MOLECULAR CLONING AND CHARACTERIZATION OF BARLEY β-GLUCANASE GENES

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A thesis submitted in fulfilment of the requirements for the degree of Master of Agricultural Science.

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ABBREVIATIONS

ABA	abscisic acid
Amp	ampicillin
ATP	adenosine 5'-triphosphate
bp	base pair
BSA	bovine serum albumin
CAT	chloramphenicol acetyltransferase
cDNA	complementary DNA
dCTP	2'-deoxycytidine 5'-triphosphate
DEPC	diethyl pyrocarbonate
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleoside triphosphates
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
GA	gibberellic acid
GUS	β-glucuronidase
IPTG	isopropylthio-β-D-galactoside
kb	kilobases
kDa	kilodaltons
mRNA	messenger RNA
PCR	polymerase chain reaction
PEG	polyethylene glycol
pI	isoelectric point
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SSC	sodium saline citrate
SSPE	sodium saline phosphate EDTA
Tris	tris(hydroxymethyl)aminomethane
TSP	transcription start point
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactoside

SUMMARY

Barley (1→3)-β-glucanases (EC 3.2.1.39) and (1→3,1→4)-β-glucanases (EC 3.2.1.73) are β-glucan endohydrolases encoded by a single super gene family. They share 44%-80% positional identities at the nucleotide sequence level. In this work, genes encoding (1→3)-β-glucanase isoenzyme GI and (1→3,1→4)-β-glucanase isoenzyme EII have been isolated from a barley genomic library with cDNA probes and specifically designed oligonucleotides.

The nucleotide sequence of a 3,327 bp genomic fragment for the (1→3)-β-glucanase isoenzyme GI gene has been determined. This DNA fragment encodes a mature protein of 310 amino acid residues. The molecular weight of the protein, deduced from the DNA sequence, is consistent with that of the purified enzyme reported previously. Nucleotide sequence analysis of the gene indicates that no targeting signals are present and that the initiating Met residue is removed from the precursor protein during post-translational processing. These observations suggest that the isoenzyme GI is a cytosolic enzyme. The mature enzyme-coding region of the gene has a high G+C content (68.9%) that is commonly found in other barley genes, and an extreme bias towards the use of G and C (99.4%) in the third position of codons. No intron-like sequence is detected in the 5' flanking region or coding region of the isoenzyme GI gene. Nucleotide sequence analysis of the promoter region of the isoenzyme GI gene reveals several *cis*-acting elements related to tissue-specific, hormonal and developmental regulation, which may be characterized by promoter deletion analysis in the future.

The genomic clone for the (1→3,1→4)-β-glucanase isoenzyme EII has been positively identified by the sequence of a 300 bp region that corresponds to the COOH-terminal encoding region of the mature enzyme and the 3' untranslated region of a previously characterized cDNA. Soon after the genomic clone for the barley (1→3,1→4)-β-glucanase was isolated in the present work, the full sequence of the gene was published elsewhere. Therefore, the complete sequence analysis of the gene was

abandoned in favour of characterizing the gene encoding the (1→3)-β-glucanase isoenzyme GI.

It is clear that (1→3,1→4)-β-glucanases function as hydrolytic enzymes during the degradation of cell walls that obstruct translocation of other hydrolytic enzymes during germination. The expression of the (1→3,1→4)-β-glucanase genes has been shown by other workers to be regulated in a tissue-specific manner, and in response to phytohormones. Expression of the isoenzyme EII gene is germination-specific, and is only detected in the aleurone of germinated grain. The expression of the isoenzyme EII gene is enhanced by gibberellic acid (GA_3) and suppressed by abscisic acid (ABA). The isolation of a genomic clone for the isoenzyme EII now allows promoter deletion analyses to be undertaken in order to define the sequences representing regulatory *cis*-elements that are responsible for the regulation of gene expression.

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CHAPTER ONE

GENERAL INTRODUCTION

In the germinating barley (*Hordeum vulgare* L.) grain, a variety of hydrolytic enzymes is synthesized in the aleurone and scutellar epithelium and subsequently translocated into the starchy endosperm, where they depolymerise cell walls, starch, storage proteins and residual nucleic acids into molecular products that are employed as a nutrient source to support the growth and development of the seedling. Among these hydrolytic enzymes, two classes of β-glucan endohydrolases, (1→3)-β-glucanases and (1→3,1→4)-β-glucanases, have been shown to perform very important roles in the process of germination, and the genes encoding those enzymes are the subject of this study. In this chapter, available information on the morphology and composition of barley grain and the involvement of hydrolytic enzymes in germination are reviewed as a basis for understanding the structural and physiological changes which occur after germination is initiated.

1.1 ANATOMY AND COMPOSITION OF THE BARLEY GRAIN

The mature barley grain consists of two major anatomical components; the embryo and the endosperm (Figure 1.1; Briggs, 1973). These are surrounded by the outer layers of the grain, which include the testa and palea, or husk (Briggs, 1978; 1983). The endosperm includes two distinct tissues; the starchy endosperm and the peripheral aleurone layer that surrounds the starchy endosperm (Briggs, 1973, 1978). The scutellum is a part of the embryo and is located between the embryonic axis and the starchy endosperm.

1.1.1 Embryo and Scutellum

The embryo, derived from the fertilized ovum, is a diploid tissue and is situated at the basal end of the grain (Cass & Jensen, 1970; Briggs, 1978). It comprises the apical meristem at one end, which is sheathed by the coleoptile; and the primary rootlet at the other end, which is surrounded by the coleorhiza (Briggs, 1978). The embryo is well stocked with molecules such as proteins and sugars to support its initial metabolic activities and growth (Briggs, 1978, 1992), but relies on the translocation of the

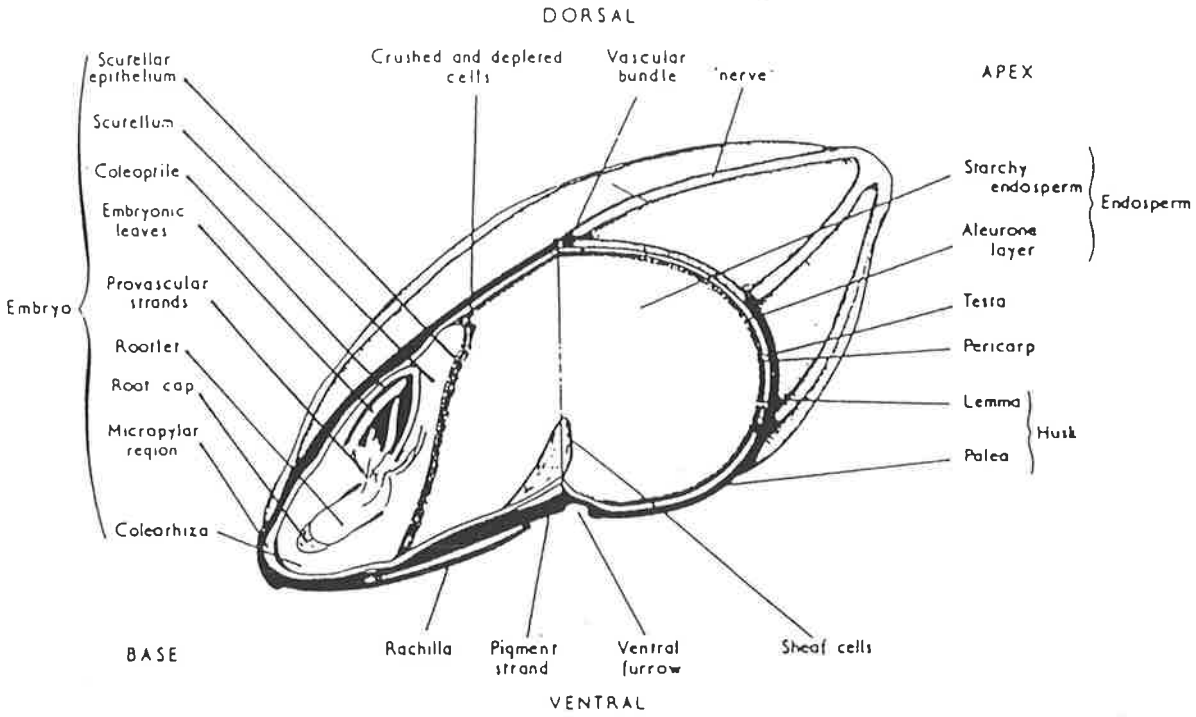


Fig 1.1 A Schematic Drawing of A Barley Grain (from Briggs, 1978).

nutrients from the starchy endosperm for growth over the longer term.

The scutellum is another major component of the embryo (Briggs, 1978). It is composed of thin-walled parenchyma cells, but at the interface of the scutellum and the starchy endosperm a single layer of cells known as the scutellar epithelium is present (Briggs, 1978). Scutellar epithelial cells in quiescent grains contain a central nucleus, mitochondria, ribosomes and some endoplasmic reticulum (Nieuwdorp, 1963; Nieuwdorp & Buys, 1964; Swift & O'Brien, 1972a), and are packed with numerous protein bodies, which are surrounded by lipid bodies and contain phytin inclusions (Smart & O'Brien, 1979a; Vance & Huang, 1988). These inclusion bodies of phytin serve as a source of phosphate during germination (Nieuwdorp, 1963; Swift & O'Brien, 1972a; Tanaka *et al.*, 1976; Aisien *et al.*, 1986; Vance & Huang, 1988). Walls of the scutellar epithelial cells feature two distinct layers that are distinguishable morphologically, particularly in the early stages of germination (Swift and O'Brien, 1972b). Histochemical studies showed that the cell walls of the scutellum consist of heteroxylans, protein and ferulic acid (Smart & O'Brien, 1979b, 1979c). No lignin and pectin are detected in the cell walls (Smart & O'Brien, 1979b), but (1→3,1→4)-β-glucans are almost certainly present (Fincher, 1992).

1.1.2 Endosperm

The aleurone and the starchy endosperm arise from the differentiation of primary endosperm cells during grain development, but only the cells of the aleurone layer are living after endosperm maturation (Cass & Jensen, 1970; Briggs, 1978). The aleurone tissue is typically three to four cells in thickness, except at the interface between the scutellum and the starchy endosperm, where it narrows to a single layer of thick-walled, somewhat flattened cells, designated "germ aleurone" (Briggs, 1978, 1987; Knudsen & Müller, 1991). Mature aleurone cells accommodate nuclei, mitochondria and endoplasmic reticulum, and are packed with specialized protein bodies, known as aleurone grains. The aleurone grain is surrounded by lipid droplets, or spherosomes (Jones, 1969; Buttrose, 1971; Morrison *et al.*, 1975). Protein deposits

in aleurone cells are relatively rich in basic amino acids (Bacic & Stone, 1981a). Two types of inclusion can be observed in the protein matrix of aleurone grains: phytin globoids (Type I inclusions) and niacytin particles (Type II inclusions) (Morrison *et al.*, 1975; Bechtel & Pomeranz, 1981; Peterson *et al.*, 1985). Phytin globoids represent the major storage form of phosphate in aleurone cells and are composed of the potassium and magnesium salts of myo-inositol hexaphosphate (Stewart *et al.*, 1988). Niacytin is a protein-carbohydrate complex containing bound niacin, o-aminophenol, protein and carbohydrate (Van der Eb & Nieuwdorp, 1967; Jones, 1969; Jacobsen *et al.*, 1971). Thus, the aleurone with its stored amino acids, lipids, phosphate, carbohydrates and vitamins serves as the source of nutrients necessary for the initiation of germination and the subsequent rapid synthesis and secretion of hydrolytic enzymes into the starchy endosperm (Fincher, 1989).

The aleurone cell walls are primary walls that consist of a relatively thin inner layer (0.4 μm) and a thicker outer layer (2-2.5 μm) (Taiz & Jones, 1970; Bacic & Stone, 1981b). The major polysaccharide components of the aleurone cell walls include arabinoxylan (67%), (1→3,1→4)-β-glucan (26%), and small amounts of cellulose and glucomannan (2%) (Bacic & Stone, 1981b). Other minor components include protein and ferulic acid (Bacic & Stone, 1981b). Small deposits of (1→3)-β-glucan that are scattered throughout the endosperm are concentrated in the subaleurone endosperm region and are associated with the inner walls of the endosperm cells (Fulcher *et al.*, 1977; Bacic & Stone, 1981b; MacGregor *et al.*, 1989).

The starchy endosperm, the major reserve tissue of whole barley grain (about 75% by volume), is formed following the fusion of the male nucleus with two polar nuclei in the embryo sac and is therefore triploid in nature (Briggs, 1978). The starchy endosperm cells are non-living, although remnants of nuclei, ribosomes and endoplasmic reticulum are present (Bechtel & Pomeranz, 1981; McFadden *et al.*, 1988). The cells of the starchy endosperm are packed with starch granules, which are embedded in a storage protein matrix (Briggs, 1978). Starch is the chief component of

Table 1.1 Composition of Polysaccharide Components of Barley Cell Walls

Tissue	Major polysaccharide components
Mature Aleurone[1]	70% arabinoxylan
	25% (1→3,1→4)-β-glucan
	2% cellulose
	2% glucomannan
	1% (1→3)-β-glucan
Mature Starchy Endosperm[2]	75% (1→3,1→4)-β-glucan
	20% arabinoxylan
	2% cellulose
	2% glucomannan

Values taken from:
1. Bacic & Stone, 1981a, b.
2. Fincher, 1975, 1976; Ballance & Manners, 1978.

the grain (58-65% by weight). Starch granules are composed predominantly of amylopectin, the branched (1→4,1→6)-α-glucan (75%) and of amylose, the linear (1→4)-α-glucan (25%) (MacGregor & Fincher, 1992). Small amounts of lipids occur within the granules and appear to contribute to their structural characteristics (Morrison, 1978; MacGregor & Fincher, 1992).

The prolamines are the alcohol-soluble proteins that are the most abundant storage proteins in cereal grains such as wheat, rye, barley and maize (Shewry & Miflin, 1985) and are known as hordeins in barley grain. They are rich in Asn, Gln and Pro, and contain repeated amino acid sequences ranging from 7 to 20 residues (Pernolet & Mosse, 1983; Brandt *et al.*, 1985; Heidecker & Messing, 1986; Kreis *et al.*, 1985). Water-soluble proteins and salt-soluble proteins are also present (Giese & Hejgaard, 1984), and comprise 20-40% of total endosperm nitrogen in barley grain.

The cell walls of the starchy endosperm are approximately 2 μm in thickness and are composed of mixed-linkage (1→3,1→4)-β-glucan, arabinoxylan, cellulose and glucomannan (Table 1.1; MacLeod & Napier, 1959; Fincher, 1975; Thompson & La Berge, 1977). The cell walls show no secondary thickening, and are unlignified (Fincher, 1975). Small amounts of protein and phenolic acids, especially ferulic acid, are also present (Fincher, 1975, 1976; Ballance & Manners, 1976; Thompson & La Berge, 1977).

1.2 GERMINATION

The germination of grain plays a central role in the life cycle of the barley plant. It involves the synthesis of hydrolytic enzymes in the aleurone and scutellum, the catabolism of nutrient reserves in the starchy endosperm, and is accompanied by major morphological and biochemical changes within the grain (Fincher, 1989). The reserves used to support the process are drawn initially from the embryo, and later from the aleurone and the starchy endosperm. Components of the starchy endosperm are degraded to yield soluble products including oligosaccharides, sugars, peptides and

amino acids, which diffuse to the embryo and are taken up by the scutellum. The aleurone appears to be the principal source of hydrolytic enzymes in germinating barley. The scutellum is also involved in enzyme secretion during the early stages of germination (McFadden *et al.*, 1988) and some hydrolytic enzymes pre-exist in the starchy endosperm, either in an active or zymogen form. Germination starts with endosperm dissolution in the region next to the scutellum and the degradation progresses towards the distal end of the grain in a front that is approximately parallel to the face of the scutellum (Gibbons, 1981; MacGregor & Matsuo, 1982). This pattern results from the spatially and temporally coordinated secretion of hydrolases from the scutellum and the aleurone, which is effected by gibberellic acid (GA) and calcium ions (Deikman & Jones, 1986; Jacobsen & Chandler, 1987; Fincher, 1989; 1992).

1.2.1 Hormone Action

In the early stages of germination, a diffusible factor identified as gibberellic acid (GA_3) is released from the embryo, resulting in the synthesis of hydrolytic enzymes by the aleurone and their secretion into the starchy endosperm (Paleg, 1960; Yomo, 1960; Jacobsen, 1983). The role of GA_3 in the process is supported by the observation that the hormone enhances *in vitro* secretion of α-amylase, β-glucanase and other hydrolases from isolated barley aleurone cells (Paleg, 1960; Yomo, 1960; Chrispeels & Varner, 1967; Stuart *et al.*, 1986). It remains uncertain whether the diffusing factors from the embryo are exclusively GA_3 (Fincher, 1989). The embryonic axis (Paleg, 1960; Yomo, 1960; MacLeod & Palmer, 1966, 1967) and the scutellum (Radley, 1967; Briggs, 1972) have been proposed as sites for GA_3 synthesis. It has been shown that the aleurone responds to other forms of GA (Radley, 1967; Atzorn & Weiler, 1983; Gilmour & MacMillan, 1984), but it is not clear that these variants originate from different tissues or are responsible for separate functions. Recent studies of regulation of gene expression using barley aleurone layers have revealed an interesting variation of responses of different genes to GA_3. The induction of mRNA for α-amylase (Chandler

et al., 1984; Rogers, 1985), (1→3,1→4)-β-glucanase (Mundy & Fincher, 1986) and a putative thiol protease (Rogers *et al.*, 1985) indicated that gibberellic acid exerts its control at the level of transcription.

It is suggested that receptors of GA might be located in the plasma membrane, where they mediate hormone action in a mechanism similar to that observed in mammalian cells (Hooley *et al.*, 1991). Alternatively, the putative receptor protein of gibberellic acid may be cytosolic and the GA-receptor complex may also be translocated to the nucleus where it might bind to DNA sequences flanking hormone responsive genes, causing their transcription (Fincher, 1989; Jones and Jacobsen 1991).

Abscisic acid (ABA), another important phytohormone that may control the maturation of the embryo and inhibit germination in immature grain, accumulates in the embryo and the endosperm of developing grain and decreases as the grain dries out (King, 1976; Goldbach & Michael, 1977). The hormone prevents elongation of apical meristems, maintains dormancy in buds and in grains, promotes stomatal closure, root growth, fruit ripening and senescence (Addicott & Carns, 1983). When isolated aleurone layers from barley grain are treated with ABA, the hormone suppresses the expression of genes specifically involved in germination which are expressed in the presence of GA, and may enhance the expression of several "ABA-specific" genes (Williamson & Quatrano, 1988). It has been suggested that ABA regulation is also at the transcription level (Jacobsen & Beach, 1985; Nolan & Ho, 1988). In barley aleurone, ABA reverses all GA-promoted changes, whether increases or decreases in protein synthesis or mRNA transcription. In addition, ABA prevents the suppression of total transcripts and ribosomal RNA synthesis by GA_3 (Jacobsen & Beach, 1985).

1.2.2 Enzyme Synthesis and Secretion

Two groups of hydrolytic enzymes are believed to be associated with barley grain germination: enzymes involved in internal aleurone and scutellar reserve mobilization, and those secreted for the mobilization of starchy endosperm reserves (Fincher, 1989).

In the early stages of germination, enzymes responsible for the mobilization of aleurone and scutellar reserves are synthesized or released. It is now clear that both the scutellum and the aleurone participate in starchy endosperm mobilization in barley, but as germination proceeds, the aleurone layer becomes the principal source of hydrolytic enzymes (Gibbons, 1981; McFadden *et al.*, 1988; Fincher, 1989).

Scutellum Function

In the scutellar epithelium of germinating grain, protein bodies and associated phytin inclusions are rapidly mobilized and their remnants coalesce into large vacuoles (Smart & O'Brien, 1979a). Lipid bodies slowly disappear (Fernandez *et al.*, 1988), mitochondria become metabolically active, and a marked development of endoplasmic reticulum and Golgi is observed (Swift & O'Brien, 1972a, 1972b; Nieuwdorp & Buys, 1964; Okamoto *et al.*, 1980; Gram, 1982; Aisien *et al.*, 1986). Unlike aleurone cells, both the parenchyma and epithelial cells of the scutellum accumulate starch granules early in the germination process (Nieuwdorp & Buys, 1964; Swift & O'Brien, 1972b; Aisien *et al.*, 1986).

The scutellar epithelium performs a dual role. In the early stages of germination, it synthesizes and secretes hydrolytic enzymes (Gibbons, 1979; 1981; Ranki *et al.*, 1983; Ranki & Sopanen, 1984; MacGregor *et al.*, 1984; Stuart *et al.*, 1986; McFadden *et al.*, 1988). Its second function is the absorption of endosperm degradation products and their translocation to the developing seedling (MacLeod & Palmer, 1966; Briggs, 1973; Gram, 1982). Hydrolysis products of reserve proteins in the starchy endosperm are taken up by the scutellum either as amino acids or as di- and tri- peptides (Sopanen *et al.*, 1978). The peptides are hydrolysed into amino acids by aminopeptidases inside the scutellum before their transport via the vascular strand to the embryonic axis (Mikola & Kolehmainen, 1972; Sopanen *et al.*, 1978; Walker-Smith & Payne, 1983).

There is good evidence that α-amylase is synthesized in the scutellar epithelium and secreted into the starchy endosperm (Briggs, 1973; Stuart *et al.*, 1986; Ranki, 1990). Similarly, evidence obtained from hybridization histochemistry in germinating

barley grain has demonstrated that expression of (1→3,1→4)-β-glucanase genes is first observed in the scutellar epithelium (MacFadden *et al.*, 1988) one day after the initiation of germination. This tissue is likely to be the major source of (1→3,1→4)-β-glucanase isoenzyme EI in the grain. Expression of (1→3,1→4)-β-glucanase genes in the aleurone is not detected until two days after the initiation of germination, and at this stage the level of expression is decreasing in the scutellum. Induction of (1→3,1→4)-β-glucanase in the aleurone layer progresses from the proximal to the distal end of the grain as a front moving away from and parallel to the face of the scutellum (McFadden *et al.*, 1988). This pattern is consistent with the suggestion that a hormonal signal inducing expression of hydrolases originates from the embryo and diffuses through the germinating grain (McFadden *et al.*, 1988).

Aleurone Function

During germination, aleurone cells undergo remarkable ultrastructural changes and are transformed from dormancy into a metabolically active tissue. The protein matrix of the aleurone grains and their phytin and niacin inclusions disappear rapidly on germination and protein bodies coalesce into vacuoles (Van der Eb & Nieuwdorp, 1967). Lipid bodies decrease in number and mitochondria become highly active (Van der Eb & Nieuwdorp, 1967; Gram, 1982). A dramatic proliferation of rough endoplasmic reticulum (ER) occurs during the mobilization of aleurone cell reserves; they develop into well-defined, fenestrated stacks. Golgi complexes also appear (Jones, 1969). In the early stage of germination, hydrolytic enzymes for the mobilization of aleurone reserves are released. These include a battery of endo- and exopeptidases (Adams & Novellie, 1975; Jelsema *et al.*, 1977), acid phosphatases (Ashford & Jacobsen, 1974; Gabard & Jones, 1986; Ching *et al.*, 1987; Jones, 1987; Polya & Haritou, 1988) and lipases (Fernandez & Staehelin, 1985; Jones, 1985).

The aleurone is the major source of hydrolytic enzymes for dissolution of the starchy endosperm. α-Amylases (Paleg, 1960; Filner & Varner, 1967; Chrispeels & Varner, 1967), proteases (Jacobsen & Varner, 1967), (1→3,1→4)–β–glucanases

(Stuart *et al.*, 1986), xylanases (Taiz & Honigman, 1976; Dashek & Chrispeels, 1977; Slade *et al.*, 1989), limit dextrinase, α-glucosidase (Hardie, 1975), peroxidases (Gubler & Ashford, 1983), (1→3)-β-glucanases (Taiz & Jones, 1970), ribonuclease (Chrispeels & Varner, 1967) and a multifunctional nuclease (Brown & Ho, 1986) are produced as germination progresses.

1.2.3 Cell Wall Degradation

During germination, hydrolytic enzymes are synthesized and secreted across the plasma membrane of scutellar or aleurone cells, where they need to overcome two physical barriers; the walls of the secretory aleurone or scutellar cells themselves, and those of the starchy endosperm cells. These cell walls are not sufficiently permeable to allow the passage of secreted enzymes, which are therefore isolated from their starch and protein substrates that are packaged within starchy endosperm cells. Removal of these cell wall barriers is therefore critically important in the early stages of germination (Fincher, 1989).

Cell walls of the starchy endosperm are broken down before the arrival of α-amylases (Gibbons, 1980). During endosperm mobilization, walls of the starchy endosperm are completely degraded. Some remnants of walls are left after the initial front of wall-degrading enzymes passes, but these subsequently disappear (Fincher & Stone, 1974; Selvig *et al.*, 1986).

During germination, the outer wall layers of scutellar epithelial cells are partially degraded and individual epithelial cells become disconnected along their lateral interface to form cylindrical papillae (Nieuwdorp & Buys, 1964). This process results in an increase in surface area of epithelial cells, which presumably enhances their ability to absorb the degradation products from the mobilization of endosperm reserves.

In the case of aleurone cells, the thick outer layer disappears but the thin, inner layer appears to resist degradation. Gubler *et al.* (1987) observed that α-amylase and other hydrolases are released from the aleurone through channels digested in the outer wall layer; eventually this outer layer is degraded completely. Secreted enzymes

appear to pass through the intact, thin inner wall of the aleurone (Taiz & Jones, 1973; Gubler *et al.*, 1987). It is possible that enzymes may move freely through the inner wall if a specific component, such as the (1→3,1→4)-β-glucan that is suggested to be located in this layer, is removed early in germination. This would result in a resistant polymeric framework through which the enzyme could move freely (Fincher, 1989). The thin, inner layer which remains intact during enzyme secretion would presumably provide continuing structural support for the aleurone protoplasts. It is still uncertain which enzymes are responsible for the degradation of the outer wall layer (Benjavongkulchai & Spencer, 1989; Fincher, 1989), how the secreted hydrolytic enzymes cross the inner wall layer, and how this inner layer remains intact in the presence of high concentrations of xylanase, β-glucanase and a battery of exo- and endo-peptidases (Fincher, 1989).

(1→3,1→4)-β-Glucans and arabinoxylans represent major polysaccharides (95%) of cell walls of the aleurone and the starchy endosperm (Table 1.1). The monosaccharides released from wall polysaccharides make a contribution to the total energy generated by metabolism of the endosperm for seedling development (Morall & Briggs, 1978). The (1→3,1→4)-β-glucans are linear chains of β-D-glucosyl residues polymerized through both (1→3)-β-linkages (30%) and (1→4)-β-linkages (70%) (Parrish & *et al.*, 1960; Woodward *et al.*, 1983b). They are members of a polysaccharide family, which vary in size, solubility and molecular structure (Bacic & Stone, 1981b; Woodward & Fincher, 1983; Woodward *et al.*, 1983a, 1988; Edney *et al.*, 1991). Enzymes capable of degrading (1→3,1→4)-β-glucans in germinated barley have been studied in detail. (1→3,1→4)-β-Glucan endohydrolases are primarily responsible for the degradation of cell wall (1→3,1→4)-β-glucan during starchy endosperm mobilization. In addition, exo-β-glucanases and β-glucosidase are also capable of degrading (1→3,1→4)-β-glucan or its breakdown products (Preece & Hoggan 1957; Manners & Marshall, 1969; Anderson *et al.*, 1976). The enzymes responsible for degradation of arabinoxylan include α-arabinofuranosidase, endoxylanase, exoxylanase

and xylobiase (Preece & MacDougall, 1958; Taiz & Honigman, 1976; Dashek & Chrispeels, 1977; Benjavongkulchai & Spencer, 1986, 1989; Slade *et al.*, 1989).

1.2.4 Mobilization of Starchy Endosperm Reserves

Endosperm dissolution in the germinating barley grain starts in the region adjacent to the scutellar epithelium and progresses as a wave which moves approximately parallel to the scutellum towards the distal end of the grain (Gibbons, 1981; MacGregor & Matsuo, 1982; Briggs & MacDonald, 1983). This pattern reflects the secretion of hydrolytic enzymes from the scutellum initially and subsequently from the aleurone (Fincher, 1989). It is clear that both the scutellum and aleurone participate in endosperm metabolism in barley. The relative contributions of the scutellum and the aleurone to total hydrolytic activity secreted into the starchy endosperm vary according to the particular enzyme and to the time after the initiation of germination (Fincher, 1989). Starch granules, protein bodies and residual nucleic acids in the starchy endosperm are depolymerized by the concerted action of many enzymes (Briggs, 1978; Enari & Sopanen, 1986).

Starch granules can be converted to glucose by the combined action of α-amylase, β-amylase, limit dextrinase and α-glucosidase during germination. It has been shown that holes form over the surface of large starch granules and once their interiors are degraded and hollow, the granules collapse (Kiribuchi & Nakamura, 1973; Maeda *et al.*, 1978; MacGregor and Ballance, 1980). Although the starch-degrading enzymes are known, the physical mechanism of attack on the granules and the role of each enzyme in the process are not fully understood. α-Amylases (EC 3.2.1.1) are Ca^{2+}-dependent endohydrolases that cleave internal (1→4)-α-glucosyl linkages of amylose or amylopectin in an essentially random fashion (Thoma *et al.*, 1971; MacGregor & Fincher, 1992). Immunocytochemical studies using labelled antibodies have shown that early in germination α-amylase is located near the scutellum (Dure, 1960; Gibbons, 1979, 1981; Briggs & MacDonald, 1983). β-Amylases (EC 3.2.1.2) are exohydrolases that cleave the penultimate (1→4)-α-linkage from the non-reducing

termini of (1→4)-α-glucans to release the disaccharide maltose (Maeda *et al.*, 1978). Both amylases need limit dextrinase (EC 3.2.1.41) to degrade the branched starch component amylopectin, because limit dextrinase specifically hydrolyses (1→6)-α-linkages and thereby increases levels of linear (1→4)-α-glucans. α-Glucosidase (EC 3.2.1.20) releases glucose from a variety of α-glucosides and presumably functions in the final conversion of maltose and other small dextrins to glucose (Jorgensen, 1965; MacGregor & Lenoir, 1987).

The reserve proteins of the starchy endosperm in barley grain are mobilized by the action of endo- and exo-peptidases (Rastogi & Oaks, 1986), which are secreted into or pre-exist in the starchy endosperm (Mikola, 1987). The major endopeptidases secreted from the aleurone are thiol endopeptidases. Several forms have been identified (Koehler & Ho, 1988). Endopeptidases may be important in the initial solubilization of storage proteins (Jones & Poulle, 1988). Serine carboxypeptidases are the major exo-peptidases involved in protein degradation (Mikola, 1987). In germinated barley, a group of five carboxypeptidases (EC 3.4.16.1) with different but complementary substrate specificities has been identified (Mikola & Kolehmainen, 1972; Mikola, 1983). Carboxypeptidases rapidly hydrolyse large peptides but depolymerize di- and tri-peptides more slowly (Mikola & Mikola, 1980). The products of hydrolysis include amino acids and di- and tri- peptides. Along with the degradation of reserve proteins in the starchy endosperm, peptidases may participate in the activation or release of β-amylase (Lundgard & Svensson, 1987), carboxypeptidase and endopeptidases that may pre-exist in a zymogen form (Doan & Fincher, 1988).

Residual RNA and DNA are found in the non-living cells of the starchy endosperm in barley grain (McFadden *et al.*, 1988). These nucleic acids are hydrolysed during germination by a multifunctional nuclease I, which produces oligonucleotides and finally 5'-mononucleotides (Brown & Ho, 1986; 1987). Levels of secreted nucleases are enhanced by GA_3 in isolated aleurone layers and in barley malt (Chrispeels & Varner, 1967; Taiz & Starks, 1977; Lee & Pyler, 1985). Other enzymes, such as

phosphodiesterases, 3'- and 5'-nucleotidases, phosphomonoesterases, and nucleosidases are also detected in germinated barley (Lee & Pyler, 1985, 1986).

1.3 (1→3,1→4)-β-GLUCANASES

(1→3,1→4)-β-Glucanases (EC 3.2.1.73) are probably the most important hydrolases in the degradation of cell wall (1→3,1→4)–β–glucans of the starchy endosperm of germinated barley grain (Woodward & Fincher, 1983; Fincher & Stone, 1993). The enzyme activity has also been correlated with the efficiency of the malting and brewing process (Woodward & Fincher, 1983; Bamforth, 1985, 1993). Two (1→3,1→4)-β-glucanases, isoenzymes EI and EII, have been purified from extracts of germinated barley grain and characterized (Woodward & Fincher, 1982a, b). The enzymes show classical endohydrolase action patterns and catalyse the hydrolysis of (1→4)-β-linkages in (1→3,1→4)-β-D-glucans when the glucosyl residue is substituted at C(O)3 (Woodward & Fincher, 1982b), as follows:

where G represents β-glucosyl residues, the numbers 3 and 4 indicate the linkage positions and RED denotes the reducing terminus of the polysaccharide chain (Fincher & Stone, 1993). They are usually assayed by their ability to decrease the viscosity of barley (1→3,1→4)-β-glucan solutions (Woodward & Fincher, 1982a). The major oligomeric products of hydrolysis released by enzyme action are 3-*O*-β-cellobiosyl-D-glucose and 3-*O*-β-cellotriosyl-D-glucose. Neither isoenzyme can hydrolyse (1→3)-β-glucans or (1→4)-β-glucans (Fincher, 1989).

Southern analysis of barley genomic DNA has confirmed that these two isoenzymes are encoded by two separate genes (Loi *et al.*, 1988; Slakeski *et al.*, 1990). Near full length cDNAs and genomic clones for isoenzymes EI and EII have been isolated and sequenced (Fincher *et al.*, 1986; Slakeski *et al.*, 1990; Litts *et al.*, 1990; Wolf, 1991). The isoenzyme EI gene is characterized by the presence of an intron of

2514 bp, which is inserted in the region encoding the signal peptide, close to the cleavage position at the NH_2-terminus of the mature enzyme. In addition, the coding regions for the mature enzymes of both (1→3,1→4)-β-glucanase genes have an high overall G+C content of 67%, which may be attributed to the use of G or C in the third base position of more than 92% of codons (Fincher *et al.*, 1986; Slakeski *et al.*, 1990).

1.3.1 Properties of the (1→3,1→4)-β-Glucanases

Properties of the two barley (1→3,1→4)-β-glucanase isoenzymes are shown in Table 1.2. Both isoenzymes are basic, monomeric proteins (Woodward & Fincher, 1982a, b). Analysis of corresponding cDNAs indicated that the enzymes both consist of 306 amino acids and that the positional identity of their amino acid sequences is 92%. They show different mobility on SDS-PAGE (Woodward & Fincher, 1982a, b), which probably results from differences in their degree of glycosylation: isoenzyme EI contains only a trace of carbohydrate, while isoenzyme EII carries 4% by weight carbohydrate, of which at least two residues are thought to be *N*-acetylglucosamine (Woodward & Fincher, 1982a). This has been confirmed by the analysis of the two cDNA sequences, which indicates that isoenzyme EII has a single, potential *N*-glycosylation site. The site is not present in isoenzyme EI because of a nucleotide substitution in the gene at that position (Fincher *et al.*, 1986; Slakeski *et al.*, 1990). Their different carbohydrate content is considered to be one important reason for the different thermostability displayed by each isoenzyme (Doan & Fincher, 1992); isoenzyme EII is significantly more stable at elevated temperatures than isoenzyme EI, both in unpurified extracts of germinated barley (Loi *et al.*, 1987) or as purified enzyme preparations (Woodward & Fincher, 1982b). This suggestion was supported by the observation that the thermostability of isoenzyme EII decreases when the carbohydrate of the enzyme is removed by PNGase treatment (Doan & Fincher, 1992). Moreover, removal of the single *N*-glycosylation site in isoenzyme EII by site-directed mutagenesis of the corresponding cDNA led to a reduction in thermostability, while the introduction of

Table 1.2 Properties of Barley (1→3,1→4)-β-Glucanases[1]

Property	Isoenzyme EI	Isoenzyme EII
Apparent molecular weight[2]	30,000	32,000
Isoelectric point	8.5	10.6
pH optimum	4.7	4.7
Amino acids	306	306
Carbohydrate	Trace	4%
N-glycosylation sites	0	1
Thermal stability[3]	~37°C	~45°C
Polyclonal antibodies	Cross react	Cross react
Monoclonal antibodies	Specific	Specific
Expression sites	Aleurone, scutellum, young leaves, young roots	Aleurone

1. Woodward & Fincher (1982a, b); Høj *et al.* (1990); Slakeski *et al.* (1990); Slakeski & Fincher (1992a).
2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
3. Temperature at which 50% of initial activity is retained after 15 minutes of incubation (Woodward & Fincher, 1982b).

this site into isoenzyme EI enhanced its stability (Doan & Fincher, 1992).

1.3.2 Regulation of (1→3,1→4)-β-Glucanase Expression

Previous experiments have shown that little or no expression of (1→3,1→4)-β-glucanases is detected in ungerminated barley grain, but enzyme activity rises to a maximum 4-6 days after the initiation of germination and then declines (Ballance *et al.*, 1976; Stuart & Fincher, 1983; Bamforth & Martin, 1983). Western blotting with monoclonal or polyclonal antibody probes has identified and quantitated levels of the individual isoenzymes in unpurified extracts of germinated grain (Stuart & Fincher, 1983; Stuart *et al.*, 1987; Høj *et al.*, 1990), indicating that isoenzyme EI develops in approximately equimolar proportions with isoenzyme EII, but appears slightly earlier than isoenzyme EII in the variety Clipper (Stuart & Fincher, 1983). Variations in absolute levels and in the ratio of individual isoenzymes are observed in other varieties (Henry, 1990).

Stuart *et al.* (1986) investigated the expression of the two (1→3,1→4)-β-glucanases in excised aleurone layers and scutella with Western blots, and these experiments showed that isoenzyme EI is predominantly secreted by isolated scutella, and is secreted at a much lower level in isolated aleurone layers, while isoenzyme EII is only secreted by the aleurone (Stuart *et al.*, 1986). The activity level of (1→3, 1→4)-β-glucanase secreted per isolated scutellum is up to 40% of that secreted by a single aleurone layer (Stuart *et al.*, 1986), suggesting that the scutellum might be a major contributor of (1→3,1→4)-β-glucanase during endosperm dissolution.

Later, a cDNA encoding (1→3,1→4)-β-glucanase isoenzyme EII (Fincher *et al.*, 1986) was used to locate the corresponding mRNA transcripts in intact, germinated barley by hybridization histochemistry of grain cryosections (McFadden *et al.*, 1988). Expression of (1→3,1→4)−β-glucanase was first detected in the scutellum after one day, when expression is not apparent in the aleurone. Two days after the initiation of germination, levels of (1→3,1→4)-β-glucanase mRNA decrease in the scutellar epithelium, but increase in the aleurone (McFadden *et al.*, 1988), and thereafter (1→3,

1→4)-β-glucanase gene expression is confined to the aleurone (McFadden *et al.*, 1988). Although the cDNA probe did not differentiate between mRNAs for isoenzyme EI and EII, it confirmed that both the scutellum and the aleurone participate in (1→3, 1→4)-β-glucanase synthesis *in vivo* (McFadden *et al.*, 1988). The hybridization also revealed a temporal and spatial co-ordination of (1→3,1→4)-β-glucanase gene regulation in intact grain that was not observed with isolated tissues.

Specific probes for the two isoenzymes, prepared from the 3' untranslated regions of their corresponding cDNA clones, are now available (Slakeski *et al.*, 1990) and have confirmed that isoenzyme EI transcripts predominate in the scutellum in germinated barley grain, whereas both isoenzyme EI and EII mRNAs are present in the aleurone (Table 1.2; Slakeski & Fincher, 1992a). In addition, isoenzyme EI mRNA was detected in developing leaves and roots. Isoenzyme EII was detected principally in the aleurone cells and appeared to be a germination-specific enzyme (Slakeski *et al.*, 1990; Slakeski & Fincher, 1992a; Fincher & Stone, 1993).

Stuart *et al.* (1986) also noted that the secretion of both isoenzymes from isolated aleurone was enhanced by treatment with gibberellic acid (GA_3) and Ca^{2+}. Enhancement of transcript levels for both enzymes in aleurone layers was observed, but abscisic acid (ABA) dramatically suppressed the relative abundance of the transcripts (Mundy & Fincher, 1986). Northern analyses with specific probes for each isoenzyme have been used to examine (1→3,1→4)–β-glucanase mRNA levels in different tissues of germinated grain and seedlings of barley upon phytohormone treatment (Slakeski & Fincher, 1992b). The two isoenzymes have different responses in different tissues and at different stages of development: GA_3 and IAA (indole acetic acid) increased levels of isoenzyme EII mRNA in isolated aleurone layers or isoenzyme EI in young leaves. Simultaneous treatment of leaves, roots and aleurone layers with GA_3 and ABA enhanced mRNA levels of isoenzyme EI. IAA inhibited expression of isoenzyme EI in young roots but GA_3 had no effect (Slakeski & Fincher, 1992b).

1.4 (1→3)-β-GLUCANASES

(1→3)-β-Glucanases [(1→3)-β-D-glucan glucanohydrolases; EC 3.2.1.39] of barley can be detected in a variety of tissues at many stages of development (Manners & Marshall, 1969; Ballance *et al.*, 1976; Xu *et al.*, 1992; Wang *et al.*, 1992; Hrmova & Fincher, 1993). The activity of (1→3)-β-glucanases is initially detected in the embryo of ungerminated barley grain, and increases markedly in the aleurone during germination (Manners & Marshall, 1969; Manners & Wilson, 1974; Ballance *et al.*, 1976). (1→3)-β-Glucanases hydrolyse (1→3)-β-glucosyl linkages in (1→3)-β-glucans in an essentially random fashion, and finally release laminaritriose ($G3G3G_{RED}$) and laminaribiose ($G3G_{RED}$) as major hydrolysis products, as follows:

$$\begin{array}{c} \downarrow \quad \downarrow \quad \downarrow \\ \cdots\cdots \mathbf{G3G3G3G3G3G3G3G3G3G} \cdots\cdots \mathbf{(RED)} \end{array}$$

where G represents a glucosyl residue, 3 represents a (1→3)-β-linkage, and **RED** indicates the reducing end of the polysaccharide chain (Fincher & Stone, 1993). The enzymes usually require a segment of unbranched, adjacent (1→3)-β-glucosyl residues for hydrolysis. Enzyme activity is generally measured using soluble (1→3)-β-glucans such as laminarin as substrates. Barley (1→3)-β-glucanases can not hydrolyse (1→3, 1→4)-β-glucans that contain no contiguous (1→3)-β-linkages (Woodward *et al.*, 1983; Høj *et al.*, 1988, 1989), suggesting that (1→3)-β-glucanases play no major role in the degradation of cell walls during germination. However, they are able to hydrolyse branched and substituted (1→3,1→6)-β-glucans that are cell wall components of plant fungal pathogens. The rate of hydrolysis decreases as the degree of substitution or branching increases (Hrmova & Fincher, 1993).

In other plants, the expression of (1→3)-β-glucanases is regulated by gibberellic acid (Taiz & Jones, 1973), ethylene (Abeles *et al.*, 1971), kinetin, abscisic acid (Moore & Stone, 1972) and auxin (Wong & Maclachlan, 1980). Despite the high levels of the enzymes produced in germinated barley, endogenous (1→3)-β-glucans make up only a small proportion of mature grain.

1.4.1 (1→3)-β-Glucans

(1→3)-β-Glucans are present as deposits (callose) in specialized cell walls at various stages during normal growth and development of plant tissues, and are deposited in response to wounding, infection or physiological stress (Stone & Clarke, 1993). (1→3)-β-Glucans also appear to be important in pollen grain development and germination (Waterkeyn, 1967). During the mechanical wounding of plant tissue, (1→3)-β-glucans accumulate at the interface of the cell wall and the plasma membrane (Eschrich & Currier, 1964). A similar response is observed when cells are subjected to chemical treatment or ultrasonic radiation (Currier & Webster, 1964; Hughes & Gunning, 1980). Physiological stress caused by plasmolysis and high or low temperatures, also result in callose deposition in the plasma membrane/cell wall region (Webster & Currier, 1968; Smith & McCully, 1977; Currier, 1957; Eschrich, 1957). Because callose often appears only transiently, it has been suggested that (1→3)-β-glucanases may be involved in the removal of the wound or stress callose (Currier & Webster, 1964).

(1→3)-β-Glucans can be measured as fluorescent complexes formed with the fluorochrome in the dye aniline blue (Mangin, 1890; Kessler, 1958). In barley, (1→3)-β-glucans are found as small bead-like deposits on the inner wall of starchy endosperm cells, and larger deposits are often seen in subaleurone regions of the endosperm (Fulcher *et al.*, 1977; Bacic & Stone, 1981a; Wood & Fulcher, 1984; MacGregor *et al.*, 1989). (1→3)-β-Glucans represent approximately 1% of the grain (Tiuova *et al.*, 1988; MacGregor *et al.*, 1989).

1.4.2 Enzyme Properties and Gene Structure

Three (1→3)-β-glucanases, designated isoenzymes GI (Høj *et al.*, 1988), GII (Ballance & Svendsen, 1988; Høj *et al.*, 1989; Leah *et al.*, 1991) and GIII (Wang *et al.*, 1992; Hrmova & Fincher, 1993) have been purified from extracts of germinated barley grain or young seedlings. Southern analysis of barley genomic DNA with a probe prepared from the cDNA for (1→3)-β-glucanase isoenzyme GII (Høj *et al.*,

1989), suggested that barley (1→3)-β-glucanase isoenzymes are encoded by a family of approximately six genes (Xu *et al.*, 1992). Genomic and cDNA clones encoding each isoenzyme have now been isolated and characterized (Høj *et al.*, 1989; Xu *et al.*, 1992). Another gene encoding an acidic isoenzyme, have designated isoenzyme GVII, has recently been isolated and characterized (Malehorn *et al.*, 1993). Complete amino sequences of barley (1→3)-β-glucanases have been deduced from either genomic clones (for isoenzymes GIII, GIV, GVI and GVII) or cDNA clones (for isoenzymes GI, GII and GIV) (Xu *et al.*, 1992; Malehorn *et al.*, 1993). The positional identities of their amino acid sequences range from 44% to 81%, which are lower than those of (1→3, 1→4)-β-glucanases (92%) and indicate a much higher degree of divergence during evolution.

Properties of genes and proteins for barley (1→3)-β-glucanase isoenzymes are listed in Table 1.3. The isoenzymes are similar in length and almost identical in size to the barley (1→3,1→4)-β-glucanases, except that the sequence of isoenzyme GIV has an extra 19 amino acids at the COOH-terminal extension. A single small intron splits a codon in a putative signal peptide in genes encoding isoenzymes GII, GIII and GVII; the intron is found in an almost identical position in the (1→3,1→4)-β-glucanase genes (Slakeski *et al.*, 1990; Wolf, 1991). The intron separates exons with distinctly different patterns of codon usage in the (1→3)-β-glucanase genes: in the 5' exon a balanced codon usage is observed, while in the coding region of the larger 3' exon there is a strong bias towards the use of G and C in the wobble base position (Xu *et al.*, 1992). The similarities in amino acid sequences, enzyme length, intron position and patterns of codon usage in the genes suggest that the barley (1→3)-β-glucanase genes share a common evolutionary origin with the (1→3,1→4)-β-glucanase genes (Slakeski *et al.*, 1990; Xu *et al.*, 1992).

Northern analyses with specific oligonucleotide and DNA probes have shown that the barley (1→3)-β-glucanase genes are subject to tissue-specific regulation (Xu *et al.*, 1992; Malehorn *et al.*, 1993; Table 1.3). (1→3)-β-Glucanase isoenzymes

Table 1.3 Properties of Barley (1→3)-β-Glucanase Isoenzymes and Genes[a]

Property	Isoenzyme						
	GI	GII[b]	GIII	GIV	GV	GVI	GVII[d]
Amino acids	310	306	305	327	312	315	308
Molecular Weight[c]	33,000	32,300	32,400	35,000	34,000	32,900	32,600
Isoelectric point	8.6	9.5	9.8	10.7	7.5	4.6	4.9
N-Glycosylation sites	1	0	5	1	1	1	1
(Amino acid number)	(67)		(75,132,133,259,296)	(317)	(119)	(26)	(290)
Codon bias in gene, %[e]	99.4	98.1	87.6	90.4	91.2	94.5	92
Transcription sites	Young roots, young leaves	Aleurone	Young roots, young leaves, shoots	Aleurone	Young roots, young leaves	Not known	Young roots

a. From Xu *et al.* (1992).

b. From Høj *et al.* (1989).

c. Excluding carbohydrate.

d. From Malehorn *et al.* (1993).

e. Percentage of codons in the coding region of the mature enzyme that have G or C in the wobble base position.

GII and GIV are detected in the aleurone layer of germinating barley grain, while isoenzymes GI (Høj *et al.*, 1988) and GIII (Wang *et al.*, 1992) originate in vegetative tissues of the developing seedling. Further, the isoenzyme GVII transcript is observed in young roots (Malehorn *et al.*, 1993).

The physiological function of the individual (1→3)-β-glucanase isoenzymes in germinated barley grain or in other tissues is not yet defined. Endogenous (1→3)-β-glucan levels are low in the starchy endosperm of barley grain (Fulcher *et al.*, 1977; MacGregor *et al.*, 1989). The enzymes may function in normal developmental processes, such as pollen formation, the removal of dormancy or wound callose, and senescence (Fincher & Stone, 1981). The enzymes have also been implicated in a general, non-specific protection of the germinating grain against pathogen invasion (Fincher, 1989; Høj *et al.*, 1989; Xu *et al.*, 1992), through their ability to hydrolyse the (1→3,1→6)-β-glucans that are major cell wall constituents of many fungi (Wessels & Sietsma, 1981). This suggestion is supported by the observation that in many other plants, (1→3)-β-glucanases are important members of the pathogenesis-related proteins (Boller, 1987; Dixon & Lamb, 1990; Linthorst, 1991) that are expressed in response to pathogen attack.

The presence of multiple isoforms of (1→3)-β-glucanases in barley would meet requirements for independent control of individual gene expression in different tissues, it would permit individual isoenzymes to be targeted to different subcellular or extracelluar sites during normal growth and development, and it would allow expression of specific genes at times of pathogen attack or stress (Xu *et al.*, 1992; Hrmova & Fincher, 1993). The evolution of several (1→3)-β-glucanases, particularly if they demonstrated slightly different or refined substrate specificities, may enable them to hydrolyse a range of fungal cell wall (1→3)-β-glucans with differing degrees of main chain substitution or branching (Hrmova & Fincher, 1993).

1.5 AIMS OF THIS STUDY

The primary aim of this work was to isolate genomic clones encoding (1→3,1→4)-β-glucanase isoenzyme EII. As described previously in this chapter, (1→3,1→4)-β-glucanases are encoded by two separate genes (Loi *et al.*, 1988; Slakeski *et al.*, 1990). The genomic clone for isoenzyme EI had been isolated and characterized (Slakeski *et al.*, 1990). At the start of this project, complete information about the gene structure of isoenzyme EII was still lacking, although a near full length cDNA clone for the isoenzyme had been isolated (Fincher *et al.*, 1986; Slakeski *et al.*, 1990). Analyses of cDNA clones for the two isoenzymes revealed sequence divergence in the 3' untranslated region of their genes. This information enabled specific oligonucleotides to be designed for use with the cDNA clone to screen the barley genomic library (Chapter II).

However, shortly after the isoenzyme EII gene was isolated in the present work, its complete sequence was published elsewhere (Wolf, 1991). The emphasis of the project was therefore shifted towards the isolation and characterization of the gene for (1→3)-β-glucanase isoenzyme GI, because this isoenzyme had been purified (Høj *et al.*, 1988) and a cDNA was available (Xu *et al.*, 1992), but the gene itself had not been cloned. Given the potential importance of (1→3)-β-glucanase gene expression during pathogen attack, the availability of the corresponding promoters would allow detailed examination of *cis*- and *trans*-acting factors that might participate in the regulation of transcription rates during pathogen invasion of barley tissues. Using an isoenzyme GII cDNA clone as a probe, together with specifically designed oligonucleotides corresponding to each gene, a barley genomic library was screened to recover genomic clones encoding (1→3)-β-glucanase isoenzyme GI (Chapter II).

Thus, in the present work, genomic clones coding for (1→3,1→4)-β-glucanase isoenzyme EII (Chapter III) and (1→3)-β-glucanase isoenzyme GI (Chapter IV) have been isolated and characterized.

CHAPTER TWO

ISOLATION OF GENES ENCODING β-GLUCANASES FROM A BARLEY GENOMIC LIBRARY

2.1 INTRODUCTION

Recently, a barley gene family of (1→3)-β-glucanases encoding seven members has been described (Xu *et al.*, 1992; Malehorn *et al.*, 1993), together with another class of genes encoding (1→3,1→4)-β-glucanases (Litts *et al.*, 1990; Slakeski *et al.*, 1990; Slakeski & Fincher, 1992a, b). Because of the similarity of the primary structures of these two classes of genes, it has been suggested that they both belong to a "super" gene family, in which genes encode enzymes with related but quite distinct substrate specificities (Xu *et al.*, 1992). The availability of genomic clones for some of the enzymes has enabled further investigations into the corresponding promoter regions with a view to understanding the regulation of the genes at a molecular level. As described in Chapter I, the objective of the present work was to isolate genes for (1→3,1→4)-β-glucanase isoenzyme EII and for (1→3)-β-glucanase isoenzymes which have not been isolated in the work described by Xu *et al.* (1992).

DNA cloning techniques have developed rapidly in recent years, in particular in the field of plant molecular biology. A number of strategies might be applied for the isolation of β-glucanase genomic clones from the barley genome. The genes of interest could be isolated from a small genomic library (gene bank) constructed from genomic DNA fragments excised from gels after the identification by Southern analysis of specific bands carrying the desired gene. Alternatively, a representative genomic library, in which all fragments and genes of the barley genome are present, could be generated and screened.

The approach used in this work was to screen a barley genomic library obtained from Clontech Laboratories Inc., and constructed from partially-digested barley genomic DNA from 7-day old seedlings of *Hordeum vulgare* L. (var NK 1558) into the *SalI* site in the cloning vector lambda EMBL3 (Frischauf *et al.*, 1983). The library contains 1.1×10^6 independent recombinant clones with an average insert size of 15 kb. A genomic clone for (1→3,1→4)-β-glucanase isoenzyme EI (Slakeski *et al.*, 1990) and three genomic clones encoding (1→3)-β-glucanase isoenzymes GIII, GIV and GVI have

previously been isolated from this library and characterized (Xu *et al.*, 1992; Xu, 1994). Using the same genomic library and different screening conditions, it should be possible to isolate genomic fragments that carry genes encoding other (1→3)-β-glucanase isoenzymes and the gene encoding (1→3,1→4)-β-glucanase isoenzyme EII.

The cDNA clones encoding (1→3,1→4)-β-glucanase isoenzyme EII (Fincher *et al.*, 1986) and (1→3)-β-glucanase isoenzyme GII (Høj *et al.*, 1989) were used to screen the barley genomic library as general probes for (1→3,1→4)-β-glucanase or (1→3)-β-glucanase genes, respectively. DNA probes prepared with a random primer labelling system generally result in strong hybridization signals and this method was therefore chosen to label the probes. In conjunction with the cDNA probes, specific probes prepared from oligonucleotides designed according to variable regions in the corresponding cDNAs, were used to identify genes encoding specific isoenzymes. The identities of putative clones were confirmed by nucleotide sequence analysis.

In summary, two experimental procedures are described in this chapter:

1) the screening of the barley genomic library and identification of genomic clones that carry genes encoding (1→3,1→4)-β-glucanase isoenzyme EII and (1→3)-β-glucanase isoenzyme GI; and

2) the subcloning of DNA fragments carrying the genes into the plasmid vectors pUC19 or pBluescript SK+ for restriction analysis and nucleotide sequencing.

The characterizations of the genes are described in Chapters III and IV.

2.2 MATERIALS AND METHODS

2.2.1 Materials

The barley genomic library and bacteriophage host strain NM538 were obtained from Clontech Laboratories Inc. (Palo Alto, CA, USA). Tryptone, yeast extract and bactoagar were from Difco (Detroit, MI, USA). Agarose, ethidium bromide, ampicillin, maltose, glucose, DTT, BSA, PEG (8000), RNase and DNase were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Urea, SDS, IPTG, and Xgal were obtained from IBI (New Haven, CT, USA). Plasmid pBluescript SK+/- and *E. coli* bacterial strain DH5α were obtained from Stratagene (La Jolla, CA, USA). Dextran sulphate and Ficoll 400 were obtained from Pharmacia-LKB (Uppsala, Sweden). Proteinase K, herring sperm DNA, and calf intestinal phosphatase were purchased from Boehringer Mannheim GmbH (Germany). GeneClean-II kits were from BIO 101 Inc. (La Jolla, CA, USA). Plasmid vector pUC19 was from Bresatec Ltd. (Adelaide, Australia). [α-^{32}P]dCTP (3,000 Ci/mmol) and [γ-^{32}P]ATP (>4,000 Ci/mmol) were obtained from either Amersham (UK) or Bresatec. Restriction enzymes were purchased from Bethesda Research Laboratories (BRL, Gaithersburg, USA), Promega (Madison, WI, USA), or New England Biolabs (Beverly, MA, USA). Nitrocellulose filters (BA 85, 0.45 μm) were from Schleicher & Schuell (Dassel, Germany). The Magic Multiprime DNA labelling kit, Klenow fragment DNA polymerase and autoradiographic film were purchased from Amersham International (UK). The DNA sequencing kit was purchased from United States Biochemical Corporation (USBC; Cleveland, Ohio, USA). Solutions were prepared using "Milli-Q" water (Bedford, MA, USA) and sterilized by autoclaving at 121°C for 20 minutes or filtering through Millex-GS 0.22 μm filter units from Millipore and 0.45 μm cellulose nitrate filters from Sartorius GmbH (Gottingen, Germany).

2.2.2 Maintenance and Growth of Bacterial Strains

As a host bacterium for plating out the barley genomic DNA library, *E.coli* strain NM538 (Sambrook *et al.*, 1989) was streaked out on a LB agar plate from a glycerol

stock. A single bacterial colony was picked to inoculate 50 ml LB medium containing 0.2% maltose and 10 mM $MgSO_4$, and grown overnight at 37°C. Cells were harvested by centrifugation at 4,000 rpm for 10 minutes. The cells were resuspended in 20 ml 10 mM $MgSO_4$ solution and stored at 4°C.

To prepare fresh competent cells for transformation of recombinant plasmids, *E.coli* strain DH5α (Sambrook *et al.*, 1989) was inoculated into 5 ml LB medium in a 10 ml sterile capped tube and grown overnight at 37°C. The overnight culture (1 ml) was transferred to 50 ml LB medium and incubated for 3-4 hours to the middle logarithm phase of growth at 37°C with vigorous shaking. Cells were recovered by centrifugation for 10 minutes at 4°C. The cells were resuspended in 15 ml ice-cold 50 mM $CaCl_2$ and stored on ice for at least one hour. After centrifugation, competent cells were resuspended in 2 ml of the same solution at 4°C prior to use.

2.2.3 Preparation of [^{32}P]-radiolabelled Probes

DNA fragment probes for screening the genomic library or for Southern blot analyses were prepared from cDNAs for (1→3,1→4)-β-glucanase isoenzyme EII (Fincher *et al.*, 1986; Slakeski *et al.*, 1990) and (1→3)-β-glucanase isoenzyme GII (Høj *et al.*, 1989). The cDNA insert of the plasmid was excised with an appropriate restriction enzyme and labelled using [α-^{32}P]dCTP and the Amersham multiprime DNA labelling system as described in the standard protocol provided by Amersham (Feinberg and Vogelstein, 1983).

End-labelled oligonucleotide probes were prepared according to Sambrook *et al.* (1989) under the following reaction conditions: 10 pmol oligonucleotide, 2 µl 10×T4 polynucleotide kinase buffer (700 mM Tris-HCl buffer, pH 7.6, 100 mM $MgCl_2$, 50 mM DTT), 10 µCi [γ-^{32}P]ATP, and 1 U T4 polynucleotide kinase. The volume of the labelling reaction was adjusted to 20 µl with sterile H_2O and incubated at 37°C for 10 minutes. The efficiency of radioactive incorporation was measured by polyacrylamide gel electrophoresis (Sambrook *et al.*, 1989).

2.2.4 Screening the Barley Genomic Library

The barley genomic library was diluted with SM buffer (10 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris-HCl buffer, pH 7.5 and 0.01% gelatin) and mixed at 37°C for 20 minutes with an overnight culture of *E.coli* NM538 cells for phage infection, prior to plating out in LB top agarose (0.7% agarose in LB medium). A total of ten plates was incubated at 37°C for no more than 16 hours. Plaques transferred onto nitrocellulose filter were denatured with 0.5M NaOH/1.5M NaCl for 4 minutes, neutralized with 0.5M Tris-HCl buffer, pH 8.0/1.5M NaCl for 8 minutes, and dried at room temperature before baking in a vacuum oven at 80°C for two hours. The conditions of prehybridization, hybridization and washing were modified from the procedure described by Xu *et al.* (1992). The filter replicas were washed in 2×SSC (20×SSC: 3 M NaCl, 0.3 M sodium citrate), 0.1% SDS at 65°C for 30 minutes or longer to remove bacterial debris, and prehybridized in 6×SSPE (20×SSPE: 3 M NaCl, 0.2 M NaH_2PO_4, 20 mM EDTA pH 7.4), 5×Denhardt's solution (50×Denhardt's solution: 1% Ficoll, 1% polyvinylpyrrolidone, 1% BSA), 0.1 mg/ml herring sperm DNA, 0.1% SDS at 65°C for at least two hours. The hybridization reaction was performed in fresh prehybridization solution containing the appropriate radiolabelled DNA probe at 65°C for 16 hours, or at 42°C when formamide was incorporated at a final concentration of 48% (v/v). The filter replicas were washed twice at 65°C in 2×SSC/1% SDS for 30 minutes, and twice in 0.2×SSC/0.1% SDS for 15 minutes. The hybridizations with oligonucleotide probes were carried out at 42°C.

The filter replicas were dried in air and autoradiographed with an intensifier screen at -80°C for 2 or 3 days. Positive plaques were selected on the basis of the alignment of autoradiographic signals from two replicas of plaque lifts and were purified by one or two further rounds of plating, plaque lifting and hybridization.

2.2.5 Quick Purification of Lambda DNA

Single positive plaques were picked as small agar plugs from LB plates using sterile disposable plastic tips with cut ends and were placed in Eppendorf tubes

containing 500 µl SM buffer and 20 µl chloroform. The tubes were mixed vigorously for 1 minute and held at room temperature for at least two hours to allow the diffusion of phage particles from the agar. After bacterial debris and agar were removed by centrifugation, the phage suspension was stored at 4°C for further analysis. For long term storage, phage stock was stored at -80°C in 7% (v/v) dimethyl sulfoxide (DMSO).

Recombinant EMBL3 DNA was purified following the procedure for preparation of bacteriophage lambda DNA from liquid culture as described by Sambrook *et al.* (1989) with minor modifications. Purified plaques (200-400 µl phage stock) were added to 2 ml overnight *E. coli* NM538 cell culture to allow infection at 37°C for 20 minutes. The mixture was added to 100 ml LB medium containing 10 mM $MgSO_4$ in a 250 ml flask prewarmed to 37°C, and incubated for 7-8 hours at 37°C until bacterial debris appeared. The culture was mixed with 2 ml chloroform and the flask was shaken for a further 10 minutes. Bacterial cell debris was removed from the phage solution by centrifugation at 10,000 rpm for 10 minutes at 4°C. DNase and RNase were added to the supernatant at a final concentration of 2 µg/ml each. The mixture was incubated for one hour at 37°C. To precipitate phage particles, an equal volume of 20% (w/v) PEG 8000/2 M NaCl in SM buffer was added. The solution was mixed well, incubated on ice for 1-2 hours and centrifuged at 10,000 rpm for 20 minutes at 4°C. The pellet was drained, resuspended in 2 ml SM and the suspension was extracted with an equal volume of chloroform to remove traces of PEG.

Lambda DNA was released from the bacteriophage particles by incubation with Proteinase K, EDTA and SDS at final concentrations of 50 µg/ml, 25 mM and 0.5%, respectively, at 56°C for one hour. The DNA was extracted once each with equal volumes of phenol and 1:1 phenol/chloroform, twice with chloroform, and precipitated with two volumes of ethanol. The threadlike DNA was recovered, washed with 70% ethanol and dried. The DNA was dissolved in 50 µl TE buffer and was then ready for analysis by restriction enzyme digestion.

2.2.6 Digestion of DNA by Restriction Enzymes

DNA digestion with restriction enzymes was generally performed in a total reaction volume of 20 µl in appropriate buffers and at appropriate temperatures, according to the manufacturers' instructions. RNase, free of DNase, was used by adding 1 µl stock solution (1 µg/µl) to DNA samples. Large-scale DNA digestions for recovery of fragments were performed by increasing the total volume, but maintaining the DNA concentration at 0.2 µg/µl. Restriction enzyme was added in a ratio of 1 unit enzyme per 1 µg DNA. Excess enzyme (or double-enzyme digestions) and longer incubation times (4 hours to overnight) were applied to ensure complete digestion as necessary.

2.2.7 Agarose Gel Electrophoresis

DNA fragments were separated by agarose gel electrophoresis as described by Sambrook *et al.* (1989). The agarose gel, routinely at concentrations of 0.7% (for lambda DNA) or 1% (for plasmid DNA), was prepared in TAE buffer (40 mM Tris-acetate buffer, pH 8.0 and 1 mM EDTA), into which ethidium bromide was incorporated at a final concentration of 1 µg/ml. All DNA samples were mixed with 5 volumes electrophoresis loading buffer (0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol, 40% w/v sucrose). Molecular weight markers were included on all gels. The DNA molecular markers were prepared by combining equal volumes of *Hin*dIII and *Hin*dIII/*Eco*RI digested, unmethylated lambda DNAs. For DNA radiolabelling and subcloning, DNA fragments were excised from the agarose gel and purified using the GeneClean II kit according to the directions of the manufacturer. The DNA fragments were recovered from glass milk with H_2O and were then ready for ligation or labelling.

2.2.8 Southern Blot Analysis

Southern blot analysis was used to identify DNA fragments carrying genes of interest. EMBL3 DNA was isolated and subjected to restriction digestion using a variety of restriction enzymes. Resulting DNA fragments were fractionated on agarose

gel and transferred onto nitrocellulose filters overnight according to Sambrook *et al.* (1989), using the procedure developed by Southern (1975). Hybridization of the filters was carried out for 2-3 hours with the appropriate [^{32}P]-radiolabelled probes, as described in Section 2.2.4. The sizes of DNA fragments were calculated by comparing migration rates with those of the DNA molecular markers.

For the quick identification of putative positive clones, dot blot analysis was also employed. Purified phage stocks or plasmids were spotted onto nitrocellulose filters. The filters were denatured and probed as described in Section 2.2.4.

2.2.9 Subcloning into Plasmid pUC19

Recombinant EMBL3 DNA purified from positive clones (Section 2.2.5) was digested with appropriate restriction enzymes and fractionated on agarose gels. DNA fragments identified by Southern blot analysis were purified and ligated into the plasmid vector pUC19, which was cleaved with a compatible enzyme and dephosphorylated. The recombinant plasmid was transformed into fresh competent cells of *E. coli* DH5α following the procedure developed by Hanahan (1983), as described by Sambrook *et al.* (1989). To transform recombinant plasmids with large inserts, the electroporation method (Chassy *et al.*, 1988) was employed. The transformed bacterial cells were plated onto LB plates containing ampicillin (100 μg/ml), X-gal (50 μg/ml) and IPTG (0.5 M). After incubation at 37°C for approximately 16 hours, white colonies were picked with sterile toothpicks and DNA was isolated as described in Section 2.2.10. Recombinant plasmids was identified by Southern analysis.

2.2.10 Small-scale Preparation of Plasmid DNA

A mini-prep method for plasmid DNA isolation was modified from the alkaline-lysis procedure (Birnboim & Doly, 1979). Bacteria harbouring plasmids were grown in 5 ml LB medium for 12-16 hours in the presence of 100 μg/ml ampicillin. The bacterial culture (1.5–4.5 ml) was gradually transferred into a sterile Eppendorf tube

and centrifuged for 30 seconds twice to remove culture medium thoroughly. The cells were resuspended in 100 µl ice-cold lysis buffer (50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl buffer, pH 8.0) and mixed with 200 µl freshly prepared 0.2 M NaOH/1% SDS. The tube was inverted gently three times and chilled on ice for exactly 5 minutes. The contents were neutralized and chromosomal DNA precipitated by mixing with 150 µl 3 M potassium acetate solution pH 4.6. After two minutes, the tube was carefully inverted three times and incubated on ice for a further 5 minutes and chromosomal DNA removed by centrifugation for 10 minutes at 12,000 rpm. The supernatant (400 µl) was transferred to a fresh tube and extracted with an equal volume of chloroform. The aqueous phase was recovered by centrifugation. Plasmid DNA was precipitated with 2 volumes absolute ethanol for 5 minutes at room temperature, centrifuged for 5 minutes and washed with 70% ethanol solution. The pellet was air dried and resuspended in 20 µl TE buffer, pH 8.0. The preparations usually contained about 90% supercoiled DNA and were competent for digestion with restriction enzymes.

2.3 RESULTS AND DISCUSSION

2.3.1 Screening of the Barley Genomic Library

The barley genomic library was plated out at high plaque density and a total of approximately 10^6 plaques were screened. This accounted for approximately one complete genome equivalent of DNA fragments. The screening of the genomic library was performed with DNA probes prepared from near full-length cDNA clones either for (1→3)-β-glucanase isoenzyme GII (Høj *et al.*, 1989) or for (1→3,1→4)-β-glucanase isoenzyme EII (Fincher *et al.*, 1986; Slakeski *et al.*, 1990).

(1→3,1→4)-β-Glucanase isoenzyme EII cDNA probe

The barley genomic library was initially screened with a full length cDNA for (1→3,1→4)-β-glucanase isoenzyme EII (Slakeski *et al.*, 1990). Eight putative clones, designated λEII-1 to λEII-8, showed relatively weak intensities on autoradiographic films (data not shown) when the screening was performed at high stringency; the hybridization solution contained 48% (v/v) formamide and the final washing was done in 0.2×SSC/0.1% SDS solution at 65°C for 30 minutes. The putative positive clones which could be aligned on two replica plaque lifts were recovered from plates and purified by secondary screening in smaller plates (90 mm petri dish) with relatively low plaque densities (30-50 plaques per plate). Six of the original clones (λEII-1 to λEII-5 and λEII-7) gave positive hybridization signals and were essentially monoclonal after the second screening. They all showed much stronger signals than in the first screening. Clones λEII-5 and λEII-7 exhibited the strongest signals under the conditions used (Figure 2.1). Attention was concentrated on these two clones; the clones showing weaker signals were not examined further.

(1→3)-β-Glucanase isoenzyme GII cDNA probe

The cDNA clone for (1→3)-β-glucanase isoenzyme GII was used as a general DNA probe to re-screen the barley genomic library in an attempt to isolate genomic

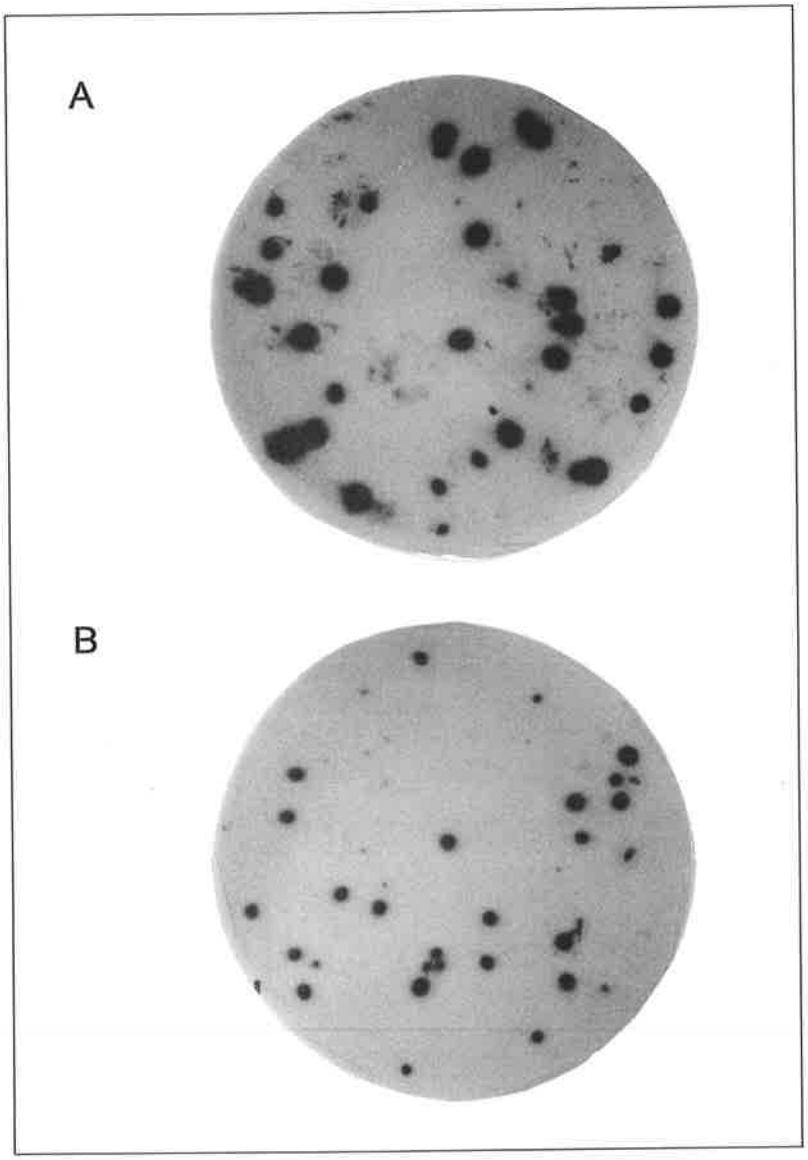


Fig. 2.1 Rescreening of positive clones selected from the first screening. The cDNA clone encoding (1→3,1→4)-β-glucanase isoenzyme EII was used as a probe. The two clones, λEII-5 (A) and λEII-7 (B) showed the most intense signals.

clones coding for one or more of the (1→3)-β-glucanase isoenzymes which had not been isolated previously by Xu *et al.* (1992).

Filter replicas were hybridized with the radiolabelled DNA probe in hybridization solution, without formamide, at 65°C for 20 hours. These hybridization conditions were slightly less stringent than those applied to the screening for genomic clones encoding (1→3,1→4)-β-glucanase isoenzyme EII, because the barley (1→3)-β-glucanases showed much lower positional identities (Xu *et al.*, 1992). Each plate produced an average of 10 positive clones, and fifty clones that gave relatively strong signals were picked and stored at 4°C. A small EMBL3 recombinant library was constructed by combining an equal volume (10 µl eluted phage) of each positive clone. This sublibrary, which was expected to contain genes for (1→3)-β-glucanase isoenzymes, was plated out on two large plates at high plaque densities. In this secondary screening, attempts were made to isolate the gene for (1→3)-β-glucanase isoenzyme GII, as follows:

A. The first of the replica lifts was probed with an oligonucleotide specific for (1→3)-β-glucanase isoenzyme GII, which is complementary to the 3' untranslated region of the cDNA (Høj *et al.*, 1989). The sequence of the oligonucleotide was,

5'-GGATGTAGGTATGTGAGCTAGGTAGCTACA-3'.

The hybridization conditions were as described in Section 2.2.4. As a result of this screening, 4-5 positive clones were detected on each plate.

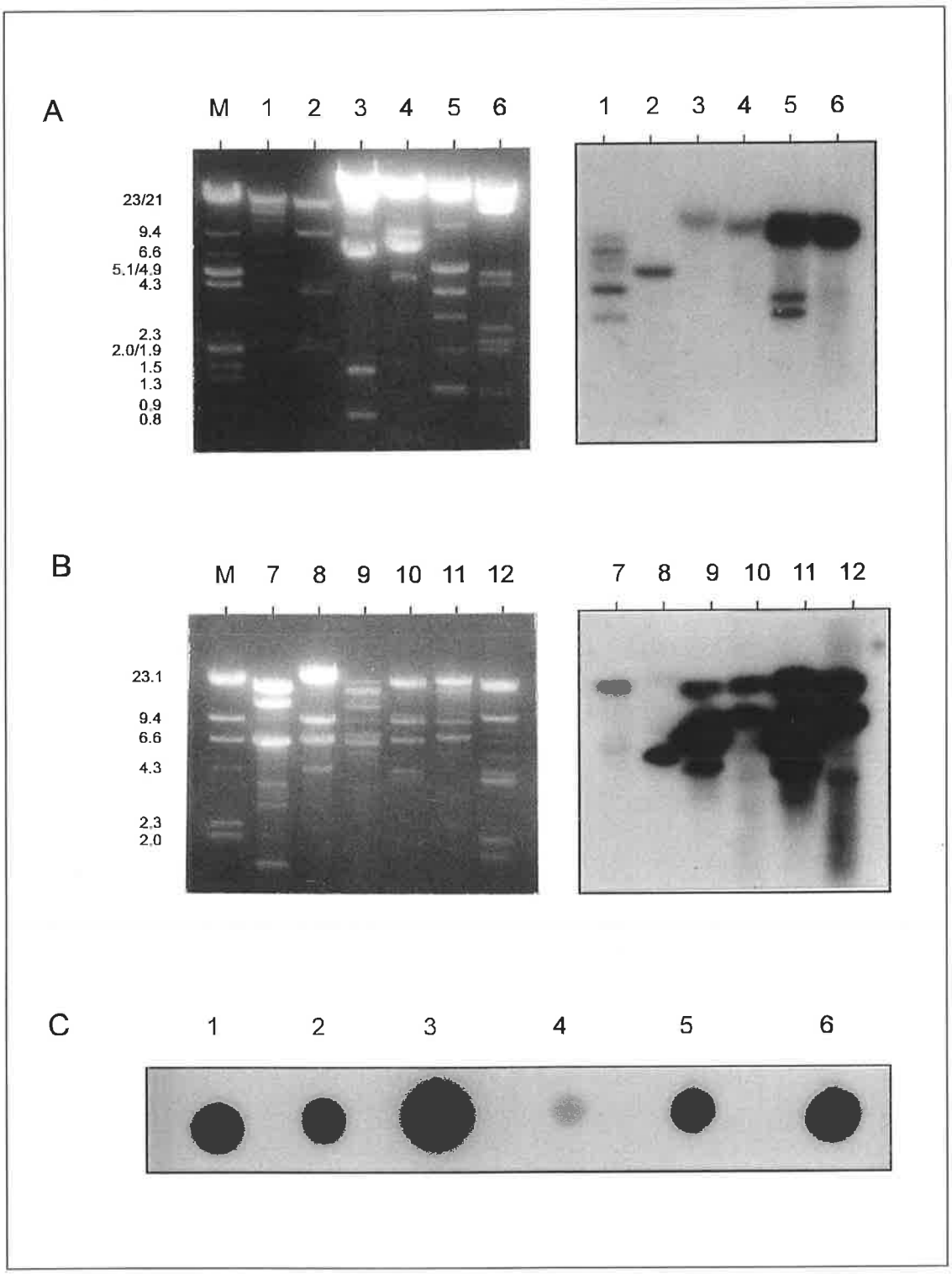
B. The second of the replica lifts was hybridized with the cDNA clone for (1→3)-β-glucanase isoenzyme GII, but at higher stringency: the hybridization solution (Section 2.2.4) with 50% (v/v) formamide was used and filters were washed with 0.1% SSC/ 0.5 SDS at 68°C for 30 minutes twice.

No clone gave strong signals with both the oligonucleotide and cDNA probes. One clone showed a very strong signal with the oligonucleotide, but a weaker signal when hybridized with the cDNA. The clone was purified and subcloned for further analysis.

Figure 2.2

Fig. 2.2 Southern analysis of putative genomic clones for (1→3)-β-glucanase isoenzymes. Recombinant EMBL3 DNAs purified from putative clones were digested with *Bam*HI (lanes 1, 3, 5, 7, 9, 11) and *Hin*dIII (lanes 2, 4, 6, 8, 10, 12) and probed with the cDNA clone for (1→3)-β-glucanase isoenzyme GII. Parts A and B represent: lanes 1-2, λG-1; lanes 3-4, λG-2; lanes 5-6, λG-3; lanes 7-8, λG-4; lanes 9-10, λG-5; lanes 11-12, λG-6. Lane M is the DNA markers, combined *Hin*d III and *Hin*dIII/*Eco*RI digests of lambda DNA. Part C illustrates the dot blot analysis of EMBL3 DNAs of the same amount from the six positive clones probed with the cDNA for (1→3)-β-glucanase isoenzyme GII .

Figure 2.2


A
M 1 2 3 4 5 6
23/21
9.4
6.6
5.1/4.9
4.3
2.3
2.0/1.9
1.5
1.3
0.9
0.8
1 2 3 4 5 6
B
M 7 8 9 10 11 12
23.1
9.4
6.6
4.3
2.3
2.0
7 8 9 10 11 12
C
1 2 3 4 5 6

In addition, six putative clones, designated λG-1 to λG-6, which showed stronger positive signals with the cDNA probe, were purified and analyzed by DNA restriction digestion (Figure 2.2). λG-5 and λG-6 probably include the same genomic DNA fragment, because both generate a 9.6 kb positive fragment after *Hin*dIII digestion. A *Bam*HI restriction site splits a putative gene carried by these two clones and the cDNA for (1→3)-β-glucanase isoenzyme GV also has a *Bam*HI site in its coding region (Xu *et al.*, 1994). A *Hin*dIII fragment of 9.5 kb was also observed in the restriction digest of λG-1 DNA. λG-3 gave the most intense signal among these positive clones (Figure 2.2) and its restriction pattern is different from that of the cDNA for (1→3)-β-glucanase isoenzyme GII, for which a *Hin*dIII fragment of 6.5 kb was detected in the Southern analysis of the barley genomic DNA (Xu, 1994).

2.3.2 Identification of a (1→3,1→4)-β-Glucanase Isoenzyme EII Gene

Sequence analysis of the (1→3,1→4)-β-glucanase cDNAs indicates divergence in their 3' untranslated regions (Slakeski *et al.*, 1990), suggesting that specific oligonucleotides for the isoenzyme EII gene may be used in identification of the putative clones (Section 2.3.1). The six purified clones were characterized by dot blot analysis with specific probes corresponding to the 3' untranslated region of the isoenzyme EII cDNA. The specific probes were prepared by end labelling oligonucleotides Oli 4 and Oli 5, kindly provided by Dr. Slakeski. The oligonucleotides, Oli 4 and Oli 5, are complementary to the 3' untranslated region of the cDNA encoding (1→3,1→4)-β-glucanase isoenzyme EII (Figure 2.3). Their nucleotide sequences are as follows:

Oli 4, 20 mer; 5'-CGAGTAGCTCGTCAAGTTCG-3'

Oli 5, 19 mer; 5'-ACGTTCTCATCCCTCATGTG-3'

The use of two oligonucleotides allowed relatively stronger signals to be obtained during *in situ* hybridization. The specificity of the probe was measured by using the cDNA for isoenzyme EII as a positive control, along with the cDNA for isoenzyme EI as negative control (data not shown).

Phage stocks for each clone were prepared, spotted onto a nitrocellulose filter and

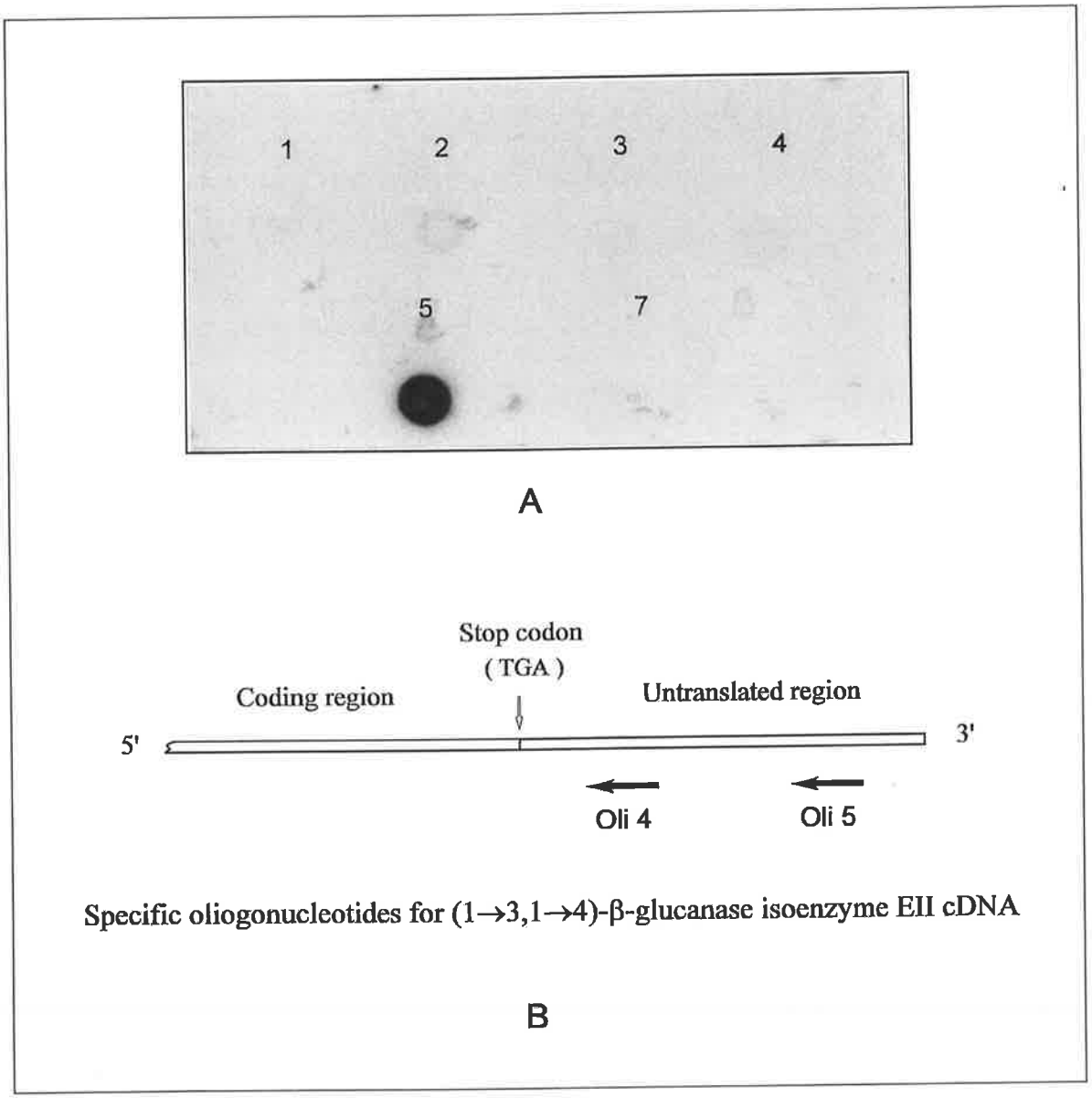


Fig. 2.3 Dot blot analysis of putative genomic clones for (1→3,1→4)-β-glucanase isoenzyme EII. Two specific oligonucleotides, Oli 4 and Oli 5, corresponding to the 3' untranslated region of the cDNA for (1→3,1→4)-β-glucanase isoenzyme EII (Part B) were used in the analysis. Phage plaque elutions from six putative clones (λEII-1 to λEII-5 and λEII-7) were spotted on a nitrocellulose filter and hybridized with probes prepared from Oli 4 and Oli 5. The positive signal given by λEII-5 (Part A) suggested it is a genomic clone for (1→3,1→4)-β-glucanase isoenzyme EII.

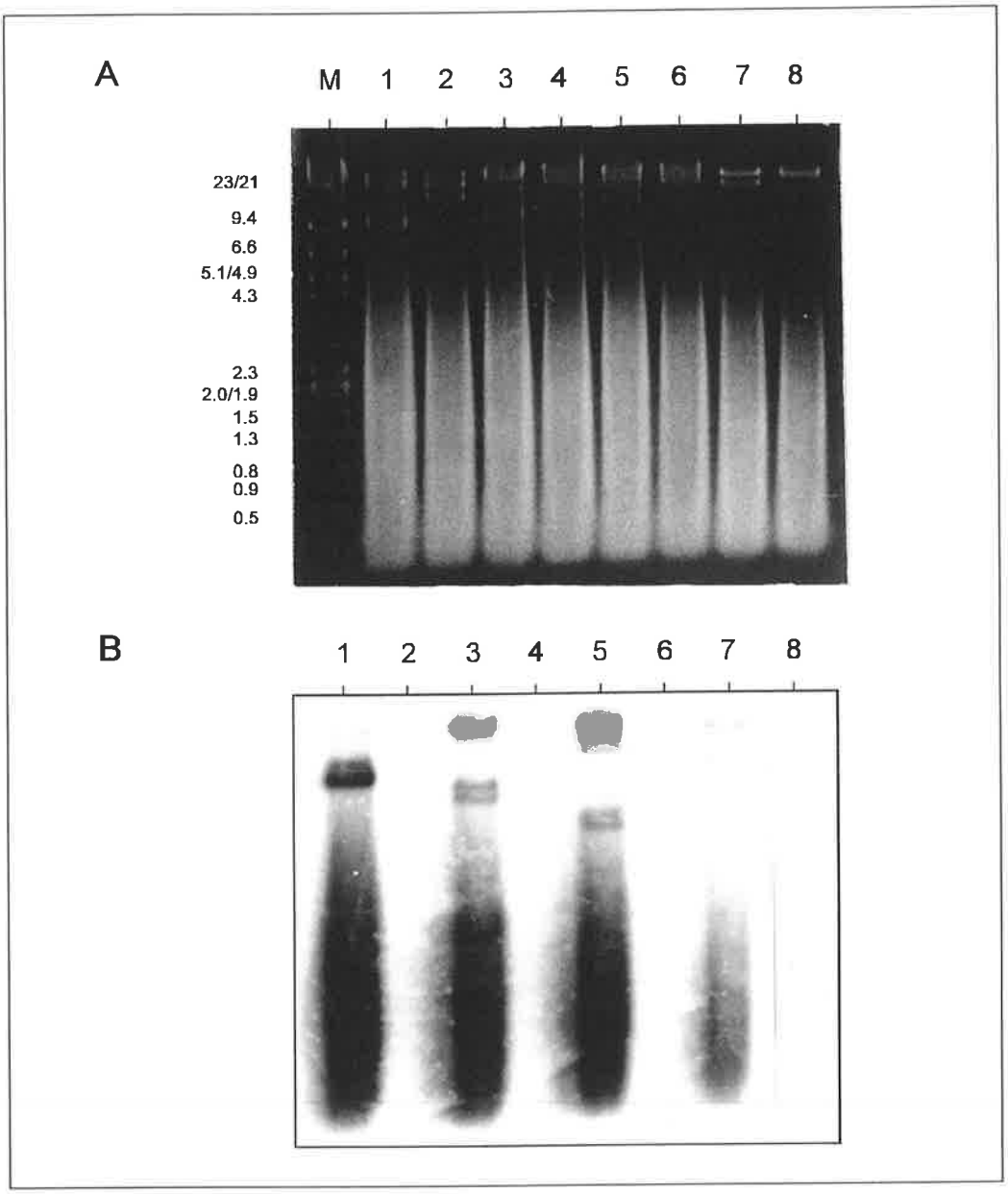


Fig. 2.4 Southern analysis of putative genomic clones (λEII-5 and λEII-7) for (1→3,1→4)-β-glucanase isoenzyme EII. Recombinat DNAs from the two clones were digested with *Bam*HI (lanes 1, 2); *Eco*RI (lanes 3, 4); *Hin*dIII (lanes 5, 6); and *Sal*I (lanes 7, 8) and the fragments were fractionated together with DNA size markers, equally combined *Hin*dIII and *Hin*dIII/*Eco*RI digests of lambda DNA (Lane M). Part A represents λEII-5 and λEII-7, loaded in lanes 1, 3, 5, 7 and lanes 2, 4, 6, 8, respectively. Part B represents a Southern blot probed with probes prepared from Oli 4 and Oli 5 together. Positive signals were obtained only with λEII-5.

hybridized with the oligonucleotide probes. Only λEII-5 showed a strong positive signal (Figure 2.3). This led to the conclusion that λEII-5 was a genomic clone carrying the (1→3,1→4)-β-glucanase isoenzyme EII gene. This was supported by Southern analysis of DNAs purified from λEII-5 and λEII-7 (Figure 2.4) using the same specific oligonucleotide mixture as a probe. It remained to be confirmed by nucleotide sequencing that this genomic clone (designated EMBL-EII) encoded the (1→3,1→4)-β-glucanase isoenzyme EII. This is described in Chapter III.

Primary structure analysis indicated that cDNA or genomic clones encoding barley (1→3)-β-glucanases have 49%-51% positional identity with the cDNA for (1→3, 1→4)-β-glucanase isoenzyme EII (Xu *et al.*, 1992). The clones that hybridized weakly with the (1→3,1→4)-β-glucanase cDNA probe might therefore encode (1→3)-β-glucanases. To check this possibility, a gene-specific oligonucleotide, corresponding to a portion of the coding region of (1→3)-β-glucanase isoenzyme GII, was obtained from Ms Peilin Xu for Southern blot analysis. λEII-4 exhibited a rather weak positive signal with this probe (data not shown). A *Hin*dIII fragment of 6.5 kb from this clone was subcloned into the plasmid vector pUC19 and was analysed by restriction digestion (data not shown). Comparison of restriction maps available for (1→3)-β-glucanase isoenzymes (Xu *et al.*, 1992), finally revealed that λEII-4 was another copy of a gene for the (1→3)-β-glucanase isoenzyme GIV (Xu *et al.*, 1992), but was different in length.

2.3.3 Identification of Clones for (1→3)-β-Glucanase Genes

The genomic fragments carried by two clones (λG-1 and λG-3) were analysed in more detail using the cDNA for (1→3)-β-glucanase isoenzyme GII as a probe. Southern analysis of restriction fragments from λG-3 (Figure 2.5) revealed a *Sal*I fragment of 1.4 kb that hybridized with the probe, together with a 1.4 kb *Acc*I fragment and a *Pst*I fragment of 2.6 kb. The 1.4 kb *Sal*I fragment was subcloned, but the nucleotide sequences obtained from each end of the fragment had no homology with those of any genomic or cDNA clones for (1→3)-β-glucanase. This may have resulted

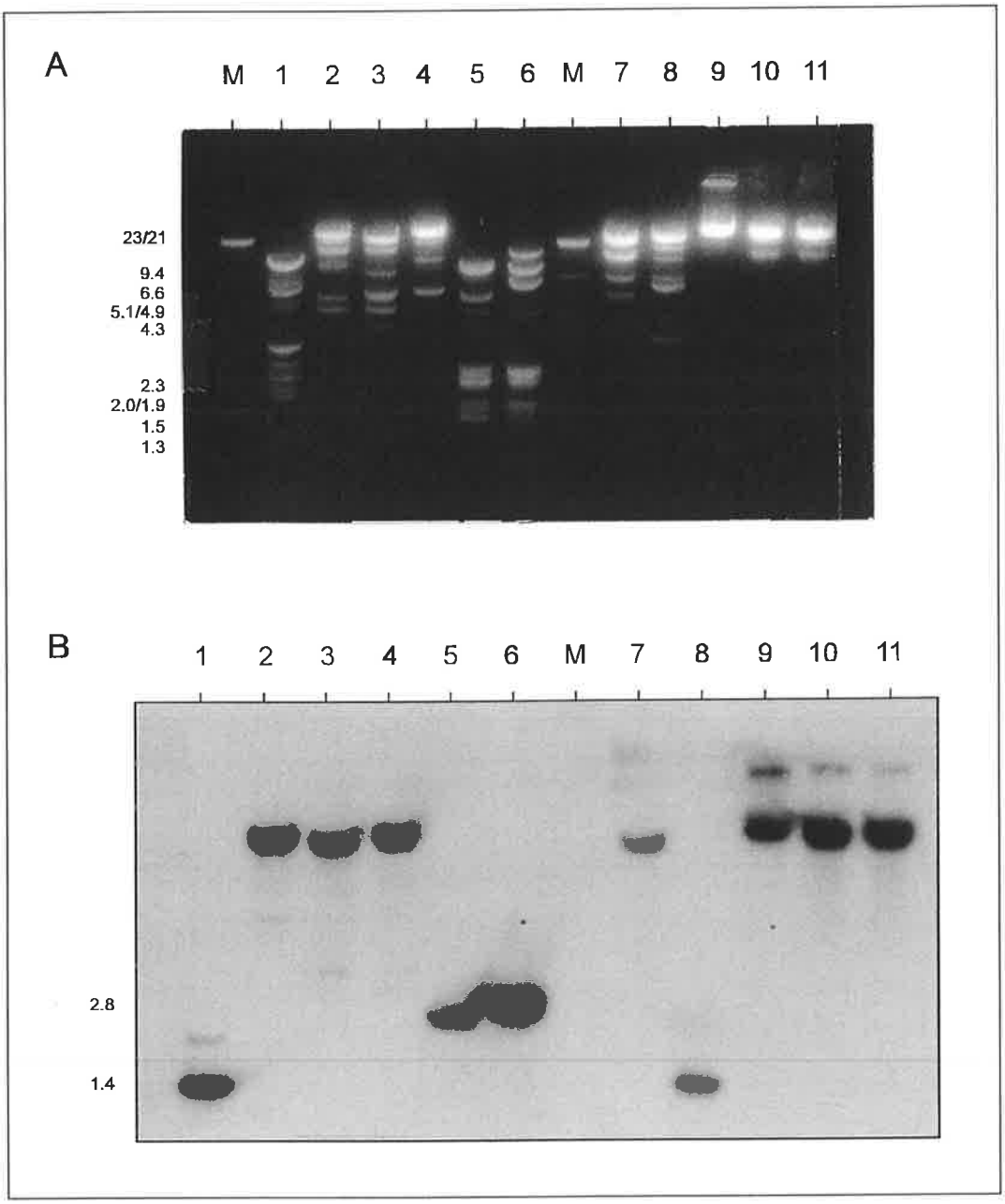


Fig. 2.5 Southern analysis of a putative genomic clone (λG-3) using a (1→3)-β-glucanase probe. Part A represents recombinant EMBL3 DNA digested with (lane numbers precedes restriction enzymes) 1, *Acc*I; 2, *Bam*HI; 3, *Bam*HI/*Eco*RI; 4, *Eco*RI; 5, *Eco*RI/*Pst*I; 6, *Pst*I; 7, *Hin*dIII; 8, *Sal*I; 9, *Xba*I; 10, *Xba*I/*Xho*I; 11, *Xho*I. Lane M is the DNA markers (sizes shown in kb), combined digests of *Hin*dIII/*Eco*RI and *Hin*dIII lambda DNA. Part B represents Southern blot analysis probed with (1→3)-β-glucanase isoenzyme GII cDNA.

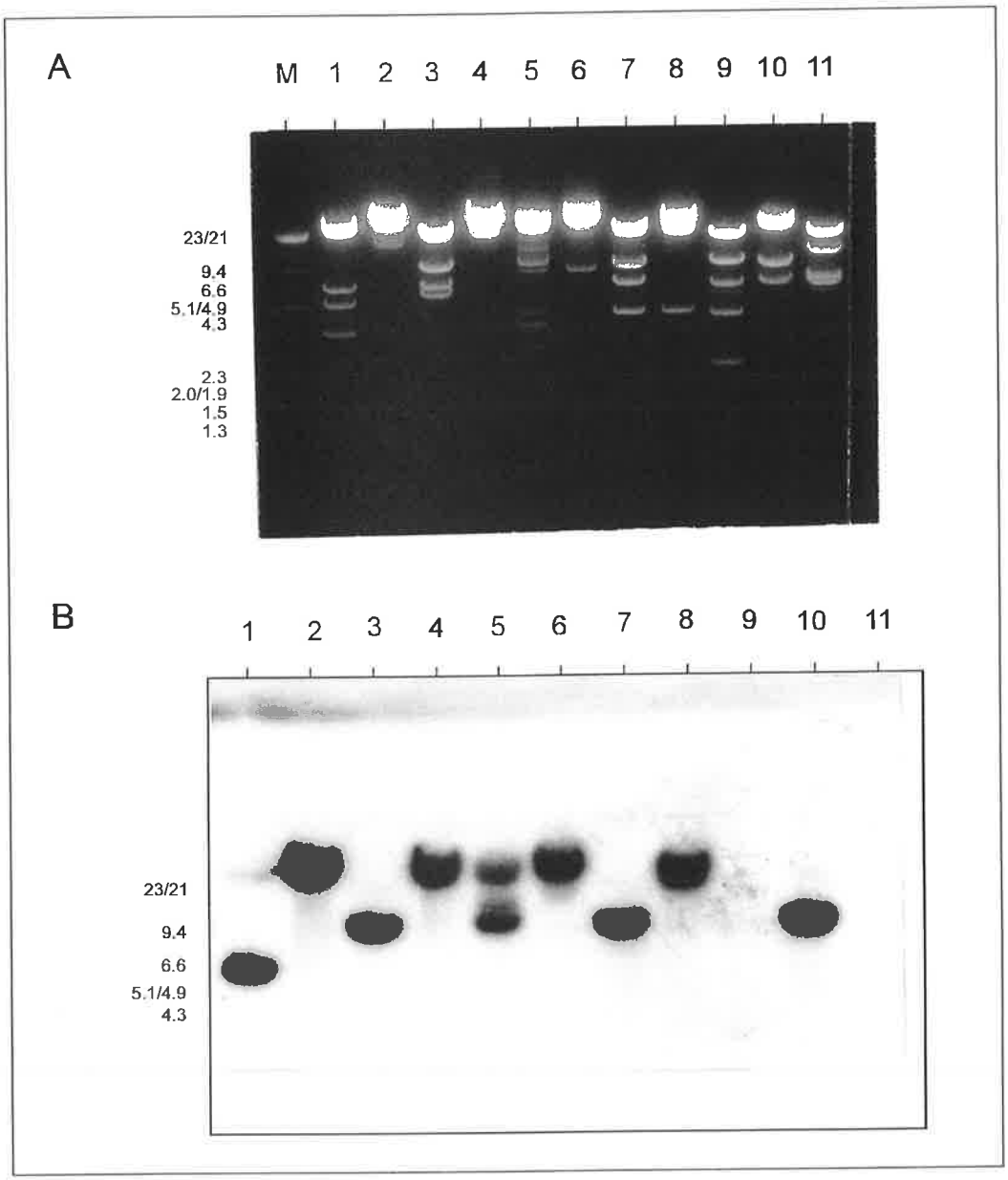


Fig. 2.6 Southern analysis of the genomic clone (λG-1) using a (1→3)-β-glucanase probe. Recombinant EMBL3 DNA (Part A) is digested with (lane numbers precede restriction enzymes) 1, *Sal*I/*Hin*dIII; 2, *Sal*I; 3, *Eco*RI/*Hin*dIII; 4, *Eco*RI; 5, *Sst*II/*Hin*dIII; 6, *Sst*II; 7, *Sst*I/*Hin*dIII; 8, *Sst*I; 9, *Bam*HI/*Hin*dIII; 10, *Hin*dIII; 11, *Bam*HI. Southern blot analysis (Part B) is probed with (1→3)-β-glucanase isoenzyme GII. Lane M is the DNA size markers (sizes shown in kb).

from the nucleotide sequences not reaching an area that could be recognised as the mature protein coding region. The *Sal*I fragment was subsequently cut into three smaller fragments: a 400 bp *Sac*II fragment, and 500 bp and 550 bp *Sal* I/*Sac*II fragments. These were subcloned into compatible sites of the pBluescript SK(+) vector. The nucleotide sequence from the one end of the 500 bp *Sal*I/*Sac*II fragment was identical to that of the 5' region of a cDNA for (1→3)-β-glucanase isoenzyme GI (Xu 1994). Furthermore, the amino acid sequence deduced from the nucleotide sequence exactly matched with the first 35 amino acids of the NH_2-terminal sequence determined directly from purified (1→3)-β-glucanase isoenzyme GI (Høj *et al.*, 1988). Therefore, λG-3 (EMBL-GI) is a genomic clone carrying the gene encoding (1→3)-β-glucanase isoenzyme GI.

The insert of λG-1 was also analysed with restriction enzymes (Figure 2.6). Southern blot analysis showed that only one band (lane 5 was incompletely digested) gave a hybridization signal in the *Sst*II (*Sac*II) restriction digests. One or more restriction sites for this enzyme are commonly detected in the coding region of genomic or cDNA clones for (1→3)-β-glucanase isoenzymes (Xu *et al.*, 1992). The λG-1 restriction patterns of *Eco*RI (>20 kb fragment) and *Hind*III (9.4 kb fragment) did not match those of the genomic clones for (1→3)-β-glucanase isoenzymes GIII (5.8 kb *Eco*RI genomic fragment), GIV (10.2 kb *Hind*III genomic fragment) or GVI (7.3 kb *Eco*RI genomic fragment) which have been isolated previously (Xu *et al.*, 1992). Thus, λG-1 appeared to represent a previously unidentified (1→3)-β-glucanase gene. Further investigations into λG-1 could be concentrated on subcloning, sequencing and expressing this clone to confirm its identity as a gene fragment encoding a (1→3)-β-glucanase gene. This was not undertaken in the present study.

2.3.4 Subcloning into Plasmid pUC19

For the manipulation of the identified genomic clones, plasmids pUC19 and pBluescript SK(+) were employed as vectors because of their high copy number for DNA preparation and their ease of use for double-stranded sequencing.

(1→3,1→4)-β-Glucanase isoenzyme EII gene

The insert of recombinant clone EMBL-EII was analysed by digestion with several restriction enzymes and Southern blotting (Figure 2.8), and this resulted in the identification of fragments that carry the entire gene or smaller fragments suitable for subcloning into plasmid pUC19 for DNA sequencing. The restriction analysis indicated that the gene for (1→3,1→4)-β-glucanase isoenzyme EII was carried on a 9.2 kb *Bam*HI fragment, or on two *Eco*RI fragments of 5.0 kb and 2.0 kb. This restriction pattern differed from that of the genomic clone encoding (1→3,1→4)-β-glucanase isoenzyme EI (Litts *et al.*, 1990; Slakeski, 1992), where the gene was on a 14 kb *Bam*HI fragment or a 12 kb *Eco*RI fragment.

The 9.2 kb *Bam*HI fragment from the recombinant clone EMBL-EII could not be subcloned into pUC19 vector, probably because the fragment was too large. The two *Eco*RI fragments of 5.0 kb (pEII50) and 2.0 kb (pEII20) were successfully subcloned. Primary structure analysis of the gene coding for (1→3,1→4)-β-glucanase isoenzyme EI indicated a large intron of approximately 2.5 kb and a coding region of approximately 1 kb (Slakeski *et al.*, 1990). Another Southern analysis (Figure 3.4) of clone EMBL-EII probed with a DNA fragment designated FP1800, the promoter region of the (1→3,1→4)-β-glucanase isoenzyme EI gene, reveals that the 5.0 kb *Eco*RI fragment is likely to carry the promoter region of the (1→3,1→4)-β-glucanase isoenzyme EII gene. Therefore, it could be concluded that the two *Eco*RI fragments were large enough to include a promoter region, a large intron and the coding region of the (1→3,1→4)-β-glucanase isoenzyme EII gene. It could be also concluded that the 2.0 kb *Eco*RI fragment contained the mature enzyme-coding region of the (1→3,1→4)-β-glucanase isoenzyme EII gene. There were no visible *Eco*RI fragments smaller than 300 bp on the Southern analysis (Figure 2.8). If there were any gaps of small fragments between the two *Eco*RI fragments, a overlapping fragment, like the 3.6 kb *Hin*dIII fragment (Figure 3.4) could be subcloned, or an oligonucleotide could be designed as a sequencing primer for nucleotide sequence linkage of the two

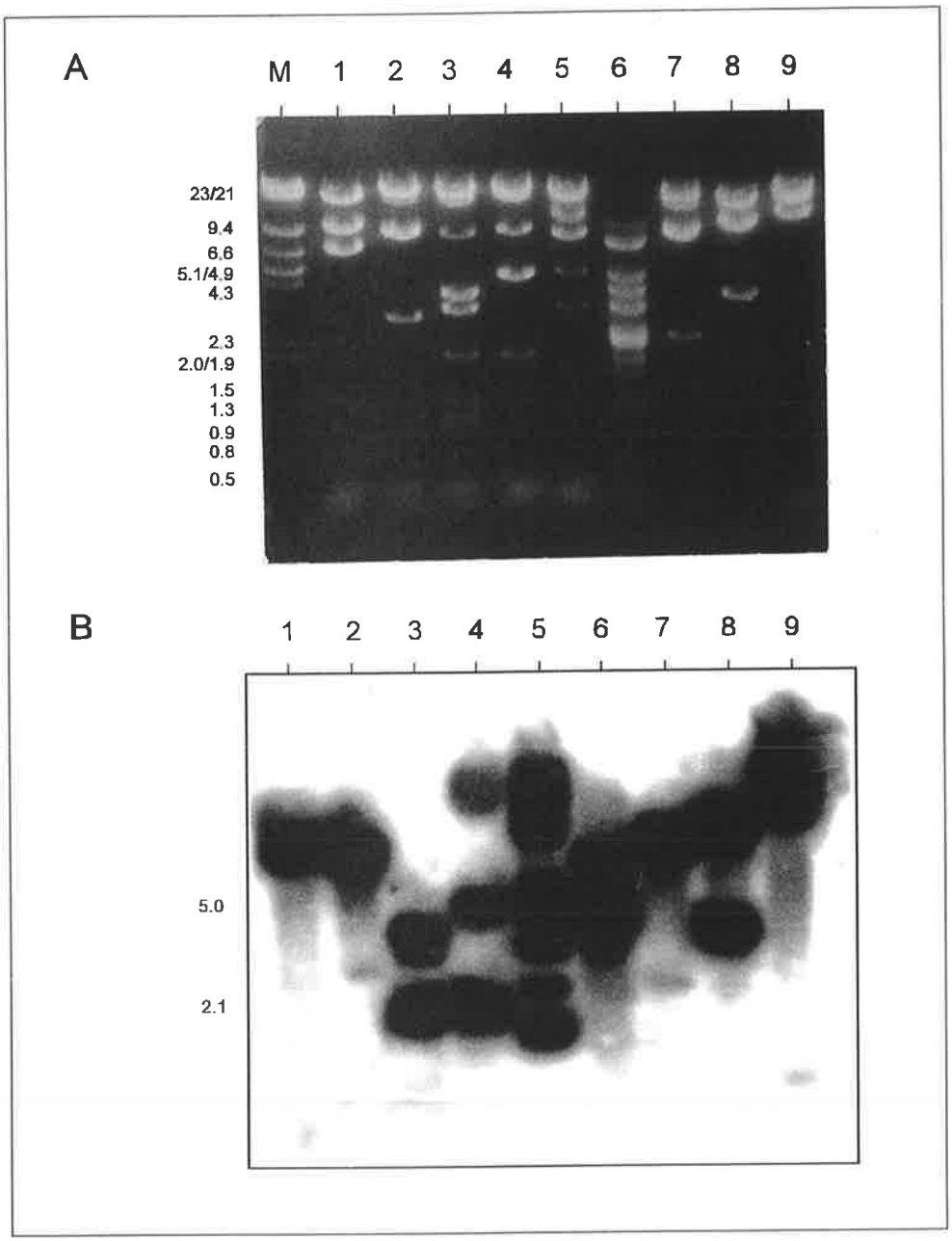


Fig. 2.7 Restriction analysis of the genomic clone EMBL-EII encoding (1→3, 1→4)-β-glucanase isoenzyme EII. EMBL-EII DNA (Part A) was digested with (lane numbers precede restriction enzymes) 1, *Bgl*II; 2, *Bam*HI; 3, *Bam*HI/*Eco*RI; 4, *Eco*RI; 5, *Hin*dIII; 6, *Pst*I; 7, *Sal*I; 8, *Sma*I; 9, *Xba*I. Southern analysis (Part B) was performed using a probe labelled with the cDNA for (1→3,1→4)-β-glucanase isoenzyme EII. Lane M is the DNA size markers.

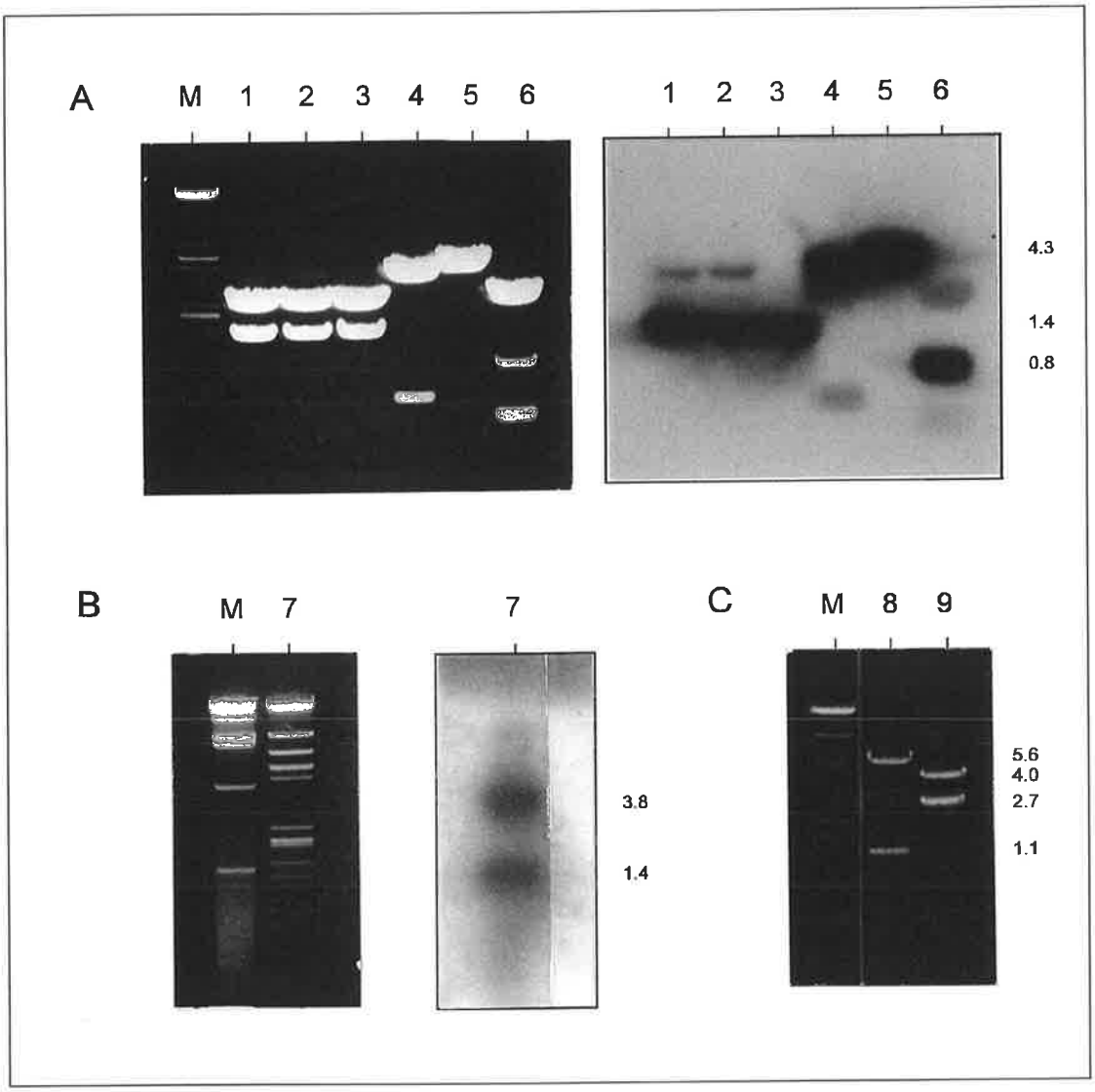


Fig. 2.8 Restriction analysis of EMBL-G1 DNA and a *Sal*I genomic fragment of 1.4 Kb. Plasmid DNA (Part A) was digested with (lane numbers precedes restriction enzymes) 1, *Acc*I; 2, *Sal*I; 3, *Pst*I; 4, *Sac*II; 5, *Sma*I; 6, *Sph*I and probed with (1→3)-β-glucanase isoenzyme GII cDNA. EMBL-G1 DNA (Part B) was digested with *Sal*I (lane 7) and probed with the 2.8 Kb *Pst*I fragment. Part C represents digestion with *Pst*I of the 3.9 Kb *Sal*I fragment subcloned into pUC19, indicating recombinant plasmids with inserts in different orientations, which were designated as pGIS39(−) (lane 8) and pGIS39(+) (lane 9), respectively. Lane M is the DNA size markers (sizes shown in kb).

*Eco*RI fragments.

(1→3)-β-Glucanase isoenzyme GI gene

A 1.4 kb *Sal* I fragment (pGIS14) of the genomic clone for (1→3)-β-glucanase isoenzyme GI was subcloned for DNA sequence analysis (Section 2.3.3). This fragment appeared to be too small to carry all of the coding region, the promoter region and, possibly, a large intron, by comparison with the partial cDNA clone of 1054 bp for (1→3)-β-glucanase isoenzyme GI (Xu *et al.*, 1992). Therefore, a 2.8 kb *Pst*I fragment (pGIP28), identified by Southern analysis among several similarly-sized fragments in the *Pst*I digestion of the EMBL-GI DNA, was also subcloned into pUC19. This fragment could be used as a probe for detecting overlapping fragments. The restriction analysis of the subcloned *Pst*I fragment subsequently showed that it included the 1.4 kb *Sal*I fragment (Figure 3.1). Southern analysis of the recombinant EMBL-GI DNA, digested with *Sal*I and probed with the 2.8 kb *Pst*I fragment (Figure 2.8), indicated a *Sal*I fragment of 3.9 kb that probably carries the promoter region of the gene and possibly a large intron of the type detected in other genes for (1→3)-β-glucanase isoenzymes. This *Sal*I fragment was successfully subcloned, and plasmids with inserts in different orientations, pGIS39(−) and pGIS39(+) were selected for sequencing analysis (Figure 2.8).

2.4 CONCLUSIONS

In this work, two barley genomic clones have been isolated from a genomic library. The clone for (1→3)-β-glucanase isoenzyme GI is now confirmed by its nucleotide sequence and is described in detail in Chapter IV. The clone for (1→3, 1→4)-β-glucanase isoenzyme EII, which hybridized with the gene-specific oligonucleotide designed from the corresponding cDNA clone is to be confirmed by nucleotide sequencing as described in Chapter III. No positive clone was identified for (1→3)-β-glucanase isoenzyme GII, although hybridization conditions had been designed to isolate this gene and clones representing more than 8 times the total barley

genome were screened in the course of this work. In other work, an acidic (1→3)-β-glucanase isoenzyme has been recently isolated from the same commercial barley genomic library using the isoenzyme GII cDNA probe (Malehorn *et al.*, 1993). These results suggest that the library may not carry the gene for isoenzyme GII.

CHAPTER THREE

IDENTIFICATION OF A GENE FOR (1→3,1→4)-β-GLUCANASE ISOENZYME EII

3.1 INTRODUCTION

The primary structure of barley (1→3,1→4)-β-glucanase isoenzyme EII has been defined from a partial cDNA clone prepared from gibberellic acid (GA_3) treated aleurone layers, and amino acid sequences of several tryptic peptides obtained from the purified protein (Fincher *et al.*, 1986). The sequence was confirmed later by a near full length cDNA clone (Slakeski *et al.*, 1990). Subsequently, the isolation of the cDNA clone and genomic clone for isoenzyme EI enabled the structure of the isoenzyme EI gene to be determined (Litts *et al.*, 1990; Slakeski *et al.*, 1990), together with the examination at a transcriptional level of tissue-specific regulation of the expression of the genes for the two isoenzymes by various phytohormones (Slakeski *et al.*, 1990; Litts *et al.*, 1990; Slakeski & Fincher, 1992a, b).

Although the two (1→3,1→4)-β-glucanase isoenzymes share 92% positional identity at both the nucleotide and amino acid sequence levels (Slakeski *et al.*, 1990), it has been observed that the two genes exhibit different developmental and hormonal regulation in various barley tissues; the gene for isoenzyme EI is transcribed at relatively high levels in young leaves and in the scutellum of germinated grain, while the transcription of the isoenzyme EII gene is restricted to the aleurone layer of germinated grain (Stuart *et al.*, 1986; Slakeski *et al.*, 1990; Slakeski & Fincher, 1992a, b). Thus, isoenzyme EII appears to be germination-specific. In view of the different expression patterns of the two isoenzymes, further studies were focussed on the investigation of the promoter regions and introns of the two genes, to identify *cis*-acting sequence elements which might function in gene regulation (Messing *et al.*, 1983; Kozak, 1984; Dean *et al.*, 1986; Joshi, 1987a, b; Lütcke *et al.*, 1987; Nussinov, 1990).

When this work was initiated, the gene for barley (1→3,1→4)-β-glucanase isoenzyme EI had been isolated and characterized (Slakeski *et al.*, 1990), but the isolation of the gene for isoenzyme EII had not been reported. A barley genomic library was therefore screened for the latter gene and a genomic clone that probably

encoded this gene was identified (Chapter II). It was expected that sequence analysis of the promoter region of the gene and its intron might allow the identification of regulatory elements important in hormone responsiveness and tissue specificity (Ou-Lee *et al.*, 1988; An *et al.*, 1990; Conner *et al.*, 1990; Guiltinan *et al.*, 1990; Huang *et al.*, 1990; Skriver *et al.*, 1991).

In this chapter, the identification and structure of the gene encoding (1→3,1→4)-β-glucanase isoenzyme EII is described. The sequence is confirmed with the corresponding cDNA clone (Slakeski *et al.*, 1990).

3.2 MATERIALS AND METHODS

3.2.1 Materials

The Sequenase™ dideoxynucleotide sequencing kit was from USBC (Cleveland, Ohio, USA). Polyacrylamide and bisacrylamide were from Merck (Darmstadt, Germany). Ammonium persulphate and analytical grade mixed bed resin [AG 501-X8(D)] were obtained from Bio-Rad (Richmond, CA, USA). [α-^{35}S]ATP (1,000 Ci/mmol) was from Bresatec (Adelaide, Australia). The GeneClean-II kit was from BIO 101 Inc. (La Jolla, CA, USA). X-ray film and autoradiography cassettes were purchased from Amersham (England, UK). Restriction enzymes, T4 ligase and TEMED were purchased from Promega (Madison, WI, USA).

3.2.2 Restriction Mapping

The restriction analysis of the fragments carrying the gene encoding (1→3,1→4)-β-glucanase isoenzyme EII was performed in more detail than described in Chapter II, to facilitate subsequent DNA sequencing. The fragments were excised from plasmids with a variety of commonly-used restriction enzymes. Where multiple enzyme digestion was necessary, the digest was performed either in one step with different restriction enzymes together in an optimal buffer, or in multiple steps. The resulting restriction fragments were separated on 1% agarose gels with molecular markers consisting of *Hin*dIII and *Hin*dIII/*Eco*RI digests of Lambda DNA. The fragments from all digestions were aligned and a unambiguous and internally consistent restriction map for the gene was constructed.

3.2.3 DNA Sequencing

Double stranded DNA sequencing protocols were based on the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) and were modified as described by Chen & Seeburg (1985). Inserts from recombinant plasmids were subjected to restriction enzymes digestion and fragments were subcloned into pUC19 or pBluescript SK(+) vectors. DNA sequencing templates were prepared according to the large-scale

alkaline extraction procedure (Birnboim & Doly, 1979) and further purified by CsCl density gradient ultracentrifugation (Sambrook *et al.*, 1989). M13 forward and reverse primers (Promega) or specific oligonucleotides were used as sequencing primers. DNA templates (5 μg per reaction) were denatured with 0.2 M NaOH/1 mM EDTA for 15 minutes and annealed with sequencing primers at 65°C for 2 minutes, followed by a period during which temperature slowly decreased to 30°C over 20 minutes. Radioactive labelling of the DNA with [α-^{35}S]ATP and the termination reactions were performed with the T7 polymerase supplied in the Sequenase™ Version 2.0 Kit (USB), according to the manufacturer's instructions. Sequencing reactions were separated on 6% polyacrylamide gels on a DNA sequencer (IBI, Pustell). Nucleotide sequence was analysed with the aid of a computer analysis program (IBI, Pustell).

3.3 RESULTS AND DISCUSSION

3.3.1 Detailed Restriction Map

Two *Eco*RI subclones, pEII50 and pEII20 (Section 2.3.4) of the genomic clone for (1→3,1→4)-β-glucanase isoenzyme EII were analysed by single and double restriction digestions (Figure 3.1). The alignment of the two fragments was determined using a *Bam*HI site and a *Hin*dIII fragment of 3.6 kb from the restriction analysis of EMBL-EII (Section 2.3.4), and by the restriction sites on the corresponding cDNA (Doan, 1992). A restriction map of the gene for (1→3,1→4)-β-glucanase isoenzyme EII was constructed (Figure 3.2) and compared with that of its corresponding cDNA clone. This showed that pEII20 contained the entire coding region of the mature enzyme, the 3' untranslated region, and part of an intron near its 5' end. As the nucleotide sequence of the (1→3,1→4)-β-glucanase isoenzyme EI gene indicated the presence of a large intron of 2514 bp (Litts *et al.*, 1990; Slakeski *et al.*, 1990), pEII50 is likely to contain most of the intron, the signal peptide-coding region and the 5' untranslated region. This has subsequently been confirmed by R Kalla, JV Jacobsen & GB Fincher (unpublished data). The restriction map was also compared with that of isoenzyme EI, and it was clear that the restriction maps are quite different (Slakeski, 1992).

3.3.2 Nucleotide Sequence

The subclone pEII20 was sequenced from both ends with forward and reverse M13 primers. The nucleotide sequence of 300 bp obtained with the forward primer was identical to the 3' end of the cDNA clone for (1→3,1→4)-β-glucanase isoenzyme EII (Figure 3.3). As sequence comparisons of the cDNA clones for (1→3,1→4)-β-glucanase isoenzymes EI and EII revealed a marked divergence in their 3' untranslated region (Slakeski *et al.*, 1990), this allowed the conclusion that the subclones pEII20 and pEII50 carry the gene for (1→3,1→4)-β-glucanase isoenzyme EII. Furthermore, the amino acid sequence deduced from pEII20 nucleotide sequence matched the COOH-terminal sequence of (1→3,1→4)-β-glucanase isoenzyme EII (Figure 3.3). Further sequencing of pEII20 and pEII50 inserts was abandonned when a 5159 bp nucleotide

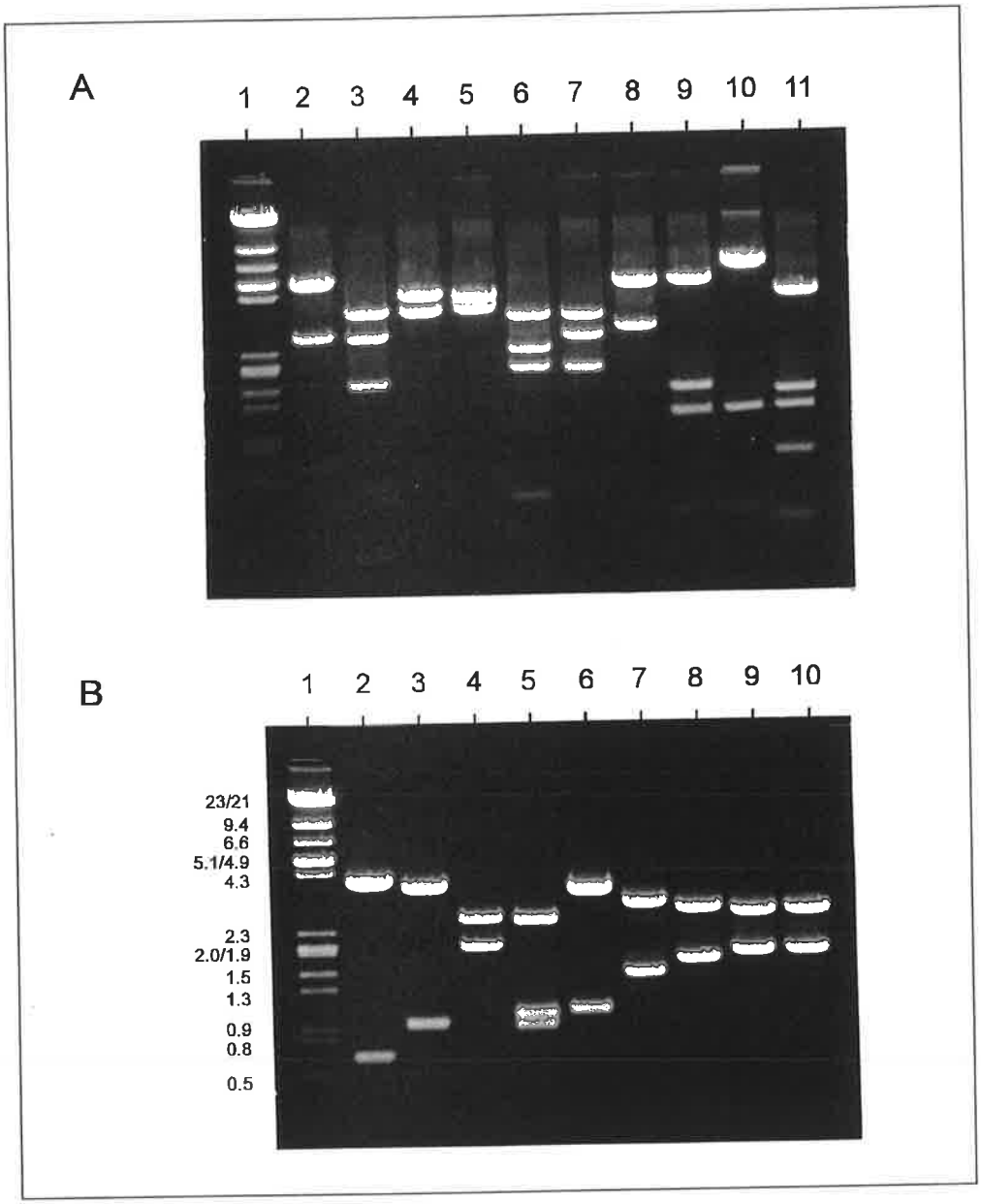


Fig. 3.1 Restriction analyses of pEII50 and pEII20 subclones for (1→3,1→4)-β-glucanase isoenzyme EII gene. Part A represents pEII50 digested with (lane number precedes restriction enzyme) 2, *Eco*RI; 3, *Eco*RI/*Pst*I; 4, *Pst*I; 5, *Bam*HI; 6, *Bam*HI /*Kpn*I; 7, *Kpn*I; 8, *Hin*dIII; 9, *Hin*dIII/*Xba*I; 10, *Xba*I; 11, *Sph*I. Part B represents pEII20 digested with 2, *Hin*dIII; 3, *Sph*I; 4, *Eco*RI; 5, *Eco*RI/*Pst*I; 6, *Pst*I; 7, *Kpn*I; 8, *Sma*I; 9, *Acc*I; 10, *Sac*I. Lane 1 was loaded with the DNA markers (sizes shown in kb), an equal combination of *Hin*dIII/*Eco*RI and *Hin*dIII lambda DNA digests.

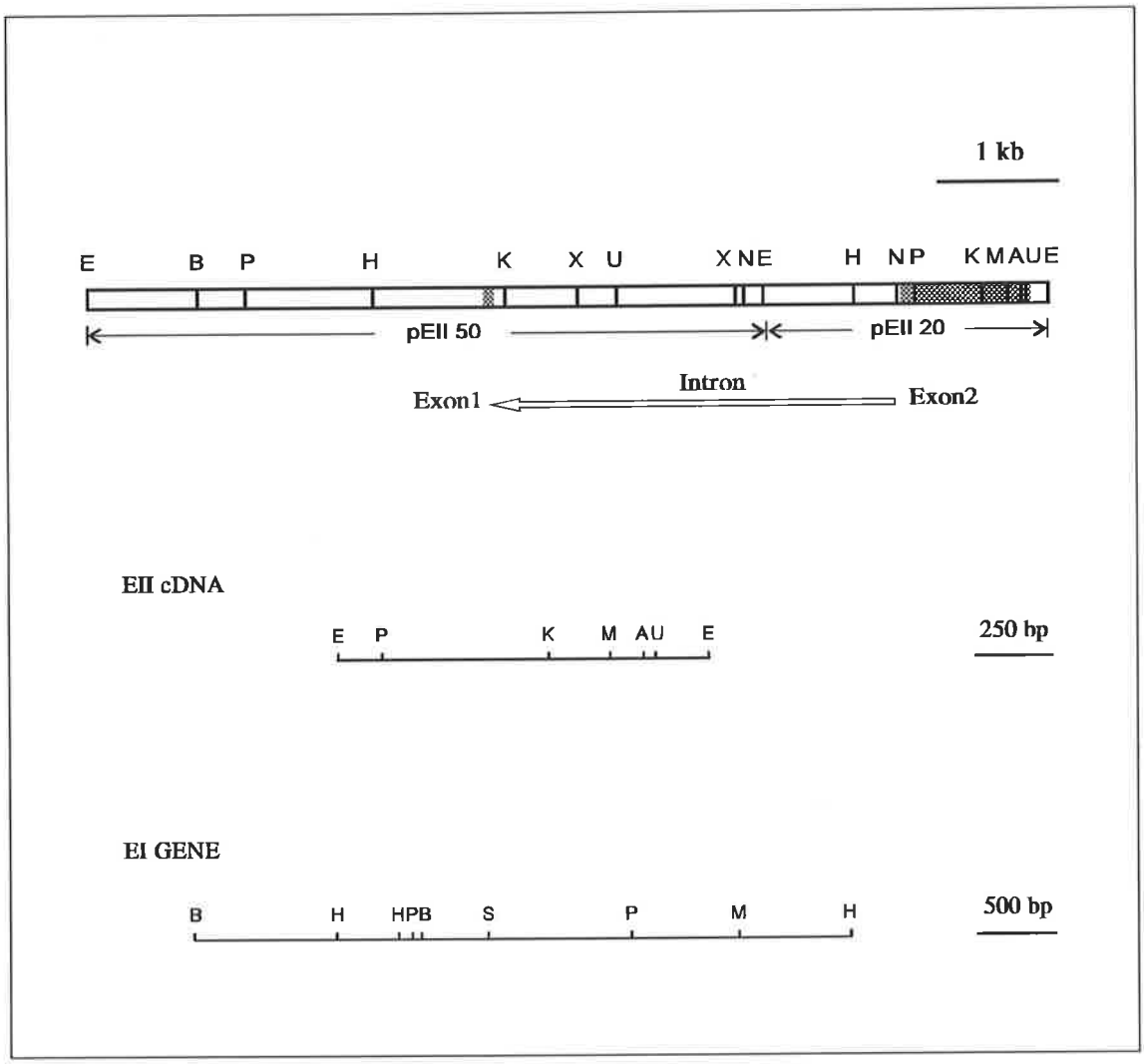


Fig. 3.2 Restriction maps of a 7.0 Kb fragment containing the gene encoding (1→3,1→4)-β-glucanse isoenzyme EII, compared with those of the cDNA clone for (1→3,1→4)-β-glucanse isoenzyme EII (Doan, 1992) and the gene for (1→3,1→4)-β-glucanse isoenzyme EI (Slakeski *et al.*, 1990). Restriction enzymes are designated as follows: A, *Acc*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; M, *Sma*I; N, *Sph*I; P, *Pst*I; S, *Sal*I; T, *Sac*II; U, *Sac*I. The large shaded region indicates the location of the mature enzyme coding region (exon2) and the short shaded region indicates the signal peptide coding region, which is located on exon1. The signal peptide coding region (exon1) and the intron location are determined from the 5159 bp nucleotide sequence for the entire gene (Wolf, 1991).

Figure 3.3

```
pEII20  CCCCACGCCA CCCGGGCGCC ATCGAGACCT ACATCTTCGC CATGTTCAAC GAGAACCAGA AGGACAGCGG CGTGGAGCAG
           P  R  H   P  G  A   I  E  T   Y  I  F  A   M  F  N   E  N  Q   K  D  S  G   V  E  Q
EI                                        V     S                          N

pEII20  AACTGGGGAC TCTTCTACCC CAACATGCAG CACGTCTACC CCATCAACTT CTGACGGAGC TCGTGCTCGT TAAGTCCCTA
         N  W  G   L  F  Y  P   N  M  Q   H  V  Y   P  I  N  F
EI                                                    S

pEII20  CTTGTTCTTG TTAACGAGTA AAAAGTCATG TTACGCGAAC TTGACGAGCT ACTCGTTTGG AGAGCCTGTT AATTACCTCC

pEII20  TCTTTCCACA TGAGGGATGA GAACGTATGA GTTAATAACC AGACCCCATT ACTGTGAATT
                                          ↑
```

Fig. 3.3 Partial nucleotide sequence of the gene encoding (1→3,1→4)-β-glucanase isoenzyme EII. The nucleotide and amino acid sequence of the genome fragment corresponded exactly to the sequences obtained from the cDNA clone for isoenzyme EII (Fincher *et al.*, 1986; Slakeski *et al.*, 1990). The deduced amino acid sequence is aligned with the (1→3,1→4)-β-glucanase isoenzyme EI sequence, showing differences only. The underlined sequence is the putative polyadenylation signal (AATAAC). The arrow shows the putative polyadenylation addition point, determined from the polyadenylated cDNA clone.

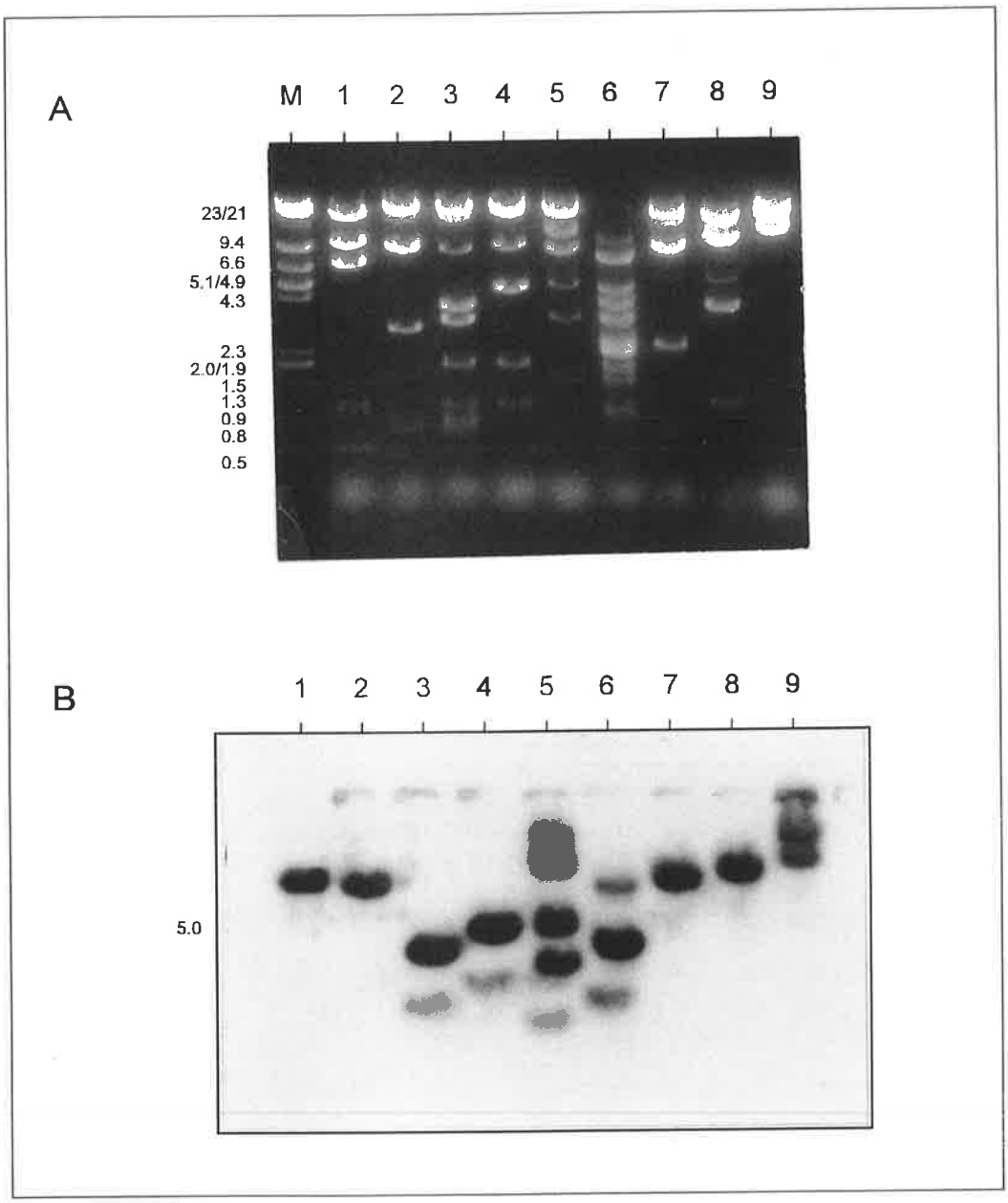


Fig. 3.4 Southern analysis of EMBL-EII DNA carrying the gene encoding (1→3, 1→4)-β-glucanase isoenzyme EII. Part A presents EMBL-EII DNA digested with 1, *Bgl*II; 2, *Bam*HI; 3, *Bam*HI/*Eco*RI; 4, *Eco*RI; 5, *Hin*dIII; 6, *Pst*I; 7, *Sal*I; 8, *Sma*I; 9, *Xba*I. Lane M was loaded with the DNA size markers (sizes shown in kb). Part B represents Southern blot analysis probed with FP1800, the promoter region of (1→3, 1→4)-β-glucanase isoenzyme EI, indicating that the *Eco*RI fragment of 5.0 Kb contains the promoter region of the isoenzyme EII gene, and revealing that the EII promoter region has relatively high homology with that of isoenzyme EI.

sequence of a genomic clone for (1→3,1→4)-β-glucanase isoenzyme EII was reported in the literature (Wolf, 1991).

3.3.3 Analysis of the Promoter Region

During the subcloning of the genomic clone of the gene for isoenzyme EII, the similarity between the promoter regions of the two (1→3,1→4)-β-glucanase genes was also investigated. The similarity was first demonstrated by Southern analysis of the genomic clone for (1→3,1→4)-β-glucanase isoenzyme EII (Figure 3.4). EMBL-EII (Section 2.3.2) was digested with restriction enzymes and hybridized with a probe prepared using the random primer method (Amersham) from a clone designated FP1800. This clone contained the promoter region of the isoenzyme EI gene, starting from the transcription start point and including 1800 bp of the upstream region of the promoter (Baulcombe, DC & Fincher, GB, unpublished data). Strong hybridization signals were observed, even when filters were washed at high stringency. This suggested a high degree of positional similarity between the promoter regions of the two (1→3,1→4)-β-glucanase genes (Figure 3.4). Later on, alignment of the nucleotide sequences of the promoter regions of the two isoenzyme genes indicated two highly conserved regions of 380 bp and 160 bp, upstream from the TATA boxes (Wolf, 1992). These were separated by a 150 bp region only observed in the isoenzyme EI gene, and no similarity was observed in regions further upstream. Relative high positional identity was also observed in the region between the TATA boxes and the translation start points of the two genes (Wolf, 1992).

3.4 CONCLUSIONS

The gene encoding the barley (1→3,1→4)-β-glucanase isoenzyme EII has been isolated and its identity confirmed by comparison with the full length cDNA (Slakeski *et al.*, 1990) and the COOH-terminal sequence of (1→3,1→4)-β-glucanase isoenzyme EII. Because the complete sequence for the gene was published by Wolf (1991), the emphasis of this work was moved to the characterization of the gene for (1→3)-β-glucanase isoenzyme GI (described in the next chapter).

CHAPTER FOUR

CHARACTERIZATION OF A GENE ENCODING (1→3)-β-GLUCANASE ISOENZYME GI

4.1 INTRODUCTION

(1→3)-β-Glucanase isoenzyme GI has been purified from germinated barley grain and characterized (Høj *et al.*, 1988). Amino acid sequence of the first 35 amino acids from the NH_2-terminus corresponded exactly with that deduced from a cDNA clone which had been isolated from a cDNA library constructed from poly(A)$^+$-RNA of both young roots (5 days old) and young leaves (10 days old) (Xu *et al.*, 1992). The cDNA clone is not full length and lacks information on both the 5' untranslated region and the sequence of the first few amino acid residues (Xu *et al.*, 1992). The (1→3)-β-glucanase isoenzymes GI and GII exhibit 78% positional identity at the amino acid level, and 86% at the nucleotide level (Xu *et al.*, 1992). The enzymes have similar kinetic properties and share the same action pattern (Hrmova & Fincher, 1993), indicating their extremely close relationship in the evolution of the barley (1→3)-β-glucanase gene family (Xu *et al.*, 1992).

The functional significance of (1→3)-β-glucanase isoenzyme GI is not yet clear. It has been demonstrated that (1→3)-β-glucanases of acidic (intracellular) or basic (vacuolar) isoforms are detected amongst pathogenesis-related (PR) proteins in higher plants, and that their activities can also be measured in healthy tissues (Kauffmann *et al.*, 1987; Jutidamrongphan *et al.*, 1991; Xu *et al.*, 1992; Beerhues & Kombrink, 1994). (1→3)-β-Glucanases have been implicated in the signalling process that leads to the hypersensitive response in plants, and probably participate by releasing elicitors from fungal cell walls (Mauch & Staehelin, 1989; Lamb *et al.*, 1989; Takeuchi *et al.*, 1990). Sequence analysis of cDNA and genomic clones has confirmed the close similarities between the acidic and basic isoforms of (1→3)-β-glucanase in plants (Meins *et al.*, 1992). In barley, both (1→3)-β-glucanase isoenzyme GII and the acidic isoenzyme *Abg2*, which has been designated isoenzyme GVII in this thesis, are expressed in response to fungal infection, induction of stress, and developmental signals (Xu *et al.*, 1992; Malehorn *et al.*, 1993). No evidence is available to indicate whether or not the (1→3)-β-glucanase isoenzyme GI responds to fungal infection (Xu *et al.*, 1992), to

developmental signals or to other stresses. In tobacco, only certain classes of (1→3)-β-glucanases have been identified as pathogenesis-related proteins (Sela-Buurlage *et al.*, 1993), suggesting that the (1→3)-β-glucanase gene family in barley may encode isoenzymes with functions other than those required to protect the plant against pathogen attack.

In the present work, the primary structure of the gene encoding (1→3)-β-glucanase isoenzyme GI is described, and comparisons with its corresponding cDNA and other members of the (1→3)-β-glucanase gene family have been undertaken. The potential physiological function of the enzyme during grain germination and development of barley seedlings is discussed.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Barley grain (*Hordeum vulgare*, cv. Clipper) was obtained from the Victorian Institute of Dryland Agriculture, Horsham, Victoria, Australia. "Erase-a-base" system (Klenow DNA polymerase, S1 nuclease, exonuclease III, T4 ligase), restriction enzymes, terminal deoxynucleotidyl-transferase, AMV reverse transcriptase, *Taq* polymerase and dNTPs, and sequencing primers were from Promega (Madison, WI, USA). The PRISM™ Ready Reaction DyeDeoxy™ Terminators sequencing Kit, a model 373A DNA Sequencer and the DNA sequence analysis program SeqEd® were from Applied Biosystems, Inc. (USA). DNA Thermal cycler and mineral oil were from Perkin Elmer Pty Ltd (PEC, USA). DEPC, PEG (8000) and CTAB were from Sigma Chemical Co. (St. Louis, MO, USA). Oligonucleotides were synthesized on a model 381A DNA synthesiser from Applied Biosystems, Inc. (USA). [^{32}P]-α-dCTP (3,000 Ci/mmol) was obtained from Bresatec (Adelaide, Australia). Speed Vac® concentrator SCII was from Savant Instruments Inc. (Farmingdale, NY, USA).

4.2.2 DNA Truncation

For nucleotide sequence analysis, unidirectional deletions of the DNA insert of the desired gene were prepared according to the procedures recommended by the manufacturer (Promega), using the "Erase-a-Base" system. Supercoiled plasmid DNA (10-20 μg) was double-digested with restriction enzymes to generate two terminal protrusions: one restriction site for protecting the primer binding site and the other susceptible to exonuclease III. Alternatively, to produce 3' terminal resistance to exonuclease activity, plasmid DNA was cleaved with restriction enzymes and treated with α-phosphorothioates and the Klenow DNA polymerase fragment. The digested plasmid DNA (1-2 μg) was incubated with an appropriate amount of exonuclease III (200-400 units) at 37°C and samples were removed at 40 second intervals. The single-stranded tails of the plasmid DNA samples were removed with S1 nuclease and flushed terminals were created with the Klenow DNA polymerase fragment. The DNA was

subjected to blunt-end ligation and transformation into *E. coli* DH5α competent cells. The recombinant plasmids were selected by screening the resulting subclones from each time point. The inserts of the plasmids were 250-300 bp difference in size and therefore convenient for overlapping sequences.

4.2.3 Preparation of DNA Template for Nucleotide Sequencing

DNA template for sequencing reactions was prepared from 10 ml overnight cultures of Terrific Broth (17 mM KH_2PO_4, 72 mM K_2HPO_4, 12% bacto-tryptone, 24% bacto-yeast extract, 4.0% glycerol by volume) according to the procedure described as section 2.2.10, incorporating an PEG 8000 precipitation at the final step. Plasmid DNA (40 µl) prepared by the alkaline-lysis method was mixed with 10 µl 4 M NaCl and 50 µl 13% PEG 8000 and kept on ice for 20 minutes. The DNA pellet was recovered by centrifugation, and the aqueous phase was discarded. High-quality DNA, of which approximately 90% was in the supercoiled form, was washed twice with 500 µl cold 70% ethanol, dried in air and dissolved in sterile H_2O at a concentration of 250 ng/µl.

4.2.4 DNA Sequencing

The Cycle Sequencing procedure was used for double-stranded DNA sequencing as described by the manufacturer (Applied Biosystems). Plasmid DNA (1µg) was mixed with a primer, a pre-mixed cocktail of dye-labelled dideoxynucleotide terminators, *Taq* polymerase, and reaction buffer in a small Eppendorf tube. The mixture was overlaid with one drop of mineral oil and placed in a DNA thermal cycler pre-warmed to 96°C. The sequencing reaction was started immediately and performed under the following conditions: denaturation at 96°C for 30 seconds, annealing at 50°C for 15 seconds and extension at 60°C for 4 minutes for a total of 25 cycles. To remove excess terminators and reduce the volume for gel loading, the reaction mixture was transferred to a fresh tube and precipitated with 5% CTAB/0.5 M NaCl. The supernatant was removed and the pellet was resuspended in 1.2 M NaCl. The DNA was precipitated with two

volumes of absolute ethanol and washed twice with 70% ethanol. The DNA pellet was dried and redissolved in loading buffer. The sample was loaded onto an Applied Biosystems 373A DNA sequencer and run for 8 hours. Nucleotide sequence data generated by the automatic sequencer were modified manually and aligned using the SeqEd™ V 1.03 software package (Applied Biosystems, USA).

4.2.5 Preparation of Total RNA

Barley grains were surface-sterilized with 2.5% sodium hypochlorite for 20 minutes, washed with 70% ethanol for 5 minutes, and soaked with sterile water containing 100 μg/ml neomycin, 100 μg/ml chloramphenicol and 100 units/ml nystatin for 16 hours at room temperature. The grains were germinated on Whatman 3MM papers moistened with sterile water in the dark at 22°C for 5 days to 10 days. Tissues for RNA preparation were removed and immediately frozen in liquid nitrogen.

Total RNA was isolated, with minor modifications, by the procedure of Verwoerd *et al.* (1989). Tissues were ground to a fine powder in liquid nitrogen and transferred to pre-cooled Eppendorf tubes. To the tube was added 500 μl phenol homogenization buffer (phenol equilibrated with an equal volume of 0.1 M LiCl, 100 mM Tris-HCl buffer, pH 8.0, 10 mM EDTA, 1% SDS); the phenol buffer was pre-heated to 80°C. The suspension was thoroughly mixed and 250 μl chloroform/isoamylalcohol (24:1) was added with additional mixing. After centrifugation for 5 minutes, the aqueous phase was transferred to a fresh tube and precipitated with an equal volume of 4 M LiCl at -20°C for 16 hours. The RNA pellet was recovered by centrifugation at 4°C for 20 minutes and dissolved in 250 μl H_2O. The RNA solutions were mixed with 0.1 volume cold 3 M sodium acetate (pH 5.2) and precipitated with 2 volumes ice cold ethanol. After centrifugation the RNA pellet was washed with 70% ethanol, dried under a stream of N_2 and redissolved in H_2O.

4.2.6 Amplification of the 5' End of mRNA

In an attempt to identify the transcription start point and to define the

5' untranslated region of the (1→3)-β-glucanase isoenzyme GI gene, RNA was amplified using the RACE protocol (Frohman *et al.*, 1988). Total RNA (200 ng–1 μg) was reverse transcribed with a gene-specific primer (15 ng) in the presence of AMV reverse transcriptase (15 units) and [^{32}P]-α-dCTP (10 μCi) at 42°C for 1 hour. Excess primer was removed from the reaction mixture on a 2 ml column of Sephadex G100 equilibrated with TE buffer (10 mM Tris-HCl buffer, pH 8.0; 1 mM EDTA), and concentrated into 20 μl on a Speed Vac concentrator (Savant Instruments Inc., Farmingdale, NY, USA). The transcript (10 μl aliquot) was tailed with poly(C) by incubation with 6 μl 1 mM dCTP and 10 units terminal deoxynucleotidyl transferase (Promega) at 37°C for 10 minutes, and at 65°C for 15 minutes. The resulting product was diluted to 100 μl and 1–10 μl was used for amplification. The first strand cDNA was amplified by PCR with an isoenzyme GI gene-specific primer (15 pmol) and a poly(G) primer (25 pmol) under the following conditions: 3 thermal cycles (quick ramp to 94°C for 40 seconds; quick drop to 55°C for 60 seconds; ramp 120 second to 72°C for 60 seconds) and 25 step cycles (94°C 40 seconds; 55°C 60 seconds; 72°C 60 seconds) on a PEC thermal cycler. The PCR products with blunt ends were subcloned into the T-vector prepared from pBluescript SK(+) and boundaries confirmed by DNA sequencing.

The T-vector was constructed for direct cloning of blunt-ended PCR products according to the procedure described by Marchuk *et al.*(1990). Plasmid pBluescript SK(+) (20 μg) was digested with *Eco*RV restriction enzyme at 37°C, and incubated with *Taq* polymerase (20 units) in 20 μl buffer containing 10 mM Tris-HCl buffer, pH 8.3, 50 mM KCl, 1.5 mM Mg_2Cl, 200 μg/ml BSA, 2 mM dTTP at 70°C for 2 hours. The plasmid was purified by extraction with chloroform and precipitation with ethanol.

4.3 RESULTS AND DISCUSSION

4.3.1 Restriction Mapping Genomic Inserts

The plasmids, pGIS39(+) and pGIP28 (Section 2.3.4), containing fragments from the genomic clone for (1→3)-β-glucanase isoenzyme GI, were analysed by multiple digestions with restriction enzymes (Figure 4.1). A restriction map of a 5.3 kb fragment containing the gene encoding (1→3)-β-glucanase isoenzyme GI was constructed (Figure 4.2), indicating that the genomic DNA fragment is long enough to include the structural gene and regulatory elements of the promoter. Comparison of the restriction map with that of the (1→3)-β-glucanase isoenzyme GI cDNA clone (Xu *et al.*, 1992) reveals that the entire coding region of the mature (1→3)-β-glucanase isoenzyme is located on the 1.4 kb *Sal*I fragment, which is included in subclone pGIP28. The restriction map enabled a sequencing strategy to be devised (Figure 4.3) and could be useful for confirmation of nucleotide sequence data.

4.3.2 Generation of Subclones for DNA Sequencing

The fragments of the gene encoding (1→3)-β-glucanase isoenzyme GI for DNA sequencing were prepared by restriction enzyme digestions or exonuclease DNA deletions. The pGIS14 (1.4 kb *Sal*I fragment) had previously been cut into three parts (Section 2.3.3); a 400 bp *Sac*II fragment, a 500 bp *Sal*I/*Sac*II fragment and a 550 bp *Sac*II/*Sal*I fragment. To generate subclones representing the 5' region of the gene, a *Sal*I/*Pst*I fragment of 2.6 kb (pGISP26) and a *Pst*I/*Sal*I fragment of 1.4 kb (pGIPS14) were excised from pGIS39(–) and pGIS39(+) (Section 2.3.4), respectively (Table 4.1). The restriction map indicated that these two fragments were linked by an *Eco*RI-ended 2.9 kb *Sal*I/*Eco*RI fragment derived from pGIS39(+). A total of ten subclones from pGISP26 and five subclones from pGIPS14 were selected from exonuclease III deletion products (Table 4.1).

4.3.3 Nucleotide Sequence

A 5.3 kb genomic DNA fragment for (1→3)-β-glucanase isoenzyme GI was

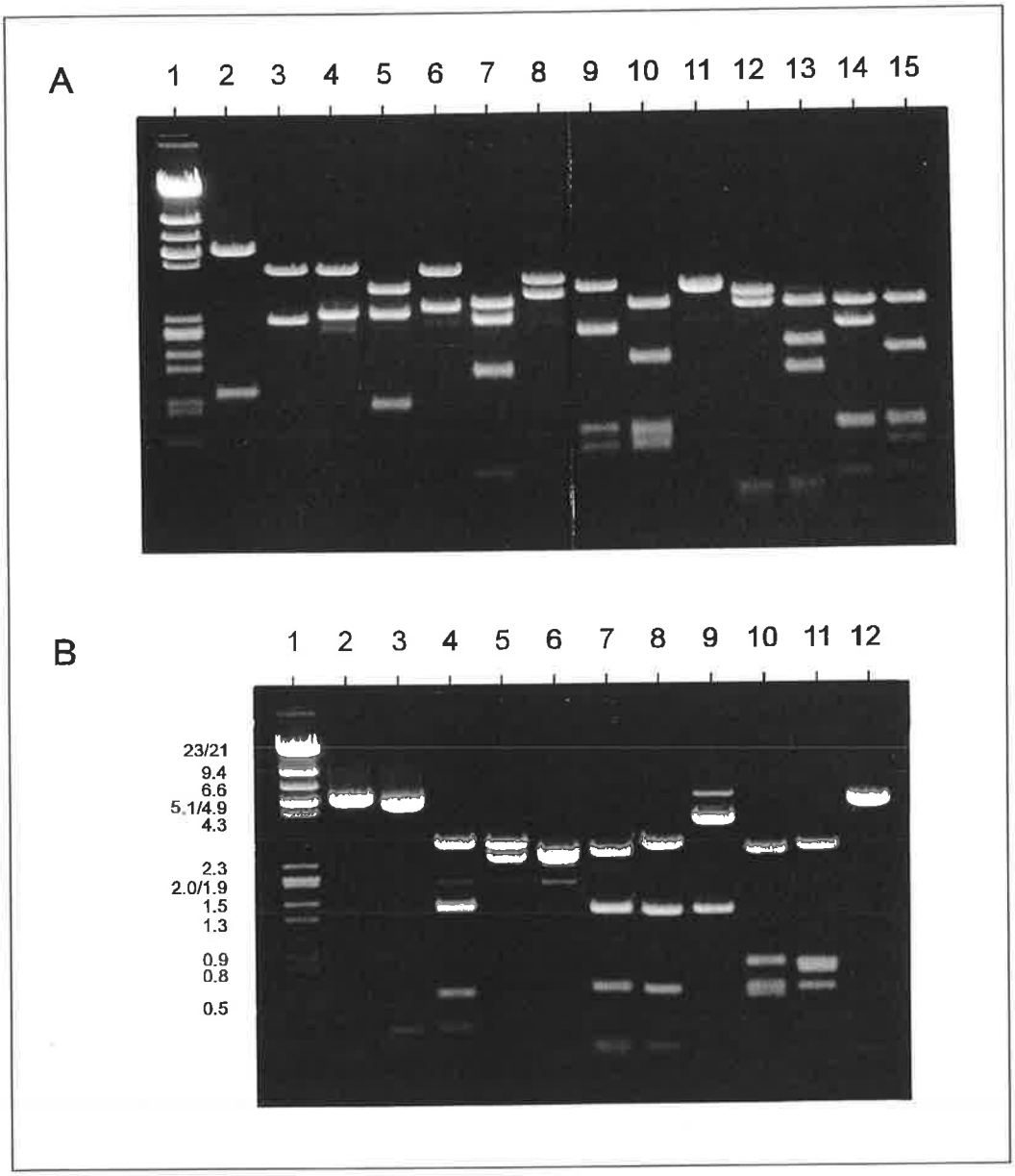


Fig. 4.1 Restriction analyses of pGIS39(+) and pGIP28 subclones of the gene encoding (1→3)-β-glucanase isoenzyme GI. Part A represents pGIS39 digested with 2, *Eco*RI; 3, *Hin*dIII; 4, *Kpn*I; 5, *Kpn*I/*Bam*HI; 6, *Pst*I; 7, *Sma*I/*Pst*I; 8, *Sma*I; 9, *Sph*I; 10, *Sph*I/*Bam*HI; 11, *Bam*HI; 12, *Sac*I; 13, *Sac*I/*Pst*I; 14, *Acc*I; 15, *Acc*I/*Bam*HI. Part B represents pGIP28 digested with 2, *Bam*HI; 3, *Sac*II; 4, *Sac*II/*Eco*RI; 5, *Eco*RI; 6, *Eco*RI/*Pst*I; 7, *Acc*I/*Pst*I; 8, *Acc*I; 9, *Sal*I; 10, *Sph*I/*Sal*I; 11, *Sph*I; 12, *Sac*I. Lane 1 is DNA size markers (sizes shown in kb), combined *Hin*dIII/*Eco*RI and *Hin*dIII digests of lambda DNA.

Figure 4.2

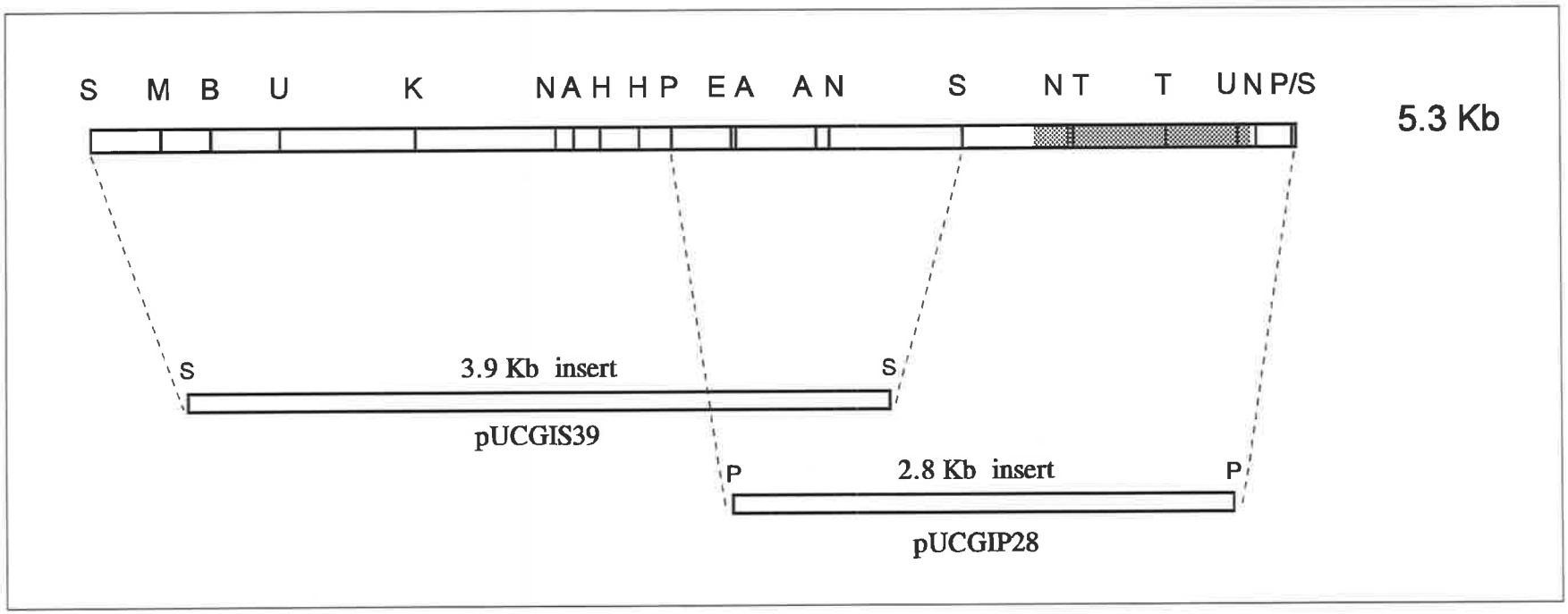


Fig 4.2 Restriction map of a 5.3 Kb genomic fragment representing the gene for (1→3)-β-glucanase isoenzyme GI. Recombinant plasmids pGIS39 and pGIP28 were digested with restriction enzymes designated as follows: A, *Acc*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; M, *Sma*I; N, *Sph*I; P, *Pst*I; S, *Sal*I; T, *Sac*II; U, *Sac*I. The shadow region indicates the location of the mature enzyme encoding region.

Table 4.1 Deletions of Fragments for DNA Sequencing

	pGISP26	pGIPS14
Size (kb)	2.6	1.4
Direction	5'→3'	3'→5'
Primer protection site	*Eco*RI	*Kpn*I
Insert deletion site	*Xba*I	*Sal*I
Subclones	10	5

Figure 4.3

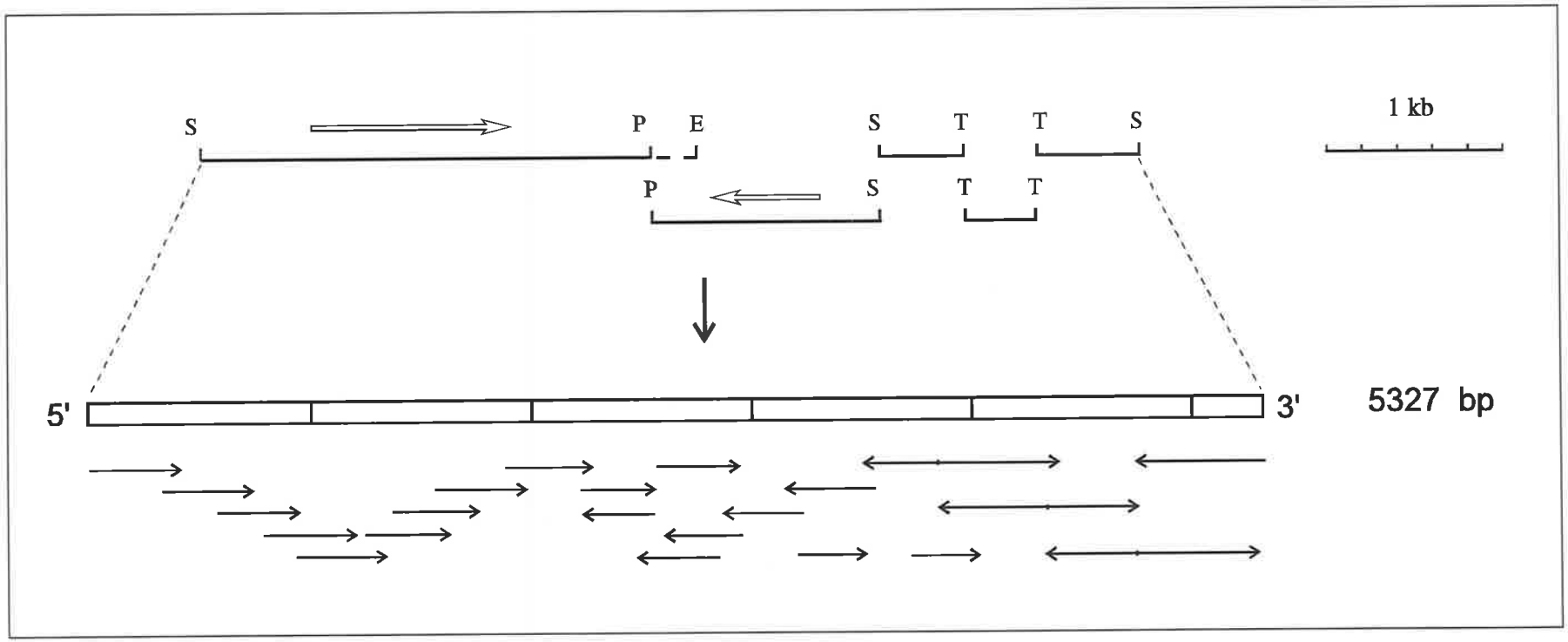
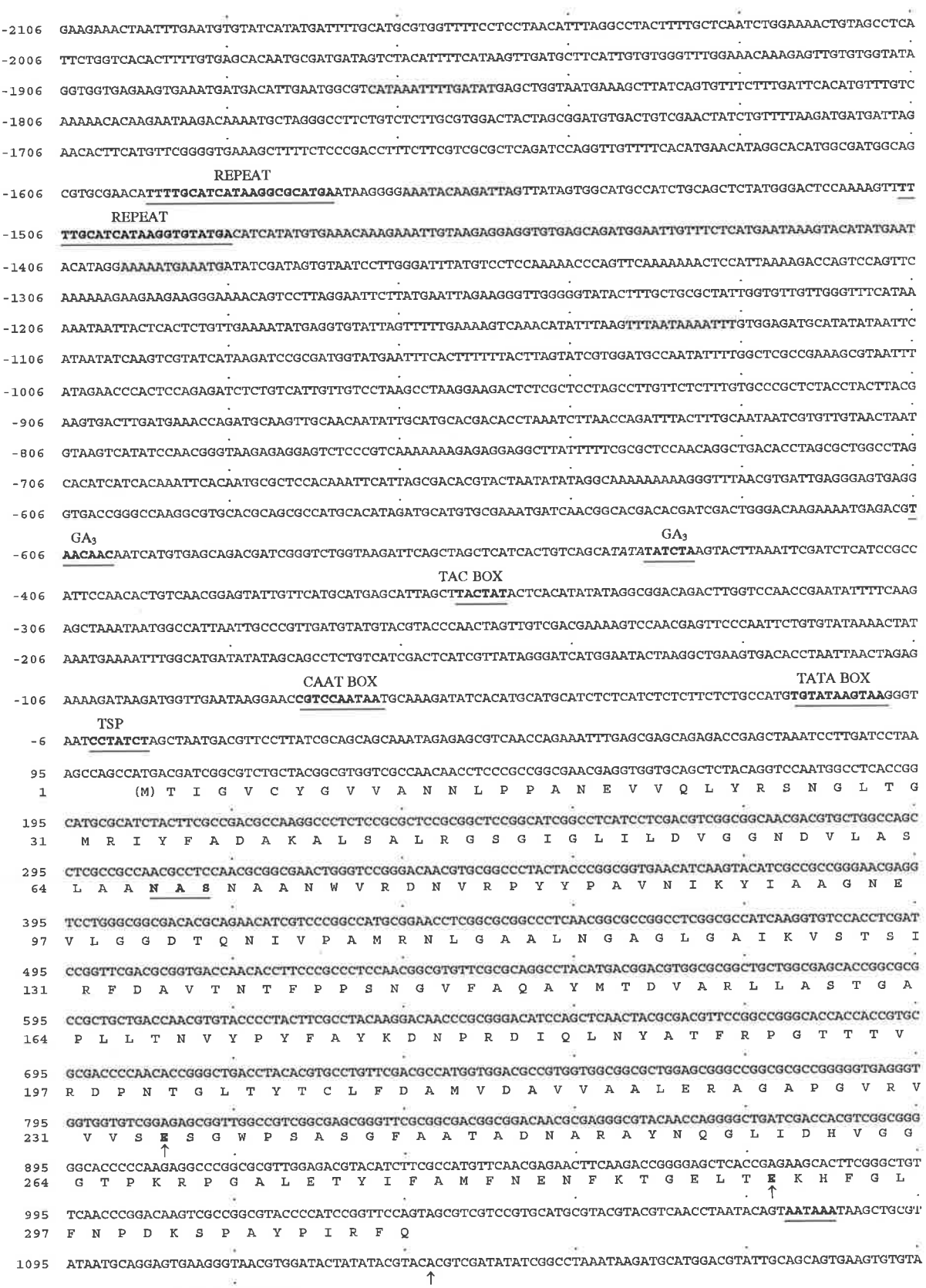


Fig. 4.3 Nucleotide sequencing strategies for the gene encoding (1→3)-β-glucanase isoenzyme GI. The direction and extent of individual sequencing reactions are marked by arrows. Restriction enzymes are designated as follows: E, *Eco*RI; P, *Pst*I; S, *Sal*I; T, *Sac*II. The unfilled horizontal arrows indicate the deletion directions of inserts of pGISP26 and pGIPS14.

Figure 4.4

Fig. 4.4 Nucleotide sequence of a 3327 bp fragment of the genomic clone carrying the gene for the (1→3)-β-glucanase isoenzyme GI. The deduced amino acid sequence is shown in single letter code. The underlined sequences in the diagram are the putative CAAT box (CGTCCAATAA, -74 bp), TATA box (TGTATAAGTAA, -19 bp), transcription start point (CCTATCT, +1 bp), translation start site (CAGCCATGAC), the potential N-glycosylation site (NAS), and the polyadenylation signal (AATAAA). The putative catalytic amino acids Glu^{234} and Glu^{291} and the poly(A) addition site are indicated by vertical arrows. The predicted *cis*-acting elements including a TAC box, direct repeat sequences and possible GA_3-responsive sites are also indicated by underlined.

Figure 4.4



sequenced according to the sequencing strategy shown in Figure 4.3, and the nucleotide sequence of a 3327 bp portion is shown in Figure 4.4. The amino acid sequence of 310 residues was deduced from an open reading frame; the NH_2-terminal sequence exactly matched the sequence determined directly from the purified enzyme (Høj *et al.*, 1988). A comparison of the enzyme coding region of the gene with its corresponding cDNA clone (Xu *et al.*, 1992) revealed several differences, which resulted in substitutions of eight amino acid residues (Table 4.2). A further comparison with amino acid sequences for other (1→3)-β-glucanase isoenzymes (Xu *et al.*, 1992) revealed that the sequence deduced from the genomic clone was more closely related to the other isoenzymes than was that deduced from the cDNA. This may be due to the fact that the gene and the cDNA were isolated from libraries that were constructed from different barley varieties.

The cDNA clone for (1→3)-β-glucanase isoenzyme GI (Xu *et al.*, 1992) provided no nucleotide sequence information for the determination of the translation start point or putative signal peptide. Nucleotide sequence analyses of the genomic clone indicate that a Met residue precedes the NH_2-terminal Thr determined by amino acid sequencing of the enzyme itself (Høj *et al.*, 1988). It is possible that this Met codon may represent the translation start point and that there is no signal peptide for the enzyme. The presence of a Thr residue next to the Met would be expected to result in the removal of the Met residue during post-translational processing (Flinta *et al.*, 1986). This prediction is strongly supported by the detection of nucleotide sequences resembling a putative CAAT box, a TATA box, and a transcription start point at appropriate positions. These were detected by a computer analysis performed on the 5' untranslated region of the gene, using eukaryotic promoter consensus sequences (Bucher, 1990). In addition, no sequence encoding a signal peptide can be detected (Watson, 1984); a Met-Ala-Arg-Lys or similar sequence has been demonstrated in the genes for (1→3)-β-glucanase isoenzymes GII, GIII and GVII (Høj *et al.*, 1989; Wang *et al.*, 1992; Malehorn *et al.*, 1993). Furthermore, no peptide extension is observed at

Table 4.2 Sequence comparison of the genomic and cDNA clones

Amino Acid	Genomic Clone	cDNA Clone	Consensus
98	CTG (L)	TGG (W)	–
117	AAC (N)	AAG (K)	–
118	GGC (G)	GCG (A)	–
119	GCC (A)	CCC (P)	A
123	GCC (A)	ACC (T)	–
205	TAC (Y)	TCA (S)	Y
206	ACG (T)	CAG (Q)	T
213	GCC (A)	TCC (S)	A

the COOH-terminal of the mature enzyme, a feature which is believed to represent a vacuolar targeting signal (Shinshi *et al.*, 1988; Van der Bulcke *et al.*, 1989; Bednarek *et al.*, 1990; Chrispeels & Raikhel, 1991; Neuhaus *et al.*, 1991; Melchers *et al.*, 1993) and is associated with barley (1→3)-β-glucanase isoenzyme GIV (Xu *et al.*, 1992).

In other plant (1→3)-β-glucanase and (1→3,1→4)-β-glucanase genes, single introns have been identified in the signal peptide-encoding regions (Litts *et al.*, 1990; Slakeski *et al.*, 1990; Wolf, 1991; Simmons *et al.*, 1992; Wang *et al.*, 1992; Lai *et al.*, 1993). These introns range from 200 bp up to 3.1 kb in size, and often split the Val codon in Val-His-Ser sequences adjacent to the signal peptide cleavage point. In the absence of a clear signal peptide for (1→3)-β-glucanase isoenzyme GI, an intron might be located in the 5' untranslated region, as observed in other plant genes (Christensen *et al.*, 1992) and in animal genes (Maichele *et al.*, 1993). A putative 3' intron splice point (AGCCAG↓) was detected just two nucleotides before the translation start Met codon, but no nucleotide sequence similar to the 5' splice point of intron could be detected. The presence of promoter elements (Figure 4.4) in the gene about 100 bp upstream from the putative translation start point also suggests there is no intron.

4.3.4 Identification of the 5' Terminal of the mRNA

To determine the 5' terminal of the cDNA for (1→3)-β-glucanase isoenzyme GI, an oligonucleotide (P1), 5'-TGGCCAGCACGTCGTTGCCGCCGA-3', complementary to a specific region (amino acids 55 to 62) of the mature enzyme coding region, was designed as a PCR primer. A fragment of around 300 bp should be amplified by PCR using the RACE protocol. Initially, four fragments were isolated, but nucleotide sequence analyses showed that none of them represented the 5' end of the cDNA. To overcome non-specific amplification encountered in the PCR experiment, an oligonucleotide 5'-GTCGAGGATGAGGCCGATGC-3' (P2) immediately next to the P1 in the same direction was required, or another oligonucleotide located downstream, near the putative transcription start point. These experiments were not completed in the

present study but could be undertaken in the future.

4.3.5 Codon Usage

Sequence analyses of the gene encoding (1→3)-β-glucanase isoenzyme GI revealed an overall G+C content of 68.9% in the coding region of the mature protein, which was mainly contributed by a strong bias towards the use of G and C in the wobble-base (third) position of codons, of which 308 of 310 codons had G or C in the third base position. This codon bias (99.4%) is also observed in the mature peptide-coding region of the genes or cDNAs encoding barley (1→3,1→4)-β-glucanases (Slakeski *et al.*, 1990; Litts *et al.*, 1990; Wolf, 1991) and other (1→3)-β-glucanase isoenzymes (Høj *et al.*, 1989; Xu *et al.*, 1992; Wang *et al.*, 1993). The use of high G+C content in the third position of the codon is widely observed in genes from monocotyledons (Murray *et al.*, 1989). The biased codon usage may contribute to mRNA stability or to translational efficiency (Fincher, 1989).

4.3.6 Protein Structure

Nucleotide sequence reveals that the gene for (1→3)-β-glucanase isoenzyme GI encodes a mature protein of 310 amino acids, as previously described (Xu *et al.*, 1992). The molecular weight calculated from the deduced amino acids is 32,785 Dalton, which is almost the same as the apparent value of 32 kDa determined from SDS-PAGE (Høj *et al.*, 1988). However, the calculated pI of 8.2 is somewhat lower than the value of 8.6 previous reported (Høj *et al.*, 1988). A single potential N-glycosylation site, Asn^{67}-Ala-Ser, is detected (Hughes, 1983). The enzyme has an extra Thr at the NH_2-terminal of the mature enzyme, when the sequence is compared with the other (1→3,1→4)-β-glucanase and (1→3)-β-glucanase isoenzymes. The positional identity of the amino acid sequence with isoenzyme GII is 89%. Based on the work of Chen *et al.* (1993), it is highly likely that Glu^{234} and Glu^{291} represent the catalytic amino acids. The thermostability of the enzyme is similar to that of isoenzyme GII, but less than that of isoenzyme GIII, where several N-glycosylation

sites have been identified (Xu *et al.*, 1992; Hrmova & Fincher, 1993) and may contribute to thermostability.

4.3.7 Analysis of the Promoter Region

In the promoter region of the gene for (1→3)-β-glucanase isoenzyme GI (Figure 4.4), sequence motifs of *cis*-acting elements that normally interact with RNA polymerases (Dynan & Tjian, 1985; Nussinov, 1990) are detected. The putative TATA box (TGTATAAGTA) is located at a position 19 bp upstream from the putative transcription start point (CCTATCT) and 121 bp upstream from the putative translation start point. A CAAT box (CGTCCAATAA) is located 55 bp upstream from the TATA motif. These motifs are relatively similar to plant consensus sequences reported previously, both in sequence and position (Messing *et al.*, 1983; Nussinov *et al.*, 1990). No sequence similar to a GC box (Wingender, 1988) could be found in the region immediately 5' to the putative TATA box.

The nucleotide sequence of over 2 kb in the 5' flanking region of the gene (Figure 4.4) allowed the identification of other gene regulation elements, which might be involved in hormonal and stress induction. A consensus sequence of TAACAAA and its cooperative element TATCCAC have been identified as gibberellic acid responsive elements (Ou-Lee *et al.*, 1988; Gubler & Jacobsen, 1992; Wolf, 1992). Similar sequences are found at position -505 bp and -437 bp from the transcription start point in the (1→3)-β-glucanase isoenzyme GI gene (Figure 4.4). However, they appear to be too far from the transcription start point. In the distal region from -1596 bp to -1487 bp, two direct repeat sequences of 22 bp, with only two different nucleotides, are present. A hexameric sequence of TACTAT at position -359 bp perfectly matches the TAC box (TAPyAT) that was observed in a *Petunia* chalcone synthase promoter, which has been shown to control both organ and developmental regulation (van der Meer *et al.*, 1992).

The functional importance of these putative *cis*-acting elements would be confirmed by transient expression in protoplasts of the gene promoter region, and

deletion mutants, fused onto reporter genes such as GUS or CAT (Dron *et al.*, 1988; Marcotte *et al.*, 1988; Wingernder *et al.*, 1990; Wolf, 1992). The barley aleurone protoplast system has been used to investigate the promoter region of α-amylase and (1→3,1→4)-β-glucanase isoenzyme EII (Gubler & Jacobsen, 1992; Rogers & Rogers, 1992; Wolf, 1992), suggesting that this system could be employed in the study of the promoter of the (1→3)-β-glucanase isoenzyme GI gene.

4.3.8 Analysis of the 3' Untranslated Region

The 3' untranslated regions of the barley (1→3)-β-glucanase genes reveal large differences (Xu *et al.*, 1992), which may reflect different regulatory roles. The 3' untranslated region of the gene for (1→3)-β-glucanase isoenzyme GI (Figure 4.4) is relatively short compared with the 3' untranslated regions of other (1→3)- and (1→3,1→4)-β-glucanase genes. A putative polyadenylation signal sequence AATAAA (Joshi, 1987b), at 1079 bp (Figure 4.4), is located a considerable distance (58 bp) from the poly(A) addition site determined from the corresponding cDNA clone (Xu, 1994). Consensus sequences for other motifs such as GT-clusters, Z-DNA and CAYTG boxes could not be detected in the 3' untranslated region (Joshi, 1987b).

4.3.9 Physiological Function

The function of the (1→3)-β-glucanase isoenzyme GI is not known. The enzyme has been purified from germinated barley grain (Høj *et al.*, 1988) and young barley leaves (Wang *et al.*, 1993; Hrmova & Fincher, 1993) grown under aseptic conditions; the transcript has also been detected in young roots (Xu *et al.*, 1992). If the enzyme is a member of the "pathogenesis-related" proteins, pathogen invasion is clearly not required for its expression. However, this does not preclude a role for the enzyme in protective strategies against microbial attack, because the gene might be expressed pre-emptively at stages in the life cycle where the plant is particularly vulnerable to pathogen invasion (Fincher, 1989).

Constitutive expression of the enzyme was confirmed recently in investigations on

the expression and subcellular localization of the barley (1→3)-β-glucanase isoenzyme GI in young barley leaves, where activity was examined at different times after infection with the scald fungus (*Rhyncosporium secalis*). The results showed that (1→3)-β-glucanase isoenzyme GI is expressed constitutively and not in response to fungal infection (S Roulin & GB Fincher, unpublished data). The experiment also showed that the enzyme was detected in extracts from whole cells, but not in extracts of extracellular fluid, which is in accordance with the suggestion that the enzyme has no targeting peptide (Figure 4.4). This, therefore, leads to the conclusion that the barley (1→3)-β-glucanase isoenzyme GI is cytosolic in origin. This has also been suggested for isoenzyme GV, another member of the (1→3)-β-glucanase gene family in barley (Xu *et al.*, 1994). A cytosolic location for the enzyme would be quite different from other (1→3)-β-glucanases from higher plants, which are targeted into vacuoles or to the extracellular space. A cytoplasmic location and pre-emptive expression of the barley (1→3)-β-glucanase isoenzyme GI suggests that the enzyme may participate in a second line of defence, which would come into play after cells are broken during microbial penetration of the tissue (Mauch & Staehelin, 1989).

4.4 CONCLUSION

The gene encoding the barley (1→3)-β-glucanase isoenzyme GI has been isolated from a genomic library and characterized. Structural analyses suggest that the enzyme is cytosolic in location and the deduced amino acid sequence confirms that the mature enzyme consists of 310 residues, without a signal peptide in its precursor form. Further investigations into the gene promoter region are required to demonstrate that the enzyme plays a role in defence against pathogen attack, and to identify regulatory sequences involved in gene regulation in a tissue-specific manner, by developmental signals and by hormonal action.

CHAPTER FIVE

FUTURE DIRECTIONS

In the present work, the isolation and characterization of genes encoding (1→3)-β-glucanase isoenzyme GI and (1→3,1→4)-β-glucanase isoenzyme EII in barley have been described. With these two genomic clones, future experiments could be undertaken to identify *cis*- and *trans*-acting regulatory elements that mediate responses to stress, phytohormones, tissue-specific expression and developmental signals. Precise information on mechanisms of gene regulation will be important for understanding physiological functions of the enzymes. It may also allow genetic manipulations of the genes which could be applied in the malting and brewing industries or may offer new opportunities for protecting the plants against pathogen attack.

Structural analysis of the (1→3)-β-glucanase isoenzyme GI gene suggested that the enzyme is probably located in the cytosol. However, direct evidence for this is lacking. Given that almost all (1→3)-β-glucanases so far described are secreted from plant cells or are targeted to vacuoles, it is important to confirm the putative cytosolic location of the barley isoenzyme GI. Leaf (1→3)-β-glucanases could be fractionated on the basis of extracellular and intracellular location and isoforms present in each could be identified. A more precise method would be to raise specific monoclonal antibodies against the isoenzyme and use gold-labelling histochemical methods to define its subcellular location.

(1→3)-β-Glucanases of other higher plants are believed to play a direct or indirect role, often in combination with chitinases and other proteins, in plant defense systems. The role of barley (1→3)-β-glucanase isoenzyme GI as a "pathogenesis-related" protein could be examined by testing any antifungal activity *in vitro*. Furthermore, this potential role could be studied in transgenic barley transformed with multiple gene copies or antisense constructs of the gene. Transgenic plants could then be compared with wild type plants for susceptibility to common fungal pathogens.

Since the genomic clones for (1→3)-β-glucanase isoenzymes GII and GV have not yet been isolated by standard screening procedures, PCR techniques could be employed to clone these genes. Barley genomic DNA fragments could be amplified by PCR with

primers based on sequences specific to these two genes.

Future experiments with the (1→3,1→4)-β-glucanase isoenzyme EII gene could be directed towards the generation of a more thermostable isoform. Production of a thermostable (1→3,1→4)-β-glucanase isoenzyme EII has been proposed as an important application of the enzyme in the malting and brewing industries. (1→3,1→4)-β-Glucanase isoenzyme levels directly influence the efficiency of malting and brewing processes through the degradation of cell walls for quick mobilization of stored nutrients, and to remove residual (1→3,1→4)-β-glucans from the final products. The thermostability of (1→3,1→4)-β-glucanases could be enhanced by site-directed mutagenesis of the protein-coding region through the introduction of more glycosylation sites, or by the substitution of specific amino acids to increase covalent or non-covalent cross-linking of the C^{α} backbone of the enzyme (Varghese *et al.*, 1994). Additionally, the levels of (1→3,1→4)-β-glucanases could be increased by the introduction into their promoter region of regulatory elements that direct high level expression of other hydrolases during the germination of barley grain. Thus, α-amylase promoter sequences could be spliced onto the (1→3,1→4)-β-glucanase-coding region.

For most of these possible future applications of the work described here, a routine barley transformation system will be required. Recent advances in this technology (Wan & Lemaux, 1994) indicate that these experiments will become feasible in the immediate future.

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