secretion was apparent after 24 h and by 48 h large amounts of the enzyme had accumulated in the medium surrounding the isolated aleurone layers (Figure 5.4 d). The same developmental patterns were observed in several independent experiments.

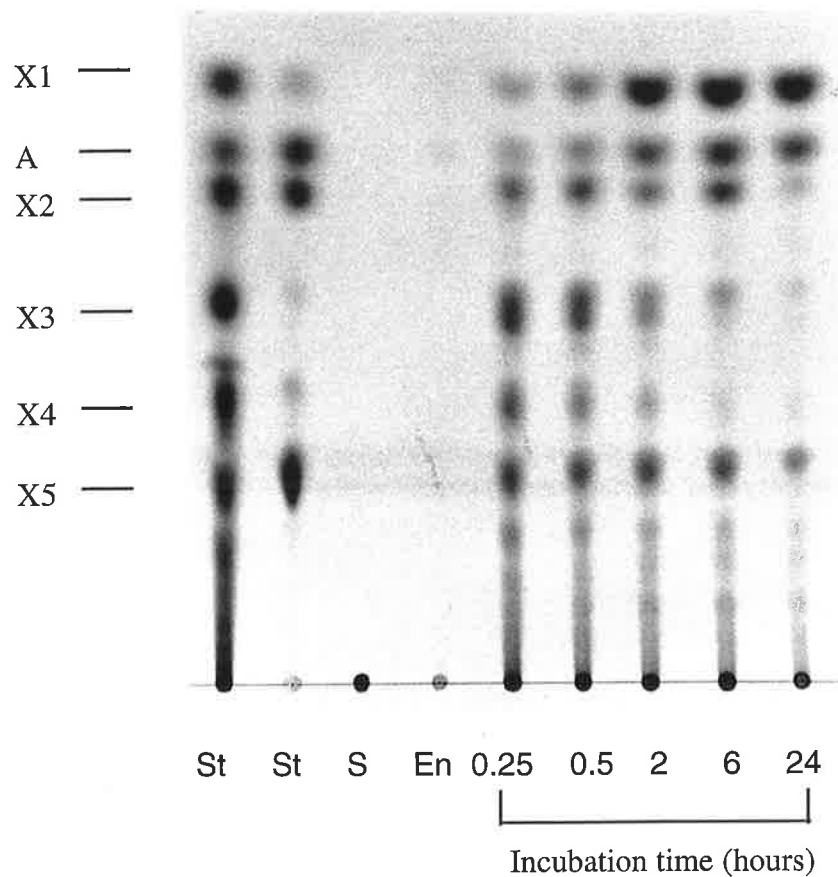
### 5.3.3 TLC analysis of hydrolysis products

Examination by TLC of the oligosaccharide products released from the arabinoxylan substrate revealed the presence of higher oligosaccharides early in the incubation period (Figure 5.5). These indicated that (1→4)-β-xylan endohydrolases were secreted by GA<sub>3</sub>-treated aleurone layers *in vitro*. The eventual accumulation of xylose and arabinose (Figure 5.5) could be attributed to the α-L-arabinofuranosidase and β-D-xylopyranosidase activities (cf. Figures 5.4 b and 5.4 c).



**Figure 5.4** Time course of secretion of cell wall-degrading enzymes from isolated barley aleurone layers. (1→4)- $\beta$ -Xylan endohydrolase (panel a),  $\alpha$ -L-arabinofuranosidase (panel b),  $\beta$ -xylosidase (panel c) and (1→3,1→4)- $\beta$ -glucanase (panel d) activities were measured in the medium surrounding aleurone layers treated with GA, ABA, GA+ABA and buffer (control) (C).





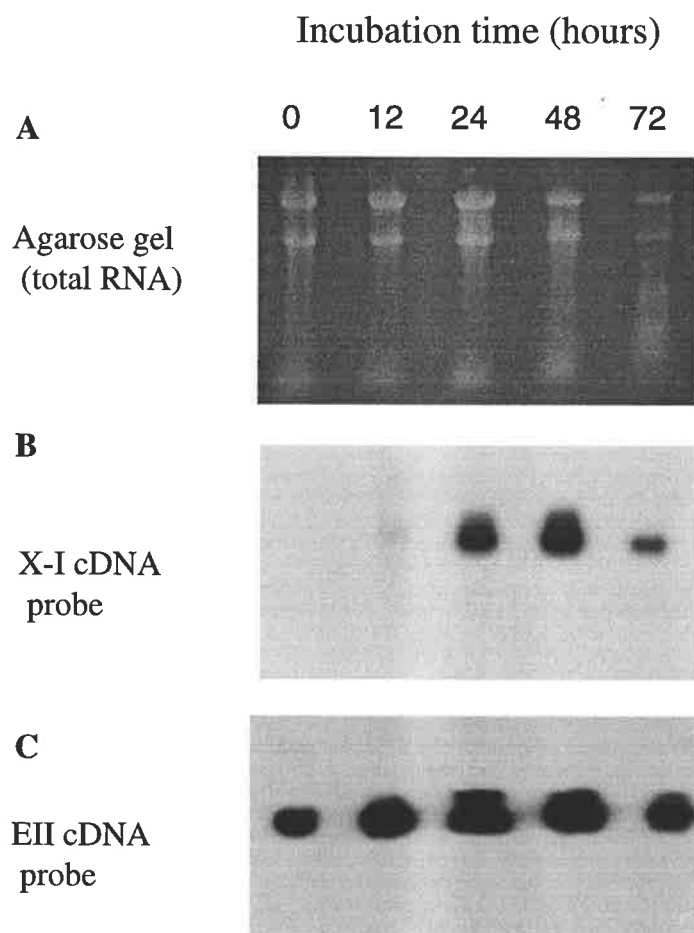
**Figure 5.5** Thin layer chromatography of products released from wheat flour arabinoxylan by enzymes secreted from  $GA_3$ -treated barley aleurone layers. Arabinose (A), xylose (X1) and oligoxyloside standards (St) of degrees of polymerization 2 to 5 (X2-X5) are shown in lanes 1 and 2. Lanes 3 and 4 show substrate (S) and enzyme (En) controls, respectively. Lanes 5 to 9 show the products of hydrolysis 0.25, 0.5, 2, 6, and 24 h after addition of media surrounding the aleurone layers.

#### 5.3.4 *Effect of hormones on the accumulation of mRNA transcripts in barley aleurone layers*

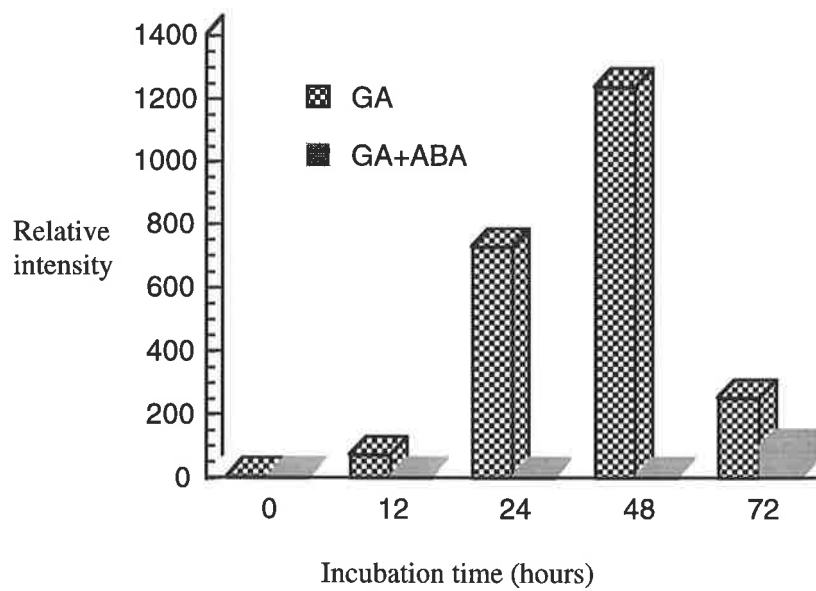
Northern blot analyses of total RNA extracted from isolated aleurone layers were used to monitor the hormonal control of (1→4)- $\beta$ -xylanase gene expression at the transcriptional level. The close similarity of nucleotide sequences in the two genes encoding isoenzymes X-I and X-II (Chapter 3 Figure 3.12), coupled with the absence of sequence information for the third gene, precluded the use of gene-specific probes in these analyses.

In GA<sub>3</sub>-treated aleurone layers, (1→4)- $\beta$ -xylanase gene transcripts could be detected at 12 h but were most abundant 24-48 h after application of the hormone (Figure 5.6 B). No (1→4)- $\beta$ -xylanase mRNA could be detected in untreated or ABA-treated aleurone layers, although low levels were evident after 72 h in layers treated with both GA<sub>3</sub> and ABA (Figure 5.8 B). Phosphorimager analysis was used to provide a quantitative measure of band intensities following Northern blotting. Based on this analysis, it is clear that (1→4)- $\beta$ -xylanase transcripts in GA<sub>3</sub>-treated aleurone layers increase in abundance until 48 h, and thereafter decline rapidly (Figure 5.7).

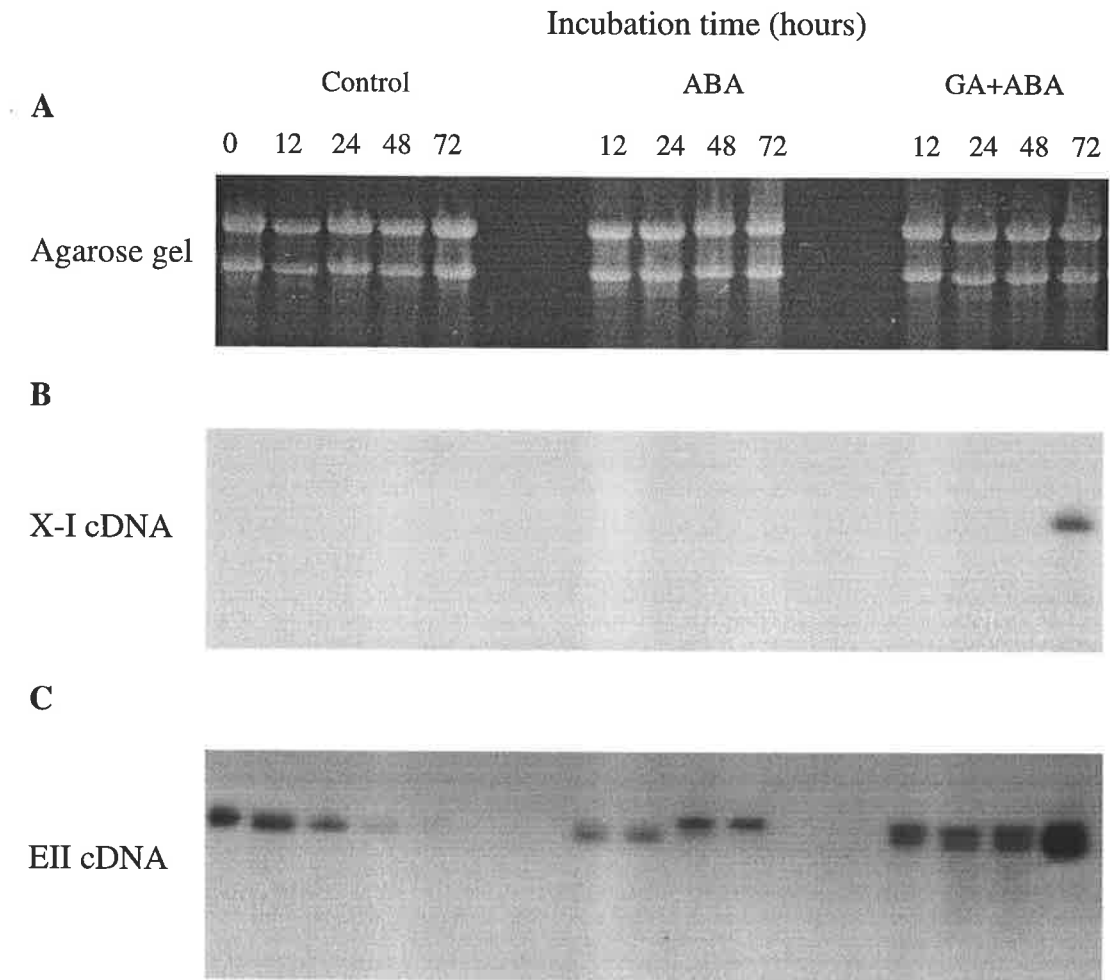
For comparative purposes, the same Northern blots were subsequently probed with a cDNA encoding barley (1→3,1→4)- $\beta$ -glucanase. It should be noted that the relative intensities of hybridizing bands on a single membrane allow meaningful comparisons to be made, provided total RNA loadings are similar, but that care needs to be exercised in comparing intensities when different probes have been used. Levels of (1→3,1→4)- $\beta$ -glucanase mRNA reached a peak 24 h after GA<sub>3</sub>-treatment of the aleurone layers (Figure 5.6 C). In untreated aleurone layers, significant levels of (1→3,1→4)- $\beta$ -glucanase RNA transcripts were also present (Figure 5.6 C). After hybridization with the (1→3,1→4)- $\beta$ -glucanase cDNA probe, two transcripts of slightly different sizes were detected. It is likely that these two transcripts correspond to (1→3,1→4)- $\beta$ -glucanase isoenzymes EI



**Figure 5.6** Northern blot analyses of total RNA from isolated aleurone layers, at various times up to 72 h after GA<sub>3</sub> treatment. In each lane 10 μg of total RNA was loaded. A. EtBr-stained gel shows approximately equal loading. B. Autoradiograph of membrane probed with (1→4)-β-xylanase isoenzyme X-I cDNA. C. Autoradiograph of the same membrane reprobbed with (1→3,1→4)-β-glucanase isoenzyme EII cDNA.



**Figure 5.7** Relative intensities of bands on the Northern blot in Figure 5.6 B probed with (1→4)- $\beta$ -xylanase isoenzyme X-I cDNA, as determined by phosphorimager analysis.



**Figure 5.8** Northern blot analyses of RNA from isolated aleurone layers treated with buffer (control), ABA and GA<sub>3</sub>+ABA. A. EtBr-stained gel showing approximately equal loadings in each lane. B. Filter probed with (1→4)-β-xylanase isoenzyme X-I cDNA probe. C. Same filter probed with (1→3,1→4)-β-glucanase isoenzyme EII cDNA.

and EII. No (1→3,1→4)-β-glucanase transcripts could be detected in ABA-treated aleurone layers until after 12 h. An increase in the levels of (1→3,1→4)-β-glucanase transcripts was observed with the combined GA<sub>3</sub>+ABA treatment (Figure 5.8 C).

## 5.4 DISCUSSION

The (1→4)- $\beta$ -xylanase isoenzyme X-I cDNA was used to investigate expression patterns in a selection of tissues, as measured by mRNA levels (Figure 5.1 B). The (1→4)- $\beta$ -xylanase mRNA transcripts were detected only in the aleurone layer (Figure 5.1 B). It is reasonable to assume that the transcripts detected in the whole grain preparations were indeed those present in the aleurone layer (Figure 5.1 B). Because cDNAs corresponding to both mRNA transcripts were screened from GA<sub>3</sub>-treated aleurone libraries, it can be concluded that both genes are transcribed in these cells. Definition of the relative abundance of the individual transcripts awaits the availability of gene-specific probes. The presence of (1→4)- $\beta$ -xylanase transcripts in GA<sub>3</sub>-treated aleurone layers is consistent with the secretion of (1→4)- $\beta$ -xylanases from isolated aleurone layers (Benjavongkulchai and Spencer, 1989; Dashek and Chrispeels, 1977; Taiz and Honigman, 1976) and with a role for the enzymes in the depolymerization of cell wall arabinoxylans in the germinated barley grain (Fincher, 1989; Slade *et al.* 1989).

Northern blots also indicated that (1→4)- $\beta$ -xylanase transcripts are present in low abundance or absent in the scutellum of germinated grain, coleoptiles, young leaves, roots and untreated aleurone layers (Figure 5.1 B). In contrast, significant levels of (1→3,1→4)- $\beta$ -glucanase are detected in the scutellum, young leaves, roots and untreated aleurone layers (Figure 5.1 C, 5.2 C). Slakeski and Fincher (1992 b) also reported that substantial amounts of (1→3,1→4)- $\beta$ -glucanase transcripts are present in untreated aleurone layers. The (1→3,1→4)- $\beta$ -glucanase isoenzyme EI gene is transcribed at relatively high rates in the scutellum of germinated grain and in young leaves and roots, where it might be involved in wall loosening during cell elongation (Slakeski and Fincher, 1992 b).

Total RNA from healthy leaves and leaves infected with the scald fungus were also subjected to Northern blot analysis. It is evident that the (1→4)- $\beta$ -xylanase X-I probe

does not hybridize to any mRNA transcripts from either infected or uninfected leaves (Figure 5.3 B). This result indicates that xylanase transcription was not induced by *Rhynchosporium secalis* infection, whereas in positive control experiments a relatively large amount of mRNA encoding the pathogenesis-related protein (1→3)-β-glucanase accumulated in response to pathogen infection (Figure 5.3 C).

Northern blot analyses of total RNA extracted from isolated barley aleurone layers were used to monitor the hormonal control of (1→4)-β-xylanase genes. Again the close similarity of nucleotide sequences in the two genes encoding (1→4)-β-xylanase isoenzymes X-I and X-II (Chapter 3), precluded the use of gene-specific probes in these analyses. Indeed, there is some doubt as to whether the genes will prove to have regions of unique sequence that might be suitable as such gene-specific probes. However, the isolation of cDNAs for both isoenzymes X-I and X-II from aleurone layer cDNA libraries, and the inability to detect (1→4)-β-xylanase mRNA in any other tissue, indicates that at least the two genes are expressed only in aleurone layers; the relative proportion of the individual transcripts is unknown.

The induction of (1→4)-β-xylanase gene expression by GA<sub>3</sub> in isolated aleurone layers and its abolition by ABA is clearly mediated at the transcriptional level (Figures 5.4 a and 5.7). As observed in Figure 5.4 a mRNA transcripts peak approximately 24 h before enzyme is secreted from the layers (Figure 5.4 a). However, it must be noted that sampling time at this stage of the experiment was 24 h and the lag between the appearance of peak mRNA levels and peak enzyme activity could be considerably less than 24 h. A similar large induction of endoxylanase activity by GA<sub>3</sub> and abolition of the effect by ABA has been observed previously in isolated barley aleurone layers (Taiz and Honigman, 1976; Dashek and Chrispeels, 1977; Bengavongkulhai and Spencer, 1986).

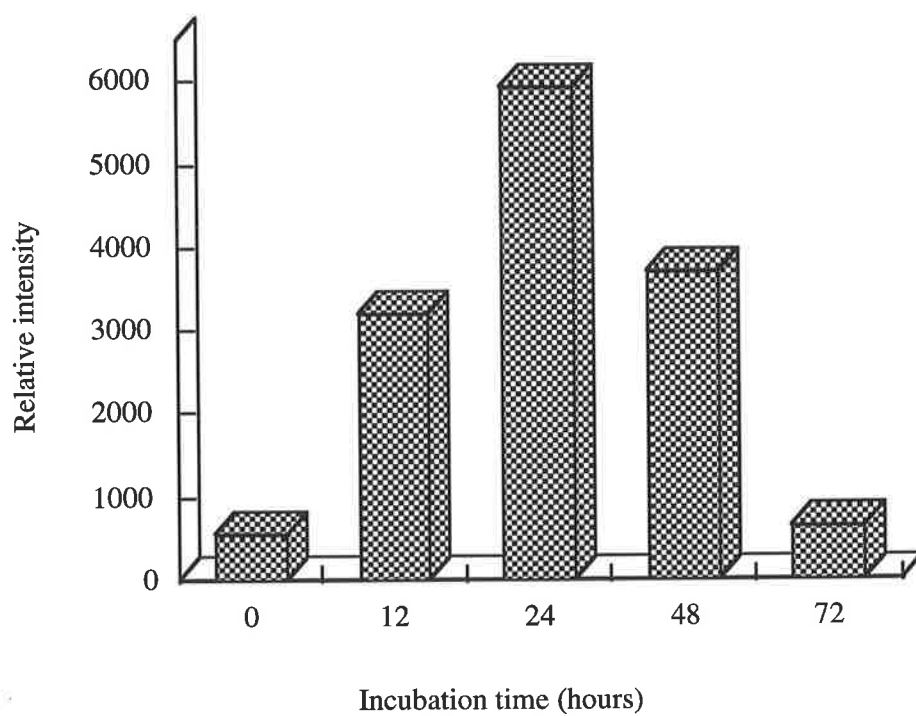
In considering the role of (1→4)-β-xylanases in cell wall degradation during endosperm mobilization, it might be anticipated that the synthesis and secretion of



endohydrolases responsible for hydrolysis of the major wall polysaccharides, namely (1→3,1→4)-β-glucanases and (1→4)-β-xylan endohydrolases, would be subject to coordinated regulation. Certainly (1→3,1→4)-β-glucanase isoenzyme EII gene transcription and secretion of the enzyme from isolated aleurone layers is induced by GA<sub>3</sub> (Stuart *et al.*, 1986 a; Slakeski and Fincher, 1992 b). However, ABA does not completely abolish this effect (Figure 4d; Slakeski and Fincher, 1992 b) and (1→3,1→4)-β-glucanase induction by phytohormones in isolated aleurone layers has features which differ from (1→4)-β-xylanase responses; the latter more closely resemble those exhibited by α-amylase genes.

An additional, important difference between (1→4)-β-xylanase and (1→3,1→4)-β-glucanase gene expression is related to the temporal control of transcription and enzyme secretion. It was observed by Slade *et al.* (1989) that xylan endohydrolases appeared in extracts of intact, germinated barley grain several days later than (1→3,1→4)-β-glucanases. This delay, albeit shorter in duration, is also seen in enzyme secretion from isolated aleurone layers (Figure 5.4 a cf. Figure 5.4 d). Slade *et al.* (1989) suggested that the (1→4)-β-xylanases might simply resist extraction because they are bound to cell walls initially and are released only after cell wall degradation is complete. However, the results shown in Figures 5.7 and 5.9, where a single Northern blot was probed with a (1→4)-β-xylanase cDNA, stripped and subsequently probed with a (1→3,1→4)-β-glucanase cDNA, indicate that the peak of transcription activity of the (1→4)-β-xylanase genes indeed occurs up to 24 h later than the corresponding peak in (1→3,1→4)-β-glucanase transcription.

The late appearance of (1→4)-β-xylanases, both in intact germinated grain and in isolated aleurone layers, raises the question as to whether arabinoxylan depolymerization is really necessary for the penetration of cell walls by α-amylases, peptidases and other hydrolytic enzymes to their substrates in starchy endosperm cells. Perhaps removal of (1→3,1→4)-β-glucan increases the porosity of walls sufficiently to allow free diffusion



**Figure 5.9** Relative intensities of bands from the Northern blot probed with (1→3,1→4)- $\beta$ -glucanase isoenzyme EII (Figure 5.6 C), as determined by phosphoimager analysis.

of the hydrolases to starchy endosperm cells. A biphasic degradation pattern, in which (1→3,1→4)-β-glucans are degraded before arabinoxylans, is consistent with evidence from electron microscopy that remnants of barley endosperm cell walls remain after the initial front of wall-degrading enzymes has passed and that subsequent degradation of the remaining components proceeds more slowly (Selvig *et al.*, 1986). It should also be noted that aleurone layer cell walls, which exhibit a relative high proportion of arabinoxylans (71%), (Bacic and Stone 1981 b), remain partially intact in the germinated grain (Jones *et al.*, 1987). Perhaps the late appearance of xylanases enables the arabinoxylan of the aleurone cell wall to escape extensive hydrolysis in the early stages of the endosperm mobilization; the retention of some cell wall material around the aleurone cells might be essential to maintain their integrity during the period of active enzyme secretion.

In any case, it is clear that there are important differences in the hormonal regulation of expression of barley genes that encode cell wall-degrading enzymes during starchy endosperm mobilization, and that comparisons of promoter regions of the genes may shed some light on these differences, particularly those responsible for the temporal control of gene expression.

## **CHAPTER 6**

# **ISOLATION OF A (1→4)- $\beta$ -D-XYLANASE GENE**

## 6.1 INTRODUCTION

In the previous Chapter, the tissue specificities and hormonal regulation of barley (1→4)-β-xylanase expression were examined by Northern blot analyses. The cDNA encoding (1→4)-β-xylanase isoenzyme X-I, which was described in detail in Chapter 3, was used as a probe for the Northern analyses. Expression was detected only in the aleurone layer of germinated grain. Furthermore, regulation of expression was mediated at a transcriptional level, where GA<sub>3</sub> enhanced levels of (1→4)-β-xylanase mRNA in a process that was antagonised by ABA (section 5.4). The induction of (1→4)-β-xylanase gene expression in isolated aleurone layers was similar to patterns described for α-amylase, but significant differences in response to hormones were observed when (1→4)-β-xylanase expression was compared with expression of (1→3,1→4)-β-glucanases. In addition, (1→4)-β-xylanase expression, as measured by either the appearance of mRNA or the secretion of active enzyme, occurred at least 1 day later than expression of (1→3,1→4)-β-glucanase and the high pI α-amylase (Figure 5.4 a and 5.6 B of 5.4 d and 5.6 C; Chandler and Jacobsen, 1991; Gubler *et al.*, 1995 ).

Because transcriptional activity of genes will be ultimately controlled by regulatory proteins that bind to promoter regions of the genes, and because both GA response elements (GARE) and ABA response elements (ABRE) have been identified in the promoters of other barley genes that are expressed in the aleurone layer of germinated grain (Skriver *et al.*, 1991; Gubler and Jacobsen, 1992; Jacobsen *et al.*, 1995), it was considered highly likely that similar sequence elements would be involved in (1→4)-β-xylanase gene regulation. Close examination of the (1→4)-β-xylanase promoter might also reveal possible explanations for the observed hormonal and temporal differences in (1→4)-β-xylanase gene regulation.

In this Chapter, the isolation of a (1→4)-β-xylanase gene from *Hordeum vulgare* genomic libraries is described. The complete sequence and structural features of the

gene are presented. In addition, the promoter region was scanned to identify possible *cis*-acting sequence elements which may be related to specific regulatory functions such as hormone-responsiveness and tissue-specificity. The number of genes encoding barley (1→4)- $\beta$ -xylanases has been defined, their chromosomal location identified and polymorphisms within the different cultivars has enabled the genes to be accurately mapped on the barley genome.

## 6.2 MATERIALS AND METHODS

### 6.2.1 Materials

Bacterial cells *E. coli* XL1-Blue (P2) and plasmid vector pBluescript II SK+ were purchased from Stratagene.  $\alpha$ -[ $^{32}\text{P}$ ]dCTP and  $\gamma$ -[ $^{32}\text{P}$ ]ATP were obtained from Bresatec. All restriction enzymes, T4 polynucleotide kinase, T4 DNA ligase, and alkaline phosphatase were from Promega or New England Biolabs. The Megaprime DNA labelling kit and autoradiographic film were purchased from Amersham International. The Gene-Clean kit and 6% (w/v) polyacrylamide mix were purchased from Bresatec. The DNA sequencing kit (version 2.0) was purchased from United States Biochemical Corporation (USBC; Cleveland, OH, USA).

### 6.2.2 Screening of barley genomic library

The barley genomic library was prepared by Mr. Ron Osmond (Department of Plant Science, University of Adelaide, South Australia) from partially digested DNA from 7-day-old seedlings of *Hordeum vulgare* L. (cv. Galleon) cloned into the *Eco*RI site of the bacteriophage vector  $\lambda$ DASH II (Stratagene, La Jolla, CA, USA). A second library, prepared from partially digested DNA from 7-day-old seedlings of cv. Boni and cloned into the *Bam*HI site of the EMBL3 vector, was obtained from Clontech Laboratories (Palo Alto, CA, USA). The libraries were plated out on lawns of *E. coli* XLI-Blue (P2) cells (Stratagene) and screened by hybridization of membrane filter plaque replicas (section 3.2.4) using the barley (1 $\rightarrow$ 4)- $\beta$ -xylanase isoenzyme X-I cDNA as a probe. Positive plaques were rescreened until it was confirmed that they were monoclonal (section 3.2.4).

### 6.2.3 Preparation of $\lambda$ DNA

Individual positive plaques were picked and phage stock was prepared as described in section 3.2.2.3. Phage stock (200  $\mu$ l) was added to 400  $\mu$ l overnight cultured *E. coli* XL1-Blue (P2) cells. The mixture was incubated for 15 min at 37°C to allow infection and transferred to a 125 ml conical flask containing 50 ml LB medium. The flask was incubated at 37°C at 200 rpm for approximately 8 h or until the bacterial cells flocculated. The culture was centrifuged for 10 min at 10,000 rpm to pellet the cell debris. The supernatant was transferred to a fresh centrifuge tube and incubated at 37°C for 30 min with 20  $\mu$ l DNase and RNase (10 mg/ml in 10 mM Tris-HCl buffer, pH 7.5, containing 15 mM NaCl). After incubation, the supernatant was adjusted with PEG 8000 and NaCl to final concentrations of 10% (w/v) and 1 M, respectively. The solution was left on ice overnight and phage particles were pelleted by centrifugation at 10,000 rpm for 20 min at 4°C. The supernatant was decanted and the tube inverted for 15 min to remove residual PEG. The drained pellet was dissolved in 700  $\mu$ l LB, transferred to an Eppendorf tube, and a further 700  $\mu$ l DEAE-cellulose DE-52 (equilibrated in LB) was added to remove bacterial proteins and DNA. The tube was thoroughly mixed by inversion and centrifuged for 5 min at room temperature. Following centrifugation the supernatant was transferred to a fresh Eppendorf tube and the process repeated. Next, the supernatant was mixed with 15  $\mu$ l proteinase K (0.1 mg/ml) and 35  $\mu$ l 10% (w/v) SDS, and incubated for 5 min at room temperature to lyse the phage particles. Following incubation, 130  $\mu$ l 3 M potassium acetate buffer, pH 4.8, was added, the solution was incubated for 20 min at 88°C and subsequently collected on ice for 10 min. After centrifugation the supernatant was transferred to a fresh tube and an equal volume of isopropanol was added to precipitate the phage DNA. After 1 h at -20°C the precipitate was pelleted by centrifugation, washed with 70% ethanol, dried, and resuspended in 100  $\mu$ l TE.



#### 6.2.4 Restriction digestion and subcloning

Purified phage DNA carrying the barley genomic DNA insert was digested with appropriate restriction enzymes and the resulting fragments were separated by electrophoresis on a 1% (w/v) agarose gel. The separated DNA was transferred to a Hybond-N<sup>+</sup> membrane (Amersham) and hybridized using [ $\alpha$ -<sup>32</sup>P]dCTP-labelled barley (1→4)- $\beta$ -xylanase X-I cDNA as a probe under the conditions described in section 3.2.3. Fragments giving a positive signal were purified from the agarose gel using Gene Clean according to the manufacturers instructions (Bresatec). The pBluescript (SK+) vector was prepared for subcloning as follows: vector DNA was digested with the appropriate restriction enzymes and extracted with phenol:chloroform (1:1 v/v) and chloroform. After centrifugation the upper phase was precipitated with 0.1 vol 3 M sodium acetate and 2.5 vol of ethanol. The pellet was washed with 70% ethanol, dried and resuspended in TE. The dissolved DNA was dephosphorylated with alkaline phosphatase as described in Sambrook *et al.* (1989) and the dephosphorylated DNA was separated on a 1% (w/v) agarose gel. The plasmid DNA band was excised from the gel and purified using Gene Clean before ligation. The procedures used for ligation and transformation were as described in section 2.2.3.3.

#### 6.2.5 Preparation of genomic DNA for Southern blot analyses

Several pieces of 5-day old leaves (approximately 1 g) were frozen in liquid nitrogen in a 10 ml plastic tube and crushed with a small spatula. This was transferred to a 2 ml cold Eppendorf tube and crushed again. The tube was filled with powder to the 800  $\mu$ l mark and 750  $\mu$ l 100 mM Tris-HCl buffer, pH 8.5, containing 1% (v/v) sarkosyl, 100 mM NaCl, 10 mM EDTA was added. Phenol/chloroform/isoamyl-alcohol (25:24:1, v/v/v) (750  $\mu$ l) was added, the tube was shaken for 40 sec, placed in a rotating platform

mixer for 15 min at room temperature, and spun for 10 min at 13,200 rpm. The upper phase was transferred to an Eppendorf tube and the chloroform and isoamyl-alcohol extraction was repeated. An equal volume of isopropanol and 0.1 vol 3M sodium acetate buffer, pH 5.2, were added to the upper aqueous phase. The contents of the tube were mixed by inversion and the DNA allowed to precipitate for 5 min at room temperature. The tube was centrifuged for 10 min at 13,200 rpm at room temperature. The supernatant was poured off, the pellet was washed with 70% ethanol (v/v) and dried for 5 min in a Speed Vac evaporator (Savant Speed Vac SC 110). The pellet was dissolved in 42  $\mu$ l 5 mM Tris-HCl buffer, pH 8.0, containing 500 mM EDTA and 40 mg/ml RNase A.

For Southern blot analyses the genomic DNA was hydrolysed with restriction enzymes, fragments were separated on agarose gels and blotted onto nylon membrane (2.2.3.5), and membranes were probed as described in section 3.2.4.

#### **6.2.6 Chromosomal location of the (1 $\rightarrow$ 4)- $\beta$ -xylanase genes**

Disomic wheat-barley addition lines having individual pairs of barley (*Hordeum vulgare*, cv. Betzes) chromosomes added to a full complement of wheat chromosomes (*Triticum aestivum*, cv. Chinese Spring) were developed by Islam *et al.* (1981). Thus, addition line 1 has a full complement of wheat chromosomes, plus barley chromosome 1; addition line 2 has all the wheat chromosomes plus barley chromosome 2, etc. The only exception is addition line 5, in which the long arm of this barley chromosome causes cytological abnormalities and male-sterility when added to wheat (Islam *et al.*, 1981). The barley chromosome 5 addition line is therefore a double monosomic containing one copy each of barley chromosomes 5 and 6 (Islam and Shepherd, 1990). On the basis of the genetic relationship of barley chromosomes with specific wheat chromosomes, barley chromosomes 1, 2, 3, 4, 5, 6 and 7 are designated as the homoeologous wheat chromosome 7H, 2H, 3H, 4H, 1H, 6H and 5H, respectively (Islam and Shepherd, 1990).

Throughout this thesis, the traditional barley chromosome numbers are followed, in parentheses, by the numbers of the equivalent wheat homoeologous group of chromosomes.

DNA samples from the addition lines and from the wheat and barley parents were kindly provided by Dr A. K. R. M. Islam. Chromosomal locations of (1→4)- $\beta$ -xylanase genes in the barley genome were defined by Southern blot analyses (section 3.2.4).

#### **6.2.7 Southern blot analysis of genomic DNA**

Genomic DNA from different barley cultivars (15-20  $\mu$ g) was digested to completion with different restriction enzymes (*EcoRV*, *BamHI*, *HindIII*, *DraI*, *EcoRI* and *XbaI*) using 1.5 U enzyme/ml, 1mg/ml BSA, 1.3-4 mM spermidine at 37°C. DNA preparations from wheat (cv. Chinese Spring), barley (cv. Betzes) and the addition lines were digested with *EcoRI*, *BamHI* and *XbaI* restriction enzymes using the conditions described above. Digested fragments were separated on a 1.2% (w/v) agarose gel at 40 V for 16 h. The gel was stained with 1mg/ml ethidium bromide for 15 min on a rocking platform. The gel was destained with milli-Q water for 20 min and DNA was transferred to a nylon membrane (Amersham) as described in section 2.2.3.5. Prehybridization, hybridization and washing of the membrane were performed as described in section 3.2.4.

## 6.3 RESULTS

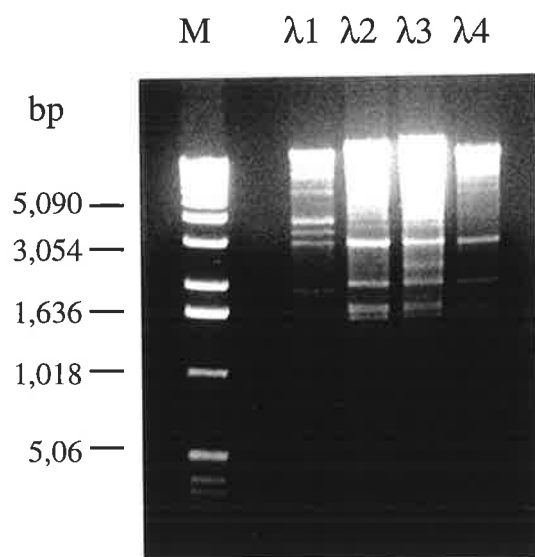
### 6.3.1 Isolation of genomic clones

From approximately  $2 \times 10^6$  plaques, four positive clones were isolated from the cv. Galleon library using the cDNA for barley (1→4)- $\beta$ -xylanase isoenzyme X-I as a probe. The four positive clones were designated  $\lambda 1$ ,  $\lambda 2$ ,  $\lambda 3$  and  $\lambda 4$ . Bacteriophage DNA was digested with *EcoRI* to isolate the genomic DNA inserts. After restriction digestion, the four clones were analysed by 1% (w/v) agarose gel electrophoresis. Insert sizes were all in the range 16-18 kb (Figure 6.1 a). On the basis of banding patterns, the four clones were grouped into three classes; class 1,  $\lambda 1$ ; class 2,  $\lambda 2$ ,  $\lambda 4$ ; class 3,  $\lambda 3$ . Southern blot analyses showed that in each case the hybridizing sequence was located on a 3 kb *EcoRI* fragment (Figure 6.1 b). This fragment was subcloned into the pBluescript vector and designated p $\lambda 2E$ . The restriction map of this gene fragment is shown in Figure 6.2.

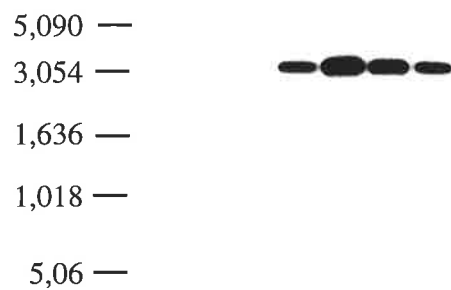
Restriction digests were performed with *PstI*, *SacI*, *SacII*, *XhoI* and *SallI*. Following digestion, fragments were separated by agarose gel electrophoresis (Figure 6.3 a) and Southern blot analysis was carried out using the 685 bp *PstI* fragment of pMX1 cDNA clone (Figure 2.8, Chapter 2) as a probe to identify the 5' end of the gene's coding region. An *EcoRI/SallI* fragment (720 bp) of p $\lambda 2E$  was found to contain the signal peptide coding region of the gene and a putative TATA box (Figure 6.3 b).

Sequence analyses indicated that all four genomic clones encoded (1→4)- $\beta$ -xylanase isoenzyme X-I, based on their high degree of identity (99.5%) with the corresponding cDNA, but that they did not extend far enough beyond the putative TATA box to allow a comprehensive analysis of the promoter region. Indeed, their 5' termini were all located at the *EcoRI* site at nucleotide 394 in Figure 6.4. To isolate the 5' region of the promoter the genomic library from the variety Boni was subsequently screened, using the 720 bp *EcoRI/SallI* fragment from p $\lambda 2E$  (Figure 6.4) as a probe. A single

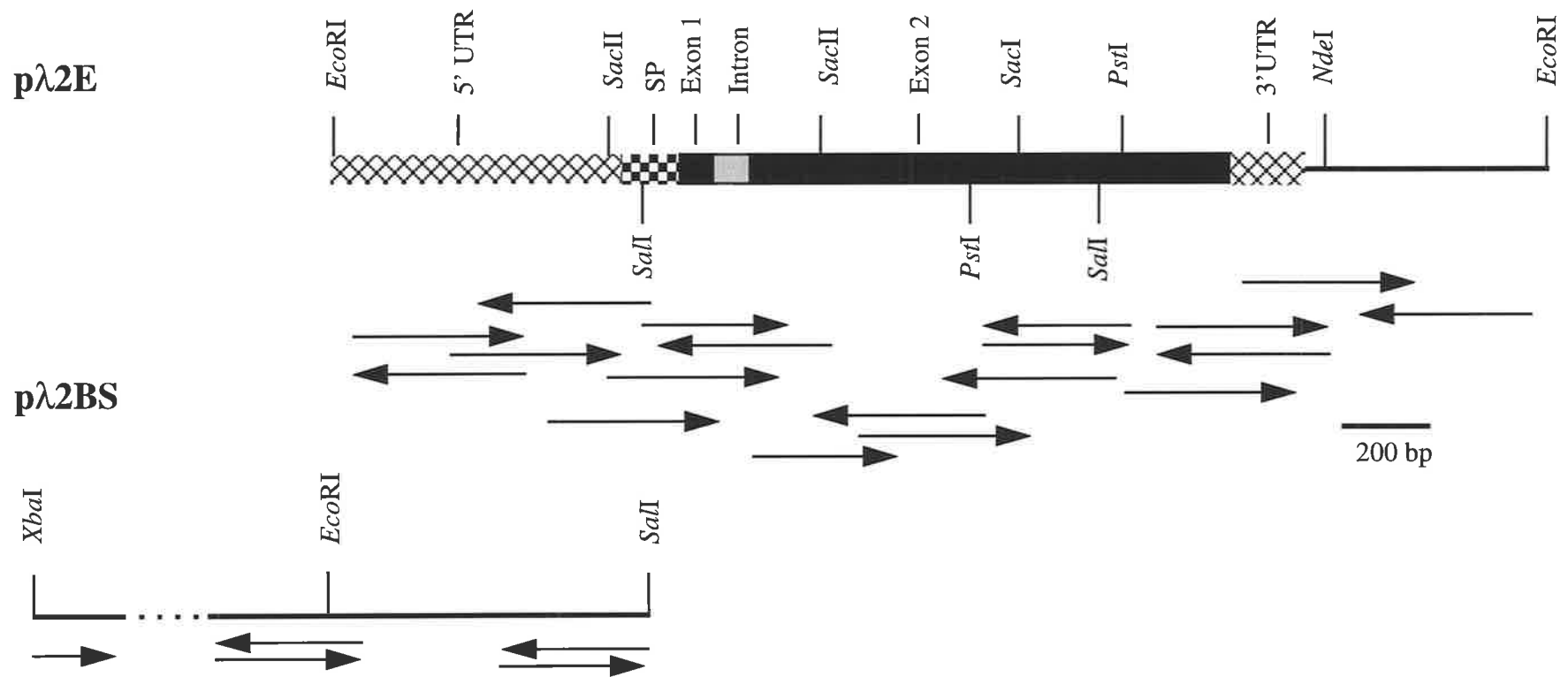
**A**



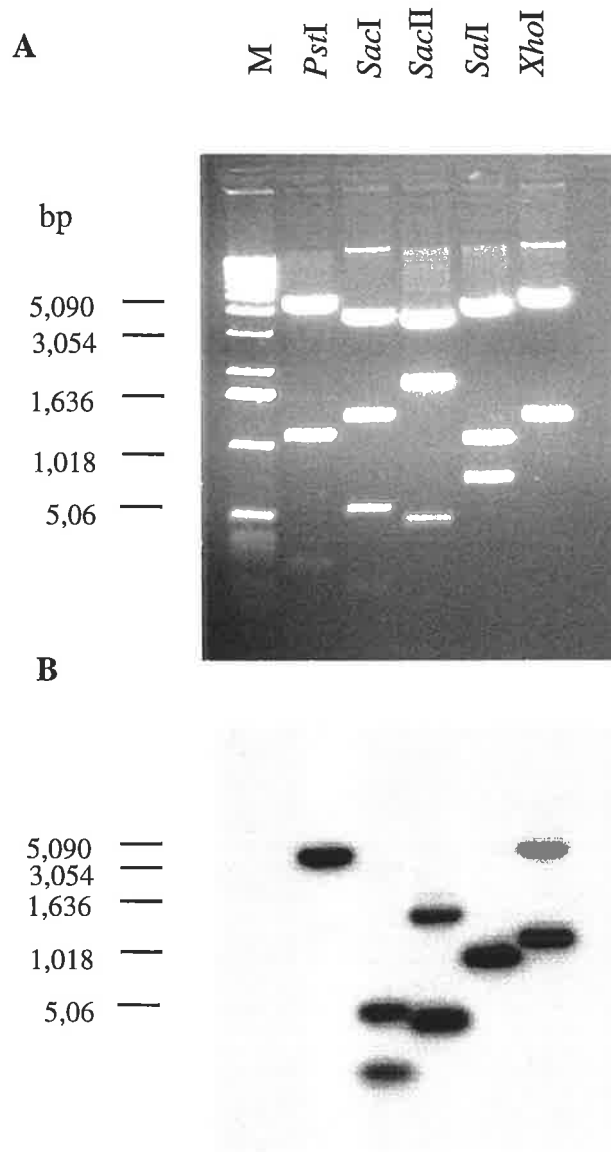
**B**



**Figure 6.1** Phage DNA from positive genomic clones containing the (1→4)- $\beta$ -xylanase insert after digestion with *EcoRI*. A. EtBr-stained B. Southern blot analysis. The DNA size markers are on the left.



**Figure 6.2 Structure and sequencing strategy of (1→4)-β-xylanase isoenzyme X-I gene.** Restriction maps of the two gene fragments of overlapping clones pλ2E and pλ2BS. The 5' untranslated region (5'UTR) and the 3' end of the promoter are hatched, as is the 3' untranslated region (3'UTR). The signal peptide-coding region (SP) is represented by the blocked area, exons 1 and 2 are shaded and the position of the single intron in the coding region of the mature enzyme is indicated. The arrows show the length and direction of the fragments sequenced during the determination of the complete nucleotide sequence.



**Figure 6.3 Restriction analysis of p $\lambda$ 2E.** A. EtBr-stained agarose gel showing separated DNA fragments before transfer to the nylon membrane. B. Southern blot analysis of the transferred DNA using the 685 bp *Pst*I fragment (from pMXI clone) as a probe.

**Figure 6.4** The complete nucleotide sequence of the gene encoding barley (1→4)-β-xylanase isoenzyme X-I. The pyrimidine box, the TAACGAC box, the TATCCAT box, the CAAT and the TATA box of the promoter region are underlined. The putative transcriptional start point at nucleotide 468 is indicated by an arrow, as is the NH<sub>2</sub>-terminal Val residue of the mature enzyme. The intron is shown in lower case letters and the putative catalytic Glu residue are in bold type. A possible polyadenylation signal in the 3' untranslated region is underlined. Amino acid numbering starts at the mature protein NH<sub>2</sub>-terminal.



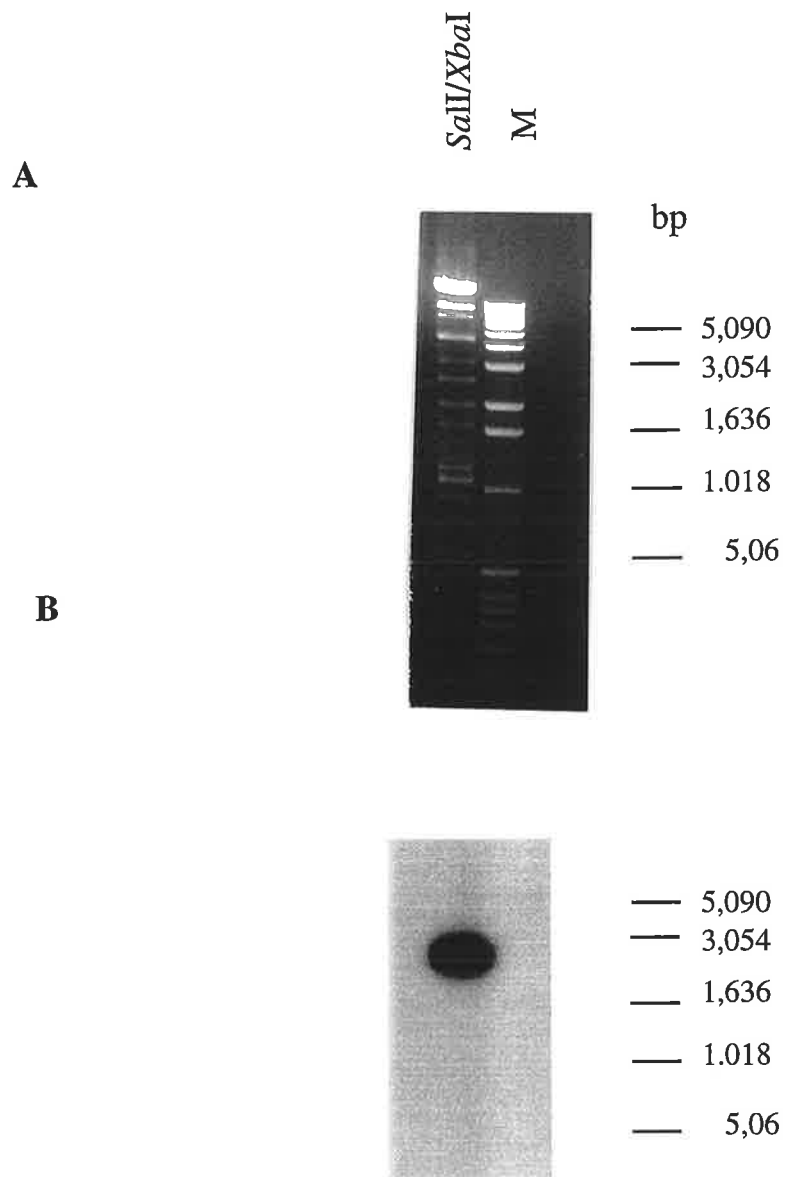
AACTATCGTGGTGCATCCATACTGACGTGTGGAGCCGTTTTCTGTAACCTCAAGCCATGCATTGCAAGGGCAGCCTCGAATTTAGCATGCAGGAATTA 100  
 GTTACATCGTTCGTCACCACAAGTGAGGGCGGCTGCAAGCAACTCGGCACGCCTCTGGTTTTCCGGTCGAGGAATTAATGCTACGTGCGTGCCTACTAC 200  
 CACGTCTCGTTTTGGAAATCGTAGCACTCGCCAGGAAGGTCACGCCCTTTGTGTGTGTGCAATCTTACTGTTACTCAAGAGCAGCAAGCATGCGAGAG 300  
 AGAGTTCGTTGCTTCCGGTTTGTGCTCGTTCGTTATTGCTCTTACCGTTACTCTTTCCATCCTGTGATAACGACTCGACTATATCCATCTCGAATTC 400  
 CGATCGACTCAACGTCGCCAGCCGCCGCAAAATTCGCCCTTTAAATACGGTGGCCACCGTGATCCATCATCCCTCACTACTCACACAGCAGAGATCAT 500  
 CAATCCGACGAACATCTTCGCAACCTCCAGGCCAGTCTGCTCTCACTAGCTAGTACCTCTCCACTCGCGTAAGATGGCAAGCACAACCTCAGGTATGTAA 600  
 CTTGCATGCAGCTAGCACACCATGAGTCCAGCTATAGCTCATTTCATGGTGCACCTTGTGTGCTGCTTCTTTCAGGACGTGAACATGGACGGCAGCCTCG 700  
 CCGGCTGCGCACCCTTTCGCACGGGCACGACGACGCTCTCCGTGCACATCGAGGAAGAGATGGCATTGCTCCCCGTCACTGTGGCCGTGGTGGCAACAA 800  
 GCCCAGCGCCGGTACGCTCTCGTGGCTGGCCGCGCGATGAGGAGGACGGCCTGCGCTGCCGATCCCGGTAGACACCTGAAGCCTCGTCTCACTTAC 900  
 CGCGTGGCCGGTGGATCAGCTTGGGAGCAGCACGGGCACCGCCCGTGCGCATCGACCTTGGCGTGGAAAGACAATGGCAACGAGACCTTGGTGG 1000  
 AGTGGCGCGGTTGTGCGCAAGGAGGGCGGGTGGTGGAGATCATGGGGCCTTCCGGCTCAGGACGGAGCCCGCAGCGCCGCGGTTTTCTGTCACGG 1100  
 M G A F R L R T E P R S A A V F V H G  
 CGCCCCGCGGGCTCGAGCTCAAGTTCATGGATCTCCGGTCTACCCGGTGGACCACAAGGCGCGTTCAAGCAGCTCAAGGACAAGACTGACAAGgtg 1200  
 A P A G V D V K V M D L R V Y P V D H K A R F K Q L K D K T D K 19  
 agagagcatgatccacgtaataaccacctgcatgcacactcgccttgatgtggcagtaacgtgatcatacagagctccattgatgcagGGCGGCAAGAGG 1300  
 A R K R 23  
 GACGTGATTCCTCAAGTGGGCACGCCGGCGGGAGCGGGAGCGGGCGGGCGGCGCTCCGTGCGCGTGGTGCAGTTGGACAACGCCCTTCCCTTCGGGACAT 1400  
 D V I L K L G T P A G A G A G A A A S V R V V Q L D N A F P F G T C 57  
 GCATCAACACGTCCTGATCCAGAAGCGGCCCTTCTCGACTTCTTACCAACCACTTCGACTGGGCGTCTTCGAGAACGAGCTCAAGTGGTACCACAC 1500  
 I N T S V I Q K P A F L D F F T N H F D W A V F E N E L K W Y H T 90  
 GGAGGTGACGAGGGCCAGCTCAACTACGCCAGCCGACGCGCTGCTCGCGTCTTGCATCGCCTGGGCAAGACCGTCCGCGGCCACTGCGTCTTCTGG 1600  
 E V Q Q G Q L N Y A D A D A L L A F C D R L G K T V R G H C V F W 123  
 TCCGTGGAGGGCAGCTGACGAGTGGGTCAAGAACCTCAACAAGGACCAGCTCAGGTCCGCCATGCAGAGCCGCCCTCGAGGGCCTCGTCTCCCGTACG 1700  
 S V D G D V Q Q W V K N L N K D Q L R S A M Q S R L E G L V S R Y A 157  
 CCGGCAGGTTCAAGCACTACGACGTCACAACGAGATGCTGCACGGCCGCTTCTTCCGGGACCGCTCGGCGACGAGGACGTCGCCGGGTACATGTTCAA 1800  
 G R F K H Y D V N N N E M L H G R F F R D R L G D E D V P A Y M F K 190  
 GGAGGTGGCGGGCTGGACCCGGAGCCCGTGTCTTCTGCAACGACTACAACGTTGGAGTGGCGCAACGACCCCAACGCGACGCCGGAGAAGTACGCCGAG 1900  
 E V A R L D P E P V L F V N D Y N V E C G N D P N A T P E K Y A E 223  
 CAGGTGCATGGCTGCAGAGCTGCGGCGGGTGGTGGCGGCATCGGGCTGCGAGGGCCAGTGCAAAACCGGTCCGGGGAGGTGATCTGCGCCGCGCTCG 2000  
 Q V A W L Q S C G A V V R G I G L Q G H V Q N P V G E V I C A A L D 257  
 ACAGGCTCGCCAAGACGGGGTGCCTATGTTTCACCGAGCTCGACATGCCGAGTACGACGTGGGCTCCGCGCCAAGGACCTGGAGGTGGTGCCTCCG 2100  
 R L A K T G V P I W F T E L D M P E Y D V G L R A K D L E V V L R 290  
 GGAGGCTACGCGACCCGGCGTGGAGGGCATCGTGTCTTGGGCTTCAATGCAGGGCACAATGTGGCGCCAGAACGCTTGGCTCGTGCAGCCCGATGGC 2200  
 E A Y A H P A V E G I V F W G F M Q G T M W R Q N A W L V D A D G 323  
 ACCGTCAACGAGCGGGCCAGATGTTCTGAACTGCAGAAAGGATGGAAGACGCGCGGGGAACTTCGACGGCAGCGGAACTTCAAGTTCAGGG 2300  
 T V N E A G Q M F L N L Q K E W K T D A R G N F D G D G N F K F R G 357  
 GCTTCTACGGCAGATACGTCGTTGGAGGTTACGACGGCGAAGGGGAAGCAGATGCTCAAGACCTTACGGTGGAGAAAGGGACAACACACCTGTCGTCGT 2400  
 F Y G R Y V V E V T T A K G K Q M L K T F T V E K G D N T P V V V 390  
 GGATTTGGCTGACGCTGACGGTGAATCTATCTAAGAACTATTTATTTATACCTATCTAATTACATGCAACACGTCAGGGATAAATGGTTGTATAATT 2500  
 D L A D A 395  
 TTCATTTCTAAGTAAAGGGTATTGTATTTGTAAAGAAATGTATGGTATTTGTACTCCTAAATCTGATGAACATGATTGAAGCAAAAAGCCCTATTG 2600  
 GTCTTACTAGCAAAATATGCCCGTGGTGGCAACTTTATACAATAAATTTCTGCACTAGCAATAATTATAAGTCAATATCGTACATATGAACATCTCT 2700  
 ATTA 2704

positive clone, which carried the hybridizing sequence on a 2.9 kb *XbaI/SalI* fragment (Figure 6.5), was isolated and the *XbaI/SalI* fragment was subcloned into the pBluescript vector and designated p $\lambda$ 2BS. The restriction map of this fragment, together with its relationship to the p $\lambda$ 2E clone, is shown in Figure 6.2. Nucleotide sequence analyses of the p $\lambda$ 2E and p $\lambda$ 2BS clones demonstrated that their sequences were identical in the 720 bp region where they overlapped, and it was therefore concluded that they represented fragments of a single gene.

### 6.3.2 Characterization of genomic clones

The complete nucleotide sequence of a 2704 bp fragment which carries the gene encoding barley (1 $\rightarrow$ 4)- $\beta$ -xy lanase isoenzyme X-I is presented in Figure 6.4. Specific sequence motifs are compared with the corresponding plant consensus sequences in Table 6.1. The putative transcription start point (TSP) TCCATCA at nucleotide 468 (Figure 6.4) is so designated because of its close similarity to the CTCATCA consensus sequence defined for plant genes (Joshi, 1987 a) and because of its proximity to 5' sequences corresponding to a TATA box, a possible CAAT box and elements of a GA response complex (Table 6.1; Figure 6.4). The cDNA sequence of isoenzyme X-I described in Chapter 3 begins at nucleotide 907 of the gene and the ATG translation start point is found at nucleotide 1045 (Figure 6.4). Thus, the 5' untranslated region is more than 550 bp in length, which is considerably longer than is observed in most plant genes (Joshi, 1987 a). However, it remains possible that the 5' untranslated region includes introns (Hawkins, 1988) and potential 5' and 3' intron splice sites can be seen in this region (Figure 6.4).

The region of the gene that encodes the mature enzyme was found to consist of two exons interrupted by a short intron of 91 bp. The intron is located between nucleotides 1198 and 1288, it has boundary sequences very similar to the plant consensus sequences



**Figure 6.5** Phage  $\lambda$ 2BS DNA after cutting with *SalI* and *XbaI*. A, EtBr- stained agarose gel of restriction fragments. B, Southern blot analysis of membrane with the 720 *EcoRI/SalI* fragment (Figure 6.4).

Table 6.1: Sequence motifs in the barley (1→4)-β-xylan endohydrolase gene

Motif	Sequence	Position
GA Response Complex (i) pyrimidine box (ii) TAACAAA box (iii) TATCCAC box	CTCTTTC TAACGAC TATCCAT	-115 (353) -98 (370) -84 (384)
CAAT Box	CCAT CAAAT	consensus xylanase gene -75 to -80 -40 (428)
TATA Box	ACTATATATAG CTTTTAAATAC	consensus xylanase gene -25 to -39 -27 (431)
Transcription Start Point (TSP)	CTC <u>A</u> TCA TCC <u>A</u> TCA	consensus xylanase gene 1 1 (468)
Translation Start Point	TAAACA <u>ATGG</u> CT GAGATC <u>ATGG</u> GC	consensus xylanase gene 1st ATG 3' to TSP not 1st ATG 3' to TSP (1045)
Intron (i) 5' junction	AG↓gtaag AG↓gtgag	consensus xylanase gene (1198)
(ii) 3' junction	ttgag↓ gatgag↓	consensus xylanase gene (1288)
Polyadenylation Signal	AATAAA AATAAA	consensus xylanase gene (2643)

Consensus sequences for plant TATA boxes, TSPs and translation start points are from Joshi (1987 a) and for intron splice sites from Hanley and Schuler (1988). Numbers in parentheses show the actual position of the motif in the nucleotide sequence in Figure 6.4.

(Table 6.1) and it contains stop codons in all three reading frames (Figure 6.4). The G+C content of the intron is 51%. However, the region of the gene that encodes the mature (1→4)- $\beta$ -xylanase enzyme and its signal peptide shows an extreme bias towards the use of G and C in the wobble base position of codons (Figure 6.4). Of the 427 codons in the translated region of the gene, only 21 have A or T in the third nucleotide position. In the 3' untranslated region, a putative polyadenylation signal AATAAA is present at nucleotide 2643 (Figure 6.4).

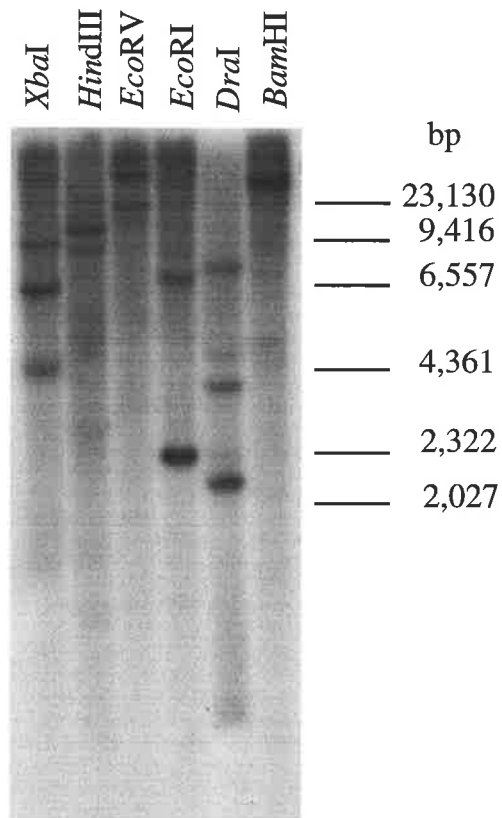
The sequence of the 30 NH<sub>2</sub>-terminal amino acids determined directly from highly purified (1→4)- $\beta$ -xylan endohydrolases (Slade *et al.*, 1989) exactly matches the amino acid sequence deduced from the gene in the region spanning nucleotides 1141-1321; the intron described above is inserted in this region of the gene (Figure 6.4). Alignment of the cDNA encoding isoenzyme X-I and the gene reveals an overall sequence identity of 99.5%; only 6 amino acid substitutions can be detected (Figure 6.6). The two (1→4)- $\beta$ -xylanase cDNA sequences which encode isoenzymes X-I and X-II (Chapter 3, Figure 3.10 and 3.11), showed considerably more sequence divergence (87% identity at the amino acid level, 91% at the nucleotide level). Furthermore the 5' and 3' untranslated sequences of the cDNA encoding isoenzyme X-I are essentially identical to the corresponding regions in the gene (Figure 3.10 cf. 6.4). It was concluded therefore that the isolated gene encodes barley (1→4)- $\beta$ -xylanase isoenzyme X-I.

### **6.3.3 Southern blot analysis and the barley (1→4)- $\beta$ -xylanase gene family**

Southern blots of barley genomic DNA probed with the barley (1→4)- $\beta$ -xylanase isoenzyme cDNA revealed the presence of at least three hybridizing DNA fragments in each restriction enzyme digest (Figure 6.7). When DNA preparations from six different barley cultivars (Clipper, Sahara, Galleon, Haruna Nijo, Chebec, Harrington) were subjected to Southern blot analysis, three or four DNA fragments again hybridized with

	↓			
MGAFRLRTEPRSAAVYVHGAPAGVDVKVMDLRVYPVDHKARFRQLKDKTDKARKRDVILK		28	X-I	cDNA
.....F.....K.....		28	X-I	gene
LGTPAGAGAGAAASVRVQLDNAFPFGTCINTSVIQKPAFLDFFT <del>N</del> HFDWAVFENELKWY		88	X-I	cDNA
.....		88	X-I	gene
HTEVQQGQLNYADADALLAFCDRLGKTVRGHCVFWSVDGDVQQWVKNLNKDQLRSSMQSR		148	X-I	cDNA
.....A....		148	X-I	gene
LEGLVSR <del>Y</del> AGRFKHYDVN <u>NE</u> MLHGRFFRDRLGDEDVPAYMFKEVARLDPEPVL <del>F</del> VNDYNV		208	X-I	cDNA
.....		208	X-I	gene
ECGNDPNATPEKYAEQVAWLQSCGAVVRGIGLQGHVQNPVGEVICAALDRLAKTGVPTWF		268	X-I	cDNA
.....I..		268	X-I	gene
<u>TE</u> LDVPEYDVGLRAKDLEVVLR <del>E</del> AYAHPAVEGIVFWGFMQGTMMWRQNAWLVDADGTVNEA		328	X-I	cDNA
...M.....		328	X-I	gene
GQMFLNLQKEWKT <del>D</del> ARGNFDGDGNFKFRGFYGRYVVEVTTAKRKQMLKTFTVEKGDNTPV		388	X-I	cDNA
.....G.....		388	X-I	gene
VVDLADA		395	X-I	cDNA
.....		395	X-I	gene

**Figure 6.6** Alignment of the complete amino acid sequence of barley (1→4)-β-xylanase isoenzyme X-I gene and isoenzyme X-I cDNA. The arrow indicates the NH<sub>2</sub>-terminal Val residue in the mature enzyme, dots indicate the identical residues and the doubly underlined Glu residues are the putative catalytic residues.



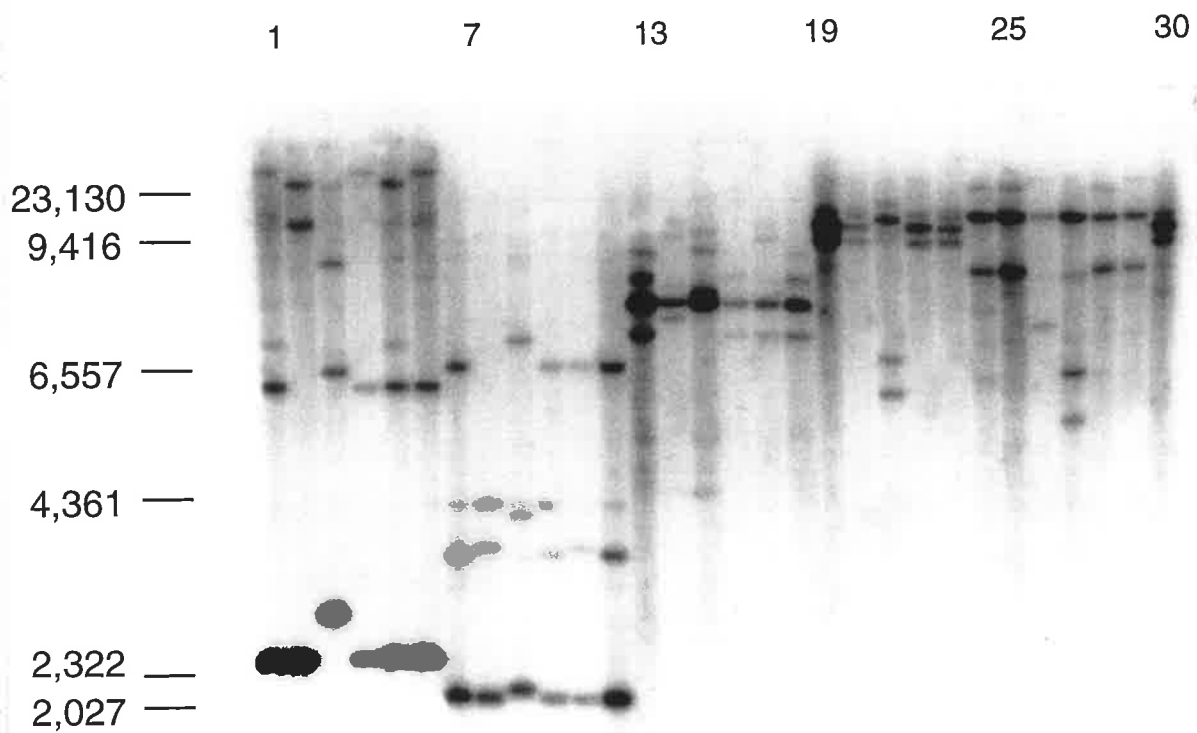
**Figure 6.7 Southern blots of barley genomic DNA.** DNA was cut with restriction enzymes *Xba*I (lane 1), *Hind*III (lane 2), *Eco*RV (lane 3), *Eco*RI (lane 4), *Dra*I (lane 5) and *Bam*HI (lane 6), and probed with the near full-length barley (1→4)- $\beta$ -xylanase cDNA isoenzyme X-I.

the (1→4)- $\beta$ -xylanase isoenzyme X-I cDNA probe (Figure 6.8), suggesting that barley (1→4)- $\beta$ -xylanases are encoded by a small gene family of at least three genes. In addition, considerable polymorphism was evident between the cultivars (Figure 6.8).

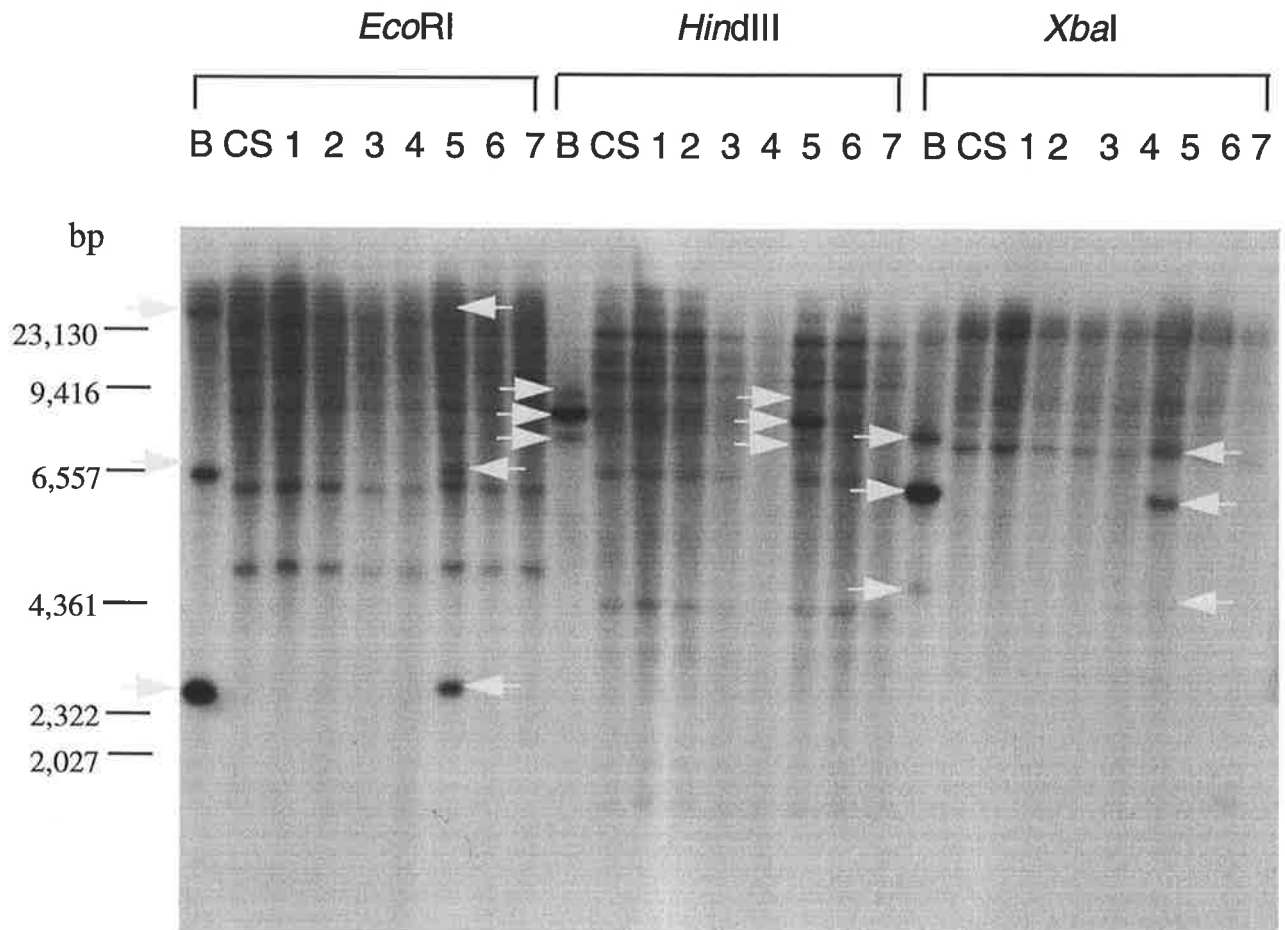
#### ***6.3.4 Chromosomal location of the (1→4)- $\beta$ -xylanase genes***

Southern blot analyses showed, wherever the bands were clearly visible, that the hybridizing DNA fragment of the parent barley DNA corresponded to the hybridizing band of addition line 5 (Figure 6.9). Three hybridizing bands (one intense and two weak) were observed. No corresponding bands were detected in any other addition line. From this result it can be concluded that the (1→4)- $\beta$ -xylanase genes are located on barley chromosome 7 (5H).





**Figure 6.8** Genomic Southern blot of DNA from 6 barley cultivars cut with *EcoR1* (lanes 1-6), *DraI* (lanes 7-12), *HindIII* (lanes 13-18), *BamHI* (lanes 19-24) and *EcoRV* (lanes 25-30). In each group of 6 digests the barley cultivars are, from left to right, Clipper, Sahara, Galleon, Harana Nijo, Chebec and Harrington.



**Figure 6.9** Genomic DNA from wheat-barley addition lines, and their wheat and barley parents were digested with *EcoRI*, *HindIII* and *XbaI* restriction enzymes. B and C represent barley (cv. Betzes) and wheat (cv. Chinese Spring), respectively; 1, 2, 3, 4, 5, 6, 7 represent wheat-barley addition lines 1, 2, 3, 4, 5, 6, 7, respectively. Arrows indicate location of barley (1→4)-β-xylanase gene in corresponding addition line.

## 6.4 DISCUSSION

In this study two overlapping genomic clones were isolated from barley gene libraries using the (1→4)- $\beta$ -xylanase isoenzyme X-1 cDNA as a probe. Comparisons between the nucleotide and amino acid sequences of the gene and the isoenzyme X-I cDNA sequence allowed the positive identification of the gene fragment as that encoding barley (1→4)- $\beta$ -D-xylanase isoenzyme X-I. The amino acid sequence of the thirty NH<sub>2</sub>-terminal residues from the previously purified protein (Slade *et al.*, 1989) are identical to the deduced amino acid sequence from the same region in the isolated gene. However, amino acid residue 11 of the mature enzyme is an Arg and although this residue is found in the gene (Figure 6.4), it is a Lys in the isoenzyme X-I cDNA (Figure 6.6). Similarly, the putative signal peptide encoded by the isoenzyme X-I gene differs from the cDNA signal peptide at the 16th amino acid position. In the gene sequence the amino acid is Phe whereas in the cDNA the corresponding amino acid is Tyr (Figure 6.5). Although the amino acid sequence of (1→4)- $\beta$ -D-xylanase isoenzyme X-I cDNA and the gene are similar, six conservative amino acid differences have been identified overall (Figure 6.6); the differences can be attributed to the different barley cultivars used to prepare the libraries.

The calculated molecular weight of the mature enzyme (without the signal peptide) from the deduced amino acid sequence of the isolated gene is 45,600, which is about 4,000 larger than the reported molecular weight of the purified protein as estimated by SDS-PAGE (Slade *et al.*, 1989). Possible reasons for this discrepancy are discussed in section 3.4. The sequences surrounding the translation start site (ATG) were found to be in reasonable agreement with the consensus sequence for the translation start site of plant genes (Joshi, 1987 a, Table 6.1).

In general, plant signal peptides are 20-40 amino acids in length and contain charged residues towards their NH<sub>2</sub>-terminus, followed by a hydrophobic core. Towards

the COOH-terminus, more hydrophilic residues are found. Small, uncharged amino acids are usually present in the cleavage site of the signal peptide; these residues are typically alanine, glycine or serine (Watson, 1984). No such cleavage site sequence was observed in the sequence deduced from the (1→4)-β-xylanase isoenzyme X-I gene, where an Arg residue is located next to the cleavage site (Figure 6.6). Barley (1→3,1→4)-β-glucanase isoenzymes EI and EII (Slakeski *et al.*, 1990; Litts *et al.*, 1990; Wolf, 1991) and (1→3)-β-glucanase (Wang *et al.*, 1992, Xu *et al.*, 1992) signal peptides contain a Ser residue in the corresponding site and exo-glucanase isoenzyme ExoII (Hrmova *et al.*, 1996) contains an Ala residue.

The open reading frame, which encodes the 32 amino acid signal peptide and a mature protein of 395 amino acids, is interrupted by an intron of 91 bp. The nucleotide sequences present at the intron/exon junctions are generally conserved in eukaryotic genes, with most intron sequences starting with GT at the 5' end and ending with AG at the 3' end (Brown 1988). The 5' plant splice junction consensus sequence is C/AAG↓gtaag and the 3' consensus sequence is ttgcag↓ [note that nucleotide sequences for introns are usually represented in lower case]. The gene for (1→4)-β-xylanase isoenzyme X-I has exon/intron boundary sequences which are in good agreement with the above-mentioned consensus sequences (Table 6.1). Furthermore, stop codons are present in all reading frames, which is also typical of plant introns. The G+C and A+T contents of the intron are 51% and 49%, respectively. Plant introns generally exhibit a higher A+T to G+C ratio (Slakeski *et al.*, 1990; Wolf, 1991; Litts *et al.*, 1990; Wang *et al.*, 1992; Xu *et al.*, 1992). The A+T rich sequence near the intron boundaries are reported to be required for effective splicing of adjacent exons. The higher A+T content is thought to be necessary for the efficient processing of plant pre-mRNA (Goodall and Filipowicz, 1989). In yeast, a consensus branch point sequence is required for the formation of a lariate intermediate in the excision of intervening sequences in pre-mRNA, and is located 18-40 bp upstream from the 3' splicing boundaries in yeast

(Brown, 1986; Pikielny *et al.*, 1983). Plant genes generally do not contain the highly conserved TACTAAC sequence found in yeast introns (Brown, 1986). The TACTAAC region of the yeast intron lies between bases -15 and -50 from the 3' splice junction (Brown 1986). However, a putative branch-point-like sequence ACGTAAC is located 33 bp from the 3' splicing zone of the intron (at nucleotide 1257, Figure 6.4) of the isolated barley (1→4)- $\beta$ -xylanase gene and an additional TAATAAC sequence is found another 35 (at nucleotide 1219) bp in the 5' direction (Figure 6.4). In (1→3,1→4)- $\beta$ -glucanase isoenzyme EI, the branch point is found in the same position (Slakeski *et al.*, 1990).

The intron observed in the isolated isoenzyme X-I gene is relatively short (91 bp). Similar short introns have been reported in the *RGA1* gene from rice (*Oryza sativa* L. IR-36), which encodes a G protein  $\alpha$  subunit (Seo *et al.*, 1995). In contrast, Brown (1986) has reported that higher plant genes contain very few short introns and almost two-thirds of known plant introns are between 100 and 2000 bp in length. Introns which are even larger than 2000 bp have been reported in (1→3,1→4)- $\beta$ -glucanases and (1→3)- $\beta$ -glucanases of barley, rice, and tobacco (Ohme-Takagi and Shinishi, 1990; Sperisen *et al.*, 1991; Slakeski *et al.*, 1990; Wolf, 1991; Simmons *et al.*, 1992; Wang *et al.*, 1992).

The promoter region of the isolated gene for isoenzyme X-I has a putative TATA sequence which shows some similarity to the plant consensus sequence; 6 residues out of 11 are identical (Table 6.1). An identical TATA box sequence has been reported in soybean heat shock protein genes (Joshi, 1987 a). In the rice *RGAI* gene, a divergent TATA (ATATGA) sequence is also observed (Seo *et al.*, 1996). Thus, considerable divergence from the consensus sequence is observed in plant TATA boxes and this is certainly the case for the (1→4)- $\beta$ -xylanase gene studied here. Unlike most other plant genes, the region between the TATA box and the transcription start site in the barley

(1→4)- $\beta$ -xyylanase gene is very GC-rich, as has been observed in the rice *PAL* (phenylalanine ammonia lyase) gene (Zhu *et al.*, 1995).

Nucleotide sequence analysis of the (1→4)- $\beta$ -xyylanase isoenzyme X-I gene in the coding region of the mature enzyme has an overall G+C content of 65% (Figure 6.4). This is mainly due to the extremely strong bias towards the use of G+C in the wobble base position of codons. Similar codon biases have been reported in the translated regions of genes or cDNAs encoding barley and wheat (1→3,1→4)- $\beta$ -glucanases (Slakeski *et al.*, 1990; Litts *et al.*, 1990; Wolf, 1991; Lai *et al.*, 1993) and barley (1→3)- $\beta$ -glucanases (Høj *et al.*, 1989; Wang *et al.*, 1992; Xu *et al.*, 1992; Qi, 1994). According to Murray *et al.* (1989) and Campbell and Gowri (1990), monocotyledonous genes generally exhibit a high G+C content in the degenerate third base of codons, whereas dicots exhibit a more balanced codon usage. In *E. coli* and yeast, codon bias is most extreme in highly expressed proteins. Biased codon usage in highly expressed genes is believed to enhance translation and may be required for maintaining mRNA stability (Murray *et al.*, 1989). In maize and wheat, the genes for ribulose 1,5 biphosphate (RuBPC) and chlorophyll a/b (CAB) almost completely avoid the use of A+T in the third position of codons, whereas in soybeans the codon usage pattern for the same genes is almost identical to the more balanced dicotyledonous pattern (Murray *et al.*, 1989). However, balanced codon usages have been reported in a barley carboxypeptidase cDNA (Doan and Fincher, 1988) and in a barley exo- $\beta$ -glucanase isoenzyme ExoII cDNA (Hrmova *et al.*, 1996).

For the isolated (1→4)- $\beta$ -xyylanase isoenzyme X-I gene, a putative polyadenylation signal AATAAA is located 222 bp downstream from the TGA stop codon (Figure 6.4). Several other potential polyadenylation signals can be identified. The barley (1→4)- $\beta$ -xyylanase isoenzyme X-I cDNA has no conserved AATAAA sequence, but this cDNA did not extend to the AATAAA sequence of the corresponding (1→4)- $\beta$ -xyylanase isoenzyme X-I gene. Whether this resulted from truncation of the cDNA during

synthesis, or whether alternative polyadenylation sites are present, remains to be demonstrated. Previous studies have demonstrated that polyadenylation signal sequences in plant genes are not as highly conserved as they are in animal genes (Joshi, 1987 b). Sequence comparisons of plant nuclear genes reveals that less than 50% of mRNAs have AAUAAA sequences near their poly(A) site (Joshi, 1987 b; Messing *et al.*, 1983; Wu *et al.*, 1995). In plant mRNAs, an additional *cis*-acting, GU-rich sequence element is required for efficient functioning of the poly(A) signal. The element is present upstream from the poly(A) signal and is much less conserved than the AAUAAA sequence. This upstream region has been found in all plant genes analyzed and is believed to be essential for 3'-end processing ( Hunt and MacDonald, 1989; Sanfacon *et al.*, 1991; Guerineau *et al.*, 1991; Mogen *et al.*, 1992; Wu *et al.*, 1993, 1994; Sanfacon, 1994). In cauliflower mosaic virus (CaMV), a direct repeat of UUUGUA is the sole sequence element essential for the polyadenylation site (Rothnie *et al.*, 1994). Similar TTTGTA sequences are seen upstream from the poly(A) site (at nucleotides 2531 and 2553) in the (1→4)- $\beta$ -xylanase isoenzyme X-I gene isolated here (Figure 6.4).

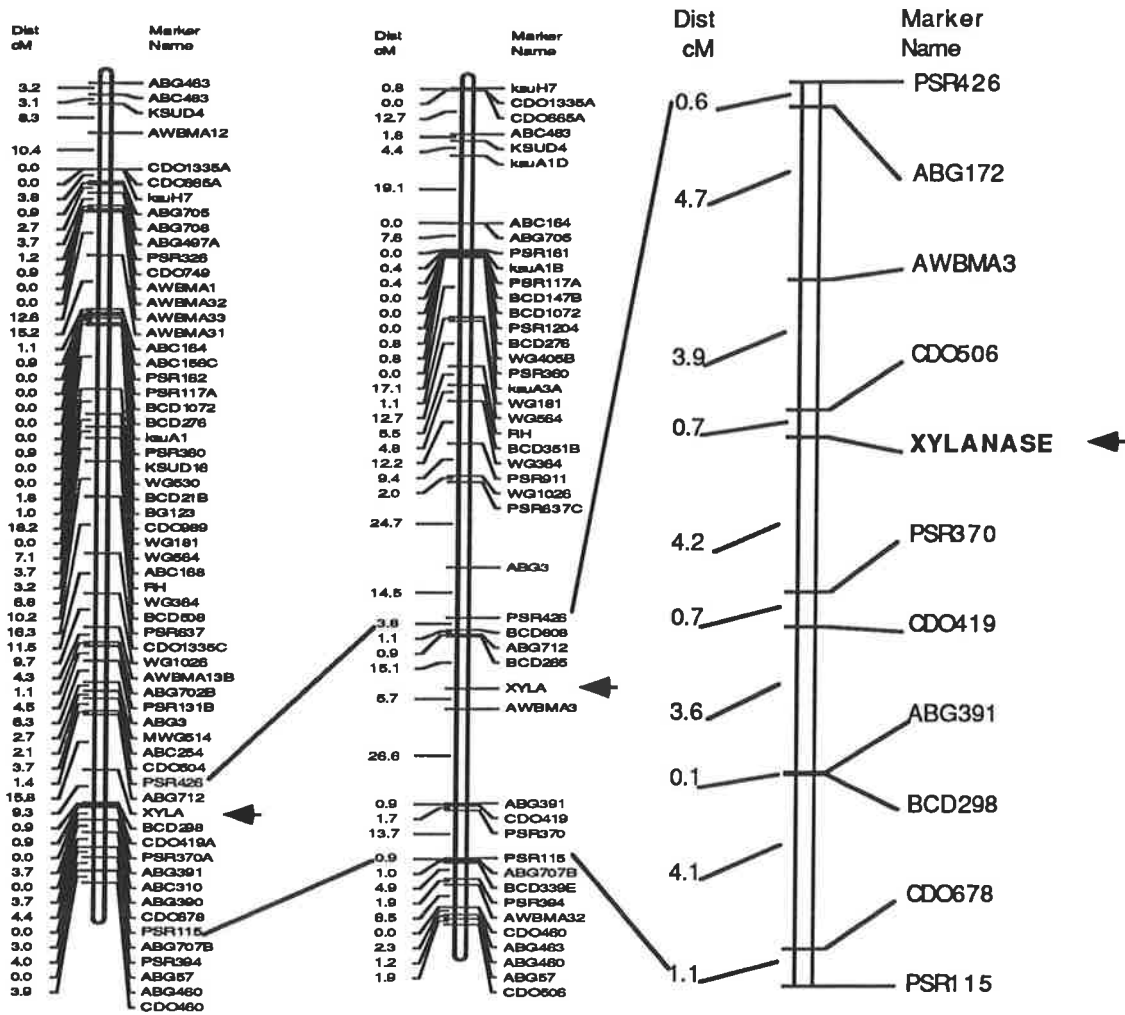
Thus, the barley (1→4)- $\beta$ -xylanase isoenzyme X-I gene contains sequence motifs for transcription, RNA processing, translation and protein processing that are typical of plant genes (Figure 6.4, Table 6.1). In addition, the promoter region has well-defined sequence elements that are very similar to components of the GA response complex that has been identified in cereal  $\alpha$ -amylase promoters in recent years (Skriver *et al.*, 1991; Rogers and Rogers, 1992; Sutcliff *et al.*, 1993; Huttly and Phillips, 1995; Gubler *et al.*, 1995). The GA response complex in the high pI barley  $\alpha$ -amylase promoter has at least three *cis*-acting elements, located in a region between -174 and -108 from the transcription start point. These elements include a pyrimidine box, a TAACAAA box and a TATCCAC box (Gubler and Jacobsen, 1992). More recent *in vitro* evidence suggests that a GA-regulated Myb protein transactivates the  $\alpha$ -amylase gene promoter through specific binding to the TAACAAA box (Gubler *et al.*, 1995).

The barley (1→4)-β-xylanase gene promoter studied here has similar *cis*-located sequences, arranged in the same order in the region -115 to -80, including a pyrimidine box, a TAACGAC box and a TATCCAT box (Figure 6.4). These components of the putative GA response complex are closer together than are the corresponding components in α-amylase promoters. The presence of a GA response complex is consistent with the GA induction of both (1→4)-β-xylanase gene transcription in isolated aleurone layers and the secretion of active enzyme by the layers (Chapter 5, Figures 5.6 B and 5.4 a). The barley (1→3,1→4)-β-glucanase isoenzyme EII gene promoter also has a pyrimidine box, a TAACAAC box and a TATCAC box (Wolf, 1991), but whether the subtle differences in hormonal responses observed with this gene result from the spacing of these elements, from minor sequence variations within the boxes, from additional *cis*-acting elements or from post-transcriptional effects, remains to be demonstrated.

Genomic Southern blot analyses suggest that (1→4)-β-xylan endohydrolases in barley are encoded by a family of at least three genes (Figure 6.7 and 6.8). In the present study a gene encoding (1→4)-β-xylanase isoenzyme X-I was isolated. No clones carrying the other two genes were found during extensive screening of two genomic libraries and, indeed, no other plant (1→4)-β-xylanase genes have been described. A Southern blot of wheat-barley addition lines indicates that all three genes for (1→4)-β-xylanases are located on barley chromosome 7 (5H) (Figure 6.9). Genomic Southern analysis of six different barley cultivars showed that (1→4)-β-xylanase gene polymorphism exists between the cultivars (Figure 6.8) and this has allowed the genes to be mapped (Banik *et al.*, 1996). Mr. Chen-Dao Li used the barley (1→4)-β-xylanase isoenzyme X-I cDNA isolated here to map the genes (Figure 6.10). The three barley (1→4)-β-xylanase genes are located on the long arm of chromosome 7 (5H), where they are so tightly clustered that no recombination is observed between them. The distribution of tightly-linked (1→4)-β-xylanase genes on barley chromosome 7 (5H) suggests that they have arisen by tandem duplication of a single ancestral gene. Several



**Figure 6.10** Map positions of the three (1→4)- $\beta$ -xylanase genes on barley chromosome 7 (5H) of the “Galleon” x “Haruna Nijo” and “Chebec” x “Harrington” DH populations. The consensus linkage map shows the position of the xylanase genes between the RFLP markers CDO506 and PSR370; the centomere is located on the CDO506 side of the genes. No recombination was detected between the three xylanase genes in 234 DH lines (Data of Mr. Chen-Dao Li).



GalleonxHaruna Nijo

ChebecxHarrington

Consensus Map

other gene families in barley are also organised as clusters in a limited region of a particular chromosome. The barley (1→3)- $\beta$ -glucanase gene family, which consists of seven members, is distributed along the long arm of chromosome 3 (3H) (Li *et al.*, 1996), while barley hordein genes are organized in a similar manner on chromosome 5 (1H) (Shewry *et al.*, 1990).

Of particular interest is the tight clustering of  $\alpha$ -amylase genes (*Amy1*) on chromosome 6 (6H) (Khursheed and Rogers, 1988; Takano and Takeda, 1987). Both the  $\alpha$ -amylase genes and the (1→4)- $\beta$ -xylanase genes studied here may represent an early stage in the evolution of gene families, where tandemly duplicated genes are still closely linked and dispersal along the chromosome by chromosome rearrangements or translocations to other chromosomes has not yet occurred. Recent duplication of (1→4)- $\beta$ -xylanase genes might also explain the relatively high level of sequence identity (91% at the nucleotide level) between the genes encoding isoenzymes X-I and X-II (Chapter 3). This level of sequence identity can be compared with values as low as 45% for seven members of the barley (1→3)- $\beta$ -glucanase gene family (Xu *et al.*, 1992), six of which are located in a region less than 20 cM in length on chromosome 3 (3H); the seventh gene lies on the same chromosome about 50 cM outside this cluster (Li *et al.*, 1996). A high level of sequence identity between the (1→4)- $\beta$ -xylanase genes might also present practical difficulties in obtaining gene-specific probes for future expression studies.

## **CHAPTER 7**

### **SUMMARY AND FUTURE DIRECTIONS**

### 7.1 Summary of work described here

The isolation, sequencing and characterization of two cDNAs encoding barley (1→4)- $\beta$ -xylanase isoenzymes X-I and X-II and the gene corresponding to isoenzyme X-I have been described in this thesis. The results of genomic Southern blot analyses indicated that the barley (1→4)- $\beta$ -xylanase gene family consists of at least 3 genes which have been mapped to a single locus on the long arm of chromosome 7 (5H). The cDNA was used to monitor tissue-specific expression, developmental regulation and hormonal control of the (1→4)- $\beta$ -xylanase genes. Expression, as measured by the appearance of (1→4)- $\beta$ -xylanase mRNA transcripts, was detected only in the aleurone layer of germinated grain. Expression could also be induced in isolated aleurone layers following treatment with the phytohormone GA. However, expression patterns of individual genes could not be defined because gene-specific probes were not available. Although heterologous expression of the barley isoenzyme X-I cDNA was achieved in *E. coli*; the expressed protein was precipitated into insoluble, inclusion bodies and no active enzyme could be recovered.

### 7.2 Potential short-term follow-up

A number of experiments could be performed in the short-term to expand on and to complete work described here. Of primary importance is the isolation of gene-specific probes for the detailed description of spatial and temporal factors which regulate the expression of the three individual (1→4)- $\beta$ -xylanase genes. Thus, cDNAs or genes encoding each isoenzyme need to be cloned, in the expectation that sequence differences might be detected in the genes or their flanking regions; these could be used as gene-specific probes. Experiments could also be conducted to identify *cis*- and *trans*-acting regulatory elements that mediate tissue specificity and the developmental and hormonal

control of expression of each gene. In particular, factors or gene sequences which are responsible for the relatively late expression of the (1→4)-β-xylanase genes in the aleurone layer of germinated grain might be pin-pointed by detailed promoter deletion analyses. Derived information on the mechanisms of gene regulation could be important not only for a thorough understanding of the physiology of cell wall degradation in germinated grain, but also could be central in future attempts to manipulate (1→4)-β-xylanase expression in barley which might be genetically engineered to improve its performance in commercial processes.

Further experiments could be designed to investigate more thoroughly the potential role of (1→4)-β-xylanases in plant-pathogen interactions. (1→4)-β-Xylanase production in plants is enhanced by pre-treating leaves with ethylene (Fuchs *et al.*, 1989; Avini *et al.*, 1994) which is generally believed to be a stress-related hormone. This leads to an increase in the accumulation of (1→4)-β-xylanase mRNA transcripts, (1→4)-β-xylanase activity and the concurrent expression of pathogenesis-related proteins (Fuchs *et al.*, 1989; Avini *et al.*, 1994; Bailey *et al.*, 1990, 1991, 1993, 1995; Lotan and Fluhr, 1990). In Chapter 5, it was observed that barley (1→4)-β-xylanase was not induced in barley leaves which were infected with the scald fungus, *Rhynchosporium secalis*, but other pathogens might induce (1→4)-β-xylanase expression and the expression of xylanase genes not detectable with the currently available cDNA probes can not yet be ruled out.

Additional studies could be initiated to express the (1→4)-β-xylanase protein in an active form in appropriate heterologous systems such as *E. coli*, yeast, or the insect cell/baculovirus system, using recently reported improvements in these expression systems. Co-expressing the proteins with the assistance of molecular chaperones might be required to obtain soluble, active protein. If such studies proved to be successful, the active, folded protein could be used in crystallization studies to resolve the three-dimensional structure of the enzyme. Such three-dimensional data could be used in conjunction with site-directed mutagenesis to elucidate substrate-binding mechanisms

and the molecular details of catalysis. It would also present opportunities for the engineering of increased stability into the enzyme, which could have commercial applications.

### 7.3 Industrial importance of (1→4)- $\beta$ -xylanases

(1→4)- $\beta$ -Xylanases and their substrates are central in many of the industrial processes described below and novel, highly purified (1→4)- $\beta$ -xylanases could provide manufacturers with useful additives which might improve process productivity or product quality. Additionally, properties of selected (1→4)- $\beta$ -xylanases might be tailored through genetic engineering to enhance their performance in industrial processes.

*Pulp and paper:* Microbial (1→4)- $\beta$ -xylanases are used in the pulp and paper industry. Cellulase-free xylanase preparations yield a range of desirable pulp characteristics by selectively hydrolyzing particular non-cellulosic polysaccharides in pulps and paper fibres (Viikari *et al.*, 1986; Sharma, 1987; Nissen *et al.*, 1992; Lavielle *et al.*, 1992; Buchert *et al.*, 1994). The use of (1→4)- $\beta$ -xylanase in pulp refining also decreases the energy demand for subsequent mechanical processes (Wong and Saddler, 1992). Incorporation of (1→4)- $\beta$ -xylanase in pre-bleaching processes allows the use of lower chlorine charges during the bleaching of kraft pulp. As a result, environmental problems associated with chlorine discharge are diminished (Senior *et al.*, 1992; Viikari *et al.*, 1991; Milagres and Duran, 1992). In the paper and pulp industry it is essential that these xylanase preparations are free of contaminating cellulase activity, which could result in costly damage to the cellulose fibres.

*Livestock nutrition:* Arabinoxylans are responsible for anti-nutritional effects in cereal-based stockfeeds for monogastric animals because they limit access of digestive enzymes

to nutrients in the digestive tract and can also slow the uptake of digestion products (MacGregor and Fincher, 1993). The addition of xylanases to poultry diets improves their nutritive value and as a consequence improves the growth performance of broilers (Paridon *et al.*, 1992; Bedford and Classen, 1992).

*Food processing:* In the food processing industries, (1→4)- $\beta$ -xylanases find applications in the clarification of fruit juices (Biely, 1985) and in the preparation of dextrans which are used as food thickeners (Biely *et al.*, 1980). (1→4)- $\beta$ -Xylanases are also utilized by the baking industry (Maat *et al.*, 1992). Although treatment of wheat dough with excessive (1→4)- $\beta$ -xylanase results in a rapid loss of dough strength and the production of a wet, sticky dough mass (McCleary, 1992), the judicious use of (1→4)- $\beta$ -xylanases in bread-making mixtures can lead to improved qualities characteristics such as increased loaf volume or a more desirable crust colour (McCleary, 1992). Clearly the large-scale production of purified xylanases is a desirable prerequisite for these applications.

*(1→4)- $\beta$ -Xylanases in malting and brewing:* Barley (1→3,1→4)- $\beta$ -glucans are widely recognized as undesirable in brewing processes, because highly viscous solutions interfere with filtration steps and because the (1→3,1→4)- $\beta$ -glucans can form precipitates in the final beer product (MacGregor and Fincher, 1993). However, the physiochemical properties of arabinoxylans are similar to those of (1→3,1→4)- $\beta$ -glucans and problems normally attributed to (1→3,1→4)- $\beta$ -glucans might also be caused by arabinoxylans. Hitherto, most work has been focused on (1→3,1→4)- $\beta$ -glucans; the arabinoxylans have received relatively little attention (Coote and Kirsop, 1976; MacGregor and Fincher, 1993).

The potential importance of arabinoxylans during malting and brewing has recently been reported by Schwarz and Han (1995), who found that arabinoxylan contents in several commercial beers ranged from 514  $\mu\text{g/ml}$  to 4,211  $\mu\text{g/ml}$ ; (1→3,1→4)- $\beta$ -glucan



levels ranged from 0.3  $\mu\text{g/ml}$  to 247  $\mu\text{g/ml}$ . Arabinoxylans accounted for 70% of non-starchy polysaccharides in pilot-brewed beer and for up to 10% of the total carbohydrates in beer (Schwarz and Han, 1995). Viëtor *et al.* (1991) have also reported that a substantial amount of arabinoxylan is present in malt wort and Han and Schwarz (1996) showed that (1 $\rightarrow$ 4)- $\beta$ -xylanase levels are positively correlated with malt extract values.

In the light of these results, it is surprising that very little attention has been paid to the role of (1 $\rightarrow$ 4)- $\beta$ -xylanases in the malting and brewing processes. Arabinoxylan-degrading enzymes could play an important role in decreasing filtration difficulties in malting and brewing. Further experiments could be undertaken to genetically manipulate barley varieties or lines to enhance (1 $\rightarrow$ 4)- $\beta$ -xylanase expression.

#### **7.4 Improving (1 $\rightarrow$ 4)- $\beta$ -xylanases for commercial processes**

An important prerequisite for the use of (1 $\rightarrow$ 4)- $\beta$ -xylanases in industry is their ability to remain active at elevated temperatures. For example, malt extracts are incubated at temperatures of about 65°C during the initial enzymic degradation of starch in the brewing process. However, Slade *et al.*, (1989) showed that barley (1 $\rightarrow$ 4)- $\beta$ -xylanases are extremely unstable. The production of more stable isoforms of barley (1 $\rightarrow$ 4)- $\beta$ -xylanases could be beneficial for a range of biotechnological applications not only in the malting and brewing industries, but also in the pulp and paper, food-processing and stockfeed industries.

Stable isoforms could be produced through the use of site-directed mutagenesis by introducing additional glycosylation sites, or by altering key amino acids (Vieille and Zeikus, 1996). Doan and Fincher (1992) have successfully increased the thermostability of barley (1 $\rightarrow$ 3,1 $\rightarrow$ 4)- $\beta$ -glucanase isoenzyme EI by the introduction of an additional glycosylation site. In the malting and brewing industries it might also be an advantage to genetically engineer increased thermostability into barley (1 $\rightarrow$ 4)- $\beta$ -xylanases, but this

would probably need to be linked with earlier and higher levels of expression of the genes.

Manipulation of the *cis*-acting elements within the 5' regions of barley (1→4)- $\beta$ -xylanase genes might be used to enhance levels of gene expression. In the present study it was observed that induction of (1→4)- $\beta$ -xylanase expression occurred one day later than that of both (1→3,1→4)- $\beta$ -glucanase and  $\alpha$ -amylase. To accelerate arabinoxylan degradation during endosperm mobilization, the entire promoter region of the barley (1→4)- $\beta$ -xylanase could be replaced by a germination-specific promoter such as the  $\alpha$ -amylase promoter, which is activated earlier than endogenous (1→4)- $\beta$ -xylanase and to relatively higher levels. In addition, to obtain higher expression rates for endogenous (1→4)- $\beta$ -xylanases, the copy number of the genes could be increased.

Any attempts to manipulate barley (1→4)- $\beta$ -xylanases will be dependent on the stable integration of genetically engineered genes into the barley genome. A major prerequisite for this is the availability of routine barley transformation protocols. Recent successes in the production of transgenic barley has opened up the possibility for the stable integration of engineered genes into barley, with the aim of producing transformed barley plants for incorporation into breeding programs. The most successful approach for the production of transgenic barley so far is particle bombardment (Jähne *et al.*, 1994; Ritala *et al.*, 1994; Wan and Lemaux, 1994; Hagio *et al.*, 1995). Fertile transgenic barley plants have also been generated by direct DNA transfer to protoplasts (Jähne *et al.*, 1991; Golds *et al.*, 1994; Kihara and Funatsuki, 1994; Funatsuki *et al.*, 1995; Salmenkallio-Marttila *et al.*, 1995) and very recent successes with a virulent strain of *Agrobacterium tumefaciens* offer possibilities for the future (Dr. R. Brettell, pers. commun.). Thus, Jensen *et al.* (1996) have introduced a genetically engineered, thermostable bacterial (1→3,1→4)- $\beta$ -glucanase into barley and it can be confidently predicted that, in the future, genetically engineered (1→4)- $\beta$ -xylanase genes encoding enzymes with enhanced performance characteristics could also be reintroduced into

barley if the industry considered such material useful. The isolated (1→4)-β-xylanase genes described in this thesis represent a source of DNA for the manipulation of both coding regions and promoters for these future experiments.

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## **APPENDICES**

## **Appendix 1**

### **Solutions**

#### **50 x Denhardt's solution**

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

#### **20 x SSC**

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

#### **20 x SSPE**

3 M NaCl, 0.2 M NaH<sub>2</sub>PO<sub>4</sub>, 20 mM EDTA

#### **SM**

10 mM NaCl, 8 mM MgSO<sub>4</sub>, 50 mM Tris-HCl (pH 7.5), 0.01% gelatin

#### **10 x TAE**

0.4 M Tris-HCl (pH 8.0), 1.15% (v/v) glacial acetic acid, 10 mM EDTA

#### **10 x TBE**

10 x 0.89 M Tris-HCl (pH 8.0), 0.89 M boric acid, 20 mM EDTA

#### **10 x TE**

0.1 M Tris-HCl (pH 8.0), 10 mM EDTA

## **Appendix 2**

### **Nutrient Media**

#### **LB media**

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

#### **Miller's Broth**

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

#### **2 x YT medium**

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

#### **NZY medium**

0.5% (w/v) Bacto-yeast extract, 0.2%  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.5% NaCl, 1% NZ amine (casein hydrolysate)