STUDIES ON (1→3)-β-GLUCAN SYNTHASES IN BARLEY

By

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STATEMENT OF AUTHORSHIP

All research reported in this thesis is original and my own work, except where due acknowledgment is made and has not been submitted for any other degree.

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ABBREVIATIONS

ABF	Aniline blue fluorochrome
AD	Activation domain
ATP	Adenosine 5' triphosphate
BAC	Bacterial artificial chromosome
BD	Binding domain
Bp	Base pair
BSA	Bovine serum albumin
BCIP	5-Bromo-4-chloro-3-indolylphosphate p-toluidine salt
CBB	Coomassie brilliant blue
cDNA	Complementary DNA
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
DEPC	Diethyl pyrocarbonate
dATP	2'-Deoxyadenosine 5' triphosphate
dCTP	2'-Deoxycytidine 5' triphosphate
DMSO	Dimethyl sulphoxide
dNTP	2'-Deoxynucleotide 5' triphosphate
DP	Degree of polymerisation
E. coli	*Escherichia coli*
EDTA	Ethylene diamine tetra-acetic acid
ELISA	Enzyme linked immuno-sorbent assay
EM	Electron microscopy
ER	Endoplasmic reticulum
GFP	Green fluorescence protein
HPLC	High performance liquid chromatography
HR	Hypersensitive response
HRP	Horse-radish peroxidase
IgG	Immunoglobulin G
IP	Immuno-precipitation
IPTG	Isopropylthio-β-D-galactoside
LB	Luria-Bertani

MALDI-TOF	Matrix assisted laser desorption ionisation-time of flight
MMC	Microsporocyte mother cell
M_W	Molecular weight
NBT	Nitroblue tetrazolium chloride
NHS	N-hydroxysuccinimide
Ni-NTA	Nickel-nitro-tri-acetic-acid
PBS	Phosphate-buffered saline
Pd	Plasmodesmata
PCR	Polymerase chain reaction
pI	Isoelectric point
PMC	Pollen mother cells
PTGS	Post-transcriptional gene silencing
PVP	Polyvinylpyrrolidine
PVX	Potato Virus X
RFLP	Restriction fragment length polymorphism
RNase	Ribonucleic acid endonuclease
RT-PCR	Reverse transcriptase-PCR
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
SSC	Sodium saline citrate
TEM	Transmission electron microscopy
TEMED	N,N,N'N',-tetramethylethylenediamine
Tm	Melting temperature
Tris	Tris (Hydroxymethyl) aminomethane
TVN	Tubulovesicular network
UTR	Untranslated region
VIGS	Virus-induce gene silencing
X-gal	5-Bromo-4-chloro-3-indoyl-β-D-galactopyranoside

ABSTRACT

(1→3)-β-Glucans are broadly distributed in higher plants. During normal growth and development they are deposited at the cell plate of dividing cells, they are components of specialised cell walls in certain floral tissues, and are associated with sieve plates and plasmodesmata. In addition, (1→3)-β-glucans are deposited between the cell wall and the plasma membrane in response to biotic and abiotic stress. Attempts to purify the (1→3)-β-glucan synthase that mediates these important processes have been frustrated by the location of the enzymes in cellular membranes and by their instability in cell extracts.

Based on the sequence similarity to the yeast (1→3)-β-glucan synthase (*FKS*) genes, a few putative higher plant (1→3)-β-glucan synthase genes have now been identified. However, in most cases a direct connection between (1→3)-β-glucan synthase activity and the protein encoded by the genes has not been demonstrated. In this study, a putative callose synthase gene, designated *HvGSL1* cDNA, has been isolated from barley, and its involvement in callose biosynthesis has been investigated.

The cloning process was initiated by screening a barley cDNA library using a yeast *FKS* homologous cDNA from *Hieracium* species. This yielded a partial cDNA, and the definition of the complete cDNA sequence was subsequently accomplished by applying a range of molecular techniques, such as polymerase chain reaction (PCR), anchor-ligated-PCR, and barley BAC library screening. A near-full length *HvGSL1* cDNA of 6055 bp, containing an open reading frame that encodes a protein of 1915 amino acids, was finally generated. The protein encoded by the gene shows approximately 30% identity with that of yeast FKS proteins at the amino acid level, and is an integral membrane protein with 15 transmembrane helices. The protein is predicted to contain three major hydrophilic portions, including 499 amino acid residues at the NH_2-terminus, 42 amino acid residues at the carboxyl terminus, and a large central region that is assumed to be oriented towards the cytoplasm.

Genetic properties and expression patterns of this gene were conducted using Southern blot analysis, Northern blot analysis and a PCR-based technique, and chromosomal

mapping in combination with searches of gene-banks and EST databases. The barley *HvGSL1* is a member of a gene family of at least seven genes. The Northern hybridisation analyses, together with the RT-PCR analysis, revealed that the *HvGSL1* gene is transcribed at low levels in all the tissues examined, including callus, roots, coleoptiles, young and mature leaves, infected leaves, stems, florets, and developing grains from barley. Expression levels, as indicated by mRNA abundance, were highest in tissues containing active dividing, undifferentiated cells, such as coleoptiles, florets, and developing grains in their early stages. These expression patterns are in agreement with expression sites reported in the literature.

As a second major component of this work, the function of the callose synthase-like gene from barley was investigated by heterologous expression, protein purification, immuno-chemistry, and mass spectrometric analysis. Initially, a recombinant protein of the gene was generated by heterologous expression of a fragment of the gene and an antibody was raised against the expressed protein. The fragment of the *HvGSL1* for expression was selected from the putative cytoplasmic region, which is conserved among the synthases identified from higher plants. The recombinant protein was approximately 17 kDa, and was designed CS17. The CS17 protein allowed the production of a specific polyclonal antibody. A two-step affinity purification of the antibody with a protein A-Sepharose and an NHS-activated column coupled with the CS17 peptide, yielded highly active antibody, and thereby provided a useful tool for functional studies of the *HvGSL1* gene.

Partial purification and characterisation of callose synthase from barley suspension-cultured cells were also performed. Applying a sequential purification procedure, which consisted of membrane fractionation, CHAPS solubilisation, $CaCl_2$ precipitation, and linear sucrose gradient sedimentation, callose synthase activity was enriched approximately 60-fold compared with that of the tissue lysate. Native gel analysis of the active fractions revealed that (1→3)-β-glucan synthase was of high molecular mass, and might be associated with regulatory ancillary proteins. Furthermore, the products synthesised under specific conditions either by a relative crude microsomal fraction (CHAPS-solubilised) or by the synthase complex resolved on a native gel, were shown by fluorescence in the presence of fluorochrome aniline blue to be (1→3)-β-glucan. The products could be hydrolysed by laminarinase, a specific (1→3)-β-glucan hydrolase, but

not by cellulase [(1→4)-β-glucan hydrolase] or by a (1→3,1→4)-β-glucan hydrolase. These chemical and enzymic data clearly demonstrated that the product was indeed a (1→3)-linked β-D-glucan.

Finally, with the availability of the antibodies and the active enzyme preparations, attempts were made to link the nucleotide sequence of the cDNA with the amino acid sequence of the active enzyme, to demonstrate unequivocally that the *HvGSL1* does indeed encode a (1→3)-β-glucan synthase gene. Western blot analysis revealed not only that the CS17 antibodies bound exclusively to the region where the callose synthase activity was detected on a native gel, but also, when the antibodies were applied to the SDS-gel of several sucrose fractions, the intensity of a single antibody-binding band correlated with callose synthase activity. This single band was of about the same molecular weight as that calculated from the *HvGSL1* cDNA. Moreover, the MALDI-TOF peptide mass finger-print data matched with some theoretically digested peptide masses of the *HvGSL1*. Some "hits" (matches) with an *Arabidopsis* callose synthase-like protein were also revealed, suggesting another isoenzyme may exist in barley. Although the data was limited by the small amount of protein present in the sample, these findings provided strong evidence that the *HvGSL1* encodes a protein which is associated with callose synthase activity, and is likely to encode the catalytic subunit of the synthase complex.

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CHAPTER ONE:

GENERAL INTRODUCTION

(1→3)-β-Glucan, a polysaccharide in which glucosyl residues are linked by (1→3)-β-glucosidic linkages, is present in higher plants, fungi and some bacteria. In higher plants, the deposition of (1→3)-β-glucan (callose) is widely associated with developmental processes. These associations can be classified into two categories. Firstly, callose is deposited as a normal developmental process at specific sites in higher plants (Fincher and Stone, 1981; Stone and Clarke, 1992), such as in the cell wall of pollen tubes and pollen mother cells (Cornish *et al.,* 1988), around the pores of phloem sieve plates, around plasmodesmata (Eschrich, 1956; Currier, 1957; Robards and Lucas, 1990; Sivaguru *et al.*, 2000) and transiently in the phragmoplast during cell division (Amino *et al.,* 1985; Northcote *et al.*, 1987; Samuels *et al.*, 1995; Verma, 2001). Secondly, callose is synthesised *de novo* in response to wounding or pathogen attack (Stone and Clarke, 1992). The deposition is often highly localised at the plasma membrane-wall interface at the site of infection. In many cases, the functional roles of callose are well established, but in others they remain undefined. Thus, cellular mechanisms of (1→3)-β-glucan biosynthesis and the effects of exogenous stimuli in triggering (1→3)-β-glucan synthesis are not well understood.

The overall aim of the work described in this thesis is to investigate molecular aspects underlying callose biosynthesis in higher plants. In this general introduction, discussion is focused on the occurrence of callose and on the major roles that callose is believed to play in higher plants. Plant cell wall structure and functions of (1→3)-β-glucan molecules are briefly discussed, together with the properties of callose and our present knowledge of callose biosynthesis in higher plants.

1.1 Cell Wall Structure and Cell Wall Polysaccharides

1.1.1 Cell Wall Structure

The cell wall is an elaborate extracellular matrix that encloses and acts as a principal structural element of the plant cell. The cell wall of a newly formed cell is generally thin, and is termed the primary cell wall. It expands as growth progresses, and is retained when growth stops. Some cell types have only a primary cell wall, whereas others may have an additional wall layer, termed the secondary wall, which is produced by deposition

of new layers of a different composition underneath the primary cell wall, after cessation of cellular growth. The cell wall is in general composed of cellulose microfibrils embedded in a matrix of polysaccharides, mainly pectins and noncellulosic polysaccharides, as well as proteins and glycoproteins (Fincher and Stone, 1981; Bacic *et al.,* 1988). As proposed by Carpita and Gibeaut (1993), there are two main types of primary cell walls in higher plants. Type I represents all dicotyledons examined and some monocotyledons, whereas Type II walls are found in the Poaceae and some related monocot families. The cellulose-xyloglucan framework is a fundamental feature of Type I walls and constitutes approximately 50% of the cell wall mass. In Type II walls, cellulose microfibrils in dividing cells are interlocked by glucuronarabinoxylans (GAXs), and type II walls also contain (1→3,1→4)-β-D-glucans (Woodward *et al.*, 1983a & b; Buliga, 1986).

Cell walls fulfil a variety of functions in higher plants. Their primary role is to provide cells, tissues and whole plants with structural strength and flexibility for normal growth and development. In addition, walls determine cell size and shape, through providing a cellular framework. Thirdly, the wall has varying degrees of porosity, allowing cell-to-cell communication and molecular exchange. Moreover, the wall is involved in metabolic events of the cells, and has important roles in plant defence mechanisms (Fincher and Stone, 1981).

1.1.2 Major Cell Wall Polysaccharides

Cellulose is the most abundant polysaccharide in plants and is a fibrillar component of all higher plant cell walls, varying from 2% to 94% of wall mass (Meinert and Delmer, 1977; Fincher and Stone, 1981). Cellulose is a polymer of (1→4)-β- linked D-glucopyranosyl residues that strongly adhere to one another in overlapping parallel arrays by intermolecular hydrogen bonds, to form a bundle of chains termed cellulose microfibrils. The network of cellulose microfibrils interacts closely with xyloglucan or glucuronarabinoxylans, and contributes the major component of strength to the normal primary wall (Shedletzky *et al.,* 1992).

(1→3,1→4)-β-D-Glucan consists of linear chains of D-glucopyranosyl residues joined by both (1→3)-β- and (1→4)-β- glycosidic linkages. The ratio of (1→3)- to (1→4)-linkages

is relatively constant at about 0.3-0.4 (Woodward *et al.,* 1983a; Woodward *et al.,* 1983b). The conformation of (1→3,1→4)-β-D-glucan is attributed to both the (1→3)- and the (1→4)-linkages. Whilst the (1→4)-β linkage is assumed to generate an extended ribbon-like shape to β-glucan polymers, the (1→3)-β linkage gives rise to an overall helical shape (Stone and Clarke, 1992). Where both linkages are present, (1→3,1→4)-β-D-glucans adopt an extended, worm-like conformation in which (1→3)-linkages introduce "kinks" in the polysaccharide chain (Woodward *et al.,* 1983a; Woodward *et al.,* 1983b; Buliga *et al.,* 1986). The (1→3,1→4)-β-D-glucans constitute an important family of polymers, which is heterogeneous with respect to molecular size and fine structure, and varies with tissue, age and species (Stone and Clarke, 1992). In barley endosperm, the (1→3,1→4)-β-D-glucans account for up to 70% of the cell wall and are important in malting and brewing (Fincher, 1975).

Xyloglucan has a (1→4)-β-D-glucan backbone with α-xylosyl residues attached to the C(O)6-position of β-glucopyranosyl residues, and it also contains galactosyl and fucosyl residues in the lateral chains. The ratio of glucosyl, xylosyl and galactosyl residues is approximately 4:3:1(Hayashi and Matsuda, 1981). Xyloglucan is an essential component of the primary wall, especially in type I walls (Hayashi and Delmer, 1988; Carpita and Gibeaut, 1993). Cross-links between xyloglucan and cellulose microfibrils are thought to play a major role in the regulation of growth and development of dicotyledonous plants, and different side-chains of xyloglucan may affect their binding to cellulose microfibrils (Levy *et al.,* 1997). Levels of xyloglucan in barley coleoptile and endosperm cell walls are relatively low (D. M. Gibeaut and G. B. Fincher, personal communication).

About one-third of the primary wall of dicots is composed of pectic substances. These substances are rich in acidic polymers, which are mostly homogalacturonan (HGA), and rhamnogalacturonan (RG). The interaction of HGA with RG and the degree of substitution of RG with side-chain have a significant impact on the physical and chemical properties of the gel matrix (Jarris, 1984; Bacic *et al.*, 1988). The current models of wall assembly suggest that a fundamental framework of cellulose and hemicellulose is embedded in a pectic gel. Nevertheless, pectic polysaccharides are relatively minor components of walls of the Poaceae.

1.1.3 (1→3)-β-D-Glucans

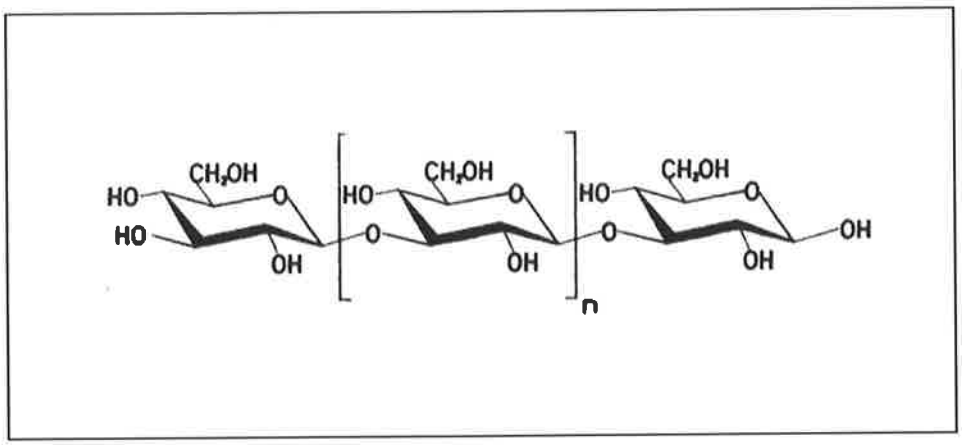
(1→3)-β-D-Glucans are not considered to be major components of walls in vegetative tissues of higher plants. However, the deposition of callose in specialised cell walls, either as wall appositions or as components of the walls themself is widely associated with physiological and developmental processes in plants. The physiochemical properties of (1→3)-β-glucans (callose), and their role and occurrence in plants are discussed in detail in the following sections.

1.2 Physical and Chemical Properties of (1→3)-β-Glucan

(1→3)-β-Glucan (callose) is a polymer of β-D-glucosyl residues joined by (1→3)-linkages (Figure 1.1 A). (1→3)-β-Glucans in nature are often associated with a small proportion of (1→6)-linkages, either within the backbone, or as substituents or branches appended to the backbone chain. Based on minimum conformational energy predictions and x-ray diffraction, the physical organisation of (1→3)-β-glucan in liquid, gel and solid states can be as a single helix (Figure 1.1 B), as a double helix with parallel or anti-parallel orientations, or as a triple helix (Rees and Scott, 1971; Bluhm and Sarko, 1977).

Studies of (1→3)-β-glucan in water or alkaline media have shown that (1→3)-β-glucans with degrees of polymerisation (DP) greater than 100 are completely insoluble in water, and the degree of solubility increases as the DP of the polysaccharide decreases (Ogawa *et al.*, 1973; Harada, 1977). Most (1→3)-β-glucan is soluble in 1 M NaOH, with the exception of some (1→3)-β-glucans from yeast and fungal cell walls (Stone and Clarke, 1992). The solubility depends on linkage arrangement, DP and degree of branching. (1→3)-β-Glucan can be dissolved in several non-aqueous solvents, such as dimethylsulphoxide, N-N-dimethylformamide and formamide (Moye, 1972). Another important property of (1→3)-β-D-glucan is its ability to form complexes with dye molecules such as fluorochromes present in the aniline-blue dye. Although the chemical basis for staining reactions has yet to be established, the (1→3)-β-glucan-aniline blue fluorochrome complex induces a brilliant yellow fluorescence under UV irradiation (Evans *et al.*, 1984).

(A)



(B)

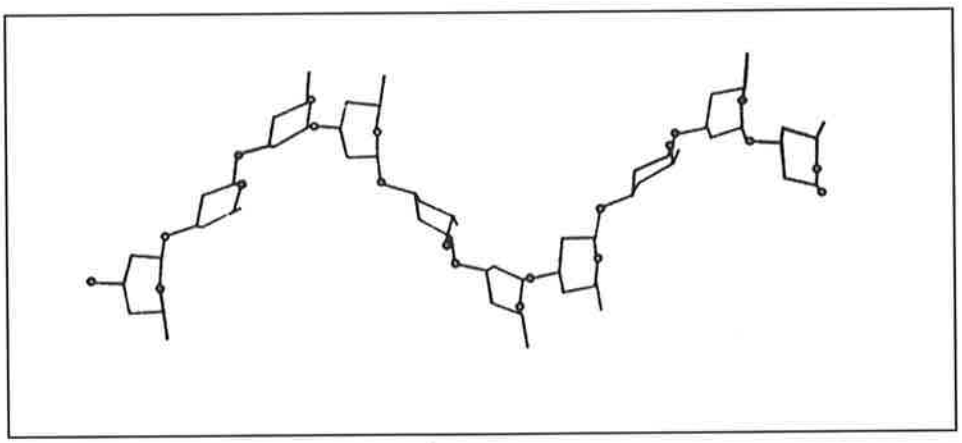


Figure 1.1 (1→3)-β-glucan molecule. (A) A linear (1→3)-β-glucan molecule. Glucose residues are joined by glucosidic linkages between the hemiacetal oxygen at C1 of one glucose and the hydroxyl at C3 on the next residue. n: number of glucose residues. **(B)** Regular, helical conformation of linear (1→3)-β-glucan chain predicted by computer-based model building. Figure is from Stone and Clarke (1992).

(1→3)-β-Glucan structure varies depending on its source. The polysaccharide is produced by a few prokaryotic microorganisms, but is widely distributed in algae, fungi, and higher plants. In prokaryotic organisms, (1→3)-β-glucans, along with a variety of polysaccharides such as (1→2)-β-glucans, are produced as components of capsules and other extracellular structures (Nakanishi *et al.,* 1976; Bauer, 1981; Kenne and Lindberg, 1983). One such polysaccharide, namely curdlan from *Agrobacterium radiobacter*, is a water-insoluble, linear (1→3)-β-glucan with only 0.8% (1→6)-linkages, and has great industrial potential as a gel-forming agent (Stone and Clarke, 1992).

(1→3)-β-Glucans, as both cytoplasmic storage material and wall components, are present in algal species such as the euglenoids and brown algae, in the form of paramylon and laminarin (Carlberg *et al.,* 1978; Marchessault and Deslandes, 1979; Markoff and Sawa, 1982). Laminarin is a component of several algal species and accounts for up to 30% or more of dry weight. The proportions and arrangement of (1→3)- and of (1→6)- linkages in these laminarins vary considerably between species (Stone and Clarke, 1992).

The β-D-glucan component of the fungal cell wall has been well characterised in several species, such as *Saccharommyces* and *Candida* (Douglas *et al.,* 1994; Mio *et al.,* 1997). β-D-Glucan in these organisms accounts for between 30-60% of the cell wall and is comprised predominately of (1→3)-β-glucan, with a few percent of (1→6)-linkages (Fleet, 1985). The (1→3,1→6)-β-glucan is often highly branched and is present as an inner wall layer. It can sometimes be associated with other wall polysaccharides, such as chitin. The (1→3,1→6)-β-glucan in the wall has important functions in modulating cell growth and morphogenesis (Bussey, 1996). Thus, (1→3)-β-glucan synthases and hydrolases involved in fungal cell wall modifications are believed to have crucial roles in establishing pathogenicity with plant and mammalian hosts (Stone and Clarke, 1992; Roulin *et al.,* 1997; Thompson *et al.,* 1999). The fungal system is so far the only system in which the biosynthesis of (1→3)-β-glucan is reasonably well understood, and is therefore regarded as a reference system for the study of mechanisms of (1→3)-β-glucan biosynthesis in higher plants.

1.3 Roles of (1→3)-β-Glucan in Higher Plants

In the following sections, callose formation in higher plants is discussed in relation to the cell plate of dividing cells, in the cell wall of pollen tubes, around plasmadosmata, and in cell walls during microsporogenesis and megasporogenesis. Important aspects of wound-induced callose, callose formed under physiological stresses, environmental stimuli and fungal, bacterial and viral invasion are subsequently presented. Evidence for the presence of callosic material is generally based on aniline blue staining, chemical analysis and immunocytochemical studies.

1.3.1 Callose in Cell Walls of Developing Tissues

1.3.1.1 Cell Plate Formation

In plant cells, cytokinesis requires additional steps compared with animal cells, because cell division in plants involves building an "extracellular compartment" within the cells (Verma, 2001). During cell division, the cytoplasm of plant cells is partitioned by construction of a new cross-wall, termed the "cell plate", which precisely determines the position where the final cell wall forms. Based on electron microscopy (EM) studies, researchers have distinguished five stages of cell plate formation (Fishkind and Wang, 1995; Otegui and Staehelin, 2000). The five stages include a) transport of Golgi-derived vesicles to the equatorial region of the cell, b) formation of a tubulovesicular network (TVN), c) TVN consolidation and callose deposition on the cell plate, d) fusion of the cell plate with the parental plasma membrane, and e) cellulose deposition and cell wall maturation. During this process, callose deposition occurs on the cell plate, a region recognised as the middle lamella following deposition of primary and secondary wall layers (Fulcher *et al.,* 1976; Verma, 2001).

Although the detailed composition of the developing cell plate is largely unknown, callose has been identified as a component in numerous plant species, by chemical analysis, aniline blue staining and immunocytochemical studies (Waterkeyn and Bienfait, 1967; Amino *et al.,* 1985; Northcote *et al.*, 1987; Samuels *et al.*, 1995; Verma, 2001).

In an early study in *Hyacinthus orientalis*, aniline blue fluorescence was evenly distributed in cell plates and young cell walls (Waterkeyn and Bienfait, 1967). Amino *et*

al. (1985) reported a transient increase in (1→3)-linked β-glucan in cell walls in synchronously growing *Cantharanthus roseus* cultures during cytokinesis. Callose has also been localised in the cell plate using antisera (Northcote *et al.*, 1987), but was not in the primary or the secondary wall. In meristematic cells of bean roots, antiserum to (1→3)-β-oligosaccharides shows the presence of the polymer in the developing cell plate. Label is observed in the centre of the plate and at its edges during early cell plate formation. In the completed cell plate, the label is dispersed fairly evenly over the whole plate. Moreover, callose is labelled in the forming plate and also detected at the T-junction of the parental cell wall in tobacco BY-2 cells and root tip cells (Samuels *et al.*, 1995).

Callose deposition on the forming plate is a transient event during cell division (Verma, 2001). It begins to accumulate as TVN is established. Despite this, little is known about how callose synthesis is initiated during cell plate formation, how it is turned off once the cell plate fuses with the parental cell wall, or about any primers or cofactors that might be required for its synthesis. However, it is known that changes in Ca^{2+} upon temporal or spatial signals represent one likely controlling factor (Verma, 2001). A high concentration of membrane-bound Ca^{2+} has been detected in forming cell plates, using chlorotetracyclin (Wolniak and Larsen, 1980).

It appears that during cell division in plants, callose and cellulose are synthesised simultaneously. Cellulose is detected as the cell plate begins to mature and fuse with the parental plasma membrane (Samuels *et al.*, 1995). Fenestrated callose sheets act only as a temporary, incomplete cell wall, and later dissolve. In the seed coat of *Gasteria verrucosa*, Wittich and Graven (1998) described callose deposition and its breakdown, followed by phytomelan synthesis, and proposed that callose probably functions as a carbohydrate source for phytomelan synthesis and/or cellulose synthesis.

The function of callose deposition during cell-plate formation and the precise mechanism as to how callose synthesis is initiated and regulated on the forming cell-plate, as well as the regulation of (1→3)-β-D-glucanase involved in its degradation while the cell wall is maturing, remain largely unknown. Possible genes associated with this complicated process are suggested by Nickle and Meinke (1998). A cytokinesis-defective mutant of

Arabidopsis-cyt1 shows altered cytokinesis and cell wall architecture during embryogenesis, and excessive callose accumulation. The authors proposed that *cyt1* might be required for normal progression through the callose-rich stage of cell-plate formation. During this process, vesicle movement and fusion are not affected in mutant embryos, but cell-plate maturation is arrested, resulting in excessive callose formation. Because of the loss of function of *cyt1*, factors that normally lead to callose degradation or removal of callose from the maturing cell plates, perhaps by (1→3)-β-D-glucanase, are somehow disrupted. Thus, precisely timed callose deposition, and in particular callose degradation, undoubtedly affect the process of cell division.

1.3.1.2 Plasmodesmata and Sieve Plates

Callose depositions are commonly associated with plasmodesmata (Pd), sieve tubes and sieve plates in higher plants (Eschrich, 1956; Northcote *et al.,* 1989; Delmer *et al.,* 1993; Brown *et al.,* 1998). Pd are the intercellular connections between plant cells that mediate cell-to-cell communication. In higher plants, living cells are connected to neighbouring cells by Pd, which form cytoplasmic channels through the intervening cell walls, facilitating intercellular movement of water, nutrients, and signalling molecules from cell to cell (Lucas *et al.,* 1993).

A classical example of callose deposition associated with Pd was given by Eschrich (1956). In epidermal cells of onion bulbs, a strong wound reaction is showed with intensively localised callose deposition in the plasmodesmata region, a result later reproduced by Delmer *et al.* (1993). The brilliant florescence of aniline blue staining was revealed, with heavier deposition in the neck region of the Pd, indicating the presence of callose (Figure 1.2). The discontinuous fluorescence actually draws outlines of the cell wall in which Pd are randomly distributed.

Using antibodies specific to (1→3)-β-oligoglucosides of DP3-8, Northcote *et al.* (1989) confirmed that callose is not only detected in the cell plate, but also is present in the Pd of primary walls. Such immunocytological analyses allow observations on particular cell walls not only from individual tissues but also for individual cells. Their data also indicated that no callose was present in the Golgi apparatus.

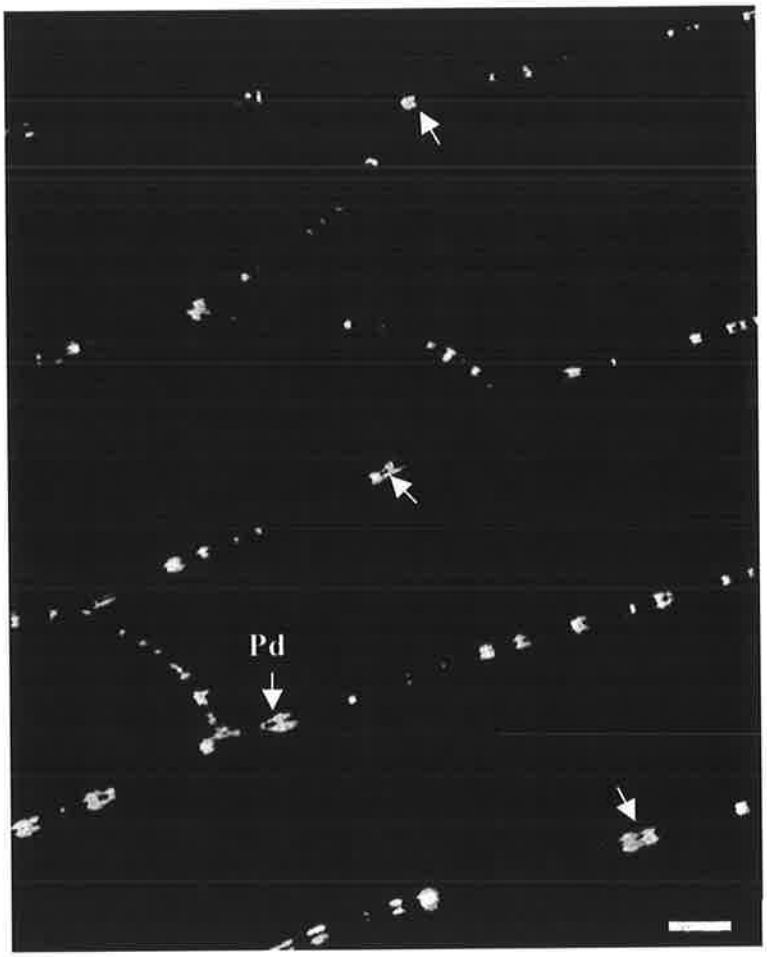


Figure 1.2 Callose deposition at plasmodesmata of onion epidermal cells. The experiment was performed with adaxial epidermal cells of onion bulbs. Aniline blue fluorescence is intense in the region of the plasmodesmata, indicating heavily localised callose deposition. The discontinuous fluorescence pattern illustrates the positions of the cell wall at which plasmodesmata are distributed. Pd: Plasmodesmata, arrows indicate the location of plasmodesmata (adapted from Delmer *et al.*, 1993).

Callose deposition at Pd may play a role in regulating cell-to-cell communication (Wolf *et al.,* 1991; Asthir *et al.,* 2001). Furthermore, callose in the Pd of cells in the crease region might be involved in regulating the movement of assimilates entering the barley endosperm (Asthir *et al.,* 2001). Evidence that callose deposition may be involved in regulating plasmodesmata properties comes from the work of Wolf *et al.* (1991) in which it was shown that, using transgenic tobacco plants expressing a virus movement protein, transient inhibition of plasmodesmatal function could be alleviated by an inhibitor of callose synthesis. Using antibodies against (1→3)-β-glucan with immuno-electron microscopic techniques, circumstantial evidence was provided that Al-induced callose deposition at Pd might be responsible for the blockage of symplastic transport and communication in higher plants. The use of 2-deoxy-D-glucose, a callose synthesis inhibitor, demonstrated that reduction in callose deposits correlated well with the improved dye-coupling and reduced root growth inhibition.

Phloem is involved in the transport of organic solutes in plants, and the main conducting cells of phloem tissues are aligned to form sieve tubes. Callose deposition associated with sieve pores of sieve tubes during phloem development has been documented using sectioned plants and tissue cultured material (Roberts, 1974; Sjolund and Shih, 1983; Sjolund *et al.,* 1983; Esau and Thorsch, 1984). During phloem differentiation, callose is observed as electron-lucent material deposited between the wall and the plasma membrane surrounding the cell plate pores (Roberts, 1974). At this stage of differentiation, callose is thought to replace cellulosic cell wall components in the middle lamella region of the cells (Esau and Thorsch, 1984). Callose platelets continue to be deposited on either side of the wall, and eventually enlarge to form a cylinder around the pores. Later, further depositions of callose replace normal cell wall components, and subsequently, the dissolution of both the wall material and callose occur, forming an enlarged pore.

Callose deposition in sieve plate pores may play a role in affecting molecule movement between cells, although interpretation of such a role in the development of sieve plate pores should be viewed with a caution (Esau and Thorsch, 1984). Evidence that callose deposition is readily induced by injuries to cells, including sample fixation, raises question as to whether callose in the phloem is an artefact or, more precisely, is wound-

induced. However, Esau and Thorsch (1984; 1985) argue that, in the development of the sieve plate pore of primary phloem of *Echium*, callose deposits in differentiating sieve tubes are in a constant and orderly arrangement. The deposition is associated with sieve elements but not with adjacent parenchyma cells. The deposits are restricted to the plasmodesmata connecting sieve elements and companion cells, and are not found in other Pd of companion cells.

1.3.1.3 Pollen Tubes

When mature pollen grains come into contact with the stigma in flowering plants, the pollen grains germinate and pollen tubes begin to grow down the style. Pollen tubes elongate and carry the sperm nuclei to the embryo sac, where fertilisation occurs. As a result of this important role, the structure and chemical composition of the cell walls of pollen tubes have been extensively studied (Stone and Clarke, 1992). These studies indicate that (1→3)-β-glucans are a major component of the pollen tube cell wall and of the callosic plugs that are deposited within the growing pollen tubes (Rae *et al.,* 1985; Anderson *et al.,* 1987; Meikle *et al.,* 1991; Ferguson *et al.,* 1998; Li *et al.,* 1999).

The cell wall of *Nicotiana alata* pollen tubes germinated *in vitro* contains (1→3)-β-glucans, arabinans and some cellulose (Stone *et al.,* 1984; Rae *et al.,* 1985). Methylation analysis of the wall polysaccharides reveals that 3-linked glucopyranosyl residues predominate (Rae *et al.,* 1985). Pollen tube walls fluoresce intensely with the aniline blue fluorochrome, but the fluorescence is characteristically absent from the tip, indicating that callose is confined to the wall behind the tube tip (Rae *et al.,* 1985). Two distinct layers are observed in the pollen-tube walls of *N. alata*, namely an inner, non-fibrillar layer and an outer, fibrillar layer (Anderson *et al.,* 1987). The inner layer is of electron-lucent appearance, indicating the presence of callose. Furthermore, immunocytochemical studies on the walls of pollen tubes in a style of *Nicotiana alata* using (1→3)-β-glucan-specific monoclonal antibodies show that (1→3)-β-glucan is located in the inner wall layer of the tubes, but not in the outer layer (Meikle *et al.,* 1991). It was also found that the immuno-gold labelling pattern in the inner wall changes with time after initiation of pollen tube growth. The labelling was restricted to the outer zone of the inner layer and became evenly distributed after 48 hours.

The co-localisation of callose and cellulose in the walls of *in vitro*-germinated pollen tubes of *Nicotiana tabacum* has been shown (Ferguson *et al.,* 1998), using the same anti-(1→3)-β-glucan antibodies (Meikle *et al.,* 1991) and a gold-labelled cellobiohydrolase that binds cellulose. Both polysaccharides are exclusively located in the inner layer of the walls, and neither cellulose nor callose is present in the outer fibrillar layer, or in the wall of pollen tube tips (Meikle *et al.,* 1991). Chemical analysis of pollen cell walls further supports the co-existence of callose and cellulose (Rae *et al.,* 1985; Schlüpmann *et al.,* 1994; Li *et al.,* 1999). Li *et al.* (1999) reported that the neutral components of the tube wall comprise 86% callose, 5% cellulose and 9% arabinan. Cellulose is synthesised and deposited approximately 5 to 15 μm behind the growing pollen tube tip, and this inner wall layer is thickened by deposition of callose from 30 μm behind the tip (Ferguson *et al.,* 1998).

Callose plugs are another form of callosic depositions that are observed in growing pollen tubes (Nakamura *et al.,* 1984; Stone and Clarke, 1992). The plugs show aniline blue-induced fluorescence at regular intervals, compared with the callose which is scattered in the inner walls of the tube. The components of callose plugs are physically and/or chemically different from those of pollen tube wall callose. Nakamura *et al.* (1984) demonstrated from a partially purified preparation from *Camellia japonica* that callose plugs consist mainly of (1→3)-β-glucan and protein, with a small portion of cellulose. Methylation analysis revealed that plug callose had a DP of at least 90 (Nakamura *et al.,* 1984), whereas the pollen tube wall callose had a DP of 21 and 37 in DMSO-extractable and alkali-extractable preparations of pollen tubes, respectively (Nakamura and Yoshia, 1980). Moreover, the distributions of callose and cellulose are notably different in plugs. Callose is abundant and evenly distributed in the plugs, whereas cellulose is present towards the outer edge of plugs (Nakamura and Suzuki, 1983).

Despite many investigations, little is known about the precise function of callose deposition in pollen tubes. However it is generally accepted that callose plugs may function in supporting growth of pollen tube by separating its active tip portion from its aged part (Nakamuka *et al.,* 1984). That is as the tip grows, callose plugs continually form behind the cytoplasm, and thereby ensure that the cytoplasm remains at the tip of the pollen tube where it is needed for fertilisation.

1.3.1.4 Microsporogenesis and Megasporogenesis

Callose is synthesised in cell walls of male and female meiocytes of most angiosperm species during microsporogenesis (Heslop-Harrison and Mackenzie, 1967; Worrall *et al.*, 1992; Fei and Sawhney, 1999) and megasporogenesis (Rodkiewicz, 1970; Tucker *et al.*, 2001).

The transient occurrence of a callosic wall around the microsporosis (pollen) mother cells (PMCs) is one striking feature of microsporogenesis in angiosperms (Heslop-Harrison and Mackenzie, 1967; Worrall *et al.*, 1992; Owen and Makaroff, 1995; Alche and Rodriguez-Garcia, 1997; Fei and Sawhney, 1999). During meiotic division, the callosic walls surrounding microsporocyte mother cells have been revealed through the aniline blue fluorochrome (Alche and Rodriguez-Garcia, 1997), light and electron microscopy (Owen and Makaroff, 1995). During the early meiotic prophase of microsporogenesis, each PMC synthesises a special cell wall consisting of callose. Callose deposition persists throughout meiosis, until the tetrad stage. At each stage, the tetrad of microspores is surrounded by callose. Finally the callosic wall is degraded and microspores are released into the locule of the anther. The callose dissolution process is accomplished by (1→3)-β-glucanases secreted by the tapetum (Scott *et al.*, 1991).

Cytological studies on a number of cytoplasmic male-sterile mutants have suggested that the presence of the callosic wall is essential for normal meiotic division in PMCs and for the final formation of microspores (Izhar and Frankel, 1971; Worrall *et al.*, 1992). Premature dissolution of the specialised cell walls might be a primary cause of male sterility. In transgenic plants that express a (1→3)-β-glucanase from tapetum-specific promoters (Worrall *et al.*, 1992), normal callose appearance and distribution are found in PMCs at prophase. In the later stages of microsporogenesis, callose is prematurely degraded by the early expression of (1→3)-β-glucanase, causing male-sterility in the transgenic plants. Delayed dissolution of callose can also result in male-sterility, as seen in several male-sterile mutants with persistent callose abnormalities in pollen development (Horner and Rogers, 1974; Katti *et al.*, 1994).

Biological functions of this callosic cell wall that have been proposed include a role in the physical isolation of PMCs during meiosis in order to prevent cell cohesion and fusion

(Heslop-Harrison and Mackenzie, 1967). The specialised wall may also serve as a template or mould for the sculpturing patterns of the exine seen on mature pollen grains (Waterkeyn and Beintait, 1970).

Megasporogenesis involves two successive meiotic divisions of the megaspore mother cell, the selection of one megaspore from a tetrad of four megaspores, and the degeneration of the remaining megaspores. The chalazal (selected functional) megaspore initiates megagametogenesis in which embryo sac development occurs. Callose deposition is known to be associated with the process of megasporogenesis (Rodkiewicz, 1970; Tucker *et al.,* 2001). Using aniline blue staining, callose was first demonstrated during megasporogenesis in ovular squashes of *Orchis maculata* (Rodkiewicz, 1970). Similar deposits were subsequently documented in other plants (Bouman, 1984; Oliver *et al.,* 1995; Peel *et al.,* 1997; Tucker *et al.,* 2001). The formation of callose in the walls during megasporogenesis is similar to that in microsporogenesis. Callose is initially deposited in a bipolar manner in early meiotic prophase. In late prophase and first metaphase, the whole meiocyte undergoing microsporogenesis is enclosed by a callosic wall. As meiosis progresses to the dyad and tetrad stages, callose fluoresces intensely in the walls of each megaspore. Callose is present in the chalazal wall of the functional megaspore, but rapidly decreases as development proceeds to the megagametogenesis stage. In the non-functional megaspores, fluorescence starts to decrease gradually once they degrade. The degeneration of the non-functional megaspores is accompanied by various cellular changes, and products of degeneration of the non-functional megaspores are used by the functional megaspore. Callose is not detected in walls of the selected spore during megagametogenesis, or in other cells of developing ovule or embryo sac. This is typical of callose deposition in megaspore mother cells of most angiosperms that produce either monosporic (which accounts for more than 90% of species) or bisporic embryo sacs (Peel *et al.,* 1997). Callose deposition patterns vary between plant species undergoing different modes of embryo sac formation. Such deposits are absent in species with tetrasporic embryo sacs, and in diplosporic species (Carman *et al.,* 1991; Naumova *et al.,* 1993; Leblanc *et al.,* 1995; Peel *et al.,* 1997). The absence of megasporocyte callose may be responsible for meiotic failure in diplosporic species (Carman *et al.,* 1991).

It is assumed that the callosic wall acts as a molecular filter to decrease the permeability of the cell wall, so that it allows the meiocytes to be temporarily isolated, to embark upon an individual course of development (Bouman, 1984). However, given the fact that different deposition patterns of callose are encountered over the range of plant species with different types of embryo sac development, the functional roles of the callose, the factors controlling its formation and degeneration, and the correlation between callose deposition and embryo sac development may vary, and await to be elucidated.

1.3.2 Callose Deposition under Wounding, Stress and Pathogenic Attack

1.3.2.1 Wounding and External Stimuli

Deposition of callose at the plasma membrane-cell wall interface is known to be one of many responses of plant tissues to wounding (Currier and Webster, 1964; McNairn, 1972; Jaffe *et al.,* 1985; Sivaguru *et al.*, 2000). Responses can be induced simply by fixation and cutting of fresh sections of tissues during experimental procedures (Hughes and Gunning, 1980), or by more drastic mechanical perturbation (Jaffe *et al.,* 1985), ultrasonic stress (Currier and Webster, 1964), or heating and cooling (McNairn, 1972; Majumber and Leopold, 1967).

External stimuli are also known to induce callose formation. In cell suspension cultures and leaves of soybean, callose formation is induced by manganese (Mn) (Wissemeier *et al.,* 1993) and the concentration of Mn correlates well with the concentration of callose induced in the cells. Callose formation induced by aluminium (Al) is a well-studied effect through which callose synthesis can be used as a rapid and sensitive marker for Al toxicity (Zhang *et al.,* 1994; Massot *et al.,* 1999; Chang *et al.,* 1999). Studies on roots of *Triticum aestivum* in response to Al toxicity using spectrofluorometry revealed that treatment with Al increases callose accumulation by 86% within 30 minutes and by 3,821% after 48 hours. More callose accumulates in roots of Al-sensitive cultivars than in Al-tolerant cultivars. Similarly, callose synthesis positively correlates with internal Al concentration in bean cultures (Massot *et al.,* 1999). Moreover, in suspension cultured-cells of tobacco, supplementation of the nutrient medium with Al and iron enhanced callose production. The activity of (1→3)-β-glucan synthase in microsomes is increased several times by the addition of Ca^{2+} to the assay medium, supporting the concept that Ca^{2+} is an activator for callose synthesis (Chang *et al.*, 1999). Al-induced callose

formation also occurs in Pd (Sivaguru *et al.,* 2000). Deposition reduces the exclusion size of the plasmodesmata, and thereby inhibits cell-to-cell molecule trafficking and results in reduced root growth.

It is poorly understood how metabolic or physiological changes accompanying mechanical perturbation or external stimuli are translated into callose deposition. Deposition triggered by wounding may involve activation of plasma membrane-bound callose synthase in plant cells, either by elevation of intracellular Ca^{2+} (Kauss, 1996), proteolytic changes to the enzyme, or by increased accessibility of the synthase to its substrate after membrane damage (Stone and Clarke, 1992). In the case of Al-induced callose deposition (Sivaguru *et al.,* 2000), it is believed that an increase in intracellular Ca^{2+} is a signal for the activation of callose synthesis at Pd. Several experiments have demonstrated that exposure to Al results in prolonged elevations of cytoplasmic free Ca^{2+} in root hairs of *Arabidopsis thaliana* (Jones *et al.,* 1998) and wheat (Zhang and Rengel, 1999). Such an event is a prerequisite for Al-induced callose synthesis. Furthermore, a decrease in the temperature of corn suspension-cultured cells results in the elevation of cytoplasmic free Ca^{2+} (Holdaway-Clark, 2000), which initiates callose production upon chilling.

1.3.2.2 Pathogenic Attack

Callose is deposited between the plasma membrane and cell wall as a part of the hypersensitive reaction to infection by fungi (Aist, 1976), viruses (Shimoura and Dijkstra, 1975) and bacteria (Brown *et al.,* 1993). These wall appositions in plants accumulate at the site of contact with bacteria or attempted penetration by fungal hyphae, and are an integral part of the response to microbial challenge. The constituents of wall appositions, the timing of the deposits and potential role of callose are discussed below.

Callose Formation Upon Fungal Infection: Deposition of callose has been implicated as a structural resistance response by plants to attempted penetration by fungi (Asit 1976; Zeyen and Bushnell, 1979; Škalamera and heath, 1995; Škalamera *et al.,* 1996; Enkerli *et al.,* 1997; Trillas *et al.,* 2000). Appositions have been observed between the plant cell wall and the invaginated plant plasma membrane. The wall appositions triggered by fungal invasion are diverse with respect to the morphology and the manner of deposition.

They can be in the shape of papilla-like depositions (Zeyen and Bushnell, 1979), or as collars located at the point of penetration (Škalamera and Heath, 1996), or they can completely encase the invading structures, depending on the host-pathogen system.

Callose-containing wall appositions are readily observed at a light microscopy level, showing aniline blue-induced fluorescence (Bushnell and Bergquist, 1975; Zeyen and Bushnell, 1979; Ward *et al.,* 1988; Bailey *et al.,* 1990). Immunocytochemical location of (1→3)-β-glucans in wall appositions further supports the presence of callose as a typical wound response of plants to pathogen attack (Hussey *et al.,* 1992; Xu and Mendgen, 1994; Enkerli *et al.,* 1997). Using (1→3)-β-glucan-specific antibodies, callose has been detected in the root cortex at the penetration site around the ring nematode *Cricomella venoplax* (Hussey *et al.,* 1992), in contact cells surrounding vessels of tomato and cotton infected with *Fusarium oxysporum* (Mueller *et al.,* 1994), and in soybean roots infected with *Phytophthora sojae* (Enkerli *et al.,* 1997). In the latter case both monoclonal and polyclonal callose antibodies labelled wall appositions (Figure 1.3). The same labelling pattern was found in all wall appositions, regardless of whether the plant was susceptible or resistant to the pathogen, and this raises questions as to whether the callose actually inhibits penetration or merely accompanies it. In the host-fungus pair of cotton root-*Fusarium oxysporum*, the cell wall appositions were heavily labelled by the (1→3)-β-glucan-specific antibodies along the plant wall after contact with fungal hyphae (Mueller *et al.,* 1994). Later, label was found around the hyphae during penetration of the fungus into the host cells. In contrast, in non-infected cells, label was only found along plasmodesmata and in the cell-plate region where non-wound induced-callose exists.

Although (1→3)-β-glucan is the major component of wall appositions induced by fungal attack, it must be emphasised that the wall appositions are of a heterogenous nature. They contain other substances, such as lignin, cellulose, protein, pectin, and suberin (Aist, 1976). Arabinogalactans (AG), AG-protein and xyloglucan have been also detected in the wall appositions (Rodrigue-Galvez and Mendgen, 1995; Enkerli *et al.,* 1997).

The precise timing of callose deposition in relation to the progress of fungal penetration varies depending on the host-parasite systems. Several fungal pathogens have been shown to induce wall appositions in the form of papillae 1-3 hours after inoculation

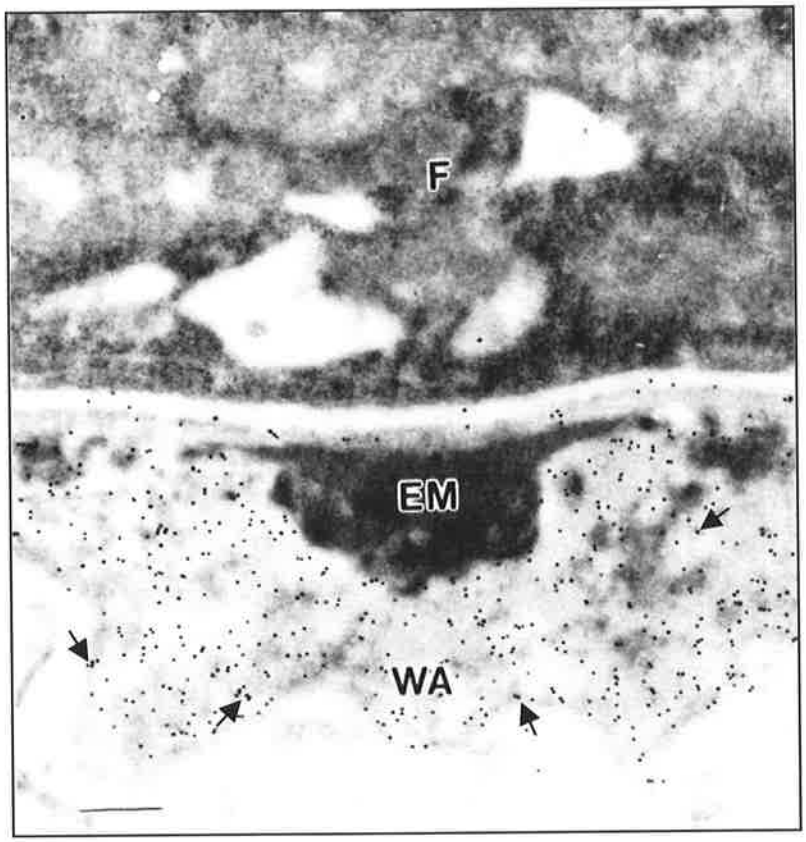


Figure 1.3 Detection of (1→3)-β-glucan in a cell wall apposition in soybean roots infected with *Phytophthora sojae*. In a resistant plant, 10 hours post-infection, extrahaustorial matrix material (EM) is seen adjacent to a hypha (F) encased by a wall apposition (WA). Immunogold label of monoclonal (1→3)-β-glucan specific antibodies are visible only on the wall apposition, an electron translucent region, but not on the extrahaustorial matrix (adapted from Enkerli *et al.*, 1997).

(Hanchey and Wheeler, 1971; Aist and Israel, 1977), whereas appositions are produced within a few minutes by accumulation of cytoplasmic aggregates at the site of fungal penetration in other systems (Zeyen and Bushnell, 1979; Kunoh, 1982; Xu and Mendgen, 1994). The data from microcinematographic time-lapse sequences of barley epidermal cells attacked by *Erysiphe graminis* (Zeyen and Bushnell, 1979) show that papilla-like depositions are formed rapidly in the first 20 to 30 minutes, and deposition continues at a fairly rapid rate for the first hour.

It appears that resistance to fungal penetration correlates with the amount of cell wall apposition. This correlation has been established in several host-parasite systems (Hachler and Hohl, 1984; Škalamera and heath, 1995; Enkerli *et al.,* 1997). For example, during the infection of a resistant cowpea cultivar with cowpea rust fungus, the majority of fungal haustoria become completely encased by callose. In contrast, in susceptible cultivars callose deposits only develop around the fungal haustorial neck in the form of collars, and the extent of deposition is much less compared with that of resistant cultivars. Moreover, the deposits are only found in about 20-30% of infection sites in susceptible cultivars, and the remaining sites show no visible response to the presence of a haustorium (Heath, 1971; Škalamera and heath, 1995). Pathogen attack does not always trigger callose deposition in higher plants (Shanker *et al.,* 1998). In a study of resistant and susceptible narrow-leafed lupin infected by *Diaporthe toxica,* callose depositions were not observed in cuticle and epidermal cells of infected tissues (Shanker *et al.,* 1998).

Callose Formation upon Bacterial Infection: Induction of large papillae has been observed in plant species from very disparate families, including *Arabidopsis*, lettuce, pepper, French bean and melon, upon bacterial infection (O'Connell *et al.,* 1990; Brown *et al.,* 1993; 1995; 1998). Ultrastructural examination of the interaction between the plant and the invading bacteria reveals that such interactions differ in different host-pathogen pairs. Furthermore, the degree of papillae formation and whether they prevent bacteria invasion or just act as a barrier to slow the infection are case-dependent.

Pepper leaves infected by both virulent strains and non-virulent *hrp* mutant strains of *Xanthomonas campestris pv. vesicatori* have been used as a model system for comparative studies of callose formation (Brown *et al.,* 1998). *Hrp* genes in bacteria

have the potential to determine basic pathogenicity in the host plant and may have roles in causing the hypersensitive reaction (HR) in resistant plants (Willis *et al.,* 1991). The *hrp* mutants may therefore lack pathogenicity and are used in conjunction with the wild-type in this study. Extensive papillae deposition is observed in adjacent cells in plants infected with the *hrp* mutant strain (non-pathogenic strain). Immunogold labelling reveals that callose is a component of papillae at all stages of their development. In un-inoculated leaf tissues, callose is detected only in sieve plates and within plasmodesmata. The restriction of bacterial colony development is associated with papillae deposition, and together with the increase in electron density of the plant cell wall, bacteria eventually become encapsulated by the amorphous electron-dense matrix attached to the plant cell wall. In plants infected by the wild-type virulent strain, papillae formation is diminished, and only minor cell wall alteration is observed. Unlike mutant strains, the virulent bacteria are pathogenic to their host plant and cause the HR in resistant plants.

Thus, papillae formation upon bacterial infection appears to serve not only as a barrier to penetration, but also to immobilise the invading microbe and potentially to expose it to anti-microbial components generated by host plants (Brown, 1995; 1998). In keeping with this role, papillae deposition is clearly regulated in some way during plant-microbe interactions, depending on the genetic make up of both the bacteria and the host plant.

Callose Formation upon Viral Infection: Callose deposition and the thickening of walls in and around viral lesions have been described in numerous investigations (Allison and Shalla, 1974; Bell, 1981; Iglesias and Meins, 2000). Callose deposition, based on enhanced fluorescence following aniline blue staining of infected tissues, can be detected in cells marginal to the necrotic zone (Allison and Shalla, 1974). Such events are known to be associated with changes of wall structure. Progressive and heavy callose deposition is pronounced around plasmodesmata, resulting in a distorted and narrowed plasmodesmatal channel, which in turn may have effects on cell-to-cell communication. It is known that successful infections of plants by viruses mostly depend on the cell-to-cell movement of viral particles via plasmodesmata (Carrington *et al.,* 1996). Thus, it is thought that callose deposition in the regions of plasmodesmata may act as a physical barrier to limit or prevent the spread of virus in the resistant host.

1.4 (1→3)-β-Glucan Biosynthesis

1.4.1 An Overview of β-Glucan Biosynthesis

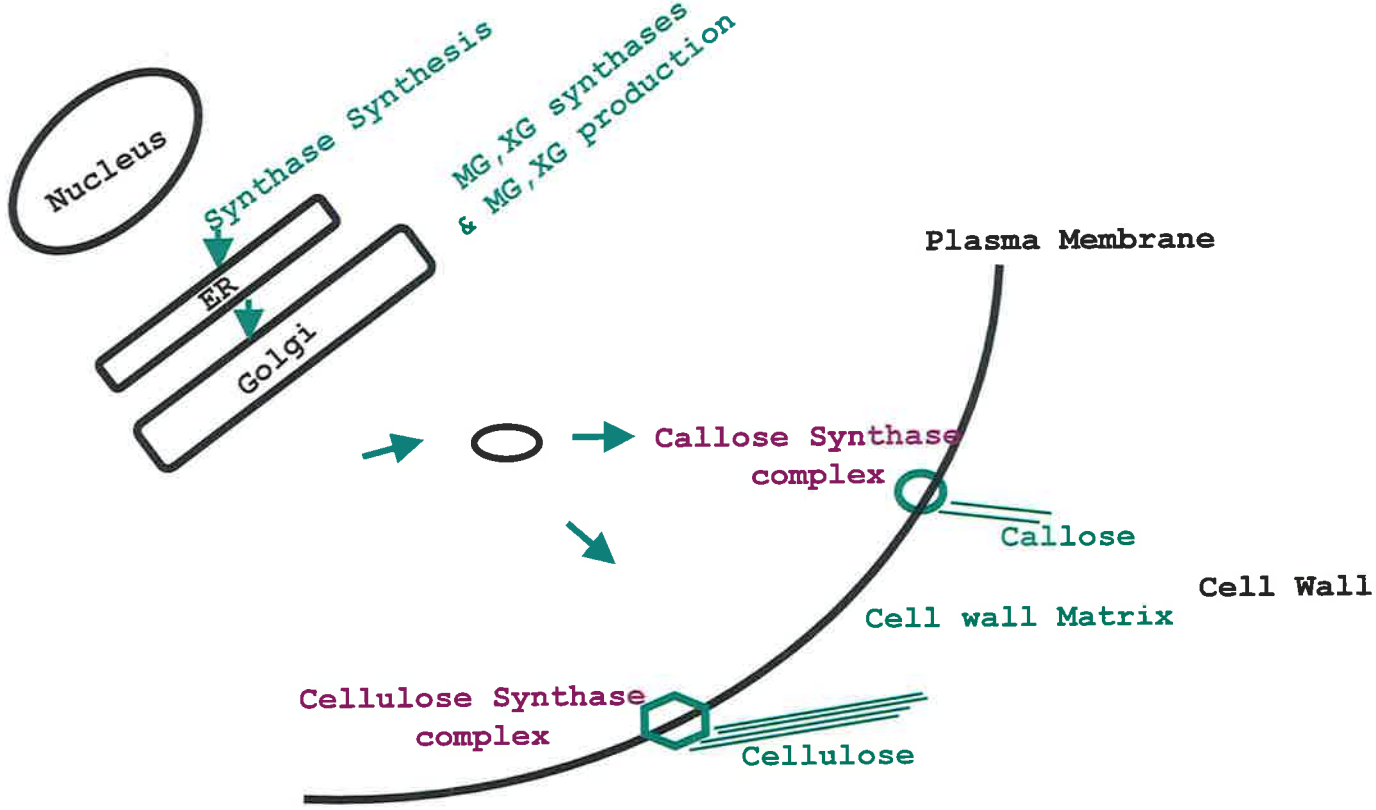
Extensive studies on β-glucan synthesis in plants have been conducted on a wide variety of plants, using genetic, biochemical, and immunochemical approaches (Gibeaut and Carpita, 1990; 1993; 1994; Fink *et al.,* 1990; Delmer, 1991; Dhugga and Ray, 1991; Driouich *et al.,* 1993; Bulone *et al.,* 1995; Kudlicka and Brown, 1997; Li *et al.,* 1997; McCormack *et al.,* 1997). Although the precise molecular details of β-glucan biosynthesis have not been established, it is generally believed that β-glucan biosynthesis pathways begin with the synthesis of β-glucan synthases in the rough endoplasmic reticulum. The synthases can be transferred to the Golgi apparatus. (1→3,1→4)-β-Glucan synthase and the (1→4)-β-glucan synthase of the xyloglucan backbone remain in the Golgi apparatus where (1→3,1→4)-β-glucan and xyloglucan production occurs. Secretory vesicles containing these two types of newly synthesised polymers bud off from the Golgi apparatus, fuse with the plasma membrane, and deposit the polysaccharides into the cell wall matrix. On the other hand, cellulose and callose synthase complexes, instead of remaining in the Golgi apparatus, are probably transported by the secretory vesicles and anchored onto the plasma membrane where cellulose and callose polymerisation occurs. A schematic diagram outlining an overview of the β-glucan biosynthesis pathway is shown in Figure 1.4 (Stone and Clarke, 1992; Gibeaut and Carpita, 1994).

It is known that nucleotide-sugars are utilised as sugar donors for the synthesis of cell wall polysaccharides (Gibeaut and Carpita, 1994). UDP-glucose is the substrate used for cellulose and callose synthesis (Feingold *et al.,* 1958; Aloni *et al.,* 1983). Two possible routes have been proposed for UDP-glucose synthesis, in which either UDP-glucose pyrophosphorylase (Kleczkowski, 1994) or sucrose synthase (Geigenberger and Stitt, 1993) are involved.

1.4.2 The (1→3)-β-Glucan Synthase Complex

Biochemical studies of yeast and fungal cell wall biosynthesis have revealed that the (1→3)-β-glucan synthase complex consists of at least two components. These are a catalytic subunit and a regulatory subunit (Kang and Cabib, 1986; Mol *et al.,* 1994). An

Figure 1.4 Overview of β-glucan biosynthesis pathway. Some synthases are synthesised in the endoplasmic reticulum, and subsequently transported into the Golgi apparatus where (1→3, 1→4)-β-glucan and xyloglucan production occurs. In contrast, the cellulose synthase complex ( ) and callose synthases complex ( ) are probably transported by secretory vesicles and anchored onto the plasma membrane where cellulose and callose are synthesised (Based on Stone and Clarke, 1992; Gibeaut and Carpita, 1994).


Nucleus
Synthase Synthesis
MG, XG synthases
& MG, XG production
ER
Golgi
Plasma Membrane
Callose Synthase
complex
Callose
Cell Wall
Cell wall Matrix
Cellulose Synthase
complex
Cellulose

activated GTP-bound Rho1p protein is associated with the catalytic subunit and has been functionally demonstrated as a regulatory subunit of the enzyme (Mol *et al.,* 1994; Drgonova *et al.,* 1996; Qadota *et al.,* 1996).

In plants, it is assumed that callose synthase is a plasma membrane-associated enzyme complex (Delmer, 1987; 1991; Gibeaut and Carpita, 1990). Studies also suggest that there might be two types of callose synthases in high plants, one of which is Ca^{2+}-activated and is found in a variety of plant tissues (Andrawis *et al.,* 1993; Delmer and Amor, 1995; Kudlicka and Brown, 1997), while the other is Ca^{2+}-independent and is found mostly in pollen tubes (Schlüpmann *et al.,* 1993). It has also been suggested that different groups of (1→3)-β-glucan synthases might be involved in different functions in a given species (Pelosi *et al.,* 2003). Moreover, it has been proposed that the enzyme complex in plants might be comprised of non-identical subunits. One line of evidence to support this suggestion is that callose synthase enrichment from plant membranes has always resulted in co-purification of many polypeptides (Fink *et al.,* 1990; Dhugga and Ray, 1991; Bulone *et al.,* 1995; Kudlicka and Brown, 1997; Li *et al.,* 1997; McCormack *et al.,* 1997). If the proteins co-purified with the synthase are indeed associated with the callose synthase complex, rather than presenting individual membrane-bound proteins, this may suggest that callose synthase requires additional proteins to function in plant cells. The different polypeptides might be involved in either priming (1→3)-β-glucan synthase reaction, in binding of the UDP-glucose substrate in the cytosol, in regulation of activity, or in the transfer of the activated glucose to a growing polymer (Delmer, 1987; 1999; Gibeaut and Carpita, 1994). Observations of Hayashi *et al.* (1987) also support the concept of an enzyme complex, in that divalent cations increase the size of callose synthase; this has been interpreted as an association with additional subunits. Furthermore, enzyme kinetic data from a microsomal fraction of *Arabidopsis thaliana* suggest the existence of positive homotropic cooperativity of (1→3)-β-glucan synthase activity with respect to the substrate UDP-glucose (Him *et al.,* 2001), from which these authors inferred that callose synthase activity is mediated by a multimeric complex containing either three catalytic subunits or a multiple of trimers of the catalytic subunits (Him *et al.*, 2001). It has been further suggested that callose synthase may well be a component of the cellulose synthase complex (Delmer and Amor, 1995; Kudlicka and

Brown, 1997). The possible relationship between callose synthase and cellulose synthase is discussed later in Section 1.5.

1.4.3 The Catalytic Subunit

1.4.3.1 Yeast and Fungi

Fungal pathogens such as *Cryptococcus neoformans*, *Paracoccidioides brasiliensis* and *Pneumocystis carinii* can cause life-threatening infections in humans (Thompson *et al.*, 1999; Pereira *et al.*, 2000; Kottom and Limper, 2000). The fungal cell wall has been an attractive target for drug therapy, because the synthesis of cell wall β-glucan is a process not found in mammalian cells. As a major component of the fungal cell wall, (1→3)-β-glucan has received particular attention. Studies on the biosynthesis of the polymer have been carried out, and genes encoding the catalytic subunits of callose synthase have been isolated from fungal species using genetic and biochemical approaches (Douglas *et al.*, 1994; Mazur *et al.*, 1995; Inoue *et al.*, 1995; Mio, 1997; Thompson *et al.*, 1999; Kottom and Limper, 2000).

A catalytic subunit of (1→3)-β-glucan synthase was first described as the product of the *FKS1* gene from *Saccaromyces cerevisiae* by complementation using echinocandin mutants that confer hypersensitivity to immunosuppressants (Douglas *et al.*, 1994). Evidence for *FKS1* gene function comes from gene disruption experiments. The mutation of *FKS1* results in a slow-growing yeast, hypersensitivity to immunosuppressants, and a significant reduction in (1→3)-β glucan synthase activity *in vitro*. Southern analysis reveals that there are two copies of the gene in yeast. The second gene, *FKS2* (Mazur *et al.*, 1995), is 88% identical to *FKS1* at the amino acid level, and was later cloned and functionally assigned as a redundant component of (1→3)-β glucan synthase. Simultaneous mutation of *FKS1* and *FKS2* is lethal, while a single disruption of either the *FKS1* or *FKS2* gene is not, suggesting that these two genes differ primarily in the manner in which their expression is controlled (Mazur *et al.*, 1995). *FKS1* is expressed in the cell cycle under optimal conditions, whereas *FKS2* expression is induced in the absence of glucose or in response to treatment with $CaCl_2$, or mating pheromones. Thus, *FKS2* is essential for cell sporulation, a process that occurs during nutritional starvation (Mazur *et al.*, 1995).

Using a biochemical approach, genes termed *GSC1* and *GSC2*, but corresponding to *FKS1* and *FKS2*, respectively, were independently identified from *Saccharomyces cerevisiae* (Inoue *et al.,* 1995). In a SDS/PAGE analysis of enriched membrane fractions obtained from product entrapment, a 200 kDa GSC1 protein was observed in parallel with an increase in specific activity of (1→3)-β-glucan synthase. Using a partially purified enzyme as antigen, an antibody that could immuno-precipitate (1→3)-β-glucan synthase activity was isolated, and the antibody recognised a 200 kDa protein on a Western blot. Sequencing data from this 200 kDa protein enabled the cloning of the *GS1* gene, for which the DNA sequence is identical to that of *FKS1*. These data provide further evidence to support the function of *FKS* genes as encoding (1→3)-β-glucan synthases, and provide a direct link between the gene sequence and enzyme activity.

In *Candida albicans*, *GSC1*, *GSL1* and *GSL2* genes have significant sequence similarities to *FKS1/GSC1* and *FKS2/GSC2* from *S. cerevisiae* (Mio *et al.,* 1997), at both the nucleotide and amino acid levels. *GSL1* and *GSL2* are probably equivalent to *FKS2/GSC2*, although the sequence of the *GSL2* gene is much shorter. Disruption of two of the three *GSC* and *GSL* genes in *Candida albicans* decreases cell wall (1→3)-β-glucan level by 50%. Other *FKS1* homologous genes have been reported for *Cryptococcus neoformans* (Thompson *et al.,* 1999), *Paracoccidioides brasiliensis* (Pereira *et al.,* 2000) and *Pneumocystis carinii* (Kottom and Limper, 2000).

The topological profiles of *FKS* gene products from *S. cerevisiae* and *C. albicans* and other species predicted from hydropathy plots showed that there are some 16 transmembrane helixes, and that the central parts of the proteins are probably cytoplasmic; the enzymes also possess signal sequences at their NH_2-terminal ends (Douglas *et al.,* 1994; Inoue *et al.,* 1995; Mazur *et al.,* 1995; Mio *et al.,* 1997; Thompson *et al.,* 1999; Kottom and Limper, 2000).

1.4.3.2 Higher Plants

Biochemical approaches have also been employed to study callose synthases in a variety of higher plants. Techniques such as detergent solubilisation, ultra-centrifugation, product entrapment, immunoprecipitation, isoelectric focusing and native gel electrophoresis have been used to enrich callose synthases from various membrane

fractions (Fink *et al.,* 1990; Dhugga and Ray, 1991; Meikle *et al.,* 1991; Pedersen *et al.*, 1993; Bulone *et al.,* 1995; Kudlicka and Brown, 1997; Li *et al.,* 1997; McCormack *et al.,* 1997; Him *et al.,* 2001; Pelosi *et al.,* 2003). However, the enzyme has never been purified to homogeneity, and many partial purifications have revealed not only the potential complexity of the enzyme, but also various apparent contradictions with respect to its properties. Thus, polypeptides ranging in size from 32 kDa to 190 kDa have been reported for (1→3)-β-glucan synthases in plants, and the presence of multiple proteins in all partially purified preparations has often led to suggestions that plant callose synthases actually consist of a multi-enzyme or multi-protein complex. However, the precise composition of the synthase complex, sequence information for proteins involved, and most importantly a definite proof of function of these peptides have not been demonstrated.

Many attempts have been made to define the relationships between individual peptides of the synthase complex, in particular on the identification of peptides which may act as catalytic subunits. Labelling of polypeptides with photoactive or radioactive analogues of UDP-glucose has enabled the identification of several potential peptides that in theory should bear the UDP-glucose binding site and therefore serve as the catalytic subunit of the synthase complex. Peptides of sizes 31 kDa, 52 kDa, 55 kDa, 57 kDa and 70 kDa have been identified from red beet, rye, cotton and *Lolium* (Frost *et al.,* 1989; Meikle *et al.,* 1991; Delmer *et al.,* 1991), and have been proposed as candidates for the catalytic subunit of the synthase.

In other studies, SDS analyses of partially purified preparations have also revealed polypeptides with molecular size ranges of 27-34 kDa, 52 kDa-57 kDa, and 65 kDa-70 kDa (Fink *et al.*, 1991; Dhugga and Ray, 1991; Fredrikson *et al.,* 1991; Delmer *et al.,* 1993; McCormack, 1997). For example, Fink *et al.* (1991) reported that antibodies raised against a 31 kDa peptide can immunopricipitate callose synthase activity from a partial purified fraction from suspension cells of *Glycine max*, suggesting that the 31 kDa peptide may be associated with the synthase complex. In a study of *in vitro* callose synthesis in Italian ryegrass (*Lolium multiflorum*) endosperm-derived suspension cultures (Bulone *et al.,* 1995), the product entrapped in enzyme preparations contained six major polypeptides, two of these were 30-31 kDa and 55-58 kDa and appeared to be involved in

callose synthesis, because these peptides were immunoprecipitated with monoclonal antibodies directed against the ryegrass (1→3)-β-glucan synthase (Meikle *et al.,* 1991). The synthase product precipitated from the reaction was verified as (1→3)-β-glucan on the basis of X-ray diffraction, electron microscopy, aniline blue staining and (1→3)-β-glucanase hydrolysis.

Kudlicka and Brown (1997) also made significant progress in identifying polypeptide components of a mung bean callose synthase, using product entrapment. The polypeptides associated with callose synthase were 32, 38, 54, 64, 78 kDa, from which the polypeptides of 54 and 64 kDa were common to both cellulose and callose synthase activity. This first separation, *in vitro,* of cellulose and callose synthase activity opened the way for dissecting the enzyme complex. Sequencing the subunits of the complex by in-gel digestion of SDS-PAGE bands would provide information on associated proteins from which a molecular approach can be employed for cloning of these synthase candidate genes, but so far this approach has not been reported.

Progress has been made on potential polypeptide components of pollen tube callose synthases (Rae *et al.,* 1985; Schlüpmann *et al.,* 1994; Ferguson *et al.,* 1998; Tuner *et al.,* 1998; Li *et al.,* 1999). The enzyme activity of this class of synthases can be distinguished from the wound-activated somatic tissues because they are not Ca^{2+}-dependant (Schlüpmann *et al.,* 1993). A polypeptide associated with developmentally regulated callose deposition in higher plants was reported from the pollen tubes of *Nicotiana alata* (Turner *et al.,* 1998). The polypeptide was 190 kDa and it was suggested that this polypeptide is a major component of the callose synthase complex (Turner *et al.,* 1998).

In summary, relatively wide range of molecular sizes of these candidates raises considerable doubt as to the veracity of their identification as (1→3)-β-glucan synthases. Furthermore, amino acid sequence data for the proteins has not been published. Similarly, little is known about genes encoding callose synthases in higher plants. Although there is sequence information available from yeast (Douglas *et al.,* 1994; Mazur, *et al.,* 1995; Inoue *et al.,* 1995; Mio, 1997), isolation of the callose synthase genes using a yeast probe has not been successful. At the time the present study was initiated there was no information describing genes encoding callose synthases in higher plants.

1.4.4 *Regulators of (1→3)-β-Glucan Synthesis*

The GTP-binding protein-Rho1 is believed to be a regulatory subunit of (1→3)-β-glucan synthase in yeast. It has been shown that Rho1 is required to activate a yeast kinase involved in the signal transduction pathway controlling cell wall integrity. Most recent studies (Drgonova *et al.*, 1996; Mol *et al.*, 1994; Qadota *et al.*, 1996) have shown that activated GTP-bound Rho1p is a positive regulatory subunit of (1→3)-β-glucan synthase, the enzyme responsible for much of cell wall glucan synthesis in yeast.

A 20 kDa GTP-binding protein, identified by photolabelling, correlated with (1→3)-β-glucan synthase activity in yeast (Mol *et al.*, 1994). A further mutation study carried out by Drgonova *et al.* (1996) and Qadota *et al.* (1996) has shown that Rho1 mutants were defective in GTP stimulation of (1→3)-β-glucan synthesis and the addition of purified or recombinant Rho1p can correct the defect. In addition, immunoblot analysis (Qadota *et al.*, 1996) revealed that Rho1 copurified with (1→3)-β-glucan synthase and associated with the Fsk1p subunit of the synthase complex *in vivo*, so that both proteins were localised at the sites of cell wall remodelling. Taken together, these data suggest that Rho1 is a potential regulatory subunit of (1→3)-β-glucan synthase.

1.5 Association with Cellulose Synthases

There have been suggestions for many years that cellulose synthase might be physically associated with callose synthase in higher plants (Delmer, 1999). There are similarities between structures of callose and cellulose synthase products, in substrate requirements, and in cellular locations. Indeed, it has been proposed that callose and cellulose synthases might be a single enzyme capable of synthesising both types of polysaccharides, depending on cellular conditions (Delmer, 1999).

Cellulose is an unbranched polymer in which glucosyl residues are linked by (1→4)-β linkages. Cellulose synthase is predicted to be located on the plasma membrane (Delmer, 1999; Saxena and Brown, 2000), which is the same location predicted for callose synthase. Cellulose synthase adopts a rosette structure on the plasma membrane of higher plants, as revealed by freeze-fracture electron microscopy (Montezinos and Brown, 1976). Such structures, termed terminal complexes, are believed to be the site where

cellulose microfibrils are initiated and elongated. It is also known that cellulose and callose synthase both utilise the same substrate, UDP-glucose, for the *in vitro* synthesis of both polysaccharides (Feingold *et al.,* 1958; Aloni *et al.,* 1983). Cellulose synthesis requires the substrate at micromolar concentration in *in vitro* assays (Ray *et al.,* 1969), whilst callose synthesis is favoured in the presence of UDP-glucose at millimolar concentrations (Van der Woude *et al.,* 1974). Moreover, it has been found that the synthesis of callose occurs coincidentally with the synthesis of cellulose when membrane preparations from mung bean, and from *Rubus fruticosus* (blackberry) are incubated with UDP-glucose (Kudlika and Brown, 1997; Him *et al.,* 2002). In some cases, little or no cellulose synthesis activity is detected above a very high background of callose synthase activity (Feingold *et al.,* 1958; Delmer, 1999). In other cases, about 20% of *in vitro* activity has been attributed to cellulose biosynthesis and over 80% to callose production, using suspension-cultured cells of *Rubus fruticosus* as an enzyme source under optimised biosynthesis conditions; this is the highest rate for *in vitro* cellulose synthesis reported so far (Him *et al.,* 2002) and indeed the only convincing demonstration of a relatively high level *in vitro* cellulose biosynthesis using plant enzyme systems.

Whilst biochemical studies with cellulose synthases from higher plants have been frustrating, and information on genes encoding cellulose synthase has become available in recent years (Pear *et al.,* 1996; Arioli *et al.,* 1998; Taylor *et al.,* 1999; Burton *et al.,* 2000). Two cotton cDNAs encoding putative cellulose synthases were reported for the first time in higher plants by Pear *et al.* (1996). The genes were isolated by random sequencing of a cDNA library made from 21 days post anthesis cotton boll tissue, where a massive amount of cotton fibre and therefore cellulose is produced. Multiple alignment of deduced amino acid sequences of the cotton *CelAs* (later called *CesAs*) and bacterial CelA proteins, suggested three homologous regions and two hypervariable regions exist between bacteria and plants. Following this success, positional cloning of a temperature-sensitive mutant *RSW1* (Root swelling) gene from *Arabidopsis* (Arioli *et al.,* 1998) was achieved by screening mutants for exaggerated radial expansion of root cells. The temperature-sensitive *RSW1* allele disassembles the cellulose synthase complexes, alters cellulose crystallinity and disrupts morphogenesis, and is therefore considered as a candidate gene for cellulose synthase.

It is known that gene transcripts for putative cellulose synthase in higher plants range in size from 3.0 kb to 3.5 kb and the proteins encoded by these genes are predicted to be membrane proteins with a number of conserved structural features. A large presumably cytoplasmic domain is separated by several transmembrane helixes, which are located at both the NH_2- and COOH- termini. Conserved motifs from the putative cytoplasmic region have been proposed to define the UDP-glucose binding domain and the catalytic sites in these glycosyltransferases (Saxena *et al.,* 1995). Evidence of the function of cellulose synthase (*CesA*) genes in plants, although lacking biochemical proof, has been obtained from several studies including gene-expression and UDP-binding (Pear *et al.,* 1996), mutant analysis (Arioli *et al.,* 1998; Delmer, 1998; Taylor *et al.,* 1999), and gene-silencing experiments (Burton *et al.,* 2000). More cellulose synthase genes have been discovered in genome sequencing projects. These large groups of genes from higher plants constitute a cellulose synthase superfamily, consisting of cellulose synthase genes (*CesA*), and cellulose synthase-like (*Csl*) genes, which are subdivided into *CslA*, *B*, *C*, *D*, *E*, and *F* subclasses. The functions of the various *Csl* groups are not known, but they may also be responsible for the synthesis of other polysaccharides found in plant cell walls (Richmond *et al.,* 2000; Richmond and Somerville, 2000), such as xyloglucans, xylans and arabinans. The involvement of some of the genes from *Csl* sub-families in callose synthesis cannot be ruled out.

As mentioned above, it is not clear whether cellulose synthase and callose synthase form part of the same enzyme, or if they are present on separate enzyme complexes (Ferguson *et al.,* 1998). Many hypotheses have been proposed, including that one enzyme, the CesA subunit, is responsible for both cellulose and callose synthesis under differential regulation conditions. The other suggestion is that there is a separate subunit, such as the FKS1-like protein, required for callose synthesis. Genes coding for (1→3)-β-glucan synthases from bacterial species show sequence similarity with the bacterial cellulose synthase (Stasinopoulos *et al.,* 1999), but not with the *FKS1* genes. This leads to the question as to whether the same catalytic subunit can perform both (1→3)-β- and (1→4)-β- glycosyltransferase activities, and what functions *FKS1*-gene homologues perform in high plants?

1.6 Aims of the Present Study

Physical and chemical analysis, ultrastructural microscopy and immunochemical studies have demonstrated that callose is deposited in specialised plant cell walls during normal growth and development, and near walls of plants after wounding and certain external stimuli. The sites, morphology, timing and the nature of constituents of callose-containing wall appositions vary between tissues, and plant species. The roles of such depositions in some cases are well defined, in others, are yet to be established. However, little is known about mechanisms of callose synthesis at the molecular level, and our understanding of callose biosynthesis has therefore been severely limited.

The principal aim of the work described in this thesis was therefore to investigate molecular mechanisms that underpin callose biosynthesis in barley. Molecular approaches have been employed to isolate candidate genes for callose synthase from barley, and biochemical approaches have been used subsequently to investigate the involvement of the genes in callose biosynthesis. The isolation and characterisation of a putative callose synthase cDNA from barley is described in Chapters 2 and 3 of this thesis. Heterogenous expression of a fragment of the putative barley callose synthase cDNA clone, and the production and affinity purification of anti-callose synthase antibodies, are described in Chapter 4. Attempts to partially purify callose synthase from barley, and the subsequent characterisation of the products synthesised by enriched membrane fractions are outlined in Chapter 5. In Chapter 6, immuno-blotting, Matrix Assisted Laser Desorption Ionisation-Time Of Flight (MALDI-TOF) mass spectrometric analysis of peptides associated with callose activity from enriched fractions, all of which were conducted to establish links between the cDNA sequence and its gene product, are described.

CHAPTER TWO:

MOLECULAR CLONING OF A BARLEY HOMOLOGUE TO A YEAST (1→3)-β-GLUCAN SYNTHASE GENE

2.1 Introduction

As described in Chapter 1, the primary objective of this study was to isolate genes encoding (1→3)-β-glucan synthases in barley in order to help understand and dissect the molecular basis of (1→3)-β-glucan synthesis in higher plants.

Since (1→3)-β-glucans are a major component of the fungal and yeast cell wall (Stone and Clarke, 1992), (1→3)-β-glucans and their biosynthesis have been extensively studied in these organisms. A *Saccharomyces cerevisiae* gene designated *FKS1* and encoding an integral membrane protein of 215 kDa, was first identified as a key component involved in (1→3)-β-glucan biosynthesis through complementation experiments (Douglas *et al.*, 1994, Section 1.4.3.1). A short time later *FKS2*, a homologue of *FKS1*, encoding a 217 kDa integral membrane protein which is 88% identical to FKS1 protein, was also cloned (Mazur, 1995). The mechanism of (1→3)-β-glucan synthesis was found to be quite conservative among yeast and fungal species. Homologues to *FKS1* and *FKS2* have since been isolated from the fungus *Candicans albicans* and designated *GSC1*, *GSL1* and *GSL2* (Mio *et al.*, 1997, Section 1.4.3.1). The amino acid sequences encoded by the *C. albicans* and the *FKS* genes show extensive structural similarity, according to their predicted topological profiles.

In bacteria, a gene encoding curdlan synthase, has been recently isolated using a curdlan deficient-mutant created by transposon insertion (Stasinopoulos *et al.*, 1999). Curdlan is also a liner (1→3)-β-glucan. Domain analysis of the protein sequence deduced from the curdlan synthase gene, however, shows a striking resemblance to the cellulose synthase (*CesA*) and the cellulose-like (*Csl*) genes (Delmer, 1999). For example, the curdlan synthase contains the putative catalytic D, D, D and QXXRW amino acid motifs, which are also found in putative cellulose synthases. However, there is little sequence similarity found between the *FKS* genes of yeast and the curdlan synthase gene of bacteria, and the conserved motifs are not found in yeast *FKS* genes (Stasinopoulos *et al.*, 1999).

In higher plants, isolation of (1→3)-β-glucan synthase cDNAs using molecular approaches, such as PCR amplification employing primers made to conserved regions of yeast and fungal (1→3)-β-glucan synthase genes, or the screening of cDNA libraries

using cDNAs encoding yeast and fungal (1→3)-β-glucan synthases as probes, had not been reported when this study was initiated in 1999. From a biochemical point of view, purifying the (1→3)-β-glucan synthase to homogeneity had proven difficult (Section 1.2). Therefore, there was no amino acid sequence information available for plant callose synthases, nor had antibodies been raised against them.

However, a homologue of the yeast (1→3)-β-glucan synthase gene was isolated from *Hieracium* (N. Peach and A. Koltunow, personal communication) during a study carried out at the CSIRO Division of Horticulture (Adelaide, Australia). The cDNA from *Hieracium* was isolated via differential screening of cDNA libraries constructed for an ovary development study. A number of clones obtained from the library were matched against the database, from which a cDNA homologous to the yeast *FKS* genes was identified.

In this Chapter, the molecular cloning of a putative callose synthase cDNA from barley, using the cDNA from *Hieracium* as a probe, is described, and approaches undertaken to obtain a full-length callose synthase cDNA are presented.

2.2 Materials and Methods

2.2.1 Materials

Barley var. (*Hordeum vulgare cv.* Clipper) for callus generation was supplied by the Barley Quality Laboratory (Department of Plant Science, University of Adelaide, Australia). A barley callus cDNA library constructed in the λZAP II bacteriophage vector was prepared by Dr. W. Gremski. The λZAPII-cDNA synthesis kit, *E.coli* strain XL1-Blue MRF', SOLR, ExAssist helper phages, and plasmid pBluesript II (SK^+) were purchased from Strategene (La Jolla, CA, USA). DNA oligonucloetide primers and the BRESA-CLEAN kit were from Geneworks (Adelaide, SA, Australia). Taq DNA polymerase, restriction enzymes and DNA phosphorylase were obtained from New England Biolabs (Beverley, MA, USA). T4 DNA ligase, T4 RNA ligase, 1 Kb DNA ladder molecular mass standards, and the pGEM T-Easy vector system were from Promega Corporation (Madison, WI, USA). The thermoscript RT-PCR kit, including avian RNase H-minus reverse transcriptase, RNA-OUT, DTT, dNTPs and the TRIZOL reagent for RNA isolation were from GIBCO BRL (Gaithersburg, MD, USA). The megaprime DNA labelling kit, Hybond-N^+ membranes, autoradiographic film, [α^{32}P]-dCTP were obtained from Amersham International. The DNA sequencing kit was provided by the Perkin-Elmer Corporation (Foster City, CA, USA), DNA sequencing was performed on an ABI 373 sequencer with stretch upgrade software (Nucleic Acid and Protein Chemistry Unit-NAPCU, University of Adelaide, Australia). The barley bacterial artificial chromosome (BAC) library and corresponding positive clones were purchased from Clemson University Genomic Institute (Clemson, SC, USA). Tryptone, yeast extract and agar were from Difco (Detroit, MI, USA).

2.2.2 Screening of the Barley Callus ZAPII cDNA Library

A ZAPII cDNA library prepared from barley callus cDNA was used initially to isolate (1→3)-β-glucan synthase-like genes. The cDNA synthesised from an oligo (dT) primer was inserted into the *Eco*RI and *Xho*I sites of the bacteriophage vector Uni-ZAP^{TM} XR using the ZAP-cDNA synthesis kit according to the manufacturer's instructions. The library was amplified once in the host strain *E.coli* XL1-Blue MRF' cells and stored at -80°C.

2.2.2.1 Preparation of [^{32}P]-Radiolabelled Probes

A 5.0 kb cDNA from *Hieracium piloselloides* (Koltunow *et al.*, 2000, and unpublished data), tentatively named *HpGSL1* and a homologue to the yeast (1→3)-β-glucan synthase gene *FKS1*, was used as a probe for screening the callus cDNA library of *Hordeum vulgare L.* The *Hieracium HpGSL1* cDNA in the pBluescript phagemid originating from the ZAPII vector was digested sequentially with restriction enzymes *Sma*I and *Sal*I at 25°C and 37°C, respectively, with an incubation time of 2 h for each digestion. The resulting fragments were separated on a 1% agarose gel, and the desired fragment was excised and purified using the BRESA-CLEAN™ DNA Purification kit. This kit is specifically designed to purify DNA longer than 200 bp, which is convenient for recovering large DNA species and leaving behind small nucleic acids such as excess DNA linkers, excess primers from PCR products and oligonucleotides, etc. Briefly, the gel piece containing the DNA fragments was mixed with 3 vol BRESA-SALT™ solution and melted at 55°C for 5 to 10 min. A 5 µl aliquot of well-vortexed BRESA-BIND™ was added to the DNA solution, mixed, and incubated for 5 min at room temperature to bind the DNA, mixing regularly to keep the BRESA-BIND™ in suspension. The BRESA-BIND™/DNA complex was pelleted for 5 sec and the pellet was washed once with BRESA-WASH™ by resuspending the pellet in a volume equivalent to the amount of BRESA-SALT™ used previously. Two additional washes were performed and the pellet was dried in a Speedivac. The DNA was recovered by resuspending the white pellet in TE (10 mM Tris-HCl buffer, pH 8.0, 1 mM EDTA) and the aqueous phase containing the DNA was removed from the residual glass milk after a brief spin.

The purified DNA fragment was labelled with [α^{32}P]-dCTP using the protocol provided with the Megaprime labelling kit. To do this, 100 ng of the 2267 bp *HpGSL1* cDNA was mixed with 10 ng of random nonamers in a total volume of 33 µl. The mixture was boiled for 5 min and cooled on ice for 5 min. A labelling mixture containing 10 µl labelling buffer, 2 µl Klenow DNA polymerase and 5 µl [α^{32}P]-dCTP was added and incubated at 37°C for 30 min. The radio-labelled fragment was fractionated through a Sephadex G-100 column using 1% (w/v) Blue Dextran and 1% (w/v) Orange G as indicators, and eluted with TE. The progress of the labelled probe was checked against the Geiger counter as it was eluted. The probe was boiled for 5 min to denature it, and added to the hybridisation buffer.

2.2.2.2 Library Plating and Plaque Hybridisation

The host strain *E.coli* XLI-Blue MRF cells were prepared in LB-medium containing 1% (w/v) NaCl, 1% (w/v) tryptone and 0.5% (w/v) yeast extract, pH 7.0, supplemented with 10 mM $MgSO_4$ and 0.2% (w/v) maltose without antibiotics. The cells were grown at 37°C until the A_{600} of the culture reached 0.6, and were pelleted at 500×g for 10 min, resuspended in 10 mM $MgSO_4$ at an A_{600} of 1, and stored at 4°C for less than 2 days before being used.

A serial dilution of the cDNA library was prepared. A total of approximately 1×10^6 plaques from the amplified library was plated onto lawns of XL1-Blue MRF' cells in pre-warmed (50°C) NZY broth (0.5% w/v NaCl, 0.5% yeast extract, 0.2% w/v $MgSO_4$ 7 H_2O, 1% w/v casein hydrolysate and 0.7% agarose) over 1.5% agar/NZY Plates (150 mm). The plates were incubated in an inverted position at 37°C for 9 h or until the plaques appeared on the plates. The plates were cooled at 4°C overnight.

Nitrocellulose filters were placed onto the chilled agarose plates for 3 min with three asymmetric locations marked. The filters were carefully peeled off and the DNA on the filters was denatured with 0.5 M NaOH containing 1.5 M NaCl, and neutralised with 0.5 M Tris-HCl buffer, pH 7.5, containing 1.5 M NaCl. The filters were air dried and subsequently baked at 80°C under vacuum for 2 h to fix the DNA to the membrane.

The filters were prehybridised in 200 ml prehybridisation buffer containing 6×SSC (1×SSC: 90 mM sodium citrate, pH 7.5, 0.9 M NaCl), 20 mM NaH_2PO_4, 0.5% w/v SDS, 5×Denhardt's reagent (0.1% w/v Ficoll, 0.1% w/v PVP, 0.1% w/v BSA), and 100 µg/ml denatured salmon sperm DNA at 65°C for 2 h. After prehybridisation, the filters were transferred to 50 ml hybridisation buffer which contained the same components as the pre-hybridisation buffer, except the SSC concentration was reduced from 6× to 3×. The denatured radio-labelled probe was added and the filters hybridised with gentle shaking in a water bath at 65°C overnight. The filters were sequentially washed at 65°C for 20 min, with 2×SSC, 0.1% w/v SDS, and with 1×SSC, 0.1% w/v SDS, 0.5×SSC, 0.1% w/v SDS, and finally with 0.1×SSC, 0.1% w/v SDS. The filters were air-dried and antoradiographed against X-ray "Hyperfilm™-MP at -80°C for a period of between 2 h to 2 days, depending on the intensity of the positive plaques as judged with the Geiger

counter. The films were developed and aligned with the filters to identify positive plaques. Such positive clones were subjected to three rounds of plaque purification using the method described above until a single, well-isolated plaque could be cored from a plate.

2.2.2.3 Rescue of Positive cDNA into pBluescript Phagemids

The purified, positive cDNA clones from the callus cDNA library were rescued directly into pBluescript phagemids according to the single-clone excision protocol described by Stratagene. The plaques of interest were cored from the agar plates and transferred to 500 µl SM buffer (50 mM Tris-HCl buffer, pH 7.5, containing 10 mM NaCl, 8 mM $MgCl_2$, 0.01% w/v gelatine) and 20 µl chloroform in microcentrifuge tubes. The tubes were votexed to release the phage particles into the SM buffer and incubated for 2 h at room temperature or overnight at 4°C prior to excision. This stock was maintained at 4°C for up to 6 months.

To excise, a 250 µl aliquot of each phage stock was combined with 1 µl of the ExAssist helper phage (>1×10^6 pfu/µl) in 200 µl XL1-Blue MRF cells at A_{600} of 1.0 (Section 2.2.2.2) and incubated at 37°C for 15 min for infection to occur. The mixture was further incubated with the addition of 3 ml LB broth for 3-16 h, heated at 65°C for 20 min and pelleted at 1000×g for 15 min. An appropriate amount of supernatant containing the excised phagemid was incubated at 37°C for 15 min with host SOLR cells and plated onto LB-ampicillin agar plates (50 µg/ml). The plates were incubated overnight at 37°C to allow the growth of the phagemid colonies.

2.2.2.4 Phagemid (Plasmid) DNA Mini Preparation

Colonies harbouring phagemid DNA of interest were picked. The alkaline-lysis method for plasmid DNA mini-prep procedure was employed for the phagemid DNA isolation as follows. A single colony was inoculated into 3 ml LB media containing 100 µg/ml ampicillin, and incubated with shaking at 37°C for 18-20 h. The cells were harvested at 5,000×g for 5 min, and resuspended in 100 µl ice-cold GTE buffer (20 mM Tris-HCl buffer, pH 8.0, containing 50 mM glucose and 10 mM EDTA). Following incubation on ice for 5 min, 200 µl freshly prepared lysis solution (0.2 M NaOH + 1% SDS) was added

to the suspension and mixed by gentle inversion several times, followed by incubation on ice for 5 min. The lysed cells were neutralised with the addition of 200 µl of 3 M potassium acetate buffer, pH 8.0. The mixture was subsequently centrifuged at 12,000×g for 10 min to pellet the cell debris. The DNA was precipitated from the supernatant with 1 ml 100% ethanol for 15 min at -20°C, and recovered by centrifugation at 12,000×g for 15 min. The recovered DNA was washed twice with cold 70% ethanol, dried, and redissolved in TE (10 mM Tris-HCl buffer, pH 8.0, 1 mM EDTA) containing 40 µg/ml RNase A.

2.2.2.5 Automated DNA Sequencing

The phagemid DNA prepared by the alkaline lysis method was further purified using a Sephadex CL-6B column prior to sequencing. The DNA was loaded onto the column, and recovered by centrifugation at 500×g for 2 min. For automated sequencing, the PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing kit was used. Four different fluorescently labelled dideoxynucleotides were used in the dideoxy chain termination reaction. A dye group is incorporated into the DNA sequence along with the terminating base when these terminators replace standard dideoxy nucleotides in the polymerase chain reaction (PCR) and is detected with a laser on the automated sequencing machine (Perkin Elmer PRISM™ Ready Reaction DyeDeoxy™ Terminator Cycle Sequencing kit protocol).

The sequencing reaction was prepared by combining 0.5 µg phagemid DNA, 3.2 pmoles primer, and 8 µl standard ABI PRISM™ Dye Terminator Ready Reaction Mix in a final volume of 20 µl. The PCR reaction was carried out in a Thermal Cycler with the following cycling profile: denaturing at 95°C for 15 sec, annealing at 55°C for 15 sec, and at 50°C extension for 4 min. The steps were repeated 25 times, and rapidly ramped to 4°C. The extension products were purified by ethanol precipitation, followed by two washes with 70% ethanol, dried, resuspended in the sequencing loading buffer (125 mM deionised formamide, 25 mM EDTA, pH 8.0, 50 mg/ml Blue Dextran), and analysed on the ABI 373 DNA sequencer (Perkin Elmer, CA, USA) in the Nucleic Acid and Protein Chemistry Unit (NAPCU, Adelaide, Australia). Sequenced products were analysed using the Seqed software.

2.2.3 Amplification of 5' End Sequence of the Barley (1→3)-β-Glucan Synthase-like cDNA by RT-PCR

DNA can be amplified from a single-stranded RNA template by combining a standard polymerase chain reaction (PCR) protocol with an initial incubation with reverse-transcriptase (Sambrook *et al.*, 1989). RT-PCR (Figure 2.1) involves the synthesis of first strand cDNA using RNA as a template, primed with either an oligo dT or a gene-specific primer, and using this cDNA with other primers in a subsequent PCR amplification. This strategy can be used to amplify DNA fragments for which the primers were readily designed for both 5' (forward primer) and 3' (reverse primer) ends as illustrated in Figure 2.1.

The detailed procedures for RNA extraction, first strand DNA synthesis, and subsequent PCR amplification, the cloning of the PCR product, and relevant DNA manipulation techniques are described in the following sections.

2.2.3.1 Extraction of RNA from Barley Callus

Care was taken during the RNA extraction to prevent the introduction of adventitious RNases into the preparation. All glassware was baked at 180°C for 8-16 h. Mortars and pestles were soaked in 0.4 M NaOH for 30 min, rinsed well with MilliQ water, and baked. Gel tanks and gel casting trays were also treated with 0.4 M NaOH. Tubes, tips and solutions used for the RNA work were made RNase-free by soaking them in 0.1% (v/v) DEPC water overnight, followed by autoclaving for 20 min at 120°C.

A method using the TRIzol reagent, a commercially available mono-phasic solution of phenol/guanidine isothiocyanate, was used to extract total RNA from barley tissues according to the manufacture's instruction (GIBCO BRL, TRIzol reagent protocol) with slight modifications as described below.

Callus (100-200 mg) grown on a solid culture plate was harvested and ground under liquid nitrogen using a mortar and a pestle and transferred to an Eppendorf tube. TRIzol reagent (1ml) was immediately added to the ground material, which was vortexed and incubated at room temperature for 15 min. The suspension was centrifuged at 12,000×g

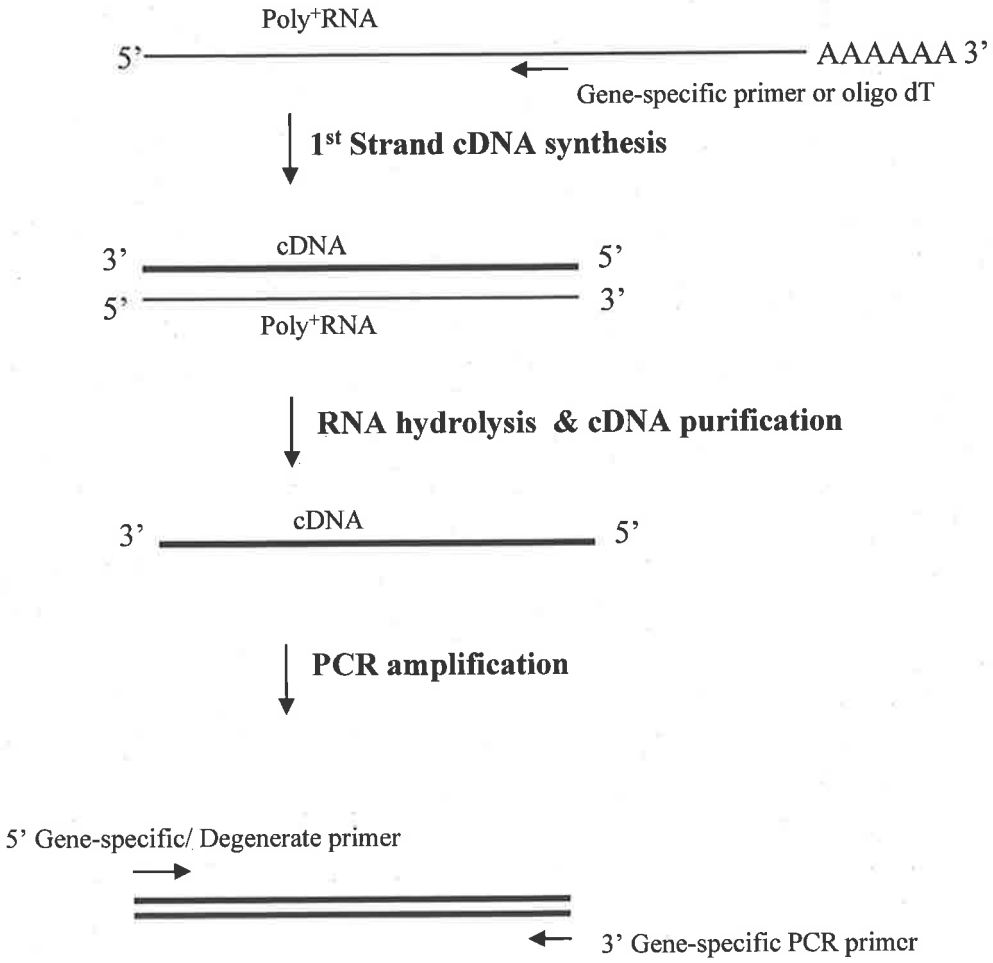


Figure 2.1 A schematic diagram of RT-PCR. The first strand cDNA was synthesised from the total RNA of barley callus using a 3' gene-specific primer. This primer and a gene-specific or degenerate 5' primer were used in a subsequent PCR reaction to amplify the cDNA (Frohman *et al.*, 1988).

at 4°C for 10 min to pellet polysaccharides and cell wall debris. The supernatant was transferred to a new tube containing 200 µl chloroform, shaken vigorously for 15 sec, and incubated at room temperature for 5 min. The aqueous phase containing the RNA was recovered by centrifuging the mixture at 11,200×g at 4°C for 15 min. The upper phase was transferred to a clean tube, 500 µl isopropanol was added and the contents mixed, incubated at room temperature for 10 min and centrifuged at 11,200×g at 4°C for 15 min to precipitate the RNA. The RNA pellet was washed with 1 ml RNase-free 75% ethanol, dried, resuspended in 10 µl sterile Milli Q water, and stored at -20°C.

The concentration of the RNA was determined by measuring the A_{260}. Calculations were carried out based on the fact that a 40 µg/ml RNA solution gives an absorbance of 1 at 260 nm (Sambrook *et al.*, 1989).

2.2.3.2 First Strand cDNA Synthesis

A 2 µg quantity of total RNA was mixed with the required primer, adjusted to a final volume of 10 µl with DEPC-treated water and incubated at 65°C for 5 min to denature the RNA. This mixture was snap-cooled in ice. To synthesise the first strand cDNA, a mixture containing 4 µl 5×synthesis buffer supplied by the manufacturer (1×buffer: 0.1 M DTT, 40 U RNase OUT, 2 µl 10 mM dNTP), and 15 U THERMOSCRIPT RT was added to the denatured RNA in a final volume of 20 µl on ice. The mixture was incubated at 58°C for 45 min, and heated to 85°C for 5 min to terminate the reverse transcription reaction. The RNA in the reaction was digested by adding 1 µl *E.coli* RNase H and incubating at 37°C for 20 min.

2.2.3.3 PCR Amplification from Single Stranded cDNA (RT-PCR)

The PCR was carried out in a 50 µl reaction mixture containing 2 µl of the cDNA synthesis reaction, 5 µl 10×PCR buffer (1×PCR buffer: 50 mM KCl, 10 mM Tris-HCl buffer, pH 8.3, 0.1% TritonX-100), and 2 µl 50 mM $MgSO_4$, 2 µl 5 mM dNTPs, 1 U Taq DNA polymerase. To perform a hot start, the mixture was mixed gently and brought to 94°C on a thermal cycler and held for 1 min, and 10 µM of each forward and reverse primer was added. The mixture was thermal-cycled using the appropriate conditions, based on such parameters as the T_m of the primers and predicted size of the amplified

products, an example of which might be 94°C for 1 min, 55°C for 3 min, and 72°C for 1 min for a total of 35 cycles with a final extension at 72°C for 5 min.

2.2.3.4 Subcloning PCR Products into T-Easy Vector

In order to confirm the identity of a PCR product, the DNA fragments were routinely sub-cloned into a suitable vector such as T-Easy, and sequenced.

To purify the PCR products, the PCR mixtures were separated on a 1% agrose gel in TAE buffer (40 mM Tris-HCl buffer, pH 8.0, 1 mM EDTA,). The gel was stained with ethidium bromide and selected bands were excised. The DNA was extracted from the gel with the "BRESA-CLEAN™ kit according to the manufacturer's instructions (Section 2.2.2.1).

The commercially available pGEM T-Easy vector was prepared by digestion with *Eco*RV followed by the addition of a 3' terminal thymidine (T) to both ends. These single 3'-T overhangs are compatible with PCR products generated by Taq polymerase since they usually bear a single 5' A overhang, they allow high efficiency of ligation of a PCR product into the plasmid, and at the same time prevent recircularisation of the empty vector.

The vector contains T7 and SP6 RNA polymerase promoters, an α-peptide coding region of the enzyme β-galactosidase and multiple restriction sites. These features allow recombinant clones to be directly identified by colour selection, the release of the insert by digestion with a single restriction enzyme, and direct sequencing of the insert using T7 and/or SP6 primers.

In practice, the purified PCR products were combined with the T-Easy vector at an optimised ratio of 3:1 in the presence of a ligation buffer (30 mM Tris-HCl buffer, pH 7.8, containing 10 mM $MgCl_2$, 10 mM DTT and 1 mM ATP) and 3 U T_4 DNA ligase. Water was added to a final volume of 10 µl, and the mixture was incubated either for 2-3 h at room temperature or overnight at 4°C. The ligation mixture was then ready for transformation.

2.2.3.5 *Preparation of* E. coli *Competent Cells and DNA Transformation*

Hanahan's method for chemical treatment was adapted for the preparation of competent cells as outlined below (Inoue *et al.*, 1990). *E. coli* DH 5α cells were used as the host strain and grown in LB overnight at 37°C with vigorous shaking. A fresh LB culture was inoculated and grown until the A_{600} of the culture reached 0.6. The culture was aliquoted into 20 ml volumes in pre-chilled centrifuge tubes, left on ice for 10 min, and spun at 5,000×g for 5 min at 4°C. To induce competence, the pellet was resuspended in 5 ml ice-cold transformation buffer A (10 mM MES buffer, pH 6.3, containing 100 mM RbCl, 45 mM $MnCl_2$, 3 mM $[Co(NH_3)_6]$ Cl_3) on ice for 10 min. Cells were harvested by centrifugation, resuspended in 200 µl fresh transformation buffer A supplemented with 7 µl ice-cold buffer B (1.53 g DDT, 100 µl KAc, 9 ml DMSO in a final volume of 10 ml). Following 15 min incubation on ice, a further 7 µl buffer B was added to the cells, and a final incubation was carried out for 15 min on ice. Competent cells were snap-frozen in 100 µl aliquots in liquid N_2 and stored at -80°C.

For transformation, 100 µl frozen competent cells was thawed on ice, one half of the ligation mixture was added to the cell suspension, and incubated on ice for 20 min. The cells/DNA mixture was heat-shocked for 90 sec at 42°C, followed by recovery of cells on ice for 5 min. LB broth (500 µl) was added to the mixture which was subsequently incubated for 1 h at 37 °C with gentle shaking. Cells were harvested by centrifugation at 5,000×g for 30 sec, resuspended in 200 µl LB, and plated on a LB agar plate containing 50 µg/ml ampicillin, 40 µg/ml X-gal, 12.5 µg/ml IPTG. The plate was incubated overnight at 37°C. Colonies harbouring plasmids with the insert DNA were white, whilst colonies containing unmodified plasmid remained blue. The white colonies were selected and used to inoculate individual overnight cultures for the preparation of plasmid DNA (Section 2.2.2.5).

2.2.4 *Amplification of 5' end of (1→3)-β-Glucan Synthase-like cDNA using Anchor-ligated PCR*

The anchor-ligated PCR method is an efficient and relatively simple technique for generating a cDNA library for the immediate cloning of the 5' ends of mRNA

(Biotechniques Euro Edition, 1993), when designing a gene specific primer becomes impossible for the region that to be cloned. This technique differs from the RT-PCR method, where a gene specific primer can be designed and used as a 5' end primer with another gene specific primer at the 3' end (Section 2.2.3.1). When a 5'primer can not be designed, the anchor-ligated PCR becomes a useful method in cloning the upstream region of the target cDNA.

The principle of the anchor-ligated PCR technique is illustrated in Figure 2.2. A unique anchor and the corresponding anchor primer were designed. The anchor was designed to have a 3' end amine group to prevent self-ligation and a 5' end phosphate group to allow ligation to cDNAs. The first strand cDNA is synthesised with a gene-specific primer (or an oligo dT), which is similar to the method described in RT-PCR method (Section 2.2.3.2). The cDNA generated is purified and the anchor is attached to the end of the first strand cDNA by ligation using T4 RNA ligase. A primary primer (this primer can be the same primer as used to prime the mRNA for the first strand DNA synthesis) and a nested secondary primer are designed for each cDNA to be amplified. The anchor-ligated cDNA can be amplified by PCR with gene-specific primer and anchor primer. The detailed procedure is as the follows.

First strand cDNA synthesis was carried out with a gene-specific primer as described in Section 2.2.3.2. Following the termination of reverse transcription, 2 µl 6 M NaOH was added to the reaction mixture to hydrolyse RNA that remained in the mixture. The cDNA was neutralised with 2 µl 6 M acetic acid and subsequently purified using the Gene-Clean kit (Section 2.2.2.1), except that the cDNA was eluted twice from the silica particles in 25 µl water in the final step of the purification. The purified cDNA was incubated in a precipitation mixture (20 µg glycogen, 5 µl 2 M sodium acetate and 100 µl 95% v/v ethanol) for 30 min at -20°C, recovered by centrifugation for 10 min, washed twice with 70% ethanol, dried, and resuspended in 5 µl water. The cDNA was stored at -20°C.

To ligate the anchor to the single-stranded cDNA, 1 µl cDNA from the cDNA stock was combined in a 10 µl ligation mixture containing 6 pmol anchor nucleotide, 10 U T4 RNA ligase, 50 mM Tris-HCl buffer, pH 8.0, containing 10 mM $MgCl_2$, 20 µM ATP, 10 µg/µl DNase-free BSA and 25% w/v PEG 8,000. The ligation was carried out for 6 h at 37°C.

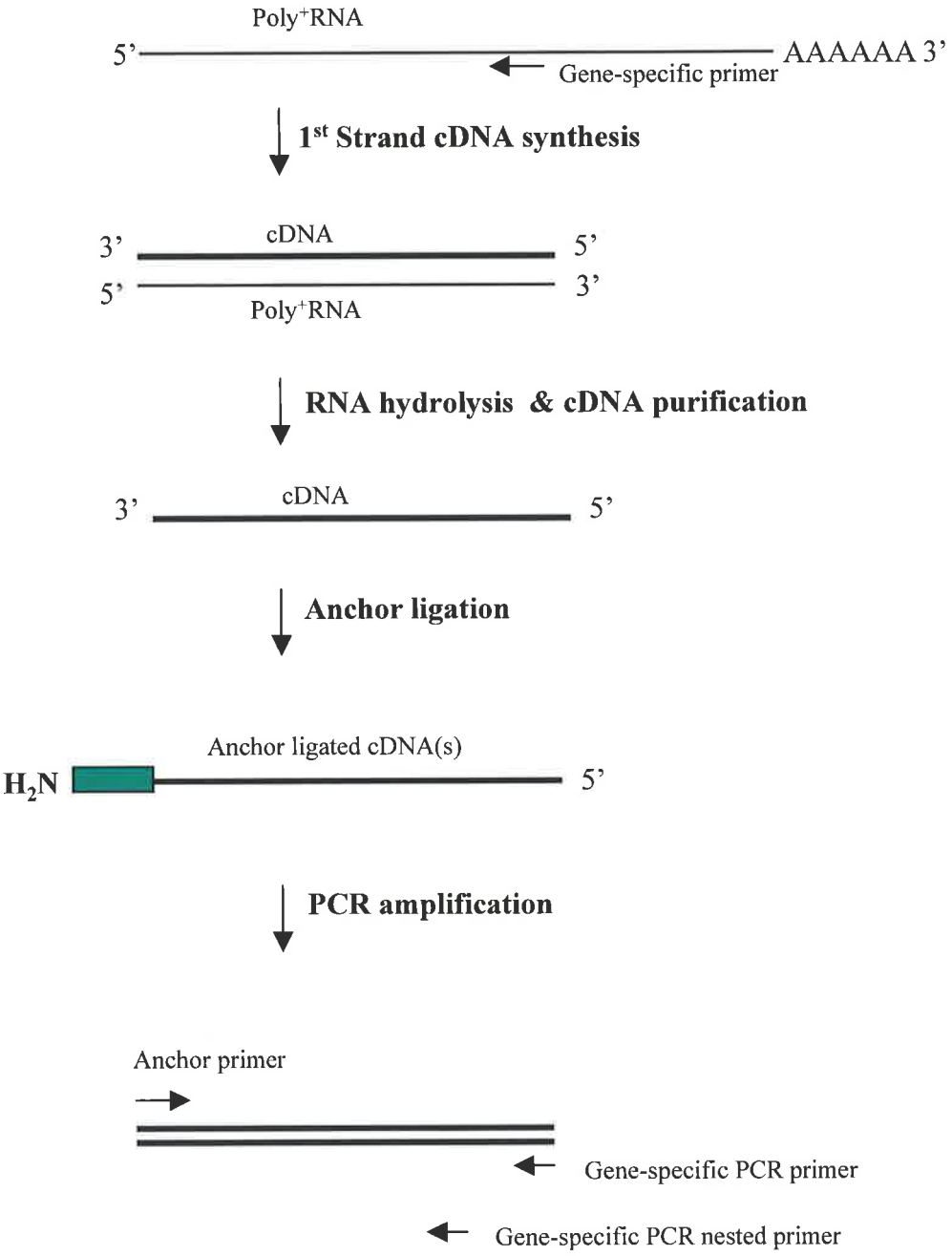


Figure 2.2 A schematic diagram of anchor-ligated PCR. The first strand cDNA is synthesised from total RNA of barley callus using a gene-specific primer. An anchor is ligated to the newly synthesised cDNA. Two rounds of PCR amplifications are performed to isolate the missing sequences at the 5' end of a target cDNA using the anchor primer and two gene-specific primers as the 5' primer and the 3' primer, respectively (Apte and Siebert, 1993). Note the differences between the methods of RT-PCR (Figure 2.1) and anchor-ligated PCR outlined in this figure.

A standard PCR reaction in a total volume of 50 µl was prepared with 1 µl of the anchor-ligated cDNA. A "hot-start" was applied to the PCR reaction to increase specificity as described in the RT-PCR section, except that the anchor primer and a different gene-specific primer were used as a forward and reverse primer in the PCR amplification, respectively. Again, the PCR parameters were determined depending on the primers used.

To improve the sensitivity and specificity of the PCR amplification, nested PCR was applied using the product of the first round PCR as a template. Approximately 1/25 to 1/100 of the first reaction was used as a template for the second round PCR in which nested primers, internal to the first primers, were used. The nested PCR amplification was performed for 35 cycles with the same parameters used in the first round.

The final PCR product was separated on a 1-1.5% agarose gel, subcloned into the T-Easy vector, examined by restriction digestion and further confirmed by DNA sequencing according the procedures described earlier. Once the products were confirmed, new primers were designed based on the newly obtained sequences for "walking" towards the up-stream region of the putative barley putative callose synthase cDNA. The same PCR-based approach was repeatedly applied.

2.2.5 Screening of Barley Bacterial Artificial Chromosome (BAC) Library

2.2.5.1 The Barley BAC Library

A barley bacterial artificial chromosome library, which consists of 17 BAC high-density replica filters, was purchased from the Clemson University Genomics Institute. The putative positive clones identified from the library were subsequently ordered from the same source for sequence analysis. The barley BAC library (cv. Morex) was constructed using DNA fragments partially digested with *Hin*dIII from megabase-size DNA inserted into a pBeloBAC11 vector shown in Figure 2.3. This most widely used BAC library has three unique cloning sites, namely *Hin*dIII, *Bam*HI and *Sph*I within the LacZ gene, allowing identification of recombinants by colony color through alpha complementation. Based on the homogenous distribution assumption, the library was mathematically determined to have 99% coverage of a 4-5 haploid genome equivalent. Therefore, the

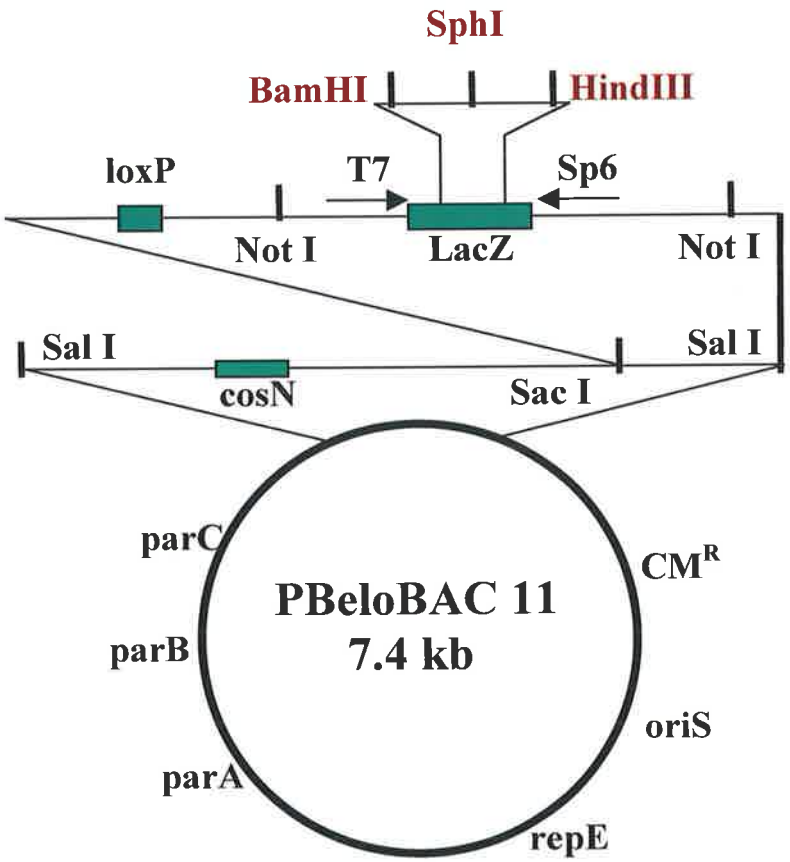


Figure 2.3 A schematic diagram of pBeloBAC 11 vector used for barley BAC library construction. The plasmid is based on a mini-F plasmid, pMB0131, and is chloramphenicol resistant. The vector backbone contains four essential regions that function in plasmid stability and copy number. It has three unique cloning sites: *Hin*dIII, *Bam*HI and *Sph*I within the LacZ gene, allowing identification of recombinants by colour selection. Megabase-size genomic DNA isolated from the barley tissue was partially digested with the restriction enzyme *Hin*dIII, and inserted into the pBeloBAC 11vector. Figure adapted from Clemson University Genomics Institute website (http://www.genome.clemson.edu/groups/bac/).

library was a good source for cloning, using colony hybridisation to identify positive inserts carrying target genes.

2.2.5.2 Screening the Barley BAC Library

A total of 10 BAC filters were divided into two groups and prehybridised in two hybridisation bottles at 65°C overnight in hybridisation buffer containing 0.5 M sodium phosphate buffer, pH 7.2, 7% SDS, 1 mM EDTA and 10 µg/ml sheared denatured salmon sperm DNA. The solution was exchanged with fresh prehybridisation buffer, and the prehybridisation was resumed for another 4 h at the same temperature. To generate a radio-labelled probe, DNA containing the 5' end of the *HvGSL1* cDNA was digested with *Eco*RV, the insert was separated on an agarose gel, purified (Section 2.2.2.1), and radio-labelled (Section 2.2.2.1). The denatured probe of approximately 500 bp was added to each of the bottles and the filters were hybridised overnight at 65°C, and washed with 0.2×SSC and 0.1% SDS three times at 65°C. The membranes were blotted dry, wrapped in plastic wrap, and exposed for 8 days to X-ray film at -80°C.

2.2.5.3 Identification of the Positive Clones and Preparation of the BAC DNA

Positive BAC clone addresses were identified according to the clone designation procedure provided by Clemson University. In brief, the membrane was divided into six fields, each containing 384 squares. Within each of the squares there were 16 positions where eight clones were spotted in duplicate. The plate address of the BAC was generated by the pattern of the spotted clones. The X-ray film was developed and lined up against the corresponding filter. The field number and the well location of the hybridisation clone were determined according to the position in the field and the number of the filter. Identification of the plate number was accomplished by determining the orientation of the duplication spots. The positive clones with a designated address were subsequently ordered from Clemson for further analysis.

The positive clones cultured in agarose in microcentrifuge tubes sent by Clemson University were streaked onto agar plates containing LB with 12.5 µg/ml chloramphenicol and incubated at 37°C overnight. A single colony harbouring the BAC plasmid was inoculated into 5 ml LB culture with the same antibiotics, and incubated with shaking at 37°C for 18-20 h. A standard alkaline lysis method (Section 2.2.2.5) was

conducted, except that a double volume of the starting culture was used, and a double amount of the reagents was also applied throughout the preparation. The plasmid was further purified by phenol/chloroform extraction and ethanol precipitation (Sambrook *et al.*, 1989).

2.2.5.4 Characterisation and Sequencing of BAC Positive Clones

To confirm the identity of the putative positive clones and verify whether they contained the sequence of the *HvGSL1*gene, the inserts of the BAC plasmid were released by digesting with *Hin*dIII, followed by Southern hybridisation performed using the 500 bp barley cDNA as a probe.

Approximately 5 μg of the BAC plasmid DNA was added to a 20 μl digestion mixture containing 2 μl 10×buffer 2 (NEB Biolabs), 2 μl 10×BSA, and 10 U *Hin*dIII restriction enzyme, and incubated for 6-16 h at 37°C. The digested DNA (20 μl) was mixed with 5 μl loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) and separated on a 1% agarose gel overnight at a constant voltage of 25 V in TAE buffer (40 mM Tris-HCl buffer, pH 8.0, 1 mM EDTA).

After electrophoresis the gel was stained with 0.1% w/v ethidium bromide and photographed under UV light. The DNA in the gel was transferred onto a Hybond-N+ nylon membrane in 0.4 M NaOH for 4 h or overnight. The DNA was fixed on the membrane by baking for 2 h at 80°C under vacuum. The membrane was prehybridised with 1.5×HSB (1×HSB buffer, pH 6.8, containing 0.6 M NaCl, 20 mM PIPES and 5 mM Na_2EDTA), 0.3 Denhardt's III (2% BSA, 2% Ficoll 400, 2% PVP), 7.5% dextran sulphate and 0.25 mg/ml denatured salmon sperm DNA at 65°C for 2 h, followed by hybridisation with a 500 bp barley DNA probe at the same temperature overnight. The membrane was washed under stringent conditions (1×SSC, 0.1% SDS; 0.5×SSC, 0.1% SDS; 0.2×SSC, 0.1% SDS; 20 min at 65°C for each wash), and exposed to X-ray film at -80°C.

Based on confirmation by Southern analysis, positive clones were subjected to DNA sequencing. The method applied was a direct sequencing approach with which the BAC plasmid was sequenced by primer walking, without subcloning. The details are as follows. Plasmid DNA (1.2 μg) was mixed in a 20 μl reaction mixture containing 8 μl

standard ABI PRISM™ Dye Terminator Ready Reaction Mix and 30 pmol primer. The PCR profile was specifically designed to accommodate BAC plasmids carrying large insert sizes, which were around 100 kb to 500 kb according to the Clemson BAC library instructions. The actual thermal cycles for the PCR reaction consisted of denaturing at 95°C for 5 min, 100 cycles of 95°C for 30 sec, 50°C for 30 sec and 60°C for 4 min. The resulting PCR products were subsequently precipitated with 2.0 µl 3 M sodium acetate, pH 4.6 buffer, 50 µl 95% ethanol, followed by an extensive wash with 70% ethanol. The pellet was vacuum dried and sequenced on the ABI 373 automated Sequencer (NAPCU, the University of Adelaide, Australia).

2.3 Results and Discussion

2.3.1 Isolation and Characterisation of Positive Clones from a cDNA Library

A barley callus cDNA library was screened in an attempt to obtain barley homologues of yeast (1→3)-β-glucan synthase using a 5.0 kb cDNA probe originally isolated from *Hieracium*. This cDNA was a homologue of the yeast *FKS1* gene and was isolated by Mr. Nick Paech from *Hieracium piloselloides* flower capitula between developmental stages 2 and 4, at which megasporogenesis occurs (N. Paech, personal communication; Tucker *et al.*, 2001). During screening, difficulties were encountered in identifying strongly hybridising plaques, probably due to a low degree of sequence homology between barley and *Hieracium*. From the second round of library screening, only very weakly hybridising plaques were observed, producing ambiguous results and making coring of the positive plaques difficult. The third round of library screening yielded stronger positives, as shown in Figure 2.4. Four positive plaques were cored and cDNA was excised into pBluescript phagemids according to the single-clone excision procedure (Section 2.2.3.3). The sizes of the cDNA inserts were determined by restriction digestion with *Eco*RI and *Xho*I, and found to be approximately 2.2 kb, 2.0 kb, 1.5 kb and 1.2 kb. DNA sequencing of the inserts and the subsequent BLAST searching against the database revealed that three out of four clones had best matches with the yeast (1→3)-β-glucan synthase *FKS* genes. Clones containing 2.2 kb and 2.0 kb inserts had identical overlapping sequence and produced significant sequence alignment to the upstream sequence of the *FKS* genes, while the Clone containing 1.5 kb insert had sequence homology to the FKS genes close to the 3' end. Clone containing 1.2 kb insert was found to be a false positive.

Using the newly obtained 2.2 kb cDNA from above as a probe, the callus cDNA library was once again screened in the hope of obtaining a full-length cDNA, which was expected to be more than 5 kb in length. This effort produced a number of new positive plaques, two of which, termed Clone 1 and Clone 2, made up a total length of 4.2 kb and had approximately 1.5 kb of overlapping sequence. Clone 1 covered the 3' untranslated region and possessed a poly A tail, whilst five other positive clones contained identical sequences to Clone 1, differing only in the length of the 3' untranslated region (UTR) between the stop codon and the poly A tail. Such a phenomenon implies that alternative

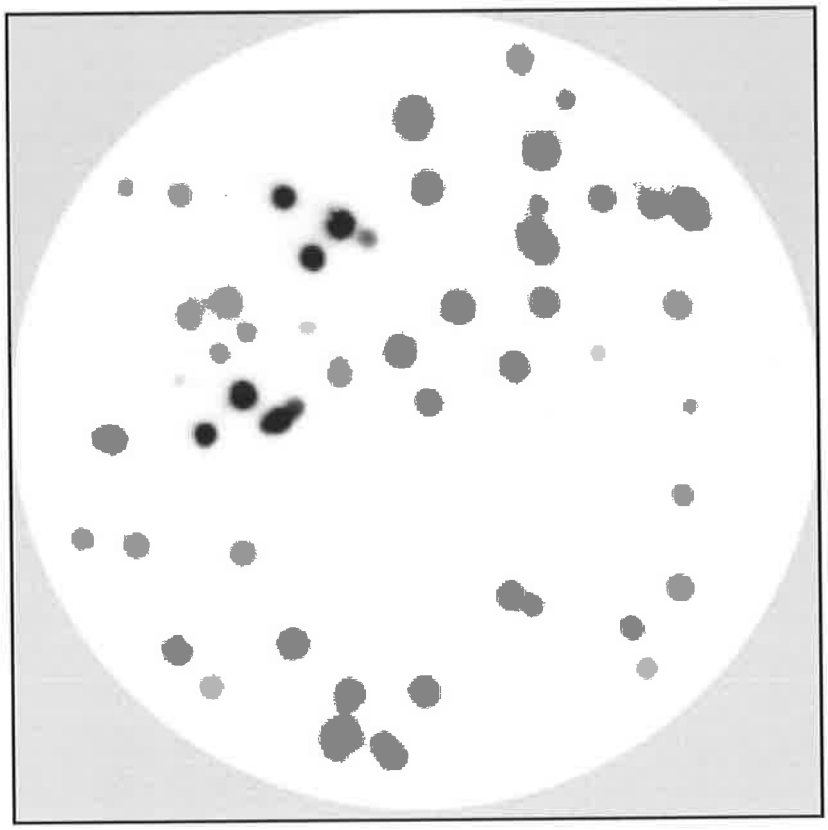


Figure 2.4 Isolation of positive plaques from the λZAPII cDNA library of barley callus using a fragment of a callose-like cDNA from *Hieracium* as a probe. A total of 1×10^6 plaques were screened giving rise to three positive clones identified as putative callose synthase cDNAs (*HvGSL1*) of barley. The library was rescreened using one of the positive clones as a probe.

polyadenylation sites are used during 3' processing of the primary transcript in callus cells, although the mRNAs probably originate from a single gene. Similar observations were reported by Banik *et al.* (1998) for xylanase cDNAs isolated from barley in this laboratory.

By comparison with the yeast *FKS1* gene, it was concluded that Clones 1 and 2 isolated from the barley cDNA library were indeed the barley homologues of the yeast (1→3)-β-glucan synthase gene. The barley cDNA had over 30% sequence identity with the yeast *FKS* gene at the amino acid level. However, this sequence represented only about 2/3 of the length of the total cDNA as estimated by comparing the barley sequence to the yeast *FKS* cDNA. This indicated that there was a significant amount of the cDNA missing at the 5' end, and further efforts to clone the remainder of the putative callose synthase gene in barley were therefore required.

2.3.2 *Amplification of 5' End of the (1→3)-β-Glucan Synthase-like cDNA using RT-PCR*

As the cloning of the barley (1→3)-β-glucan synthase-like cDNA progressed, Cui *et al.* (1999) deposited a cDNA sequence, designated *CFL1* and encoding a putative (1→3)-β-glucan synthase catalytic sub-unit from cotton (*Gossypium hirsutum L.*), into the databases. These data, together with the *Hieracium* cDNA *HpGSL1* (Section 2.2.2.1), provided the first two sequences from genes which may encode (1→3)-β-glucan synthase proteins in higher plants. In addition, 9h3ara, an open reading frame (ORF) sequence of *Arabidopsis* translated from a BAC clone was also present in the databases (Federspiel *et al.*, 1999). Alignment of these three sequences displayed regions of identity at the amino acid level and a conserved region close to the 5' end of the existing sequences, FGFQKDNV, was targeted as the basis for primer design for RT-PCR. The corresponding nucleotide sequences from cotton and *Arabidopsis* are shown below:

Cotton: 5' TTT GGA TTT CAG AAA GAC AAT GTT 3'
(F G F Q K D N V)

Arabidopsis: 5' TTC GGT TTC CAG AAA GAT AAC GTT 3'
(F G F Q K D N V)

Therefore, a degenerate primer (named 5'Hv1) was designed with the following sequence:

5' TTY GGW TTY CAG AAA GAY AAY GTT 3' (forward primer)

Where Y=T/C and W=A/T.

A gene-specific primer designed from Clone 2, a positive clone obtained from the cDNA library screening (Section 2.3.1), was chosen as a reverse primer, and designated 3' Hv1. Its sequence was:

5' TTA GCC ATA GTT CAT CAA 3' (reverse primer)

These two primers were used in reverse transcription-PCR (RT-PCR) as described in Section 2.2.3. The total RNA used in RT-PCR was isolated from callus because a library made from this tissue had already yielded a number of positive cDNAs. The RNA was extracted using the TRIzol reagent and its quality was checked on a 1.5% (w/v) agarose gel by confirming the integrity of the rRNA present in the RNA extracts (Figure 2.5 A). The reverse transcription was performed using the 3'Hv1 primer, with the THERMOSCRIPT TRANSCRIPTASE kit.

The result of the RT-PCR experiment is shown in Figure 2.5 B & C. The pair of primers 5'Hv1 and 3'Hv1 yielded a PCR product of approximately 1.4 kb, which was the length predicted from the sequences of the putative callose synthase cDNA of cotton and *Arabidopsis* between the regions at which those two primers were expected to bind. The 1.4 kb PCR product was isolated on a 1% agarose gel, and cloned into the T-Easy vector, transformed into the *E. coli* DH 5α strain and named Clone 3. Positive colonies were picked and the inserts of plasmids were fully sequenced, confirming that they contained a section of the barley homologue of the (1→3)-β-glucan synthase cDNA. Clone 3 and Clone 2 shared 288 bp of overlapping sequence, and Clone 3 extended the putative (1→3)-β-glucan synthase cDNA of barley, designated *HvGSL1*, to a length of 5208 bp. However, the total length of Clones 1, 2, 3 combined (Figure 2.5 C) was still shorter than

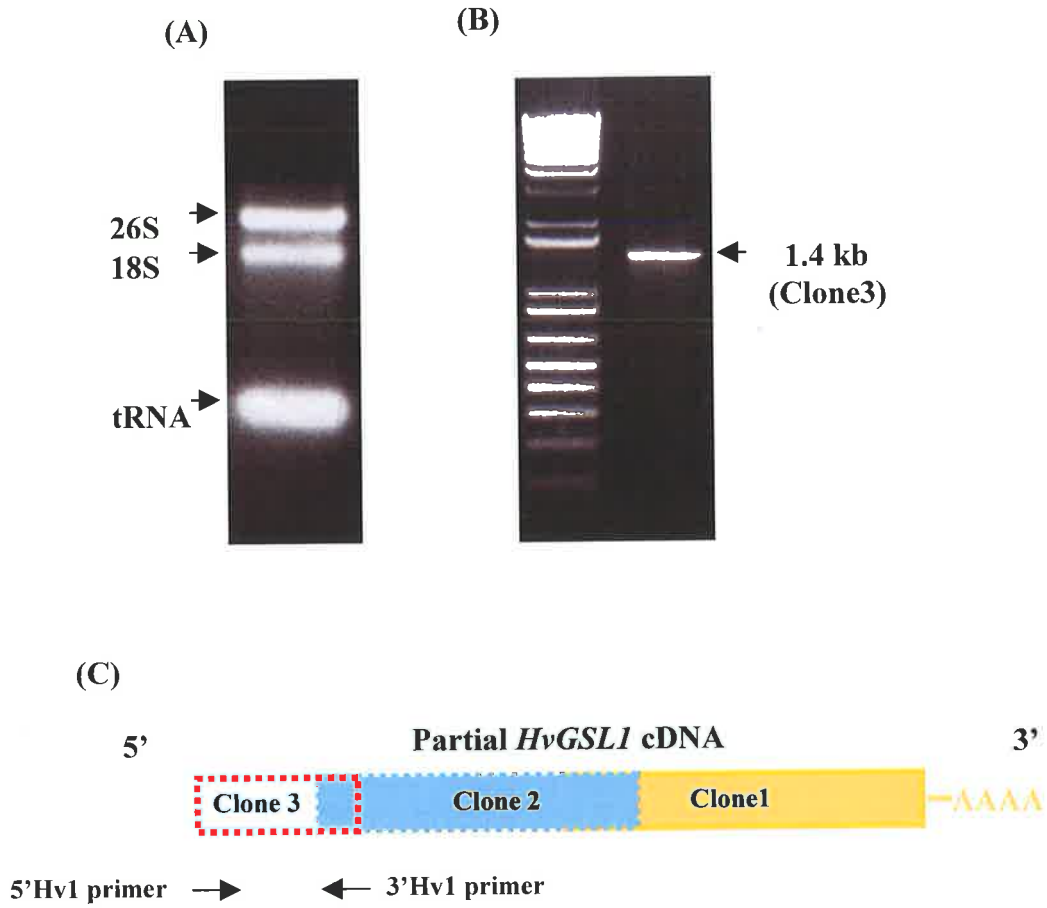


Figure 2.5 Amplification of barley (1→3)-β-glucan synthase-like cDNA by RT-PCR. **(A)** Total RNA was extracted from barley callus using the TRIzol reagent. The integrity of the RNA was checked on a 1.5% agarose gel which shows the two ribosomal RNA species at 26S and 18S and the smaller tRNA species. **(B)** One PCR fragment of approximately 1.4 kb was generated by RT-PCR. The first-strand cDNA was synthesised from the total RNA of barley callus with the 3' gene-specific primer 3'Hv1 (which was based on clone 2 sequence). This primer and a degenerate 5' primer 5' Hv1(which was based on the conserved sequences of callose synthase-like cDNAs from cotton and *Arabidopsis*) were used in the subsequent PCR reaction to produce Clone 3 shown in B. The 1.4 kb product was cloned into the T-Easy Vector and sequenced to confirm its identity. Clone 3 extended the existing *HvGSL1* cDNA to a total length of 5208 bp. **(C)** A schematic diagram of the composite barley callose synthase-like cDNA which contained Clones 1 and 2 from the cDNA library and Clone 3 from RT-PCR. The 1.4 kb product was cloned into T-Easy Vector.

the full-length sequences of the putative (1→3)-β-glucan synthase cDNAs from *Arabidopsis* and *Gossypium hirsutum L.*

2.3.3 Amplification of 5' End of the (1→3)-β-Glucan Synthase-like cDNA using Anchor-Ligated PCR

Alignment of the 5' ends of the putative callose synthase cDNAs from *Arabidopsis* and *Gossypium hirsutum L.* revealed no regions of strong sequence conservation to which primers could be designed for repeating the RT-PCR process described above. An anchor-ligated RT-PCR approach described in section 2.2.4 was therefore employed as an alternative method in an attempt to isolate the missing 5' region of the *HvGSL1* cDNA. Primers designed and subsequently used in the anchor-ligated RT-PCR together with the sequences of the unique anchor and anchor primer are summarised in Table 2.1.

Based on the sequence of Clone 3, a barley gene-specific primer called 3'1.4PCR was designed. The first-strand cDNA was synthesised using this primer and after ligation of the anchor onto the cDNA, the first round of anchor-ligated PCR was carried out with this primer at the 3' end and the anchor primer at the 5' end. This was followed by a semi-nested PCR using the 3'1.4PCR nest at the 3' end and the anchor primer at the 5' end, as shown in Figure 2.6 B. One product, of approximately 650 bp (Figure 2.6 A), was obtained from this semi-nested PCR amplification. The PCR product was cloned into the T-Easy vector, and the insert was fully sequenced in both directions. Sequence analysis of Clone 4 revealed that the 650 bp fragment was a correct sequence of the *HvGSL1* cDNA and therefore extended this sequence towards the 5' end.

With the same method, a pair of newly designed primers to the sequence of Clone 4, called 3'600PCR and 3'600PCRnest (Table 2.1 and Figure 2.6 B) was used in a second PCR amplification, from which a fragment of approximately 500 bp was generated (Figure 2.6 A). The PCR fragment was cloned, named Clone 5, and sequenced, and was found to extend the *HvGSL1* cDNA to a total length of 6033 bp. Clone 4 and Clone 5 shared 138 bp of identical overlapping sequence. However, comparing this 6033 bp of the *HvGSL1* cDNA sequence with that of *CFL1* from cotton, it appeared that the *HvGSL1* cDNA was still not full-length; it was about 21 bp (7 amino acids) shorter at the NH_2-terminus than the *CFL1* sequence.

Table 2.1 Oligonucleotide sequences designed and used in anchor-ligated PCR for isolating the 5' end of a putative barley (1→3)-β-glucan synthase cDNA. The anchor was ligated to the 5' end of the single-stranded cDNAs synthesised from the total barley callus RNA using THERMOSCRIPT RT and a gene-specific primer 3'1.4 PCR. The anchor primer was designed to bind to the anchor and used as a forward primer for two rounds of PCR amplifications, whereas two pairs of primers, 3' 1.4 PCR, 3' 1.4 PCR nest and 3' 600 PCR, 3' 600 nest, were used as the reverse primers for the first round and the second semi-nested rounds of PCR amplifications, resulting in the isolation of two PCR fragments at the 5' end of the *HvGSL1* cDNA.

Oligonucleotide	
Anchor	5' PO_3 CAC GAA TTC ACT ATC GAT TCT GGA ACC TTC AGA GG NH_2 3'
Anchor Primer	5' CTG GTT CGG CCC ACC TCT GAA GGT TCC AG ATC GAT AG 3'
3' 1.4 PCR	5' TTG CTA CGT GAT GAA ATA TGT A 3'
3' 1.4 PCRnest	5' ACA GGA CGC AGT GGT AAA TAG TT 3'
3' 600 PCR	5' TAC GCC ACA AAA TCC TCT GTC CT 3'
3' 600 PCRnest	5' CCT CTA TTA CTG ACC ATA AGA C 3'

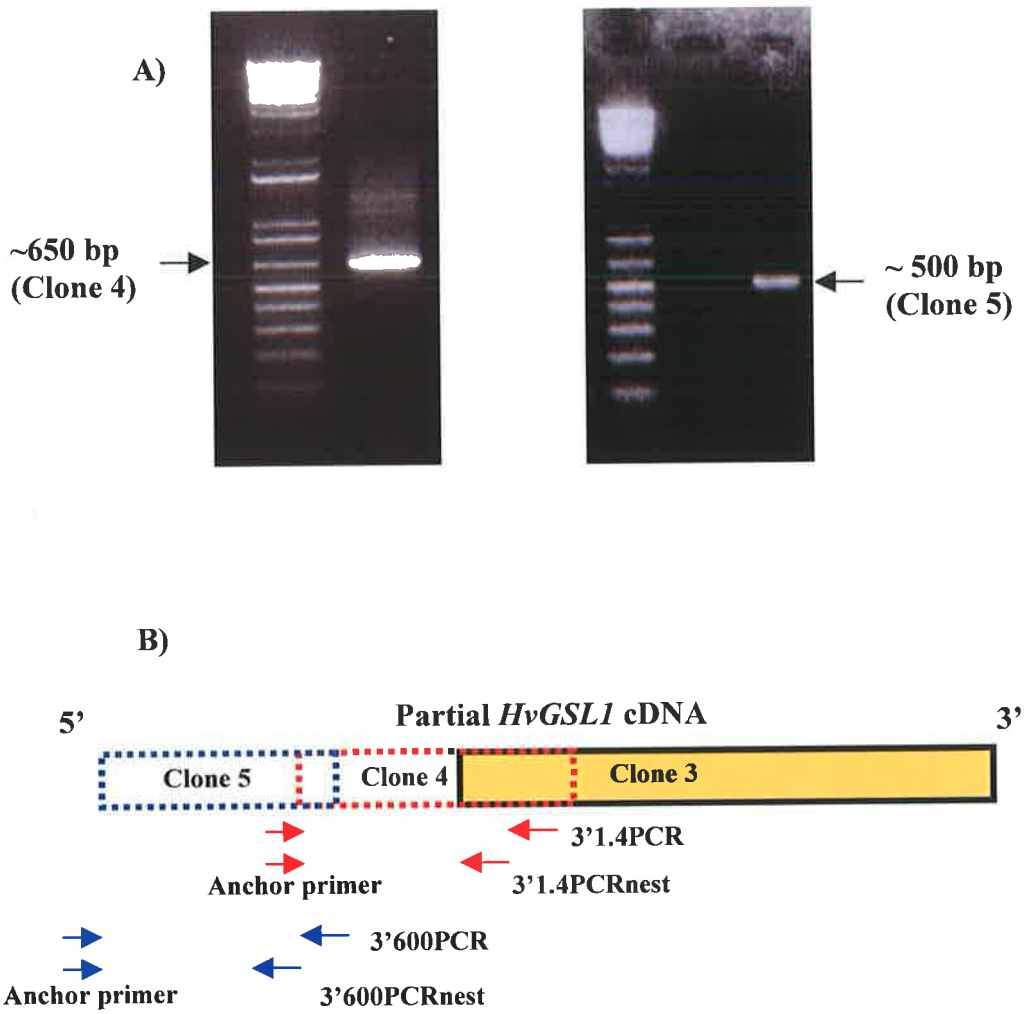


Figure 2.6 Extension of a barley synthase-like cDNA by anchor-ligated PCR. A) Two PCR fragments, of approximately 500 bp and 600 bp shown on the above gels were sequentially generated by anchor-ligated PCR. The first-strand cDNA was synthesised from the total RNA of barley callus using the 3' gene specific primer 3'1.4PCR. The anchor primer and two pairs of gene-specific primers, 3'1.4 PCR and 3'1.4 PCRnest, 3'600 PCR and 3'600 PCRnest, were used in the PCR amplifications as shown. **B)** A schematic diagram of the barley callose synthase-like cDNA containing the two newly obtained PCR fragments. The 500 bp and 600 bp PCR products were cloned into the T-Easy Vectors and their sequences were confirmed. They extended the existing *HvGSL1* cDNA to a total length of 6033 bp.

Continuing efforts to amplify the rest of the cDNA using the anchor-ligated PCR method were not successful. A number of other strategies were therefore employed to clone the last portion of the gene. These included inverse-PCR (Ochman *et al.*, 1988) and genomic-walking (Siebert *et al.*, 1995). These methods are generally used on a genomic template for walking from a known region to an unknown region in cloned or uncloned genomic DNA. Trials of the inverse-PCR method produced some PCR fragments, which were subsequently found to be false positives. Genomic walking also failed to work in this case, although it was reported to be an efficient technique for cloning in other systems (Siebert *et al.*, 1995). Therefore, the strategy of using a barley BAC library as a source of positive clones was employed, as described in the next section.

2.3.4 *Positive Clone Identification from the Barley BAC Library*

The barley BAC library was screened in an attempt to obtain the full-length callose synthase-like gene from which a full-length corresponding cDNA sequence could be deduced. In particular, the less than 100 bp of missing sequence at the 5' end of the *HvGSL1* cDNA was sought from a positive BAC clone. Thus, Clone 5, which contained sequence at the furthermost 5' end of the *HvGSL1* cDNA, was used as a probe to screen the barley BAC library.

After screening the library, five strongly hybridising clones were identified. According to the clone designation procedure (Section 2.2.4), these were subsequently ordered from Clemson with identification addresses as plate 239-C24, plate 807-F11, plate 201-I4, plate 213-G11 and plate 562-M8. Figure 2.7 shows an autoradiogram image of the BAC high-density replica filter Q hybridised with the 500 bp probe derived from Clone 5. The clone, addressed as 807-F11 on the filter, shown in Figure 2.7, gave a very strong signal. This hybridisation plaque almost covered half of the square, signalling that it was less likely to be a false positive. The fact that only one hybridising spot shown on the filter was caused by the strongly hybridising signal, such that two replicated plaques originally fixed on the filter had merged to form one spot. According to the position of this hybridisation dot on the film and the specific patterns of 16-dot array defined in this square, addressing of this clone was clearly defined.

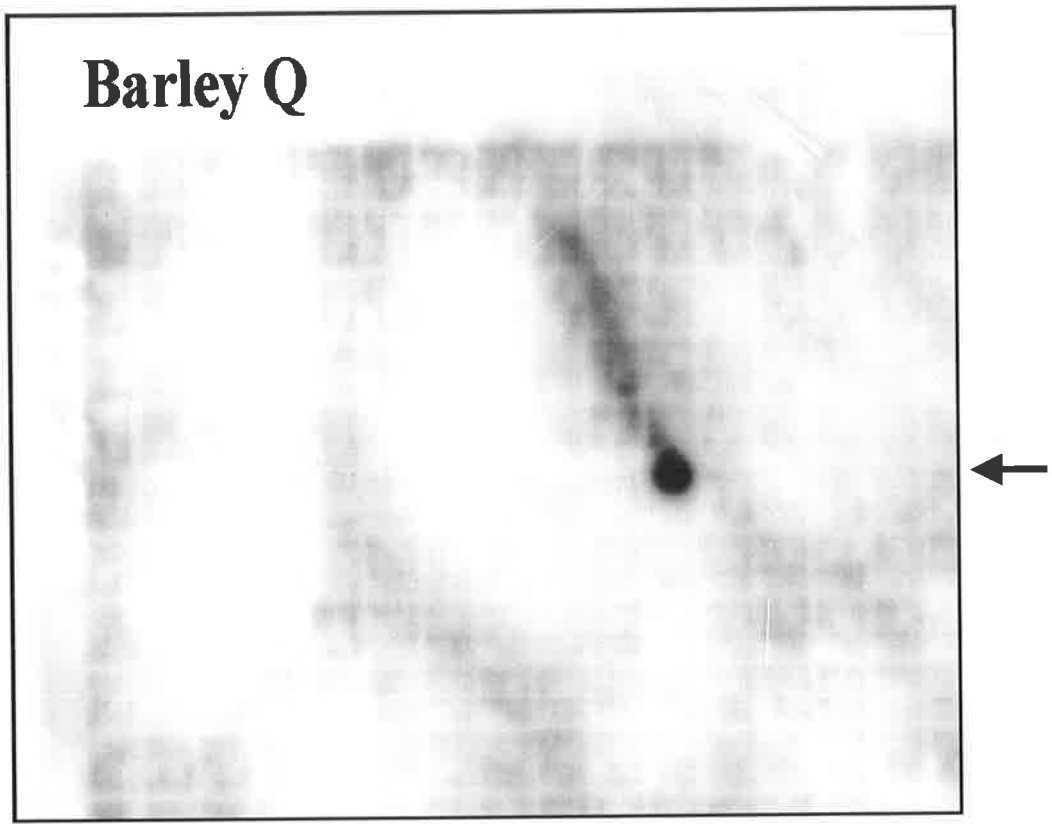


Figure 2.7 Barley BAC library screening by colony hybridisation. This figure shows an autoradiogram image of a corner of the BAC high-density replica filter Q which had been hybridised with an approximately 500 bp cDNA fragment. This fragment contained the sequence of the 5' end of the barley callose synthase-like cDNA (*HvGSL1* cDNA) as shown in Figure 2.5. A positive clone was found on membrane Q, in Field 3, position 7, thus, the address of this clone was identified as plate 807-F11. This and other positive clones were therefore ordered from the Clemson University for further characterisation. Note that due to the strongly hybridising signal, the single spot shown on the filter was the image of two replicated plaques originally fixed on the filter. However, according to the position of this spot and the specific patterns of the 16-dot array in each square, the addressing of the clone was possible.

The positive clones were initially verified via Southern blot analysis of the BAC plasmid DNA. The DNA was prepared, purified, and digested with the *Hin*dIII restriction enzyme, and separated on a 1% agarose gel containing ethidium bromide. Figure 2.8 A shows several examples of digested BAC plasmid DNA from the positive clones. Most of the fragments are still of large sizes, confirming that the inserts in the BAC clones are likely to range from 100 kb to 250 kb. Southern blot analysis of BAC plasmid DNA, using the 500 bp cDNA fragment of Clone 5 as a probe, showed that two of the five clones were positive (Figure 2.8 B). They are designated F11 (plate 807-F11) and C24 (plate 239-C24), respectively. Direct sequencing (Section 2.2.5.4) of the F11 and C24 clones was carried out using a gene-specific primer 3'103 (5' CCT CGT CGA TGT GGG TGT TGT T 3'), which was located 103 bp downstream of the current 5' end of the existing *HvGSL1* cDNA, and confirmed that F11 and C24 contained an identical *HvGSL1* gene. Using the 3'103 primer, F11 and C24 yielded an extra 197 bp of sequence. With this sequence, a putative translation start point methionine was identified. The missing sequence at the amino acid level is shown in Figure 2.9 as MARAENA. Therefore, the putative open reading frame sequence encoding the barley callose synthase-like enzyme was finally completed, although the transcript start point has not been confirmed using alternative molecular methods such as RNase protection experiments.

In order to obtain further upstream sequence of the *HvGSL1* gene and its promoter region sequence from the clones F11 and C24, new primers were designed according to the sequence of the newly acquired 5' end. However, direct sequencing with these new primers did not produce further sequence data of good quality. Analysis of the sequencing gels performed on the ABI Sequencer revealed that some of the sequencing lanes only showed the dye front, which indicated that some of the primers failed to prime. Other reactions were of a very short length which generated a highly noisy background on the chromatograms. Direct sequencing from BAC clones has not yet been established as a routine procedure by many laboratories or sequencing services. An effort was made to improve the sequence data through alternative methods for BAC plasmid DNA purification, altering the quantity of DNA used in the sequence reactions, and adjusting thermal cycling protocols. However, none of these modifications yielded improved data. Clones F11 and C24 were finally sent to the Australian Genome Research Facility (AGRF) for sequencing. Again, a number of different procedures were tried at AGRF,

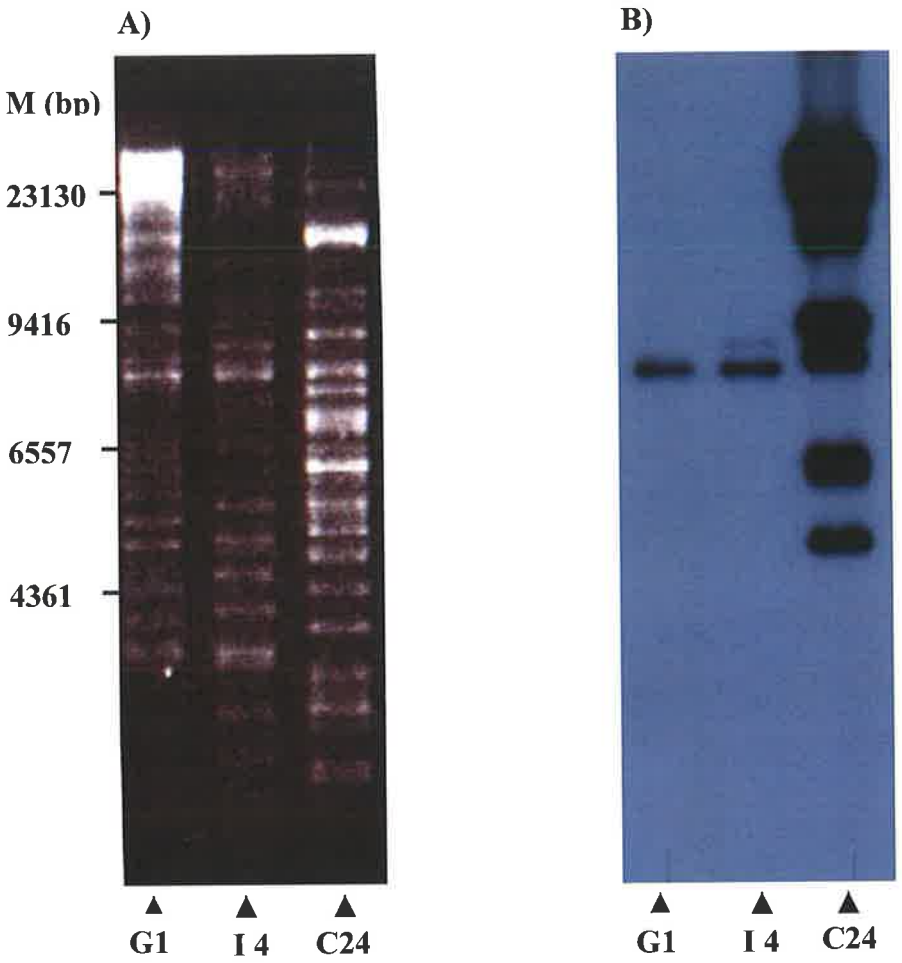


Figure 2.8 Southern blot analysis of positive barley BAC clones. A) Profiles of BAC plasmid DNA prepared from positive clones after *Hin*dIII digestion. BAC plasmid DNAs, G11, I4, and C24 were digested with the *Hin*dIII restriction enzyme, and separated in a 1% agarose gel containing ethidium bromide. This DNA was transferred onto a nylon membrane by capillary blotting for use in B. M, DNA markers. **B)** Southern blot analysis of BAC plasmid DNA from plaques G11, I4 and C24 using a cDNA fragment prepared from Clone 5 as a probe. C24 showed significant positive signals as compared to clones G11 and I4 confirming that C24 is a positive clone. Weak hybridisation bands in G11 and I4 indicate that these two clones are likely to be false positives. Clone F11 has a similar hybridisation signal in Southern analysis, an experiment carried out later with another batch of putative positive BAC clones (data not shown).

but no additional sequence was obtained. Study of the upstream sequence of the *HvGSL1* gene obtained from F11 and C24 (Figure 2.9), indicated that the problem might be attributed to the GC richness of this region. Over a fragment of 160 bp shown in Figure 2.9, the GC content was 77.5%.

2.3.5 *Sequence of a Putative Barley Synthase (HvGSL1) cDNA and Its Restriction Map*

As described in the above sections, a near full length, putative barley synthase cDNA (*HvGSL1*) was assembled by various methods. Figure 2.10 shows the nucleotide sequence and the deduced amino acid sequence of this putative barley callose synthase. The total length of the *HvGSL1* cDNA from the putative start codon to the polyA tail is 6055 bp, and it contains an open reading frame of 5745 bp, that encodes a protein of 1915 amino acids. As underlined in Figure 2.10, the putative translation start codon ATG and the putative stop codon TGA are predicted at 1-3 bp and 5746-5748 bp in the sequence. Numbering of amino acid residues of the protein therefore begins at the putative translation start Met residue (Figure 2.10). Because there are no obvious targeting signals in the sequence and no information from the enzyme itself, the NH_2-terminal residue of the mature enzyme cannot be identified.

The sizes and positions of the six clones, which were combined to produce the near full length *HvGSL1* cDNA, and a restriction map of the full-length cDNA are shown in Figure 2.11. In summary, Clone 1 of 2558 bp (from 3497 bp to 6055 bp) and Clone 2 of 2603 bp (from 1919 bp-4522 bp) were isolated from the barley callus cDNA library; Clone 3 of 1360 bp (from 847 bp to 2207 bp) was an RT-PCR product obtained using primers designed according to the conserved sequence of the putative callose synthases from cotton and *Arabidopsis*; Clone 4 of 638 bp (from 360 bp to 998 bp) and Clone 5 of 476 bp (from 22 bp to 498 bp) were the anchor ligated PCR products, and Clone 6 containing the missing 5'end (0-22 bp), including the putative start point, and was obtained from the barley BAC library. It appears that the BAC clone F11 contains the entire *HvGSL1* gene. This was proven by sequencing the F11 clone, using primers which flank the very 5' end and the 3' end, together with some of the internal regions (data not shown).

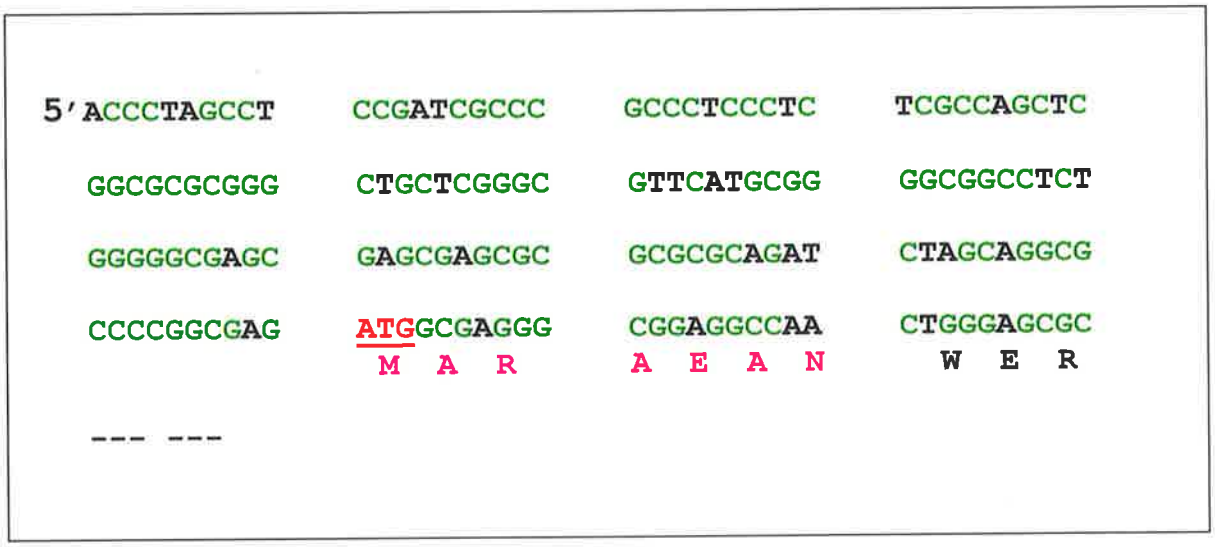


Figure 2.9 The upstream sequence including the putative translation start codon (M) of the *HvGSL1* cDNA. Characteristically the 5' untranslated region is GC rich. The BAC clones F11 and C24 were both sequenced with 3' 103 primer (Section 2.3.4). The sequence of the two BAC clones was identical and contained the missing 5' end of the *HvGSL1* cDNA in which a putative start codon was identified as underlined in red. Sequencing the promoter region failed to produce data of good quality, due to the GC richness (marked in green) in the upstream sequence of the *HvGSL1*.

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ATGGCGAGGGCGGAGGCCAACTGGGAGCGCCTGCTGCGGGCGGCGCTGCGCGGGGACCGGATGGGCGGCGTCTACGGGGTCCCCGCCAGCGGCATCGCCG   100
M A R A E A N W E R L L R A A L R G D R M G G V Y G V P A S G I A G                                          34

GGAACGTCCCCTCCTCGCTCGGCAACAACACCCACATCGACGAGGTGCTCCGCGCCGCCGACGAGATCCAGGACGAGGATCCCACCGTCGCCAGGATTCT   200
  N V P S S L G N N T H I D E V L R A A D E I Q D E D P T V A R I L                                          67

GTGTGAGCACGCATATGCCCTCGCCCAAAATCTGGATCCAAATAGCGAAGGGAGAGGTGTGCTTCAGTTCAAGACCGGTTTAATGTCAGTAATCAGGCAA   300
 C E H A Y A L A Q N L D P N S E G R G V L Q F K T G L M S V I R Q                                          100

AAACTAGCTAAGAGGGAAGGCGGTGCTATAGACCGAAGTCGGGATATCGCTAAACTGCAAGAATTTTATAAGCTATACAGAGAAAAACATAAAGTTGATG   400
K L A K R E G G A I D R S R D I A K L Q E F Y K L Y R E K H K V D E                                          134

AGTTGTGTGAAGACGAAATGAAGCTGAGGGAATCTGGTGTGTTCAGCGGTAACCTCGGAGAGCTGGAGCGCAAAACTCTGAAGCGCAAAAAAGTACTTGC   500
  L C E D E M K L R E S G V F S G N L G E L E R K T L K R K K V L A                                          167

AACTCTCAAGGTCTTATGGTCAGTAATAGAGGATATAACAAAGGAAATTTCCCCTGAGGATGCAGCAAATTTGATTTCTGAAAAGATGAAAGAATTCATG   600
 T L K V L W S V I E D I T K E I S P E D A A N L I S E K M K E F M                                          200

GAAAAGGATGCGGCAAGGACAGAGGAT.TTGTGGCGTATAATATCATTCCTCTAGATTCCTTGTCTACAACTAACCTGATTGTCACTTTTCCAGAGGTGA   700
E K D A A R T E D X V A Y N I I P L D S L S T T N L I V T F P E V R                                          234

GGGCAGCAATATCATCTTTGCAGTACCATAGGGATCTGCCCAGGCTTCCGAATACCATTTCAGTTCCTGATGCTAGGATTTCAAATATGCTGGACTTGGT   800
  A A I S S L Q Y H R D L P R L P N T I S V P D A R I S N M L D L V                                          267

GCACTGCGTGAGTGGTTATCAGAAAGACAATGTGAGCAATCAACGGGAGCACATTGTTCACCTGTTGGCAAATGAGCAGTCTCGATTAGGCAAACTATCA   900
 H C V S G Y Q K D N V S N Q R E H I V H L L A N E Q S R L G K L S                                          300

GGGAATGAACCGAAAATTGACGAGGGTGCTGTACATGTTGTGTTCTCTAAGTCCCTGGACAACTACATAAAATGGTGCAACTATTTACCACTGCGTCCTG   1000
G N E P K I D E G A V H V V F S K S L D N Y I K W C N Y L P L R P V                                          334

TCTGGAATAACATTGAATCATTGACCAAAGAAAAGAAGTTGCTATATGTTTGTTTATACTACTTGATCTGGGGAGAGGCTGCCAACGTACGATTTCTTCC   1100
  W N N I E S L T K E K K L L Y V C L Y Y L I W G E A A N V R F L P                                          367

AGAAGGCTTATGCTACATATTTCATCACGTAGCAAGAGAACTAGAGGTGATTATGCAGAAACAGACTGCCGAGCCAGCTGGAAGCTGCATCTCTAATGAT   1200
 E G L C Y I F H H V A R E L E V I M Q K Q T A E P A G S C I S N D                                          400

GGCGTATCATTTCTTGACCAAGTCATTTATCCTCTATATGAAATCGTTGCAGCTGAAGCAGGCAACAATGACAATGGGCGGGCAGCACATTCTGCATGGA   1300
G V S F L D Q V I Y P L Y E I V A A E A G N N D N G R A A H S A W R                                          434

GAAACTATGATGATTTCAATGAGTTCTTCTGGTCTGAGAAATGTTTTCAGCTGGGTTGGCCGTGGAAACTGAGCAATCCATTTTTCTCAAAGCCTAATAG   1400
  N Y D D F N E F F W S E K C F Q L G W P W K L S N P F F S K P N R                                          467

GAAAGAGCAGGGCTTGATAAGTAGGAATCACCATTATGGAAAGACATCTTTCGTTGAGCACAGAACTTTTCTGCATCTTTACCATAGCTTTCACCGCCTC   1500
 K E Q G L I S R N H H Y G K T S F V E H R T F L H L Y H S F H R L                                          500

TGGATGTTCCTACTTTTGATGTTTCAGGGACTTACTATCATTGCTTTTAACAATGGCAGTTTTGACACAAATACTGTATTGGAACTTCTTAGCCTGGGCC   1600
W M F L L L M F Q G L T I I A F N N G S F D T N T V L E L L S L G P                                          534

CAACTTATATCATAATGGAATTTATTGAGAGTGTATTGGACATTCTAATGATGTATGGCGCCTATTCAACATCTCGTGGTTCTGCAATCACTAGAGTGAT   1700
 T Y I I M E F I E S V L D I L M M Y G A Y S T S R G S A I T R V I                                          567

CTGGCGATTCTGTTGGTTTACCGCAGCTTCATTGGTCATCTGTTACCTATATATCAAGGCACTTCAAGATGGGGTGCAATCTGCACCTTTTAAGATATAT   1800
 W R F C W F T A A S L V I C Y L Y I K A L Q D G V Q S A P F K I Y                                          600

GTTGTTGTCATCAGCGCGTATGCGGGTTTCCAGATAATCATCAGCCTTCTCATGAGCGTTCCCTGCTGCCGTGGTATTACCAATGCTTGCTACAGCTGGT   1900
V V V I S A Y A G F Q I I I S L L M S V P C C R G I T N A C Y S W S                                          634

CTTTTGTACGCCTTGCCAAGTGGATGCATCAGGAACATAATTATGTTGGAAGAGGCTTGCATGAAAGGCCTCTAGATTATATCAAATATGCCGCTTTCTG   2000
  F V R L A K W M H Q E H N Y V G R G L H E R P L D Y I K Y A A F W                                          667

GCTTGTTATTTTTGCTGCGAAATTTTCATTCACCTATTTCCTCCAGATTAGACCTCTTGTAAAACCAACAAGACTGATAATCAGTTTCAAAGGCTTACAG   2100
 L V I F A A K F S F T Y F L Q I R P L V K P T R L I I S F K G L Q                                          700

TATCAATGGCATGACTTTGTTTCAAAGAATAACCATAATGCGATTACAATCCTTTCTTTATGGGCTCCAGTGGCCTCAATCTATCTTTTGGACATCCATG   2200
Y Q W H D F V S K N N H N A I T I L S L W A P V A S I Y L L D I H V                                          734

TTTTTTACACCATCATGTCTGCTCTTGTTGGATTCCTCCTTGGTGCACGTGATCGCCTGGCAGAGATTAGGTCTGTTGAAGCAGTTCACAGGTTCTTTGA   2300
  F Y T I M S A L V G F L L G A R D R L G E I R S V E A V H R F F E                                          767

GAAGTCCCTGAAGTATTCATGGATAAACTTCCATGTTGCTGTTCCAAAAAGGAAACAACTGCTATCATCTGGTCAGCATGCAGAGTTAAACAAGCTTGAT   2400
 K S L K Y S W I N F H V A V P K R K Q L L S S G Q H A E L N K L D                                          800

GCATCTAGATTCGCTCCTTTCTGGAATGAAATTGTGAAGAATTTGCGGGAAGAGGACTACATTAGCAACACTGAGCTGGATTTACTCTTGATGCCAAAGA   2500
A S R F A P F W N E I V K N L R E E D Y I S N T E L D L L L M P K N                                          834

ACATAGGTGGTCTTCCAATTGTGCAGTGGCCACTTTTTTTGCTTGCTAGCAAGGTTTTCTTGGCCAAAGATATTGCCGTTGATTGCAACGACTCACAAGA   2600
  I G G L P I V Q W P L F L L A S K V F L A K D I A V D C N D S Q D                                          867

TGAACTATGGCTAAGCATCTCAAAGGACGAATATATGCAATACGCTGTTGAGGAGTGCTTTCATAGCATCAAGTATATCCTGTCAAATATTCTAGATAAA   2700
 E L W L R I S K D E Y M Q Y A V E E C F H S I K Y I L S N I L D K                                          900

GAAGGCCATCTCTGGGTGCAAAGGATTTTTGATGGTATTCAAGAAAGCATTTCAAAGAACAACATCCAGAGTGATATTCATTTCAGCAAATTGCCTAATG   2800
E G H L W V Q R I F D G I Q E S I S K N N I Q S D I H F S K L P N V                                          934
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TCATTGCCAAGCTTGTTGCTGTAGCGGGAATACTGAAAGAAACGGAATCTGCTGATATGAAGAAGGGGGCAGTTAATGCTATTCAAGATCTATACGAAGT   2900
  I  A  K  L  V  A  V  A  G  I  L  K  E  T  E  S  A  D  M  K  K  G  A  V  N  A  I  Q  D  L  Y  E  V   967

TGTTCATCACGAAGTACTTTTTGTTGATTTGAGTGGTAACATTGACGATTGGAGTCAGATAAATAGAGCAAGAGCCGAAGGCCGCCTCTTCAGTAATCTC   3000
  V  H  H  E  V  L  F  V  D  L  S  G  N  I  D  D  W  S  Q  I  N  R  A  R  A  E  G  R  L  F  S  N  L   1000

AAGTGGCCAAATGAACCTGGATTGAAGGACATGATCAAACGATTGCATTCACTTCTGACCATCAAGGAATCAGCTGCAAATGTTCCTAAAAACCTGGAAG   3100
  K  W  P  N  E  P  G  L  K  D  M  I  K  R  L  H  S  L  L  T  I  K  E  S  A  A  N  V  P  K  N  L  E  A   1034

CCAGCCGGAGACTGCAGTTCTTCACGAACTCTTTGTTCATGCGAATGCCTGTTGCAAGGCCTGTTTCAGAAATGCTTTCCTTTAGCGTATTTACTCCATA   3200
  S  R  R  L  Q  F  F  T  N  S  L  F  M  R  M  P  V  A  R  P  V  S  E  M  L  S  F  S  V  F  T  P  Y   1067

TTGCTCTGAGACAGTGCTTTATAGCATCGCCGAACTCCAAAAGAAAAATGAAGATGGTATATCTACACTATTTTATCTTCAGAAGATTTATCCAGATGAA   3300
  C  S  E  T  V  L  Y  S  I  A  E  L  Q  K  K  N  E  D  G  I  S  T  L  F  Y  L  Q  K  I  Y  P  D  E   1100

TGGAAGAACTTCCTTACTCGCATCAACAGGGATGAAAATGCAGCAGACAGTGAGCTTTTTAGCAGTGCGAATGACATACTAGAACTTCGGCTATGGGCAT   3400
  W  K  N  F  L  T  R  I  N  R  D  E  N  A  A  D  S  E  L  F  S  S  A  N  D  I  L  E  L  R  L  W  A  S   1134

CTTACCGAGGGCAGACATTAGCGCGAACAGTTCGTGGGATGATGTACTACAGAAAGGCCCTTATGTTGCAAAGTTATTTGGAGAGAATGCATTCTGAAGA   3500
  Y  R  G  Q  T  L  A  R  T  V  R  G  M  M  Y  Y  R  K  A  L  M  L  Q  S  Y  L  E  R  M  H  S  E  D   1167

CCTCGAATCTGCATTGGATATGGCTGGTCTGGCTGACACACATTTTGAGTACTCCCCTGAAGCACGCGCACAGGCTGATTTGAAATTTACGTATGTGGTT   3600
  L  E  S  A  L  D  M  A  G  L  A  D  T  H  F  E  Y  S  P  E  A  R  A  Q  A  D  L  K  F  T  Y  V  V   1200

ACCTGCCAAATCTACGGAGTGCAGAAAGGTGAAGGGAAGCCAGAAGCTGCAGATATAGCCCTTCTGATGCAAAGAAATGAAGCTCTCAGAATTGCTTACA   3700
  T  C  Q  I  Y  G  V  Q  K  G  E  G  K  P  E  A  A  D  I  A  L  L  M  Q  R  N  E  A  L  R  I  A  Y  I   1234

TCGATGTTGTTGAGAGCATTAAGAATGGAAAGTCTAGCACCGAGTATTACTCAAAGCTTGTGAAAGCTGACATCCATGGAAAGGACAAGGAAATTTATTC   3800
  D  V  V  E  S  I  K  N  G  K  S  S  T  E  Y  Y  S  K  L  V  K  A  D  I  H  G  K  D  K  E  I  Y  S   1267

TGTTAAATTGCCTGGCAATCCAAAGCTTGGGGAGGGTAAACCCGAAAATCAAAACCATGCTGTAATATTCACTCGCGGAAATGCTGTACAAACCATTGAT   3900
  V  K  L  P  G  N  P  K  L  G  E  G  K  P  E  N  Q  N  H  A  V  I  F  T  R  G  N  A  V  Q  T  I  D   1300

ATGAATCAGGACAACTATTTCGAGGAGGCACTCAAAATGCGAAATCTGCTTGAGGAATTCTCTCAAAATCATGGCAAGTTCAAGCCTTCAATTCTTGGTG   4000
  M  N  Q  D  N  Y  F  E  E  A  L  K  M  R  N  L  L  E  E  F  S  Q  N  H  G  K  F  K  P  S  I  L  G  V   1334

TTAGAGAACATGTCTTCACCGGAAGTGTTTCCTCCCTTGCTTCATTTATGTCAAATCAGGAAACTAGTTTTGTGACATTAGGACAGCGTGTTCTTTCTAA   4100
  R  E  H  V  F  T  G  S  V  S  S  L  A  S  F  M  S  N  Q  E  T  S  F  V  T  L  G  Q  R  V  L  S  N   1367

TCCACTGAAAGTGAGAATGCATTATGGTCACCCAGACGTGTTTGATAGAATATTTCATATTACGAGGGGTGGCATCAGTAAGGCGTCCCGTATCATCAAT   4200
  P  L  K  V  R  M  H  Y  G  H  P  D  V  F  D  R  I  F  H  I  T  R  G  G  I  S  K  A  S  R  I  I  N   1400

ATCAGTGAGGATATATTTGCAGGGTTTAATTCTACTCTGCGTCAAGGGAACATAACTCACCATGAGTATATCCAGGTTGGTAAAGGAAGAGATGTTGGGC   4300
  I  S  E  D  I  F  A  G  F  N  S  T  L  R  Q  G  N  I  T  H  H  E  Y  I  Q  V  G  K  G  R  D  V  G  L   1434

TTAATCAGATCGCACTATTTGAAGGAAAAGTTGCGGGAGGAAACGGCGAACAAGTTCTTAGCAGAGATATATACAGACTTGGGCAACTTTTTGACTTTTT   4400
  N  Q  I  A  L  F  E  G  K  V  A  G  G  N  G  E  Q  V  L  S  R  D  I  Y  R  L  G  Q  L  F  D  F  F   1467

CAGGATGTTATCCTTCTATGTGACTACTGTTGGGTTTTACTTCTGTACGATGCTAACTGTACTGACAGTGTACATATTTCTCTATGGTAAAACCTATCTG   4500
  R  M  L  S  F  Y  V  T  T  V  G  F  Y  F  C  T  M  L  T  V  L  T  V  Y  I  F  L  Y  G  K  T  Y  L   1500

GCTTTATCTGGTGTTGGAGAATCAATTCAAAATAGGGCGGATATACAGGGAAATGAAGCATTGAGCATAGCTCTGAACACCCAGTTTCTTTTCCAGATTG   4600
  A  L  S  G  V  G  E  S  I  Q  N  R  A  D  I  Q  G  N  E  A  L  S  I  A  L  N  T  Q  F  L  F  Q  I  G   1534

GTGTGTTTACTGCAATTCCTATGATTCTAGGTTTCATCCTGGAAGAAGGTGTCCTGACGGCTTTTGTCAGCTTCATTACCATGCAGTTCCAACTATGCTC   4700
  V  F  T  A  I  P  M  I  L  G  F  I  L  E  E  G  V  L  T  A  F  V  S  F  I  T  M  Q  F  Q  L  C  S   1567

CGTATTTTTCACGTTCTCCCTTGGGACAAGGACTCACTATTTTGGTCGCACAATACTGCATGGAGGTGCAAAGTATAGAGCAACTGGTAGGGGTTTTGTG   4800
  V  F  F  T  F  S  L  G  T  R  T  H  Y  F  G  R  T  I  L  H  G  G  A  K  Y  R  A  T  G  R  G  F  V   1600

GTACGGCATATTAAGTTTGCTGAGAATTACCGTCTTTATTCCCGAAGCCATTTTGTGAAAGGGCTGGAGGTTGCACTTCTGTTGGTTATCTTTCTAGCTT   4900
  V  R  H  I  K  F  A  E  N  Y  R  L  Y  S  R  S  H  F  V  K  G  L  E  V  A  L  L  L  V  I  F  L  A  Y   1634

ATGGTTTTAACAACAGTGGAGCAATTGGCTATATCTTACTTTCCATAAGTAGCTGGTTTATGGCTCTTTCTTGGCTTTTTGCTCCATACGTATTCAACCC   5000
  G  F  N  N  S  G  A  I  G  Y  I  L  L  S  I  S  S  W  F  M  A  L  S  W  L  F  A  P  Y  V  F  N  P   1667

GTCTGGATTTGAATGGCAAAAGGTCGTCGAGGATTTCAGAGACTGGACAAACTGGCTTTTTTACCGAGGTGGTATTGGGGTTAAAGGAGAAGAAAGCTGG   5100
  S  G  F  E  W  Q  K  V  V  E  D  F  R  D  W  T  N  W  L  F  Y  R  G  G  I  G  V  K  G  E  E  S  W   1700

GAAGCTTGGTGGGATGAAGAACTGGCACATATTCACACCTTCCGCGGGAGGATACTGGAAACTATACTTAGTTTAAGATTTTTTATTTTCCAGTATGGAG   5200
  E  A  W  W  D  E  E  L  A  H  I  H  T  F  R  G  R  I  L  E  T  I  L  S  L  R  F  F  I  F  Q  Y  G  V   1734

TTGTTTACCACATGAAAGCAAGCAATGAAAGTACAGCATTACTGGTATATTGGGTATCATGGGCTGTGCTTGGAGGGCTTTTTGTCCTGCTAATGGTATT   5300
  V  Y  H  M  K  A  S  N  E  S  T  A  L  L  V  Y  W  V  S  W  A  V  L  G  G  L  F  V  L  L  M  V  F   1767

TAGTTTAAACCCCAAGGCCATGGTTCATTTCCAGTTGTTCCTGCGTCTGGTCAAAAGCATTGCACTGTTAGTGGTTTTGGCAGGTTTGGTTGTGGCAATT   5400
  S  L  N  P  K  A  M  V  H  F  Q  L  F  L  R  L  V  K  S  I  A  L  L  V  V  L  A  G  L  V  V  A  I   1800

GCAATTACAAGACTCGCTGTTGTAGATGTACTTGCTTCCATCCTAGCATATGTGCCTACTGGATGGGGAATTCTTTCGATTGCTGTGGCATGGAAACCAA   5500
  A  I  T  R  L  A  V  V  D  V  L  A  S  I  L  A  Y  V  P  T  G  W  G  I  L  S  I  A  V  A  W  K  P  I   1834

TTGTGAAGAGACTGGGTTTGTGGAAAACAGTGCGCTCGTTGGCTCGCCTGTATGATGCTGGCATGGGAATGATCATTTTTGTGCCCATAGCTATCTGCTC   5600
  V  K  R  L  G  L  W  K  T  V  R  S  L  A  R  L  Y  D  A  G  M  G  M  I  I  F  V  P  I  A  I  C  S   1867

GTGGTTTCCCTTCATCTCCACCTTCCAGACACGGCTACTGTTCAACCAGGCTTTCAGCAGAGGTTTGGAGATTTCTCTCATCCTGGCTGGCAACAATCAG   5700
  W  F  P  F  I  S  T  F  Q  T  R  L  L  F  N  Q  A  F  S  R  G  L  E  I  S  L  I  L  A  G  N  N  Q   1900
```

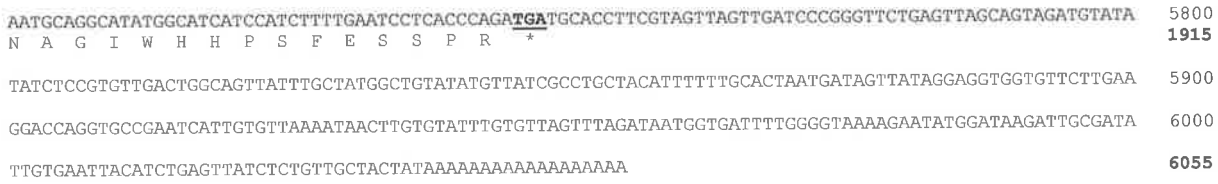


Figure 2.10 The nucleotide sequence and the deduced amino acid sequence of a putative barley callose synthase. The putative translation start codon and stop codon are underlined and shown in bold.

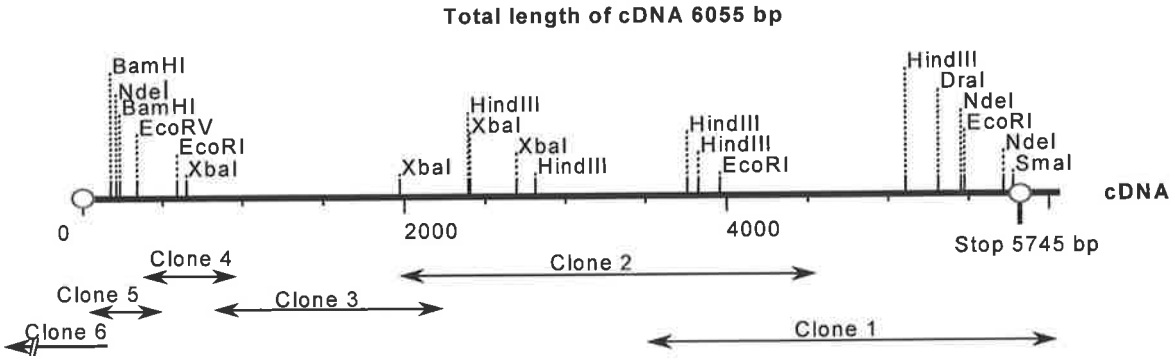


Figure 2.11 Restriction map of the *HvGSL1* cDNA. The total length of the *HvGSL1* cDNA from the putative start codon to the poly A tail is 6055 bp and contains an open reading frame of 5745 bp, which encodes a protein of 1915 amino acids. The putative start and stop points for translation are illustrated by red circles. The sizes of the six composite clones and their sources are as follows. Clone 1 (3497 bp-6055 bp): 2558 bp from the barley callus cDNA library; Clone 2 (1919 bp-4522 bp): 2603 bp from the same cDNA library as clone1; Clone 3 (847 bp-2207 bp): 1360 bp, RT-PCR product; Clone 4 (360 bp-998 bp): 638 bp, anchor ligated PCR product; Clone 5 (22 bp-498 bp): 476 bp, anchor ligated PCR product; Clone 6 contains the rest of the sequence of the missing 5'end (0-22 bp), including the putative start point and was obtained from a barley BAC library. Sequencing results from this BAC clone revealed that it appears to carry the entire *HvGSL1* gene.

2.4 Summary and Conclusions

The molecular cloning and assembly of a putative near full-length (1→3)-β-glucan synthase cDNA from barley is described in this Chapter. A cDNA of *Hieracium* homologous to a yeast *FKS* gene was initially used to screen a barley cDNA library, resulting in the isolation of a partial barley cDNA. The subsequent application of a range of molecular cloning techniques, such as RT-PCR, anchor-ligated PCR, and BAC library screening, led to the isolation of a number of clones which in composite defined a near full-length callose synthase-like cDNA, designated *HvGSL1*, from barley. The total length of the *HvGSL1* cDNA from the putative start codon to the poly A tail is 6055 bp, containing an open reading frame of 5745 bp (Figure 2.9). The deduced amino acid sequence (1915 amino acid residues) of the *HvGSL1* shows approximately 30% sequence identity to that of the yeast (1→3)-β-glucan synthase. Further sequence analysis and genetic characterisation of the *HvGSL1* will be presented in the following Chapters.

CHAPTER THREE:

THE GENETICS AND TRANSCRIPTION PATTERNS OF CALLOSE SYNTHASE GENES IN BARLEY

3.1 Introduction

The putative barley (1→3)-β-glucan synthase cDNA *HvGSL1*, containing an open reading frame sequence which encodes a 1915 amino acid protein, was compiled as the first step of this study, as described in Chapter 2. Further properties of the *HvGSL1* gene remained to be defined, including its overall sequence similarity to its yeast counterpart, the *FKS1* gene, the structure of *HvGSL* genes, the copy number of the gene in the barley genome, and chromosomal locations of the gene. In addition, it was important to define transcription sites of *HvGSL* genes in various barley tissues. These objectives are addressed in the experiments described in this Chapter.

In the yeast *Saccharomyces cerevisiae*, two genes (*FKS1* and *FKS2*) are believed to encode the catalytic subunit of the (1→3)-β-glucan synthase, as defined using echinocandin mutants (Douglas *et al.*, 1994; Mazur *et al.*, 1994). As mentioned in Chapter 1, simultaneous disruption of both *FKS1* and *FKS2* is lethal, whilst a single insertional disruption of either gene is not, suggesting that *FKS1* and *FKS2* are alternative subunits which possess essentially overlapping functions (Douglas *et al.*, 1994; Mazur *et al.*, 1994). A similar mechanism was reported in the fungal species *Candida albicans*, where the synthesis of (1→3)-β-glucan in the fungal cell wall is governed by either two or three genes, *GS1, GSL1* and *GSL2*, whose sequences show significant homology to *FKS1* (Mio *et al.*, 1997). However, the *Cryptococcus neoformans FKS1*, a gene closely related to the *FKS1* of *Saccharomyces cerevisiae*, appears to be present as a single copy and is essential for (1→3)-β-glucan synthesis (Thompson *et al.*, 1999). The same is true in *Aspergillus nidulans* (Kelly *et al.*, 1996) as well as in *Paracoccidioides brasiliensis* (Pereira *et al.*, 2000). Thus, evidence from a number of fungal species suggests that (1→3)-β-glucan synthases are encoded by a small family of genes in fungi and yeast.

In higher plants, a single gene designated *CFL1*, which is homologous to the yeast *FKS1*, has been isolated from cotton fibres (Cui *et al.*, 2001). There is no further data describing the copy number of the gene in the cotton genome. However, a family of 12 genes, which are putative homologues of the yeast *FKS1* genes, has been identified from *Arabidopsis* (Hong *et al.*, 2001; http//cellwall.stanford.edu/gsl/index.html). They are structurally classified into two groups according to the number of the introns in the genes; one group has two to three introns, while the other has over 40 introns. The evolutionary history and

transcription patterns of these genes remain largely unknown. Similarly, a family of 13 rice genes showing sequence homology to the yeast *FKS* genes has been found, but no evidence for gene function has been reported (http//cellwall.stanford.edu/gsl/index.html).

Since the isolation of a putative callose synthase gene, *CFL1*, which shows striking similarity to the yeast synthase subunit FKS1 and to a number of other fungal (1→3)-β-glucan synthase genes (Cui *et al.*, 2001), the nomenclature system for the callose synthase genes from higher plant has been inconsistent. At that time, the Somerville group annotated putative callose synthase genes from the *Arabidopsis* genome (http//cellwall.stanford.edu/gsl/index.html), although there was no published report on the function for the genes. Rather than designating these genes as "callose synthase", Richmond and Somerville (2000) instead called them putative β-glucan synthases or *GSL* (glucan synthase-like) genes. However, a later paper published by Verma's group (Hong *et al.*, 2001), showed that the product of the *Arabidopsis* genes was located at the growing cell plate, interacted with phragmoplastin, and enhanced callose synthase activity in transgenic tobacco cells. While there is no direct biochemical evidence that these proteins are callose synthases, it seems likely that this gene family encodes plant callose synthases. Accordingly Verma's group designated their gene *AtCalS1*, and named the other genes in this family *AtCalS2-AtCalS12*. Other groups, however, have continued to use the *GSL* naming convention (Doblin *et al.*, 2001). The term *GSL* is therefore used throughout this thesis, as a designation of putative callose synthases and their genes in higher plants.

In this chapter, general aspects of the barley callose synthase-like gene *HvGSL1* are defined, using conventional methodology. This includes Southern blot analysis of barley genomic DNA using a probe derived from the barley *HvGSL1* cDNA and analysis of EST databases for gene number studies, Northern blot analysis of total RNA extracted from a range of barley tissues, and a RT-PCR approach employed to gain further insights into the transcriptional patterns of the *HvGSL* genes. Furthermore, two strategies, barley double-haploid population lines from six different varieties, and wheat-barley additional lines, were used to define the chromosomal location of the *HvGSL* genes. Finally, using available computer programs, the *HvGSL1* gene was studied at the protein level *in silico* by comparing its sequence with known sequences of putative callose synthase (*GSL*)

genes described amongst other higher plants, to define the likely topology of the protein, and by rationalising the possible domain functions of the enzyme encoded by the *HvGSL1* cDNA.

3.2 Materials and Methods

3.2.1 Materials

Genomic DNA from barley varieties Clipper, Sahara, Galleon, Haruna niji, Chebec and Harrington were kindly provided by Professor Peter Langridge (Department of Plant Science, University of Adelaide, Australia). Samples of DNA from wheat variety Chinese Spring, barley variety Betzes, and wheat-barley addition lines were generously provided by Dr. I. M. R. Islam and Associate Professor K. W. Shepherd (Department of Plant Science, University of Adelaide, Australia). All the enzymes, reagents and related materials used in DNA and RNA manipulations, and in hybridisation experiments outlined here were obtained from the same sources as described in Chapter 2.

3.2.2 Determination of Copy Number of HvGSL1

3.2.2.1 Southern Blot Analysis of Barley Genomic DNA

Genomic DNA had been isolated from barley leaves using a caesium chloride extraction method (Sambrook *et al.*, 1989). The DNA (10 μg) was subjected to single restriction enzyme digestions with *Eco*RI, *Bam*HI, *Dra*I, *Eco*RV and *Hin*dIII, and separated on 1% agarose gels at a constant voltage of 25 V. The DNA was transferred onto a Hybond membrane by capillary blotting in 0.4 M NaOH and fixed by UV light. The membrane was hybridised with a 1513 bp radiolabelled cDNA fragment obtained from Clone 1 by *Eco*RI digestion, using the procedure described in section 2.2.5.4.

3.2.2.2 Searching EST Databases for HvGSL1 *Homologues*

To investigate the number of the genes in the *HvGSL1* family in barley, Expression Sequence Tag (EST) databases were searched for sequences homologous to the *HvGSL1* and a web-site including callose synthase genes named tentatively as Glucan Synthase Like (GSL) generated by C. Somerville's research group (Richmond and Somerville, 2000; http//cellwall.stanford.edu/gsl/index.shtml) was also used as a reference.

3.2.3 *Chromosomal Localisation of the* HvGSL1 *Gene*

3.2.3.1 *Mapping by Barley Doubled Haploid Population Lines*

Genomic DNA samples from six parental lines (Clipper, Sahara, Galleon, Haruna niji, Chebec and Harington), digested with five restriction enzymes, *Eco*RI, *Dra*I, *Hin*dIII, *Bam*HI, *Eco*RV and transferred to nylon filters, were hybridised with a 1513 bp probe (Section 3.3.2) to screen for restriction fragment length polymorphisms (RFLPs). The subsequent progeny screening was carried out by probing the filters from the populations that yielded polymorphisms. For example, for the cross Clipper x Sahara, the DNA of 120 population lines was digested with *Eco*RV, transferred to a membrane and the filter was hybridised with the partial *HvGSL1* probe. The results from these population lines were scored based on the parental genotype. The linkage scores were analysed using the Map-Manager software (Lander *et al.*, 1987). The chromosome location of the *HvGSL1* gene was assigned to the barley linkage map (Langridge *et al.*, 1995).

3.2.3.2 *Mapping by Wheat-Barley Addition Lines*

Wheat (var. Chinese Spring)-barley (var. Betzes) addition lines generated by Islam and Shepherd (Islam *et al.*, 1981; Islam and Shepherd, 1990) were used as an alternative strategy to determine the map location of the *HvGSL1* gene. In these addition lines, one pair of barley chromosomes was added to the wheat Chinese Spring genome, so that the wheat genome contains an extra barley chromosome, designated as 1H, 2H, 3H, 4H, 5H, 6H and 7H (Islam and Shepherd, 1990). Due to a toxic effect incurred by the addition of barley 1H to the wheat genome, a wheat addition line carrying both 1H and 6H was generated (Islam and Shepherd, 1990). Thus, the occurrence of an extra hybridising fragment in DNA digests from a particular line enables bands hybridising to the *HvGSL1* probe to be assigned to a specific barley chromosome. Genomic DNA preparations of wheat, barley and the wheat-barley addition lines were digested with restriction enzyme *Eco*RI, and separated on a 1% agarose gel. The DNA fragments were transferred to a membrane, the DNA was fixed, and the membrane was prehybridised and hybridised with a 1513 bp probe (Section 3.3.2) using the procedure described in Section 3.3.3.1.

3.2.4 *Transcription Patterns of the* HvGSL1 *Gene*

3.2.4.1 *Northern Blot Analysis*

Total RNA was extracted from a range of tissues, which included callus, roots, coleoptiles, first leaves, mature leaves, leaves infected by powdery mildew, stems, florets and early developing grains, using the TRIzol reagent according to the manufacturer's instructions (Section 2.2.3.1). The gel tank and the casting tray were pre-treated with 0.4 M NaOH to denature RNases. A 1.5% agarose gel was prepared in 1×MOPS buffer (50 mM MOPS buffer, pH 7.0, containing 1 mM EDTA and 3% v/v formaldehyde). The gel was pre-run in MOPS buffer at a constant voltage of 60 V for 30 min. RNA samples (20 μg) from a variety of barley tissues were prepared in 20 μl 50 mM MOPS buffer, pH 7.0, containing the denaturing agents 18% formaldehyde and 50% formamide, incubated at 65°C for 10 min and mixed with 1 μl loading buffer (50 mM MOPS buffer, pH 7.0, containing 1 mM EDTA, 40% sucrose, 0.5% xylene cyanol, 0.5% bromocresol green, 18% formaldehyde and 50% formamide). After the samples were loaded, the gel was run at 100 V for 2 to 4 h in 1×MOPS buffer until the first dye front ran off the gel. After electrophoresis, the gel was stained with ethidium bromide for 10 min, and destained in water. The RNA was subsequently transferred to a Hybond N membrane and fixed by UV light.

Two types of probes were generated for the Northern blot analysis. Type 1 was a gene-specific probe that contained a short fragment covering the 3' untranslated region of the *HvGSL1* cDNA. It was expected that probing with the 3' untranslated region fragment, where other possible callose synthase genes would share little sequence similarity, would specifically detect *HvGSL1* mRNA. To generate this probe, a forward primer 3UTR-F with the sequence 5'-GCA CCT TCG TAG TTA GTT-3' and a reverse primer 3UTR-R with the sequence 5'-TAG CAA CAG AGA TAA CTC A-3' were synthesised (refer to 5' end untranslated region sequence in Figure 2.10). Clone 1 was used as a template for the PCR amplification. A fragment of 284 bp was obtained.

The type 2 probe consisted of two fragments, which were generated from the cDNA inserts of Clone1 and Clone 2. Both cDNAs were digested with the restriction enzyme *Eco*RI, the inserts were purified and the two fragments were combined for use as the probe. These two fragments together covered the cDNA from the 3' UTR and extended towards the 5' end to a total length of 4.2 kb. The "double" probe was used in the

Northern blot analyses in the expectation that a stronger hybridising signal would be obtained. The RNA filter was prehybridised in pre-hybridisation solution (7.5×Denhardt's, 5×SSPE, 0.5% SDS, 0.5 mg/ml denatured salmon sperm DNA, 45% formamide) at 42°C overnight. For hybridisation, the filter was transferred to the hybridisation buffer (5×Denhardt's, 5×SSPE, 0.5% SDS, 0.5 mg/ml denatured salmon sperm DNA, 45% formamide and 2.5% dextran sulphate), and the denatured radio-labelled probe, either Type 1 or Type 2, was added. The filter was incubated at 42°C, with rotation, for 48 h. The washing and exposure steps were identical to those described for Southern blot analysis (Section 3.2.2.1).

3.2.4.2 Analysis of HvGSL1 *Gene Transcription using RT-PCR*

The RT-PCR method was also employed to examine the transcription patterns of the *HvGSL1* gene. In comparison to standard Northern hybridisation analysis, RT-PCR is highly sensitive, and allows the detection of very low levels of mRNA (Frohman *et al.*, 1988), whilst a standard Northern blot analysis is semi-quantitative if performed carefully and if loadings are equal in all lanes, and can provide an indication of relative levels of mRNAs in the tissues analysed.

The total RNA preparations from the tissues described above (Section 3.3.4.1) were used as templates for the first strand cDNA synthesis in which oligo dT (20) was used as the primer. For the PCR amplification, two primers, 5'HvClone1 primer with the sequence 5'-GCTGGC ATG GGA ATG ATG ATC AT-3' and 3UTR-R (Section 3.3.4.1), were used as the forward and reverse primers, respectively. These two primers were expected to produce a PCR fragment of 477 bp in tissues where the *HvGSL1* gene is transcribed. The PCR reaction, which contained the reaction mixture given in Section 2.2.3.2, was thermocycled for 25 cycles of 94°C for 30 sec, 55°C for 45 sec, and 72°C for 60 sec. The resulting fragments were analysed by electrophoresis on a 1% agarose gel (Sambrook *et al.*, 1989). To ensure the integrity of cDNAs which were synthesised from all RNA samples, control PCR reactions were performed with the same cDNAs from all tissues examined, using the two primers specific for constitutively-expressed mRNA encoding the glycolytic pathway enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from barley. The forward and reverse primers for GAPDH were 5'-CCA CCG GGT CTT CAC TGA CAA GG 3, and 5'-GCC TTA GCA TCA AAG TGA TGG-3', respectively.

The PCR product produced from these two primers was expected to have a size of approximately 550 bp. The PCR reaction conditions and subsequently thermocycling parameters were the same as those described above.

3.2.5 *Sequence Analysis of the HvGSL1 Polypeptide*

The coding region of the putative barley callose synthase protein, designated HvGSL1, was deduced from the cloned cDNA (*HvGSL1*) for sequence analysis. The TBLASTP program (Stephen *et al.*, 1997) was used to search the *Arabidopsis thaliana* database and the National Centre for Biotechnology Information (NCBI) database for homologues of the barley HvGSL1, and a web-site containing callose synthase genes generated by C. Somerville's research group described in Section 3.3.2 was also referenced. Barley EST analysis was conducted by searching EST databases (A. Harvey, personal communication). Kyte-Doolittle hydrophobicity plots (Kyte and Doolittle, 1982) were completed with a 9-residue window at the http://fasta.bioch.virginia.edu/ofasta/grease.htm web site.

A topological profile of the putative callose synthase was modelled using the computer program TopPred (von Heijne, 1992; http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html) to predict possible trans-membrane helices of the protein. Multiple sequence alignments and other computer analyses of the callose synthases were generated with PILEUP, PRETTYBOX and other programs from the University of Wisconsin Group (GCG) software (Devereux *et al.*, 1984) in the ANGIS suite of programs at the University of Sydney (http://www.angis.org.au/) with GeneBank accession numbers (CFL1: AF085717, CalS1: AF237733, CalS12: AC005142), in order to analyse the sequence similarities between the plant putative callose synthases. Hydrophobic cluster analysis (HCA) was performed using DrawHCA (Callebaut *et al.*, 1997; http://www.lmcp.jussieu.fr/~callebau/hca_method.html). Multiple sequence alignments were generated using ClustalW (http://www2.ebi.ac.uk/clustalw; Thompson *et al.*, 1994) for domain analysis.

3.3 Results and Discussion

3.3.1 Number of Callose Synthase Genes in Barley

Southern blot analysis of barley genomic DNA, probed with a partial *HvGSL1* cDNA fragment of 1513 bp, revealed the presence of one to three hybridising bands in each restriction digestion (Figure 3.1B). One strong signal dominated in the *Bam*HI, *Eco*RV and *Hin*dIII cut DNA, whilst two additional faint bands were present in the *Hin*dIII digestion and three bands were observed in the *Eco*RI digestion. This implies either that there are several copies of the *HvGSL1* gene in the barley genome, or that there are internal sites for the restriction enzymes used. Similarly, one to three hybridisation bands were observed in a Southern blot analysis when DNA samples from six different barley cultivars (Clipper, Sahara, Gallen, Haruna nijo, Chebec and Harrington) were probed, as described in the mapping section below (Section 3.3.2), and three to four major bands were evident in DNA from the wheat-barley addition lines (Figure 3.5, Section 3.3.2).

Because the Southern analyses might not reveal some of the *HvGSL1* genes at the relatively high stringencies used, and because the *Arabidopsis* and rice genomes contain 12 and 13 *GSL* genes, respectively, it was likely that the Southern analyses were under-estimating the number of *GSL* genes in barley. Another approach was to analyse the EST databases, which currently contain about 314,000 barley sequences, and to define the number of unique *HvGSL* sequences. This work was performed with Dr. Andrew Harvey. Searching EST databases using the sequence of the *HvGSL1* cDNA identified a number of potential callose synthase sequences in barley EST libraries. Eighty callose synthase EST sequences were found in barley, of which at least six independent genes can be identified (A. Harvey, personal communication). In Figure 3.2, the positions of EST sequences are indicated in relation to the sequence of the 6.1 kb *HvGSL1* cDNA (top) and each block represents a potentially different gene. Contig one, named *HvGSL1,* contains 18 EST sequences that match the sequence of the *HvGSL1* cDNA, but each EST is only approximately 600 bp in length. Contigs 2, 3 4, 5, 6, 7, 8 have 40, 2, 3, 2, 3, 5, 2 EST sequences, respectively. In each contig, the ESTs have some overlapping, identical sequence but differ in length. In addition to the eight contigs, ESTs BE559011, BJ479194, BJ484184, BQ484184 and BU982241 are also homologous to the *HvGSL1* cDNA and may represent further genes or may be different segments of the contigs

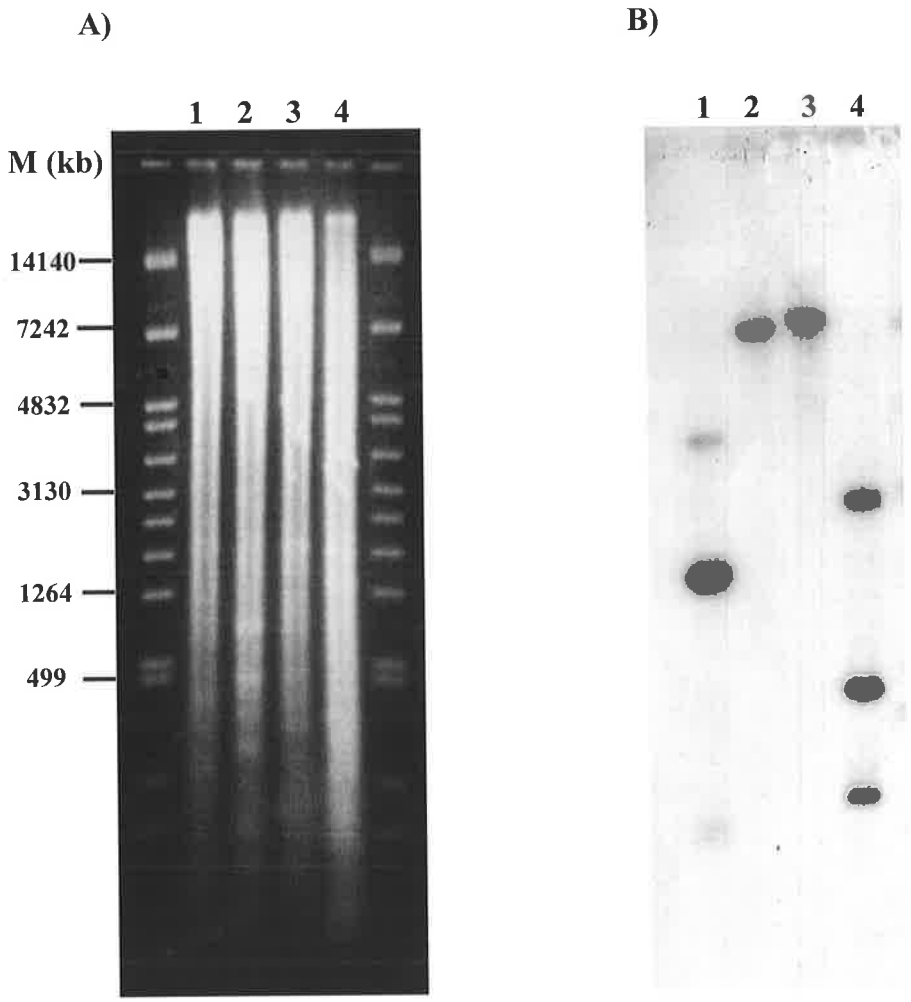
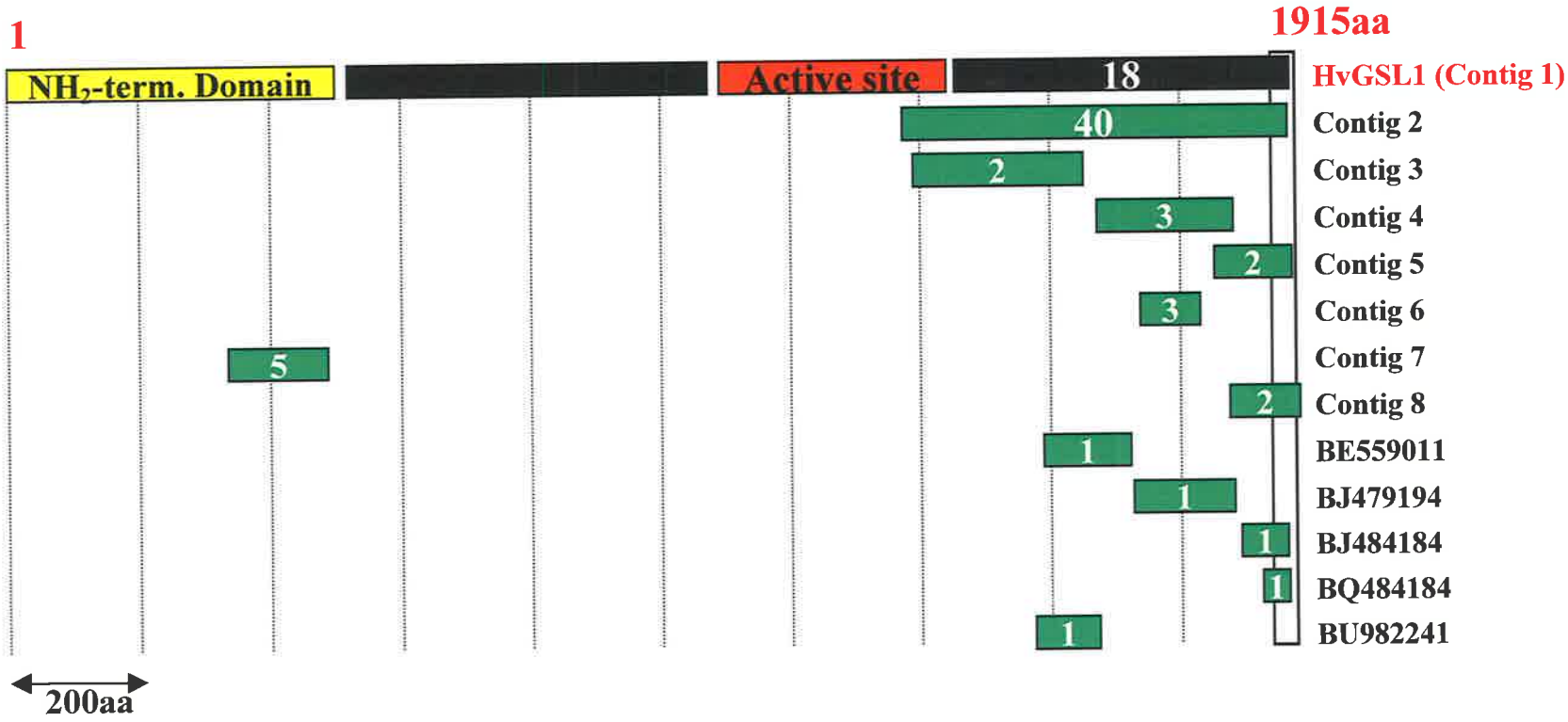


Figure 3.1 Southern blot analysis of barley genomic DNA. A) Barley genomic DNA was digested with the restriction enzymes *Hin*dIII (1), *Bam*HI (2), *Eco*RV (3), and *Eco*RI (4) and run on a 1% agarose gel. λ DNA cut with *Bst*EII was used as DNA molecular markers seen in lanes adjacent to samples 1 and 4. **B)** The DNA in gel A was transferred to a nylon filter, and hybridised with a partial *HvGSL1* cDNA Probe.

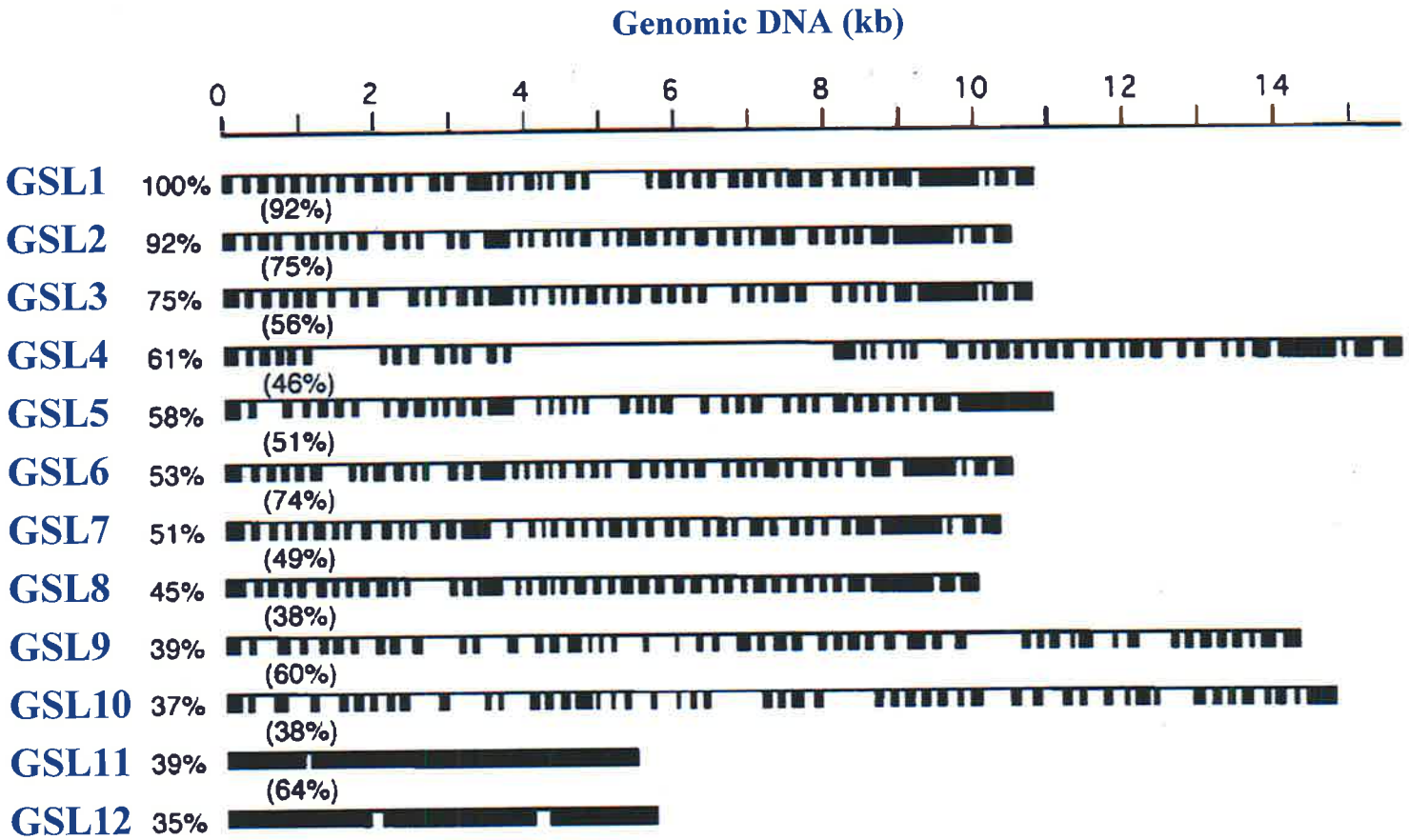
Figure 3.2 Analysis of the barley callose synthase gene family from EST databases. The positions of EST sequences are shown in relation to the amino acid sequence of the 1,915 residues (HvGSL1 enzyme). Each block represents a potentially different gene, and numbers in the blocks indicate the total number of ESTs for that segment. Where there are more than one ESTs the block is labelled as a Contig, and where there is only one EST, its database accession number is given. The six blocks that extend to the COOH-terminal regions of the enzyme are known to represent six individual genes (Figure prepared by A. Harvey).


1
1915aa
NH2-term. Domain
Active site
18
HvGSL1 (Contig 1)
40
Contig 2
2
Contig 3
3
Contig 4
2
Contig 5
3
Contig 6
5
Contig 7
2
Contig 8
1
BE559011
1
BJ479194
1
BJ484184
1
BQ484184
1
BU982241
200aa

described above. Sequence identity values for the individual barley callose synthase genes range from 40 to 60% (data not shown) and this presumably explains why most of them were not detected when Southern hybridisations were performed at high stringency. It can be concluded that there are at least six *HvGSL* genes in barley (Figure 3.2).

As discussed in Section 3.1, genes encoding the (1→3)-β-glucan synthase catalytic subunit in yeast and fungal species consist of a small gene family of one to three genes (Douglas *et al.*, 1994; Mazur *et al.*, 1994; Kelly *et al.*, 1996; Thompson *et al.*, 1999; Pereira *et al.*, 2000), although definitive proof of gene function has not yet been presented. Several lines of evidence suggest that these genes encode the catalytic subunit of the enzyme and may have overlapping functions. In higher eukaryotic systems, the presence of multiple putative callose synthase genes has been reported (Hong *et al.*, 2001). In the *Arabidopsis* genome, 12 homologous sequences were identified using the deduced amino acid sequence of HvGSL1 to search the available databases. Figure 3.3, adapted from Hong *et al.* (2001), outlines the genomic organisation, the size of each gene, and the relative sequence homology of these *Arabidopsis GSL* genes. These genes are distributed over five *Arabidopsis* chromosomes, and can be classified into two main categories based on gene structure. One group has two to three introns (*GSL11-12*), and the other has 40 to 49 introns (*GSL1-10*). The cloned gene *AtGSL1* of *Arabidopsis* in group one has been examined for its possible involvement in callose synthesis, and it appears that this synthase is associated with cell-plate formation and interacts with phragmoplastin, and in transgenic tobacco cells, enhanced callose synthase activity was observed (Hong *et al.*, 2001). To date, the functions of the other *GSL* genes remain unclear and it is unknown as to whether they are truly genes encoding callose synthases, and what specific function each gene may have in the process of synthesising a specific type of callose in specific plant tissues. Functional redundancy also remains a possibility. Similarly, Somerville's group independently performed an alignment of homologous sequences from *Arabidopsis* and other higher plants (http://cellwall.stanford.edu). On their website, a number of EST sequences are presented from a variety of higher plants, ranging from soybean, cotton, tomato, rice, wheat, and maize. At this stage, the number of independent plant *HvGSL1* sequences in the EST databases is at least 800, although the final copy number of *HvGSL1*-like genes in barley is yet to be determined.

Figure 3.3 The putative callose synthase gene family of *Arabidopsis*. Based on the sequences of the yeast (1→3)-β-glucan synthase *FKS* gene, 12 putative callose synthase genes were identified from the databases. Exons are shown as boxes and introns as lines. According to their gene structures, these 12 genes are classified into two categories; one group of genes contains only 1-2 introns, whilst the other group has up to 49 introns. The figure was adapted from Hong *et al.* (2001).


Genomic DNA (kb)
0
2
4
6
8
10
12
14
GSL1
100%
(92%)
GSL2
92%
(75%)
GSL3
75%
(56%)
GSL4
61%
(46%)
GSL5
58%
(51%)
GSL6
53%
(74%)
GSL7
51%
(49%)
GSL8
45%
(38%)
GSL9
39%
(60%)
GSL10
37%
(38%)
GSL11
39%
(64%)
GSL12
35%

3.3.2 *Chromosomal Location of the* HvGSL1 *Gene*

To facilitate the assignment of the *HvGSL1* gene to a specific chromosomal location in barley, Southern blot analysis was initially performed on DNA from parental lines of six different barley varieties digested with five restriction enzymes. Several polymorphisms were revealed between these parental lines. This included Clipper and Sahara digested with *Eco*RI, *Bam*II, *Eco*RV, and Galleon and Haruna nijo digested with *Eco*RV. Two polymorphisms were therefore selected for mapping in the Clipper × Sahara and Galleon × Haruna nijo mapping populations.

Linkage analysis of these RFLP banding patterns in both populations resulted in assignment of the polymorphisms to a single site on the long arm of chromosome 4H. The position of the locus, designated *HvGSL1*, is shown in Figure 3.4 A and Figure 3.4 B in the Clipper × Sahara and Galleon × Haruna nijo mapping populations, respectively.

The chromosomal location of the *HvGSL1* was confirmed by hybridising DNA from the wheat-barley addition lines with the same probe as used in the doubled-haploid lines. Southern blot analysis of wheat-barley addition lines (Figure 3.5) revealed that the banding pattern in the barley parent Betzes from which the addition lines were derived were only detectable in the wheat-barley addition line carrying barley chromosome 4H. Therefore, the location of the *HvGSL1* gene was confirmed to be on barley chromosome 4H, in agreement with the data obtained from the barley doubled-haploid lines described above.

3.3.3 *Transcription Patterns of* HvGSL1 *mRNA*

3.3.3.1 *Northern Blot Analysis*

Northern blot analysis of a range of RNA samples extracted from different barley tissues using the 3'untranslated region fragment of *HvGSL1* (type 1 probe, Section 3.3.4.1) failed to reveal any hybridisation bands. To investigate possible causes for this result, trials involving modified radiolabelling procedures, increased hybridisation times and increased amounts of total RNA used for the hybridisation, as well as increased exposure times of the film, were carried out. The amount of total RNA loaded on the gel from each tissue type was increased from 10 μg to 15 μg, and the amount of labelled probe was also

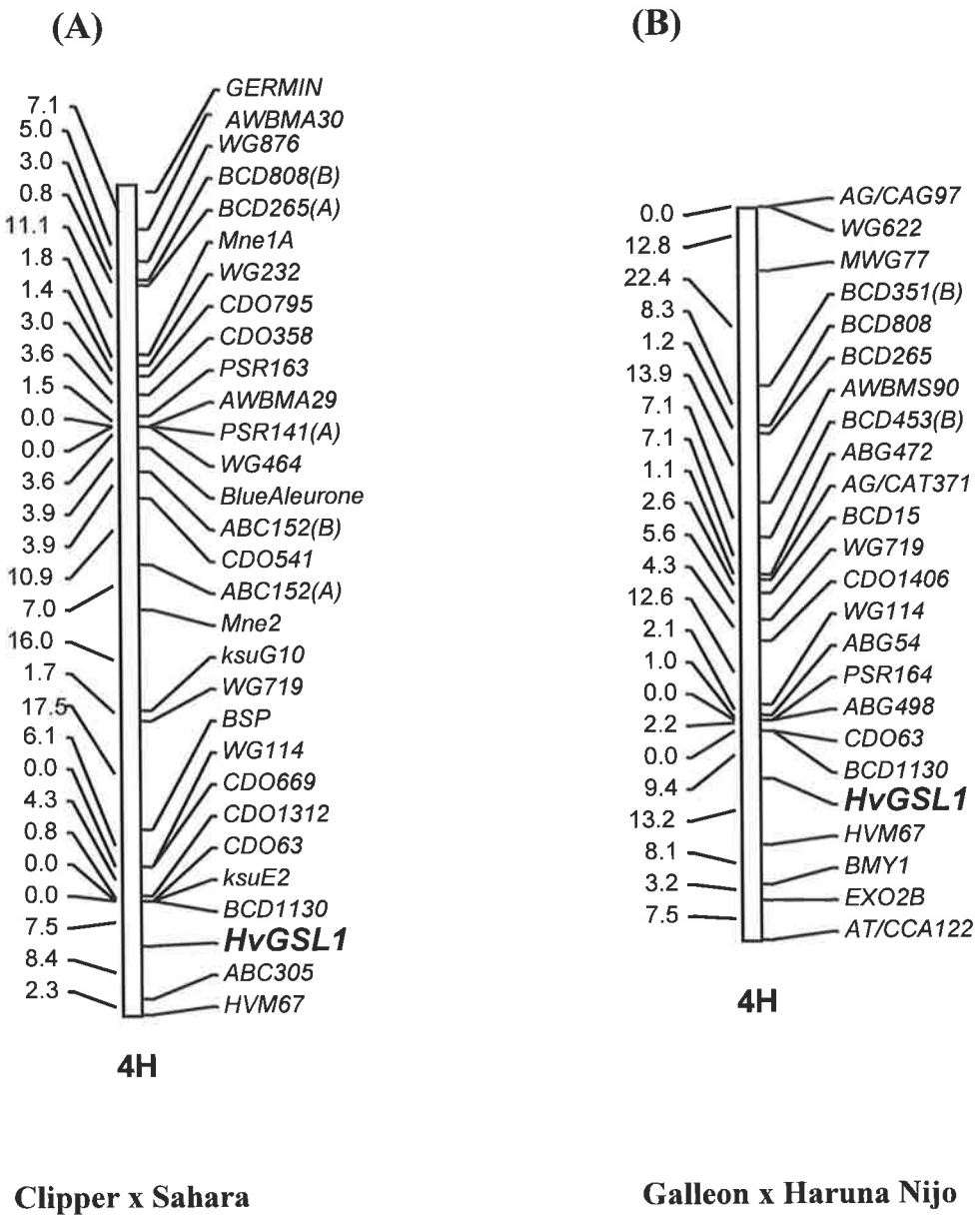


Figure 3.4 Chromosomal location of the barley *HvGSL1* gene. Two linkage maps, between Clipper×Sahara and Galleon×Haruna Nijo, show an identical position of the *HvGSL1* gene on the long arm of barley chromosome 4H, between the RFLP markers BCD1130 and HVM67. The *HvGSL1* locus is shown in bold and colored in red. The linkage analysis was performed using the Map-Manager software, and is graphically represented using the JoinMap software.

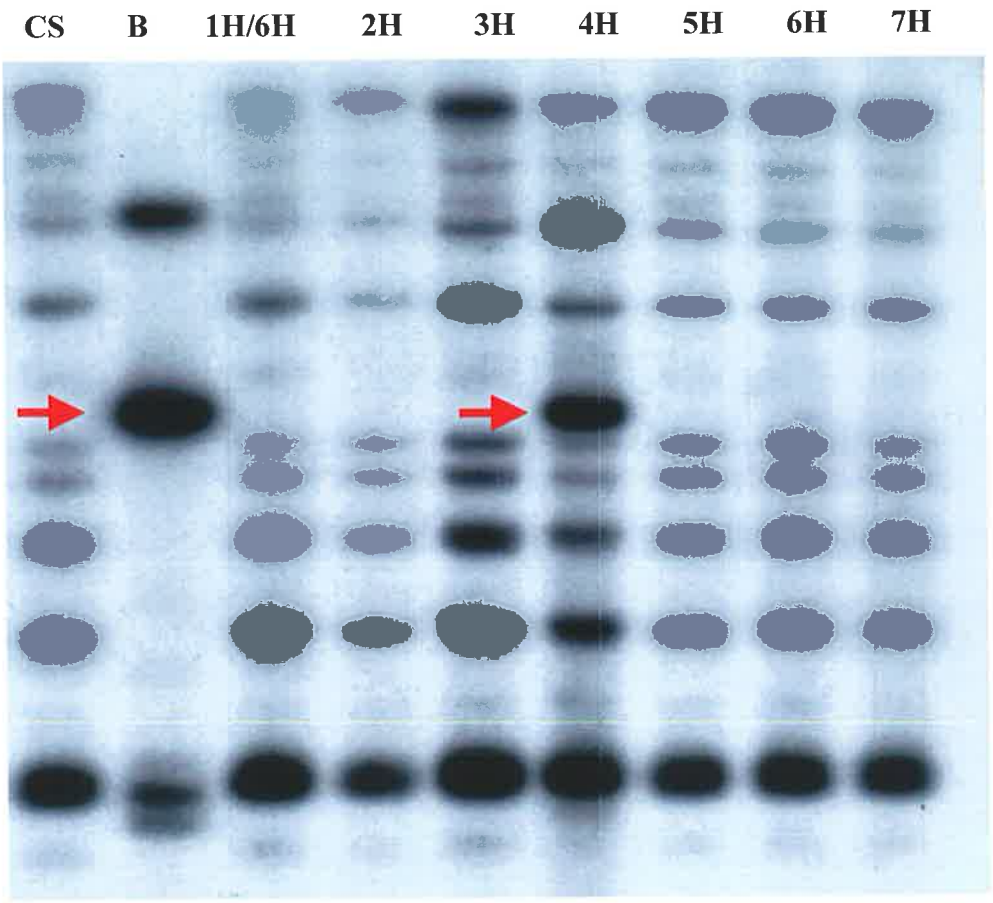


Figure 3.5 Southern hybridisation analysis of wheat-barley addition lines. Genomic DNA from wheat var. Chinese-spring (lane 1), barley var. Betzes (lane 2), and seven Chinese-spring wheat lines carrying chromosome 1H to 7H (lanes 3-9), respectively, was digested with *Hin*dIII, and probed with a *HvGSL1* fragment. The arrow indicates the band unique to barley, which is located on chromosome 4H.

increased. To achieve the latter, both [α^{32}P]-dCTP and [α^{32}P]-dATP were added to the labelling mixture. However, these measures, together with extended hybridisation and exposure times, still failed to produce any hybridising bands. This implied that the *HvGSL1* gene is not transcribed in great abundance in the barley tissues examined.

In order to reveal detectable bands for the Northern blot analysis, the stringency of hybridisation conditions was reduced by using a longer probe, 4.2 kb in length (Type 2 probe, Section 3.3.4.1). However, this would mean that the probe would no longer be specific for *HvGSL1*, but would reveal mRNA from other *HvGSL* genes. The longer probe did indeed produce a single hybridising band in each sample, as shown in Figure 3.6. The RNA samples from different barley tissues all produced a band of approximately 6.2 kb, which is consistent with the size of the *HvGSL1* cDNA. A relatively strong hybridisation signal was observed with RNA samples from early developing grains (3 days after anthesis), florets, coleoptiles and young roots (3 days after gemination), while a weaker signal was detected in stems and leaves. Thus, *HvGSL* mRNA levels are higher in tissues of rapidly dividing or expanding cells, such as young roots and florets, and early developing grains, supporting the hypothesis put forward by Stone and Clarke (1992), that callose deposition was mostly found in these types of tissues. A similar expression pattern was also reported in cotton tissues (Cui *et al.*, 2001). It is worth noting that the transcript size detected in first leaves (lane 4, Figure 3.6) is relatively smaller than that in other tissues, which is probably because an alternative polyadenylation site was used in first leaves during 3' processing of the primary transcript, as discussed in section 2.3.1.

3.3.3.2 RT-PCR Analysis

The more sensitive RT-PCR approach was applied to further examine transcription levels of the *HvGSL1* gene in barley, and to confirm the results obtained from the Northern blot analysis described in 3.3.3.1. Total RNA (2 μg) from the same tissues was used to synthesise first-strand cDNA with an oligo dT (20) primer. The forward primer 5'HvClone1 and reverse primer 3UTR-R were used to specifically amplify a region of the *HvGSL1* transcript. As shown in Figure 3.7, a single fragment of the expected size of 477 bp was amplified from cDNA prepared from all tissues, and its identity with the callose synthase gene was confirmed by direct sequencing of the PCR fragment. This result

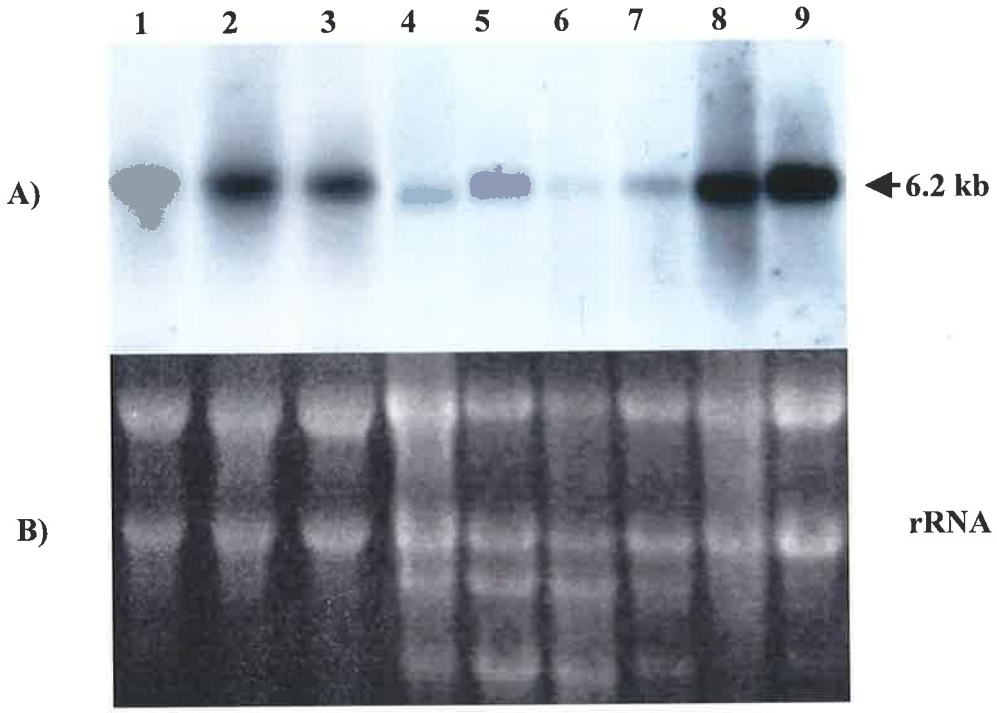


Figure 3.6 Northern blot analysis of RNA from a range of barley tissues. A) RNA blot probed with a 4.2 kb *HvGSL1* probe. **B)** Ethidium bromide-stained RNA gel to demonstrate equal loading as judged by the amount of rRNA in each lane. Lane 1: callus; Lane 2: roots; Lane 3: coleoptiles; Lane 4: first leaves; Lane 5: mature leaves; Lane 6: infected leaves by powdery mildew; Lane 7: stem; Lane 8: florets; and Lane 9: early developing grains (3 days post anthesis).

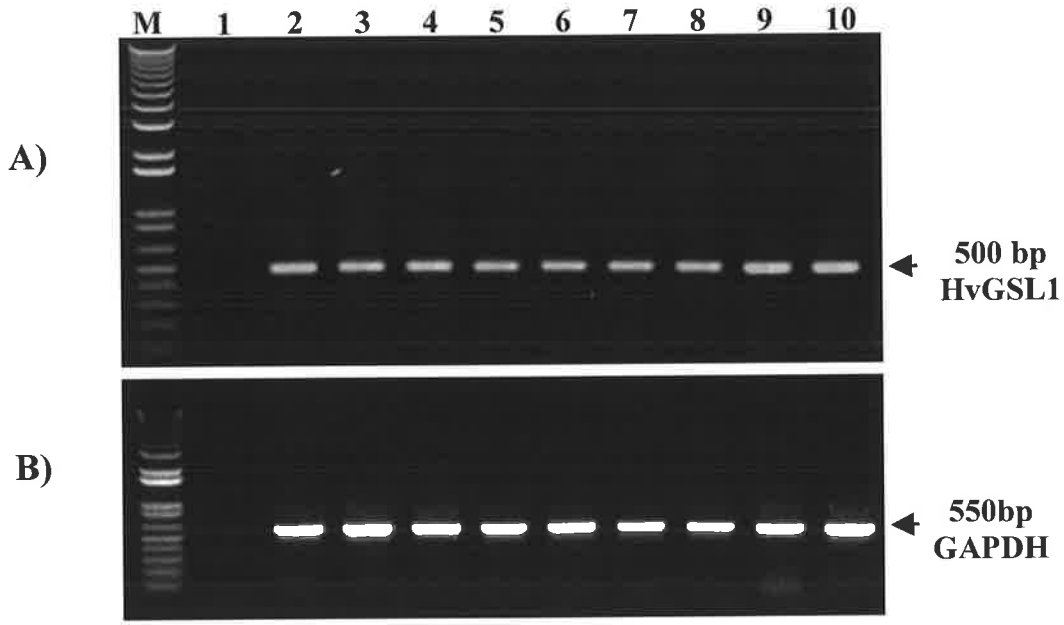


Figure 3.7 RT-PCR analysis of *HvGSL1* gene expression. Total RNA for the RT-PCR reaction was extracted from tissues callus (2); roots (3); coleoptiles (4); first leaves (5); mature leaves (6); leaves infected with powdery mildew (7); stem (8); florets (9); and developing grains (10). A negative control shown in (1) contained primers and the reaction mixture for PCR reaction but no cDNA template was added. M represents DNA molecular weight markers. **A)** Products amplified with primers specific for the barley *HvGSL1* gene. **B)** Products amplified with primers specific for the constitutively expressed GAPDH gene in barley.

indicated the presence of the *HvGSL1* mRNA in all tissues examined. Meanwhile, primers specific for the constitutively expressed barley GAPDH gene yielded a single fragment of 550 bp of similar abundance for all tissues, confirming the integrity of the cDNA synthesised from each tissue.

As discussed previously, the increased sensitivity and specificity for a single gene are two advantages of using RT-PCR, where an mRNA expressed at a very low level can be detected. The RT-PCR method can therefore discriminate directly between the presence or absence of transcript in the tissues and the results from this RT-PCT experiment indicates that mRNA of the *HvGSL1* gene is expressed in all tissues tested here. However, RT-PCR did not provide reliable values for the relative levels of *HvGSL* mRNA in the various tissues.

3.3.3.3 Searching EST Databases for Transcription of HvGSL1 *Genes*

Transcription patterns of *HvGSL1* were further studied using available barley EST databases. A similar search to that described in Section 3.3.1 was performed with the expectation that *HvGSL1*-homologous sequences and their tissue of origin could be identified. Several EST sequences that are homologous to the *HvGSL1* cDNA were indeed found from barley EST libraries and the respective tissues from which the libraries were made included seedling leaves, green leaves, seedling shoots, 20 DAP (days after pollination) spikes, testa/pericarp, etc. The majority of the ESTs were obtained from second leaf stage seedling leaves libraries, with a considerable number in tissues such as heading stage top three leaves and testa/pericarp. ESTs were also evident in libraries made from seedling green leaves infected with or without *Erysiphe graminis*. Moreover, some ESTs were found from seedling shoots grown under dehydration or cold stress, and with etiolation treatment. These data suggest the existence of several barley *HvGSL1* genes, at least six copies, as summarised in Section 3.3.1 and their transcription in many different tissues.

Further investigation of the EST databases among higher plants revealed that over several hundred sequences from 46 plant species show sequence similarity to the *HvGSL1* of barley, *GSL* of *Arabidopsis*, and *CFL1* of cotton. The mRNA of these genes were found

in a broad range of tissue types such as callus, immature seed, shoot, shoot tips, green seedling, leaf, stem, ear tissue and mixed stages of anther and pollen development.

3.3.4 Sequence and Domain Analysis of the HvGSL1 Polypeptide

3.3.4.1 Sequence Analysis and Predicted Topology of the HvGSL1 Polypeptide

A hydropathy analysis of the HvGSL1 protein predicted that the protein has several highly hydrophobic regions, and the topology predicted by the computer program TopPred for the barley HvGSL1 enzyme indicates that there is a 499 amino acid NH_2-terminal region, which is assumed to be oriented towards the cytoplasm, followed by a group of six transmembrane helices. A central region of 618 amino acid residues, again assumed to be cytoplasmic, is located between this group of six transmembrane helices and another group of transmembrane helices, the predicted number of which depends on computing parameters (Figure 3.8). The overall topology predicted for HvGSL1 is similar to that predicted for the yeast FKS1 protein (Figure 3.9), although the overall sequence identity between HvGSL1 and FKS1 is as low as 26%.

Figure 3.10 shows an alignment of amino acid sequences from putative callose synthases from barley, cotton and *Arabidopsis*. Two *Arabidopsis* sequences, AtGSL1 and AtGSL12, were selected to represent the two different classes of *Arabidopsis* callose synthase genes (Section 3.3.1). The sequence alignment reveals that the deduced amino acid sequence of the HvGSL1 displayed sequence identities with CFL1, AtGSL1 and AtGSL12 of 70.5%, 46.5%, and 48%, and sequence similarities of 85%, 68%, and 69%, respectively. These proteins have been classified together as family GT48 glycosyl transferases (Coutinho and Henrissat, 1999). The alignments showed that sequence identity in the NH_2-terminal region and in transmembrane helices was not high, but that much higher identities were present in the central "cytoplasmic" loop of the protein (Figure 3.10). This region has been implicated in substrate binding and catalysis (Delmer, 1999), and high identity values would therefore be expected in this region.

3.3.4.2 Prediction of Secondary Structure and Catalytic Residues

To predict secondary structural elements of the HvGSL1 protein, hydrophobic cluster analysis (HCA) was used in conjunction with several other programs within the META

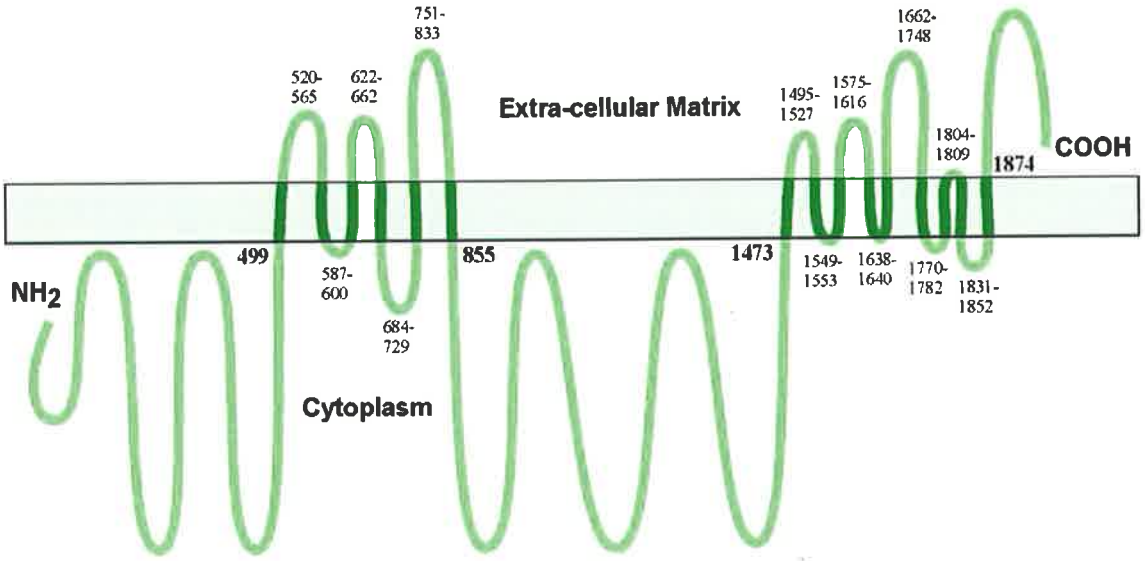


Figure 3.8 Predicted topology of the putative barley callose synthase HvGSL1 protein. The schematic topological profile of the HvGSL1 protein was predicted by the computer program TopPred (Section 3.3.5). The long rectangle represents the membrane. Numbers indicate the amino acid residues at the boundaries of transmembrane helices and cytoplasmic or extracellular domains of the enzyme. Two groups of transmembrane helices are separated by a central segment of the protein. Although the likely extracellular and cytoplasmic orientations are indicated, the computing programs used to generate the topology diagram cannot discriminate between the two possibilities.

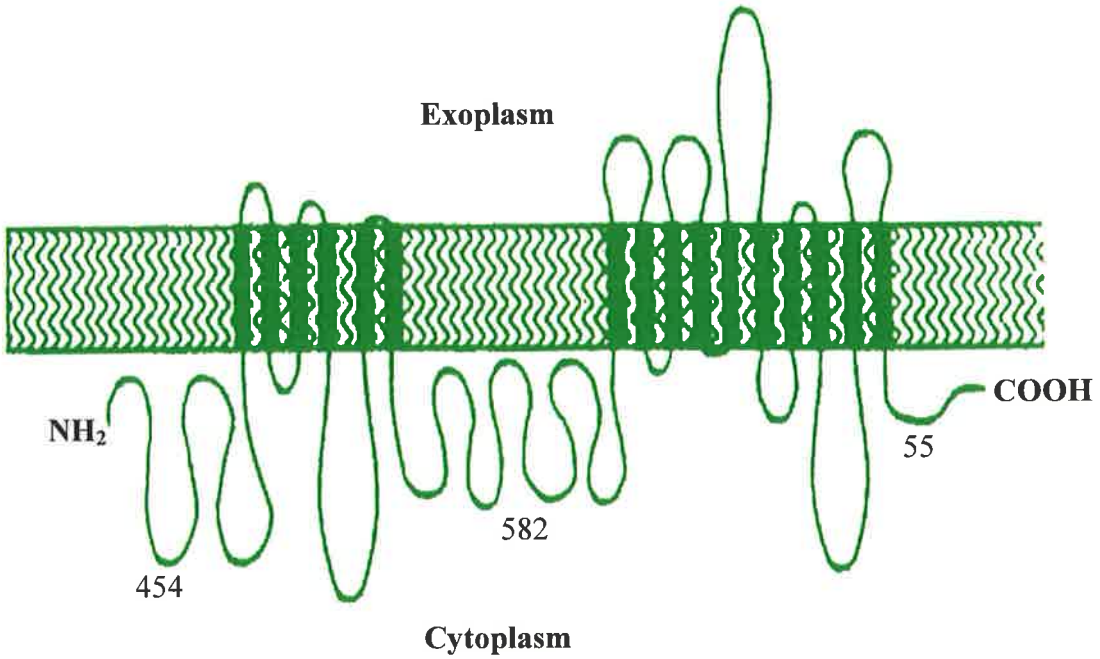
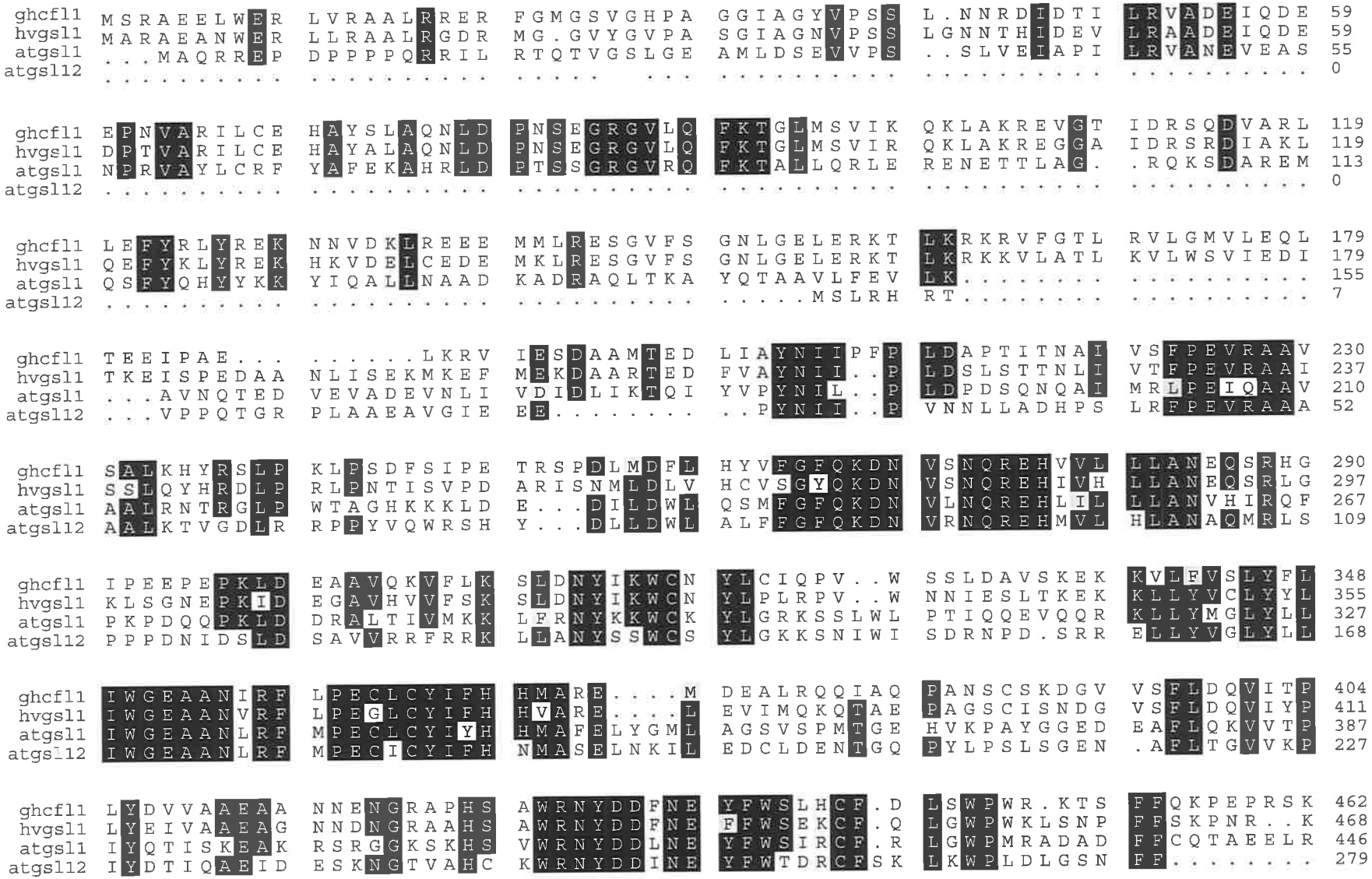


Figure 3.9 Predicted membrane topology of the yeast (1→3)-β-glucan synthase FKS1 protein. The 16 transmembrane helices are presented as vertical solid bars. Three major hydrophilic portions of the proteins are predicted to be cytoplasmic, with a length of 454 amino acid residues at the NH_2-terminus, 582 amino acid residues in the central region, and 55 amino acid residues at the COOH-terminus. The central cytoplasmic segment is implicated in substrate binding and catalysis. Figure adapted from Douglas *et al.* (1994).

Figure 3.10 Protein sequence alignment of putative callose synthases from higher plants. The amino acid sequence deduced from the barley *HvGSL1* was aligned with the sequences encoding putative callose synthases from *Gossypium hirsutum* and *Arabidopsis*, derived from Genbank under the following accession numbers, AF085717 (CFL1), AF237733 (AtGSL1), and AC005142 (AtGSLS12), respectively. AtGSL1 and AtGSL12 were chosen to represent two classes of the synthases in *Arabidopsis*. The alignments were performed using the PILEUP program, and represented graphically using the PRETTYBOX program in the suite of ANGIS programs.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hvgsl1 MARAEANWER LLRAALRGDR MG.GVYGVPA SGIAGNVPSS LGNNTHIDEV LRAADEIQDE 59
atgsl1 ...MAQRREP DPPPPQRRIL RTQTVGSLGE AMLDSEVVPS ..SLVEIAPI LRVANEVEAS 55
atgsl12 0
ghcfl1 EPNVARILCE HAYSLAQNLD PNSEGRGVLQ FKTGLMSVIK QKLAKREVGT IDRSQDVARL 119
hvgsl1 DPTVARILCE HAYALAQNLD PNSEGRGVLQ FKTGLMSVIR QKLAKREGGA IDRSRDIAKL 119
atgsl1 NPRVAYLCRF YAFEKAHRLD PTSSGRGVRQ FKTALLQRLE RENETTLAG. .RQKSDAREM 113
atgsl12 0
ghcfl1 LEFYRLYREK NNVDKLREEE MMLRESGVFS GNLGELERKT LKRKRVFGTL RVLGMVLEQL 179
hvgsl1 QEFYKLYREK HKVDELCEDE MKLRESGVFS GNLGELERKT LKRKKVLATL KVLWSVIEDI 179
atgsl1 QSFYQHYYKK YIQALLNAAD KADRAQLTKA YQTAAVLFEV LK........ 155
atgsl12 ....MSLRH RT........ 7
ghcfl1 TEEIPAE... ......LKRV IESDAAMTED LIAYNIIPFP LDAPTITNAI VSFPEVRAAV 230
hvgsl1 TKEISPEDAA NLISEKMKEF MEKDAARTED FVAYNII..P LDSLSTTNLI VTFPEVRAAI 237
atgsl1 ...AVNQTED VEVADEVNLI VDIDLIKTQI YVPYNIL..P LDPDSQNQAI MRLPEIQAAV 210
atgsl12 ...VPPQTGR PLAAEAVGIE EE........ ..PYNII..P VNNLLADHPS LRFPEVRAAA 52
ghcfl1 SALKHYRSLP KLPSDFSIPE TRSPDLMDFL HYVFGFQKDN VSNQREHVVL LLANEQSRHG 290
hvgsl1 SSLQYHRDLP RLPNTISVPD ARISNMLDLV HCVSGYQKDN VSNQREHIVH LLANEQSRLG 297
atgsl1 AALRNTRGLP WTAGHKKKLD E...DILDWL QSMFGFQKDN VLNQREHLIL LLANVHIRQF 267
atgsl12 AALKTVGDLR RPPYVQWRSH Y...DLLDWL ALFFGFQKDN VRNQREHMVL HLANAQMRLS 109
ghcfl1 IPEEPEPKLD EAAVQKVFLK SLDNYIKWCN YLCIQPV..W SSLDAVSKEK KVLFVSLYFL 348
hvgsl1 KLSGNEPKID EGAVHVVFSK SLDNYIKWCN YLPLRPV..W NNIESLTKEK KLLYVCLYYL 355
atgsl1 PKPDQQPKLD DRALTIVMKK LFRNYKKWCK YLGRKSSLWL PTIQQEVQQR KLLYMGLYLL 327
atgsl12 PPPDNIDSLD SAVVRRFRRK LLANYSSWCS YLGKKSNIWI SDRNPD.SRR ELLYVGLYLL 168
ghcfl1 IWGEAANIRF LPECLCYIFH HMARE....M DEALRQQIAQ PANSCSKDGV VSFLDQVITP 404
hvgsl1 IWGEAANVRF LPEGLCYIFH HVARE....L EVIMQKQTAE PAGSCISNDG VSFLDQVIYP 411
atgsl1 IWGEAANLRF MPECLCYIYH HMAFELYGML AGSVSPMTGE HVKPAYGGED EAFLQKVVTP 387
atgsl12 IWGEAANLRF MPECICYIFH NMASELNKIL EDCLDENTGQ PYLPSLSGEN .AFLTGVVKP 227
ghcfl1 LYDVVAAEAA NNENGRAPHS AWRNYDDFNE YFWSLHCF.D LSWPWR.KTS FFQKPEPRSK 462
hvgsl1 LYEIVAAEAG NNDNGRAAHS AWRNYDDFNE FFWSEKCF.Q LGWPWKLSNP FFSKPNR..K 468
atgsl1 IYQTISKEAK RSRGGKSKHS VWRNYDDLNE YFWSIRCF.R LGWPMRADAD FFCQTAEELR 446
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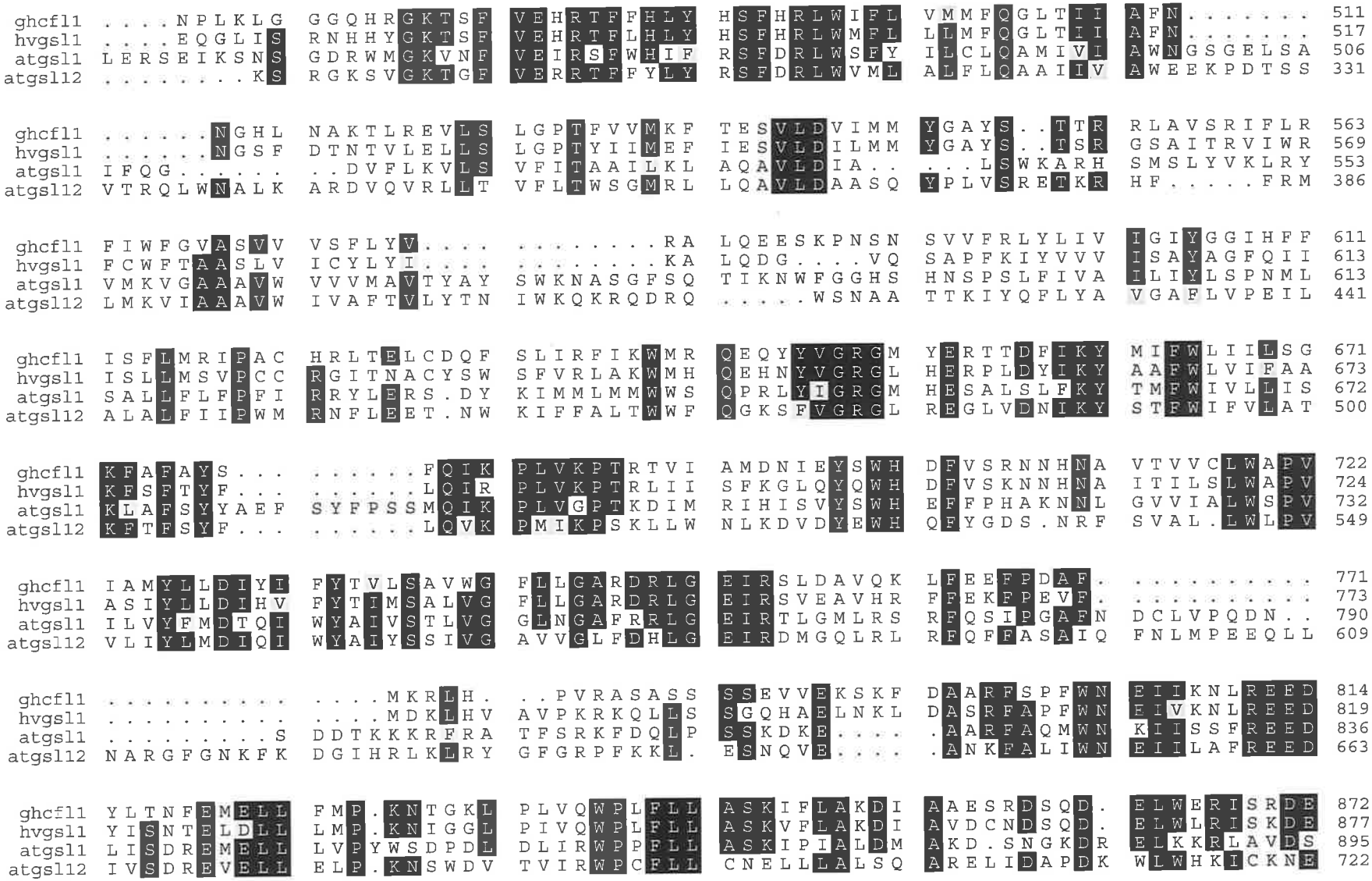
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hvgsl1 . . . . E Q G L I S R N H H Y G K T S F V E H R T F L H L Y H S F H R L W M F L L L M F Q G L T I I A F N 517
atgsl1 L E R S E I K S N S G D R W M G K V N F V E I R S F W H I F R S F D R L W S F Y I L C L Q A M I V I A W N G S G E L S A 506
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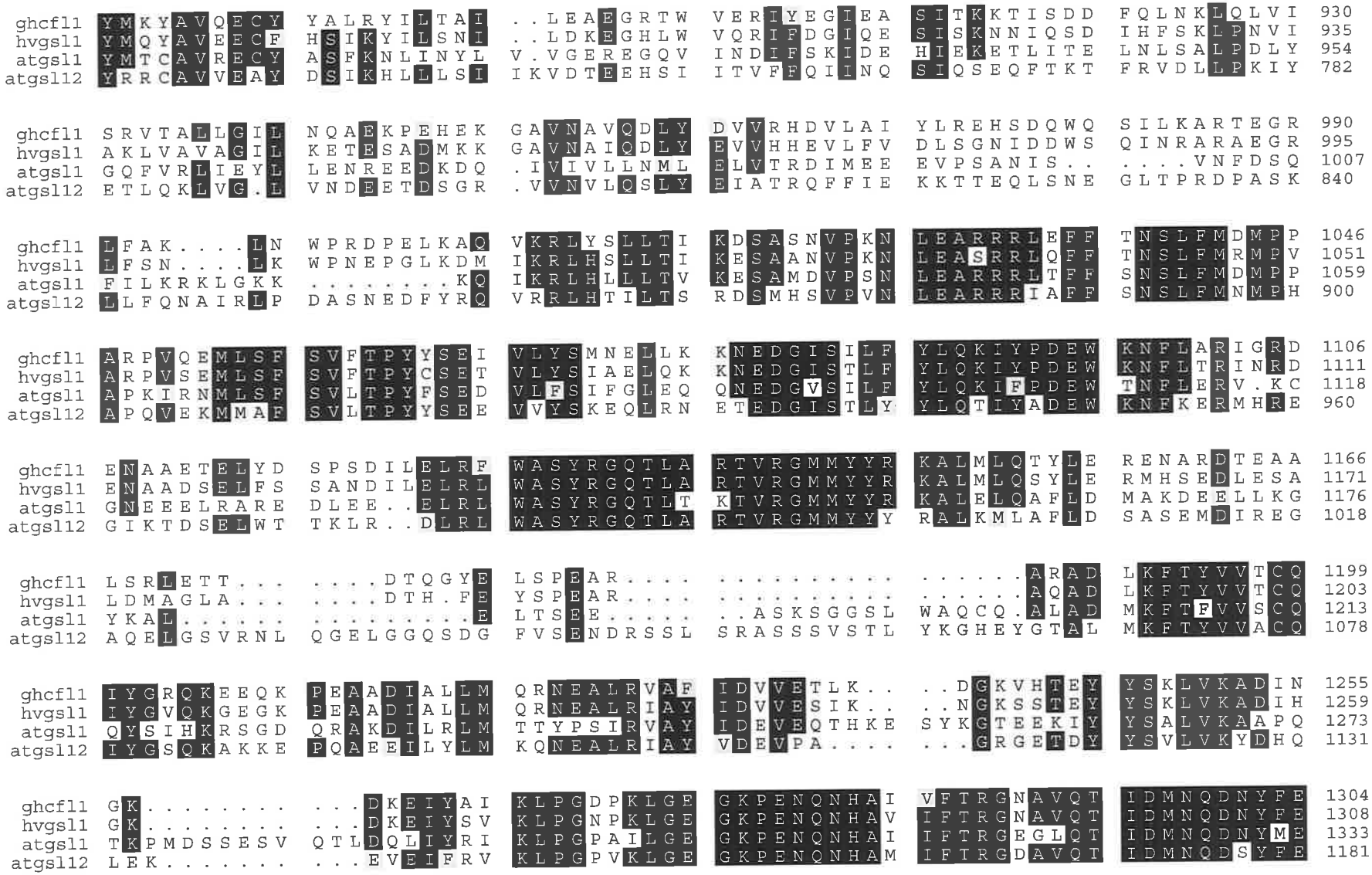
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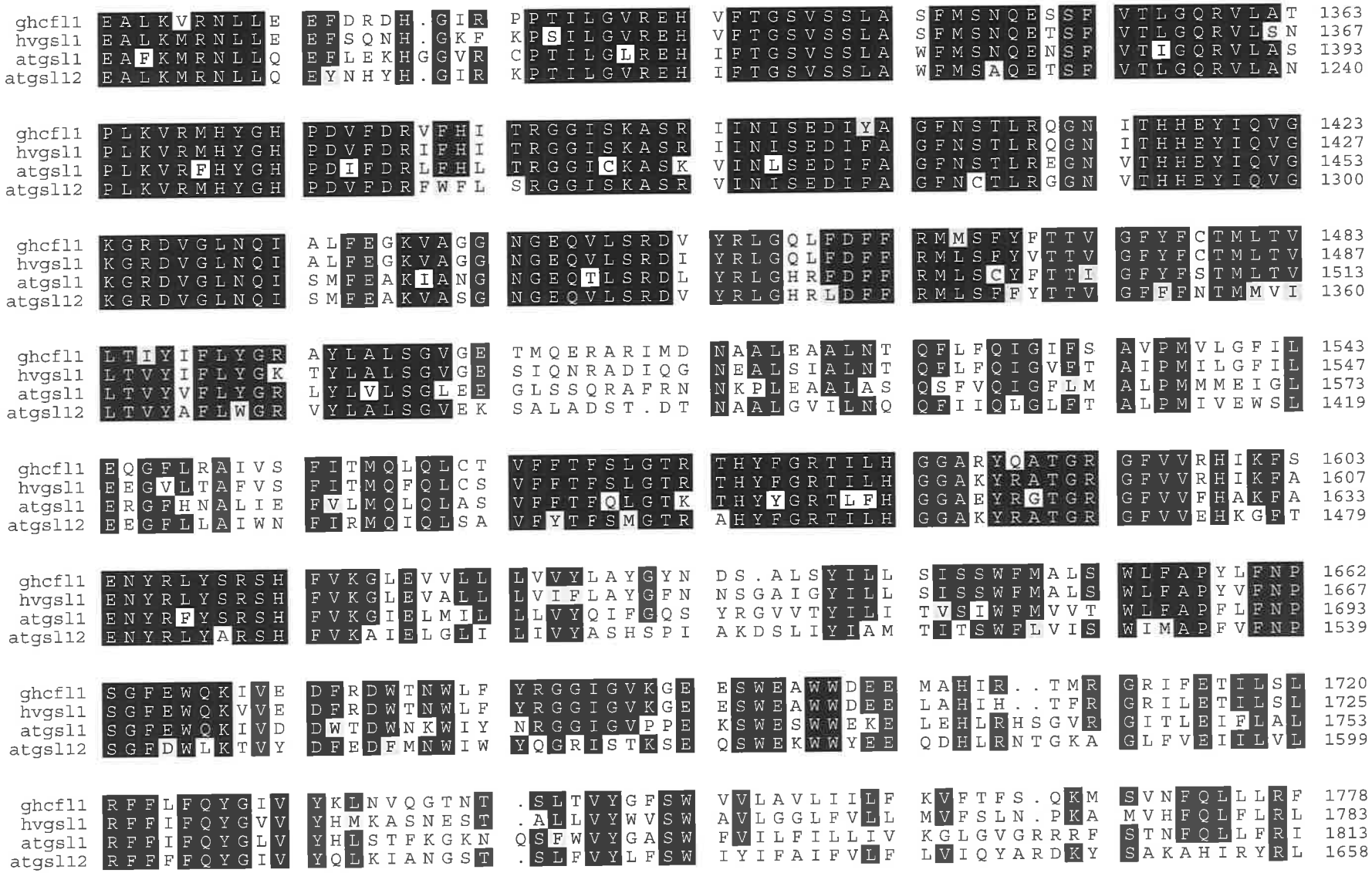
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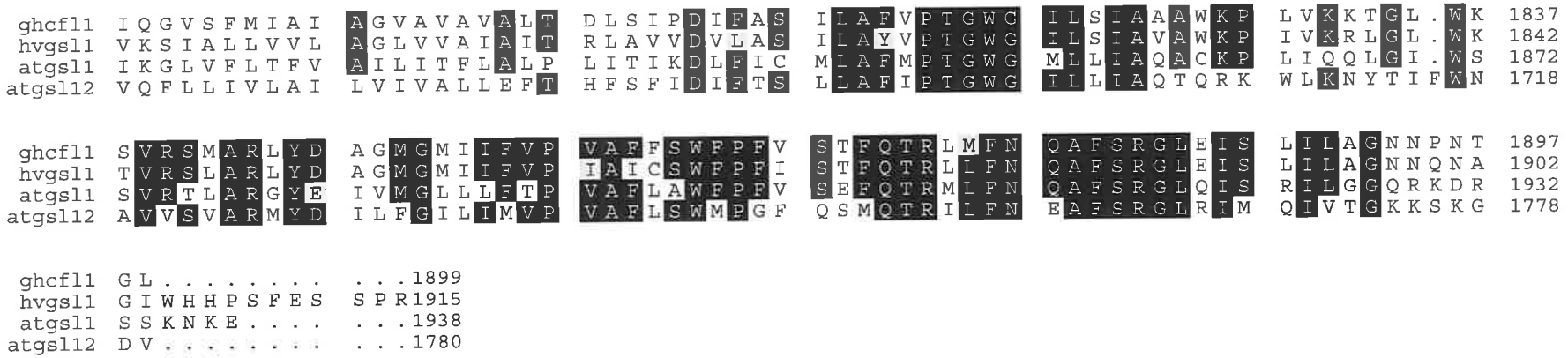
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ghcfl1 GL.....
hvgsl1 GIWHHPSFES
atgsl1 SSKNKE....
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server (Koretke *et al.*, 1999). Dr. Maria Hrmova performed this work. In each case, the HCA assignments of secondary structural elements agreed with predictions from the various algorithms within the META server (Bujnicki *et al.*, 2001). HCA plots generated from the barley family GT48 callose synthase, a family GT2 nucleotide-diphospho-sugar transferase from *Bacillus subtilis* (Charnock and Davies, 1999) and a family GT28 glycosyl transferase from *E. coli* (Ha *et al.*, 2000) were used to compare secondary structural elements in the active sites of these proteins (Figure 3.11). The family GT2 nucleotide-diphospho-sugar transferase from *Bacillus subtilis* is representative of the GT-A group of protein folds of glycosyl transferases and the family GT28 glycosyl transferase from *E. coli* is representative of the GT-B group of protein folds in glycosyl transferases (Bourne and Henrissat, 2001).

In the family GT2 nucleotide-diphospho-sugar transferase (GT-A fold), highly conserved MDDN, TDDN, IDH and GDA motifs are positioned on sheet, sheet, loop and helix structural elements, respectively (Figure 3.11; Charnock and Davies, 1999). In the family GT28 glycosyl transferase (GT-B fold), three glycine-rich motifs GGxGG, GxGG and GGxxGG motifs and an SEI motif, are recognised on loop, loop, loop and helix structural elements, respectively (Figure 3.11; Ha *et al.*, 2000).

In a region predicted to correspond to the active site of the putative callose synthase from barley, absolutely conserved motifs, including SET, DEW, PGxPxxGxGKP, IDxNQDxxxEE and SED, are positioned on loop, helix, loop, helix/loop/helix, and loop structural elements, respectively (Figure 3.11). It is noteworthy that the multiple glycine-rich motifs of the family GT28 enzymes are not present in the barley sequence. In summary, it can be concluded that the barley enzyme is more closely related to the family GT2 enzyme, and is therefore more likely to adopt a GT-A-like fold.

Amino acid sequence alignment, HCA and hydrophobicity plots confirmed that the barley HvGSL1 protein is a family GT48 glycosyl transferase (M. Hrmova, personal communication). The absence of solved three-dimensional structures for any family GT48 enzymes means that the identification of catalytic amino acid residues and donor- and acceptor-binding sites will not be straightforward. So far, only two three-dimensional structural types have been identified for glycosyl transferases, and these have been designated folds GT-A and GT-B (Bourne and Henrissat, 2001). In phylogenetic

Figure 3.11 Prediction of putative active site of barley (1→3)-β-D-glucan synthase. **Panel a.** Putative active site region of the barley (1→3)-β-D-glucan synthase, showing the S/T-rich, possible carbohydrate-binding regions (pink), the P/G-rich, possible linker region (boxed), and the absolutely conserved amino acid residues likely to be involved in UDPGlc- and acceptor-binding, and catalysis (highlighted yellow). **Panel b.** HCA plots of putative active sites of barley (1→3)-β-D-glucan synthase (family GT48), and the known active sites of glycosyl transferases of families GT2 and GT28. Hydrophobic clusters are shown in the vicinity of amino acid residues that are involved in binding of donors and acceptors, or in coordination of divalent cations. The two major folds found in glycosyl transferases (GT-A and GT-B) are represented by the *Bacillus subtilis* SpsA enzyme of family GT2 and an *E. coli* glycosyl transferase of family GT28, respectively. The colour-coded regions in the sequences represent the absolutely conserved regions in the glycosyl transferases. Numbers in the HCA plots indicate the amino acids of the mature enzymes (Figure 2.10). Standard *one-letter codes* for amino acid residues are used except symbol ★ is used for Pro, ♦ for Gly, or Ser and □ for Thr. This figure and the data were generated by Dr. Maria Hrmova.

a

GT48 (1,3)-β-D-Glucan synthase (residues 1056-1406; putative active site)

```
                1060        1070        1080        1090        1100        1110
1056 SEMLSFSVFTPYCSETVLYSIAELQKKNEDGISTLFYLQKIYPDEWKNFLTRINRDENAA
1116 DSELFSSANDILELRLWASYRGQTLARTVRGMMYYRKALMLQSYLERMHSEDLESALDMA
1176 GLADTHFEYSPEARAQADLKFTYVVTCQIYGVQKGEGKPEAADIALLMQRNEALRIAYID
1256 VVESIKNGKSSTEYYSKLVKADIHGKDKEIYSVKLPGNPKLGEGKPENQNHAVIFTRGNA
1296 VQTIDMNQDNYFEEALKMRNLLEEFSQNHGKFKPSILGVREHVFTGSVSSLASFMSNQET
1356 SEVTLGQRVLSNPLKVRMHYGHPDVFDRIFHITRGGISKASRIINISEDIF
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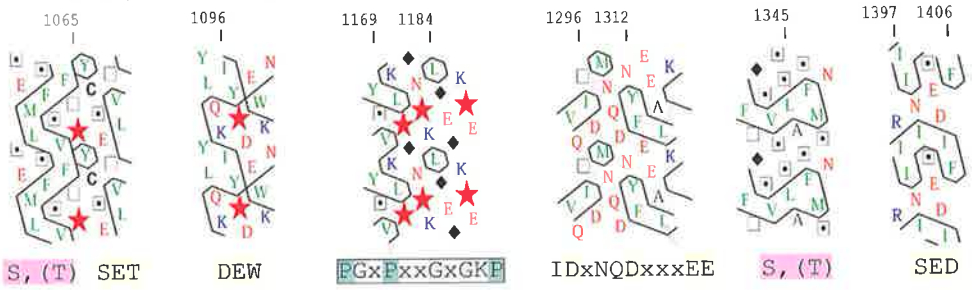
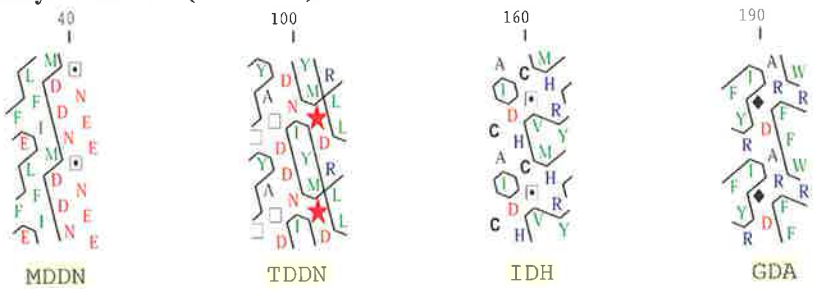
S,(T) rich regions
P, G rich region
Regions with conserved D or E

b

GT48 (1,3)-β-D-Glucan synthase



GT2 Glycosyltransferase (GT-A fold)



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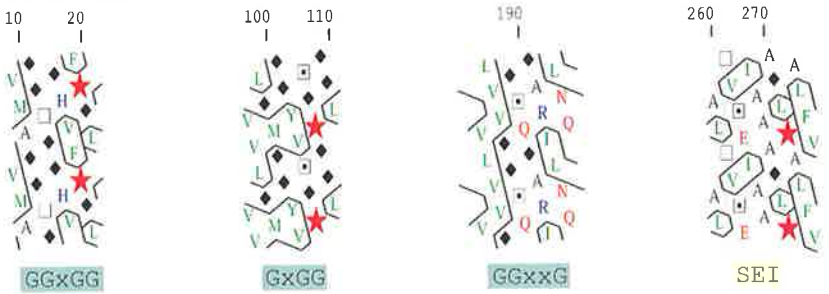


analyses of amino acid sequence alignments, the barley HvGSL1 sequence groups with the GT2, GT7 and GT43 clan of families, which have GT-A type folds (Charnock and Davies, 1999; Bourne and Henrissat, 2001; Tarbouriech *et al.*, 2001). The availability of a GT-A-type three-dimensional structure for a family GT2 glycosyl transferase, namely the SpsA enzyme from *Bacillus subtilis*, has enabled the structural details of nucleotide-diphospho-sugar binding in this and related glycosyl transferase families to be defined (Tarbouriech *et al.*, 2001; Charnock *et al.*, 2001). Three Asp residues in the NH_2-terminal region of the family GT2 enzymes are involved in coordination of the divalent metal cation/nucleotide-diphospho-sugar substrate, while a fourth Asp residue in the COOH-terminal region acts as the catalytic base. The acceptor molecule is bound on the COOH-terminal region of the enzyme and there is only one catalytic reaction centre per enzyme molecule (Tarbouriech *et al.*, 2001).

Our analysis further suggested that the catalytic region of the barley putative (1→3)–β-glucan synthase is likely to be located between amino acid residues 1056 and 1406 (Figure 3.11). Within this 350 amino acid region, two distinct 'domains' are separated by a potential linker of 13 amino acid residues that is rich in Pro and Gly residues. Two potential carbohydrate-binding motifs, which are 13 and 19 residues long, rich in Ser/Thr and aromatic residues, and low in charged residues, are observed at the NH_2- and COOH-terminal ends of the putative catalytic section of 350 amino acid residues (Figure 3.11).

No UDPGlc binding motifs of the type found in family GT2 cellulose synthases, in particular D,D,D,QXXRW (Pear *et al.*, 1996; Saxena *et al.*, 2001), are identifiable in callose synthases. However, absolutely conserved DE and EE dipeptides in the central region of the putative catalytic section are candidates for the coordination of the UDPGlc/metal ion complex, and additional absolutely conserved, single Glu or Asp residues elsewhere in the region are candidates for the catalytic base (Figure 3.11; Tarbouriech *et al.*, 2001). An RATG motif, which has been implicated in UDPGlc binding (Inoue *et al.*, 1996), is found in a small loop between transmembrane helices 11 and 12, but it should be noted that this is predicted to be on the opposite side of the membrane from the putative catalytic region (Figure 3.8). A similar motif is found in other higher plant callose synthases (Østergaard *et al.*, 2002).

In summary, we were able to identify absolutely conserved SET, PDEW, IDxNQDxxxEE and SED motifs clustered in all family GT48, putative (1→3)-β-D-glucan synthases, of both higher plant and fungal origin, and it is proposed that these are involved, in part at least, in substrate binding and/or catalysis.

3.4 Summary and Conclusions

The genetic, transcriptional and sequence characteristics of the barley putative (1→3)-β-glucan synthase gene *HvGSL1* have been characterised using conventional methodologies such as Southern analysis, Northern analysis, RT-PCR, chromosomal mapping, and sequence analysis of the *HvGSL1* cDNA.

Southern blotting analysis using a partial *HvGSL1* cDNA as a probe, together with the grouping of barley EST sequences that are homologous to the yeast (1→3)-β-glucan synthase gene *FKS1* (Douglas *et al.*, 1994), suggested that the *HvGSL* family may have at least six members in the barley genome. One *HvGSL* gene was mapped to the long arm of chromosome 4H and this result was confirmed by the use of the wheat-barley addition lines. Northern blots and RT-PCR analysis of transcription patterns of the *HvGSL* gene indicated that the *HvGSL* genes are highly transcribed in barley florets, and early developing grains. The transcription levels of mRNA were lower in coleoptiles and roots, while the genes are transcribed in all other tissues examined at low levels, in agreement with the literature (Stone and Clarke, 1992; Cui *et al.*, 2001; Hong *et al.*, 2001). Searching EST databases revealed that callose synthase-like genes are members of multi-gene families and are transcribed in a wide range of tissues from over 28 plant species, supporting the concept that different genes might be responsible for tissue-specific callose depositions.

Sequence analysis of the HvGSL1 protein indicated that it is a membrane-bound protein, which has low sequence homology to the yeast (1→3)-β-glucan synthase, but is strikingly similar to the predicted topology of the yeast synthase. Sequence comparisons between higher plant genes revealed that all GSLs showed significant sequence identity, which is much more pronounced in the central domain of the enzymes, for which a crucial catalytic role has been suggested. The active site of the enzyme, which is likely to include absolutely conserved SET, PDEW, IDxNQDxxxEE and SED motifs, has been predicted by a combination of multiple sequence alignment, hydrophobicity plots, hydrophobic cluster analysis and secondary structural element prediction.

In the following chapters, experiments aimed at more precisely defining the functions of the barley *HvGSL* genes are described, given that there remains some debate as to whether or not the genes participate in callose biosynthesis (Dijkgraaf *et al.*, 2002).

CHAPTER FOUR:

HETEROLOGOUS EXPRESSION AND ANTIBODY PRODUCTION AND PURIFICATION

4.1 Introduction

In the previous two chapters, the genetic evidence obtained has suggested that the barley *HvGSL1* is homologous to the yeast (1→3)-β-glucan synthase gene. Its transcriptional patterns coincide with sites of callose deposition, although the functions of the *HvGSL1* product have yet to be demonstrated. A key objective of the work described in this thesis was to link the sequence of the putative callose synthase gene with the protein responsible for (1→3)-β-glucan synthase activity. This required the generation of antibodies to expressed fragments of the putative callose synthase cDNA, so that protein corresponding to *HvGSL1* could be detected during purification of the enzyme. This, in turn, would be enable the determination of amino acid sequence from preparations of purified and active (1→3)-β-glucan synthase and reconciliation of that amino acid sequence with the cDNA sequence. Thus, in this Chapter an attempt to express fragments of the *HvGSL1* cDNA for subsequent antibody production are described. The partial purification of callose synthase from barley for amino acid analysis will be presented in the next Chapter.

To perform the experiments proposed, several heterologous hosts for the expression of barley *HvGSL1* cDNA fragments were evaluated. These included bacterial cells, fungal cells and insect cells. The advantages and disadvantages of these expression systems are briefly discussed below.

Yeast is a eukaryote that represents an ideal system to express eukaryotic proteins. With well-defined molecular genetics, this organism is known to have the necessary "machinery" to mediate correct folding and post-translational modifications of expressed proteins (Romanos *et al.*, 1992; Giga-Hama and Kumagai, 1999). In spite of its advantages, the expression process can be hampered by the need for maintenance of cell cultures and the manipulation of expression constructs. The transformation efficiency is often low, and this results in low yields of the expressed protein. In addition, the mechanism of protein expression in yeast is not exactly the same as that of higher eukaryotes. Errors in post-translational modifications of the expressed proteins can occur. For example, the expressed proteins may be glycoslated with different sugars. Likewise, acetylation and isoprenylation of proteins at an incorrect position is common (Giga-Hama and Kumagai, 1999). Such features can have a significant impact on the biological functions of the expressed proteins.

The baculovirus insect cell expression system is another option for the expression of eukaryotic proteins (Doan and Fincher, 1992; Grabherr *et al.,* 2001). Compared with the yeast system, the baculovirus system has proven to be more efficient in terms of producing biologically active eukaryotic proteins, and has been used in a range of applications. Several hundred animal, plant and human proteins that require folding, subunit assembly and extensive post-translational modification have been successfully expressed in the insect cell/baculovirus system (Miller *et al.,* 1989; Grabherr *et al.,* 2001). The most widely used method for gene insertion is via homologous recombination, utilising infection of cultured *Spodoptera frugiperda* cells with the baculovirus *Autographa califonica* (AcMNPV). A plasmid containing the gene of interest, flanked by sequences homologous to the baculovirus genome, is co-infected with viral DNA into the insect cells. Virus-encoded envelope glycoprotein (gp64), as well as gp64-fusion proteins containing a target protein, are expressed during infection and transported to the cell surface, where they can be detected on the surface of virally infected insect cells. However, the insect cell expression system can be technically demanding and time-consuming. In addition, it runs the risk of diminishing or even losing viral infectivity (Grabherr *et al.,* 2001), and changes or addition of amino acids to an essential viral protein have been observed. Furthermore, the yield of the recombinant protein is sometimes very low.

Escherichia coli, a prokaryotic organism, represents the simplest and most popular host cell for heterologous expression (Gold, 1990; Kruszewska, 1999). Compared with the insect and yeast cell systems, the *E. coli* system has many advantages, including the speed and adaptability, its simple and rapid replication, and ease of transformation. Another advantage is that a wide range of vectors, designed to meet different requirements of expression, are commercially available. The major limitations of the system are that bacteria cells do not possess the necessary "machinery" to process expressed eukaryotic proteins, or to meet the requirements of protein secretion and post-translational modification. Over-expression in *E. coli* cells often leads to the production of insoluble inclusion bodies (Kopito, 2000), which contain the recombinant protein in insoluble, inactive forms. In some cases, inclusion body formation can be significantly reduced by optimising the experimental conditions (Schein and Noteborn, 1988; Moore *et al.,* 1993; The QIAexpressionist literature, QIAGEN). In the worst cases, there are means to refold

denatured proteins to their active state (Brown *et al.,* 1993; Friedhoff *et al.,* 1994; Pear *et al.,* 1996).

With these advantages and disadvantages in mind, the *E. coli* QIAexpression system was finally chosen as a preferred host system for the present study. The decision was based on the fact that *E. coli* is a very well characterised system which is easy to manipulate, and therefore is efficient in terms of time and cost. In addition, there are examples where active recombinant barley proteins have been successfully generated using this system (Liu *et al.*, 1993; Stewart *et al.*, 1999; S. Rutten, personal communication).

The QIAexpression system offers high-level protein expression in *E. coli*, because it features an optimised, regulable T5 promoter and two Lac operator sequences, an NH_2-terminal poly-histidine-tag and a multiple-cloning site with translation stop codons in all frames. The expression of the target protein is tightly regulated by inclusion of the lac repressor in the vector and can be rapidly induced by the addition of isopropylthio-β-D-galactoside (IPTG), which inactivates the repressor and clears the promoter. The presence of the poly-histidine tag on the heterologously-expressed protein has an added advantage for subsequent purification of the protein. Employing a novel metal chelate adsorbent Ni-NTA (Nickel-nitro-tri-acetic-acid) resin, the expressed protein can be readily purified through a simple affinity purification method (Hochuli *et al.,* 1987).

In this Chapter, the heterologous expression of a fragment of barley *HvGSL1* cDNA in *E. coli* is described. The production of polyclonal antibodies using the recombinant protein as an antigen, together with the affinity purification of the active antibodies, are also presented.

4.2 Materials and Methods

4.2.1 Materials

Fragments of a near full length cDNA encoding putative callose synthase *HvGSL1* isolated from *Hordeum vulgare L.* (Chapter 2) were used for the generation of an expression construct. The QIAexpressionist™ expression and purification system, including vector pQE30, *E. coli* strain M15[pREP4], Ni-NTA Spin Columns, and Ni-NTA Superflow were acquired from QIAGEN GmbH (Hilden, Germany). Nitrocellulose filters were obtained from Amersham Pharmacia (NJ, USA). The Anti-rabbit IgG conjugated to alkaline phosphatase, alkaline phosphatase substrate buffer containing nitroblue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (BCIP) were from Bio-RAD (Hercules, CA, USA). The low molecular weight protein markers, Protein A-Sepharose CL-4B, the HiTrap NHS-activated column, and the ECL-plus fluorescent detection system were purchased from Amersham Phamacia (NJ, USA). The PR648 slot blotter was from Hoefer Scientific Instruments (San Francisco, CA, USA). The STORM 860 optical scanner was supplied by Molecular Dynamics (Sunnyvale, CA, USA).

4.2.2 Construction of the Expression Vector

4.2.2.1 Choosing a Fragment of the HvGSL1 *cDNA for Expression*

Based on the *HvGSL1* cDNA sequence, a topological profile of the putative barley callose synthase was predicted (Section 3.3.5). Non-transmembrane regions were primarily targeted for protein expression, to avoid the presence of hydrophobic regions which could cause a possible toxic effect on the host cells (Miroux and Walker, 1996). In addition, a multisequence alignment of the callose synthases among higher plants was generated by the multiple sequence analysis programs from the Australian National Genomic Information Service (Section 3.3.5). A coding fragment from a non-transmembrane region of the *HvGSL1* cDNA that contained a highly conserved sequence (Figure 4.1, and Figure 3.10: Section 3.3.4.1) was chosen for protein expression in *E. coli.* Expression of the possible catalytic region of the protein was proposed, with the expectation that the resulting recombinant protein could be used for UDP-glucose binding assays, if it were biologically active. In addition, if the recombinant protein were not active, the protein could still be used as an antigen for raising antibodies. Such antibodies would be

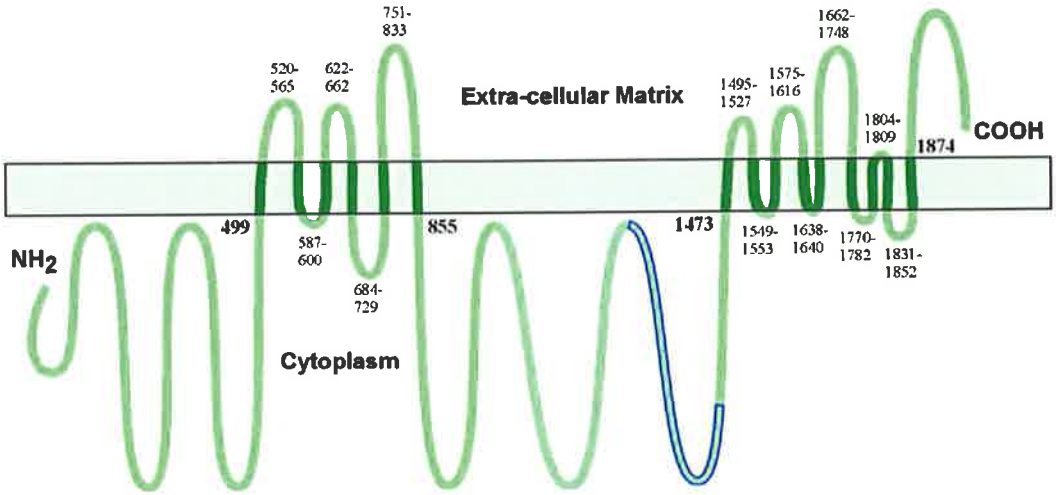


Figure 4.1 The position of a fragment chosen for protein expression on the predicted topology of the HvGSL1. A coding fragment from a non-transmembrane region of the *HvGSL1* cDNA highlighted in blue was chosen for expression. This region showed sequence identity greater than 95% at the amino acid level among the putative plant callose synthases CFL1, AtGSL1 and AtGSL12 (Figure 3.10).

expected to recognise not only the antigen from the barley extracts, but also callose synthases from other plant sources, because they were generated against a conserved region of the protein.

4.2.2.2 *Preparation of the* HvGSL1 *cDNA Fragment*

All DNA manipulations were performed in accordance with the standard conditions described in Chapters 2 and 3, unless otherwise specified. The coding sequence from amino acids 1280 (**K**PEN…) to 1478 (…TTV**G**) (Figure 2.10) of the *HvGSL1* cDNA was used as a template and amplified by PCR with Primer 1 and Primer 2 as follows:

Primer 1-forward 5' CGG GGA TCC AAA CCC GAA AAT CAA AAC CA 3'
*Bam*HI Coding Region…

Primer 2-reverse 5' GGC AAG CTT ACC CAA CAG TAG TCA CAT AGA 3'
*Hin*dIII Coding region…

These two oligonucleotide primers were designed to set the correct reading frame, with inclusion of the *Bam*HI site in the upper primer and *Hin*dIII in the lower primer. Additional bases overhanging at both ends were also added for convenient cloning. The end of the coding fragment was modified by PCR with the addition of a stop codon TAA, to minimise the number of extra codons beyond these codons presented in the putative callose synthase. This was achieved by designing the reverse primer (primer 2) containing TTA, which is complementary to TAA. Figure 4.2 shows a section of the multiple-cloning site region of the expression vector. The PCR was carried out on a Gene Amp PCR system from Perkin Elmer using 2.5 U Taq DNA polymerase, 1×PCR buffer (10×buffer: 100 mM Tris-HCl buffer, pH 8.3 and 500mM KCl), 1.5 mM $MgCl_2$, 200 µM each dNTP and 100 µM each primer. The PCR profile had an initial denaturing step at 94°C for 1 min, followed by 34 cycles of 94°C, 1 min; 55°C, 1min; 72°C, 1min; with a final 8 min extension time at 72°C. The amplified fragment was Gene-cleaned, digested with *Bam*HI and *Hin*dIII at 37°C for 4 h, and further purified by phenol-chloroform extraction and ethanol precipitation before ligating into the expression vector.

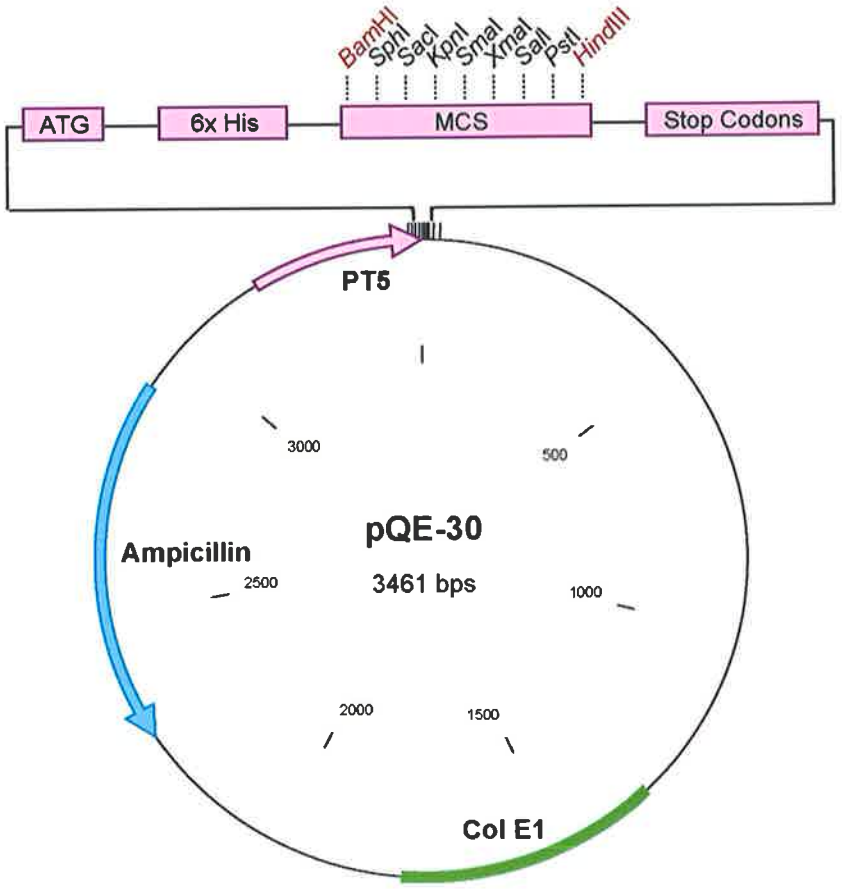


Figure 4.2 Schematic diagram of a pQE-30 expression vector. The vector features a multiple-cloning site (MCS) with translation stop codons in all frames, an optimised T5 promoter and two Lac operator sequences, and an NH_2-terminal polyhistidine-tag. *Bam*HI and *Hin*dIII sites were chosen for insertion of the cDNA. Expression can be rapidly induced with the addition of IPTG. The expressed protein can be purified by one-step affinity purification with Ni-NTA resin. Figure adapted and modified from QIAexpressionist™ expression manual.

4.2.2.3 Ligation of Insert into the pQE-30 Expression Vector

To linearise the pQE-30 expression vector, the plasmid was digested with *Bam*HI and *Hin*dIII restriction enzymes at 37°C for 3 h, separated on 1% agarose gel electrophoresis, and purified with the Gene-clean kit. To prevent self-ligation, 2 μg vector DNA was dephosphorylated with 10 U phosphatase (CIP) in NEB buffer 3 (50 mM Tris-HCl buffer, pH 7.9, containing 100 mM NaCl, 10 mM $MgCl_2$ and 1 mM dithiothreitol) in a final volume of 50 μl, at 37°C for 1 h. The reaction was stopped by heat-inactivation at 75°C for 10 min in the presence of 5 mM EDTA. The DNA was subjected to further purification with Gene-clean. For ligation, the dephosphorylated vector DNA and insert DNA were mixed at a molar ratio of approximately 1:3 and incubated with T4 DNA ligase in 1× DNA ligation buffer (50 mM Tris-HCl buffer, pH 7.5, containing 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP and 25 mg/ml BSA) at room temperature for 2 to 3 h, and the ligation products were transformed into *E. coli* strain M15 cells using standard transformation protocols (Section 2.2.3.4). Colonies containing the proposed construct were identified by restriction enzyme digestion, and verified by DNA sequence analysis (Section 2.2.2.5).

4.2.3 Expression of Recombinant Protein

A single *E. coli* colony carrying the expression construct was inoculated and grown at 37°C overnight with shaking in LB medium containing both ampicillin (100 μg/ml) and kanamycin (25 μg/ml). The overnight culture was diluted 50-fold with pre-warmed fresh media containing antibiotics, and growth was resumed under the same conditions until the A_{600} of the culture reached 0.6. Before induction, 2 ml culture was taken as an un-induced control. The expression was induced by addition of isopropylthio-β-D-galactoside (IPTG) at a final concentration of 1 mM. The incubation was at either 23°C or 37°C for up to 4 h. Aliquots (2 ml) of the final culture were centrifuged and the pellet was resuspended in 1 × SDS buffer. The remaining cells were harvested in 50 ml centrifuge tubes by centrifugation at 3, 000×g for 10 min at 4°C, and the pellets were stored at -20°C for protein purification.

4.2.4 *Purification of Recombinant Protein under Native and Denaturing Conditions*

The frozen cell pellets containing recombinant proteins were thawed on ice for 15 min and resuspended in 50 mM sodium phosphate buffer, pH 8.0, containing 300 mM NaCl and 10 mM imidazole, at approximately 1/10 of the culture volume. The cell suspension was sonicated for 30 sec on ice and centrifuged at 10,000×g for 15 min at 4°C to separate soluble proteins and insoluble proteins.

To purify the expressed protein under native conditions, 600 µl supernatant containing soluble proteins of the cell lysate was loaded onto a Ni-NTA column equilibrated with the lysis buffer, centrifuged at 700×g for 2 min, and washed twice with the lysis buffer adjusted to 20 mM imidazole. Bound protein was eluted from the Ni-NTA column using lysis buffer with the imidazole concentration increased to 250 mM. The eluted protein, "flow through" from the washing steps, and the pellets were dissolved in SDS loading buffer and analysed by SDS-PAGE.

To purify the expressed protein under denaturing conditions, the pellets containing insoluble proteins after sonication were resuspended in 0.001 M Tris-HCl buffer, pH 8.0, containing 8 M urea and 0.1 M sodium phosphate (buffer I) at 1/10 of the culture volume. The suspension was mixed gently for at least 4 h at room temperature to solubilise the proteins from the pellet, and centrifuged at 14,000 × g for 5 min. Supernatant (600 µl) containing the denatured proteins was loaded on a Ni-NTA column. The purification procedure was performed according to the procedure described under native condition, except that the buffer used for washing steps was replaced with Buffer I with pH adjusted to 6.3, and the recombinant protein was eluted using buffer I with pH adjusted to 5.9, based on a pH gradient purification principle rather than using imidazole.

Purification of the expressed protein was initially attempted on a small scale (10 ml) in both native and denaturing conditions, and subsequently scaled up to 1 litre culture for preparative purification under denaturing conditions. Supernatant (5 ml) containing denatured protein solubilised from the pellet (50 ml culture) in 0.001 M Tris-HCl buffer, pH 8.0, containing 8 M urea and 0.1 M sodium phosphate was mixed with 0.5 ml of Ni-NTA slurry, and purified with 5 ml buffer I with pH adjusted to 6.3 for the washing steps. The bound protein was eluted with 1 ml buffer I with pH adjusted to 5.9. The eluted

protein, flow through from the washing steps, and the pellets were solubilised in SDS loading buffer and analysed by SDS-PAGE.

4.2.5 SDS-PAGE Analysis of Recombinant Protein

The method described by Laemmli (1970), using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), was employed for protein analysis. Protein samples were mixed with an equal volume of 2×SDS sample loading buffer [5× buffer: 0.125 mM Tris-HCl buffer, pH 6.8, containing 4% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 20% (v/v) glycerol and 0.0005% (v/v) Bromophenol Blue], boiled for 5 min, loaded onto either a 7.5% or a 12.5% polyacrylamide gel with a 4% stacking gel. The gel was run at a constant current of 25 mA, using either the Bio-RAD Mini-Protein apparatus or the Hoefer Mighty Small II, SE250/SE260, in electrophoresis buffer (25 mM Tris buffer, pH 8.3, containing 192 mM Glycine, and 0.1% SDS). After electrophoresis, the gels were stained with 0.2% (w/v) Coomassie Brilliant Blue R-250 solution containing 40% (v/v) methanol, 10% (v/v) glacial acetic acid, and de-stained in destain solution containing 30% (v/v) methanol and 10% (v/v) glacial acetic acid. For Western blotting, proteins from the gels were transferred without staining.

4.2.6 Generation of Polyclonal Antibodies Against Recombinant Protein-CS17

4.2.6.1 Generation of Antibodies

Polyclonal antibodies were raised against the recombinant protein, designated CS17 (CS-callose synthase; 17- the size of the expressed protein is approximately 17 kDa), in rabbits at the Institute of Medical and Veterinary Science (Gillies Plain, SA, Australia), using a standard inoculation protocol. After Ni-NTA affinity purification, 2 mg denatured CS17 in urea buffer was extensively dialysed against phosphate buffered saline (PBS), 10 mM sodium phosphate buffer, pH 7.4, containing 150 mM NaCl, using 3 kDa cut-off dialysis tubing. The protein was concentrated in a Centricon 3 micro concentrator, divided into four tubes, and the identity of the protein was confirmed by SDS-PAGE. Primary inoculation of the rabbits was conducted with 500 μg CS17 protein mixed with an equal volume of Freund's Complete Adjuvant, followed by three boosts with the same amount of antigen and Freund's Incomplete Adjuvant mix at intervals of three weeks. Pre-bleed (pre-immune serum) and test bleeds (immune serum) at approximately 9 days after the 3rd

and 4th doses were tested for specificity against the antigen CS17 by Westerns blot analysis, according to the procedure described below. From the final bleed-out, approximately 50 ml serum was obtained.

4.2.6.2 Examination of Antibody Specificity by Western Analysis

Protein CS17 was run on a 12% polyacrylamide gel under the conditions described in Section 4.2.5. Proteins were electrophoretically transferred to nitrocellulose membranes in transfer buffer (25 mM Tris-HCl buffer, pH 8.3, containing 192 mM glycine and 20% methanol) at a constant current of 180 mA for 6-16 h. The membranes were stained with Ponceau S for 30 min to examine the efficiency of the transfer, and destained with water. The membrane was blocked with 5% (w/v) skim milk powder in TBS buffer (10 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl) for 2 h. After three washes with TBST (TBS containing 0.05% v/v Tween-20) for 10 min each, the membrane was incubated at room temperature with either the polyclonal antibody serum or pre-immune serum at 1:2000 dilution for 2 h with gentle shaking. Following a further three washes with TBST, the anti-rabbit IgG alkaline phosphatase conjugate (1:4,000 dilution in TBST) was applied to the membrane, and incubated at room temperature for 2 h or overnight. Binding between the antigen and antibodies was observed by the colour reaction, after incubating the membrane with the alkaline phosphatase colour development reagent containing 0.34 mg/ml NBT (nitroblue tetrazolium) and 0.16 mg/ml BCIP (5-bromo-4-chloro-3-indolyl phosphate). Colour development was terminated by rinsing the membranes with water.

4.2.7 Affinity Purification of Antibodies

To increase the specificity of the antibody preparation for a range of analyses, such as immunoprecipitation and *in-situ* hybridisation, the immune serum was initially purified by protein A affinity to obtain the IgG fraction (Kagel *et al.,* 1989), and subsequently purified on an affinity column carrying immobilised CS17.

4.2.7.1 Preparation of HiTrap Affinity Column and Ligand Coupling

A 1 ml HiTrap NHS-activated Sepharose high performance column was used for coupling CS17, the peptide expressed in *E. coli* and inoculated into a rabbit for polyclonal antibody

production. The NHS-activated gel is designed for the covalent coupling of ligands containing primary amino groups and is based on highly cross-linked agarose beads with appropriate spacer arms attached to the matrix. The CS17 peptide contained seven lysines and should allow efficient amine coupling of the antigen to the NHS-activated column.

For coupling, CS17 protein was dialysed against PBS buffer, pH 7.4, containing 0.01% Tween-20 and 10 mM DTT. However, the CS17 precipitated during dialysis. Thus, 4 M guanidine-HCl was used to solubilise the precipitated CS17. A 3mg/ml solution of CS17 (1 ml) in 4 M guanidine-HCl, pH 7.8, was applied to the NHS-activated column (1 ml capacity), followed by three washes (1ml each wash) with cold 1 mM HCl. Care was taken to avoid trapping air bubbles in the column. To couple the antigen, the column was allowed to stand up-right at room temperature for 1 h. To deactivate excessive active groups that were not coupled to the ligand and remove non-specifically bound CS17, the column was washed and blocked sequentially with: 3×2 ml buffer A (0.5 M ethanolamine buffer, pH 8.3, containing 0.5 M NaCl) (fraction 1-3); 3×2 ml buffer B (0.1 M sodium acetate buffer, pH 4, containing 0.5 NaCl) (fraction 4-6); and 3×2 ml buffer A (fraction 7-9). The column was allowed to stand for 15 min, and subjected to further washes with 3×2 ml buffer B (fraction 10-12), 3×2 ml buffer A (fraction 13-15), and 3×2 ml buffer B (fraction 16-18). The column was stored in 50 mM sodium phosphate buffer, pH 7.0, and was ready to use for the affinity purification.

The coupling efficiency was determined by comparison of the A_{280} reading of 1 ml starting material with A_{280} readings of eluted fractions 1-3 defined above. To do this, 1 ml of the antigen and fractions 1-3 collected from the coupling steps were both adjusted to 10 ml in PBS, and centrifuged at 3000×g for 20 min. The precipitates were dissolved in 6 M guanidine-HCl and A_{280} was measured.

4.2.7.2 Affinity Purification of Anti-CS17 Antibody using the Protein A Column

Approximately 18 ml CS17 anti-serum was defrosted, mixed with 2/3 vol of chloroform, and centrifuged at 1500 × g for 10 min to remove lipids. The aqueous phase was collected, residual chloroform was removed, and the aqueous phase was filtered through a 0.45 μm Amicon filter. The serum was adjusted to pH 8.0 with 1.0 M Tris-HCl buffer,

pH 8, and loaded onto a 1 ml BioRad Econoac Protein A column in 5 ml batches, using an automated Biologic HPLC detection system at 254 nm. The system was programmed to wash the column sequentially with 10 ml 0.1 Tris-HCl buffer, pH 8.0, 10 ml 0.1 sodium citrate buffer, pH 5.0, and 10 ml 0.1 Tris-HCl buffer, pH 8.0, at a flow rate of 1ml/min. The IgG fraction, which bound to the protein A, was eluted using 100 mM glycine buffer, pH 2.5, at a flow rate of 2 ml/min. The purified antibodies were immediately adjusted to pH 7.0 with 1 M Tris-HCl buffer, pH 8.0 in order to retain antibody activity. Four batches of purification were conducted using this protocol.

The protein A-purified antibodies were subsequently exchanged into PBS buffer using a Pharmacia fast desalting FPLC column packed with Sephadex G-25 Superfine. The antibodies were finally concentrated in a 10 ml Amicon ultra-filtration cell with an YM 10 membrane at 30 psi.

4.2.7.3 Purification of Protein A-Purified Antibodies using the HiTrap NHS-Column

The antibodies purified from the Protein A column were applied to the CS17 affinity column, recycled over the column at 0.2 ml/min using the BioRad Biologic HPLC system to achieve maximum binding. Unbound antibodies were collected and measured for protein concentration. The column was washed with 100 mM sodium citrate buffer, pH 5.0, the bound proteins were eluted with 100 mM glycine buffer, pH 2.5. Fractions were collected into 1/10 volume of 1.0 M Tris-HCl buffer, pH 8.0. Both citrate buffer fractions (4-6) and glycine buffer fractions (15-17) were collected, concentrated using the Amicon ultrafiltration cell, and exchanged into PBS using the Pharmacia fast desalting FPLC column (Section 4.2.7.2). Fractions from the desalting column were pooled and further concentrated using Centricon 50 kDa cut-off micro concentrators.

4.2.7.4 Examination of Affinity Purified Antibody using a Slot-Blot Assay

Serially diluted CS17 was transferred to nitrocellulose filters using a PR648 slot blotter. Filters were blocked with PBS buffer, pH 7.4, containing 2% (w/v) BSA and 0.1% (v/v) Tween-20, and probed with either protein A-purified or CS17 affinity-purified polyclonal antibodies at an appropriate dilution rate for 2 h with gentle shaking. After three washes with PBST, the anti-rabbit IgG (whole molecular)-horseradish peroxidase conjugate at 1: 3,000 dilution in TBST was applied to the membranes and incubated at room temperature

for further 2 h. Finally, the filters were developed with the ECL-plus fluorescent detection system and imaged with the STORM 860 optical scanner using a blue fluorescence mode. Signal from the bands were quantified using the ImageQuant densitometry software.

4.3 Results and Discussion

4.3.1 Construction of Expression Vector

A cDNA fragment corresponding to the central cytoplasmic region of the callose synthase enzyme, was amplified by PCR using clone 2 (Section 2.3.1) as a template. The resulting DNA fragment contained *Bam*HI and *Hin*dIII restriction sites at its 5' and 3' ends, respectively. The DNA fragment and the pQE30 vector were digested with *Bam*HI and *Hin*dIII, and ligated (Figure 4.3). The resulting expression vector was used to direct the expression of the recombinant protein under the control of the T5 promoter. A poly-histidine affinity tag was located at the NH_2-terminal of the expressed protein for affinity purification of the expressed protein (Figure 4.2).

The restriction enzyme digestion and DNA sequence of the expression plasmid showed the correct orientation and correct sites of insertion. However, a single nucleotide deletion of "T" was revealed in the DNA sequence, resulting in the early termination of protein translation. The deletion could have been caused by a PCR error during the amplification process. As a result, the expected recombinant peptide based on the sequence examined at the nucleotide level was shortened to 150 amino acids, and had 12 amino acids at the COOH-terminal that did not match the HvGSL1 peptide sequence. The peptide sequence was searched against protein databases, but no matches were found other than the putative callose synthase. It was therefore considered that the expressed protein carrying extra 12 amino acids would have no significant effect on the identity of the expressed protein, given the fact that expression constructs containing a number of extra restriction enzyme sites, and hence a few extra amino acids at the protein level, generated from the multiple-cloning site of the expression vectors are a common feature of protein expression constructs. Likewise, constructs containing fusion proteins have been widely adapted to express protein of small sizes and it was found that extra amino acids attached to a target protein would not unduly interfere with the behaviour of the target protein (Frangioni and Neel, 1993; Stewart, 1999).

4.3.2 Expression and Purification of Recombinant Protein

The construct was transformed into *E. coli* M15 strain containing the pREP4 repressor plasmid for protein expression (Section 4.2.3). To monitor expression, a time course with

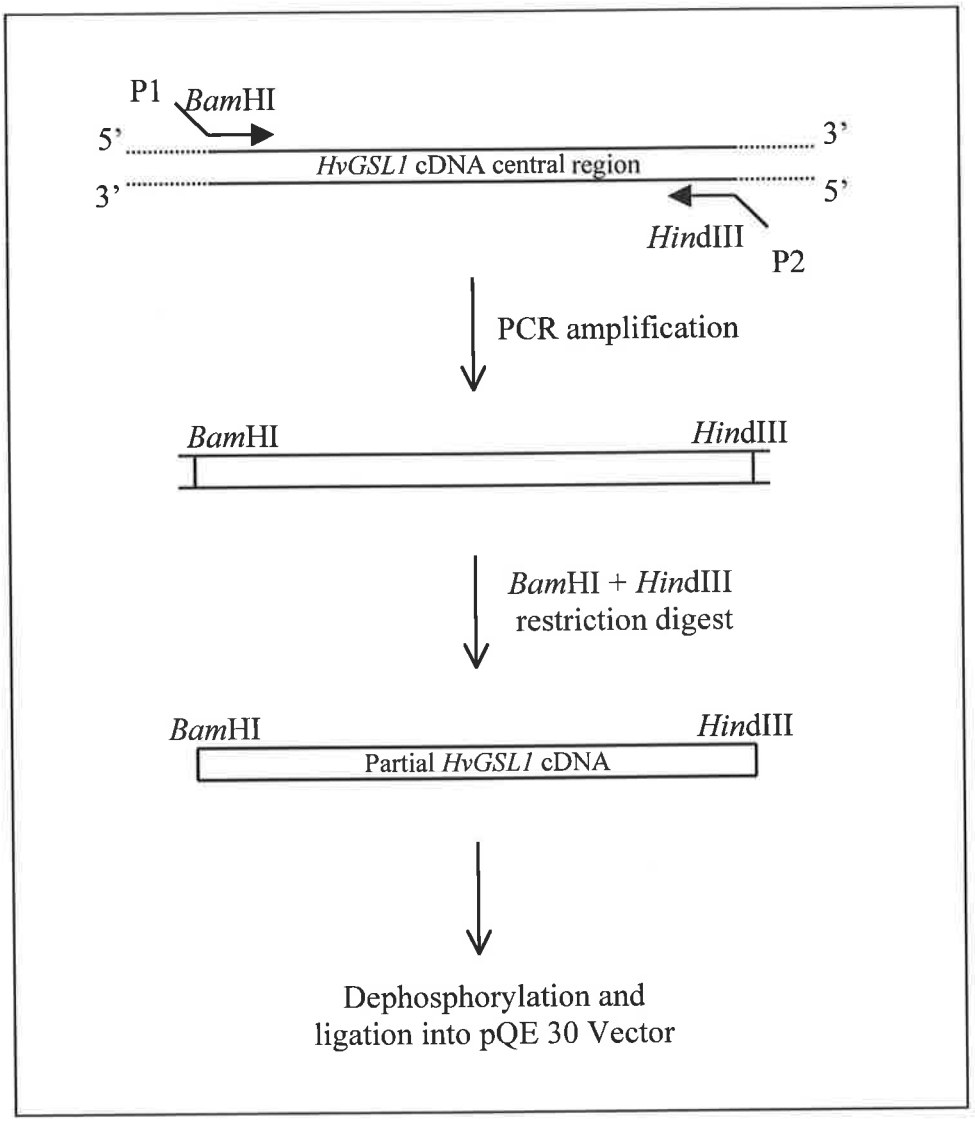


Figure 4.3 Construction of an expression vector using the pQE-30 system. Primers 1 and 2 were designed to amplify a fragment of the *HvGSL1* with *Bam*HI and *Hin*dIII restriction sites at the 5' and 3' ends, respectively. Following restriction digestion with *Bam*HI and *Hin*dIII, the amplified cDNA was dephosphorylated and ligated into the pQE-30 expression vector as illustrated in Figure 4.2.

1 ml cultures taken 0, 1, 2, 3, 4 h after induction was conducted. SDS-PAGE analysis of the time course samples from the total lysate of the cells suggested the expression construct, the pQE-30-HV plasmid, directed the synthesis of a recombinant protein of approximately 17 kDa. This value corresponds to the calculated molecular mass for the shortened peptide fragment predicted according to the DNA sequence (Section 4.3.1). The same size band was induced in all samples, with highest expression 4 h after induction, both at 23°C and 37 °C (Figure 4.4). The four-hour incubation period after induction was therefore used in large-scale protein expression.

Purification under native conditions did not lead to the isolation of the recombinant protein, due to the fact that the expressed protein was deposited into the insoluble fraction (Figure 4.5). There was only a trace of recombinant protein detected in the soluble fraction, but no protein could be recovered from the soluble fraction following affinity purification. This result was not surprising, since inclusion bodies containing the products of highly expressed foreign proteins are commonly found in *E. coli* system (Kopito, 2000). The over-expressed proteins in the cytoplasm of *E. coli* cells often do not fold efficiently, and aggregate into the so-called cytoplasmic inclusion bodies. This was also a case when expressing a barley xylan endohydrolase gene in this laboratory (Banik, 1996). In addition, the expressed protein represented only 10% of the entire HvGSL1 protein, and this short fragment lacks proximal domains, which may be required for correct protein folding or stabilisation, resulting in the production of insoluble protein.

The insoluble protein presented in the inclusion bodies was solubilised in 8 M urea, and purified by one-step affinity chromatography with the Ni-NTA resin. To confirm the identity of the expression protein, NH_2-terminal protein sequencing was conducted by automated Edman degradation (Edman and Begg, 1967). A single sequence was found, MRGSH HHHHH GSKPE NQN, which corresponds exactly with the first 18 amino acid residues located at the NH_2-terminal of the recombinant protein. MRGS is the translation start sequence after the T5 promoter, HHHHHH is the poly-histidine tag, GS was encoded by the restriction enzyme *Bam*HI recognition sequence, and KPENQN was the NH_2-terminal sequence of the HvGSL1 protein fragment selected for expression. With evidence from the DNA sequence, the protein sequence and the molecular weight

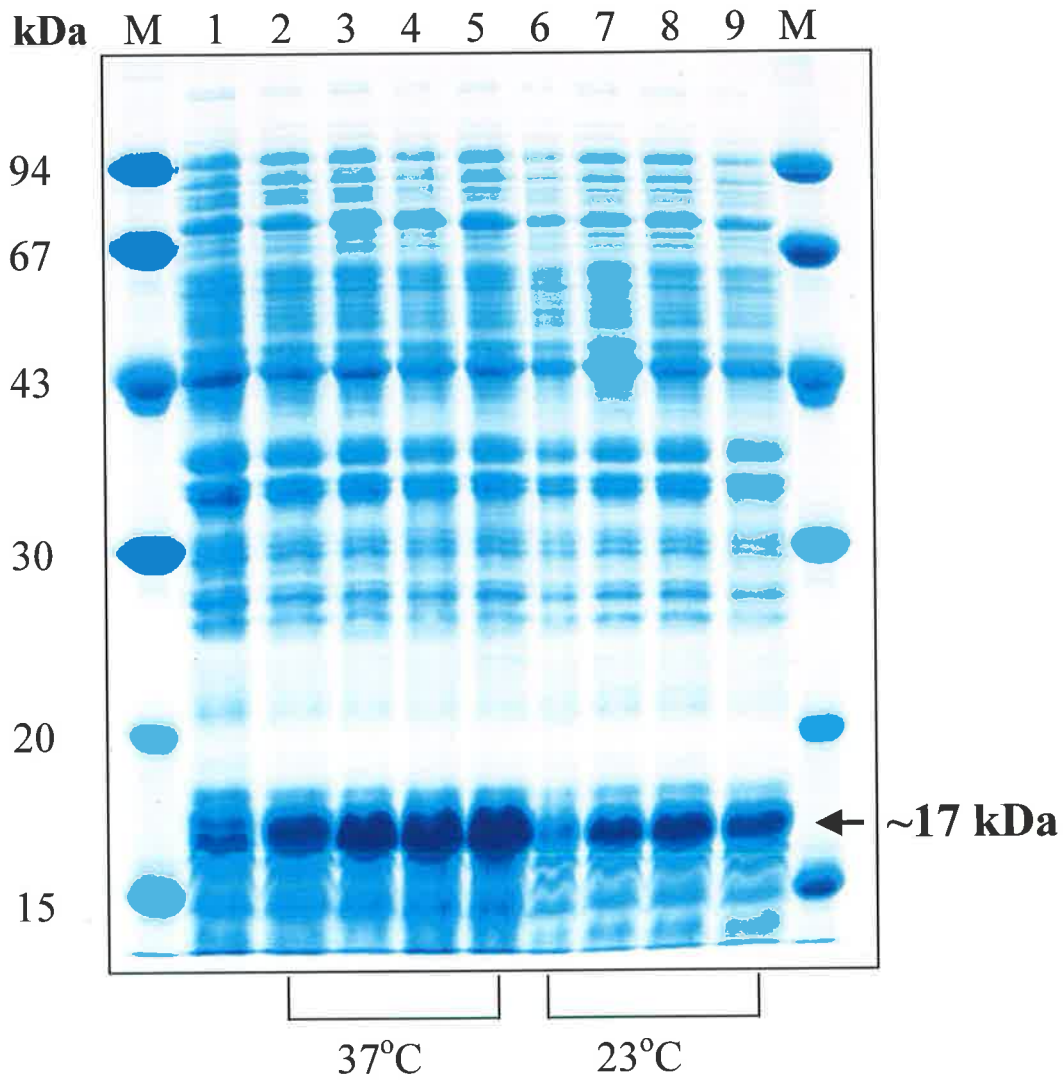


Figure 4.4 Time course expression of partial *HvGSL1* cDNA. SDS-PAGE analysis of proteins in the total lysate of the *E. coli* expression cells. M: protein molecular mass (kDa) standard markers; Lane 1: cell lysate before induction; Lanes 2-5: cell lysate after induction at an expression temperature of 37°C with post induction times of 1, 2, 3 and 4 hr, respectively; Lane 6-9: cell lysate after induction at an expression temperature of 23°C with the same time courses as in Lanes 2-5. Note that a protein of approximately 17 kDa was predominantly expressed in both cases. Expression levels were higher at 37°C than at 23°C.

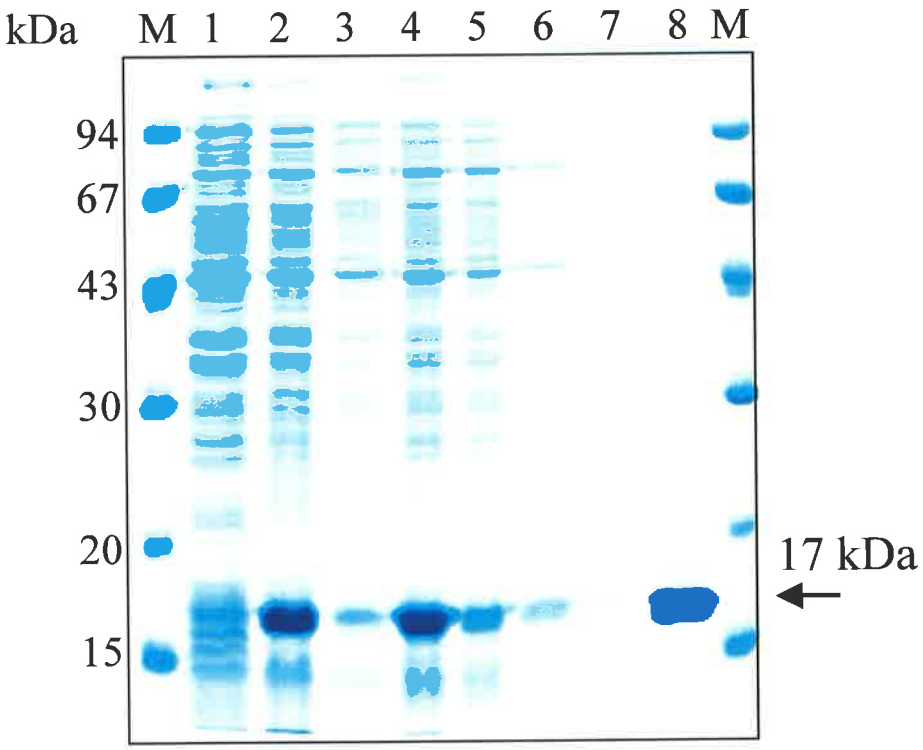


Figure 4.5 The heterologous expression and purification of partial HvGSL1 protein. A 17 kDa protein was expressed in *E. coli* and purified by one-step affinity chromatography using a Ni-NTA column. SDS-PAGE analysis was used to analyse the following fractions: Lane 1: the total cell lysate before induction; Lane 2: the total cell lysate after induction; Lane 3: the soluble fraction of the cell lysate; Lane 4: the insoluble fraction of the cell lysate; Lanes 5, 6, 7: Ni-NTA column unbound fractions, as 3 flow throughs; Lane 8: bound protein as the purified protein; M: protein molecular mass (kDa) standard markers.

estimated by SDS-PAGE, it was concluded that the correct recombinant protein was produced.

Meanwhile, several experiments were conducted in an attempt to enhance the production of the expressed protein in active form. Lowering expression temperature is known to have directly affects on both expression levels and protein solubility (Schein and Noteborn, 1988). In the present work, cells were also incubated after induction at a temperature of 23°C, instead of 37°C. The change in temperature seemed to have no effect on the solubility of the protein expressed. Because some *E. coli* strains tolerate some proteins better than the others, the host strain was changed from M15 [pREP4] to SG13009 [pREP4], but this made no difference in terms of solubility of the protein. Furthermore, reduction of IPTG levels during induction and metal supplements in the media (Moore *et al.*, 1993) were also carried out with the expectation that the former would reduce expression level and thereby decrease the chance of occurrence of inclusion bodies, while the latter would assist in maintaining protein solubility. Nevertheless, these modifications again failed to produce a soluble protein.

Attempts to refold the denatured protein from the inclusion bodies using a urea-gradient (8 M to 0.2 M) solubilisation method (Stern *et al.*, 1993) also proved unsuccessful, as precipitation always occurred during the gradual dilution of the denaturing agent. In contrast, numerous other studies have shown that inclusion bodies can be successfully renatured to their native or active state (Brown *et al.*, 1993; Friedhoff *et al.*, 1994; Pear *et al.*, 1996).

4.3.3 Generation of Polyclonal Antibodies

Purified recombinant protein of 17 kDa, designated CS17, was dialysed against PBS buffer, pH 7.4, and used to generate polyclonal antibodies that recognise the protein encoded by the *HvGSL1* cDNA. The purified protein CS17 (2 mg) was inoculated into a rabbit in 500 µg batches. Pre-immune serum and two test bleeds were collected before the final bleed for the polyclonal antibodies. Western blot analysis of CS17 antigen with the test bleeds were performed to monitor antibody production, and showed a positive result. In addition, the specificity of the polyclonal antibodies was tested with the serum of the final bleed, and confirmed the ability to recognise the original recombinant peptide

CS17, whilst the pre-immune serum exhibited no-binding at all on the Western blot (Figure 4.6).

4.3.4 *Purification of Polyclonal Antibodies using Protein A*

Microscopic studies of the subcellular locations of callose synthase, such as *in situ* immunogold labelling, require highly specific antibodies against the antigen. To achieve this, the crude rabbit serum was firstly subjected to protein A affinity chromatography to purify the IgG fraction (Section 4.2.7.2). Figure 4.7 shows the elution profile of the protein A column. The IgG fraction bound to the protein A was eluted with 100 mM glycine buffer, pH 2.5, and stored in 1.0 M Tris-HCl buffer, pH 8.0 to retain its activity. This fraction was retained because it contained purified antibodies, while the unbound fractions including serum flow through (peak1) and citrate fraction (peak 2) were discarded (Figure 4.7). The concentrated glycine buffer-eluted fraction was subsequently applied to the desalting column. The proteins in the peak shown in Figure 4.8 were collected and the concentration of the antibodies was measured. A total of 9.8 mg antibodies was obtained from five batches of purification.

4.3.5 *Purification of Protein A-Purified Antibodies using a HiTrap NHS-Column*

The protein A-purified antibodies were subjected to a second affinity purification using the HiTrap NHS column, which carried the CS17 peptide covalently attached to the column. In preparation for the NHS column, the expressed protein CS17 was initially dialysed in PBS buffer, pH 7.4, which resulted in precipitation of CS17. An alternative buffer, 4 M guanidine-HCl, was used instead to solubilise the CS17 protein. CS17 peptide (3mg/ml) solubilised in guanidine-HCl was injected onto the NHS column, followed by subsequent coupling and washing steps (Section 4.2.7.1). Examination of the coupling efficiency was performed by measuring the absorbances at A_{280} on both the unbound material from fractions 1 to 3 (Section 4.2.7.1) and the starting material CS17 protein. Approximately 70% coupling efficiency was achieved. Thus, coupling the CS17 peptide to the NHS column was considered satisfactory, and the column was subsequently used for further purification of the antibodies.

Using the Biologic HPLC system, antibodies were continuously recycled through the NHS-CS17 column overnight to ensure efficient binding and to maximise the yield of

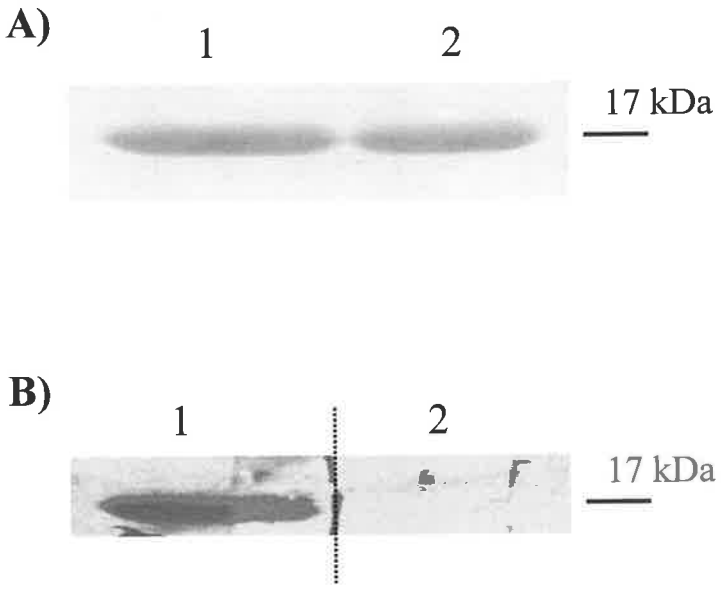
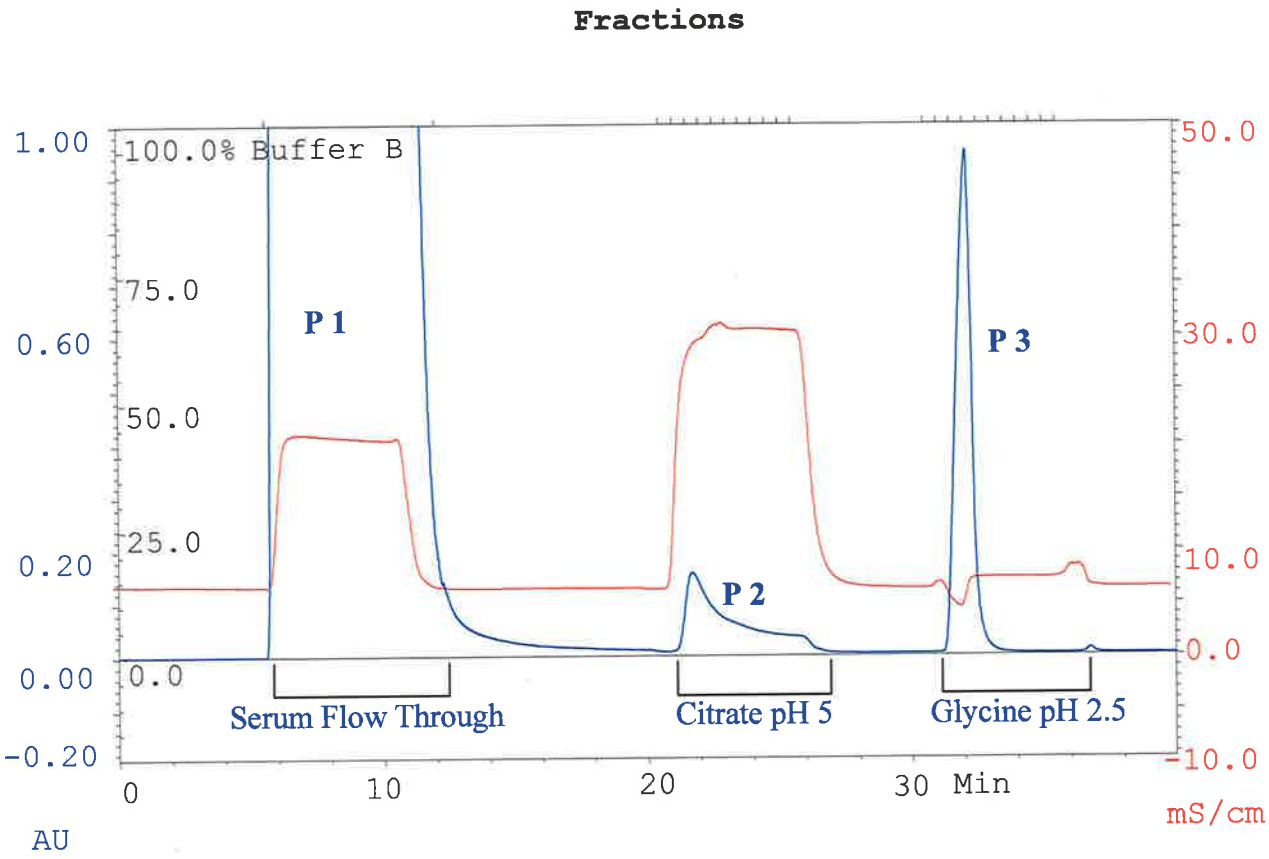


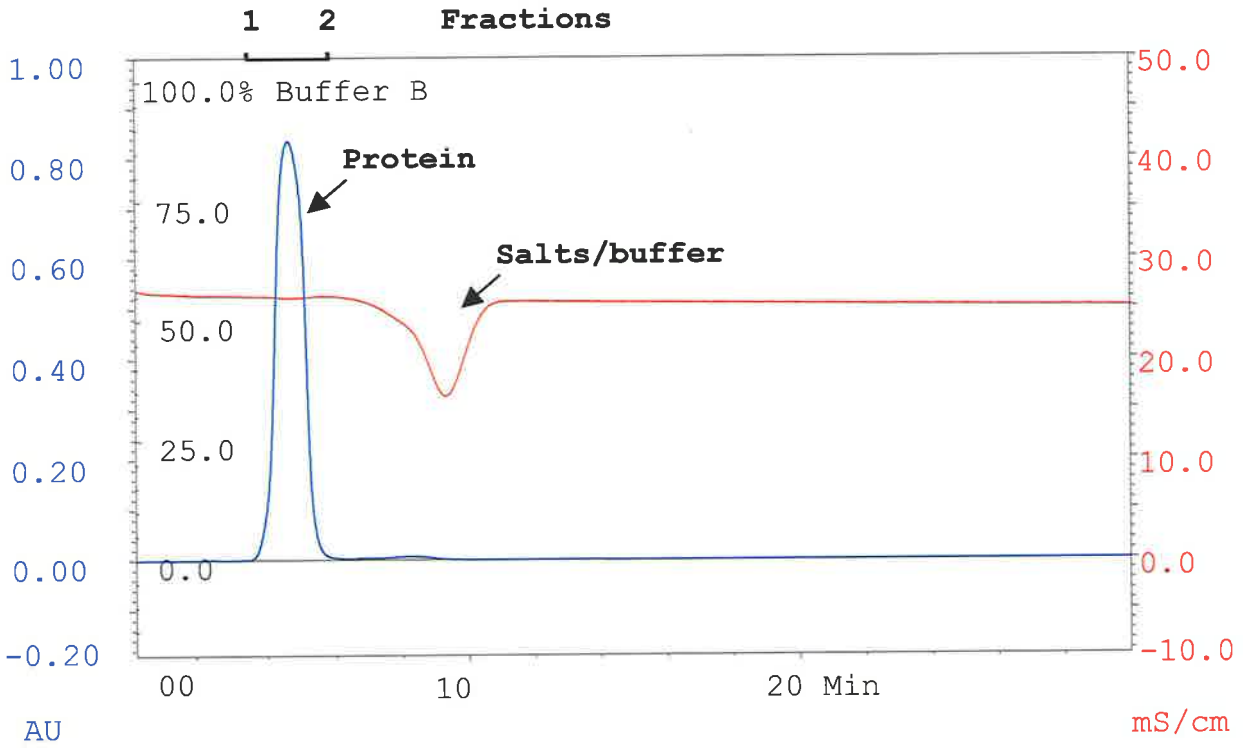
Figure 4.6 Specificity assessments of the polyclonal antibodies generated from a fragment of the *HvGSL1*. **A)** SDS-PAGE analysis of recombinant protein CS17. The recombinant protein CS17 expressed from a fragment of barley *HvGSL1* cDNA was equally loaded into Lane 1 and Lane 2 and stained with Coomassie Brilliant Blue R-250. **B)** Western analysis of the above samples using immune serum (Lane 1) and pre-immune serum (Lane 2). A strong hybridisation was observed with Anti-CS17 antibodies (immune serum), and no signals were detected with the pre-immune serum.

Figure 4.7 Protein elution profile of Protein A affinity chromatography. Antibodies were loaded on a protein A affinity column and eluted with 100 mM glycine buffer, pH 2.5. The column was washed sequentially with 0.1 M Tris-HCl buffer, pH 8.0, and 0.1 M Citrate buffer, pH 5.0, prior to elution. The conductivity is shown in red. The eluant monitored continually at 280 nm is shown in blue. Peak 1(P1): serum flow through; Peak 2 (P2): in sodium citrate buffer; Peak 3 (P3): in Glycine buffer. Glycine fractions containing IgG fractions of the antibodies were pooled for further treatment and purification. Four batches of 5 ml purification were conducted.



Protein elution profile in blue (AU); Solvent conductivity in red (mS/cm).

Figure 4.8 Protein elution profile of the fast desalting column (Sephadex G-25 superfine). The IgG fraction purified from the protein A column was desalted on a HR fast desalting column in a final buffer of PBS buffer, pH 7.4, containing 0.1% Tween-20 (buffer B). The eluant monitored continually at 280 nm is shown in the blue line, from which Fractions 1-2 (protein peaks) containing only IgG were pooled for further purification. The conductivity of the solvent including the salts is showed in red. Five batches of 5 ml chromatography were conducted.



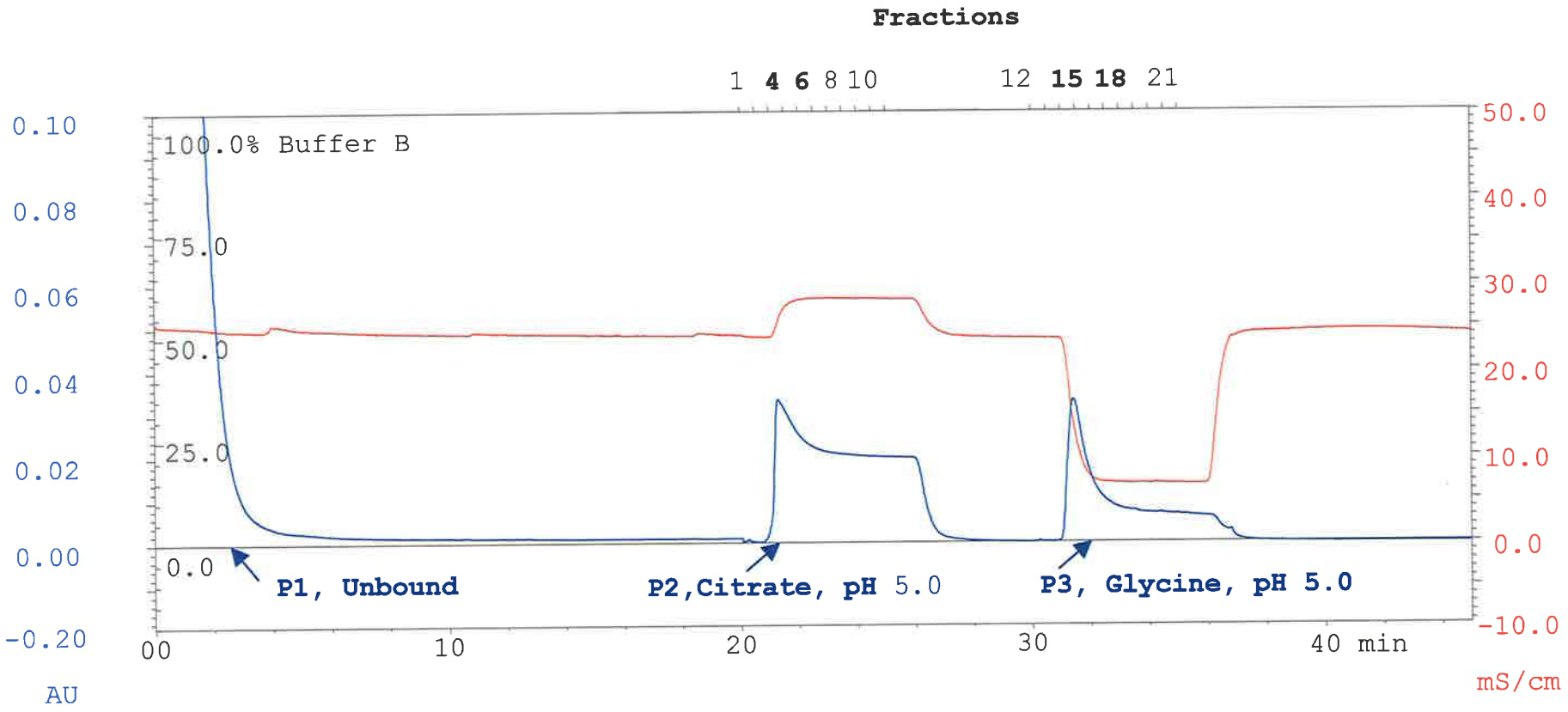
Protein elution profile in blue (AU); Solvent conductivity in red (mS/cm).

purified antibodies. An elution profile is shown in Figure 4.9. Fractions 4-6, and 15-17 in citrate and glycine buffers were pooled, whilst the unbound material was discarded. Antibodies from both citrate and glycine fractions were considered to be specific to the CS17 peptide, because they bound to the CS17 column, and perhaps represent different IgG isotypes. Following the desalting process (Section 4.2.7.3), the purified antibodies collected from the citrate and glycine peaks were concentrated to 188 μg/ml and 304 μg/ml, respectively.

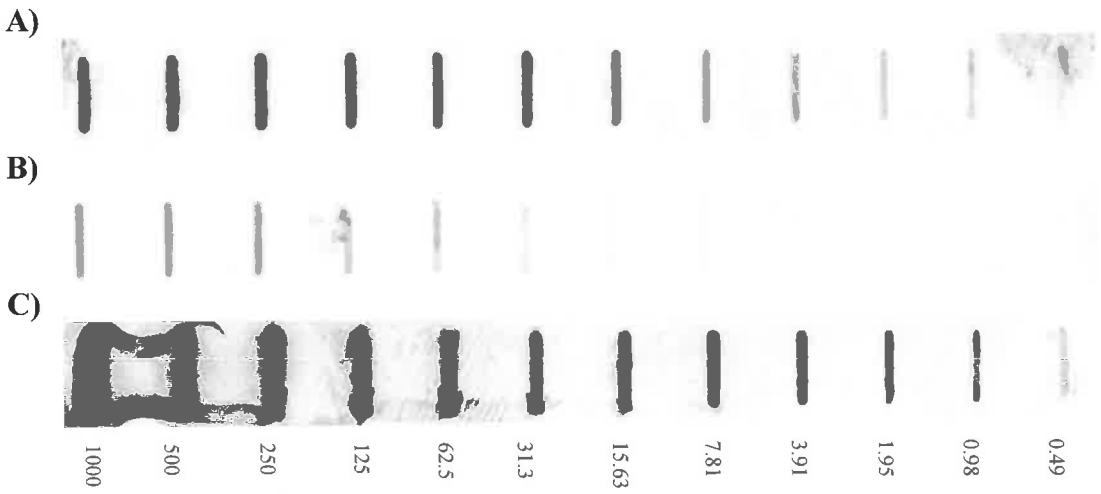
4.3.6 Examination of Affinity Purified Antibodies using a Slot-Blot Assay

Both the IgG from anti-serum purified using protein A affinity and the purified IgG specific for the CS17 peptide from the CS17 Sepharose affinity column were examined for binding to CS17, in a slot–blot assay. The CS17 peptide, at concentrations ranging from 500 pg to 1000 ng was tested using an antibody concentration of 100 ng/ml. The CS17 unbound IgG fraction was analysed to determine anti-CS17 IgG remaining in the sample. The intensity of the bands of the Western blot (Figure 4.10) from the protein A-purified material, and CS17-specific antibodies revealed enhanced activity in the final product after the two-step affinity purification (A and C). Very little binding activity was observed in the unbound fraction (B). Densitometry (Figure 4.11) of these bands suggested that the antibodies purified from the CS17 column had been enriched by approximately 10-fold for anti-CS17 activity. It was therefore concluded that two steps of affinity purification indeed produced a highly pure polyclonal antibody preparation with improved specificity and enhanced activity. This provided a useful tool in studying the function of the *HvGSL1* gene.

Figure 4.9 Protein elution profile of CS17 affinity chromatography. The desalted IgG fraction purified from the protein A column was loaded into the CS17 affinity column, and fractions 4-6 were pooled from the citrate peak, and fractions 15-17 were pooled from the glycine peak. The unbound material was discarded. The CS17 purified antibodies from the glycine fractions were used for further analysis. The eluant monitored continually at 280 nm is shown in blue. The conductivity is shown in red.



Protein elution profile in blue (AU); Solvent conductivity in red (mS/cm).



Immobilised CS17 peptide

Figure 4.10 Examination of affinity purified antibodies using a Slot Blot assay. A serial dilution of recombinant protein CS17 in the concentration range of 500 pg to 1000 ng was immobilised in a slot blot, and incubated with anti-CS17 antibodies at a concentration of 100 ng/ml. The bound antibodies were detected with goat-anti-rabbit IgG conjugated to HRP, and developed with ECL-plus using the STORM blue fluorescence detection mode. **A)** Western Blot using protein A purified anti-CS17 antibodies. **B)** Western Blot using protein A purified, CS17 column unbound fraction (peak 1, Figure 4.9). **C)** Western Blot using antibodies bound to CS17 column.

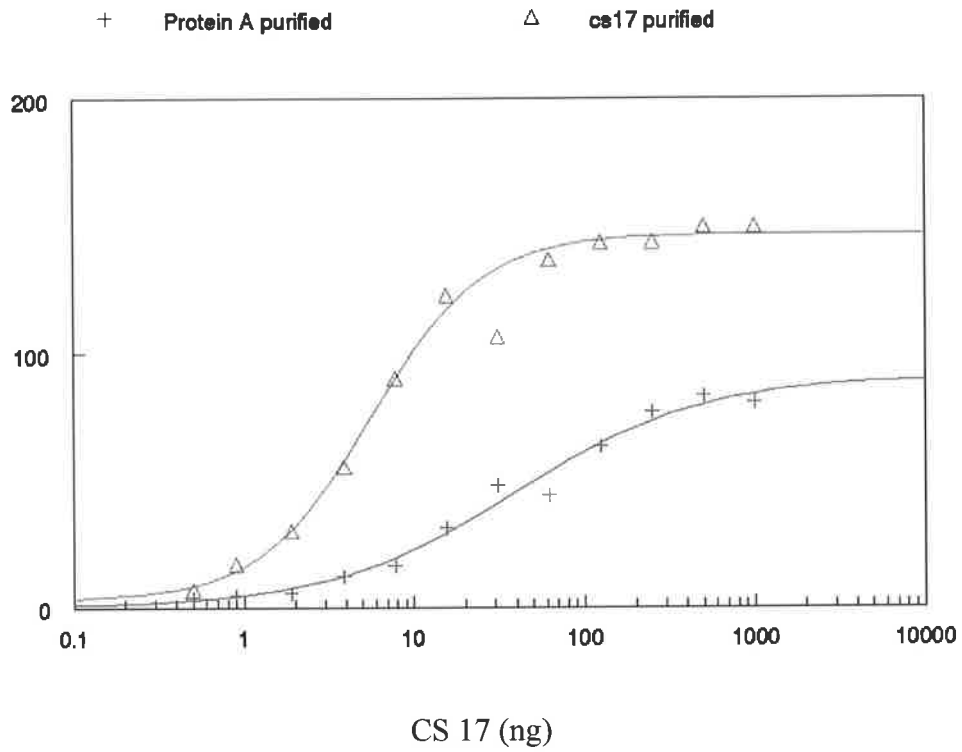


Figure 4.11 Densitometry scan of the slot blot. Densitometry scan on Western bands from both protein A and CS17 column purified antibodies. On the X-axis, the amount of CS17 peptide (ng) immobilised is shown; while the Y-axis shows fluorescence of the Western band. The Densitometry readings of the Western bands using Protein A and CS17 column purified antibodies were marked as "+" in red and "▷" in green, respectively. This figure indicates that 10-fold enrichment in anti-CS17 activity was found in CS17 column purified antibodies as compared to that of the protein A-purified antibodies.

4.4 Summary and Conclusions

In this Chapter, two experiments have been described. Firstly, a cytoplasmic fragment of the barley (1→3)-β-glucan synthase-like cDNA (*HvGSL1* cDNA) was expressed using the QIAexpressionist System, and the expressed peptide (CS17) was subsequently purified with a poly-histidine tag by single-step affinity chromatography. The recombinant protein was found exclusively in the insoluble fraction, and could not be renatured to a soluble form under a range of conditions.

The recombinant protein (CS17) was successfully used as an antigen for the production of a polyclonal antibody preparation against the putative callose synthase, which was one of the major objectives of the present study. Western blot analysis showed no binding of the CS17 peptide with the pre-immune serum. Furthermore, in order to obtain ultra-pure antibodies for microscopy studies, two-step affinity purification was conducted by firstly purifying the IgG fraction of the antibodies on a protein A column, and secondly by applying the IgG fraction to a HiTrap column coupled with CS17 peptide. With a slot-blot assay and quantification using densitometry, it was revealed that the final purified antibodies displayed enhanced anti-CS17 activity. It is therefore concluded that the CS17-specific antibodies can be used for the functionality studies of the *HvGSL1* gene.

A series of experiments designed to partially purify active (1→3)-β-glucan synthase from barley tissues and suspension cultured-cells, and subsequent enzyme characterisation will be described in the next chapter. The antibodies purified here are used to identify and follow HvGSL proteins during the partial purification of the enzyme.

CHAPTER FIVE:

PARTIAL PURIFICATION AND CHARACTERISATION OF (1→3)-β-GLUCAN SYNTHASES FROM BARLEY

5.1 Introduction

Genetic information about a putative barley (1→3)-β-glucan synthase gene, *HvGSL1,* has been presented in previous Chapters. As the cloning of the *HvGSL1* progressed, a number of putative higher plant (1→3)-β-glucan synthase genes were reported, including those from cotton and *Arabidopsis* (Cui *et al.,* 1999; Richmond and Somerville, 2000; Hong *et al.*, 2001). Nevertheless, there has been no compelling evidence to directly link putative callose synthase genes with an involvement in (1→3)-β-glucan synthesis. Verma's group has provided indirect evidence that a putative *Arabidopsis* callose synthase (AtGSL6) is located at the growing cell plate, that it interacts with phragmoplastin and that in transgenic tobacco, the *Arabidopsis* callose synthase gene causes some increase in callose synthesis on the growing cell plate (Hong *et al.*, 2001). Similarly, a direct connection between the protein encoded by the genes and (1→3)-β-glucan synthase activity has not been demonstrated. To make these connections and to explore the underlying mechanism of the biosynthesis of (1→3)-β-glucan in barley, attempts were made here to purify (1→3)-β-glucan synthases from barley tissues and to obtain amino acid sequence of active enzyme preparations.

As discussed earlier (Section 1.4.2), much effort has been devoted to purifying callose synthases from plants, but they have never been purified to homogeneity. In the present study on callose synthases from barley, the availability of the polyclonal antibody raised against an *E. coli*-expressed fragment of the barley *HvGSL1* cDNA (Chapter 4), and related genetic information on the gene (Chapters 2 & 3), increased the feasibility of these biochemical approaches. A range of analytical methods, such as immunoprecipitation, affinity chromatography, and Western blot analysis could be employed. In particular, the antibodies could be used to track proteins with amino acid sequence corresponding to the *HvGSL1* cDNA, and hence to demonstrate whether or not (1→3)-β-glucan synthase activity was associated with HvGSL1 protein during the purification process.

The second issue to be addressed in this Chapter was the characterisation of product synthesised by protein extracts enriched in callose synthase activity from barley suspension cultures. Activity assays for callose synthase involve the incorporation of UDP-[^{14}C] glucose or UDP-[^{3}H] glucose into ethanol-insoluble polysaccharide products.

The starting material is usually a total or partially fractionated membrane preparation, but these contain both cellulose synthase and callose synthase, and both use UDP-glucose as a substrate (Feingold *et al.,* 1958; Aloni *et al.,* 1983). Cellulose synthesis is favoured at micro-molar concentrations of UDP-glucose, whereas callose synthesis is favoured at milli-molar concentrations of the substrate (Delmer, 1999). Thus, rigorous analysis of the polysaccharide synthesised by enzyme extracts is essential, and a number of analytical strategies are available for product characterisation. These include fluorescent detection of (1→3)-β-glucan products using the aniline blue fluorochrome (Evans, 1984), quantification of radio-labelled substrate incorporated into the ethanol-insoluble product (Smith and Stone, 1973b), and analysis of the product through hydrolysis with highly specific (1→3)-β-glucan hydrolases.

The focus in this Chapter is on the enzyme purification process. Because the target (1→3)-β-glucan synthases are plasma membrane-bound proteins and may require multiple components for activity (Fink *et al.*, 1990; Amor *et al.,* 1991; Delmer *et al.*, 1991; Dhugga and Ray, 1991; 1994; Fredrikson and Kjelbom, 1991; Meikle *et al.*, 1991; Wasserman *et al.*, 1992; Wu and Wasserman, 1994; Bulone *et al.*, 1995; Kudlicka and Brown, 1997; Turner *et al.*, 1998), membrane structure, the properties of transmembrane proteins, detergents used for protein solubilisation, methodologies for cellular disruption, and the purification of membrane proteins are briefly discussed here.

The first consideration relates to interactions between membrane lipids and membrane-bound proteins. Plant membranes generally consist of a phospholipid bilayer, in which a broad range of integral proteins are embedded. Cell wall polysaccharide synthases are likely to be integral membrane proteins, which in many cases will bind sugar nucleotides on one side of membrane and vectorially secrete the nascent polysaccharide chain to the other side of the membrane (Carpita and Vergara, 1998; Delmer, 1999). As integral membrane proteins, the polysaccharides synthases will have hydrophilic regions in contact with the aqueous media on either side of membrane, and hydrophobic regions that interact strongly with the aliphatic chains of phospholipid fatty acids in the core of the lipid bilayer. These amphipathic properties of integral membrane proteins mean that their complete dissociation from the phospholipid bilayer will usually result in loss of activity, either through aggregation of the disassociated proteins or through their un-folding or re-

folding on exposure to aqueous conditions. As a consequence, membrane-bound callose synthases cannot be purified by conventional, aqueous-based procedures of column chromatography. To overcome these difficulties and to protect membrane-bound enzymes against inactivation, the initial disruption of the plant tissue can be effected in the presence of detergents (Maire *et al.,* 2000). Detergent will disperse membrane-bound proteins in a fine suspension of micelles or small liposomes, activity is often retained and further purification can be undertaken.

The second consideration in purifying membrane proteins is therefore the choice of an appropriate detergent. The principle of "solubilisation" is that small amphipathic detergent molecules form micelles in water. When they are mixed with membranes, the hydrophobic ends of the detergents bind to the hydrophobic regions of membrane proteins, while water and the hydrophilic end of the detergents bind to the hydrophilic regions of the proteins. The hydrophilic region of the detergents can be either charged (ionic) or uncharged (non-ionic). Ionic detergents, such as sodium dodecyl sulfate, are efficient solubilisers but almost always denaturing. Zwitteronic detergents include a heterogeneous group of compounds, which in general are more inactivating than non-ionic detergents. Polyoxyethylene and other non-ionic detergents are relatively mild, and are assumed to have little effect on important 3D structural features (Maire *et al.,* 2000). Steroid-based compounds, such as 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), form another class of detergents, and are also relatively "mild". The use of non-ionic detergents quite often leads to less inactivation of membrane-bound proteins (Li *et al.*, 1997; Maire *et al.,* 2000).

Nevertheless, the choice of an appropriate detergent remains a difficult task for purifying the desired target proteins. Specific detergents might be best for individual target proteins and concentrations might need to be adjusted empirically. For "solubilising" (1→3)-β-glucan synthase from membrane preparations, CHAPS has been widely used and has proven to be efficient for a number of plant membrane-bound proteins (Fredrikson and Kjelbom, 1991; Merikle *et al.,* 1991; Wu and Wasserman, 1994; Bulone *et al.*, 1995; Li *et al.*, 1997; McCormack *et al.*, 1997; Him *et al.,* 2001; Pelosi *et al.,* 2003). For example, Bulone *et al.* (1995) used CHAPS to disperse membrane preparations of Italian ryegrass (*Lolium multiflorum*), and this allowed considerable purification of callose synthase

activity by subsequent product entrapment techniques. Further evidence to support CHAPS for purification of callose synthase was provided by a comparative study on pollen tubes of *Nicotiana alata* (Li *et al.*, 1997). A range of non-ionic and Zwitteronic detergents was used and it was found that CHAPS increased the callose synthase activity in pollen tube membranes by more than 10 fold, but only 3-4 fold increases were observed with other detergents. Thus, in this study, CHAPS was chosen in preference to other detergents as an agent for (1→3)-β-glucan synthase purification from barley suspension cultures.

It is also worth emphasising that dispersal of plant membranes into micelle or small vesicles through detergent treatment carries a number of the features that might ultimately limit the success of the purification procedure. For example, small vesicles might entrap soluble cytoplasm proteins which could be carried through into the final, "purified" fraction of membrane-bound protein. In addition, abundant membrane-bound proteins might be distributed throughout micelles and small vesicle bilayers, together with the protein of interest. Because the proteins are physically trapped within these lipid structures, purification of a single membrane-bound protein may be extremely difficult, or impossible (Fink *et al.*, 1990; Delmer *et al.*, 1991; Dhugga and Ray, 1991; Driouich *et al.*, 1993; Bulone *et al.*, 1995; Kudlicka and Brown, 1997; Li *et al.*, 1997; McCormack *et al.*, 1997; Him *et al.*, 2001).

Cellular disruption is the first step in preparing membranes, prior to protein purification. Appropriate disruption treatments should be chosen carefully and applied in such a way to efficiently release the protein of interest in its active form (Maire *et al.*, 2000). Tissue disruption can be conducted in various ways, including osmotic shock, sonication, forcing the cells through a small orifice (French press), or mechanical grinding. After cellular disruption, the homogenate contains a variety of membrane-bound particles, each with a distinctive size, shape, charge and density. These can be separated by ultracentrifugation at an appropriate sedimentation force. For membrane preparation, the homogenate is usually subjected to between 80,000 and 150,000×g centrifugation, to pellet microsomes and small vesicles. Higher resolution separation is achieved using sucrose gradient sedimentation. When centrifuged, different cell components separate into distinct bands

according to their densities, sizes and shapes, and can be collected individually for further analysis (Claude, 1975; de Duve and Beaufay, 1981).

These methodologies have been applied to the purification of plant (1→3)-β-glucan synthases, (1→4)-β-glucan synthases and (1→3,1→4)-β-glucan synthases, with modifications for each individual enzyme, tissue and species (Gibeaut and Carpita, 1990; 1993; 1994; Tuner *et al.*, 1998; Li *et al.*, 1999). Although none of the synthases has been purified to homogeneity, the methodologies are useful tools for obtaining considerable enrichment of these enzymes, and provide valuable biological information on enzymic properties. In an effort to purify (1→3)-β-glucan synthase from barley tissues, various approaches for cellular disruption, membrane fractionation, solubilisation and enrichment for synthase activity were tested. The results are presented in this Chapter.

5.2 Materials and Methods

5.2.1 Materials

Barley suspension cultures were kindly supplied by Dr. E. Newbigin, Plant Cell Biology Research Centre (CBRC), School of Botany, and the University of Melbourne, Australia. The French pressure cell was from Aminco Bowman Corp. (Silver Spring, MD). UDP-glucose, CHAPS, and MOPS were purchased from Sigma (Australia). Pre-cast 4-20% Tris-glycine gels were from NOVEX, USA. Protein Rainbow markers (250 kDa-blue, 160 kDa-red, 105 kDa-green, 75 kDa-yellow, 50 kDa-purple, 35 kDa-blue, 30 kDa-orange, 25 kDa-green, 15 kDa-blue, 10 kDa-red), and UDP-D-[6-^{3}H] glucose with specific activity of 29.0 Ci/mmol were from Amersham International, UK. Laminarinase [endo-(1→3)-β-D-glucanase, from *Trichoderma* sp.), Lichenase [endo-(1→3,1→4)-β-D-glucanase, from *Bacillus subtilis*), cellulase (endo-1→4-β-D-glucanase, from *Trichoderma longibrachiatum*) were from Megazyme, Australia. Aniline blue fluorochrome (ABF) was generously provided by Professor Bruce Stone, School of Biochemistry, La Trobe University, Bundoora, Australia. The protein purification work described in this Chapter was mainly conducted in Professor Stone's laboratory.

5.2.2 Microsomal Preparations

All the steps for protein extraction described hereafter were carried out at 4°C, unless otherwise stated.

5.2.2.1 Barley Coleoptiles, Young Leaves and Shoots

Barley grain cv. Sloop (50 g) was imbibed in 2 l of aerated deionised water at 20°C for 24 h, and sown evenly onto 2.5 l of saturated vermiculite in a tray. Another 1.5 l of saturated vermiculite was overlayed onto the seedbed and pressed firmly. The tray was covered loosely with foil, and incubated in darkness at 20°C for 3 days. The barley coleoptiles, first leaves excised from the barley shoots, as well as whole shoots, were harvested and snap frozen in liquid nitrogen. The frozen materials were mixed with pre-chilled homogenisation buffer of 100 mM MOPS-NaOH buffer, pH 7.5, containing 2 mM EDTA and 2 mM EGTA at a ratio of 1 ml per gram tissue, and disrupted three to four times in a French pressure cell at 60 Mpa.

The homogenate was centrifuged at 20,000×g for 10 min and the supernatant filtered through two layers of Miracloth. To obtain the microsomal fraction, the filtrate was subjected to centrifugation at 100,000×g in a Beckman Ultracentrifuge (Ti 60 rotor) for 1 h. The resulting pellet contained microsomes and small vesicles, and was termed the microsomal fraction. An aliquot of 50 µl in homogenisation buffer was set aside for activity assays.

5.2.2.2 Barley Suspension Cultures

Eight 50 ml flasks of barley suspension cultures grown to the midlog-phase were harvested, washed three times with ice cold water through a filtration unit and suspended in a 100 mM MOPS-NaOH buffer, pH 7.5, containing 2 mM EDTA and 2 mM EGTA at a ratio of 1 ml per gram cell mass. The microsomal fraction was prepared according to the procedure described in Section 5.2.2.1.

5.2.3 CHAPS and $CaCl_2$ Treatments

To produce the CHAPS "solubilised" membrane fraction, microsomal pellets were resuspended at approximately 5 mg/ml in homogenisation buffer with the addition of 0.6% (w/v) CHAPS and gently stirring for 30 min. A small portion of the pellet was set aside for activity assays (Section 5.2.2). The suspension was pelleted at 100,000×g for 1 h. The supernatant, designated the CHAPS solubilised microsomal fraction, was collected. A small portion was set aside for concentration determinations and activity assays.

The CHAPS-extract was treated with $CaCl_2$ to a final concentration of 16 mM, incubated at 4°C for 30 min, and pelleted at 13,000×g for 15 min. This step is believed to remove proteinaceous material, such as inhibitors, from the CHAPS-extract, with the expectation of producing a fraction with enhanced activity (Bulone *et al.*, 1995). The supernatant was stored in aliquots at -80°C for protein determinations, activity assays, and Western analysis.

To test the effectiveness of the $CaCl_2$ precipitation, the CHAPS solubilised fraction without $CaCl_2$ treatment was also subjected to the sucrose gradient centrifugation, using the method described below.

5.2.4 Sucrose Gradient Fractionation

A continuous linear sucrose (10-60% w/v sucrose) density gradient cushion (10 ml) in 100 mM MOPS buffer, pH 7.5 was made by filling the centrifuge tube with a progressive gradient mixture of solutions A: 100 mM MOPS buffer, pH 7.5, containing 10% w/v sucrose and B: 100 mM MOPS buffer, pH 7.5, containing 60% w/v sucrose (Crawford and Beckerle, 1991).

Supernatant from the $CaCl_2$-treated microsomal homogenate (1 ml) was overlayed on a 10 ml sucrose density gradient cushion, and centrifuged at 100,000×g for 3 h. After centrifugation, a series of aliquots (1 ml) were carefully collected from the top of the centrifuge tube. The fractions were named F1 to F10. Protein concentrations (Bradford, 1976) and enzyme activity were determined as described below.

The sequential procedure for (1→3)-β-glucan synthase enrichment from barley suspension cultured-cells is summarised in Figure 5.1.

5.2.5 (1→3)-β-Glucan Synthase Activity Assay

An aliquot (50 µl) from each fraction of the callose synthase preparation was assayed in a total volume of 200 µl 50 mM MOPS-NaOH buffer, pH 7.5, containing 20 mM cellobiose, 8 mM $CaCl_2$, 1.0 mM UDP-glucose, and 0.15 µCi UDP-D-[6-^{3}H] glucose with a specific activity of 29.0 Ci/mmol. Assays were performed in duplicate at room temperature. Reactions were stopped after 30 min by heating to 100°C for 5 min (Stone and Clarke, 1992).

(1→3)-β-Glucan synthase activity was determined by measuring the incorporation of radioactive glucose into 66% (v/v) ethanol-insoluble products (Smith and Stone, 1973b). To measure incorporated glucose, the reaction mixture was applied to glass fibre filters (Whatman, 25 mm, GF/C), which were washed successively with 5 ml 66% ethanol, 5 ml chloroform: methanol (2:1, v/v), and 5 ml absolute ethanol. The filter paper carrying newly synthesised polymers was placed in a scintillation vial and dried under a lamp. Finally, scintillant (3 ml) containing 0.5% PPO (diphenyl oxazole) and 0.03% POPOP {p-

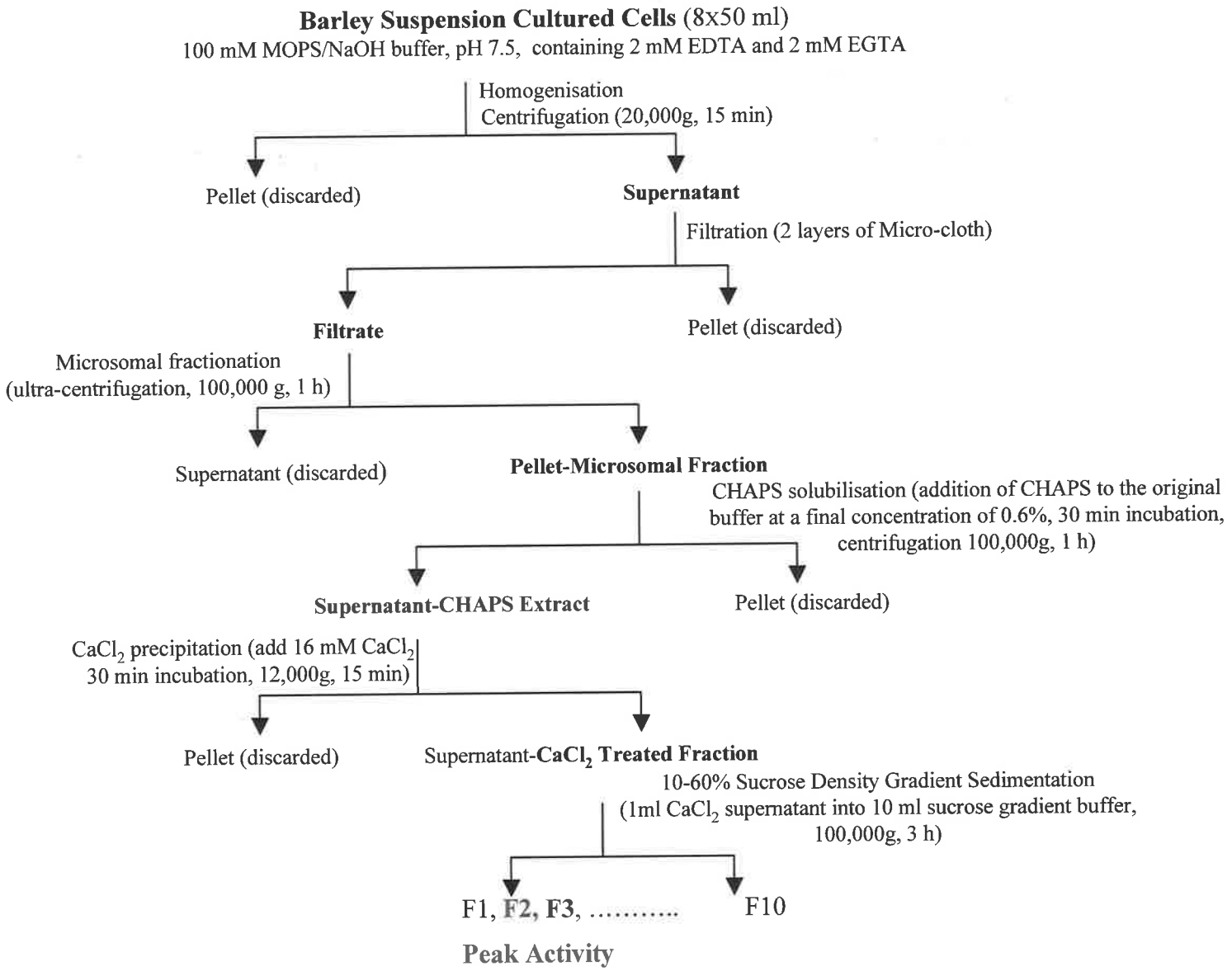


Figure 5.1 Summary of procedures for (1→3)-β-glucan synthase enrichment from barley suspension cultured-cells.

bis[2-(5-Phenyloxazolyl)]} was added to each vial for the scintillation counting (Beckman LS 6000 Scintillation Counter). The readings were recorded in duplicate.

5.2.6 SDS-PAGE Analysis

SDS-PAGE analysis was conducted on each fraction, including the CHAPS solubilised fraction, the $CaCl_2$ precipitated fraction, and sucrose gradient fractions F1 to F10, using 4% to 20% Tris-glycine gradient gels and run under conditions described in Section 4.2.5.

5.2.7 Product Characterisation by Agarose Gel System

To examine the linkage type in polysaccharides synthesised by the active fractions, the crude extract from the CHAPS solubilised microsomal fraction was initially chosen as a starting material for *in-vitro* callose synthesis. Products of the (1→3)-β-glucan synthase assays were subjected to hydrolysis with a purified (1→3)-β-glucan endohydrolase, in which a heat-inactivated enzyme sample was used as a negative control. An aliquot of the extract (50 µl) was heat-inactivated by boiling for 5 min (denoted as Sample 1), which meant that no new synthesis was expected in this tube. Sample 2 and Sample 3 were 50 µl aliquots of the extract in which substrate mix (Section 5.2.5) was added, the synthesis was carried out by incubation at room temperature for 30 min, and terminated by heating to 100°C for 5 min. For hydrolysis of newly synthesised polysaccharides, 200 mM sodium acetate buffer (15 µl), pH 5, was added to both Samples 2 and 3, and endo-(1→3)-β-D-glucanase (1U) was added to Sample 3 only. Samples 2 and 3 were subjected to 30 min incubation at 37°C. Prior to agarose gel analysis, 10 M NaOH (7 µl) was added to each tube, and the mixture was concentrated to 20 µl using the Speedivac. Products were loaded on a 0.3% agarose gel containing 50 mM NaOH and 0.0005% aniline blue fluorochrome (ABF), and separated in 50 mM NaOH at 120 mA for 2 h. The gel was viewed under UV light for fluorescence (Evans, 1984). In this experiments, Samples 1, 2 and 3 were carried out in duplicate. A positive control of curdlan was used for florescence detection, while a blank lane containing loading buffer was used for background comparison.

5.2.8 Detection of (1→3)-β-Glucan Synthase Activity in Non-Denaturing Gels

Polypeptide components involved in (1→3)-β-glucan synthesis could be identified by resolving the active extract by native PAGE. During electrophoresis, the proteins were separated according to their size and charge. After electrophoresis, a substrate mix was applied to the native gel, and incubated for in-gel (1→3)-β-glucan synthesis. The CHAPS extract and F2 fraction of the sucrose density gradient were chosen for in-gel activity detection. The regions contributing to (1→3)-β-glucan synthesis were detected using the ABF.

Two aliquots (50 µl) of the CHAPS or F2 fractions, one of which was heat inactivated, were mixed with 10 µl 5×native loading buffer (1×native loading buffer: 100 mM Tris-HCl buffer, pH 8.6, containing 10% v/v glycerol and 0.0025% w/v Bromophenol Blue), and loaded on a pre-cooled (4°C) 4% to 20% Tris-glycine gradient gel. The gel was run at 4°C at a constant current of 20 mA for 4.5 h in 25 mM Tris-HCl buffer, pH 8.3, containing 192 mM glycine. Rainbow protein markers covering sizes up to 250 kDa were used for monitoring during the electrophoresis.

For the in-gel activity assay, the native gel was washed in 50 mM MOPS buffer, pH 7.5, at 4°C for 15 min with gentle shaking, and incubated with the substrate mixture of 50 mM MOPS buffer, pH 7.5, containing 20 mM cellobiose, 8 mM $CaCl_2$, and 1.0 mM UDP-glucose (non-radio-labelled) at room temperature for 2 to 16 h. To detect (1→3)-β-glucan in-gel synthesis, the gel was rinsed with water several times and stained with 0.0005% ABF for 15 min. After several rinses with water, the gel was exposed to UV-light to detect fluorescent bands.

5.2.9 Hydrolysis of In-Gel Product by Laminarinase, Cellulase and Lichenase

The products synthesised in-gel were subjected to enzymic hydrolysis. Three types of hydrolases, laminarinase (endo-1→3-β-D-glucanase, from *Trichoderma* sp.), lichenase (endo-1→3,1→4-β-D-glucanase, from *Bacillus subtilis*), and cellulase (endo-1→4-β-D-glucanase, from *Trichoderma longibrachiatum*) were chosen to digest the synthesised products. The gel was stained with ABF and fluorescence examined. The hydrolysis was only performed on the CHAPS-extract rather than on the more enriched fraction F2. This

was limited by the small amount of (1→3)-β-glucan synthase in F2 that could be practically loaded on to a gel.

Native gel electrophoresis and the in-gel synthesis were conducted following the same procedure as described in Section 5.2.8. Five gel pieces containing the synthesised products were excised from the gel. A control gel piece in which no UDPGlc or other cofactors were added, and which was not stained with ABF, was also examined. The gel pieces were placed in test tubes containing 100 µl 50 mM sodium acetate buffer, pH 4.8, hydrolase (1 U) was added and incubated at 37°C for 4 h. Another control was a gel piece that had undergone the same treatment without the hydrolase enzyme. After digestion, gel pieces were rinsed three times with MilliQ water, stained in 0.0005% ABF for 30 mins, and rinsed again with MilliQ water. All gel pieces were placed on the same glass plate for examination of callose/ABF complexes under UV light.

5.3 Results and Discussion

5.3.1 Membrane Preparations

For callose synthase purification, membrane preparations from barley coleoptiles, first leaves and barley 3-day-old shoots were chosen, because levels of the *HvGSL1* mRNA were high in these tissues (Sections 3.3.3.1 & 3.3.3.3). The expression patterns are in agreement with the literature (Stone and Clarke, 1992), where it has been shown that callose deposition occurs in rapidly growing cells such as the coleoptile and young leaves. These tissues have also been used for other β-glucan synthases, in particular, for examination of (1→3)-β-glucan synthases from maize and barley (Gibeaut and Capita, 1990; 1993; 1994; Becker *et al.*, 1995).

It is also worth mentioning here that assay conditions for callose synthesis need to be carefully optimised. These conditions have been reviewed in detail by Stone and Clarke (1992) for plant systems, and further vigorously examined by Him *et al.* (2001). The latter study indicated that the optimal reaction mixture for the particular membrane preparation consisted of 50 mM UDP-glucose, an activator such as cellobiose, and one or several bivalent cation such as Ca^{2+}. The pH is maintained in the range of 7-8. This group also emphasised the need for careful selection of the detergent used to 'solubilise' the callose synthase preparation (Him *et al.*, 2001).

Membrane fractions from barley coleoptiles, first leaves and shoots showed callose synthase activity. Table 5.1 compares total protein and callose synthase activity from these three tissues. After high force sedimentation of microsomal particles, callose synthase activity was almost exclusively detected in the microsomal fraction. The membrane fraction from the coleoptiles yielded a markedly higher activity (17.7 nmol glucose incorporated/mg protein) than these of first leaves (3.57 nmol/mg) and shoots (3.94 nmol/mg). Solubilising these membrane fractions with 0.6% CHAPS and subsequent centrifugation resulted in inactivation of the callose synthases. It was believed that CHAPS itself should have no detrimental effect on activity (Bulone *et al.*, 1995; Maire *et al.*, 2000). Nevertheless, the concentration of CHAPS, the conditions such as the time and buffer for solubilisation, and the parameters for the subsequent centrifugation could have been contributing factors in causing inactivation. It was

therefore concluded that the production of an active enzyme preparation from barley coleoptiles, young leaves or shoots would require considerably more work, and would probably be a difficult task to accomplish in a limited time period.

The second approach to produce membrane fractions for callose synthase purification was starting with barley suspension cultured-cells. Suspension cultured-cells have proved to be a rich source of callose synthases (Meikle *et al.*, 1991; Rodgers and Bolwell, 1992; Boulone *et al.*, 1995; McCormack *et al.*, 1997). A microsomal fraction from suspension-cultured barley cells was obtained following the sedimentation of cell homogenates prepared with the French Pressure cells.

As shown in Table 5.1, a typical membrane preparation yielded a much higher specific activity of 73.9 nmol/mg for (1→3)-β-glucan synthesis, which was 4-, 19- and 21-fold greater than in extracts from coleoptiles, shoots and first leaves, respectively. The solubilisation of the membrane fraction with 0.6% CHAPS produced a supernatant with a specific activity of 210 nmol/mg (Table 5.2), a further 3-fold enrichment compared with the microsomal fraction. Thus, the microsomal preparation from barley suspension cultures was deemed to be the best starting material for (1→3)-β-glucan synthase purification.

5.3.2 *Effect of $CaCl_2$ Precipitation*

Applying the $CaCl_2$ precipitation procedure to the CHAPS-extract of membranes from the barley suspension cultures resulted in an increase in callose synthase activity of 22%. As shown in Table 5.2, once the CHAPS solubilised fraction was precipitated with $CaCl_2$, the specific activity increased from 632 nmol/mg protein to 773 nmol/mg. Although this increase did not seem to be particularly high, a pronounced increase was observed at the next stage when the supernatant was subjected to sucrose gradient sedimentation, as detailed in the following section.

5.3.3 *Sucrose Gradient Fractionation*

The fractions from $CaCl_2$-treated and non-treated membrane extracts were separated by a linear sucrose gradient centrifugation. Ten fractions collected from the sucrose gradient after centrifugation, designated F1 to F10, and were assayed for callose synthase activity.

Table 5.1 Comparison of callose synthase specific activity of membrane fraction extracted from barley tissues and barley suspension culture cells. Material from coleoptiles, first leaves and shoots (all 3 days after germination), and barley suspension culture cells were treated under the same conditions for membrane fractionation (Section 5.2.2). Callose synthase activity was measured according to Smith and Stone (1973 b).

Plant material		Protein (mg/ml)	Activity (nmol/50µl)	Specific activity (nmol/mg)
1.	Coleoptile	5.4	4.79	17.7
2.	First young leaves	10.2	1.82	3.57
3.	Shoots	14.0	2.75	3.94
4.	**Barley suspension culture cells**	**2.8**	**10.17**	**73.9**

Table 5.2 Callose synthase enrichment from barley suspension cultured-cells. The protein concentration, average (nmol/50 µl) and specific activity (nmol/mg) from crude lysate, CHAPS solubilised, and $CaCl_2$ precipitation are presented. The specific activity of the callose synthase increased approximately 14-fold, as highlighted in purple.

Purification step	Protein Concentration mg/ml	Activity nmol/50µl #1	Activity nmol/50µl #2	Activity nmol/50µl Average	**Specific Activity nmol/mg**	**Purification Fold**
1. Crude lysate	3.5	9.13	10.69	9.91	**57**	**1**
2. CHAPS solubilised fraction	1.6	49.32	51.88	50.60	**632**	**11**
3. $CaCl_2$ precipitation	1.1	43.72	41.35	42.54	**773**	**14**

Table 5.3a & 5.3b lists callose synthase activity and protein concentrations from the sucrose gradient fractions, with and without prior $CaCl_2$ precipitation. The sucrose gradient profiles are further illustrated in Figures 5.2 and 5.3. The protein concentration was highest in fraction F1 in both cases, and gradually decreased towards the bottom of the centrifuge tube. The peak of callose synthase activity was detected in fraction F3, whereas there was virtually no activity in fraction F1. The removal of approximately 65% total protein in the first fraction from the most active fractions F2, F3, and F4, led to a further 5-fold enrichment in callose synthase activity.

In summary, the purification procedure led to a final enrichment in callose synthase activity of approximately 60-fold, compared with that of the original cell lysate (Figure 5.4). Each purification step of this purification procedure, from crude microsomal extraction, CHAPS solubilisation, $CaCl_2$ precipitation to sucrose gradient centrifugation, led to increased specific activity. In particular, the $CaCl_2$ precipitation aided in the removal of proteases and impurities caused by proteolysis during the course of the extraction, and was later found to be a crucial purification step (Chapter 6).

5.3.4 Characterisation of the Product Synthesised by Partial Purified Fractions

A range of analyses including aniline blue staining and digestion with hydrolases that are specific for different linkage types of polysaccharides was used for the characterisation of polysaccharide products of the putative callose synthase reactions.

5.3.4.1 Product Characterisation in Agarose Gels

The CHAPS-extract from barley suspension cultured-cells was used for *in vitro* synthesis of (1→3)-β-glucan. Curdlan, a (1→3)-β-glucan from *Agrobacterium* sp. *biovar* (Stasinopoulos *et al.,* 1999), was loaded in the agarose gel containing the ABF, a compound purified from aniline blue (Sirofluor) dye, which gives greater specificity for (1→3)-β-glucan binding in aqueous solution (Evens *et al.,* 1984). This lane was used as a positive control (Figure 5.5), and shows a bright fluorescence band under UV light. The heat-inactivated fraction had no fluorescence (samples 1 and 1'). The active fraction exhibited fluorescent bands (lanes 2 and 2'). In addition, the product was subjected to laminarinase digestion (samples 3 and 3') and hydrolysis led to the disappearance of the

Table 5.3 Protein concentration and callose synthase activity of sucrose gradient fractions. **a:** The CHAPS membrane fraction was treated with $CaCl_2$ and subjected to sucrose gradient centrifugation. **b:** The CHAPS membrane fraction was subjected to sucrose gradient centrifugation without the $CaCl_2$ treatment. The specific activity of callose synthase (nmol glucose incorporated per mg protein) was highest in fraction F3, as highlighted in red. The protein and activity profiles are presented graphically in Figures 5.2 and 5.3 according to the data list in these tables.

Table 5.3a ($+Ca^{2+}$)

Fraction number	Protein concentration mg/ml	Activity (average) nmol/50µl	Specific activity nmol/mg
F1	0.32	0.38	24
F2	0.09	10.48	2331
F3	0.04	6.58	3293
F4	0.015	1.76	2342
F5	0.005	0.28	1140
F6	0.005	0.10	397
F7	0.005	0.10	392
F8	0.005	0.10	396
F9	0.005	0.11	444
F10	0.005	0.09	342

Table 5.3b ($-Ca^{2+}$)

Fraction number	Protein concentration mg/ml	Activity (average) nmol/50µl	Specific activity nmol/mg
F1	0.47	2.68	114
F2	0.22	14.44	1312
F3	0.14	19.71	2815
F4	0.09	3.33	741
F5	0.04	0.08	40
F6	0.015	0.15	198
F7	0.01	0.08	151
F8	0.01	0.21	422
F9	0.01	0.08	165
F10	0.01	0.04	84

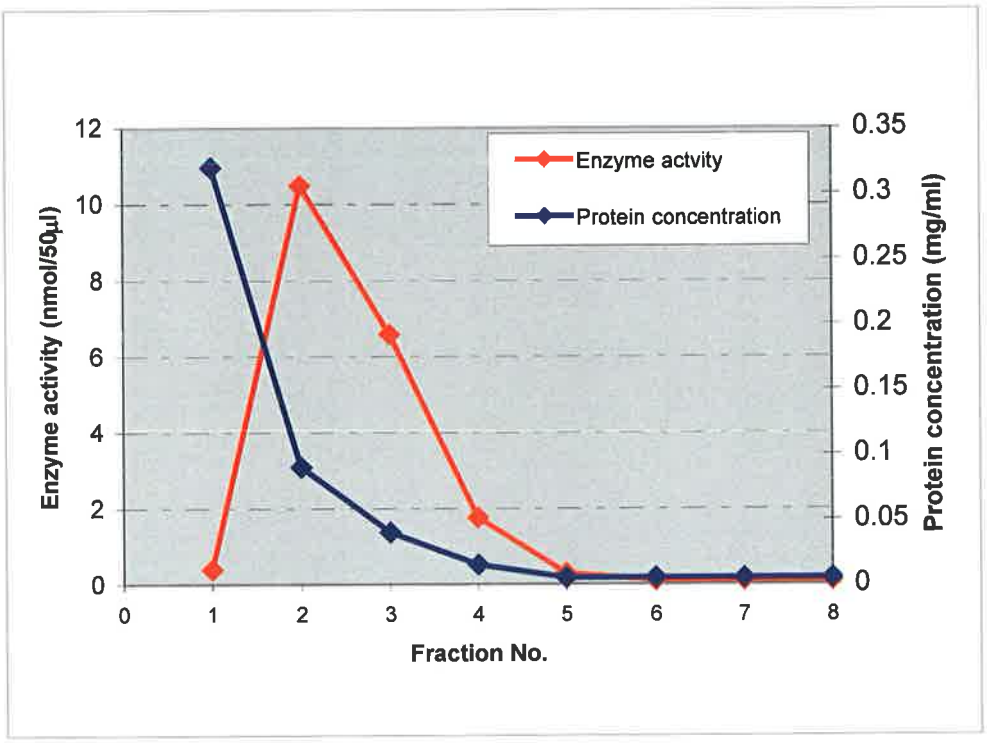


Figure 5.2 Profiles of enzyme activity and protein concentrations of sucrose gradient fractions separated using the CHAPS solubilised membrane fraction treated with $CaCl_2$. The supernatant of the CHAPS fraction precipitated with $CaCl_2$ was overlayed onto a linear sucrose gradient (10-60% w/v). Following high-speed centrifugation, the gradient was fractionated into 10 fractions, designated F1 to F10, and each fraction was subjected to protein concentration determination and activity assays (Section 5.2.5). The specific activity of the substrate UDP-D-[6-^{3}H] glucose used in activity assay was 10-30 Ci/mmol. The protein content (mg/ml) and activity (nmol/50µl) profiles of the fractions are presented as the blue line, and orange line, respectively.

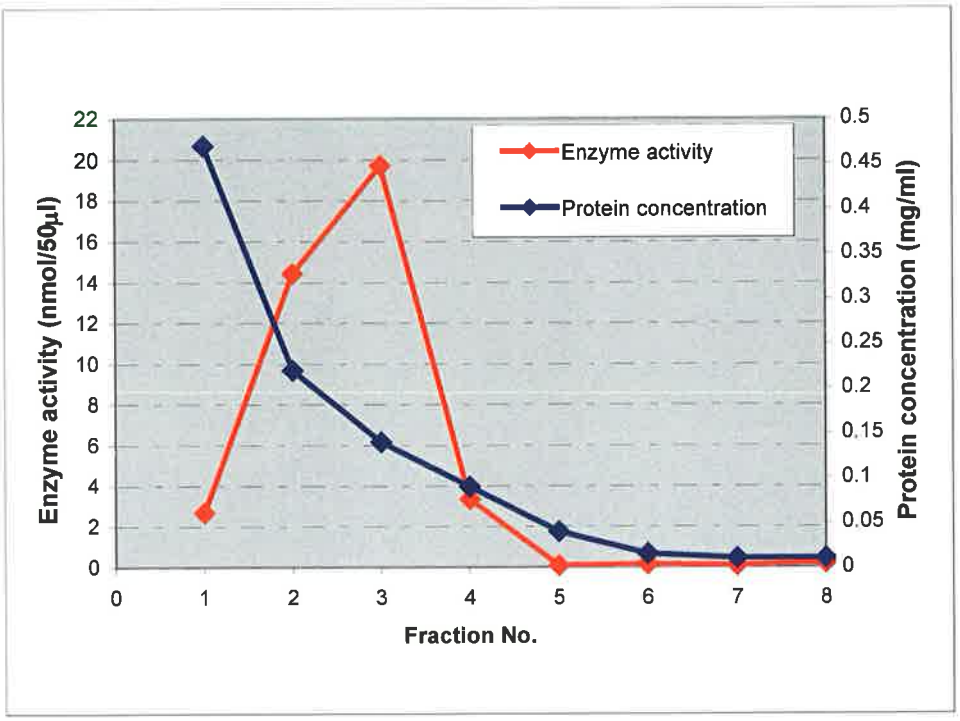
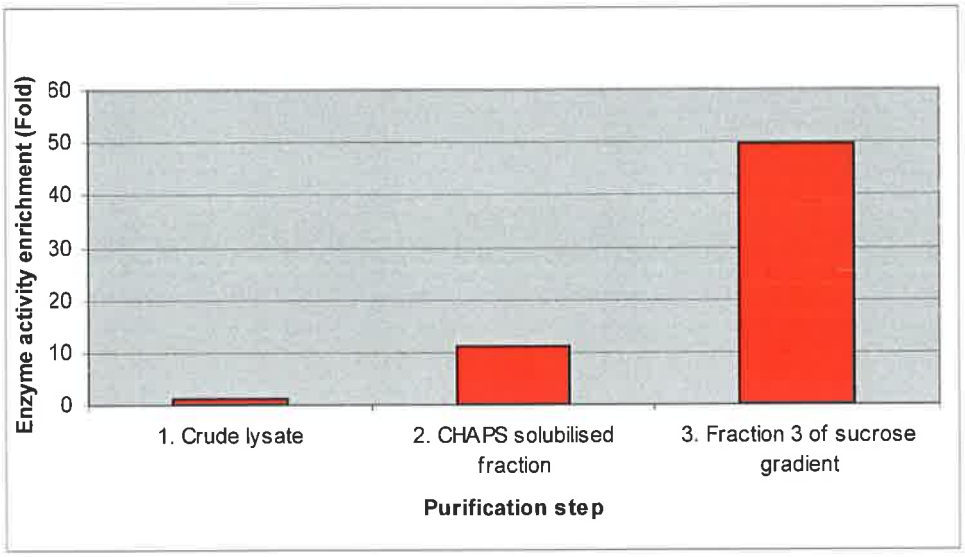


Figure 5.3 Profiles of enzyme activity and protein concentrations of sucrose gradient fractions separated using the CHAPS solubilised membrane fraction without $CaCl_2$ treatment. The CHAPS solubilised membrane fraction was directly applied to a linear sucrose gradient (10-60% w/v sucrose) without $CaCl_2$ treatment for comparison with these of $CaCl_2$ treated samples (Figure 5.2). The sedimentation procedure was conducted in the same way as described in Figure 5.2. The protein content (mg/ml) and activity (nmol/50µl) profiles of the fractions are presented in blue and in orange, respectively.

A) -Ca^{2+}



B) +Ca^{2+}

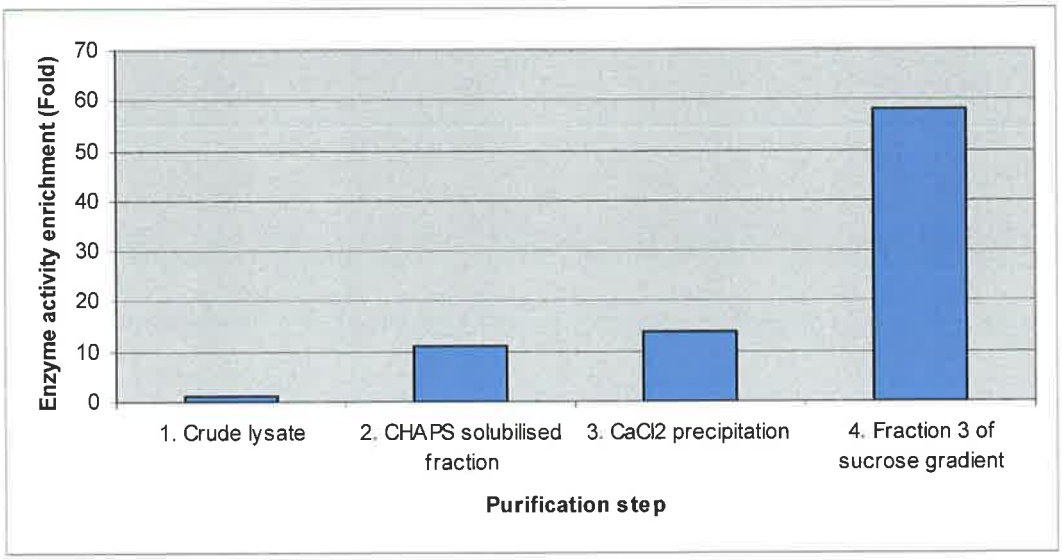


Figure 5.4 Comparison of callose synthase activity purification with and without $CaCl_2$ precipitation. **A)** Purification procedures included microsomal extraction, CHAPS solubilisation and sucrose gradient fractionation. **B)** An extra purification step, using $CaCl_2$ precipitation, was conducted before the CHAPS-extract was subjected to sucrose gradient centrifugation. Note that with this change the final enrichments in callose synthase of the fractions with and without $CaCl_2$ precipitation were 58-fold and 50-fold, respectively. The significance of the inclusion $CaCl_2$ precipitation is discussed in Section 5.3.2.

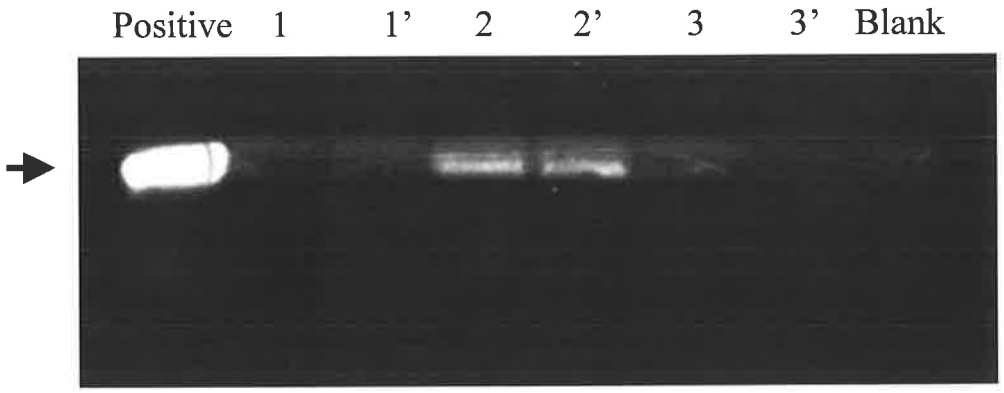


Figure 5.5 Product characterisation by the agarose gel. The CHAPS solubilised fraction was used as a source for *in vitro* glucan synthesis in test tubes. The products being synthesised were either directly loaded onto a 0.3% agarose gel containing 0.0005% ABF for fluorescence detection of (1→3)-β-D-glucan, or were subjected to (1→3)-β-D-glucanase hydrolysis before loading onto the gel (Section 5.2.7). Sample 1 (duplicates 1 & 1'): heat-inactivated CHAPS fractions, no synthesis occurred; Sample 2 (duplicates 2 & 2'): (1→3)-β-D-glucan was synthesised by active fractions, seen as fluorescent bands; Sample 3 (duplicates 3 & 3'): the synthesised (1→3)-β-D-glucan by the active fractions was digested by (1→3)-β-D-glucanase, and showing no fluorescence. "Positive" refers to curdlan, a (1→3)-β-D-glucan that was used as a positive control; "Blank" refers to loading buffer only.

fluorescence. The observation indicated that the product being synthesised by the CHAPS extract was indeed (1→3)-β-glucan.

5.3.4.2 Product Characterisation in Native PAGE Gels

In-Gel (1→3)-β-Glucan Synthesis Detected by ABF: Products synthesised by the CHAPS microsomal fraction and the F2 fraction from the sucrose gradient centrifugation were assessed using a native PAGE gel. The F2 fraction was chosen for further analysis, because it contained considerably more protein than that in the F3 fraction, although the F3 fraction was the most active fraction (Figure 5.3). The in-gel activity of callose synthase was detected by staining the gel with ABF.

As shown in the Figure 5.6, the CHAPS extract and sucrose gradient fraction F2 were both associated with in-gel deposition of ABF-positive material, which is indicative of (1→3)-β-gluan. The presence of smaller amounts of the ABF-positive material in the heat-inactivated CHAPS extracts (Figure 5.6, lane 1A), and to a lesser extent in heat-inactivated fraction F2 (Figure 5.6, lane 2A), is probably attributable to callose synthesised during enzyme purification, or to callose trapped in the synthase complex.

It is worth noting that the active band barely entered the stacking gel after approximately four and half hours electrophoresis, while a protein molecular marker of 250 kDa moved further down from the stacking gel (Figure 5.6). This was also true on a lower percentage gel (3-8%) and suggests that the molecular mass of the synthase complex must be relatively high, in agreement with the results reported from peas (Dhugga and Ray, 1994) and in hypocotyl extracts from mung bean (Kudlicka and Brown, 1997), where the active synthase complex just entered the stacking gel following prolonged electrophoresis. It is likely that the active enzyme exists as a liposome-associated complex, possibly in non-covalent association with other proteins that may or may not be required for activity.

The ABF:(1→3)-β-Gluan Complex Inhibits Enzyme Hydrolysis: An assessment as to whether ABF staining would affect the hydrolysis was conducted. (1→3)-β-Glucan hydrolysis was carried out with the gel piece either before or after ABF staining. As observed in Figure 5.7, the fluorescence was markedly decreased in the gel piece in which (1→3)-β-glucan hydrolase was applied before the gel was stained with the ABF, whereas

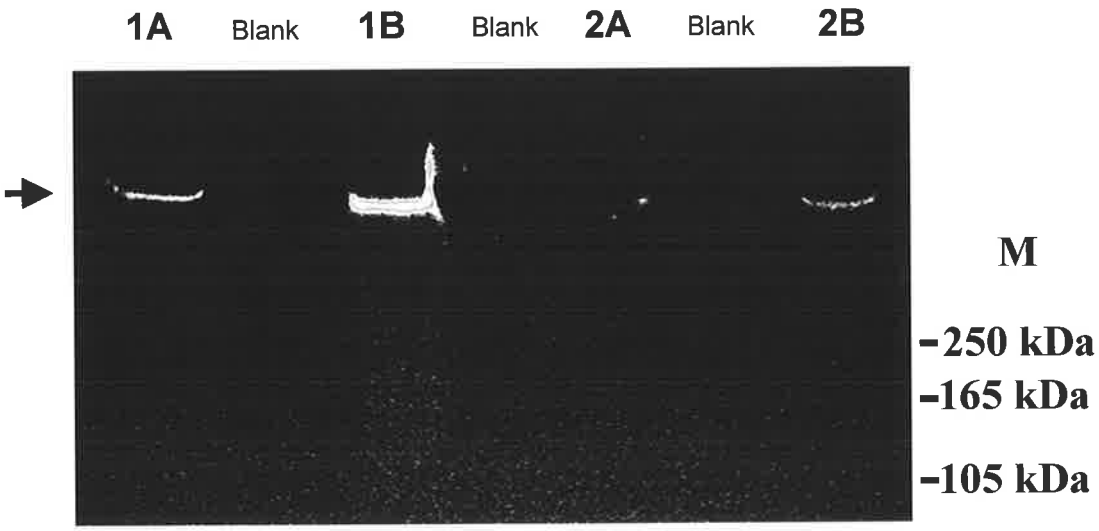


Figure 5.6 Assay of callose synthase activity in non-denaturing gels. Four fractions were run on 4-20% Tris-glycine gel under native conditions, incubated with UDPGlc, stained with ABF, and observed under UV light. 1A: CHAPS-extract heat inactivated prior to loading (50 µl); 1B: active CHAPS-extract (50 µl); 2A: sucrose gradient F2 heat inactivated prior to loading (50 µl); 2B, active sucrose gradient F2 (50 µl). M: protein molecular markers. "Blank" indicates lanes in which no protein was added.

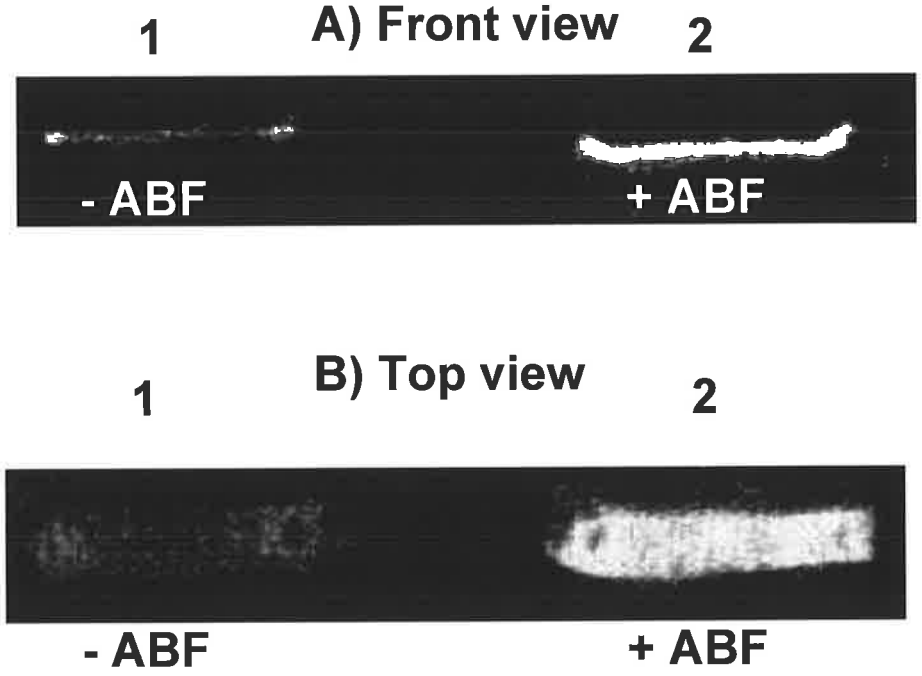


Figure 5.7 Enzymic hydrolysis inhibited by ABF staining. (1→3)-β-D-Glucan and the aniline blue fluorochrome form a strong fluorescent complex, which inhibits enzymic digestion of the polymer by (1→3)-β-D-glucan endohydrolase (laminarinase). Two aliquots of 50 µl CHAPS-extract were separated on a native gel and incubated with the substrate mix for in-gel (1→3)-β-D-glucan synthesis as described in the text. The gel slice was either hydrolysed by laminarinase prior to ABF staining (lane 1) or after ABF staining (lane 2), and viewed under UV-irritation with ABF staining. **A)** Front view of the gel slices. **B)** Top view of the gel slices.

the fluorescence remained strong in the gel piece where hydrolysis occurred after the gel was stained. This result suggests that the (1→3)-β-glucan-aniline blue fluorochrome complex has an inhibitory effect on the enzyme hydrolysis. The interaction between ABF and (1→3)-β-glucan may interfere with enzyme binding, so that enzyme accessibility to the (1→3)-β-glucan chain was diminished.

5.3.4.3 In-Gel Product Hydrolysis

As shown in Figure 5.8, the greatly decreased intensity of ABF staining of in-gel callose synthase products after hydrolysis with laminarinase (lane 2) confirmed that the products synthesised in the gel by the active fraction were (1→3)-β-glucans. This faint band of the laminarinase digest was approximately the same level of intensity as the original fraction (lane 1), which was not used for in-gel synthesis, reflecting the background of the sample. Conversely, samples digested by either (1→3, 1→4)-β-glucan hydrolase (lichenase, lane 3) or (1→4)-β-glucan hydrolase (cellulase, lane 4) had no effect on the intensity of ABF staining. Furthermore, a control sample where the hydrolase was omitted also displayed a strong fluorescence band (lane 5). This indicates that the product did not diffuse out of the gel piece during hydrolysis. The result further supports the conclusion that loss in fluorescence caused by laminarinase (lane 2) was solely due to hydrolysis of the synthase product.

The series of the experiments described above demonstrated that the products synthesised by the enzyme extracts, either in crude or enriched fractions, were (1→3)-linked glucans, as shown by specific ABF staining and (1→3)-β-glucan endohydrolase hydrolysis. Thus, the fractions used in these experiments, which were extracted from barley suspension culture cells, contained enzymes that catalyse (1→3)-β-glucan synthesis.

Figure 5.8 Product characterisation by enzymic hydrolysis using non-denaturing gels. Enzymic digestion of material deposited in the non-denaturing gel by the putative callose synthase from CHAPS-extract with (1→3)-β-D-glucan endohydrolase (2), (1→3,1→4)-β-D-glucan endohydrolase (3) or (1→4)-β-D-glucanase (4), prior to ABF staining. Piece 1 is a control gel in which no UDPGlc or other cofactors were added. Piece 5 is a control to which no hydrolase was added.

5.4 Summary and Conclusions

The work described in this Chapter demonstrated that (1→3)-β-glucan synthase can be readily enriched from barley suspension cultures, and that activity could be detected in microsomal preparations extracted from barley coleoptile, young leaves and shoots 3 days post germination. However, the latter sources failed to retain activity after CHAPS extraction and subsequent ultracentrifugation. Because of this, suspension cultured cells were chosen for further (1→3)-β-glucan synthase purification. The purification procedure included microsomal isolation, solubilisation using CHAPS, $CaCl_2$ precipitation and sucrose density gradient sedimentation, and resulted in a 58-fold enrichment in (1→3)-β-glucan synthase activity.

The native gel analysis of the active fractions revealed that (1→3)-β-glucan synthase is probably of a large molecular mass, because it barely entered the stacking gel. This implied that the active enzyme exists as a liposome-associated complex, possibly in non-covalent association with other proteins that may or may not be required for activity. Furthermore, the products synthesised either by a relatively crude microsomal fraction (CHAPS solubilised) or the synthase complex resolved on a native gel were characterised by UV fluorescence in the presence of the (1→3)-β-glucan-specific aniline blue fluorochrome. The products could be hydrolysed by laminarinase, a specific (1→3)-β-glucan hydrolase, but not by cellulase [(1→4)-β-glucan hydrolase] or by lichenase [(1→3,1→4)-β-glucan hydrolase]. This physical and enzymic data clearly demonstrated that the product was indeed a (1→3)-linkage glucan.

It is therefore concluded that the extracts from the barley suspension cultured-cells were biochemically active in (1→3)-β-glucan synthesis, and were considered appropriate for immunoprecipitation, immunoasorption, Western blotting and MALDI-TOF (Mass Assisted Laser Desorption Ion-Time Of Flight) analysis in order to establish direct links between the *HvGSL1* cDNA and the active enzyme. These investigations are described in the next Chapter.

CHAPTER SIX:

LINKING AMINO ACID AND NUCLEOTIDE SEQUENCES OF BARLEY (1→3)-β-GLUCAN SYNTHASES

6.1 Introduction

The primary aim of the work described in this thesis was to investigate the molecular basis for (1→3)-β-glucan synthase biosynthesis in barley. The work started with the isolation of yeast (1→3)-β-glucan synthase homologue cDNAs from barley. Following the cloning process, the general properties of the cloned cDNA, *HvGSL1,* were studied, from which transcription patterns of the *HvGSL* genes were consistent with callose deposition patterns reported in the literature. As the work proceeded, polyclonal antibodies and membrane preparations enriched in callose synthase activity were obtained. The combined use of the antibodies and preparations enriched in callose synthase activity provided a route to linking the nucleotide sequence of the *HvGSL1* cDNA with the protein component of the enriched membrane fraction that exhibited (1→3)-β-glucan synthase activity.

There have been no reports of antibodies produced against plant homologues of the yeast FKS protein. In the past, antibodies have been raised against callose synthase membrane preparations and could immunoprecipitate the synthase activity from plant extracts. Immunoprecipitated proteins included a 30 kDa protein from *Lolium multiflorum* (Meikle *et al.,* 1991), 55 kDa and 70 kDa proteins from pea (Dhugga and Ray, 1994), and 65 kDa proteins from mung bean, French bean and developing cotton fibres (Delmer *et al.,* 1993; McCormack *et al.,* 1997). Some of the antibodies co-localise with the callose synthase complex (Delmer *et al.,* 1993). However, the peptides associated with callose synthase activity in the past are all much smaller than the 250 kDa protein predicted from the *HvGSL1* cDNA sequence.

Here the purified antibody preparation against the HvGSL1 polypeptide was used in attempts to immunoprecipitate callose synthase activity. Further, it was used to track the HvGSL1 protein through the purification process. The final putative callose synthase band, which was associated with enzyme activity in the native gels (Chapter 5), was excised from the gel for amino acid sequence analysis. New techniques in mass spectrometry have been developed in recent years that permit the characterisation and measurement of molecules at sensitivities far beyond what could be achieved a decade ago. Specifically, matrix assisted laser desorption-time of flight mass spectrometry (MALDI-TOF-MS) and electro-spray ionisation-MS (ESI-MS) allow direct detection of

proteins in complex mixtures of biological origin (Nilsson and Davidsson, 2000; Dalluge, 2000). These techniques provide gentle ionisation needed for the volatilisation of large molecules, without inducing extensive thermal decomposition of the analytes. The analytical procedure of MALDI-TOF in general is that, following ionisation of the analytes, the ionised molecules are delivered to the mass chambers, and mass spectra of molecules are electronically collected and analysed (Hillenkamp and Karas, 1990). Furthermore, by switching to MS/MS mode, peptides of selected masses can be analysed for amino acid sequence.

In this Chapter, immunoprecipitation, immuno-blot analysis and mass spectrometry have been used to link the nucleotide sequence of the *HvGSL1* cDNA to callose synthase activity.

6.2 Materials and Methods

6.2.1 *Materials*

A rabbit polyclonal antibody preparation was raised using a 17 kDa *E. coli*-expressed fragment (CS17) of the putative barley callose synthase cDNA (Section 4.3.3). The IgG fraction was purified from antiserum using Protein A (Section 4.3.4). Pre-cast 3-8% and 4-20% Tris-glycine gels were from Novex (USA). The protein mass rainbow markers were from Amersham International (UK). Nitrocellulose membranes were obtained from Bresatec (Adelaide, Australia). Protein A-agarose beads, anti-rabbit IgG Horseradish Peroxidase Conjugate, Luminol (5-amino-2, 3-1,4-phtalazinedione) and the rapid silver staining kit were purchased from Sigma. Sequencing grade trypsin was from Promega Corporation (Madison, WI, USA). MALDI-TOF mass spectrometry analysis was conducted by Dr. Peter Hains, Australian Proteome Analysis Facility (Macquarie University, Sydney, Australia).

6.2.2 *Immunoprecipitation using Anti-CS17 Polyclonal Antibodies*

Immunoprecipitation was carried out on active membrane fractions, either on the crude lysate or on the CHAPS-solubilised microsomal preparation from barley suspension cultured-cells. Due to the difficulties in determining an accurate amount of antigen, callose synthase, that was present in the fractions, the polyclonal antibodies and protein A-agarose beads used in the reactions of the immunoprecipitation could only be estimated.

To bind callose synthase antigen in the active fractions, polyclonal antibodies (20 µl-protein A purified, Section 4.3.4) was incubated with either 200 µl crude lysate or 100 µl CHAPS-extract with gently shaking for 2 h at 4°C. To precipitate the antigen-antibody complex, protein A-agarose beads (10 µl suspension) were added to the reaction mix and incubated for a further 3 h at 4°C. After the incubation, the mixture was centrifuged at 3,000×g for 2 min at 4°C to collect the immunoprecipitated complex. The supernatant was set aside for activity assay, whilst the pellet was washed three times with PBS buffer, pH 7.4, containing 0.1% (w/v) CHAPS.

The immunoprecipitation was subsequently evaluated by assaying for callose synthase activity both in the supernatant and in the pellet. A Western blot analysis was performed on the proteins precipitated in the pellet. To assay the callose synthase activity, the washed pellet was resuspended in 50 µl PBS buffer, pH 7.4, containing 0.1% (w/v) CHAPS, and a sample (50 µl) was taken from each of the supernatant and pellet, and assayed under standard conditions (Section 5.2.5). In the Western analysis, the pellet from immunoprecipitation was treated with 50 µl SDS sample running buffer, from which 20 µl was loaded on a 4-20% Tris-glycine gradient gel. The immunoblot was performed as described in Section 4.2.6.2.

6.2.3 SDS-PAGE and Immunoblot Analysis

SDS-PAGE was conducted on 4%-20% Tris-glycine gradient gels (Novex, USA) using the method described by Laemmli (1970). Fractions obtained from various steps of purification, in particular fractions 1-8 from the sucrose gradient centrifugation, were mixed with an appropriate volume of either 2×or 5×SDS sample loading buffer (Section 4.2.5), incubated at room temperature for 2 h, boiled for 5 min, loaded onto a polyacrylamide gel and subjected to electrophoresis at a constant current of 25 mA until the dye-front moved off the gel. After electrophoresis, the gels were stained with 0.2% (w/v) Coomassie Brilliant Blue R-250 (CBB) containing 40% methanol, 10% glacial acetic acid and destained in 30% (v/v) methanol and 10% (v/v) glacial acetic acid.

For immunoblotting, proteins were electrophoretically transferred from non-stained SDS gels to nitrocellulose membranes (Section 4.2.5). The electrophoresis was conducted at a constant current of 180 mA for 16 h to allow complete transfer. Because the protein predicted from the *HvGSL1* cDNA would have a very large molecular mass, longer transfer times were required. Filters were stained with Ponceau S for protein profiling and were blotted with PBS buffer, pH 7.4, containing 2% (w/v) BSA and 0.1% (v/v) Tween-20, and probed with the polyclonal antibody serum or antibodies purified from protein A at an appropriate dilution for 2 h with gentle shaking. After washing three times, anti-rabbit IgG-Horseradish Peroxidase conjugate at 1:3,000 dilution in TBST (TBS containing 0.05% v/v Tween-20, Section 4.2.6.2) was applied to the membranes and incubated at room temperature for a further 2 h. The ECL-plus fluorescence detection system was used for immunochemical detection (Section 4.2.7.4).

6.2.4 Native PAGE and Immunoblot Analysis

The CHAPS-extract and the F2 fraction of the sucrose gradient sedimentation were mixed with an equal volume of native loading sample buffer [100 mM Tris-HCl buffer, pH 8.6, containing 10% (v/v) glycerol and 0.0005% (w/v) bromophenol blue], and loaded onto either a 3-8% NuPAGE Tris-acetate or a 4-20% Tris-glycine gradient gel, and separated under the conditions described in Section 5.2.8.

After electrophoresis, the native gel was treated in a solution containing 1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol at room temperature for 2 h, allowing complete denaturing of the proteins prior to transfer. An identical gel without the denaturing treatment was carried out in parallel. These two gels were transferred to nitro-cellulose membranes and immonoblotting was conducted according to the procedures described above (Section 6.2.3).

6.2.5 MALDI-TOF Mass Spectrometric Analysis

To facilitate identification of the proteins contributing to the (1→3)-β-glucan synthase activity detected in the native gels. MALDI-TOF PMF analysis was employed to generate the peptide mass fingerprints (PMF). This analysis involved digestion of the unknown proteins in the samples with a proteinase of known cleavage specificity. In this study trypsin was chosen for the digestion, and the resulting peptides were analysed by mass spectrometry. The experimentally measured mass data of such proteins was used as the basis of searches against both the tryptic peptide fragments of the HvGSL1 protein, according to theoretical digestions, and a specific protein database search for identification of matching masses.

6.2.5.1 Sample Preparation

The enriched fraction in callose activity F2 was chosen for MALDI-TOF PMF analysis as it was the purest fraction of all preparations. In addition, the CHAPS-solubilised fraction was examined because of its relatively high protein concentration. Both the active fraction F2 and the CHAPS-extract were separated on a 4-20% Tris-glycine gradient gel under native conditions as described in Section 5.2.8. Using the in-gel activity assay, the

regions where (1→3)-β-glucan was detected by ABF were excised from the gel, dried and used for the MALDI-TOF PMF analysis.

6.2.5.2 MALDI-TOF PMF Analysis

The gel piece was washed three times with 120 µl 50% acetonitrile, 25 mM ammonium bicarbonate at 37°C for 10 min, and the solution was removed. The gel piece was dried in the Speedivac and subjected to in-gel tryptic digestion for 16 h, using 8 µl 15 ng/µl sequencing grade trypsin in 25 mM ammonium bicarbonate buffer, pH 7.8 to a gel size equivalent to a 2-D gel spot. The resulting peptides were extracted from the gel with 50% (v/v) acetonitrile, 1% (v/v) TFA. The sample was subjected to ZipTip (C18) clean up, and peptides were eluted from the column in the matrix (8 mg/ml cyano-4-hydroxycinnamic acid in 70% v/v acetonitrile and 1% v/v trifluoroacetic acid) directly onto the MALDI target and air-dried. MALDI-TOF mass spectrometry was performed with a Micromass TofSpec 2E Time of Flight Mass Spectrometer from which a spectra profile of the peptide masses was generated. Using program ProteinProbe from Micromass' ProteinLynx software (MicroMass, Manchester, UK), the peptide profile was subsequently used as the basis of a search against the sequence of the HvGSL1 protein being theoretically digested.

6.2.5.3 Theoretical Tryptic Digestion of the HvGSL1 Protein

The HvGSL1 protein sequence deduced from the *HvGSL1* cDNA (Section 2.3.5) was used as a sequence for tryptic digestion. The theoretical tryptic peptide fragments were generated with a program called "proteins" from the "MassLynx" package (v3.4).

6.2.5.4 Searching MALDI-TOF PMF Data against the HvGSL1 Protein

The theoretically digested sequence was used as the basis of a search, and MALDI-TOF analysis with the Micromass' Proteinlynx Software was used for the identification of matches between the masses generated from MALDI-TOF and peptide fragments of the HvGSL1 protein. In addition to the automated searching, the spectra of the samples were also manually compared to the digested sequence in order to find further matching peptides.

6.2.5.5 *Searching MALDI-TOF PMF Data against Protein Databases*

The peptide masses generated by MALDI-TOF analysis were also searched against *viridiplanta* using SWISS-PROT and TREMBL with peptIdent (http://www.expasy.cn/tools/peptident.html). The experimentally measured mass data of the samples were compared with the theoretical peptides based on a tryptic digestion for all proteins in this protein database. The parameters entered for searching included [M+H] mass with a mass tolerance of 150 ppm, and a single missed cleavage. For low molecular weight proteins (< 20 kDa), a mass range of 100% was used, with the minimum peptides to match as three rather than the default of four peptides in the program.

6.2.5.6 *ESI-TOF MS/MS Analysis of the Matching Peptides*

Samples that gave raise to matching peptide masses to the HvGSL1 protein, were further subjected to MS/MS analysis to obtain amino acid sequence information, so that the sequence of the matching peptides could be directly compared to the sequence of the HvGSL1 protein at the amino acid level. The sample underwent 16 h of tryptic digestion at 37°C (Section 6.2.5.2). The resulting peptides were desalted and concentrated with a ZiP TiP. The cleaned sample was analysed by ESI-TOF MS/MS using a Micromass Q-TOF MS equipped with a nanospray source. Data were acquired over the range 400-1800 m/z, to select peptides for MS/MS analysis. After peptides were selected, the mass spectrometer was switched to MS/MS mode, data were collected and analysed.

6.2.5.7 *Analysis of Callose Synthase Complex Excised from a Native Gel*

Callose synthase enriched fraction F2 (50 µl/lane) was fractionated on a 4-20% Tris-glycine gradient gel under native conditions as described in Section 5.2.8, and the gel was stained with Coomassie Brilliant Blue. The regions showing callose synthase activity were excised from the gel. In order to avoid protein degradation and maximise digestion of protein aggregates or complexes into a solution of individual peptides, the gel pieces were subjected to reduction and alkylation treatments prior to SDS-PAGE separation. For reduction, the gel piece was immersed in 200 µl SDS equilibration buffer (50 mM Tris-HCl buffer, pH 8.8, containing 6 M urea, 30% glycerol, 2% SDS, and a trace bromophenol with freshly added 65 mM DTT), placed on a rocker, and equilibrated for

15 min at room temperature. For alkylation, the gel piece after reduction was transferred to 200 µl SDS equilibration buffer, pH 8.8, with 135 mM iodoacetateamide, and incubated for a further 15 min at room temperature. After the treatments, three gel pieces were carefully stacked into one well of a 4-8% Tris-glycine gradient gel, and run under denaturing conditions as described in Section 4.2.5. Major protein bands resolved on the gel were excised, cleaned and concentrated using the ZipTip procedure, and subjected to an ESI-TOF MS/MS analysis as described in Section 6.2.5.6.

6.3 Results and Discussion

6.3.1 Immunoprecipitation of Callose Synthase Activity

The CS17 polyclonal antibodies raised against a 17 kDa *E. coli*-expressed HvGSL1 fragment were used to test binding with the active callose synthase in barley membrane extracts. The effect of the immunoprecipitation on the crude lysate and CHAPS-extract using either CS17 antiserum or IgG fraction purified from Protein A was examined by assaying callose synthase activity. Callose synthase activity in the precipitated pellets from these two types of fractions showed no increase, compared with a control sample where the CS17 antibodies were replaced by pre-immune serum (data not shown). There was no decrease in callose activity observed from the supernatant, suggesting that the CS17 polyclonal antibodies were not able to bring down the callose synthase complex from its active preparations under the experimental conditions described here.

To identify a protein corresponding in size with the HvGSL1 sequence, the material precipitated by the anti-CS17 antibodies from either the crude lysate or the CHAPS-solubilised membrane fraction was redissolved in SDS buffer, and analysed by Western blotting. There was no band detected at molecular weights between 210 kDa-250 kDa, which would be expected if the antibodies could bind to its antigen in its native form in the active extracts. Proteins with molecular masses of approximately 23 kDa and 50 kDa, representing the light and heavy chain of the antibodies, respectively, were found on the Western blot. This indicates that the conditions used for protein A beads and the antibody interactions for immunoprecipitation were efficient (data not shown).

It was therefore concluded that the CS17 polyclonal antibodies could not precipitate callose synthase activity under native conditions. Such an outcome could be attributed to a number of factors. Because the antibody was raised against an antigen in its denatured form (Section 4.2.6.1), folding of the expressed peptide may have been different from that of the enzyme in its nature state, thus affecting the recognition of epitopes of the active enzyme in the barley extracts. Another factor could be related to the membrane-bound nature of the callose synthase. Accessibility of the antibodies to available binding sites of the synthase in the extracts might have been limited by interference by lipid molecules

and other contaminants, which prevented the antibody from binding to the antigen in the immunoprecipitation experiments.

6.3.2 *Western Blot Analysis of the Sucrose Gradient Fractions*

Proteins from various steps of the purification procedure, including the microsomal fraction, the CHAPS-extract, the $CaCl_2$ precipitated fraction and the sucrose gradient fractions (fractions 1-8), were resolved by SDS-PAGE for protein profiling. No proteins with molecular masses of approximately 220 kDa were detectable by conventional protein staining with Coomassie Brilliant Blue, or with Ponceau S staining (data not shown). In particular, there were no sharp bands at this molecular mass observed in fractions F2 and F3, which were enriched in callose synthase activity (Figure 6.1 A). This was consistent with numerous studies on callose synthase purifications from higher plants (Dhugga and Ray, 1994; Bulone *et al.,* 1995; McCormack *et al.,* 1997; Kudlicka and Brown, 1997; Hong *et al.,* 2001).

To investigate whether *HvGSL1* cDNA encodes the catalytic subunit of callose synthase in barley, the CS17 antibodies was used to reveal the putative barley callose synthase protein on Western blots of the denaturing SDS gels and native gels. Figures 6.1 A and B show the profiles of proteins and a Western blot analysis of the sucrose gradient fractions 1 to 8. When $CaCl_2$-treated CHAPS extracts were subjected to separation on a 10-60% (w/v) sucrose gradient, (1→3)-β-glucan activity was highest in fraction 2 (Figure 6.2). Although no high proteins molecular mass proteins were apparent in this fraction after SDS gel electrophoresis and Ponceau S staining (Figure 6.1 A), a strong band of apparent molecular mass 250,000 Da was subsequently revealed in Western blot analyses (Figure 6.1 B). This single sharp band of approximately 250 kDa was also present in fractions 3, 4 and 5 with decreasing intensity (Figure 6.1 B). In addition, the callose synthase activity is found to be the greatest in fractions 2 and 3 and decreases in fractions 4 and 5 (Figure 6.2 A). This pattern is found to be consistent with the pattern of the Western blot analysis, which is seen to decrease from fractions 1 to 8 (Figure 6.2 B), and strongly suggests that *HvGSL1* cDNA encodes a protein that may be involved in callose synthesis. It is noteworthy that the antibody also binds to lower molecular mass proteins in sucrose gradient fraction 1, where most of the protein is found (Figure 6.1 B). In experiments in which the $CaCl_2$ precipitation step was omitted, the amount of low molecular mass

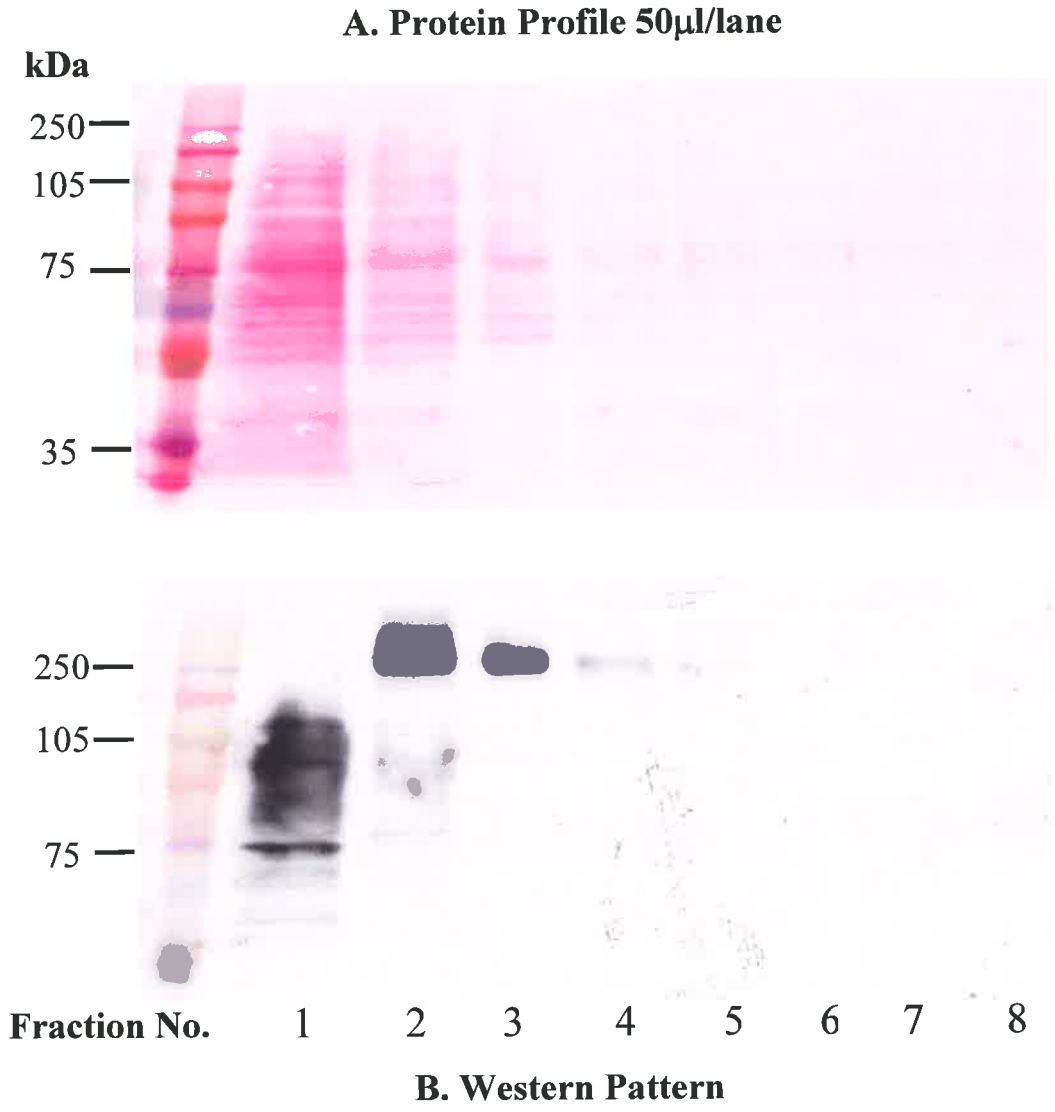


Figure 6.1 Proteins in the sucrose density gradient fractions. The top panel **(A)** shows a Ponceau S stained SDS gel loaded with 50 µl of each sucrose gradient fraction (fractions 1-8). The corresponding Western blot of the same gel, probed with the CS17 anti-callose synthase antibody, is shown in the lower panel **(B)**. Protein size markers are shown in the left lanes of each panel and molecular sizes of standard proteins are indicated.

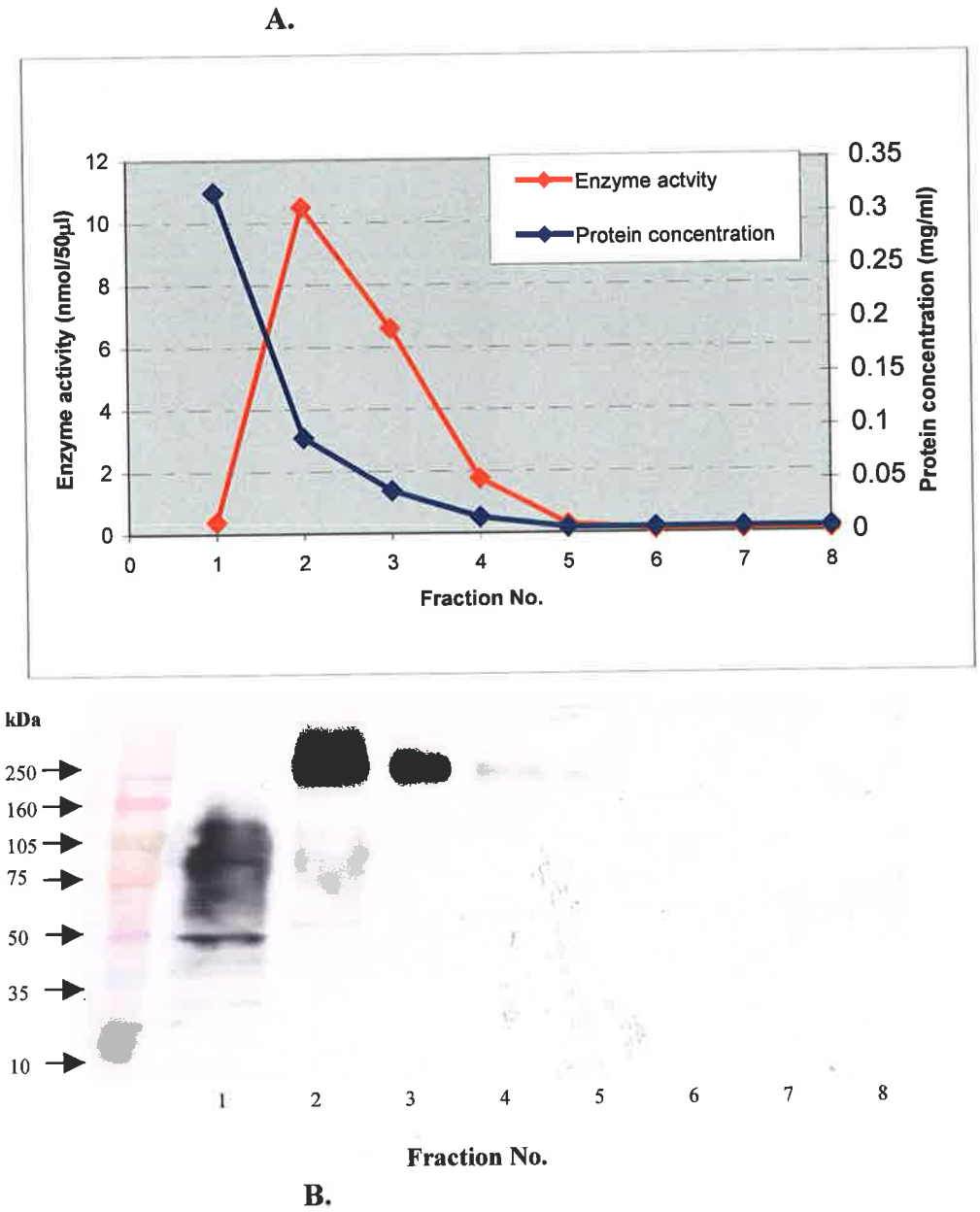


Figure 6.2 Callose activity profile of the sucrose gradient fractions visus a Western blot analysis. **(A)** Sucrose density gradient purification of the barley callose synthase. A CHAPS-extracted and $CaCl_2$-treated microsomal membrane fraction was loaded onto a 10 ml linear sucrose density gradient cushion containing 10-60% (w/v) sucrose in 100 mM MOPS buffer, pH 7.5. After centrifugation at 100,000×g for 3 h, 1 ml fractions were collected from the top of the centrifuge tube. Activity (♦) shown in orange and protein content (♦) shown in blue were measured in fractions of the gradient. **(B)** Western blot analysis of the sucrose gradient fraction using CS17 antibodies as described in Figure 6.1.

material recognised by the antibody increased markedly (Figure 6.3). This is consistent with earlier reports that $CaCl_2$ precipitation removes peptides (Bulone *et al.*, 1995). When Western blots and standard CS17 protein were probed with pre-immune serum, no signal was visible (data not shown). On this basis, it can be concluded that the low molecular mass material in fraction F1 (Figure 6.1 B) probably represents proteolytic degradation products of the callose synthase enzyme. If this is true, the sucrose gradient step had separated degraded products from active callose synthase and resulted in enrichment in fractions 2 and 3 (Figures 6.2 B and 6.3 B). Figure 6.4 (Lane 9) is also an example of a Western blot from the $CaCl_2$-treated microsomal fraction prior to sucrose gradient centrifugation. In contrast to the Western blot obtained from the sucrose fractions (Figure 6.4: Lanes 1-8), a 250 kDa band and several protein bands with sizes ranging from 75 to 150 kDa were detected by the antibodies in the $CaCl_2$ fraction. Sucrose gradient centrifugation separated these smaller proteins into fraction 1.

In contrast to the Western blot of a putative cell-plate-specific callose synthase recently reported by Hong *et al.* (2001), the Western blot obtained here with the sucrose gradient fractions of barley extract provided a direct connection between the *HvGSL1* cDNA and a protein which is part of callose synthase activity in the fractions tested. For the putative cell-plate-specific callose synthase (referred to as CalS), a membrane fraction was extracted from tobacco BY-2 cells expressing green fluorescence protein (GFP) and a callose synthase fusion protein (35S::GFP-CalS1). A Western band of approximately 250 kDa was subsequently detected from GFP-CalS1 cells using GFP antibodies, as plant callose synthase antibodies were not available (Hong *et al.*, 2001). This result suggested that the GFP-CalS1 chimeric protein formed part of the active CalS complex, thus providing indirect evidence that the CalS1 itself may be associated with the callose synthase complex.

6.3.3 Native PAGE and Western Blot Analysis

The CS17 antibodies used to reveal the putative callose synthase protein on Western blots of denaturing gels had been prepared from a heterologously expressed portion of the HvGSL1 protein and were therefore expected to bind to a protein of about 220 kDa. Although the protein of approximately 250 kDa was found as a single sharp band on the Western blot of fractions 2 and 3, under denaturing conditions, and the Western pattern

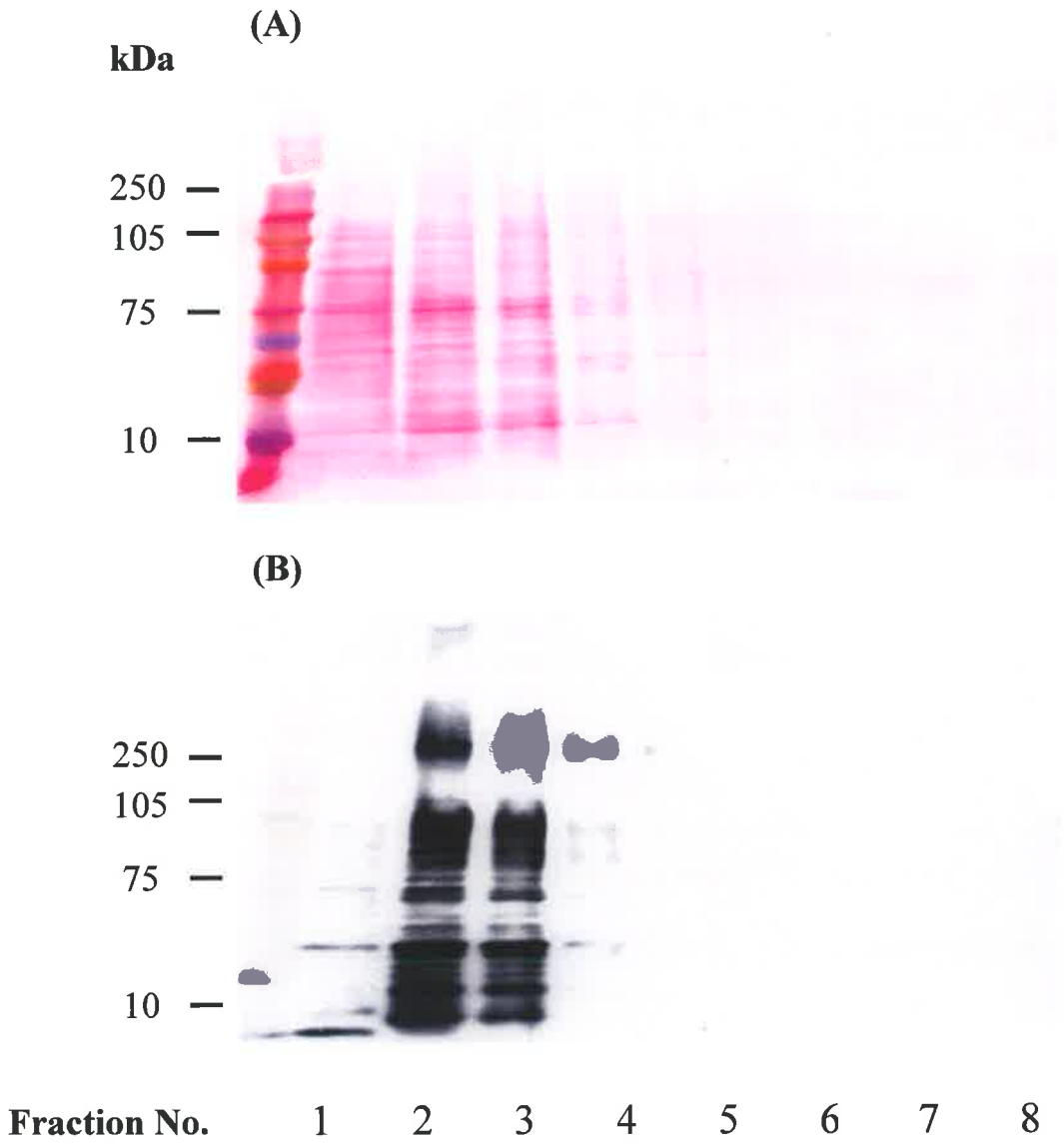


Figure 6.3 Immunoblot analysis of the sucrose gradient fractions (Non-$CaCl_2$ treated) using the CS17 antibodies. **(A)** Protein profile: 50 µl each of fractions 1-8 (Lanes 1-8) from a liner (10-60% w/v) sucrose gradient centrifugation and CHAPS fraction (Lane 9) was resolved on a 4-20% Tris-glycine gel. The protein was transferred to a nitro-cellulose membrane, stained by ponceau S and scanned. **(B)** Western blot analysis using the CS17 antibodies according to the procedure described in Figure 6.1.

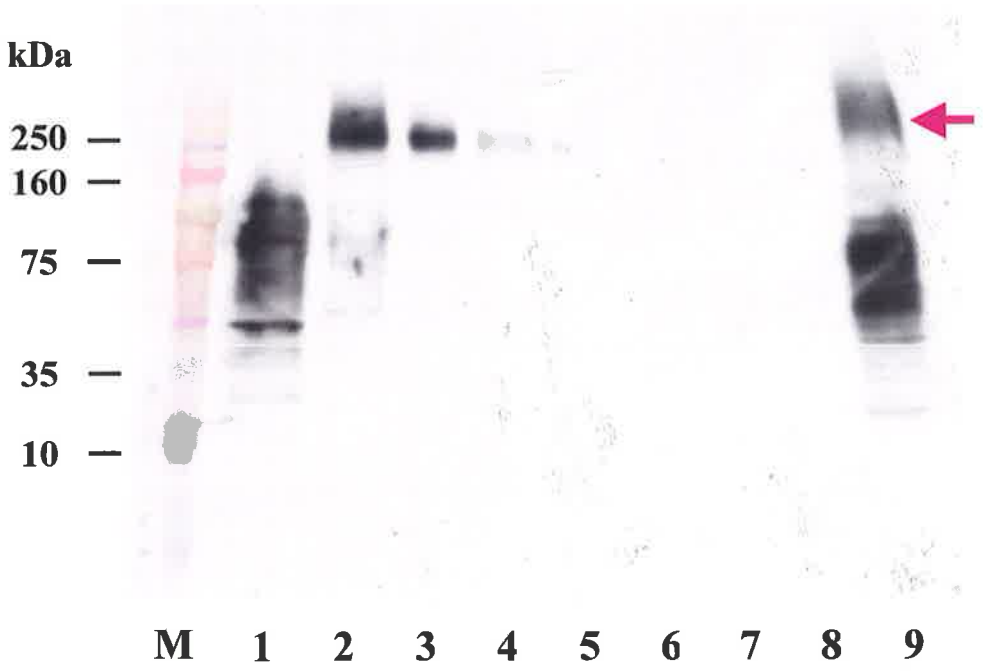


Figure 6.4 Immunoblot analysis of $CaCl_2$ treated microsomal fractions. $CaCl_2$ treated microsomal fraction (Lane 9) and its sucrose gradient fractions (Lanes 1 to 8) were separated on a gradient gel and the proteins were transferred and probed with the CS17 antibodies. M: rainbow protein molecular markers.

exhibited a strong correlation with callose synthase activity, a direct link with enzyme activity was still to be established. The next requirement was therefore to demonstrate that the high molecular mass band (Figure 6.5 A), exhibiting enzyme activity, could be recognised by the CS17 antibodies. Western blot analysis was performed on active fractions resolved by PAGE under non-denaturing conditions. Active fractions of the CHAPS-extract and fraction 2 from the sucrose gradient centrifugation were tested. No protein of high molecular mass, similar to those predicted from the *HvGSL1* cDNA, were detectable by Ponceau S staining procedures (Figure 6.5 B), but again, a high molecular mass protein that reacted strongly with the CS17 antibody was revealed by Western blot analysis (Figure 6.5 C). This protein barely entered the stacking gel, and had a much higher molecular mass than the 250 kDa protein standard (Figure 6.5 A, B and C). The high molecular mass protein was not stained with pre-immune serum (data not shown). The same is also true with the F2 fraction (Figure 6.6 A, B and C), where a protein with a very high apparent molecular mass was detected with the CS17 antibody (Figure 6.6 C) and the in-gel assays showed that ABF-positive material was deposited in the gel at the same position (Figure 6.6 A). Incubation of gel pieces with a range of substrate-specific β-D-glucan endohydrolases confirmed that the product of the in-gel enzyme activity was (1→3)-β-D-glucan (Section 5.3.4.3).

In summary, the Western data both from the CHAPS fraction and the F2 fraction demonstrated that protein encoded by the *HvGSL1* cDNA is co-located with callose synthase activity. The Western band detected on the SDS gel correlated well with callose synthase activity, and was close to the molecular weight deduced from the *HvGSL1* cDNA. It is therefore concluded that the *HvGSL1* is likely to encode the catalytic subunit of callose synthase in barley.

6.3.4 MALDI-TOF PMF Spectra

Although the high molecular mass protein in the non-denaturing gel associated with (1→3)-β-glucan synthase activity was recognised by the CS17 antibody, it was also necessary to show that its amino acid sequence corresponded with the nucleotide sequence of the *HvGSL1* cDNA. The active, high molecular weight mass bands both from the CHAPS-extract and from sucrose gradient fraction 2 were excised from the non-denaturing gel and subjected to tryptic digestion, and analysed by the MALDI-TOF mass

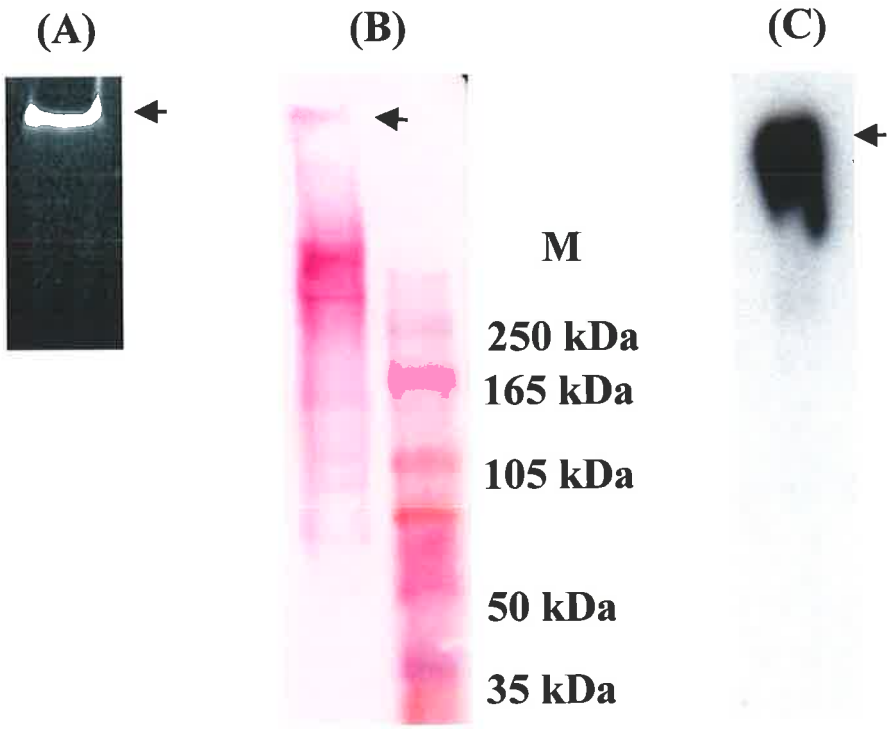


Figure 6.5 Native gel electrophoresis and immunoblot analysis of the CHAPS microsomal fraction. Two aliquots of the CHAPS fraction were separated on a 4-20% Tris-glycine gel under native conditions. One slice of the gel was used for in-gel callose synthase activity detection and the other was used for immunoblot analysis. **(A)** The gel slice was incubated with the substrate mix for callose in-gel synthesis and stained with the ABF for callose detection. **(B)** Proteins in the gel slice was transferred to a nitrocellulose membrane and stained by Ponceau S. M: Rainbow protein molecular weight markers. **(C)** A Western blot of the same gel as in B, probed with the CS17 antibodies. Arrows indicate the regions where the putative callose synthase were detected.

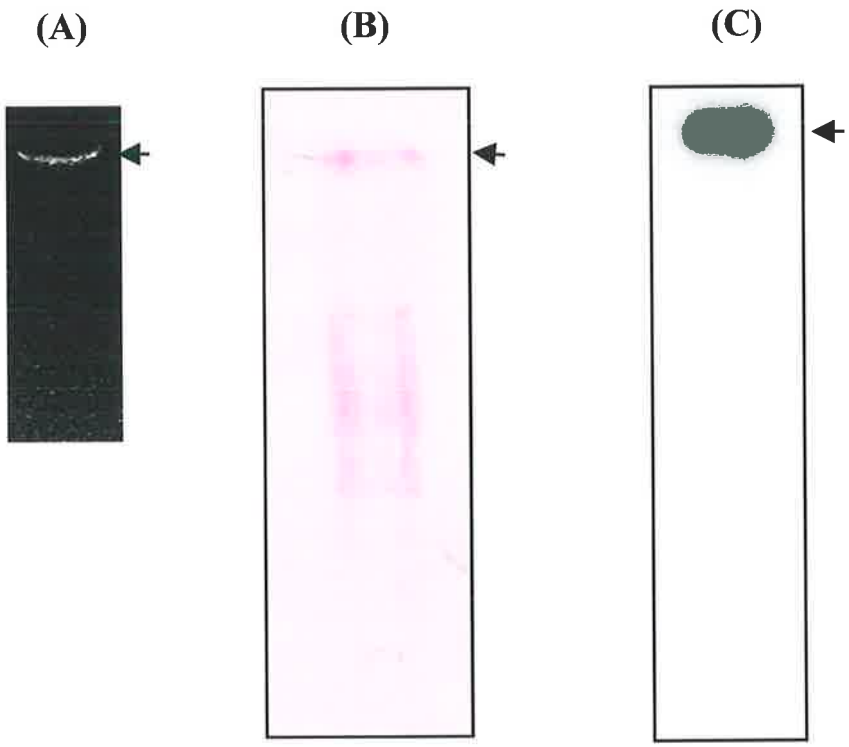


Figure 6.6 Native gel electrophoresis and immunoblot analysis of the sucrose gradient fraction 2. Two aliquots of the sucrose gradient fraction 2 were separated on a 4-20% Tris-glycine gel under native conditions, and two gel slices were subjected to the treatments as described in Figure 6.5. **(A)** In-gel activity detection by ABF. **(B)** Proteins in the gel slice transferred to a nitro-cellulose membrane. **(C)** Western blot of gel B. Arrows indicate the regions where the putative callose synthase were detected. It is notable that the Western band detected in the F2 fraction is much sharper than in that of the CHAPS fraction.

spectrometry. MS spectra profiles were generated (data not shown). The major mass peaks generated from all samples were selected for searches against both theoretical peptide fragments of the *HvGSL1* protein and a protein database, as described below.

6.3.5 *Searching for Matching Masses using MALDI-TOF PMF Data*

Table 6.1a & b and Figure 6.7 highlight the various tryptic peptides sequences found by MALDI-TOF PMF. In yellow are the sequences that match exactly with theoretical peptides from the barley *HvGSL1* cDNA sequence. Seven such peptides were detected in the F2 fraction of the sucrose gradient, following excision of the region exhibiting in-gel activity (Table 6.1a). The amount of material available for these experiments was approaching the lower limit of sensitivity for the mass spectrometer, and no other peptides could be defined. Searching the protein databases revealed six other peptides in the CHAPS fraction that matched the sequence from the *Arabidopsis* "callose synthase catalytic subunit-like protein" (Table 6.1b). Homologous sequences could be recognised in the barley *HvGSL1* cDNA and are highlighted in green in Figure 6.7. Matching the barley (1→3)-β-glucan synthase peptides with putative *Arabidopsis* callose synthase sequences could be explained if the partially purified barley (1→3)-β-glucan synthase consisted not only of enzyme corresponding to the *HvGSL1* cDNA, but also contained one or more other isoforms of the barley enzyme that happened to have some sequences matching the related *Arabidopsis* enzymes. Biochemical studies of (1→3)-β-glucan biosynthesis in *Saprolegnia monoïca* also suggested the co-existence of different callose synthase isoforms (Pelosi *et al.,* 2003). Using membrane preparations for *in vitro* callose synthesis under different pH conditions, the (1→3)-β-glucan products showed important morphological differences. At pH 6, the products consisted of very long ribbon-like microfibrils and the average degree of polymerisation (DP) of the product was more than 20,000, whereas at pH 9, the microfibrils had a length of between 200 and 300 nm and the DP was about 5,000. In addition, the shorter microfibrils obtained at pH 9 were relatively more susceptible to hydrolysis by an endo-(1→3)-β-glucanase than the longer ones obtained at pH 6. The authors suggested that in higher plants, as in *S. monoïca*, several (1→3)-β-glucan synthase isoforms might be involved in different specific functions in a given species (Pelosi *et al.,* 2003).

Table 6.1 Matching data of the MALDI-TOF MFP to HvGSL1 sequence and to an AtGSL sequence. Using MALDI-TOF MFP data to search against both HvGSL1 protein and a protein database, matched peptides were found. **A)** MS data obtained from F2 sucrose fraction purified by non-denaturing gel: seven matches with the barley protein deduced from the *HvGSL1* cDNA are highlighted in yellow. **B)** MS data obtained from CHAPS-extract purified by non-denaturing gel electrophoresis: six matches to a putative *Arabidopsis* callose synthase (AtGSL, Q9LXT9) from protein databases are highlighted in green.

Table 6.1a

MW (Da)	Delta (ppm)	Start - End (AA)	Sequence
1209.4893	-74.78	132 - 41	(K)VDELC EDEMK(L)
1398.7700	36.38	663 - 674	(K)YAAFW LVIFA AK(F)
1474.6432	-44.61	130 - 141	(K)HKVDE LCEDE MK(L)
1478.6745	-0.04	132 - 143	(K)VDELC EDEMK LR(E)
1620.8107	0.041	44 - 158	(R)ESGVF SGNLG ELERK(T)
1810.9155	43.56	1681 - 695	(R)DWTNW LFYRG GIGVK(G)
1276.6750	2.07	773 - 1782	(K)AMVHF QLFLR(L)

Table 6.1b

MW (Da)	Delta (ppm)	Start - End (AA)	Sequence
804.393	12.04	68 - 73	FYAFEK
832.416	-18.19	77 - 84	LDPTSSGR
1002.445	19.74	892 - 899	IESDTYMK
1402.598	-105.81	996 - 1006	DIMMEDYNISR
1475.667	-119.96	1868 - 1879	HNCVYPCLYAHR
1582.838	46.51	822 - 834	FAQLWNTIISSFR

Figure 6.7 Matching peptides found between HvGSL1 protein sequence and MALDI-TOF PMF data. The HvGSL1 protein sequence is shown as the backbone. Using MALDI-TOF PMF data to search against both HvGSL1 protein and a protein database. The exact matches with the barley protein deduced from the *HvGSL1* cDNA are highlighted in yellow. In the sequence KHKVDELCEDEMKLRESGVFSGNLGELERKT, four matched peptide fragments overlapped, namely KHKVDELCEDEMKL, KVDELCEDEMKL, KVDELCEDEMKLRE and RESGVFSGNLGELERKT. Regions similar to the *Arabidopsis* putative callose synthase catalytic subunit-like protein "hits": LDPTSSGR, IESDTYMK, and FAQLWNTIISSFR are highlighted in green.

MARAEANWERLLRAALRGDRMGGVYGVPASGIAGNVPSSLGNNTHIDEVL
RAADEIQDEDPTVARILCEHAYALAQNLDPNSEGRGVLQFKTGLMSVIRQ
KLAKREGGAIDRSRDIAKLQEFYKLYREKHKVDELCEDEMKLRESGVFSG
NLGELERKTLKRKKVLATLKVLWSVIEDITKEISPEDAANLISEKMKEFM
EKDAARTEDFVAYNIIPLDSLSTTNLIVTFPEVRAAISSLQYHRDLPRLP
NTISVPDARISNMLDLVHCVSGYQKDNVSNQREHIVHLLANEQSRLGKLS
GNEPKIDEGAVHVVFSKSLDNYIKWCNYLPLRPVWNNIESLTKEKKLLYV
CLYYLIWGEAANVRFLPEGLCYIFHHVARELEVIMQKQTAEPAGSCISND
GVSFLDQVIYPLYEIVAAEAGNNDNGRAAHSAWRNYDDFNEFFWSEKCFQ
LGWPWKLSNPFFSKPNRKEQGLISRNHHYGKTSFVEHRTFLHLYHSFHRL
WMFLLLMFQGLTIIAFNNGSFDTNTVLELLSLGPTYIIMEFIESVLDILM
MYGAYSTSRGSAITRVIWRFCWFTAASLVICYLYIKALQDGVQSAPFKIY
VVVISAYAGFQIIISLLMSVPCCRGITNACYSWSFVRLAKWMHQEHNYVG
RGLHERPLDYIKYAAFWLVIFAAKFSFTYFLQIRPLVKPTRLIISFKGLQ
YQWHDFVSKNNHNAITILSLWAPVASIYLLDIHVFYTIMSALVGFLLGAR
DRLGEIRSVEAVHRFFEKSLKYSWINFHVAVPKRKQLLSSGQHAELNKLD
ASRFAPFWNEIVKNLFEEDYISNTELDLLLMPKNIGGLPIVQWPLFLLAS
KVFLAKDIAVDCNDSQDELWLRISKDEYMQYAVEECFHSIKYILSNILDK
EGHLWVQRIFDGIQESISKNNIQSDIHFSKLPNVIAKLVAVAGILKETES
ADMKKGAVNAIQDLYEVVHHEVLFVDLSGNIDDWSQINRARAEGRLFSNL
KWPNEPGLKDMIKRLHSLLTIKESAANVPKNLEASRRLQFFTNSLFMRMP
VARPVSEMLSFSVFTPYCSETVLYSIAELQKKNEDGISTLFYLQKIYPDE
WKNFLTRINRDENAADSELFSSANDILELRLWASYRGQTLARTVRGMMYY
RKALMLQSYLERMHSEDLESALDMAGLADTHFEYSPEARAQADLKFTYVV
TCQIYGVQKGEGKPEAADIALLMQRNEALRIAYIDVVESIKNGKSSTEYY
SKLVKADIHGKDKEIYSVKLPGNPKLGEGKPENQNHAVIFTRGNAVQTID
MNQDNYFEEALKMRNLLEEFSQNHGKFKPSILGVREHVFTGSVSSLASFM
SNQETSFVTLGQRVLSNPLKVRMHYGHPDVFDRIFHITRGGISKASRIIN
ISEDIFAGFNSTLRQGNITHHEYIQVGKGRDVGLNQIALFEGKVAGGNGE
QVLSRDIYRLGQLFDFFRMLSFYVTTVGFYFCTMLTVLTVYIFLYGKTYL
ALSGVGESIQNRADIQGNEALSIALNTQFLFQIGVFTAIPMILGFILEEG
VLTAFVSFITMQFQLCSVFFTFSLGTRTHYFGRTILHGGAKYRATGRGFV
VRHIKFAENYRLYSRSHFVKGLEVALLLVIFLAYGFNNSGAIGYILLSIS
SWFMALSWLFAPYVFNPSGFEWQKVVEDFRDWTNWLFYRGGIGVKGEESW
EAWWDEELAHIHTFRGRILETILSLRFFIFQYGVVYHMKASNESTALLVY
WVSWAVLGGLFVLLMVFSLNPKAMVHFQLFLRLVKSIALLVVLAGLVVAI
AITRLAVVDVLASILAYVPTGWGILSIAVAWKPIVKRLGLWKTVRSLARL
YDAGMGMIIFVPIAICSWFPFISTFQTRLLFNQAFSRGLEISLILAGNNQ
NAGIWHHPSFESSPR

The only other consistent "hit" (match) seen in the MALDI-TOF PMF data of the CHAPS-extracts was an acetyl-CoA carboxylase from wheat and maize. This enzyme has multiple subunits and a molecular mass of approximately 500-700 kDa, depending on the species (Evenson *et al.*, 1994; Reverdatto *et al.*, 1999). These peptides were only in the CHAPS fraction, where one would expect many more proteins than in the F2 fraction after non-denaturing gel electrophoresis.

The important result here is that no peptides matching any other protein in the databases were detected in the high molecular mass band excised from the region of the non-denaturing gel of fraction F2 with (1→3)-β-glucan synthase activity. Nevertheless, it was not possible to conclude that the gel band contained pure (1→3)-β-glucan synthase. Further investigations of the gel band will be described later in this Chapter.

6.3.6 *Amino Acid Analysis of the Matching Peptides-MS/MS Analysis*

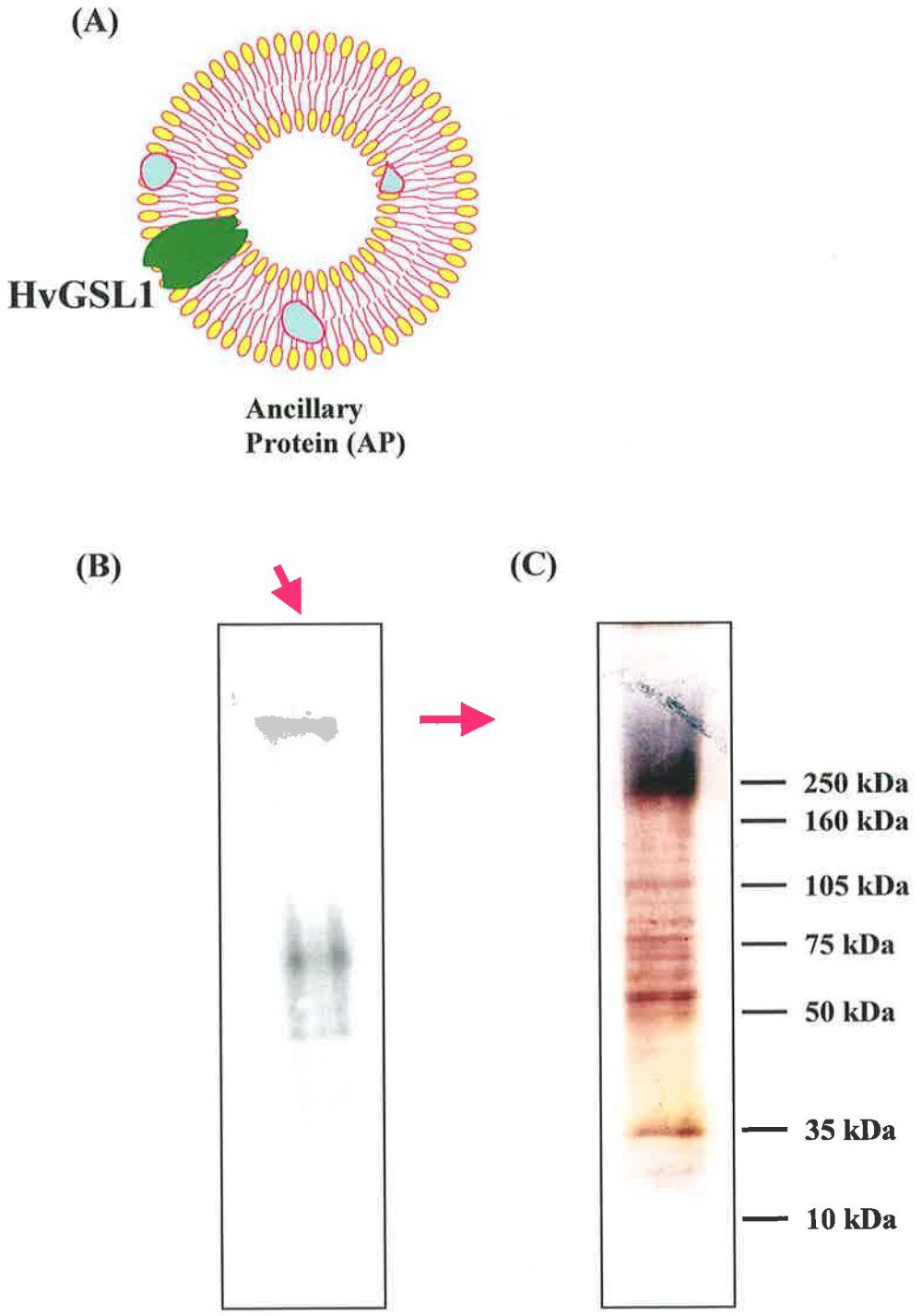
The F2 fraction from the sucrose gradient was chosen for MS/MS analysis to obtain amino acid sequences of the matching peptides. After electrophoresis under native conditions, regions corresponding to in-gel callose activity were excised and analysed by ESI-TOF MS/MS (Section 6.2.5.3). Following the MALDI-TOF mass finger print analysis, four peptides were selected, the mass spectrometer was switched to MS/MS mode to analyse amino acid sequences of these selected peptides. Only 3-4 amino acid residues could be called from each peptide, with very low confidence (data not shown).

6.3.7 *MS/MS Analysis of Peptide Components Associated with the Synthase Complex*

In an effort to identify potential peptide components associated with the synthase complex, an alternative approach was employed by analysing masses of the peptides separated from SDS-PAGE using the F2 fraction as starting material. The band with (1→3)-β-D-glucan synthase activity excised from the region of the non-denaturing gel of fraction F2 was subjected to SDS gel electrophoresis. The synthase complex, together with its ancillary proteins after the reduction and alkylation treatments, was subsequently separated by SDS-PAGE.

Figure 6.8 A is a schematic diagram of the synthase complex in the membrane preparation. The enriched fraction F2 in the "solubilising" detergent (CHAPS) is presented as a liposome-like structure, in which callose synthase, ancillary proteins required for callose synthesis and other proteins might be physically trapped in the liposome. Figure 6.8 B is a native gel profile of the sucrose gradient F2 fraction stained with Ponceau S. Figure 6.8 C shows the SDS-PAGE profile of the synthase complex excised from the native gel following silver staining, where it is apparent that there are other proteins present, albeit at much lower concentrations than the 220 kDa protein (Figure 6.8 C). It is formally possible that one of these other proteins may actually correspond to the catalytic unit of the callose synthase from barley, and some of the smaller proteins detected on the SDS gel were similar in size to those described in earlier biochemical studies of protein preparations with (1→3)-β-D-glucan synthase activity (Slay *et al.*, 1992; Meikle *et al.*, 1991; Pederson *et al.*, 1993; Dhugga and Ray, 1994). However, in many cases very high molecular mass proteins might not have been detected in the electrophoretic systems used in these earlier studies. Another explanation for the low molecular mass protein bands in Figure 6.8 C is that they might be ancillary proteins required for the synthesis of (1→3)-β-D-glucans. For example, GTP-binding proteins are regulators of (1→3)-β-D-glucan synthases in yeast (Cabib *et al.*, 1998), and have been implicated in the regulation of (1→3)-β-D-glucan synthases in cotton fibres (Delmer *et al.*, 1995). A 65 kDa membrane polypeptide that associates with a cotton callose synthase complex in a cation-dependent manner but does not bind UDPGlc (Delmer *et al.*, 1993) has also been suggested as a possible participant in (1→3)-β-D-glucan synthase activity in higher plants. Finally, the low molecular mass proteins might simply be contaminants that are physically trapped in the CHAPS-generated micelles or vesicles that carry the membrane-associated (1→3)-β-D-glucan synthase through the purification procedure (Figure 6.8 A). Morphological studies of membrane preparations for callose synthase in *Arabidopsis* (Him *et al.*, 2001) support these conclusions. Cryo-transmission electron microscopy experiments showed that the membrane extract solubilised by CHAPS resulted in the production of vesicles. This is probably one of reasons why it has been so difficult to purify plant callose synthases to homogeneity. Proteins are likely to be trapped in such a structure and abundant membrane-bound proteins will probably co-purify with callose synthase activity (Him *et al.*, 2001).

Figure 6.8 MALDI-TOF analysis of peptide components associated with the callose synthase complex in F2 fraction. (A) A schematic diagram of the callose synthase and its ancillary proteins in liposomes that are likely to be present in membrane preparations. **(B)** A native gel profile of the sucrose gradient F2 fraction stained with Ponceau S. **(C)** A SDS-PAGE profile of the synthase complex stained with silver. The high molecular mass protein seen in (B) exhibits in-gel callose activity. This band was excised, subjected to reduction and alkylation treatments, and separated by SDS-PAGE. Peptides from the SDS gel were subsequently used for the MALDI-TOF analysis.


(A)
HvGSL1
Ancillary
Protein (AP)
(B)
(C)
250 kDa
160 kDa
105 kDa
75 kDa
50 kDa
35 kDa
10 kDa

These major protein bands resolved on the SDS gel (Figure 6.8 C) were excised, cleaned and subjected to ESI-TOF MS/MS analysis of amino acid sequence. However, reliable data was not obtained, because the amount of protein from each band was approaching the lower limit of sensitivity of the equipment.

In summary, we were not completely successful in purifying the barley callose synthase to homogeneity, which is consistent with results from numerous previous studies on plant callose synthases. However, after several purification steps the barley (1→3)-β-D-glucan synthase activity co-migrated during high-resolution gel electrophoresis with a high molecular mass protein band that was recognised by the CS17 antibody. The most abundant protein by far in that band had a molecular mass close to that predicted from the *HvGSL1* cDNA, and the sequences of tryptic peptides generated from the band corresponded with sequences predicted from the nucleotide sequence of the barley *HvGSL1* cDNA. It is believed that this is the first time a link between callose synthase activity and gene sequence has been reported, and we conclude that the weight of current evidence supports a role for plant *GSL* genes in callose synthesis. Whether this is a catalytic role remains to be demonstrated, but this would appear likely. Furthermore, we cannot rule out the possibility that ancillary proteins are required for activity, although the data suggest that if this is the case the ancillary proteins are more likely to be regulatory in nature rather than proteins that are required stoichiometrically in a multi-protein complex with the *GSL* gene product.

6.4 Summary and Conclusions

Several techniques were employed and described for functional analysis of the *HvGSL1* cDNA. These include immunoprecipitation, SDS-PAGE analysis under native and denaturing conditions, immuno-blot analysis and MALDI-TOF PMF and MS/MS analysis.

Collective data obtained from this series of experimental studies has demonstrated that the *HvGSL1* cDNA encodes a protein which is indeed associated with callose synthesis in barley. The Western result of the sucrose gradient fractions correlated very well with the callose activity found from corresponding fractions. The single Western band was of about the same molecular mass predicted from the *HvGSL1* cDNA. Furthermore, the CS17 antibodies bind exclusively to the region where the callose synthase in-gel activity was detected by ABF. Such results are consistent between both the crude fraction (the CHAPS-extract) and an enriched fraction (the sucrose gradient F2 fraction) examined. The MALDI-TOF mass finger print analysis of these fractions revealed that tryptic peptides produced by in-gel digestion of the active enzyme match peptides predicted from the gene sequence. Thus, the amino acid sequence predicted from the *HvGSL1* gene has been linked with the actual amino acid sequence of an active (1→3)-β-D-glucan synthase fraction from barley.

CHAPTER SEVEN:

SUMMARY AND FUTURE DIRECTIONS

7.1 Summary of the Work Described in this Thesis

The principal aims of the work described in this thesis were to isolate and analyse the function of a putative callose synthase gene from barley. Employing a range of molecular techniques, such as cDNA library screening, polymerase chain reaction (PCR), anchor-ligated PCR, and barley BAC library screening, a putative barley callose synthase cDNA, designated *HvGSL1*, was isolated from barley. The barley gene is homologous to the yeast *FKS* gene, which is believed to encode a (1→3)-β-glucan synthase (Douglas *et al.*, 1994). The near full-length *HvGSL1* cDNA is 6055 bp in length and contains an open reading frame encoding a protein of 1915 amino acids. The protein encoded by the *HvGSL1* gene shows approximately 30% sequence identity to that of the putative yeast (1→3)-β-glucan synthase at the amino acid level. Further characterisation of the *HvGSL1* cDNA was conducted, using Southern blot analysis, Northern blot analysis, PCR-based techniques for transcriptional patterns, and chromosomal mapping. Southern blot analysis and EST database searches suggested that the barley *HvGSL* gene family has at least six members. The *HvGSL1* gene is found to be located on the long arm of chromosome 4H. Transcriptional activity of the *HvGSL1* and related barley genes could be detected in all tissues examined. These include callus, roots, coleoptiles, young and mature leaves, infected leaves, stems, florets, and developing grains. The *HvGSL1* mRNA transcripts in most of the tissues are of low abundance, but relatively high levels were detected in early developing grain three days after fertilisation and in florets harvested 2-3 days pre-anthesis, which coincides with the onset of cellularisation of the endosperm and the deposition of callose in specialised cell walls formed during micro- and macrosporogenesis, respectively.

Initial attempts to define the function of the HvGSL1 protein involved the production of recombinant protein in an *E. coli* protein expression system and the generation of an antibody against the recombinant protein. A fragment of the *HvGSL1* was successfully expressed. This was designated the CS17 protein and was used for the production of a polyclonal antibody. Subsequent affinity purification of the antibody against the CS17 antigen yielded an ultra-pure and highly active antibody, providing a useful tool for the functional studies of the *HvGSL1* gene; the gene product could be specifically tracked with the antibody during purification procedures.

The second approach in the functional analysis was the partial purification and characterisation of callose synthase from barley suspension-cultured cells. The purification procedure included membrane fractionation, CHAPS solubilisation, $CaCl_2$ precipitation, sucrose gradient centrifugation and native gel electrophoresis. Callose synthase activity was enriched approximately 60-fold in the final extract of the sucrose gradient fraction F2 compared to that of the crude extract, and considerably more after the native gel electrophoresis. Subsequent characterisation of the product synthesised by the active extracts, using the aniline blue fluorochrome as well as enzyme hydrolysis, confirmed that the product of the enzyme reaction was indeed a (1→3)-linked β-glucan.

Finally, with the availability of both polyclonal antibodies and membrane preparations enriched in callose synthase activity, attempts were made to establish links between activity and the *HvGSL1* sequence, both at the DNA and protein levels. Western blot analysis revealed that the CS17 antibodies bind exclusively to the region showing callose synthase activity on a native gel. When the antibodies were applied to a Western blot of sucrose density gradient fractions, pronounced correlations between the intensity of the Western bands and callose synthase activity were found. The single band with enzyme activity was approximately the size predicted from the *HvGSL1* cDNA. Furthermore, peptide masses matching the HvGSL1 protein were found from MALDI-TOF data, and some "hits" (matches) with *Arabidopsis* callose synthase catalytic-like protein were also revealed. Thus, these data have collectively demonstrated that the *HvGSL1* encodes a protein that is associated with callose synthase activity in barley.

7.2 Future Directions

7.2.1 Amino Acid Sequence of the Catalytic Subunit and Ancillary Proteins Associated with Callose Synthase Complex

The MALDI-TOF mass spectrometry data from barley enriched membrane fractions were found to match the theoretical tryptic peptides of the HvGSL1 protein, suggesting the *HvGSL1* cDNA may encode a callose synthase. However, the number of peptides detected was relatively low and without information on amino acid sequences of the matching peptides, the connection between the nucleotide sequence and enzyme activity remains tenuous. To date, the identification of putative callose synthase sequences from

other plant species has been solely based on nucleotide sequence similarity to the yeast *FKS* genes. No definitive proof of the function of these genes has been provided. Thus, it will be crucial to obtain amino acid sequence from the catalytic subunit of the synthase and sequence information on ancillary proteins associated with the synthase complex. Approaches to achieving this are discussed below.

MS/MS analysis of enriched extract obtained by product entrapment: The failure to obtain amino acid sequence by MS/MS analysis of the fraction F2 protein band that was excised from the native gel (Chapter 6) was attributable to insufficient protein for the analysis. Further enrichment of the callose synthase and larger scale preparations are therefore needed. The enrichment of the extract may be achieved by applying product entrapment, a technique developed and introduced by Kang *et al.* (1984) for purifying chitin synthase from yeast, and also applied to plant callose synthases (Dhugga and Ray, 1994; Bulone *et al.*, 1995). This technique involves incubation of solubilised synthase extracts with UDP-glucose and effectors such as cellobiose and Ca^{2+}, centrifugation of the incubated mixture to pellet the (1→3)-β-glucan product, and re-solubilising the callose synthase from within the pellet with an appropriate buffer. Thus, the synthase complex is "entrapped" by the polysaccharide products synthesised. The synthase is brought down by the centrifugation, and subsequently recovered from the pellet. The amount of callose synthase entrapped in the product can be assayed, and approximately one third to one half of the entrapped activity can be released in soluble form, depending on the plant material and buffer used (Dhugga and Ray, 1994). This procedure may achieve enrichment of several hundred times than that of the starting material (Dhugga and Ray, 1994). Using such an enriched fraction, combined with the use of native gel electrophoresis and SDS-PAGE separation of the synthase complex excised from the native gel (Chapter 6), the proteins associated with the complex could be analysed by MALDI-TOF mass spectrometry, from which not only peptide sequences would be expected to match that of sequence derived from the *HvGSL1* cDNA, but also the identification of the associated proteins within the synthase complex could become possible. The latter would provide direct information on protein identities by searching protein databases. If the protein sequences found were not known in databases, the amino acid sequence of the peptides would provide information for the isolation of genes that encode these proteins.

Identification of ancillary proteins via the yeast two-hybrid system: To identify proteins that interact with callose synthase *in vivo*, the yeast two-hybrid system could be used. Since its original description (Fields and Song, 1989), this system has had a major impact on the study of protein-protein interactions (Boeke and Brachmann, 1997; Frederickson, 1998). The basic principle is that a two-hybrid system is created by fusing a separate binding domain (BD) and an activation domain (AD) to a pair of interacting proteins, interaction of proteins X (bait) and Y (prey) tether the DNA binding domain to the activation domain, and this activates a downstream reporter. By far the most powerful application of the system has been its use to isolate cDNAs encoding partners for a protein of interest.

Identifying proteins that interact with the putative callose synthase catalytic subunit would involve construction of a binding domain fused with the HvGSL1 protein, and an activation domain fused with cDNA fragments from an appropriate cDNA library of barley. Several fragments selected from different regions of the HvGSL1 protein, such as those from the NH_2- or COOH- termini, or cytoplasmic domains, could be amplified by conventional PCR method and inserted into a vector containing the BD. Similarly, cDNA fragments from a library in which callose synthase is highly expressed could be ligated into a vector containing AD. The AD-containing cDNA fragments could be transformed into the reporter strain expressing the HvGSL1 protein-BD fusion. Positive growth selection could be made on the detection of colonies expressing interacting proteins among a large number of non-growing transformants. Thus, isolation of cDNAs encoding interacting proteins with the HvGSL1 protein becomes possible.

Availability of DNA sequences obtained from the two-hybrid system will facilitate the identification of proteins involved in callose biosynthesis. The sequences could be used to search databases, to compare with sequences generated by the MALDI-TOF spectrometry, and to study proteins that have been implicated in callose synthesis, such as peptides co-purified with callose synthases in plant preparations (Kudlicka and Brown, 1997), proteins that are immunoprecipitated with callose synthase activity (Meikle *et al.*, 1991), and those identified by photo-labelling techniques (Frost *et al.*, 1989; Li *et al.*, 1993).

7.2.2 Subcellular Immuno-Location of Callose Synthase

Callose synthase subcellular location: Plasma membrane and /or Golgi apparatus? Although it has long been realised that callose biosynthesis takes place at the cell surface and that callose synthase might therefore be plasma membrane associated, much of this evidence was based on early biochemical studies employing subcellular fractionation (Delmer, 1987; Ohana *et al.,* 1992). Because it is difficult to purify Golgi and plasma membranes, this process would not avoid cross contamination with membranes from other cellular compartments (Gibeaut and Carpita, 1990). The CS17 antibodies could help to verify the plasma membrane location of callose synthases through *in-situ* immunocytochemistry, without the need to fractionate cellular membranes (Delmer *et al.*, 1993). Detection of the protein at other locations could provide crucial information in the understanding of callose biosynthesis, since there are indications that components of the synthesis pathway may be spatially and temporally separated (Verma, 2001). The techniques required for this experimentation would involve tissue section preparation, gold labelling of affinity purified antibodies, *in-situ* hybridisation and electron microscopy (Northcote *et al.,* 1989; Delmer *et al.,* 1993).

Callose synthase association with the terminal cellulose-synthesising rosette complex: One phenomenon that has been encountered by various researchers is that membrane fractions that catalyse cellulose synthesis *in vitro* almost inevitably produce (1→3)-β-glucan, although the synthesis conditions was optimised to favour cellulose synthesis. For example, membranes from *Rubus fruticosus* produced large amounts of polysaccharide from UDP-glucose, about 20% of the product was cellulose and 80% was callose (Him *et al.,* 2002). It has been suggested either that one enzyme is responsible for both synthase activities, but the synthesis for either glucan is regulated by different developmental and environmental conditions, or that the synthesis of (1→4)-linked and (1→3)-linked β-glucans is governed by two distinctive enzymes, encoded by *CesA* genes and *FKS*-like genes, respectively (Ferguson *et al.,* 1998; Delmer, 1999; Richmond and Somerville, 2000; Him *et al.,* 2002). The antibodies produced against the HvGSL1 protein, which is homologous to the FKS protein, could provide a useful tool to confirm a callose synthase location in plasma membranes, and to determine whether the protein is associated with the rosette terminal complex (TC). This could be achieved through immunochemical approaches described in the previous section, coupled with the freeze-

fracture technique. However, the success of these experiments will rely on the production of new antibodies that are able to recognise the native protein. Immunogold labelling of rosette terminal cellulose-synthesising complexes has been reported using this technique (Kimura *et al.,* 1999; Kimura *et al.,* 2001), using a polyclonal antibody raised against an *E. coli*-expressed CesA fragment from cotton. With the freeze-fracture replica labelling technique, the CesA antibodies were specifically localised to rosette TCs in plasma membranes on the P-fracture face, which confirmed the long-held hypothesis that cellulose synthase is a component of the rosette TC in vascular plants. Thus, applying the fracture labelling technique with new antibodies raised against to the putative barley callose synthase, it would be possible to examine the location of the enzyme in the plasma membrane. If the immuno-gold labels were found on the rosette, a close relation between cellulose and callose synthase would be established. If the labels were found elsewhere on the plasma membrane, it would suggest that these two enzymes have distinct, independent roles in either cellulose and/or callose synthesis in plant cells.

7.2.3 Gain- or Loss-of-Function Studies

The function of the *HvGSL1* gene could be investigated in a number of genetic systems that have been developed in recent years, including both gain-of-function and loss-of-function approaches. These systems include virus-induced gene silencing (Ruiz *et al.,* 1998; Burton *et al.,* 2000), dsRNA interference (Waterhouse *et al.,* 1998; Chuang and Meyerowitz, 2000), transposon-tagged libraries/complementation (Adachi *et al.,* 2002), complementation of yeast *fks* mutants (Douglas *et al.,* 1994; Zhao *et al.,* 1998; Østergaard *et al.,* 2002), and transformation of *Arabidopsis*, tobacco or barley itself (Birch, 1997; Fagard and Vaucheret, 2000).

Yeast complementation: Functional examination of the *HvGSL1* encoding protein could be achieved by gain-of-function in an *fks* mutant of yeast (Douglas *et al.,* 1994; Zhao *et al.,* 1998; Østergaard *et al.,* 2002). Yeast complementation in mutant lines has been successfully employed to test *in vivo* the function of homologous genes from other eukaryotic cells (Wongwathanarat *et al.,* 1999; Seron *et al.,* 2000). It is a system in which a yeast mutant lacking a known function is functionally complemented by a homologous gene from another species, whose function is not defined. The yeast system is a relative well-characterised system and mutants that are defective in many genes are

available (Seron *et al.,* 2000). Functional analysis of the protein encoded by the *HvGSL1* gene would involve utilisation of a yeast mutant deficient in the *FKS* gene(s), construction of the full length *HvGSL1* cDNA in a yeast expression vector, and transformation of the expression plasmid into the yeast mutant strain. If the viability of the original mutant were not an issue, the function of (1→3)-β-glucan synthase in transformants could be restored. These could be reflected through the transformants with reduced hypersensivity to immnosuppressants and increased sensitivity to inhibitors of (1→3)-β-glucan synthase. Østergaard *et al.* (2002) employed a yeast complementation system with limited success, to establish a functional role of AtGSL5 in (1→3)-β-glucan synthesis. A putative *Arabidopsis* callose synthase gene, *AtGSL5*, encodes a plasma membrane-localised protein homologous to yeast (1→3)-β-glucan synthase (Østergaard *et al.*, 2002). In the functional complementation experiments, the yeast *fks1* mutant was unable to grow in the presence of the immunosuppressant cyclosporin A (Zhao *et al.,* 1998), but transformants harboring the *AtGSL5* ORF (open reading frame) only partially rescued the mutant phenotype (Østergaard *et al.*, 2002).

Virus-induced gene silencing (VIGS): Alternatively, the virus-induced gene silencing system provides a means for loss-of-function analysis of the *HvGSL1* gene. The VIGS system, first reported in *Nicotiana spp* (Ruiz *et al.,* 1998), has proven to be a valuable system for gene knockout studies. Operated under a putative mechanism of post-transcriptional gene silencing (PTGS), a candidate gene can be silenced in the plant upon inoculation with Potato Virus X (PVX) carrying a construct that contains a fragment of homologous DNA to the endogenous gene. The system is remarkably efficient, and offers rapid analysis of the role of a gene product. It is a very attractive approach, particularly because a full-length cDNA is not required. A fragment of 300 to 500 bp of the highly conserved region barley *HvGSL1* cDNA could be inserted into a modified PVX vector, RNA transcripts of the construct could be prepared *in vitro* for inoculation of *Nicotiana* seedlings. PTGS would result in less mRNA for endogenously expressing plant genes that have a sequence similarity of approximate 80% to that carried by the virus, resulting in silencing of the homologous genes. Plants exhibiting phenotypic differences could be examined for reduced (1→3)-β-glucan synthesis. The VIGS system has been successfully employed for functional studies of several barley cellulose synthase genes (Burton *et al.,* 2000).

Barley transformation: With rapid improvements in plant transformation in recent years (Birch, 1997; Fagard and Vaucheret, 2000), barley transformation is now a feasible option for functional studies of a candidate gene. Currently, stable introduction of novel genes into the genome of barley can be achieved by microprojectile bombardment, although transformation frequencies are much lower than 9% frequency obtained when barley is transformed by *Agrobacterium*-mediated methods (K. Gatford and A. Gierlich, personal communication). Efforts to improve transformation efficiency have been focused on aspects of regeneration frequency, and transgene integration into the plant genome. Ultimately, fragments of the barley *HvGSL1* cDNA could be introduced into the barley genome in the sense or anti-sense orientation or using dsRNAi constructs (Waterhouse *et al.,* 1998; Chuang and Meyerowitz, 2000) to produce plants either over-expressing or under-expressing putative callose synthase genes. Using wild type barley as a control, the callose synthesis could be examined from these transgenic plants at the phenotypic and cellular levels and at the DNA, mRNA and enzymatic levels, and therefore the effects and functions of putative callose synthase genes as the trans-genes could be defined.

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