Molecular and cellular studies of early endosperm development in barley (Hordeum vulgare L.)

Wilson, Christine M.

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A THESIS PRESENTED BY

CHRISTINE M. WILSON

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Department of Biological Sciences, University of Durham

Collaborating Establishment: IACR-Long Ashton Research Station
Department of Agricultural Sciences
University of Bristol
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Barley grain is an important commercial crop, being used mainly as an animal feed and in the production of malt for the brewing and distilling industries. The protein and carbohydrate composition of the endosperm (the major storage tissue) determines the grain quality and suitability for different end uses. The differentiation and maturation stages of endosperm development have been extensively studied. However, little is known about the cellular and molecular biology of the syncytial and cellularisation stages of development which occur within the first 8 days post anthesis (DPA). Events occurring during this period of development are particularly important as the overall pattern for the development and structure of the grain is laid down. Patterns of gene expression during the syncytial and cellularisation stages were investigated. A cDNA library was constructed from whole caryopses aged between 1 and 10 DPA. This cDNA library was then differentially screened using mRNA from 3 and 10 DPA caryopses. Northern and dot blot analysis led to the isolation of a number of clones which appear to show variation in level of expression. Partial sequencing of some of these clones and FASTA analysis (Genetics Computer Group, 1991) has shown four clones to have significant identity to sequences in the databases. These clones were clone 27B which showed identity to Ketol acid reductoisomerase (KARI) sequences, clone 16D which showed identity to Caffeoyl CoA-O-methyltransferase (CCoAOMT) sequences, clone 3B which showed identity to sucrose synthase sequences and clone 16B which showed identity to blue copper-binding protein sequences. A further 4 clones which were sequenced showed no significant identity to data base entries following FASTA analysis (Genetics Computer Group, 1991). The temporal and spatial distribution of these clones within tissues of barley caryopses was then analysed by *in-situ* hybrdisation. None were found to be associated uniquely with the endosperm tissues of barley caryopses. However, there were indications that the expression of the genes represented by the cDNA clones might vary during the course of development. Immunolocation studies utilising a set of JIM (John Innes Monoclonal) antibodies (and MAC207), which recognise carbohydrate epitopes of arabinogalactan proteins (AGP) were also carried out. AGPs have been associated with the plant cell surface and have been ascribed a number of possible functions related to developmental processes. The temporal and spatial distribution of AGPs within barley endosperm was analysed using sections from fixed and embedded barley caryopses and immunolocalisation techniques at the light microscope level. This revealed that at least one AGP epitope, recognised by JIM13, was expressed during early barley grain formation. JIM13 binding was observed in developing barley caryopses at the beginning of endosperm cellularisation. It was localised to the first anticlinal and then periclinal endosperm cell walls, to the crease region and the nucellar/endosperm boundary. It was not observed in any caryopsis tissue at the earlier stage of syncytial endosperm and unfortunately its distribution could not be studied at later stages of endosperm development because of poor structural integrity within the sections.

Dedication

I would like to dedicate this to my mum and Granddad Charlie
who have always believed in me.

Acknowledgments

I would like to thank both Nick Harris and Peter Shewry for their supervision and support throughout the past five years. I would also like to thank Nigel Halford and all of the Wallace Laboratory people for their help and advice.

My thanks also go to Charles Brennan (especially for his continual support) and Jackie Spence for their advise on immunolocalisation and photomicroscopy.

Bob Hughs and his crew are given a special thank you for planting and tending the barley crops.

Declaration

I declare that while registered for the degree of Doctor of Philosophy I have not been registered for any other award of the University of Durham or other academic or professional institution and except where acknowledgment is made the work presented in this thesis is my own.

The views expressed in this dissertation are those of the author and not the University of Durham

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Abbreviations

A	adenine
AHARI	acetohydroxy acid reductosiomerase
AGP	arabinogalactan protein
AMV	avian myeloblastosis virus
ATP	adenosine triphosphate
β-GlcY	β-glucosyl Yariv reagent
bp	base pairs
BSA	bovine serum albumin
C	cytosine
°C	degrees centigrade
$CaCl_2$	calcium chloride
CCoAOMT	caffeoyl CoA-3-O-methyltransferase
cDNA	copy deoxyribonucleic acid
CTAB	hexadecyl(cetyl)trimetylammonium bromide
DAF	days after fertilisation
DAP	days after pollination
dATP	deoxyadenosine triphosphate
dCTP	deoxycytosine triphosphate
DEPC	diethylpyrocarbonate
DMF	dimethyl formamide
DPA	days post antheisis
DPF	days post fertilisation
DPP	days post pollination
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
dTTP	deoxythymine triphosphate
EDTA	ethylenediaminetetraacetic acid
EM	electron microscope
Fig	figure
FITC	fluorescein isothiocyanate
G	guanine
g	gram
GuHCl	guanadine hydrochloride
HCl	hydrochloric acid
HRGP	hydroxyrich glycoprotein
IPTG	isopropyl-thio -D-galactopyranoside
JIM	John Innes Monoclonal
KARI	Ketol acid reductoisomerase
kb	kilobases
KCl	potassium chloride
kg	kilogram
KOAc	potassium acetate

LB	Luria Broth
LiCl	lithium chloride
M	molar
mA	milliamps
Mab	Monoclonal antibody
MES	2-[N-morpholino]ethanesulfonic acid
mg	milligram
$MgCl_2$	magnesium chloride
$MgSO_4$	magnesium sulphate
ml	millilitre
mM	millimolar
MMLV-RT	moloney murine leukemia virus reverse transcriptase
MOPS	3-[N-morpholino]propanesulfonic acid
mRNA	messenger ribonucleic acid (poly $(A)^+$ RNA)
NaCl	sodium chloride
NaH_2PO_4	sodium dihydrogen orthophosphate
Na_2HPO_4	di-sodium hydrogen orthophosphate
NaOAc	sodium acetate
NaOH	sodium hydroxide
NAMP	naphthol AS-MX phosphate
NaP	sodium phosphate
ng	nanogram
OD	optical density
OMT	O-methyltransferase
PBS	phosphate buffered saline
pfu	plaque forming units
pH	$\log_{10}[H^+]$
PIPES	1,4-Piperazinediethanesulfonic acid
poly $(A)^+$ RNA	poly adenylated ribonucleic acid
psi	pounds per square inch
PVP	polyvinylpyrrolidone
rDNA	ribosomal DNA
RNA	ribonucleic acid
rpm	revolutions per minute
SB	sample buffer
SDS	sodium dodecyl sulphate
SSC	saline sodium citrate
T	thymine
TBE	tris borate EDTA buffer
TBS	tris buffered saline
TCA	trichloroacetic acid
TE	Tris EDTA
TEM	Transmission electron microscope
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
TESPA	3-aminopropyltriethoxy-silane
u	units

µCi	microcurie
µg	microgram
µl	microlitre
UV	ultra violet
v/v	volume for volume
w/v	weight for volume
X-gal	5-bromo-4-chloro-3-indolyl-D-galactopyranoside

Abbreviations used in Plates and Figures

CV	central cell vacuole
V	vacuole
Sy	syncytium
N	nucleus
nu	nucellus
SE	starchy endosperm
A	aleurone
P	prismatic starchy endosperm cell
I	irregular starchy endosperm cell
aw	anticlinal cell wall
pw	periclinal cell wall
cr	crease region
cc	cross cells
FECL	first endosperm cell layer
T	testa

Line scale on all photomicrographs represents 100μm.

Chapter 1

Introduction

1.1 Importance of the Barley Grain Crop

The barley grain crop is commercially important, world-wide. In terms of production it ranks fourth among cereals and tenth among all crops (Nilan and Ullrich, 1993). The annual production figures for barley within the leading 10 barley-producing countries are given in Table 1. Adding to its importance is the fact that barley can be grown in a wide variety of habitats (many of them extremes of temperature and dryness) making it an ideal crop for marginal environments. For example, barley has been found surviving and producing grain at 4200m above sea level in Bolivia, at 65°N in Alaska, on the fringe of the Sahara Desert and near the equator in Equador and Kenya (Nilan and Ullrich, 1993). In the past, barley was a staple food source in both western and eastern countries. However, in more recent times barley as a human food has been largely replaced by crops such as wheat, rice and potatoes, with only some eastern countries (Korea, West Asia, North Africa and Morocco) maintaining it as a principal part of their diet (Bhatty, 1993) (see Table 2). At present the main uses of barley grain are in the production of malt for the brewing, distilling and food industries, as a livestock feed, as a human food source and in the food and pharmaceutical industries. Within the food industries, barley is utilised as food colouring, thickening and flavouring agents and has been used in the production of ethanol, glucose, maltose syrups and ß-amylase (Bhatty, 1993). Table 3 shows some

of the uses to which malt is put within the food industry. The end use of a barley grain crop is primarily determined by the composition of the grain at maturity. The endosperm tissue constitutes 80% of the grain at maturity. Hence the protein and carbohydrate composition of this tissue is very important in determining the 'quality' of the grain. The ideal 'quality' parameters for barley usage in the feed, food and malting industries are outlined in Table 4. The mature grain is typically composed of approximately 8-15% protein and 70-80% carbohydrate. Table 5 gives the typical composition as percentage dry weight of components in the mature grain. The types and ratios of protein and carbohydrate within the grain impart different qualities which make it suitable for particular uses. For example, in the brewing industry, cultivars with lower protein levels are preferred as high levels of protein within the endosperm reduce access of the amylolytic enzymes to the starch granules during the mashing process which leads to excessive head retention and cloudiness in the final product. Thus, to obtain optimal brewing capabilities from the barley used for malting, the carbohydrate and protein content of the grain must be balanced. Similarly, the variety of barley used for non-ruminant feed can affect growth. This is because barley is typically low in the essential amino acids lysine and threonine. The amount of cell wall carbohydrate (1-3),(1-4),ß-D glucan in the grain or malt extracts can also have consequences for both the brewing and feed industries: high levels of soluble (1-3),(1-4),ß-D glucans confer a high viscosity which reduces the digestibility of the feed in the animal and reduces the flow rate in the brewing process.

Due to the wide array of uses to which barley grain can be put there is considerable interest in its improvement for particular end uses via modern molecular biology techniques. The recent advances in transformation technology now allows easier (but

still difficult) genetic manipulation of barley. However, before any such projects can be undertaken it is necessary to have a good understanding of the structural, compositional and molecular changes which occur throughout grain development. This is especially important with regard to the endosperm as it constitutes the majority of the grain and has the largest influences on end use. Many studies on the biochemistry of protein and carbohydrate synthesis and deposition, composition and the structure and developmental processes have already been carried out (see Duffus and Cochrane, (1993) for details) and act as a basis on which to develop molecular projects. Much of the research on endosperm development has concentrated on later stages during which carbohydrate and protein synthesis and deposition are occurring and on the mature grain; relatively little has been carried out on the earliest stages of development when the cellular basis of the overall grain structure is laid down.

Table 1. Annual barley production estimates for the 10 leading barley producing countries, five-year averages, 1985-1989.

Rank	Country	Production (1000 t)	Area (1000 ha)	Yield (t/ha)	World Production (%)
1	Russia	50 452	29 482	1.7	28.8
2	Canada	12 580	4 687	2.7	7.2
3	USA	10 524	4 007	2.6	6.0
4	France	10 320	1 991	5.2	5.9
5	Spain	9 716	4 283	2.3	5.5
6	W. Germany	9 395	1 866	5.0	5.4
7	Great Britain	9 149	1 858	4.9	5.2
8	Turkey	6 360	3 270	1.9	3.6
9	China	6 218	3 369	1.8	3.5
10	Denmark	5 050	1 058	4.8	2.9

Adapted from Nilan and Ullrich (1993), data from US. Feed Grains Council (1990).

Table 2. Estimates of barley consumed as food by people of different countries in the period 1986-1988.

Country	Food consumed (kg/person/year, 1986-1988)
Morocco	68.3
Ethiopia	19.0
Algeria	18.1
Afghanistan	15.4
Iraq	11.5
Tunisia	10.6
Libya	8.9
Korean republic	7.5
Iran	7.1
Poland	6.1
Peru	4.8
Japan	1.1
Netherlands	0.9
New Zealand	0.9
Germany	0.7
USA	0.7
Canada	0.5
France	0.4
Australia	0.3
Denmark	0.3
Italy	0.3
Great Britain	0.3

Adapted from Nilan and Ullrich (1993)

Table 3. Uses for malt in the food industry.

Foodstuff	Use of Malt				
	colour	enzymes	flavour	sweetness	nutrition
Biscuits and crackers	x	x	x	x	x
Bread	x	x	x	x	x
Breakfast cereal			x	x	x
Cakes	x		x	x	
Coffee alternative	x		x		
Confectionery	x		x	x	x
Desserts	x		x		
Gravy	x				
Ice cream	x		x		
Infant food		x	x	x	x
Malted food drinks	x	x	x	x	x
Meat products	x				
Mincemeat	x				
Pickles	x				
Preserves	x				
Sauces	x		x	x	
Soft drinks	x		x	x	x
Soups	x				
Stock cubes	x				
Type of malt product used	soluble extract	soluble extract or flour	soluble extract, flour and flake	soluble extract	soluble extract, flour and flake

Adapted from Bamforth and Barclay (1993).

Table 4: Ideal 'quality' parameters in food, feed and malting barley for end use.

Parameter	END USE		
	Food	Feed	Malting
Starch content	high	high	high
Proteins			
Total	high	high	low
Lysine	high	high	NA
Lipids	NA	high	NA
β-Glucan	high	low	low
Enzymes (malt)	NA	NA	high
Phenolics	NA	NA	low
Husk	absent	absent	present, thin

From Kashsa *et al.*, (1993).

Table 5. Chemical composition of mature barley grain.

COMPONENT	DRY WEIGHT (%)
CARBOHYDRATES	78-83
Starch	63-65
Sucrose	1-2
other sugars	1
water soluble polysaccharides	1-1.5
alkali soluble polysaccharides	8-10
cellulose	4-5
LIPIDS	2-3
PROTEIN	10-12
albumins and globulins	3.5
hordeins	3-4
glutelins	3-4
NUCLEIC ACIDS	0.2-0.3
MINERALS	2
OTHERS	5-6

From MacGregor and Fincher (1993) (Data From Harris (1962)).

1.2 Overview of Barley Grain Structure and Development

The barley grain consists of the embryo and the endosperm enclosed within the testa, pericarp and husk. Both the embryo and endosperm begin to develop following the double fertilisation event within the mature embryo sac. Prior to fertilisation the embryo sac contains the egg apparatus consisting of a single egg cell and two synergids at the micropylar end, a large vacuolate central cell containing two polar nuclei (fused) and approximately 50 antipodal cells at the chalazal end (Duffus and Cochrane, 1993). A diagrammatic representation of the mature embryo sac is given in

Figure 1. Approximately 40 minutes after pollination, fertilisation occurs such that one male nucleus fuses with the egg cell to form a diploid zygote and the other male nucleus fuses with the two polar nuclei of the central cell to form a triploid primary endosperm nucleus. The zygote and the endosperm then follow separate developmental pathways. Figure 2 shows a schematic representation of each developmental path.

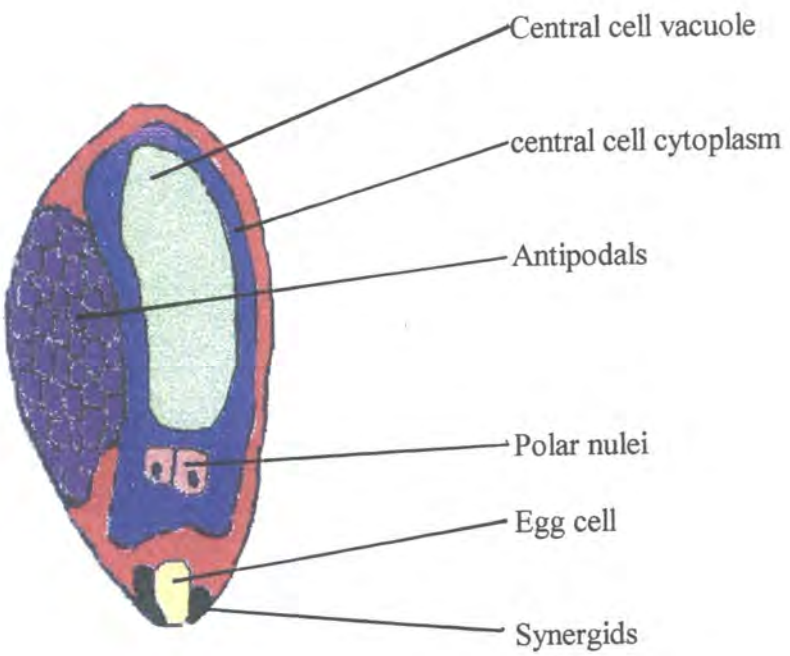


Figure 1. Diagrammatic representation of the mature embryo sac. Adapted from Engell (1989).

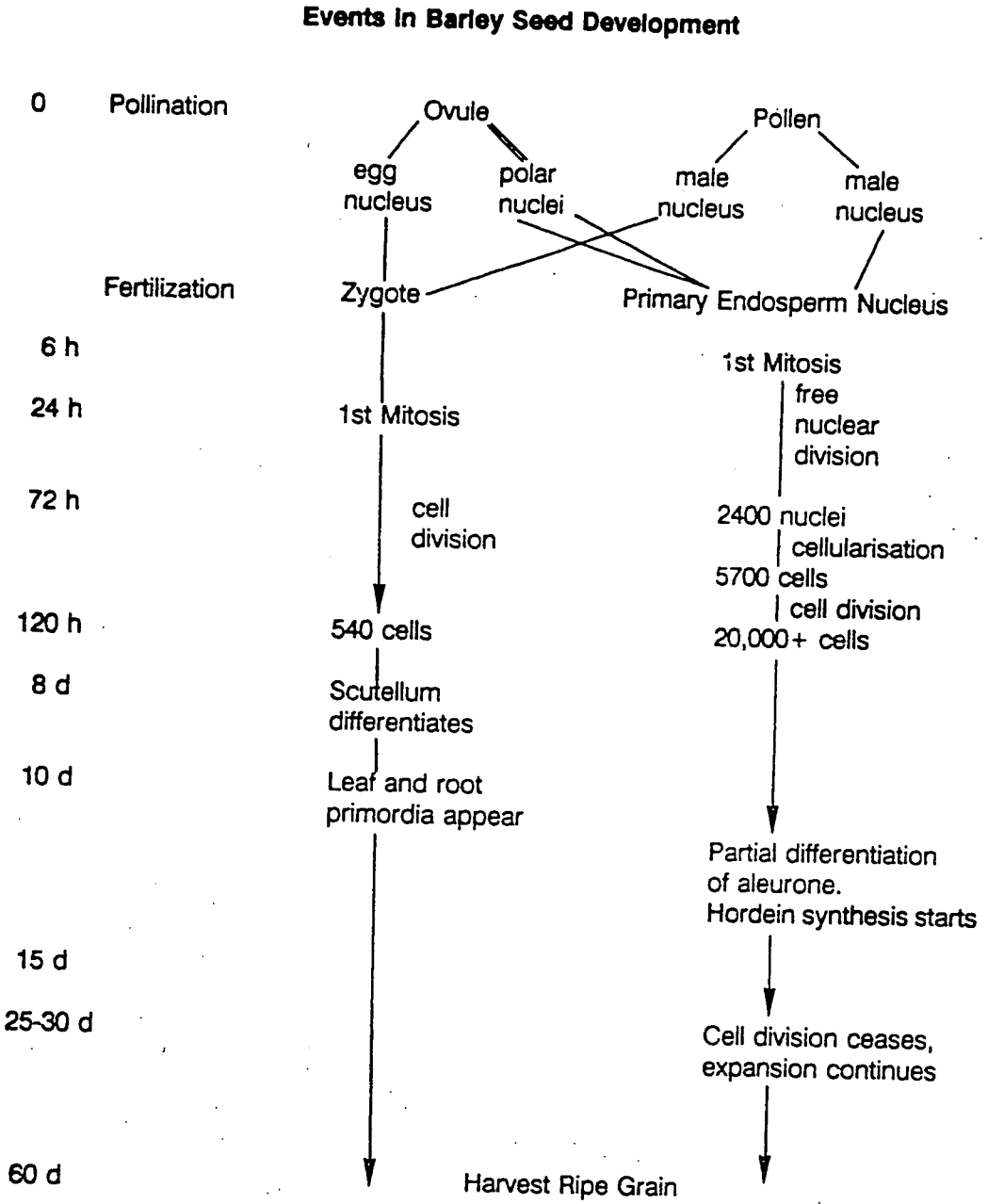


Figure 2. Schematic overview of the separate pathways of embryo and endosperm development. (Personal communication, Peter Shewry)

1.3 Timing of events during embryo and endosperm development

It is important to note that timing of events during the development of barley grains (or for that matter any grain crop) is greatly influenced by genotype and by environmental factors such as temperature, and nitrogen availability, making it difficult to state specific timings for events during endosperm/grain development. Each study requires the grains to be assessed for timing of events. Much recent work on barley endosperm development has been based on the methods and conditions of growth used by Bosnes *et al.* (1992). Their timing of developmental events is therefore used in this chapter as a general guide. It should be noted, however, that other authors do not always report similar timing for various developmental events.

1.4 Embryo development

In the past, many aspects of the development of the barley embryo have been studied in detail by a number of workers (Lerner and Holzner, 1888; Merry, 1941; Norstog, 1965, 1972; Bennett and Smith, 1976; Cass and Jensen, 1970; Mogensen,1982, 1984; Engell, 1989). The embryo has not been the subject of any detailed analysis in the project reported within this thesis and so only a basic description is given here and the reader is refered to the above mentioned authors for further details.

It should be noted that different authors use different start points; for example, days post anthesis (DPA), days after fertilisation (DAF) and days after pollination (DAP).

Following fertilisation the zygote undergoes a lag period in which no cell division occurs. Hence it begins to develop later than the endosperm. Merry (1941) indicates

that the first division of the zygote occurs within the first two days after fertilisation, whilst Engell (1989) indicates that the first division occurs within 21 hours of pollination and that the lag phase is 6-7 hours following fertilisation. The first division of the zygote is asymmetrical and gives rise to a small apical cell and a large basal cell. The apical cell develops into the embryo proper whilst the basal cell forms the suspensor. The following divisions are also asymmetrical and lead to the formation of a club or torpedo shaped embryo. Differentiation of organs is first observed at approximately 7-8 days after fertilisation (DAF), at which point the scutellum and stem meristems are noticeable. The root and leaf primordia begin to develop by between 10 days after pollination (DAP) and 14-16 DAP (Lerner and Holzner, 1888; Merry, 1941). By 16 DAP three leaf primordia enclosed within the coleoptile are recognisable as are the four pairs of seminal roots (alongside the primary root) held within the coleorhiza (Lerner and Holzner, 1888; Merry, 1941). Pro-vascular bundles within the scutellum are visible from 10 DAP. There is no further differentiation of these vascular bundles until germination is under way. The embryo is initially held within the seed coat such that the coleorhiza end lies next to the micropyle and is attached to the nucellus by the suspensor. At the 20 cell stage of development the embryo becomes detached from the nucellus/suspensor and lies within the free nuclear endosperm (Norstog, 1972).

1.5 Endosperm formation and development

The mature endosperm acts as a nutrient reserve, consisting of a mixture of proteins and carbohydrates which are utilised by the embryo during germination. Although the endosperm is commercially important and later stages have been well characterised in

terms of structure, composition and gene regulation, relatively little is known about its early development.

At maturity the endosperm consists of 2 main tissue types: the aleurone layer and the starchy endosperm. Within these there are only 5 cell types. The aleurone layer is composed of aleurone and modified aleurone cells and the starchy endosperm of, sub-aleurone, prismatic and irregular starchy endosperm cells (Figure 3). This low structural complexity of the endosperm makes it an ideal system in which to study developmental events. Problems do, however, arise in its study as during the early stages of development the tissues are soft with a milky consistency, making them difficult to isolate from the rest of the caryopsis tissues.

The development of the endosperm of cereals, especially wheat, has been well documented and it would appear that there is a certain amount of interspecies conservatism in the mode of development. Recently Bosnes *et al.*, (1992), Olsen *et al.* (1995) and Duffus and Cochrane, (1993) described endosperm development in barley in some detail. Bosnes *et al.* (1992) identified 4 main stages in the development of the barley grain and described the major events occurring in each stage. This study makes an ideal basis for further studies of structural and molecular changes during grain development. The four stages of endosperm development identified by Bosnes *et al.* (1992), each consisting of definable events at the light microscope level which can be attributed to specific age classes (for each individual crop), are: 1. syncytial formation, 2. cellularisation, 3. differentiation and 4. maturation. Diagrammatic representation, with a summary of events at each stage, is given in Table 6 and is discussed in detail below.

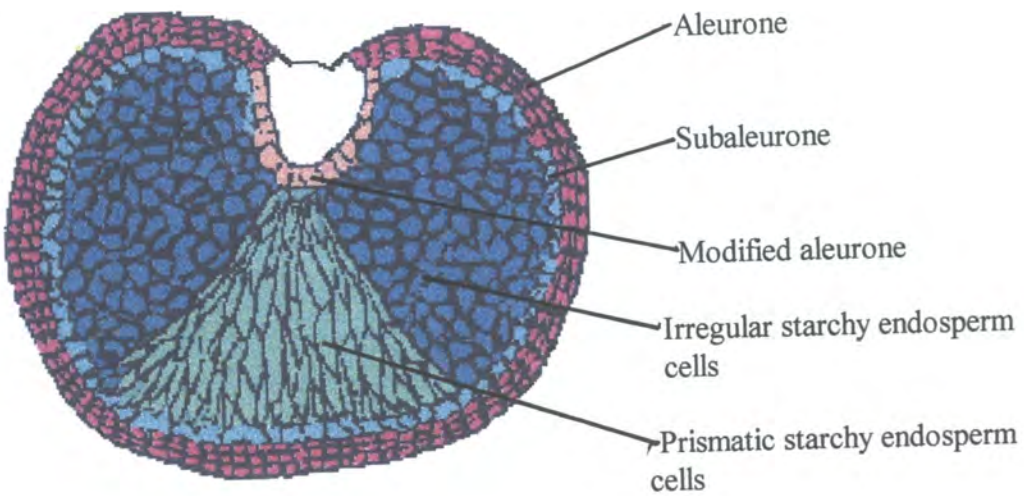


Figure 3. Diagrammatic representation of mature endosperm.

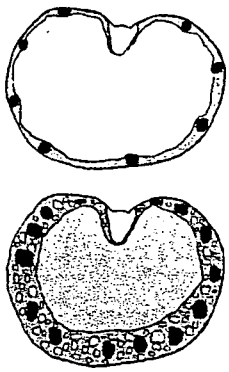
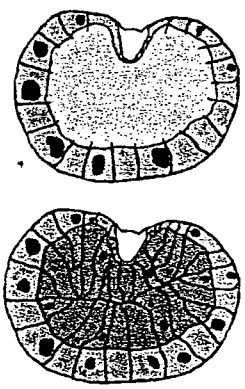
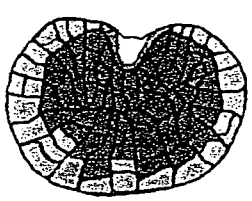
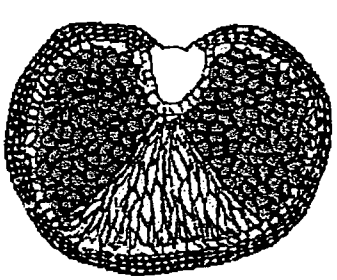
Developmental Stage	DAP	Diagramatic representation	Main events
I. Syncytial	1 3 4		Free nuclear division and nuclear migration, giving rise to a multinucleate syncytium of approx. 2000nuclei. Vacuolarisation of the syncytium cytoplasm. 6 fold increase in nuclear transcription. Anticlinal wall formation begins.
2. Cellularisation	5 8		Anticlinal and periclinal wall formation leading to the first endosperm cell layer. Continued anticlinal wall formation. Normal mitosis giving rise to complete cells. Endosperm completely cellular.
3. Differentiation	8 21		Vacuolation of the aleurone layer. Central mitotic activity ceases at 14 DAP Differentiation of prismatic, irregular and subaleurone starchy endosperm cells. Synthesis and deposition of storage products begins. Mitotic activity in the periphery stops at 21 DAP
4. Maturation	22 60		Storage product accumulation continues. Starchy endosperm cells senesce. Dessication of starchy endosperm cells. Harvest-ripe grain

Table 6. Overview of barley endosperm development.

1.5.1 Stage 1 - Syncytial formation

The development of the endosperm begins following the formation of the primary endosperm nucleus by the fusion of a male sperm nucleus with the central cell polar nuclei. From this primary endosperm nucleus, a coelenocyte or syncytium of ~2000 nuclei is formed by the process of free nuclear division. These nuclei are held within the thin cytoplasmic layer of the central cell, between the central cell wall and vacuole tonoplast. According to Engell (1989) the primary endosperm nucleus first divides at ~14 hours after pollination, and to Bennett *et al.* (1975) at ~7 hours after pollination. The daughter nuclei generated by this initial nuclear division orientate such that one lies on either side of the dorsal ventral midline and gives rise to endosperm nuclei in each corresponding wing of the developing grain (Bosnes *et al.,* 1992). The daughter nuclei undergo several rounds of free nuclear division and then begin to migrate through the central cell cytoplasm until they meet on the dorsal side (Bosnes *et al.*, 1992). Nuclei are evenly spaced within the cytoplasm. During the first 24 hours of the syncytial phase, nuclear division is synchronous with a cycle time of ~4 hours. Following this synchrony is lost. A similar process of free nuclear division and syncytial formation during endosperm development has been observed in other cereal species such as wheat (Brenchley, 1909; Gordon, 1922; Evers, 1970; Mares *et al.*, 1975; Morrison and O'Brien, 1976; Fineran *et al.*, 1982) and also in some other angiosperm species. Vacuolation of the cytoplasm marks the end of the syncytial phase and the onset of the stage 2 cellularisation (Bosnes *et al.*, 1992).

A number of aspects of the syncytial stage are of particular interest including, the occurrence of free nuclear division (the uncoupling of cell division and wall formation

from nuclear division), nuclear migration and spacing, and vacuolation of the cytoplasm prior to the onset of wall formation and cellularisation.

A number of suggestions have been made about the significance of free nuclear division. These include the suggestion that it acts as an efficient mechanism for populating the cytoplasm with numerous nuclei without the need for formation of walls or membranes or extra cytoplasm (Bennett *et al.*, 1975) and hence reduces the need for nutrients due to the simplification of the cell cycle. It also allows the simplification of the microtubule cycle as components of the cytokinetic apparatus such as the preprophase band (PPB) and functional phragmoplasts are absent (Brown *et al.*, 1994).

Nuclear migration and spacing and the influence of microtubule dynamics have been investigated by Brown *et al.* (1994). They conducted an immunoflourescent study of sectioned barley imaged by confocal scanning laser microscopy which provided three-dimensional data on the relationship of microtubules to the cytoplasm, nuclei microtubules and cell walls during development from 4-21 DAP. In their study they described the organisation of microtubules and correlated aspects of cell wall development in the endosperm of barley (cv. Bomi). Figure 4 summarises their findings in relation to the time course of events established by Bosnes *et al.* (1992). During the syncytial stage the mitotic apparatus is typical of plant cell division, having broad spindle poles composed of numerous foci of microtubules (Brown *et al.*, 1994). During this phase, microtubules of telophase nuclei proliferate around the two groups of daughter chromosomes; those coming from the poles form asters whilst those on the opposing side interact at the equatorial region, forming distinct interzonal arrays.

These distinct arrays of microtubules emanating from the nuclei appear to function in the spacing of the nuclei and in the organisation of the cytoplast into discrete units.

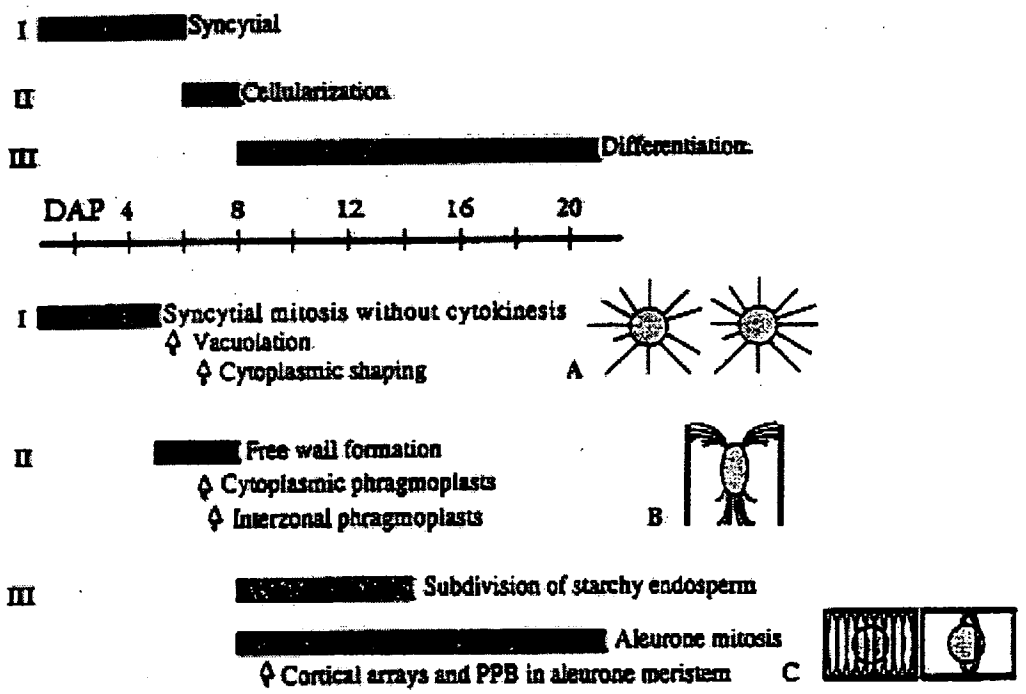


Figure 4. Stages and timing of the main events of barley endosperm morphogenesis.

The time course expressed in days after hand pollination for the first three stages of endosperm development (maturation excluded) as described by Bosnes *et al.* (1992) is shown in the top three bars. The lower bars show the main morphogenetic events based on the immunofluorescence study of microtubules and cell walls in Brown *et al.* (1994). Symbols represent interphase microtubule systems characteristic of the major stages.

A - nuclear-based radial microtubules in the syncytial stage.
B - modified system of nuclear-based microtubules during formation of the initial anticlinal walls.
C - hooplike cortical microtubules and PPB during aleurone development.

From Brown *et al.* (1994).

This organisation becomes important during the cellularisation phase. The interzonal arrays continue to increase in complexity (structural organisation) until a distinct column-shaped phragmoplast separates the nuclei. These syncytial phragmoplasts are short lived and break down to form a radial system of microtubules around nuclei which interconnect with those of neighbouring nuclei. The nuclei become evenly spaced in association with the development of the radial microtubule systems (RMSs). The RMSs appear to maintain the even spacing of interphase nuclei in the cytoplasm and define distinct domains of cytoplasm around each nucleus. The phragmoplasts of the syncytial stage do not function in the deposition of a functional cell plate leading to cytokinesis. Based on studies of endosperm mutants B7, B15 and B16 by Bosnes *et al.,* (1992), vacuolation of the cytoplasm is believed to be associated with the end of the syncytial stage. In these endosperm mutants, vacuolation of the central cell cytoplasm does not occur and cell wall formation and cellularisation do not proceed. This indicates a requirement for the vacuolation process prior to initiation of cell wall formation. This process is also seen in *Arabidopsis thaliana* (Mansfield and Briarty, 1990). Prior to the vacuolation phase of stage 1 there is a 6-fold increase in transcription levels (Bosnes and Olsen, 1992). Bosnes and Olsen (1992) suggest that this increase is representative of *de novo* transcription of genes required for cell wall synthesis, some of which may be involved with the transition from the syncytial to cellular stage.

1.5.2 Stage 2 - Cellularisation.

The scheme devised by Bosnes *et al.* (1992) indicates that cellularisation of the endosperm tissue begins following vacuolation of the syncytial cytoplasm. At the light microscope level, cellularisation is first observed as the formation of anticlinal cell walls between nuclei of the syncytium. The walls form between the central cell wall and the tonoplast of the central cell vacuole and compartmentalise nuclei and cytoplasm into discrete open ended units (Fineran *et al.*, 1982). Anticlinal wall formation begins in the wide region of syncytial cytoplasm lying next to the crease region and then proceeds to spread laterally round the edges to the dorsal side in wheat and barley (Mares *et al.*, 1975, 1977; Morrison and O'Brien, 1976; Fineran *et al.*, 1982; Bosnes *et al.*, 1992). Following the formation of the anticlinal walls, periclinal walls form between daughter nuclei via normal division of the syncytial nuclei (Bosnes *et al.*, 1992). Further anticlinal walls develop, growing towards the central cell vacuole and new cells are produced via mitosis and cytokinesis. Eventually walls developing from each side of the endosperm meet centrally causing the closure of the central cell vacuole (Bosnes *et al.*, 1992; Fineran *et al.*, 1982; Morrison and O'Brien, 1976; Mares *et al.*, 1975, 1977). This point in development was termed the closing stage by Fineran *et. al.* (1982) and marks the end of the cellularisation stage. This description of cellularisation represents the current generally accepted view of the basic pattern of barley endosperm cellularisation.

There is, however, still controversy over the exact manner in which cellularisation occurs. Questions remain about how anticlinal walls first arise and how cellularisation proceeds from that point. There is also debate as to whether or not normal

phragmoplast development and cytokinesis are involved in initiation of anticlinal walls, and whether the Golgi apparatus, vesicles and rough endoplasmic reticulum often seen associated with microtubules act in the deposition of wall material. A number of descriptions of endosperm cellularisation in the cereals, especially wheat, have been reported. Due to the conserved nature of endosperm development throughout the cereals it is likely that barley endosperm development may follow a similar pattern of development. Hence descriptions of wheat endosperm development are included here. Note that wheat differs from barley in that it has only one layer of aleurone cells whilst barley has three.

An early study on wheat by Brenchley (1909) concluded that wall formation was initiated at the periphery of the central cell and developed inwards very rapidly until the central cell vacuole became completely diminished. Unfortunately no mention is made of how this happens and it is assumed that random mitotic activity creates new cells and fills the vacuolar space.

The concept of an endospermic cambium has been suggested by Gordon (1922) for wheat, oat and barley endosperms and was later upheld by Sanstedt (1946) and Evers (1970). A cambial layer was thought to originate from the first formed layer of peripheral endosperm cells and produce a cellular endosperm through periclinal divisions. Gordon (1922) proposed that this cambial layer later developed into the aleurone layer of the mature grain.

Later, the ideas of Brenchley (1909) and Gordon (1922) were combined by Buttrose (1963) and Jennings and Morton (1963) to suggest that there is an initial growth of

walls towards the central cell vacuole followed by cambial-like divisions of the peripheral endosperm cells, leading to the differentiation of the aleurone and outer endosperm tissues.

Mares *et al.* (1975, 1977) and Morrison and O'Brien (1976), proposed similar ideas for wheat endosperm development. They concluded that anticlinal walls arose independently of phragmoplast formation and normal cytokinesis. Initial cell walls were described as arising from minute peg-like projections which occurred sporadically along the inner edge of the central cell wall (Morrison and O'Brien, 1976) and as free-ended cell wall projections (Mares *et al.*, 1975, 1977). In both cases, the walls extended into the central cell cytoplasm from the central cell wall towards the tonoplast of the central cell vacuole. However their views then differ in that Mares *et al.* (1975, 1977) suggest that inward furrowing of the initial cell walls leads to the formation of open ended compartments, while Morrison and O'Brien (1976) suggest that the initial cell walls branch as they grow out towards the central cell vacuole and that opposing branches grow towards one another, eventually meeting and forming completely closed cells. Mares *et al.* (1975, 1977) noted that the first cell layer became complete following the formation of periclinal walls via normal nuclear division and phragmoplast formation. Development in this way led to the formation of a layer of complete cells next to the central cell wall and a new layer of open ended compartments on the central cell vacuole side. The embryo sac then becomes completely cellularised by further divisions of walled cells and inward growth of free walls. In contrast, Morrison and O'Brien (1976) suggest that complete cellularisation arises as a result of tangential and radial cellular divisions of the first peripheral cell layer.

In comparison to these views of endosperm development, Fineran *et. al.* (1982), also working on wheat, reported that initial anticlinal cell walls arise as a result of normal cell division (cytokinesis) and phragmoplast formation. The outer margin of the resulting cell plate fuses with the plasmalemma of the central cell whilst the inner margin grows towards the central cell tonoplast and remains free. The result of this is the formation of open ended compartments between which the cytoplasm is continuous. Periclinal walls are again initiated by phragmoplast formation during normal cytokinesis of compartmentalised nuclei. Hence a complete cell layer is formed next to the central cell wall and an open-ended compartment layer next to the central cell vacuole. Anticlinal walls continue their inward growth and complete cells divide normally and the central cell vacuole is eventually closed when anticlinal walls growing from each side meet centrally. Similar observations were made by Bosnes *et al.* (1992) and Olsen *et al.* (1992) on studies of barley endosperm development.

Recent work on barley endosperm development has helped to clear some of the controversy over the involvement of the mitotic spindle in the formation of the first anticlinal walls. van Lammermen (1988) suggested that radial arrays of microtubules emanating from nuclei in the syncytium could lead to the development of phragmoplasts which could control the formation of initial anticlinal walls, however, he could not substantiate this with evidence.

Brown *et al.* (1994) used immunofluoresence, with a yeast anti-tubulin primary antibody, to show the importance of microtubule dynamics in nuclei spacing and migration and definition of cytoplasmic domains during the syncytial stage and in the positioning and deposition of wall material during anticlinal wall formation (for a

summary see Figure 4). Their observations did not support the development of anticlinal walls from peglike ingrowths (Mares *et al.*, 1975, 1977; Morrison and O'Brien, 1976; Morrison *et al.*, 1978) nor their initiation via normal phragmoplast formation (Fineran *et al.*, 1982). They noted that walls initially formed at the boundaries of the nuclear cytoplasmic domains (NCDs) and grew into the central vacuole from the peripheral cytoplasm such that compartments were formed, closed over by cytoplasm. Concurrent with the initiation of the anticlinal cell walls, the cytoplasm of the NCDs became more vacuolate and bulged into the central vacuole. The interphase microtubules then became rearranged such that they flared from each end of the nuclei, forming a plate at the base nearest the wall of the central cell and a crown in a thin layer of cytoplasm adjacent to the central vacuole. At the point of interaction of these 'crowns' of microtubules, between adjacent nuclei, adventitious phragmoplasts formed in the cytoplasm. The microtubules of the phragmoplasts consisted of cone-like subsets of microtubules on either side of a distinct nonfluorescent midzone. Mares *et al.* (1977), Fineran *et al.* (1988) and van Lammerman (1988) have also reported the association of phragmoplast-like microtubule arrays with the tips of free growing cell walls in wheat endosperm development. The free-growing walls continue to elongate in the advancing canopy of cytoplasm in association with the cytoplasmic phragmoplasts until the entire central cell becomes cellularised at closure (Brown *et al.*, 1994). The free growing walls occur between 5 and 8 DAP. At 7 DAP wall deposition also occurs, with cell plates being laid down in association with normal phragmoplasts following mitosis. At the same time nuclei in the columns of cytoplasm undergo a nearly synchronous round of mitosis followed by cytokinesis. As prophase continues the microtubules encasing the nuclei become more organised and the cytoplasm becomes otherwise devoid of

microtubules. Divisions in the columns are orientated with the spindle axes at right angles to the periphery of the embryo sac. Division of nuclei followed immediately by cytokinesis occurs and phragmoplasts develop in the interzones and cell plates result in the formation of periclinal walls. There is an overlap of normal cytokinesis with free growing wall formation which continues until the central cell vacuolar space becomes closed (Brown *et al.*, 1994). Brown *et al.* (1994) suggest that there are two types of phragmoplast involved with barley endosperm formation. These are primary and cytoplasmic or adventitious. The primary phragmoplasts are those which form in the interzone following mitosis and give rise to periclinal walls between daughter nuclei in the elevated columns. Cytoplasmic phragmoplasts are those which form adventitiously in areas other than the equatorial region of the mitotic apparatus, such as zones of interaction between opposing nuclear microtubule arrays.

From studies in which sections of grain were stained with aniline blue (Brown *et al.*, 1994) or subjected to immunolocalisation using antibodies to (1-3)β-glucans (Brown *et al.*, 1994), the material which is deposited during initiation of anticlinal cell walls appears to be callose.

Golgi, vesicles and/or rough endoplasmic reticulum are often seen, either associated with microtubules or standing alone in the area of wall deposition, and probably provide precursors to the initial anticlinal walls. There is, however, considerable controversy over the occurrence and involvement of Golgi, vesicles and rough endoplasmic reticulum during anticlinal wall formation.

Although a lot of work has been carried out on the cellularisation stage of cereal endosperm development, the initial cell wall synthesis and deposition remains incompletely understood and much more work in this area needs to be carried out. Similarly, confusion as to the mode of further development and events occurring at the closing stage still have to be defined.

The cellularisation stage appears to take place in the period between 3 and 8 DPA (ie begin and end in).

1.5.3 Stage 3 - Differentiation

According to Bosnes *et al.* (1992) the differentiation stage begins at 7-8 DAP, following closure of the central cell vacuole, and continues until approximately 21 DAP when mitotic activity in the peripheral region ceases.

Each tissue is composed of a number of different cell types. Peripheral and modified aleurone cells make up the aleurone layer whilst irregular, prismatic and sub-aleurone cells constitute the starchy endosperm. The aleurone layer lies beneath the nucellar layer and in barley is typically composed of three layers of cuboidal cells. In other cereals, such as wheat, rice and oats, one layer of aleurone cells is more typical. Modified aleurone cells only lie inside the nucellar projections of the crease region whilst peripheral aleurone cells occur between the starchy endosperm sub-aleurone cells and the nucellar layer. The sub-aleurone layer lies between the aleurone layer and the main body of the starchy endosperm tissue. The two wings are composed of irregular starchy endosperm cells, with the prismatic cells separating the irregular

starchy endosperm cells of each wing and occupying the area between the modified aleurone on the ventral side to the sub-aleurone on the dorsal side. Figure 3 shows a diagrammatic representation of the positions of these cells in the endosperm at the end of the differentiation stage.

During the differentiation stage mitotic activity acts to divide the large cells produced during the cellularisation stage into smaller cells. Mitotic activity occurs in two areas of the endosperm for different time periods. Mitosis in the central region of the endosperm occurs until 14 DAP and in the peripheral cells until 21 DAP (Randolph, 1936; Evers, 1970; Kvaale and Olsen, 1986). Termination of division in the peripheral area, has been implicated to mark the end of the differentiation period (Bosnes *et al.*, 1992). It has been estimated that the final number of cells in the endosperm tissues at the end of the differentiation stage is approximately 170,000 (Kvaale and Olsen, 1986). The timing of mitotic activity and estimations of final cell numbers are, however, debatable as different methods have been used by different workers (Cochrane and Duffus, 1981, 1983; Kvaale and Olsen, 1986). Due partly to interpretations based on differences in cell shape, irregular starchy endosperm cells are believed to be derived from mitotic activity in the central region whilst prismatic starchy endosperm cells are thought to arise via cell division in the peripheral area over the crease region (Bosnes *et al.*, 1992; Duffus and Cochrane, 1993). Differentiation of starchy endosperm cells (both prismatic and irregular) begins as soon as cellularisation is complete. The accumulation of hordein protein transcripts which are specific to starchy endosperm cells (Sorensen *et al.*, 1989) and the formation of starch granules (Bennett *et al.*, 1975) are early signs of differentiation.

Aleurone cells are first seen to differentiate soon after mitosis becomes confined to the peripheral areas (Duffus and Cochrane, 1993) and are identified based on their cuboidal shape. As seen with the light microscope, the first sign of aleurone cell differentiation is the appearance of a granular cytoplasm with many vacuoles and lipid bodies in cells of the peripheral area (Cameron-Mills and von Wettstein, 1980). The vacuoles are electron-lucent but may contain a single electron-dense globule. Later in development, after the aleurone cell walls have thickened, protein deposition occurs into vacuoles which, later, develop into aleurone grains (Duffus and Cochrane, 1993). Aleurone grain composition and development have been described in detail for various types of cereal grain (Buttrose, 1963; Fulcher *et al.*, 1972; Morrison *et al.*, 1975; Cameron-Mills and von Wettstein, 1980 and Peterson *et al.*, 1985). Thickening of the aleurone walls takes place when expansion of starchy endosperm cells has ceased (Cochrane and Duffus, 1981; Hoshikawa, 1984). The aleurone cells lying over the crease region are characterised by thicker walls and are termed modified aleurone. They are believed to play roles in the transport of nutrients from the maternal vascular tissues, which lie nearby, to the developing endosperm (Cochrane and Duffus 1980; Olsen *et al.*, 1992). Plasmodesmata have been observed throughout the endosperm, indicating that symplastic pathways are available for solute transfer (Duffus and Cochrane, 1993). Hoshikawa (1984) described undulations observed in endosperm cell walls during the grain filling phase as modifications that increase the surface area of the plasma membrane, allowing more rapid transfer of solutes.

Sub-aleurone cells are the last type of endosperm cell to differentiate. Based on the observation that they resemble aleurone cells it is believed that they form by a process of dedifferentiation from the aleurone cell type and simultaneous differentiation into

starchy endosperm type cells. Sub-aleurone cells resemble aleurone cells during the early part of their formation, but later lose some aleurone characteristics, such as the autofluorescent properties of the aleurone cell walls and type 1 aleurone inclusions in the vacuoles, and gain hordein proteins and starch granules which are characteristic of starchy endosperm cells (Cameron-Mills and von Wettstein, 1980). Sub-aleurone cells probably arise from the inner daughter cell from a periclinal division of the inner most layer of aleurone cells. This daughter cell redifferentiates into a starchy endosperm cell type (Duffus and Cochrane, 1993). Support for this view of sub-aleurone differentiation comes from wheat which has only one layer of aleurone cells (Morrison *et al.,* 1975). From work on endosperm mutants in barley, Bosnes *et al.*, (1992) came to the conclusion that starchy endosperm cells must be present before sub-aleurone cells could differentiate.

1.5.4 **Stage 4 - Maturation**

According to Bosnes *et al.* (1992) the differentiation phase is over and maturation begins, when cell division in the peripheral area stops. At this point, all 5 cell types are present within the endosperm. Storage products in the form of proteins, including hordeins, and starch, which are synthesised from about 8 DPA, continue to be synthesised and accumulate in the cells. Note that starch synthesis begins earlier than 8 DPA. Once peripheral division ceases, endosperm growth occurs as expansion of cells due to the accumulation of storage products. Towards the end of this stage the starchy endosperm cells begin to loose water and eventually die. The aleurone cells, however, remain alive, possibly due to a desiccation tolerance procedure similar to that

found in the embryo (Bartels *et al.,* 1988). A diagrammatic representation of a cross section of the mature grain is given in Figure 3. At harvest -ripeness the grain has typically only 15% moisture, with the dry matter being composed of 80% carbohydrate, 10% protein, 3% lipid and 2% minerals (Duffus and Cochrane, 1993). These values are, however, dependant on growing conditions. Carbohydrate content has been reviewed by Henry (1988). Depending on growth conditions the starch content varies from 51.5% to 72.1%. The oligosaccharides, fructans and raffinose, and also sucrose, account for 2% of the grain dry weight, whilst maltose, fructose and glucose only occur in smaller amounts. (1-3),(1-4),β-D glucans and arabinoxylans account for between 3% and 6% each of the grain dry weight. Cellulose, which is present mainly in the husk, accounts for only 1-3% of the grain dry weight.

1.6 Aspects of Molecular Biology of early Endosperm development.

At present, no genes involved with the regulation of early development of barley endosperm have been characterised. However, it could be expected that genes involved with regulation of the cell cycle, microtubule dynamics, vacuolation and cell wall formation would all play important roles. Bosnes *et al.* (1992) and Olsen *et al,* (1992) have proposed a hierarchical model of gene regulation during endosperm development in barley, due to its sequential mode of development. The model is based on the observations made for gene regulation in *Drosophila* embryogenesis, which also undergoes a syncytial style of development during the early phases (Ingham, 1988), similar to that which occurs in barley endosperm. It is proposed that maternal transcription factors initiate zygotic gene transcription and the start of the hierarchical mode of regulation by activating genes in the syncytial nuclei. The influence of

maternal factors at the early stages of development of barley endosperm is upheld by the occurrence of maternal *shrunken* endosperm mutants (*seg* mutants) (Felker *et al.*, 1985). Following the initial activation of genes Bosnes *et al.* (1992) propose that endosperm development is driven by transcriptional activator proteins encoded by genes regulated in a hierarchical manner.

1.7 The plant cell surface - AGP localisation and function (in relation to barley endosperm development.)

Throughout growth and development the plant cell surface, which includes the plasma membrane, the cell walls, and intercellular spaces (extracellular matrix), is important in defining the structure, shape and function of cells and ultimately tissues and organs. During development, the cells communicate with each other such that the pattern and form of the tissue is determined and controlled. Many aspects of development are regulated and co-ordinated, such as cell division, wall assembly and modification, and differentiation. Irrespective of the mode of communication a signal has to pass via the plant cell surface, either through plasmodesmata (symplastic transport) or through the cell walls (apoplastic transport).

Studies on the endosperm cell walls of barley have revealed much about their composition. It has been established that barley starchy endosperm cell walls (Fincher, 1975) and aleurone cell walls (Bacic and Stone, 1981a, 1981b) are almost entirely devoid of pectins and pectic substances and have only small amounts of cellulose. Ballance and Manners (1978) indicate that 70% of the barley endosperm cell wall is composed of hemicelluloses, namely mixed linkage (1-3)(1-4),β-glucans which are covalently bound to other cell wall components via proteins and or phenolics. Despite the fact that only small amounts of cellulose are found, electron microscopical and chemical extraction procedures have revealed that the microfiber component of barley endosperm is most likely to be cellulose and the matrix in which they are embedded composed of approximately 25% arabinoxylans and 75% (1-3)(1-4)β-glucans. Table 7 shows the composition of barley endosperm cell walls at maturity. Although the composition of endosperm cell walls at maturity has been determined (MacGregor and Fincher, 1993), little work has been carried out on initial composition of early

Table 7. Composition of Barley endosperm cell walls at maturity

Tissue	Neutral monosaccharide composition of total polysaccharides, % Ara Xyl Glx Man Gal	Protein %	Phenolic Acids %	Major polysaccharide components	References
Aleurone	24 47 26 2 2	16	1.2	71% Arabinoxylan 26% (1-3,1-4)-B-Glucan 2% cellulose 2% glucomannan 1% (1-3)-B-glucan	Bacic and Stone (1981a,b)
Starchy endosperm	11 11 75 3 0	5	0.05	75% (1-3,1-4)-B-glucan 20% arabinoxylan 2% cellulose 2% glucomannan	Fincher (1975, 1976); Ballance and Manners (1978)

Note: Values for polysaccharide components were estimated from compositional analyses presented in the references cited by MacGregor and Fincher (1993)

From MacGregor and Fincher (1993)

endosperm walls nor of changes in their composition throughout development and differentiation. Work in this area is particularly important as changes in components of the plant cell surface have been shown to be involved in many aspects of development and differentiation of tissues in other plant systems.

Interest in the cell wall of the endosperm of barley arises from a number of sources. Firstly, the composition of the cell walls affects the production and quality of high gravity beers, in that incomplete hydrolysis of the (1-3)(1-4)β-glucan content during production leads to slow filtration and haze formation. Secondly, it is now believed that the endosperm cell walls not only function structurally but also some components may act in storage of sugars which are released following hydrolysis of polysaccharide components, during germination and malting (Stone, 1986; Mieir and Reid, 1982). The sugars are used by the developing seedling and can affect the quality of the grain in terms of its use. For commercial reasons, these factors warrant investigation of the cell wall during the endosperm development. The manner in which the initial cell walls form within the endosperm is not well understood and their initiation and deposition offer interesting opportunities for investigation. Not only do the walls provide a unique cellular architecture for the development of specific cell and tissues types, which is exemplified by the variety of cell types 'that have unique cell wall compositions enabling them to perform diverse functions' (Suzuki *et al*, 1993), but they also act as sites of protein and carbohydrate metabolism (Carpita and Gibeaut, 1993; McCann and Roberts, 1991). They have been shown to contain markers that foretell patterns of development and mark positions within the plant (Knox, 1990; Knox *et al.*, 1989; Pennell and Roberts, 1990), and act in the regulation of cell division, gene expression and cell differentiation (McCann and Roberts, 1991).

In recent years a lot of interest has centred around one particular group of cell surface-associated molecules called arabinogalactan proteins (AGPs), which contain hydroxyproline-rich polypeptides and are a subset of the hydroxyproline rich glycoprotein (HRGP) group. The two other subsets of the HRGPs are the extensins (Cassab and Varner, 1988) and the solanaceous lectins (Allen and Neuberger, 1973). AGPs have been found to be differentially regulated throughout development and differentiation of a range of plant tissues and are believed to play important structural and organisational roles. They have been associated with cell plasma membranes, cell walls and as secretions in the extracellular matrix (Clarke *et al.*, 1978; Fincher *et al.*, 1983; Samson *et al.*, 1984; Basile and Basile, 1987; Pennell *et al.*, 1989; Komavalis *et al.*, 1991; Knox *et al.*, 1991).

AGPs can occur as either membrane bound or secreted molecules. Membrane bound AGPs are believed to be glycoproteins containing oligosaccharides whilst secreted AGPs are believed to be proteoglycans due to their linkage to polysaccharides (Clarke *et al.*, 1979, Clarke *et al.*,1978; Fincher *et al.*, 1983; Samson *et al.*, 1984; Basile and Basile, 1987; Pennell *et al.*, 1989; Showalter and Varner, 1989; Roberts, 1990; Komavalis *et al.*, 1991; Knox *et al.*, 1991; Baldwin *et al.*, 1993; Showalter, 1993; Du *et al.*, 1994). Analysis of AGPs from a number of sources shows that they are typically composed of between 90 and 98% carbohydrate and 2 - 10% protein. The carbohydrate moiety consists predominantly of galactose (Gal) and arabinose (Ara) residues, typically a 1,3-linked β-galactan backbone with side branches of 1,6-linked β-galactosyl residues which carry terminal residues of α-L-arabinofuranosides (Du *et al.*, 1994; Baldwin *et. al.*, 1993; Pennell, 1992; Fincher *et al.*, 1983; Clarke *et al.*,

1979). Study of AGP form and function has been hampered in the past by inability to isolate the protein core and hence most work on AGPs has concentrated on the isolation and characterisation of the carbohydrate moieties and the production of monoclonal antibodies for use in immunolocalisation studies. Over the past 2-3 years advances in isolating cDNA of the protein core have been made and should now contribute to the more rapid identification and characterisation of AGPs and their roles. The protein core is characteristically rich in serine (Ser), glycine (Gly) and alanine (Ala). Du *et al.* (1994) have isolated and characterised the protein core (AGPNa1) from a secreted AGP of *Nicotiana alata* styles. AGPNa1 was found to consist of 132 amino acids with three domains: a N-terminal secretion signal sequence, a central sequence which contains most of the hydroxyproline/proline residues and a C-terminal hydrophobic region. Similar protein cores have been isolated and characterised from pear suspension cultures (Chen *et al.,* 1994) and *N. alata* stigma and style AGPs (Bacic *et al.,* 1988). The protein backbone and carbohydrate moieties are bound via O-linkages of the sugar groups to hydroxy amino acids of the core protein. Although AGPs are widespread and abundant no clear definition of their role has been proposed. However, a number of possibilities have been proposed based on their structure, location and expression patterns.

In root meristems (Knox *et al.,* 1989: Knox *et al.*, 1991; Smallwood *et al.,* 1994), floral meristems (Pennell and Roberts, 1990; Pennell *et al.,* 1991) and embryos (Stacey *et al.,* 1990), immunolocalisation studies utilising a range of monoclonal antibodies (Mab) raised against the carbohydrate moieties of AGPs have shown the epitopes to have temporally and spatially developmentally-regulated patterns of expression which relate to cell position and cell fate (Knox *et al.*, 1991), and a role in cell-cell

interactions during plant development has been suggested (Knox *et al.*, 1989; Knox *et al.*, 1991; Pennell and Roberts, 1990; Pennell *et al.*, 1991).

Herman and Lamb (1992) suggested that a family of plasma membrane-associated arabinogalactan-rich glycoproteins reactive to Mab 16.4B4 function in a pathway for the internalisation of periplasmic material for vacuole-mediated degradation. They examined the distribution of the epitope reactive with Mab 16.4B4 using immunogold electron microscopy (EM). They showed that the epitope is widely distributed along the external face of the plasma membrane and that dense immunogold labelling of multivesicular plasma membrane invaginations (plasmalemmasomes) and similar structures sequestered within the cytoplasm and central vacuole occurred. From this they suggest that the distribution of an AGP recognised by Mab 16.4B4 defines a pathway for internalisation of periplasmic material mediated by plasma tubules and plasmalemmasomes. Plasma tubules are evaginations of the plasma membrane which are abundant in transfer cells and other cell types which exhibit high rates of solute transfer (Robards and Kidwai, 1969; Marchant and Robards, 1968), whilst plasmalemmasomes define concave shaped invaginations of the plasma membrane which contain numerous vesicles and are found in a number of tissue and cell types (Marchant and Robards, 1968). The functions of such plasma membrane modulations have not been defined but they have been implicated in roles for cell wall biogenesis (Marchant and Robards, 1968), endocytosis (Robards and Kidwai, 1969) and transport (Nishizawa and Mori, 1977). As barley endosperm and crease region cells show modulation of the cell walls (and plasma membranes) as undulations, and have high rates of solute transfer it is possible that a similar distribution of AGPs could occur which function in a manner similar to that proposed.

There is also evidence for a possible role for AGPs in growth regulation. This is supported by work on rose suspension cell cultures by Serpe and Nothnagel (1994). Treatment of suspension cultured rose cells with a phenylglycoside, which binds to AGPs, caused inhibition of cell division in a reversible manner, whilst treatment with a phenylglycoside which does not bind AGPs had no effect on cell proliferation. This is indicative of a possible role for AGPs in the regulation of cell division. However, although there is a correlation between the occurrence of AGPs and cell division no causal relationship has been established between changes in levels of AGPs and changes in the rate of cell division.

Further evidence for an influence of AGPs on cell growth comes from Schopfer (1990) who proposed that AGPs are involved in cell wall loosening. This hypothesis is based on the observation that auxin stimulated the secretion of osmophilic particles, putatively identified as containing AGPs, into the outer epidermal walls of maize coleoptiles. Also a correlation was observed between changes in AGPs and changes in cell wall extensibility in tobacco. Tobacco cells adapted to sodium chloride (NaCl) had lower levels of AGPs and they exhibited a lower rate of cell enlargement (Zhu *et al.*, 1993). It must, however, be noted that adaptation to NaCl has a variety of effects on cell wall composition and metabolism and physiological properties of the cell (Iraki *et al.*, 1992). Therefore, it is uncertain whether there is a causal relationship between changes in AGPs and changes in cell growth.

AGPs have also been implicated in responses to wounding which suggests functions for them in plant defences (Showalter and Varner, 1989).

Many of the proposed functions (especially those involving signalling, cell-cell interactions, determination cell fate, solute transfer, cell proliferation and cell growth) could theoretically be applied to certain aspects of barley grain formation including the development of the endosperm tissue. AGPs have been localised, using Yarivs reagent histochemistry, in the aleurone layer of barley endosperm but not in the starchy endosperm tissue (Anderson *et al.*, 1977). To my knowledge, no studies on AGP content of or distribution in immature endosperm have been carried out.

AIMS

The aims of the project were to study the early development of the endosperm of barley (*Hordeum vulgaes* L. cv. Sundance) at the structural, biochemical and molecular levels.

The syncytial and cellularisation stages (as defined by Bosnes *et al.*, 1992) were the stages which were studied. Within these two stages a number of important, readily defined events take place. During stage 1 in which a syncytium is formed, free nuclear division, nuclear migration and vacuole formation occur. In stage 2, cellularisation, the main events are the formation of the first anticlinal cell walls and completion of the first endosperm cell layer and a period of mitotic activity leading to closure of the central cell vacuole. These events take place within the first 8 days post anthesis (DPA), when the endosperm is of a milky liquid consistency.

Many aspects of the structural and molecular biology of mature endosperm and details of the events which take place during early endosperm development have already been well characterised. However, the molecular biology and fine structural organisation of early endosperm development have yet to be characterised. An aspect of early endosperm development which has not been looked at are the characteristics of the cell surface, including components of the plasma membrane and cell wall. Recent developments have suggested AGPs are important components of cell surface structures and they have been attributed a number of possible functions, many of which could apply to aspects of endosperm formation. The work presented here aims to elucidate whether a set of AGP molecules/epitopes occur during early endosperm

development and locate the cells in which they may arise using immunolocalisation techniques.

At the molecular level the objective was to clone cDNAs which were differentially expressed during the syncytial and cellularisation stages of early endosperm development, and to analyse their distribution in developing endosperm tissue using *in-situ* hybridisation techniques.

Chapter 2

Materials and Methods

2.1 Immunolocation and histochemistry

2.1.1 Plant Material

Both greenhouse grown and field grown *Hordeum vulgare* L. cv. Sundance were used. Greenhouse and field grown material were grown at Long Ashton Research Station. Field-grown material used for immunolocalisation studies was planted in late March, 1993 and came to anthesis June of 1993. Field grown material used for molecular biological studies (library construction and screening) was planted in March (1994) and came to anthesis June of 1994. Weather conditions throughout these two periods can be obtained from IACR - Long Ashton (Institute of Arable Crop Research - Long Ashton). For greenhouse-grown material, daylight hours were maintained at 16 hours using supplementary high pressure sodium lights. Greenhouses had a minimum day time temperature of 18°C which could fluctuate up to 23°C. Night-time temperatures had a minimum of 15°C which could fluctuate to 17°C. Leaf and root material for molecular and *in-situ* hybridisation studies was collected from greenhouse-grown plants which were 3 weeks old. To facilitate easy collection of root material plants were grown in vermiculite. Greenhouse grown barley other than this, was sown in John Innes soil based compost. For each crop, flowering spikes were labelled at anthesis and caryopses collected on a daily basis from the mid region of the spike. Caryopses were collected as sets of specific ages based on days post anthesis (DPA):

1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 DPA sets. Material for molecular studies was collected directly into liquid nitrogen and stored at -80°C until use. Material to be used for immunolocalisation and *in-situ* hybridisation studies was collected into fixative. Prior to fixation either, the tops (stigma end) of larger caryopses were cut off or they were cut in half whilst immersed in fixative. This was to allow better penetration of the fixative. Young caryopses (1-4 DPA) were fixed whole as cutting would disrupt the endosperm due to its milky consistency.

Caryopses collected and fixed by John Davies from a field grown crop at Long Ashton were also used for histochemical Toluidine Blue staining. These had been fixed in 3% paraformaldehyde (Sigma-Aldrich Company LTD), 1.25% glutaraldehyde (Sigma-Aldrich Company LTD) and had been stored in 50mM phosphate buffer at 4°C.

2.1.2 Buffers and Solutions

Contents and preparation of all solutions and buffers are given in Appendix A.

Chemicals for buffers and solutions were mainly purchased from Sigma-Aldrich Company LTD or BDH-Merck Ltd unless supplied with kits or otherwise stated. Suppliers addresses are given in appendix B.

2.1.3 Kits

IntenSEM Silver enhancment Kit (Amersham International plc)

2.1.4 Antibodies

Immunolocation studies using a number of monoclonal antibodies termed JIM antibodies and MAC207 were carried out. The characteristics of each antibody used are outlined in Table 8. The AGPs from which the JIM antibodies were derived, were extracted from the conditioned media of two carrot cell culture lines: L1 and L2 (Lloyd *et al.*, 1979 and Cole *et al.*, 1990, respectively). Carrot cell line L1 gave rise to AGP1 and cell line L2 to AGP2 (Knox *et al.*, 1989). Rats were immunised with one or other AGP. Monoclonal antibodies were then prepared by production of hybridoma of spleen cells from the rats and the IR983F myeloma cell line (Bazin, 1982) (Knox *et al.*, 1989). JIM13 and JIM14 were derived from AGP2 (Knox *et al.*, 1991), JIM4 (Knox *et al.*, 1989) and JIM15 and JIM16 from AGP1 (Knox *et al.*,1989). MAC207 was originally prepared from immunisation with peribacteroid membrane from pea root nodules (Bradley *et al.*, 1988) and production of hybridoma gave rise to the monoclonal antibody MAC207 (Pennell et al., 1989). All AGP antibodies were supplied by Professor K. Roberts, John Innes Centre, Department of Cell Biology, Norwich.

2.1.5 Fixation of samples

Tissues were fixed overnight (approximately 16 hours) at room temperature in a solution of 3% paraformaldehyde and 1.25% glutaraldehyde, buffered with 50mM phosphate buffer, pH7.0. Samples were then rinsed for 5 minutes in 50mM phosphate buffer.

Table 8. Description of antibodies against the plant cell surface AGP epitopes used in the immunolocalisation studies.

Antibody	derivation/ reactivity	Area of localisation	References
JIM 4	AGP 1	plasma membrane	Knox *et. al.* (1989) Stacey (1990)
JIM 13	AGP 1/AGP 2	plasma membrane	Knox *et. al.* (1991)
JIM 14	AGP 2	cell wall plasma membrane tonoplast	Knox *et. al.* (1991)
JIM 15	AGP 1	cell wall plasma membrane	Knox *et. al.* (1991)
JIM 16	AGP 1/AGP 2	plasma membrane	Knox *et. al.* (1991)
MAC207	AGP 1	plasma membrane	Pennell *et. al.* (1989)

2.1.6 Dehydration of fixed samples

Fixed tissues were dehydrated through a graded alcohol series of 10%, 25%, 40%, 60%, 75%, 90%, 100% ethanol (BDH-Merck Ltd.) for 2 periods of 30 minutes each, followed by dry ethanol for 60 minutes or overnight.

2.1.7 Sample embedding

2.1.7.1 Embedding in Paraplast Plus (paraffin) Wax

Dehydrated samples were first incubated at room temperature in a 1:1 solution of ethanol/Histoclear overnight. The ethanol/Histoclear was then replaced with 100% Histoclear (BDH-Merck Ltd.) for a period of 24 hours, at room temperature with two changes into fresh Histoclear. An equal volume of molten Paraplast Plus (Agar Scientific Ltd) (60°C) was added to the samples and Histoclear and incubated

overnight at 60°C. Samples were then moved into fresh molten Paraplast Plus and incubated for a further 36 hours at 60°C with frequent changes of Paraplast Plus wax. Samples were then blocked out in Paraplast Plus wax in plastic moulds at room temperature. All infiltrations at room temperature were carried out on a 45 degree rotating platform.

2.1.7.2 Embedding in LR White Resin

Dehydrated samples were incubated in a 1:1 volume of ethanol/LR White resin for 24 hours at room temperature. Samples were then infiltrated in 100% LR White resin (Agar Scientific Ltd) for 48 hours, with 3 changes of LR White resin, at room temperature. Samples were then placed in polypropylene capsules with LR White resin and polymerised at 60°C for 24 hours.

2.1.8 Sectioning

2.1.8.1 Slide Preparation.

TESPA (3-aminopropyltriethoxysilane, Sigma-Aldrich Company LTD) coated slides were prepared as follows. Clean glass slides were rinsed with 45% ethanol, 1% acetic acid solution and air dried. Slides were then dipped for 15 seconds in a 2% solution of TESPA in dry acetone, rinsed twice in acetone (BDH-Merck Ltd.), once in distilled water and allowed to air dry.

2.1.8.2 Sectioning of Paraplast Plus embedded samples.

Tissues embedded in Paraplast Plus were sectioned, using a Leitz microtome, at a thickness of 10μm. Sections were floated on to a drop of distilled water on TESPA-coated slides and dried down overnight at 40°C on a hotplate.

2.1.8.3 Sectioning of LR White embedded samples

LR White embedded samples were sectioned, using an Sorvall MTB2 ultramicrotome, to a thickness of 1μm. A glass knife was used and sections were floated onto water held in a plastic boat attached to the knife with wax. Sections were flattened by wafting chloroform over them, then floated on droplets of distilled water on TESPA-coated slides and allowed to dry down on a warm hot-plate (40°C) for at least 1 hour.

2.1.9 Histochemical Staining

2.1.9.1 Toluidine Blue O

Toluidine Blue O was used as a general histological stain. Sections were stained for 1-3 minutes in a filtered solution of 0.1% Toluidine Blue (Sigma-Aldrich Company LTD) in 1% boric acid (BDH-Merck Ltd.), then rinsed with distilled water, air dried and coverslipped with DPX mountant.

2.1.9.2 Acridine Orange

Acridine Orange (Sigma-Aldrich Company LTD) staining was used to give an indication of the RNA content of cells in sections. Sections were stained for 30 seconds in an aqueous solution of 0.01% acridine orange. Stained sections were rinsed with distilled water, mounted in Citifluor anti-fade mountant and viewed under fluorescence using a Nikon blue filter (excitation 450-490, barrier 520).

2.1.10 Immunolocalisation method

Samples embedded in both Paraplast Plus and LR White Resin were used in immunolocalisation experiments.

All wax sections were first dewaxed by incubating in Histoclear for 10 minutes. Dewaxed sections were then rehydrated through a graded ethanol/water series of 100% ethanol, 75% ethanol, 50% ethanol, 25% ethanol, 100% water to TBS (Tris buffered saline). Resin embedded material did not require this pretreatment. From this point on, resin and wax embedded material were treated to similar protocols. Optimisation of immunolocation procedures was carried out using different concentrations of primary and secondary antibodies, blocking reagents and buffers as indicated in Table 9. The following protocol was used with the relevant changes for optimisation purposes. To reduce non-specific binding, all sections were first blocked with 0.05% BSA (Bovine Serum Albumin, Sigma-Aldrich Company LTD) in TBS for 15 minutes at room temperature. Excess blocking solution was then removed by knocking the slides on the bench and then gently dabbing excess liquid away from the edges with tissue. Primary antibody was applied at the relevant concentration, diluted in TBS/0.1% Tween 20 (BDH-Merck Ltd). The primary antibody was incubated on the sections at either 4°C overnight or at room temperature for 2 hours in a humidity chamber. An even spread of primary antibody solution was achieved by covering the sections with a Parafilm strip. After the primary incubation excess liquid was then knocked off and the slides washed 3 times for 5 minutes each in fresh solutions of TBS/0.1% Tween 20. Excess liquid was wiped off the slides, and the secondary antibody conjugated to a visualisation system applied.

2.1.10.1 Secondary Antibody application

2.1.10.1.1 FITC conjugated antibodies

FITC (fluorescein isothyocyanate) conjugated secondary antibody (Sigma-Aldrich Company LTD) was diluted in TBS as Table 9. The diluted FITC conjugated antibody was pipetted onto the sections and a Parafilm cover placed on top. Sections were incubated at room temperature for 1-2 hours in a humidity chamber. Excess liquid was knocked off and the slides washed for 15 minutes in 3 changes of fresh TBS and rinsed briefly in distilled water. Excess liquid was removed and the slides coverslipped with Citifluor antifade mountant (Agar Scientific Ltd). Sections were then viewed under UV light with a Nikon blue filter (excitation 450-490, barrier 520). Green fluorescence was looked for.

2.1.10.1.2 Alkaline Phosphatase Conjugated antibodies.

Alkaline phosphatase conjugated secondary antibodies (Sigma-Aldrich Company LTD) were diluted in TBS/0.1% Tween 20 and pipetted onto the sections. Sections were covered with Parafilm and incubated at room temperature for 1 - 3 hours in a humidity chamber. Excess liqiud was removed and slides washed in 3 changes of TBS/0.1% Tween 20 for 5 minutes each then rinsed briefly in water. Alkaline phosphatase was then developed under NAMP (naphthol AS-MX phosphate, Sigma-Aldrich Company LTD) and Fast Red TR (Sigma-Aldrich Company LTD) substrate solution until colouration (red) was observed. The substrate was applied 3-4 times for 5 minute periods until colouration was observed.

2.1.10.1.3 Colloidal Gold conjugated antibodies

Colloidal gold (5-15nm) conjugated secondary antibody (Sigma-Aldrich Company LTD) was diluted as per manufacturer's instructions and pipetted onto the sections. Sections were covered with Parafilm strips and incubated for 2 hours at room temperature in darkness, in a humidity chamber. Slides were then washed in 3 changes of TBS for 5 minutes each, distilled water and Milli-Q water (heavy metal-free). Silver enhancement of immunolocated gold was then carried out using the IntenSEM Silver Enhancment Kit (Amersham International plc) following manufacturer's instructions. Silver enhancment solution was applied to sections for a number of 3-5 minute periods, with thorough washing with Milli-Q water between each application. Sections were air dried mounted with DPX and viewed under epi-flourescence illumination using a Nikon IGGS filter.

2.1.10.2 Preparation of NAMP and Fast Red TR salt for alkaline phosphatase colour development.

100X stocks of NAMP and Fast Red TR salt were prepared as follows. 51.4mg of Fast Red TR salt was added to 1ml of 70% DMF (N-N,Dimethylformamide, Sigma-Aldrich Company LTD). 20.8mg of NAMP were added to 1ml of 100% DMF. Each was stored under light excluded conditions at -20°C. A working solution of NAMP/Fast Red TR substrate was prepared by dissolving 10µl of each in 5ml of Buffer 2, pH9.0.

2.1.10.3 Optimisation of the Immunolocation Procedure.

Optimisation of the immunolocation procedure was necessary for the immunolocation experiments. A number of parameters in each experiment were altered sequentially to

try to obtain the best immunolocalisation result. The parameters changed are outlined in Table 9.

2.1.11 Photomicrography

Photomicrographs were taken using a Nikon Optiphot microscope with attachable camera on AGFA Colour film (50, 200 or 400 ASA). Processing and printing was carried out by Boots plc.

Embedding Medium	Primary Antibody Dilution	Primary Incubation Time and Temp.	Washes	Secondary Antibody Conjugate	Secondary incubation Time and Temp.	Washes	Visualisation
LR White resin Paraplast Plus	1:250 1:100 1:10 in TBS, 0.01% Tween 20, 1:100 sheep serum	4°C overnight room temperature 2-4 hours 37°C 2 hours	3X 5 min. 3X 10 min. in TBS, 0.01% Tween 20 with gentle agitation	FITC 1 1:10, 1:60, 1:100 Alkaline Phosphatase. 1:10, 1:50, 1:250 immunogold 1:100, 1:250	4°C overnight room temperature 2-4 hours	3 X 5min. 3 X 10min. 3 X 15min.	FITC - UV excitation with a blue filter. Alkaline Phosphatase - develop colour signal and view Immunogold - silver enhance and view using epipolarisaing light (Nikon IGS system)

Table 9. Parameters in the Immunolocalisation procedure which were changed for optimisation purposes.
A combination of one entry from each column was used for each localisation experiment.

2.2 cDNA Library Construction and Screening

2.2.1 Introduction

The aim here was to isolate genes showing increased or unique expression during the syncytial and cellularisation stages of barley grain development and find genes expressed within the developing endosperm. To do this a cDNA library of mRNA isolated from whole grains of barley aged between 1 and 10 DPA was made. Differential screening of the library using total and poly $(A)^+$ RNA of specific ages could then be used to isolate clones enhanced in one age group as compared with others.

The library was made in the Uni-ZAP XR vector (Stratagene Ltd). This insertion vector had a number of features which made it ideal. These included: ability to clone up to 10kb of foreign DNA, unidirectional cloning of cDNA, nucleic acid screening, *in-vivo* subcloning, blue/white colour selection, presence of the T3 and T7 RNA polymerase promoter sequences, and a number of sequencing primer sites (eg. M13, forward and reverse sequencing primer sites).

2.2.2 Materials for cDNA library construction and screening

2.2.2.1 Kits

Lambda Uni-ZAP XR cDNA Cloning Kit (Stratagene Ltd)

Gigapack II Packaging Extracts (Stratagene Ltd)

Universal Riboclone® cDNA Synthesis System (Promega Ltd)

NucTrap Push Columns (Stratagene Ltd)

Oligo d(tex) mRNA Isolation Kit (Qiagen Ltd)

Prime It II Random Priming Kit (Stratagene Ltd)

Wheat Germ System (for *in-vitro* translation) (Promega Ltd)

Sequenase Version 2.O Sequencing Kit (Amersham International Plc)

Gene Clean II Kit (Biosciences)

2.2.2.2 Vectors and Host Bacterial Strains

Vectors and host bacterial strains supplied in the cDNA cloning Kit were used and are outlined below:-

Uni-Zap XR vector

pBluescript II SK - phagemid

ExAssist Helper Phage

XL1-Blue MRF': Δ(*mcrA*)*183*, Δ(*mcrCB-hsdSMR-mrr*)*173*, *endA1, supE44, thi-1, recA1, gyrA96, relA1, lac*[F'*proAB, lacPZΔM15*, Tn*10*(tet^r)]

SOLR™ Strain: e14$^-$(*mcrA*), Δ(*mcrCB-hsdSMR-mrr*)171, *sbcC, recB, recJ, umuC*::Tn*5*(kan^r), *uvrC, lac, gyrA96, relA1, thi-1, endA1*, λ^R,[F'*proAB,lacPZΔM15*]Su^-(nonsuppressing).

2.2.3 RNA extraction

A number of total RNA and poly $(A)^+$ RNA isolation methods were tried in order to optimise the quality and quantity of RNA obtained for use in the construction and screening of the cDNA library. The following method of total RNA extraction and the Oligo-d(tex) mRNA Isolation Kit, were finally chosen.

2.2.3.1 Total RNA extraction, Guanadine Hydrochloride method

Adapted from Logemann *et al.* (1987)

Ten grams of frozen tissue (whole barley caryopses aged 1-10 DPA) were ground to a fine powder under liquid nitrogen with a mortar and pestle. To prevent freezing of the extraction buffer the powder was allowed to thaw to -20°C. The powder was then homogenised in the mortar with 5ml of GuHCl (Guanidine hydrochloride, Sigma-Aldrich Company LTD) extraction buffer (Appendix A). The resultant homogenate was transferred to a 30ml corex tube and centrifuged at 10000rpm at 4°C for 10 minutes. The supernatant was transferred to a fresh 30ml corex tube and an equal volume of phenol/chloroform (BDH-Merck Ltd) added and mixed by vortexing. The solution was centrifuged at 10000rpm at 4°C for 10 minutes. The aqueous phase was transferred to a clean corex tube and an equal volume of chloroform added, vortexed and centrifuged at 10 000 rpm at 4°C for 10 minutes. This step was repeated until no interphase material was seen. The final aqueous phase was transferred to a clean corex tube. To precipitate RNA, 0.2 volumes of 1M acetic acid (BDH-Merck Ltd) and 0.7 volumes of cold ethanol were added, mixed by inversion and chilled at either -20°C overnight or -80°C for 4 hours. RNA was pelleted by centrifugation at 10000 rpm at 4°C for 20 minutes. The pellet was washed twice with 3M sodium acetate (BDH-Merck Ltd) pH5.3. Following each wash the RNA was repelleted by centrifugation at 4°C for 5 minutes. The RNA was then washed with 70% ethanol, repelleted, allowed to air dry for 30 minutes and dissolved in an appropriate volume of DEPC- (BDH-Merck Ltd) treated sterile distilled water.

2.2.3.2 mRNA Isolation

The Qiagen Oligo-d(tex) mRNA Isolation Kit was used as per manufacturers instructions to isolate mRNA.

2.2.4 RNA quantification and quality.

The quality of the RNA sample is of great importance, especially that of the mRNA component. Total RNA quality and quantity was assessed by separation on a denaturing agarose gel and also by spectrophotometry. Use of the Qiagen mRNA isolation kit precludes the use of spectrophotometry to assess mRNA as residual oligo-d(tex) occurs in the sample. mRNA quality was assessed by denaturing agarose gels and by the ability of the mRNA to encode polypeptides in a cell free translation system (Promega Wheat Germ System).

2.2.4.1 Spectrophotometric analysis of total RNA

Total RNA samples were diluted 1:500 in sterile DEPC/distilled water and the OD_{260} and OD_{280} measured. Acid-washed quartz cuvettes were used. The quality of total RNA was assessed by the 260:280 ratio ($A_{260/A280}$). A ratio of 2+/- 0.05 was indicative of a pure RNA sample. Quantification of total RNA was carried out using the following calculation:

$$\text{RNA } \mu g/ml = A_{260} \times 44.19$$

where A_{260} = absorbance (in optical densities) at 260nm

44.19 = extinction coefficient of RNA

2.2.5 Denaturing Agarose gel for RNA

Horizontal 1% agarose (Gibco-BRL, Life Technologies) denaturing gels were used to separate RNA populations. These were prepared by melting 1g agarose in 100ml 1X MOPS (3-[N-Morpholino]propanesulfonic acid, Sigma-Aldrich Company LTD)in a microwave oven. This was allowed to cool to 55°C and 5.4ml formaldehyde (BDH-Merck Ltd) (~40%) was added and mixed prior to casting (in a fume hood). Samples were diluted (by the respective amount to allow equal loading) in SB (sample buffer), heat denatured at 65°C for 5 minutes, snap-cooled on ice and loaded into the gel wells. Gels were run in 1X MOPS. Gels were then viewed under UV (ultra-violet) light on a transilluminator.

2.2.6 *In-vitro* translation of mRNA

In-vitro translation of isolated mRNA was carried out using Promega Wheat Germ Extract System. The following components were added to a microfuge tube and incubated at 25°C for 1 hour: 25µl wheat germ extract, 1µl RNasin Inhibitor (Promega Ltd), 4µl 1mM amino acid without methionine (as supplied with the kit), 2µl RNA substrate, 2µl ^{35}S methionine (10mCi/ml) (Amersham International plc) and sterile distilled water to a final volume of 50µl. Immediately prior to use the RNA substrate was heated to 67°C for 10 minutes and snap cooled on ice. The translation products were assessed by SDS polyacrylamide protein gel electrophoresis and autoradiography.

2.2.7 *In-vitro* translation product separation on SDS - polyacrylamide gels.

10% SDS-polyacrylamide gels with a 5% SDS-polyacrylamide stacking gel was used along with electrophoresis to separate proteins. To prepare the 10% SDS-

polyacrylamide gel the following reagents were mixed in order: 1.9ml water, 1.7ml 30% acrylamide mix (BDH-Merck Ltd), 1.3ml 1.5M tris pH 8.8, 50µl 10% SDS (sodium lauryl sulphate, BDH-Merck Ltd), 100µl 10% ammonium persulphate (Sigma-Aldrich Company LTD) and 9µl TEMED (N,N,N′,N′-Tetramethylethylenediamine, Sigma-Aldrich Company LTD). The gel was poured into the vertical gel apparatus and a layer of butanol (BDH-Merck Ltd) placed on top to prevent drying and to give a straight edge. A 5% stacking gel was prepared by mixing 1.4ml water, 330µl 30% acrylamide mix, 250µl 1M tris pH 6.8, 20µl 10% SDS, 30µl 10% ammonium persulphate and 3.5µl TEMED in order. The butanol was removed from the 10% gel. This lower gel was then rinsed with water and dried before the stacking gel was applied. A comb was inserted into the stacking gel to make wells. Samples were mixed with 2X SDS-gel loading buffer prior to loading into the wells. Gels were run in 1X tris-glycine electrophoresis buffer. Following electrophoresis gels were fixed in TCA (trichloroacetic acid, BDH-Merck Ltd) and autoradiography was carried out using X-ray film, overnight at room temperature.

2.2.8 Northern Blot - transfer of RNA from gel to nylon membrane.

Equally loaded RNA samples (10µg) were separated in denaturing agarose gel, then transferred by capillary action to Hybond N membranes (Amersham International plc) using a blotting station. A blotting station which consisted of a stack composed of two layers of Whatman 3MM (Whatman International Ltd) wicks soaked in blotting buffer, on top of which the gel, Hybond N membrane, two layers of dampened Whatman 3MM paper, dry Whatman 3MM paper and a wad of paper towels placed on a tank of transfer buffer was constructed. Care was taken to remove air bubbles between each layer. A 1kg weight was placed on top of the stack to aid transfer and

blotting was carried out overnight. RNA was fixed to the membrane by UV crosslinking using the Stratagene Stratalinker UV Crosslinker on optimal setting.

2.2.9 cDNA Library Construction

The Stratagene Uni-ZAP XR cDNA Synthesis Kit was used to prepare a cDNA library from mRNA isolated from whole barley caryopses ranging in age from 1 - 10 DPA. cDNA was cloned into the lambda Uni-ZAP XR vector and packaged using Stratagene Gigapack II packaging extracts. The kits were used as per manufacturers' instructions.

2.2.10 Library Screening

The cDNA library of mRNA from whole grains aged between 1 and 10 DPA was differentially screened for plaques showing unique or enhanced expression at 3 DPA as compared to 10 DPA. Reverse transcription of total RNA (and messenger RNA) from 3 and 10 DPA whole grains was used to produce [α ^{32}P]-dATP radiolabelled cDNA probes. These probes were hybridised against duplicate plaque lifts of almost confluently plated library phage (~ 40 000 plaques per 22X22cm plate). Plaques showing differential expression were picked into SM buffer and stored for secondary screening.

Following the initial success of producing cDNA probes by reverse transcription, further attempts failed. A number of different ways of producing probes from total RNA were tried but each failed to give good labelling. Therefore differential screening using cDNA probes was not used for secondary screening. Instead, Northern blotting was used to immobilise total RNA from 1, 3, 6 and 10 DPA caryopsis, leaf and root total RNA on Hybond N membrane. *In-vitro* subcloning from the lambda ZAP vector to pBluescript II SK- phagemid was carried out to facilitate

easier handling of the clones from the primary plaque screen. Inserts cut from these were used to produce randomly primed [α ^{32}P]-dATP labelled DNA probes. These probes were hybridised against the Northern blotted total RNA to identify clones showing differential expression. Clones showing differential expression at this level were then used in *in-situ* hybridisation experiments to further clarify timing of expression and locate the area of the grain in which they were expressed. Clones identified in this way were then sequenced either manually or by automated sequencing methods.

A positive control using Maize Histone 1 and Histone 2 clones was carried out to test the library quality and the labelling and hybridisation methodology. Inserts from the Maize Histone 1 clones were excised and labelled with [α ^{32}P]-dATP using the Stratagene Prime It II Random Priming Kit .

2.2.10.1 Preparation of Host *E. coli* Cells for Library Plating

A single colony of the host *E.coli* strain XL1-Blue MRF' was grown in 20ml 0.2% maltose (Sigma-Aldrich Company LTD), 50mM $MgSO_4$ (magnesium sulfate, BDH-Merck Ltd) supplemented LB (luria broth) media. The culture was grown overnight at 37°C in an orbital shaker at 250 rpm. Cells were then pelleted by centrifugation at 4000 rpm for 5 minutes at 4°C. The pellet was drained and resuspended in 4ml of 10mM $MgSO_4$. Cells were stored at 4°C for a maximum of 2 days prior to use.

2.2.10.2 Plating of Lambda Bacteriophage.

The bacteriophage library was titred to determine the concentration. It was then plated at a density of 40000 pfu (plaque forming units) per 22x22cm plate. 4 X 10^4 pfu of

lambda bacteriophage were mixed with 1.5ml of prepared XL1-Blue MRF' cells at an OD_{600}=0.5 (OD = optical density). The cell/bacteriophage mixture was then incubated for 15 minutes at 37°C to allow infection of the host cells. The mixture was then mixed with 30ml molten top NZY agarose (equilibriated to 42°C) and poured evenly onto plates containing 300ml hardened bottom NZY agar. Plates were then incubated overnight at 37°C. This gave near to confluent plaque density.

2.2.10.3 Bacteriophage Lambda Plaque Lifts

This method was used to transfer plated bacteriophage particles to Hybond N membrane for subsequent screening with radio-labelled probes. The plated bacteriophage was chilled for 2 hours to prevent the top agarose from sticking to the membrane. A sheet of Hybond N was placed directly onto the lawn surface and left for 90 seconds. During this time a needle loaded with ink was used to stab the membrane and agar in an asymmetric pattern to record the position of the membrane on the plate. Membranes for duplicate plaque lifts were orientated with the same ink pattern and left on the lawn for 3-5 minutes. Membranes were then transferred to denaturing solution for 2 minutes then submerged in neutralising solution for 5 minutes before being air dried. DNA was fixed onto the membrane by UV crosslinking using a Stratalinker UV Crosslinker on optimal setting. Plates of transfers were sealed and stored at 4°C.

2.2.11 Preparation of Radiolabelled Probes for Library Screening

2.2.11.1 Method 1. Promega Labelling of First strand cDNA

Labelled first strand cDNA was produced using the Universal Riboclone® cDNA Synthesis System (Promega Ltd). A reaction mix consisting of 20µl 25mM $MgCl_2$

(magnesium chloride), 10µl 10X reverse transcription buffer (as supplied with the kit), 10µl dNTP (deoxynucleotide triphosphate) mix containing 10mM each dGTP, dTTP, dCTP (deoxyguanosine triphosphate, deoxythymine triphosphate, deoxycytosine triphosphate) and 120µM dATP (deoxyadenosine triphophate), 1.5µl RNasin Inhibitor, 5µg total RNA, 10µl oligo dT primers, 1.5µl 20000u/ml AMV (avian myloblastosis virus) reverse transcriptase, 400µCi[α^{32}P]dATP (Amersham International plc) was mixed in a microfuge tube. The reaction mix was then incubated at 42°C for 1 hour and then the reaction was stopped by heating at 95°C for 3 minutes. Stratagene Nuctrap Purification Columns were used as per manufacturer's instructions to remove unincorporated dNTPs.

2.2.11.2 Method 2. Labelling of second strand cDNA :- Stratagene RNase H$^-$ method.

Synthesis of first strand cDNA was carried out by making a reaction mix consisting of 5µl 10X 1st Strand buffer (as supplied with Stratagene Rnase H$^-$ reverse transcriptase), 3µl dNTP mix consisting of 10mM each of dGTP, dCTP and dTTP, 2µl oligo dT primer (1.4µg/ml), 1µl RNase Inhibitor (40000u/ml). Total RNA (1µg) was heat denatured at 65°C for 10 minutes and added to the reaction mix. To allow the primer to anneal to the template the mixture was allowed to incubate at room temperature for 10 minutes, then 2.5µl Stratascript RNase H$^-$ reverse transcriptase was added and mixed by gentle vortexing. A control reaction was undertaken by removing 5µl of the reaction mix to a fresh tube with 0.5µl 10µCi/Mol [α^{32}P]dATP. Both reactions were then incubated at 37°C for 1 hour. The control reaction mix was stored at -20°C until the second strand reaction was complete.

Labelling of second strand cDNA was carried out by addition of 20µl 10X DNA polymerase 1 buffer (as supplied with the DNA polymerase, Stratagene Ltd), 3µl

dNTP mix (10mM each of dATP, dCTP, dTTP, dGTP), 122µl distilled water, 5 µl 10µCi/Mol [α ^{32}P]-dATP to the first strand reaction mix. The second strand reaction mixture was then mixed gently and spun in a bench top microfuge to consolidate. Then 2.5µl RNase H$^-$ reverse transcriptase and 4.5µl DNA polymerase 1 were added and mixed by gentle pippetting. The reaction mix was then incubated at 16°C for 2.5 hours. Unincorporated label was removed using Stratagene Nuctrap Columns as per manufacturer's instructions.

2.2.11.3 Method 3. Labelling of cDNA by random priming - Stratagene Prime It II Random Priming Kit

Unlabelled double stranded cDNA was made using the Universal Riboclone® cDNA Synthesis System (Promega Ltd) with 3 and 10 DPA total RNA as the templates. The double stranded cDNA was then labelled by the random priming method using the Prime It II Random Priming Kit (Stratagene Ltd).

2.2.12 RNA Hydrolysis

After making labelled cDNA the RNA template was removed by hydrolysis. To hydrolyse RNA, 14µl 2M NaOH (sodium hydroxide) was mixed with the cDNA sample and incubated at 60°C for 30 minutes. To neutralise the alkali 14µl 1M HCl (hydrochloric acid, BDH-Merck Ltd) and 14µl Tris HCl (Tris-hydroxymethylaminomethane hydrochloride, BDH-Merck Ltd) pH8.3 were added to the reaction mix. Phenol/chloroform extraction was then carried out and the cDNA precipitated by addition of 4M ammonium acetate (BDH-Merck Ltd), and 2.5 volumes of ethanol followed by chilling overnight at -20°C or for 20 minutes at -80°C. The cDNA was then pelleted, washed with 2M ammonium acetate and 2.5 volumes of

ethanol, then repelleted. The pellet was then aspirated, dried and resuspended in an appropriate volume of sterile distilled water.

2.2.13 Alkaline agarose Gel

A 1% w/v agarose gel was prepared by melting 0.8g agarose in 72ml water. This was cooled to 55°C and 8ml alkaline buffer (Appendix A) added, mixed by swirling and poured immediately into a gel cast. An equal volume of 2X loading buffer was added to the samples and each loaded into the gel wells. Gels were run in 1X alkaline buffer at 100mA, blotted to remove excess liquid and sealed in a polythene bag. Care was taken not to trap any air. Gels were then exposed to X-ray film overnight at -80°C. The X-ray film was then processed in an automatic film processing unit (X-ograph Compact X2).

2.2.14 Sequencing

Both manual and automated sequencing methods were used to partially sequence clones of interest.

2.2.14.1 Manual sequencing

DNA of the clones (pBluescript II SK phagemid subclones) to be sequenced were prepared using the caesium chloride maxi plasmid prep method.

As the template DNA was double stranded, the alkali denaturation method was used to denature it prior to the sequencing reaction. The Sequenase Version 2.0 Kit was then used as per manufacturer's instructions, except that M13 forward or reverse primers

were used instead of the -40 primer supplied with the kit. 4µl of each reaction were then run on an acrylamide sequencing gel.

2.2.14.2 Alkali denaturation of double stranded DNA

4µg of template DNA were diluted with sterile distilled water to a final volume of 52µl. Alkali denaturation was then carried out by the addition of 1M NaOH (sodium hydroxide, BDH-Merck Ltd.) to a final concentration of 0.2M and incubation at 37°C for 30 minutes. The mixture was then neutralised by addition of 6.5µl 3M NaOAc (sodium acetate, Sigma-Aldrich Company LTD) pH5.2 and the DNA precipitated with ethanol and redissolved in 7µl of sterile distilled water.

2.2.14.3 Preparation and running of acrylamide sequencing gels

Sequencing gels were made by mixing 60ml Sequencing Gel Solution (NBL Gene Sciences Limited) with 30µl TEMED and 30µl of 25% ammonium persulphate. The mixture was then poured between two clean glass plates separated with spacers and sealed with tape. Combs to form wells were inserted and the gel allowed to set. Prior to use, the combs were removed, the gel pre-run for 30 minutes and the wells rinsed with 1X TBE buffer. Each of the sequencing reactions (4µl) was loaded into adjacent wells in the gel. The gel was run at 1500-2000v for 2-6 hours. Gels were then fixed in 10% TCA in methanol (BDH-Merck Ltd) for 30 minutes, transferred to Whatman 3MM paper, blotted to remove excess liquid and dried in a gel drier at 60°C for 1 hour. Dried gels were then exposed to X-ray film overnight at room temperature. The film was then developed.

2.2.14.4 Automated sequencing

DNA of clones to be sequenced was prepared using the caesium chloride maxi prep method. Sequencing was carried out at The University of Durham using an PE Applied Biosystems 373 'Stretch' Automated Sequencer.

2.2.15 Horizontal agarose gel electrophoresis

Horizontal 0.7% and 1% agarose gels, buffered with 1X TBE were used. The relevant amount of agarose was melted in 100mls of 1X TBE buffer, allowed to cool to 55°C and cast in the gel tank apparatus. DNA samples were diluted in 6X DNA loading buffer and loaded into the gel wells and electrophoresed in potential fields of 3-5v/cm. Bromophenol blue in the loading buffer acted as tracker dye for the movement of the front. Following electrophoresis DNA was visualised in the gel by staining with ethidium bromide (Sigma-Aldrich Company LTD) (0.5μg/ml final concentration) and viewing under UV illumination.

2.2.16 Restriction Enzyme Digests

Restriction of DNA was carried out in an excess of enzyme following manufacturer's recommendations. All restriction enzymes were purchased from Promega Ltd.

2.2.17 Mini Plasmid DNA preparation

Individual colonies of host *E.coli* cells were picked into 2ml LB supplemented with the appropriate selective agent and grown overnight at 37°C. Following this, 1.5ml of culture was transferred to a new tube and the cells pelleted by centrifugation at low speed in a microfuge. The remaining cell culture was stored at 4°C until further use. The supernatant was removed from the pelleted cells and the pellet resuspended in

100µl of ice cold solution I by vortexing. Then 200µl of solution II was added, mixed by inversion and the mixture allowed to stand on ice for 2 minutes. After this 150µl ice cold solution III was added and gently mixed by vortexing and the solution was left to stand on ice for 5 minutes. Cell debris was then pelleted by centrifugation at 12000 rpm for 5 minutes at 4°C and the supernatant containing the plasmid DNA transferred to a fresh microfuge tube. Contaminating proteins were removed by phenol/chloroform extraction. An equal volume of phenol/chloroform was vortexed with the supernatant and the mixture centrifuged at 12000 rpm for 5 minutes. The aqueous phase was then transferred to a fresh microfuge tube and the DNA precipitated by addition of 2 volumes of ethanol. The ethanol was mixed by inversion and the solution allowed to stand at room temperature for 2 minutes before recovery of the DNA by centrifugation at 12000 rpm for 5 minutes at 4°C. The supernatant was removed and the pellet washed with 70% ethanol. The pellet was recovered by centrifugation at 12000rpm for 2 minutes, the supernatant removed and the pellet dried using a Speedivac before being resuspended in TE buffer. 20µg/ml RNase A (Promega Ltd) was added, the solution vortexed and stored at -20°C until required.

2.2.18 Caesium Chloride Maxi Plasmid Prep

A single host colony was picked and grown overnight in 100ml selective LB medium at 37°C in an orbital shaker at 250 rpm. Cells were then harvested by centrifugation at 3000 rpm for 10 minutes at 4°C, resuspended in 4ml ice cold solution 1 by vortexing and allowed to stand on ice for 5 minutes. Alkaline lysis of cells was then carried out by addition of 8ml solution II. The solution was mixed by inversion then left on ice for 5 minutes. It was then neutralised by addition of 6ml ice cold solution III which was mixed by gentle vortexing and left on ice for a further 5 minutes. Cell debris was

removed by centrifugation at 5000 rpm for 10 minutes at 4°C. The supernatant was transferred to a fresh tube and nucleic acids precipitated by addition of 12ml isopropanol (BDH-Merck Ltd) and incubation at room temperature for 15 minutes. DNA was then pelleted by centrifugation at 10000 rpm for 15 minutes at 4°C. The supernatant was removed and the pellet washed with 70% ethanol. The pellet was then air dried for 10 minutes at room temperature and resuspended in 5ml (precisely) of TE (Tris-EDTA) buffer, pH8.0. Further purification of plasmid DNA was carried out by caesium chloride density gradient. Caesium chloride (Sigma-Aldrich Company LTD) (5g) was added to the DNA solution and dissolved by swirling. To allow visualisation of the DNA bands in the gradient, 100µl of 10mg/ml ethidium bromide was mixed in and the solution stood on ice for 10 minutes until the ethidium bromide and caesium chloride were fully dissolved. Unwanted debris was pelleted by centrifugation at 8000 rpm for 10 minutes and the supernatant transferred to ultracentrifuge tubes. The gradient was established by centrifugation at either 80000 rpm for 3 hours or 55000 rpm for 18 hours at room temperature. The plasmid DNA band was removed from the ultracentrifuge tube using a syringe and hypodermic needle. To remove the ethidium bromide, plasmid DNA was extracted 4 times with water-saturated butanol. Following the final extraction, the supernatant was transferred to a corex tube and 700µl 3M NaOAc pH6.0, 7ml water and 15ml ethanol were added, mixed and the solution chilled to precipitate DNA. The DNA was recovered by centrifugation at 12000 rpm for 30 minutes at 4°C. The supernatant was removed and the pellet air dried for 30 minutes. The pellet was redissolved in 400µl sterile distilled water, then 40µl 3M NaOAc pH6.0 and 880µl ethanol added and the solution chilled at -20°C for 20 minutes to reprecipitate the DNA. DNA was again recovered by centrifugation at 12000 rpm for 20 minutes. The supernatant was removed, the pellet washed with 70%

ethanol and recovered by centrifugation. The supernatant was drained, the pellet dried in the Speedivac and resuspended in 200µl sterile distilled water.

2.3 *In-situ* Hybridisation

2.3.1 Production of Digoxigenin UTP-11 labelled riboprobes by *in-vitro* transcription

pBluescript II SK phagemids containing cloned cDNAs of interest were linearised by restriction enzyme digest. Linearised template DNA was then cleaned using either Gene Clean II Kit or phenol/chloroform extraction. Following this, *in-vitro* transcription reactions were carried out using T7 and T3 RNA polymerases (Promega Ltd). For the reaction, 1μg of linearised template DNA, 2μl Dig RNA Labelling Mix (Boerhinger Mannheim UK), 2μl transcription buffer (as supplied with each RNA polymerase), 1μl RNase Inhibitor (Promega Ltd), sterile distilled water to a final reaction volume of 20μl and 2μl of either T7 or T3 RNA polymerase, were mixed in a microfuge tube on ice and incubated at 37°C for 2 hours. The reaction was stopped by addition of 2μl 0.2M EDTA (Ethylenediaminetetraacetic acid, Sigma-Aldrich Company LTD) pH8.0. Incorporation of the digoxigenin label was tested by dot spotting 1μl of each probe on Hybond N membrane and carrying out an immunolocation reaction.

2.3.2 Dot Blot Analysis of Dig-labelled riboprobes.

Hybond N membrane was cut to the required size and soaked for 20 minutes in 10X SSC (standard saline citrate), blotted to remove excess liquid and air dried. 1μl of each riboprobe was pipetted onto the membrane and allowed to air dry before being crosslinked under UV light. The membrane was then soaked in buffer 1 for 5 minutes, then blocked by soaking in 2% normal sheep serum (Sigma-Aldrich Company LTD), 0.05% Tween 20 in buffer 1 for 30 minutes with gentle shaking. The membrane was drained and incubated in a 1:1500 dilution of sheep anti-digoxigenin alkaline

phosphatase conjugated polyclonal Fab fragments (Boehringer Mannheim UK) diluted in 1% normal sheep serum in buffer 1 (Appendix A) for 30 minutes. The membrane was then washed 3 times in buffer 1 for 5 minutes each then transferred to buffer 2 (Appendix A) for 1 minute. Enzymatic colour precipitation was then carried out by incubating the membranes in freshly prepared Fast Red TR/NAMP substrate under light-excluded conditions until colour reaction was seen. The substrate was prepared by adding 10µl each of Fast Red TR and NAMP stock solutions to each 5ml of buffer 2, pH9.0 used.

2.3.3 *In-situ* Hybridisation

Method adapted from Leitch *et al.* (1994)

2.3.3.1 Pretreatment

Paraformaldehyde/glutaraldehyde fixed grains embedded in Paraplast Plus wax were used. Sections between 5 and 10µm thick were cut and adhered to poly-L-lysine slides (BDH Merck Ltd) by incubating on a warming plate at 40°C overnight. The sections were heated to 60°C for 10 minutes immediately prior to use. The sections were dewaxed through two changes of Histoclear and then rehydrated through 100%, 70% and 50% ethanol for 5 minutes each to DEPC water. Slides were then incubated in PBS (phosphate buffered saline) for 5 minutes then sequentially soaked in 5µg/ml proteinase K (Sigma-Aldrich Company LTD) (100mM Tris, 5mM EDTA) for 15 minutes at 37°C, 0.2% glycine (Sigma-Aldrich Company LTD) in PBS for 10 minutes at room temperature and then 2 changes of PBS for 5 minutes at room temperature.

2.3.3.2 Prehybridisation

Pretreated sections were incubated for 1 hour at 42°C in *in-situ* prehybridisation buffer in a humidity chamber moistened with 50% formamide (Sigma-Aldrich Company LTD)/4X SSC.

2.3.3.3 Hybridisation

The riboprobe was heat denatured at 60°C for 5 minutes and snap chilled before being added to *in-situ* hybridisation buffer prewarmed to 42°C. The probe was used at a concentration of 10μg/ml in *in-situ* hybridisation buffer. 50 - 100μl of probe plus *in-situ* hybridisation buffer was pipetted onto each slide and sections covered with Parafilm strips then incubated in a humidity chamber overnight at 42°C.

2.3.3.4 Post Hybridisation Washes.

The Parafilm covers were removed and slides washed briefly in 2X SSC. Slides were then washed sequentially in 2X SSC/50% formamide, 1X SSC/50% formamide and 0.5X SSC/50% formamide for 30 minutes each at 50°C. Sections were then rinsed in 2X SSC for 10 minutes at room temperature. Immunological detection of the probe was then carried out.

2.3.4 Immunological detection of digoxigenin-labelled riboprobes.

2.3.4.1 Detection using sheep Anti-digoxigenin-AP fab fragments (Boehringer Mannheim UK)

Sections were rinsed in buffer 1 for 5 minutes then blocked by incubating in 2% normal sheep serum, 0.05% Tween 20 in buffer 1 for 30 minutes at room temperature. Sections were then incubated in a 1:500 dilution of alkaline phophatase conjugated

polyclonal sheep anti-digoxigenin antibody diluted in 1% normal sheep serum, 0.05% Tween 20 in buffer 1 for 3 hours at room temperature, then washed twice in buffer 1 for 15 minutes each. Sections were then soaked in buffer 2 before being incubated in light-excluded conditions with freshly prepared Fast Red TR/NAMP substrate (2.1.10.2). Sections were monitored for colour development. Fresh substrate solution was added every 15-20 minutes.

2.3.4.2 Immunogold detection.

Detection using immunogold conjugated antibodies and silver enhancement was carried out as previously described in 2.1.10.1.3 (Secondary antibody application, Colloidal Gold conjugated antibodies).

Chapter 3

Results and Discussion

3.1 Early development of the endosperm

3.1.1 Observations on barley endosperm development

3.1.1.1 Results

Individual spikes of barley were tagged at anthesis. Caryopses from the mid-region of each spike were collected into fixative on a daily basis so that a range of caryopses of varying ages, in days post anthesis, were available up to 10 DPA. Caryopses were fixed and embedded in either Paraplast plus or LR White resin. To establish the age at which key events in the syncytial and early cellular endosperm occurred, and to compare morphological changes with other authors, embedded caryopses were sectioned and stained with the metachromatic stain Toluidine Blue. It was established that the syncytial stage lasted from 1-3 days post anthesis, with the beginning of cellularisation being at around 3 DPA. Transverse sections from the mid region of caryopses between 1-3 DPA, in the syncytial stage, are shown in Plates 1, 2 and 4a. The large central cell vacuole predominates, with nuclei being seen in the extremely thin cytoplasm which appears to be thickest next to the crease (vascular) region. The nucellus layer, integuments, testa, cross cells and pericarp can be clearly seen. Vacuolation of the central cell cytoplasm was observed in transverse sections of grains 2/3 DPA, giving an indication that cellularisation was about to proceed (Plate 2b). Anticlinal cell walls were seen in transverse sections from caryopses of 3/4 DPA (Plates 3-5), with the first complete endosperm cell

layer being observed by 4 DPA (see Plate 6). Further growth of anticlinal walls growing into the central cell vacuole and further cellularisation were only observed in sections from a few caryopses. For those sections in which cellularisation past the first cell layer was observed, some reduction in specimen quality was also noted. This was probably due to incomplete (poor) fixation. Complete cellularisation was observed in sections from caryopsis of 7/8 DPA (Plates 7 and 8 show cellularised endosperm from grains 10+ DPA).

3.1.1.2 Discussion

The primary structural observations made here on early endosperm development, which were to act as a basis for identifying the developmental stages of tissues used for molecular studies show similarities to more detailed structural studies made by other authors such as Fineran *et al.* (1982) on wheat endosperm development, and Bosnes *et al.* (1992) and Brown *et al.*(1994) on barley endosperm development. These authors tend to hold the more widely accepted views on cereal endosperm development during the early stages, as described in the Introduction (1.5). The morphology and progression of development of the syncytial stage shown here is similar to that observed by Fineran *et al.* (1982), Bosnes *et al.*(1992), Mares *et al.* (1975, 1977) and Morrison and O'Brien (1976). These authors observed that, following fertilisation of the central cell diploid polar nucleus, a syncytium of nuclei develops within the cytoplasm which is present as a thin layer held between the central cell vacuole tonoplast and the central cell membrane; the layer is slightly thicker over the crease region. As endosperm development proceeds the cytoplasm of the syncytium becomes vacuolate. From developmental studies of barley with mutant endosperm, Bosnes *et al.* (1992) proposed that vacuolation was necessary for

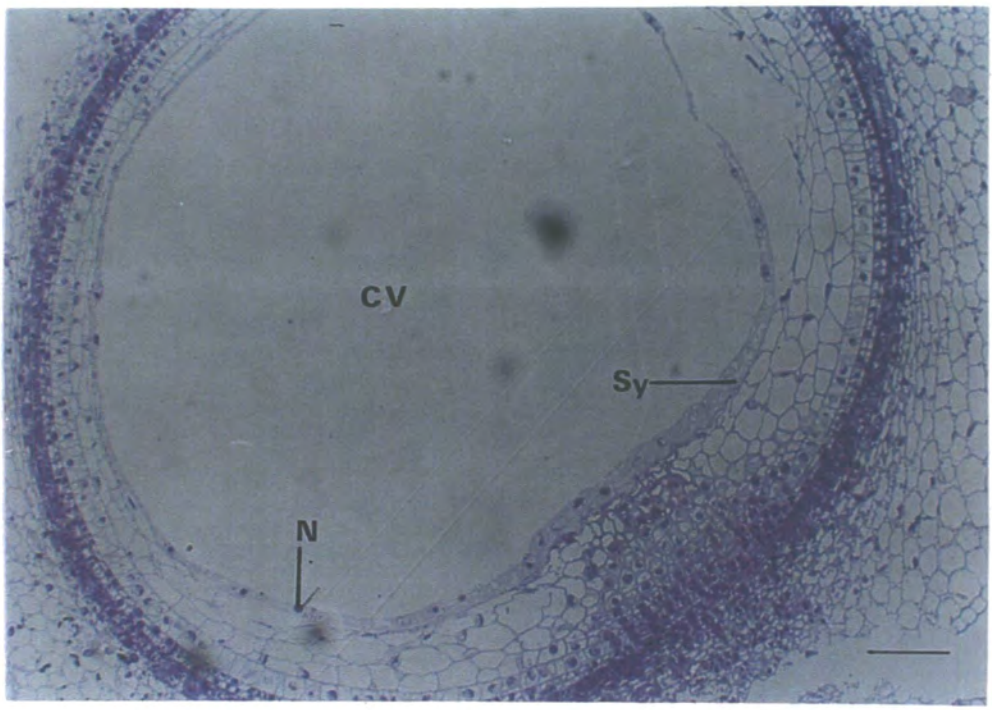
transition to the cellularisation stage. The observations of morphological changes during the syncytial stage by others are similar to those seen here. However, the observations made here on progression into and through the cellularisation stage do not correspond to those made by any other single author. There has been some controversy over morphological aspects and mechanics of the progression of the cellularisation stage; how anticlinal walls arise and whether phragmoplasts are involved are major questions. The most widely held and accepted views are based on those of Fineran *et al.* (1982) (on wheat), which have been more recently developed by Brown *et al.*(1994) (on barley). Fineran *et al.* (1982) showed that during wheat endosperm development, anticlinal walls formed between nuclei of the syncytium following normal mitosis, and the formation of a typical phragmoplast and cell plate between daughter nuclei. This contrasts with the proposals of Mares *et al.* (1975, 1977) and Morrison and O'Brien (1976) who suggest that cellularisation results from free growing walls which developed (independently of a phragmoplast) from peg-like structures protruding from the central cell membrane. The growth of these first anticlinal walls resulted in the formation of open ended compartments; such open ended compartments were also observed by Fineran *et al.*(1982) and Brown *et al.*,(1994) and are also shown here. From their study of microtuble rearrangements during endosperm formation Brown *et al.* (1994) have further clarified the importance and involvement of phragmosplasts, microtuble arrays and cytoskeletal rearrangement for the cellularisation process.

The work presented here shows that anticlinal walls form open ended compartments. However it is not possible from these micrographs to say whether a phragmoplast is involved (as proposed by Fineran *et al.* (1982) and Brown *et al.* (1994)) or if they develop, as proposed by Mares *et al.* (1975, 1977) and Morrison and O'Brien

(1976), from peg-like structures. Fineran *et al.* (1982) propose that, following formation of the anticlinal walls, periclinal walls again developed as a result of a normal phragmoplast between daughter nuclei, the cell plate growing out and meeting the anticlinal walls (perpendicularly) such that a peripheral layer of closed cells is formed, and subsequently a new layer of open ended compartments (with the open end facing into the central cell vacuole) is formed. Contrary to this view Morrison and O'Brien (1976) indicate that the periclinal walls are also free growing and form as a result of the anticlinal walls branching and growing towards one another to form a closed compartment with no overlying layer of open compartments, with further cell formation occurring as a result of mitotic activity. Fineran *et al.* (1982), however, disagrees with this indicating that both cellular division of the peripheral layer and further free growth of the anticlinal walls of the open ended compartments leads to complete cellularisation of the central cell space/endosperm. The contradictory views presented by Fineran *et al.* (1982), Mares *et al.* (1975, 1977) and Morrison and O'Brien (1976) have had some clarification from Brown *et al.* (1994). Brown *et al.* (1994) indicate that phragmoplast and microtubule arrays which are constantly rearranged depending on stage or phase of development are important for the cellularisation phase as well as the syncytial stage and that walls form in association with both conventional phragmoplasts (ie. those which form between dividing nuclei) and what they term 'adventitious phragmoplasts' which form as a result of interaction between subsets of microtubules which are not associated with mitotic processes (see Brown *et al.*,(1994) for details). The micrographs presented here in Plates 4 and 6 tend towards the view of Morrison and O'Brien (1976). This is because, following formation of the anticlinal walls and compartmentalisation of nuclei, in most

samples periclinal walls are seen to form such that only a single layer of completely-enclosed cells is observed with no overlying open-ended compartments. In comparison, Plate 5 indicates some open-ended layer lying on the first closed layer as observed by Mares *et al.* (1975, 1976), Fineran *et al.* (1982), Bosnes *et al.* (1992) and Brown *et al.* (1994). The nuclei appear to be pushed forward to the open end of the compartment which is a characteristic noted by both Fineran *et al.* (1982) and Brown *et al.* (1994). The detail of wall formation seen here, and resembling the observations made by Morrison and O'Brien (1976) may be due to fixation problems (artifacts or poor quality fixation). It is notoriously difficult to fix caryopses adequately, particularly from the early stages of endosperm development which have a 'milky', wet consistency of endosperm. The fact that other authors have observed different developmental patterns and that different patterns are represented in the micrographs presented here, suggests that some errors in fixation may have occurred here. In many of the micrographs very little or no cytoplasm is seen in association with the newly formed cells. Problems with tissue preservation meant that no reliable samples were obtained between 6 and 12 DPA; tissue blocks were either very difficult to section, or sections showed very distorted cellular arrangements. The later stages of development (ie completely cellularised endosperm) appear to be less or unaffected by poor fixation. In Plates 7 and 8 completely cellularised endosperm can be seen with examples of each cell type annotated.

The fixation methods used in this work were chosen because they had been successful in preserving tissue structure in similar experiments by previous workers within the laboratory and had been deemed as being adequate for identifying general stages of development in relation to parallel molecular studies.



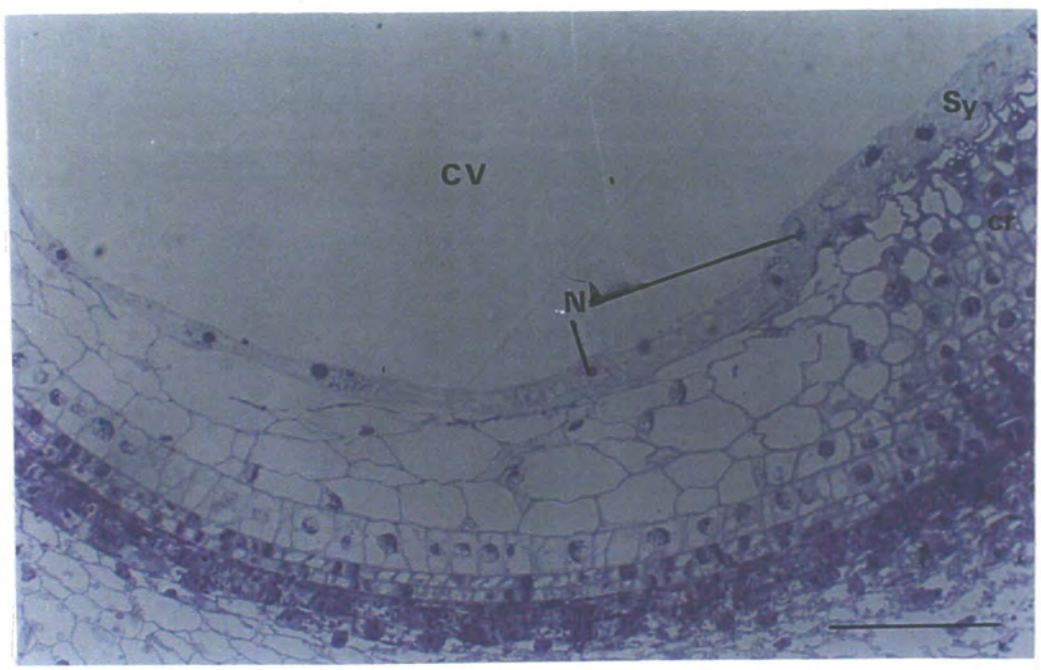
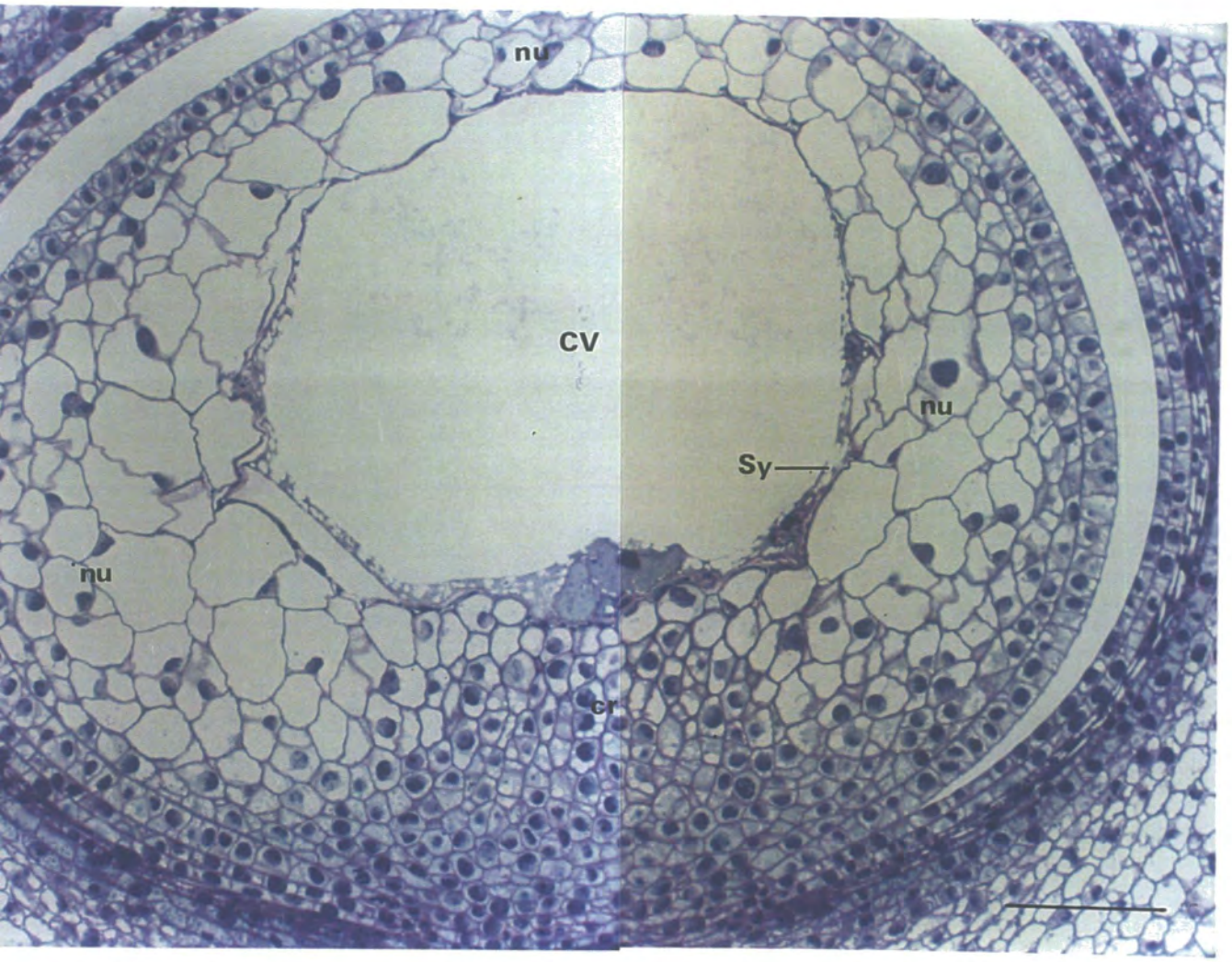


Plate 1. Transverse section through a caryopsis aged 2 DPA stained with Toluidine Blue, showing the endosperm at the syncytial stage.



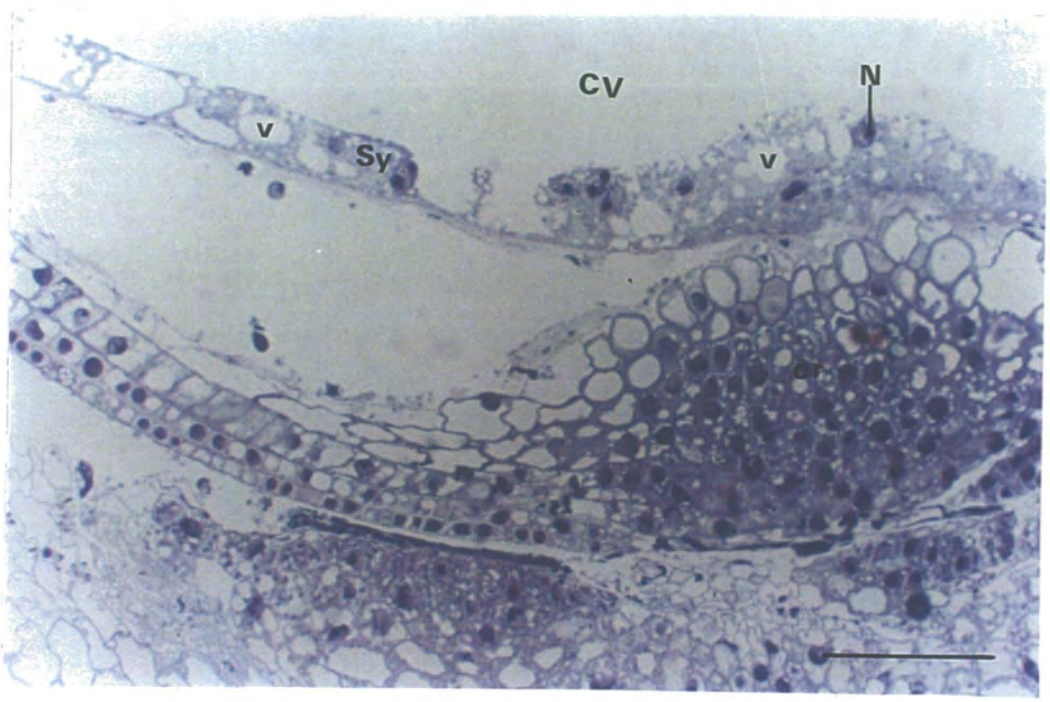


Plate 2. Toluidine Blue stained transverse section of (a) a 6 DPA caryopsis at the cellularisation stage and (b) a 2/3 DPA caryopsis at the syncytial stage, showing vacuolation of the syncytium cytoplasm.

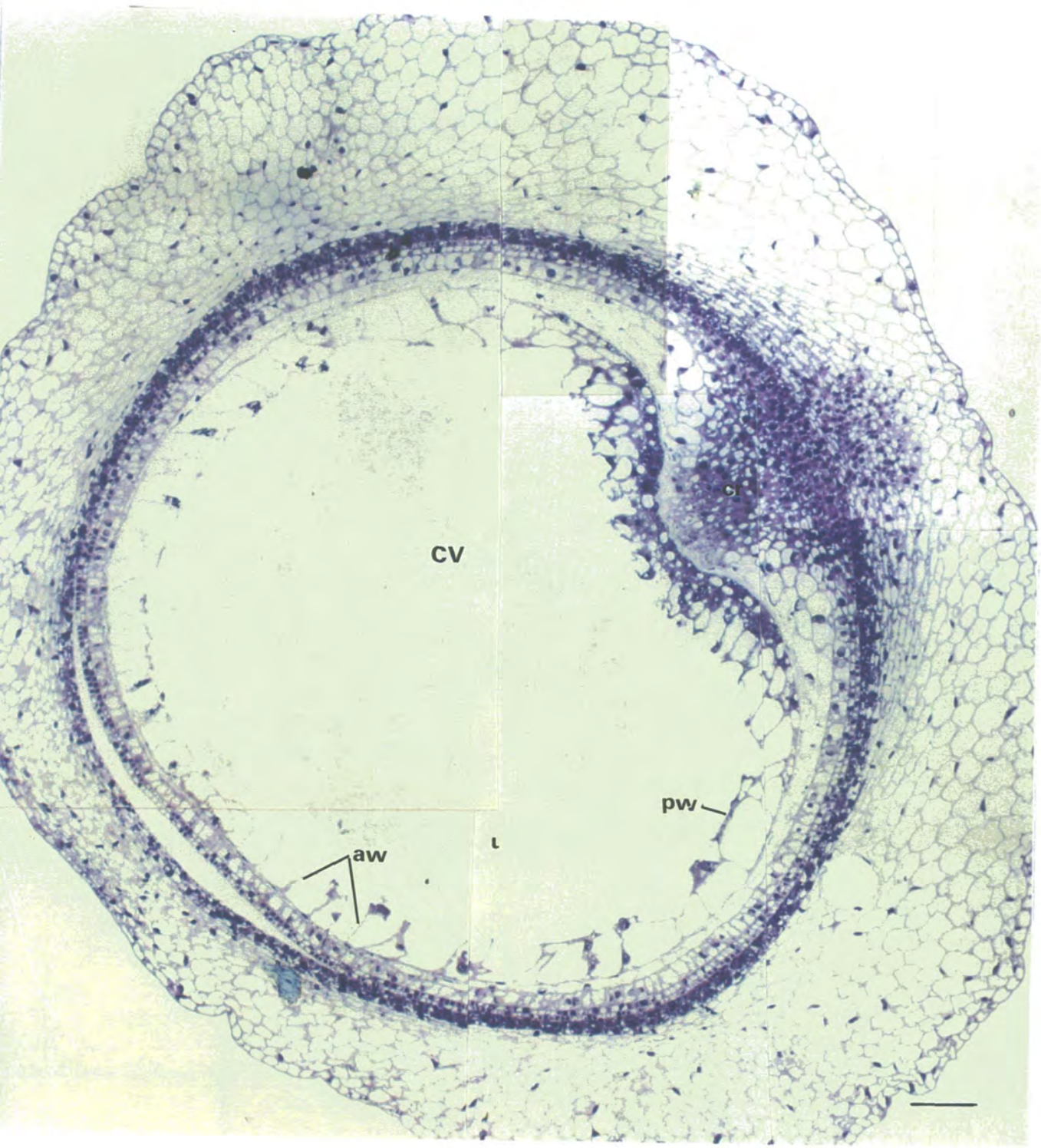


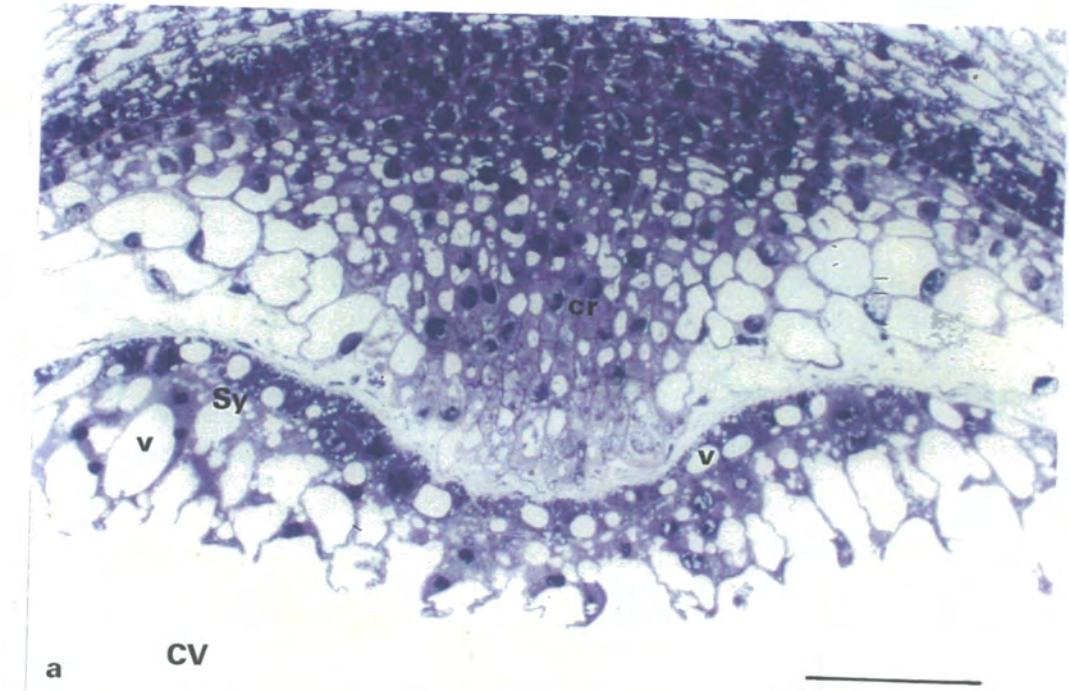
Plate 3. Toluidine Blue stained transverse section of 3 DPA caryopsis showing endosperm in the cellularisation stage at the point of anticlinal wall formation.

Plate 4. Toluidine Blue stained transverse sections of 3/4 DPA caryopses showing initial stages of endosperm cellularisation.

a. Vacuolar endosperm cytoplasm overlaying the crease region and initial anticlinal wall formation.

b. Anticlinal wall formation.

c. Formation of both anticlinal and periclinal walls giving the first complete endosperm cell layer.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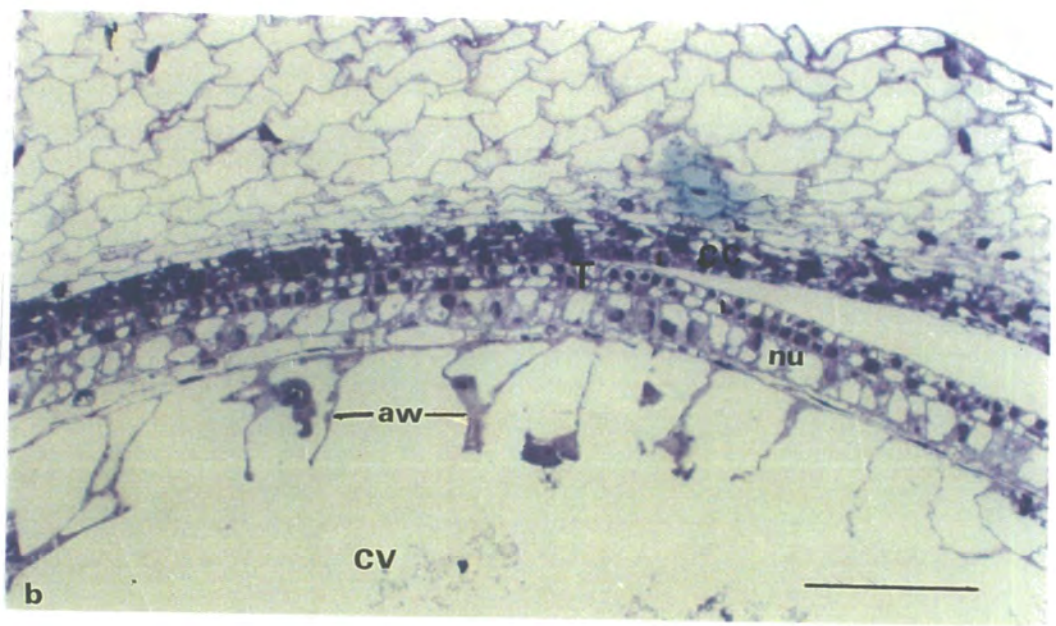
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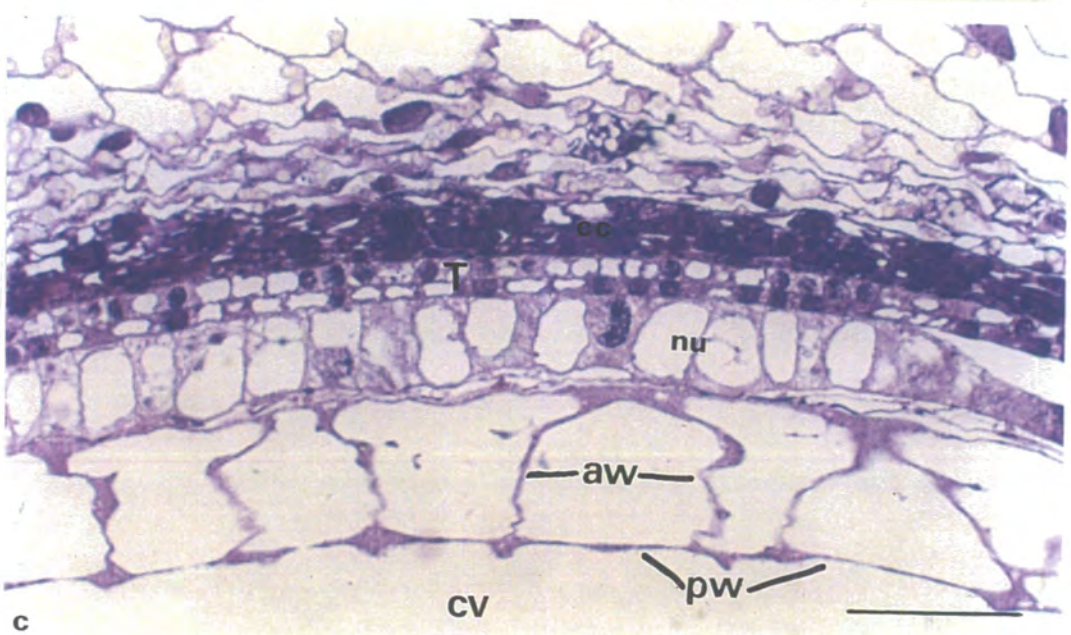
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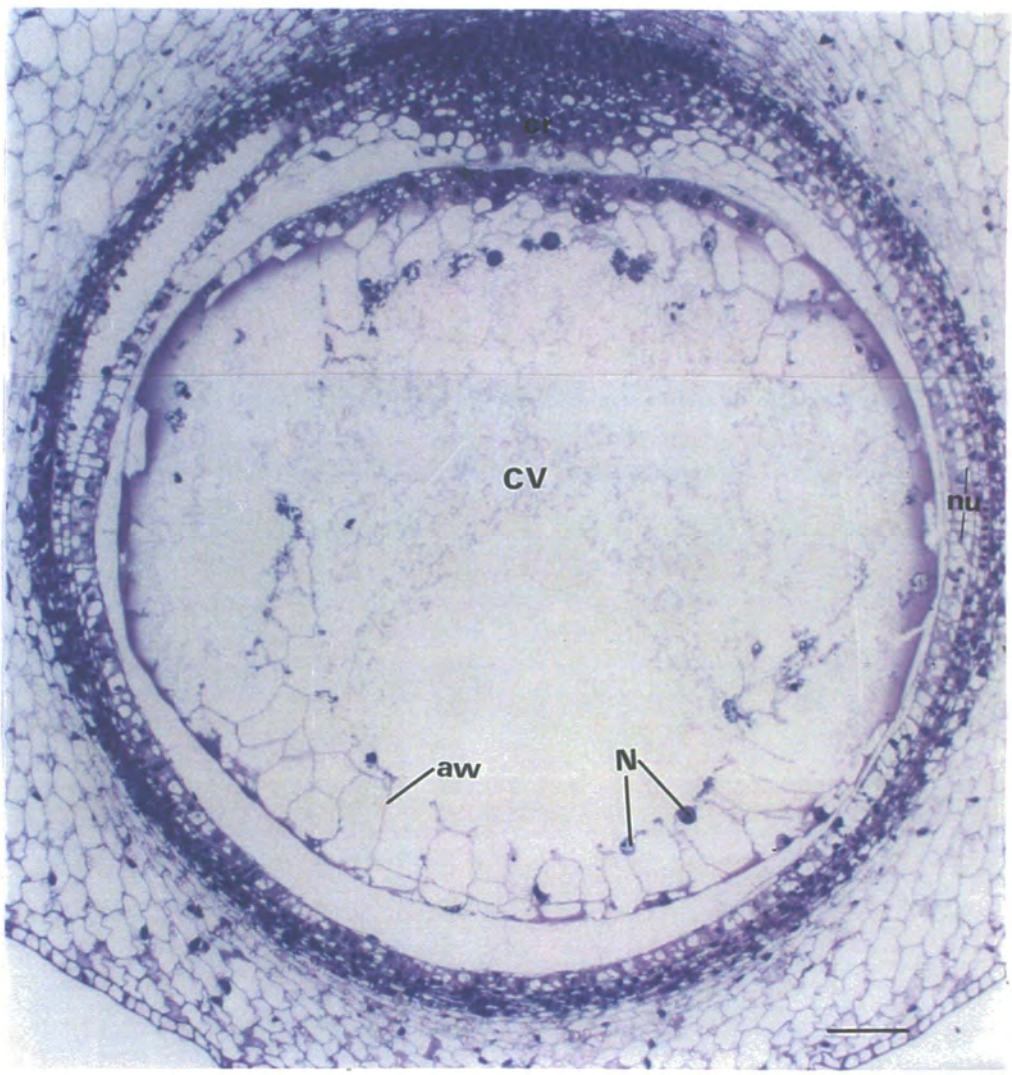


Plate 5. Toluidine Blue stained transverse section of a 3/4 DPA caryopsis.

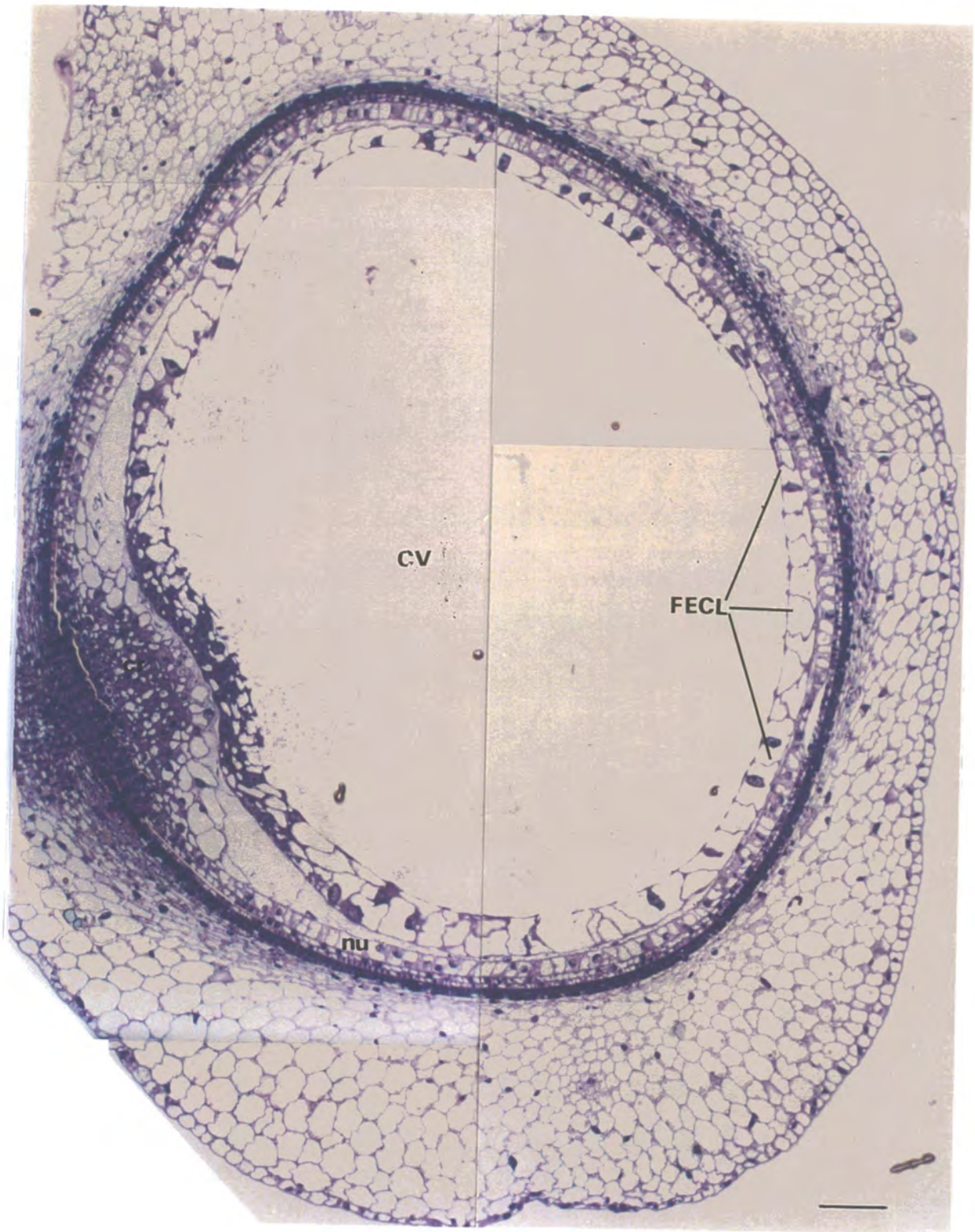
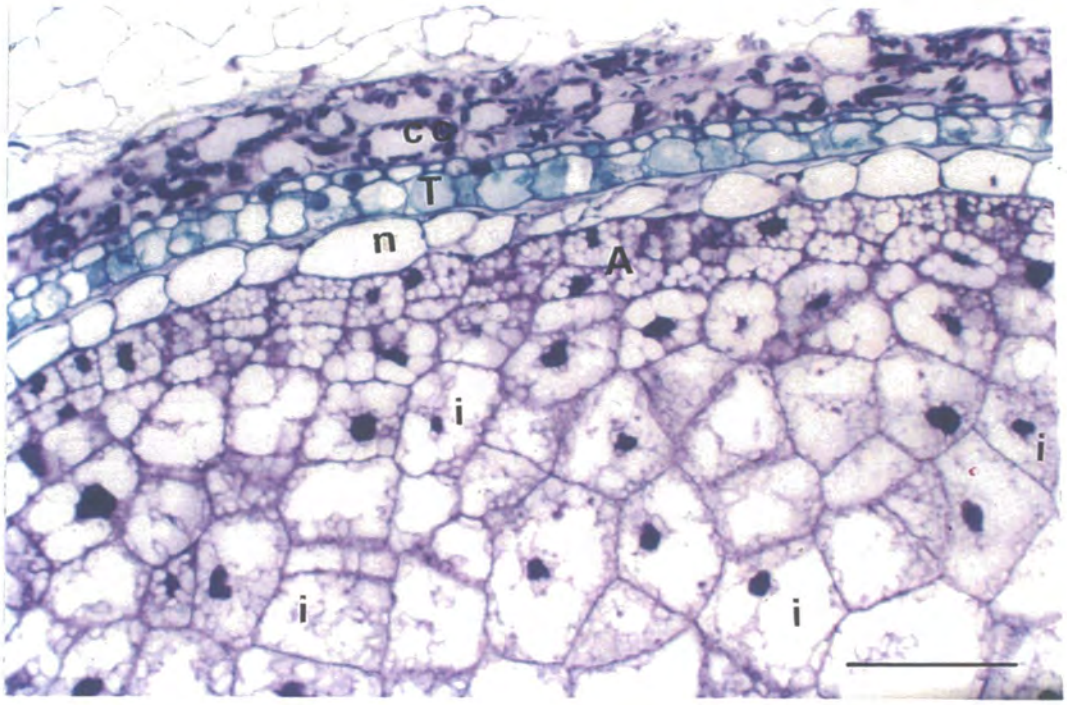


Plate 6. Toluidine Blue stained transverse section of a 4 DPA caryopsis, at the initial stages of cellularisation, showing the first complete endosperm cell layer.



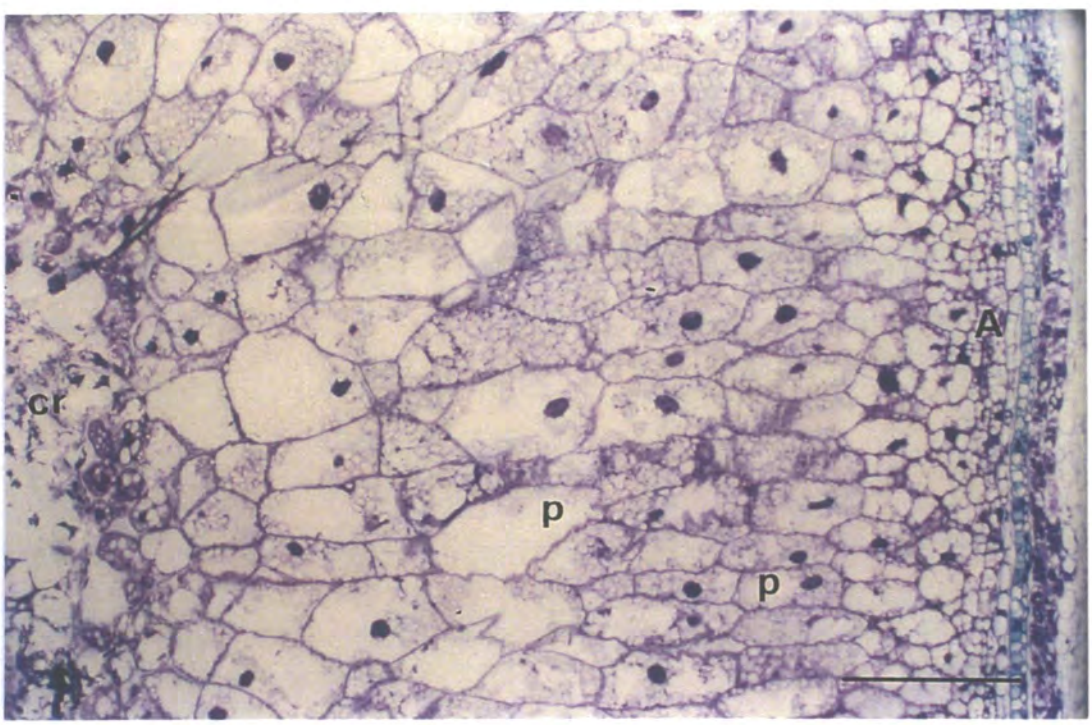


Plate 7. Toluidine Blue stained transverse sections of caryopses aged 10+ DPA.
a. Irregular starchy endosperm cells from the wing area of the endosperm.
b. Prismatic starchy endosperm cells from the mid region of the endosperm.

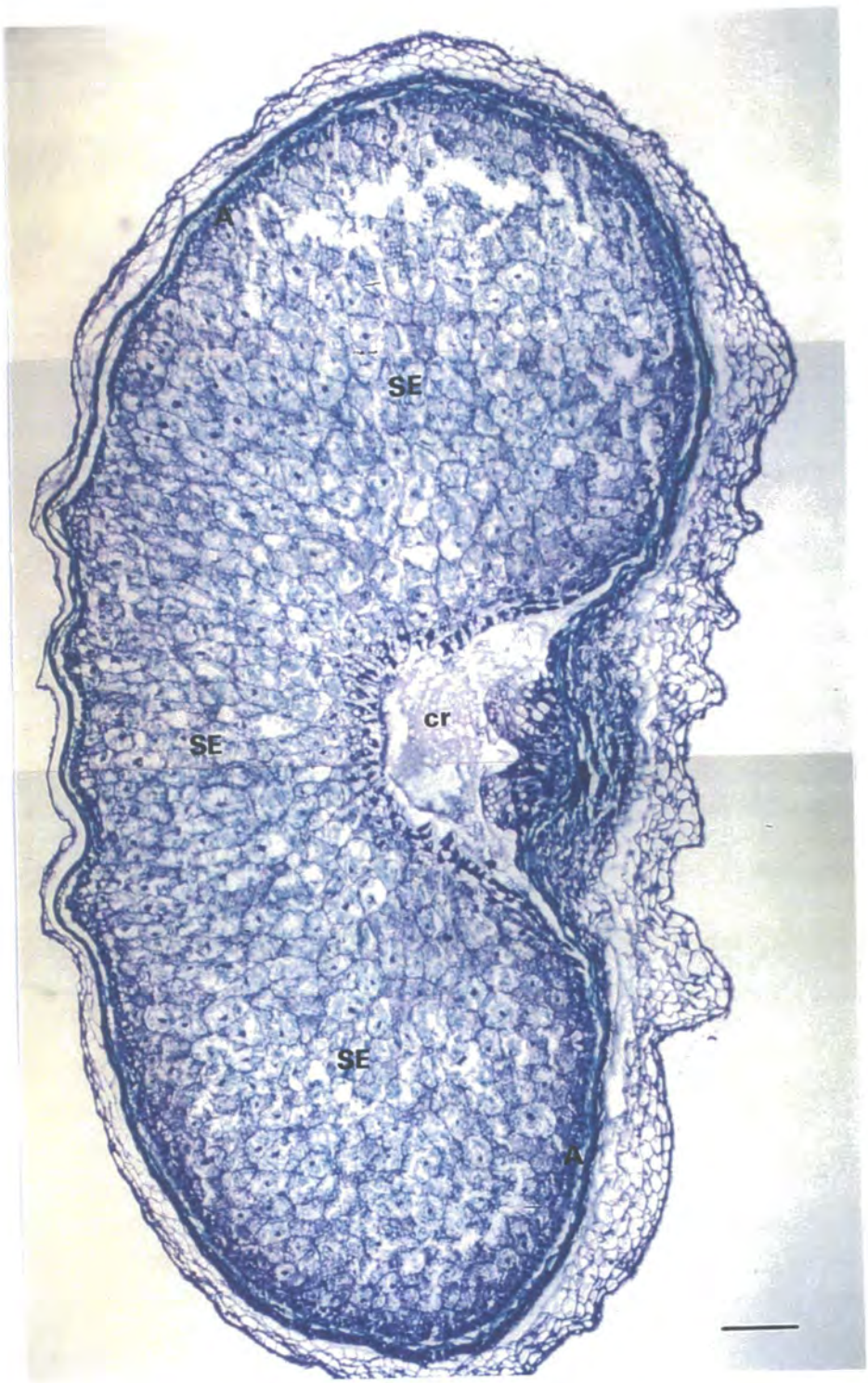


Plate 8. Toluidine Blue stained transverse section of a 16 DPA caryopsis.

3.1.2 Localisation of epitopes to JIM Antibodies

3.1.2.1 Results

Toluidine Blue tissue staining of mid-grain sections from wax-embedded and resin-embedded grains it was decided that wax-embedded grains had not maintained good structural integrity. Sections of wax-embedded grains tended to fall to pieces, with the area representing the central cell vacuole often falling out or the central region of the grain crumbling whilst sectioning. As resin-embedded material proved more amenable to sectioning, tissue embedded in LR White resin was cut for use in immunolocalisation studies. To optimise the immunolocation procedure for each primary antibody a number of combinations of dilutions, incubation times and temperatures, wash times and secondary conjugated antibody/visualisation systems were used. These are outlined in Table 9. From the combinations used, JIM 13 showed reactivity to sections of caryopses with endosperm development showing the initial anticlinal, and periclinal, wall formations. The final experimental procedures for the JIM 13 antibody are outlined in Table 10.

Table 10. Final conditions which showed reactivity of antibody JIM 13 to sections of barley caryopses.

	OPTIMISATION CONDITIONS	
	1	2
Block	BSA 0.05%	Fish gelatin 0.05%
Primary dilution	1:10	1:10
Primary incubation	room temperature 4 hours	room temperature 4 hours
Wash	3X 10 minutes	3X 10 minutes
Secondary application	1:100 immunogold 1:250 immunogold	1:100 immunogold 1:250 immunogold
Wash	3X 15 minutes-final wash MilliQ Water	3X 15 minutes - final wash MilliQ Water
Visualisation	Silver enhancement	Silver enhancement

From the micrographs in Plates 9-14, it can be seen that the JIM13 antibody locates predominantly to the area of the first anticlinal endosperm walls and the cytoplasm and nuclei at this stage, and also to the anticlinal and periclinal wall regions of the next stage ie. formation of the first endosperm cell layer. (There is also a high degree of staining over the crease region and the nucellar epidermis of both substages early in the cellularisation phase.)

Some specific immunostaining was also seen associated with the developing crease region (Plates 9, 10c, 13a and 14a) and at the interface between the endosperm and nucellar layer. The accumulations of silver stain over the pericarp and within the central cell vacuole are considered to be the result of non-specific nucleation. With shorter silver enhancement times such background is minimal but the low level of signal over the endosperm wall areas was difficult to record (results therefore not

shown).

Some staining occurs over the nucellar tissue, with slightly less over the pericarp and the central cell vacuole. This is especially noticeable at higher magnifications; at lower magnifications, however, the overall picture is that there is more staining on the anticlinal and periclinal wall areas and also over the nuclei. JIM13 showed no reactivity to any of the structures in sections of syncytial stage endosperm (results not shown). It was not possible to carry out immunolocalisation experiments on the sub-stages of cellularisation between formation of the first endosperm cell layer and full cellularisation as the endosperm of embedded caryopses at these stages had very poor structural integrity.

Unfortunately extracts from hybridomas of similar origin to those from which the monoclonal antibody was raised were not available for control purposes. Therefore, controls utilising normal serum from a similar animal (rat) were initially used. It is recognised that this is not an entirely suitable control since it is polyclonal in nature. However no specific immunolabelling was observed with this (Plate13b), nor was there any immunolabelling with either JIM14 (Plate 13c), or any of the monoclonals JIM 4, JIM8, JIM12, JIM15 and JIM16 (results not shown but as Plate 13c). These monoclonal antibodies therefore can be considered as appropriate controls to the very specific labelling observed on sections cut from the same block with the monoclonal antibody JIM13. All other factors were the same as for the primary antibody localisation procedure for each experiment. Only limited, random staining was seen on sections of caryopses at any of the stages examined in the control immunolocalisation experiments. This was taken to be background staining.

To identify low levels of specific immunogold labelling, silver enhancement was performed to the point where silver nucleation was beginning to be seen and give a

low level of background signal over the tissue and slide. Such patterns of nucleation are essentially random over a large scale, although some evidence of clustering is occasionally observed at higher LM magnifications. Such clusterings are not associated with specific structural features in serial tissue sections but again appear to be random. These may be a consequence of a chain reaction within the nucleation chemistry.

3.1.2.2 Choice of visualisation system

Three different visualisation systems were used in the immunolocation of the JIM antibodies to barley endosperm. These were FITC (flourescein) conjugated secondary antibodies, alkaline phosphatase conjugated antibodies, and immunogold conjugated antibodies. Each is an indirect method of localisation and they are known to be relatively sensitive systems for visualisation at the light microscope level. With sections of barley grain, localisation utilising FITC conjugated secondary antibodies gave poor results due to masking of any potential positive signal by autofluorescence of the tissues, particularly in the nucellar and testa regions. This may have resulted from the aldehyde fixation which is known to be a cause of autoflourescence. Hence it was decided not to use flourescein conjugated secondary antibodies. Alkaline phosphatase conjugated antibodies gave weak colouring upon development and again it was decided not to use this system for visualisation. Hence, immunogold localisation was used. This system allows intensification of the gold signal and can be very sensitive, although it can, however, also give high background staining.

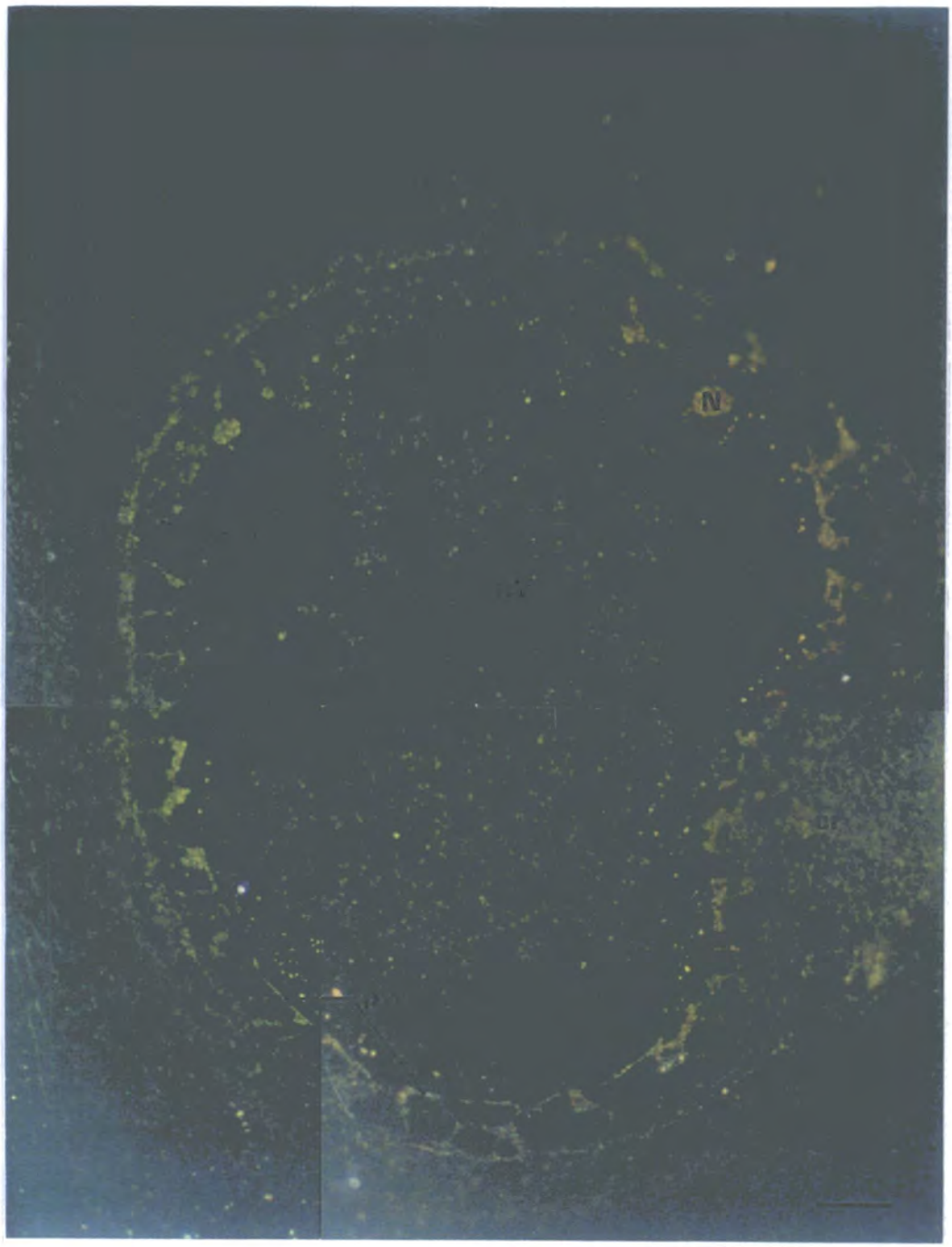
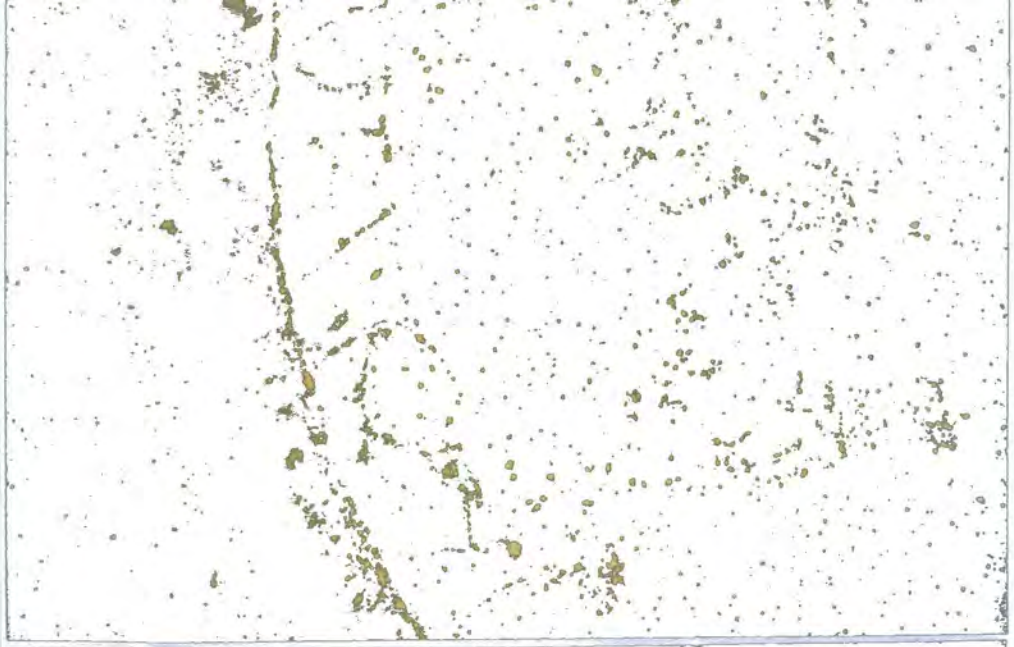
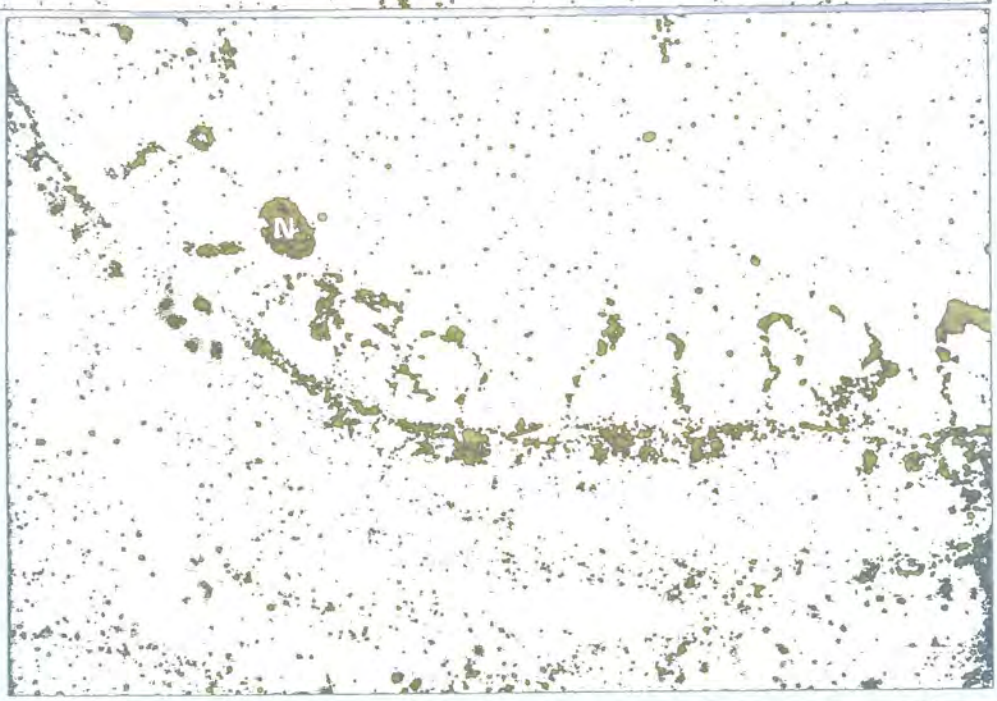


Plate 9. **An example of immunolocalisation of monoclonal antibody JIM13 to a cross section of a barley caryopsis at the initial phases of the cellularisation stage (anticlinal/periclinal wall formation). Visualised using colloidal gold conjugated secondary antibody and silver enhancement.**

Plate 10. **Examples of immunolocalisation of monoclonal antibody JIM 13 to cross sections of a barley caryopsis at the initial phases of the cellularisation stage (higher magnification of areas of the section in Plate 9). Visualised using colloidal gold conjugated secondary antibody and silver enhancement.**
(a) and (b) - wing region and central vacuole, (c) crease region.

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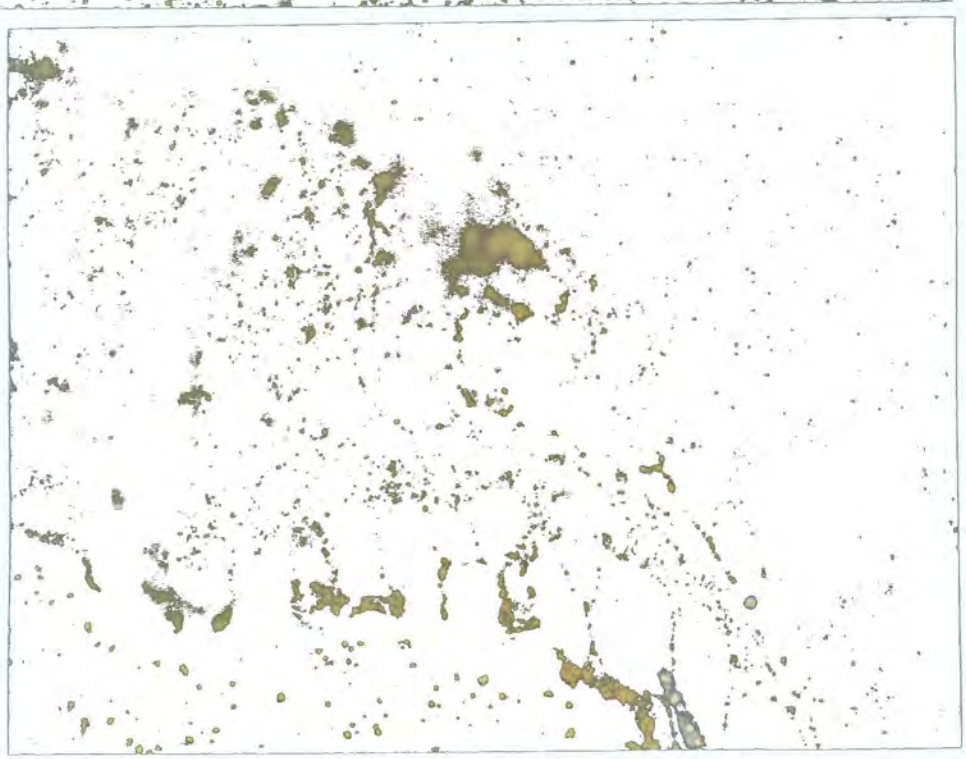
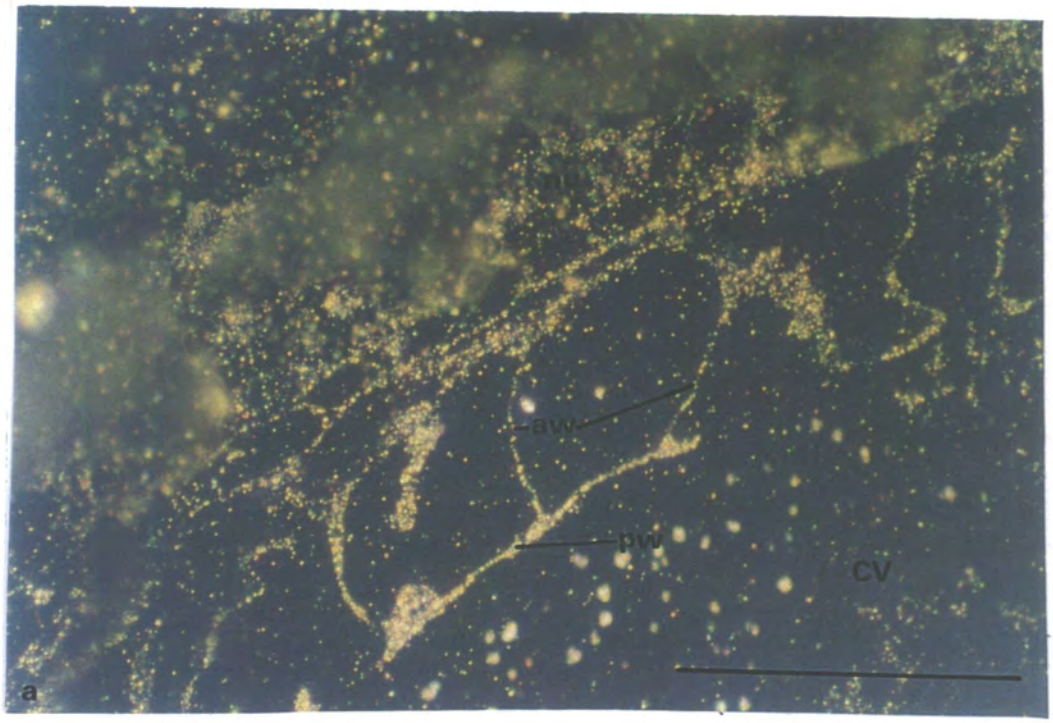


Plate 11. **Immunolocalisation of monoclonal antibody JIM13 to a cross section of a barley caryopsis at the initial phases of the cellularisation stage (initial anticlinal/periclinal wall formation). Visualised using colloidal gold conjugated secondary antibody with silver enhancement.**
(a) localisation of JIM13 to early cellularisation stage endosperm.
(b) Nomarski image of (a) showing the cell layers in more detail.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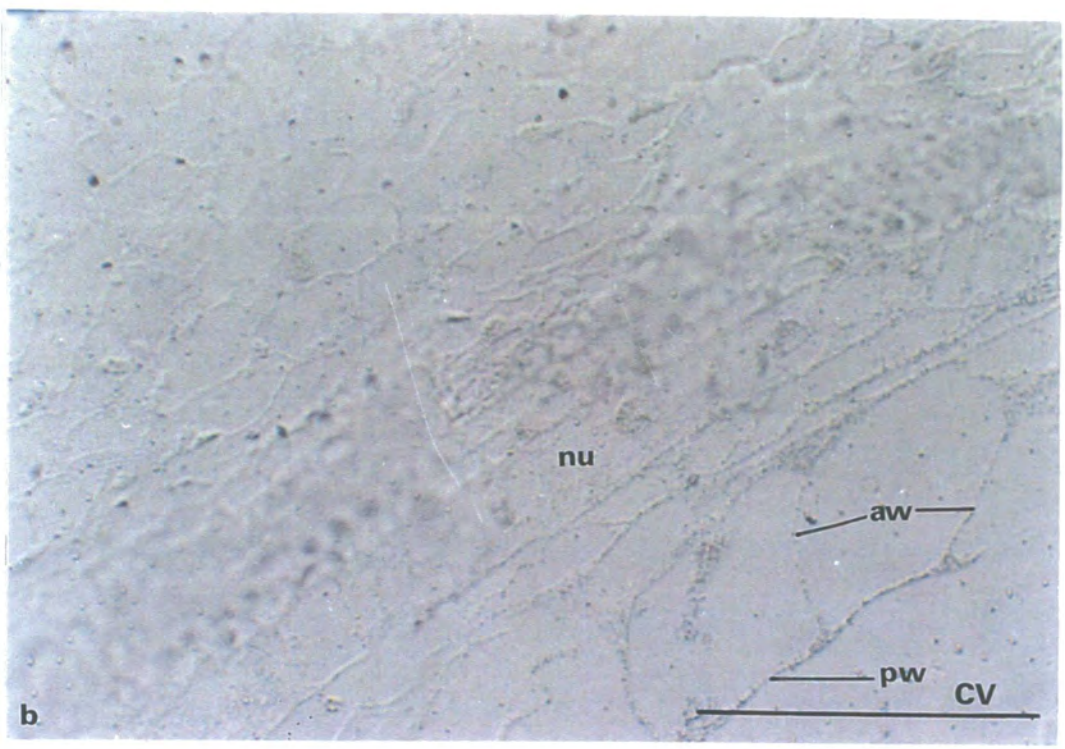
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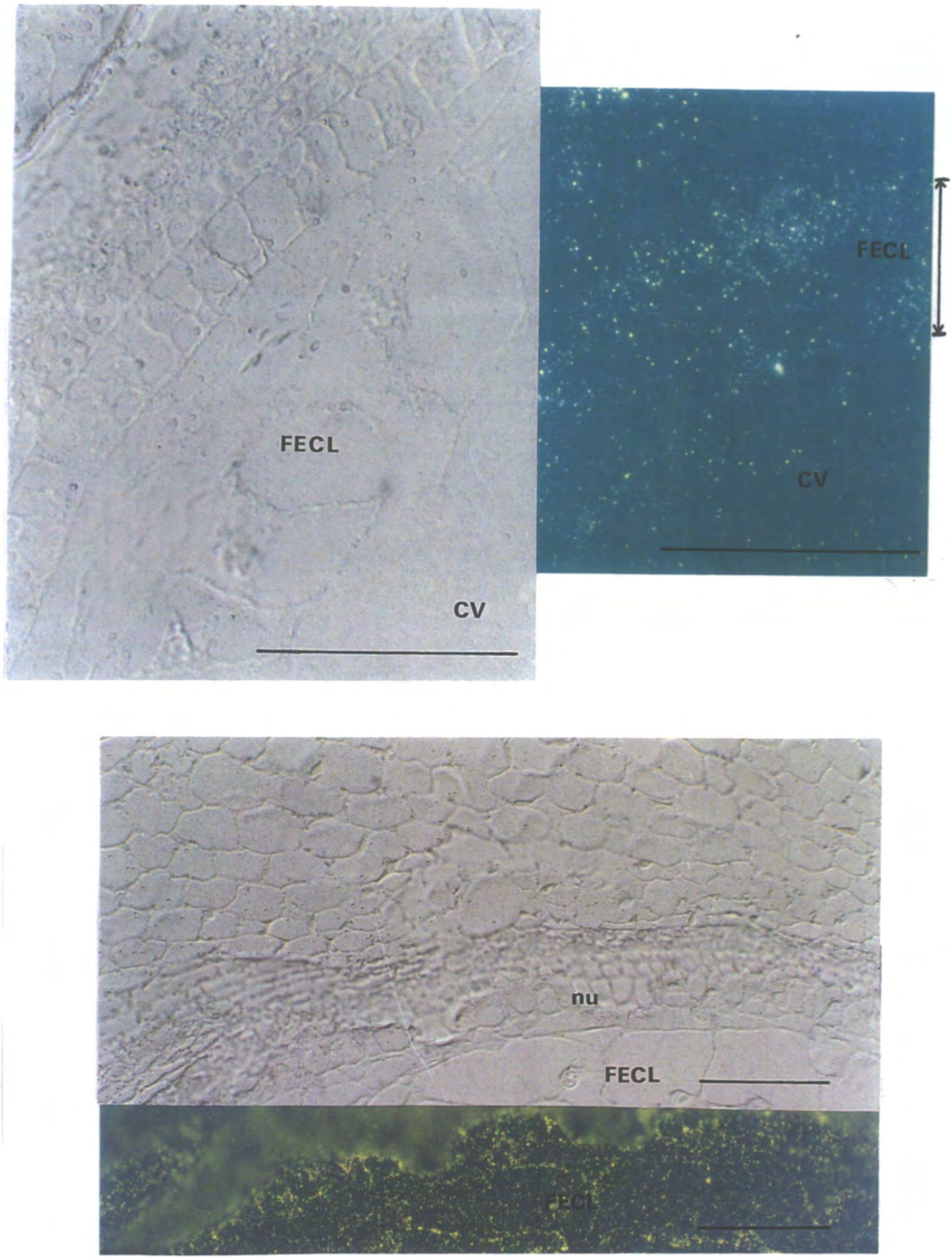
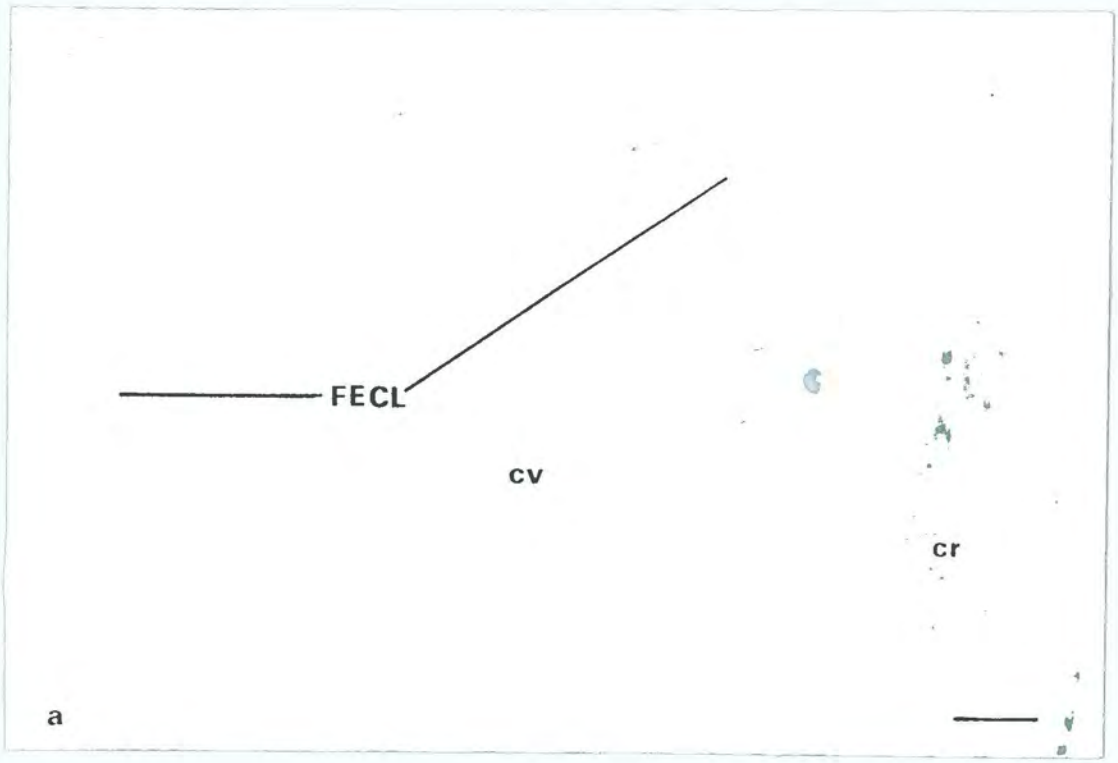


Plate 12. **Immunolocalisation control, showing details of high background staining by immunogold and silver enhancement and details of the sections' structure in equivalent Nomarski images.**

Plate 13. Example of immunolocalisation of monoclonal antibodies JIM13 and JIM14 to cross sections of a barley caryopsis at the phase of the cellularisation stage at which the first endosperm cell layer is complete. Visualised using colloidal gold conjugated secondary antibody and silver enhancement.
(a) immunolocalisation of JIM13
(b) Control using serum as the primary antibody application instead of JIM13, showing only background staining.
(c) immunolocalisation with JIM14 shows no specific staining.


FECL
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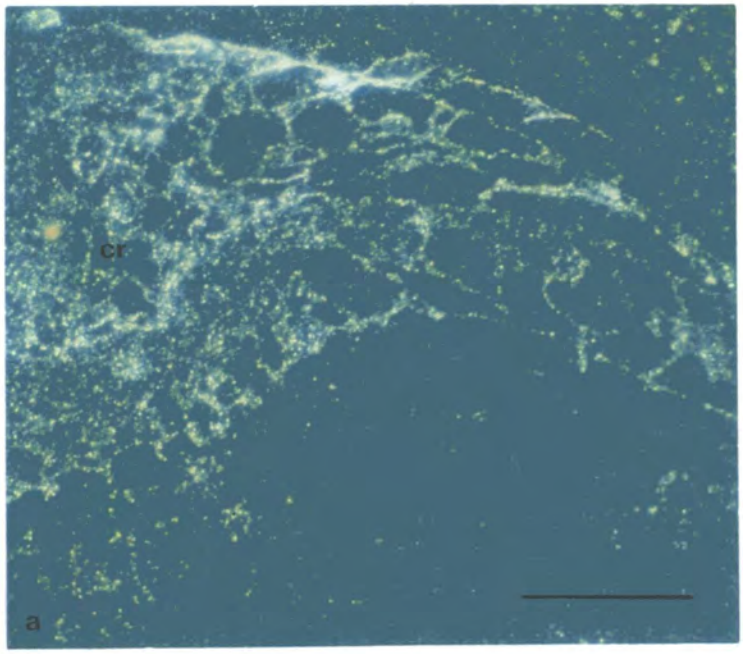
c

Plate 14. Higher magnification of the section in Plate 13 showing localisation of JIM13 to the cell wall regions of the first endosperm cell layer. Visualised using colloidal gold conjugated secondary antibody and silver enhancement. (a) and (b) show immunogold localisation of JIM13. (c) Control using serum as the primary antibody application instead of JIM13, showing only background staining.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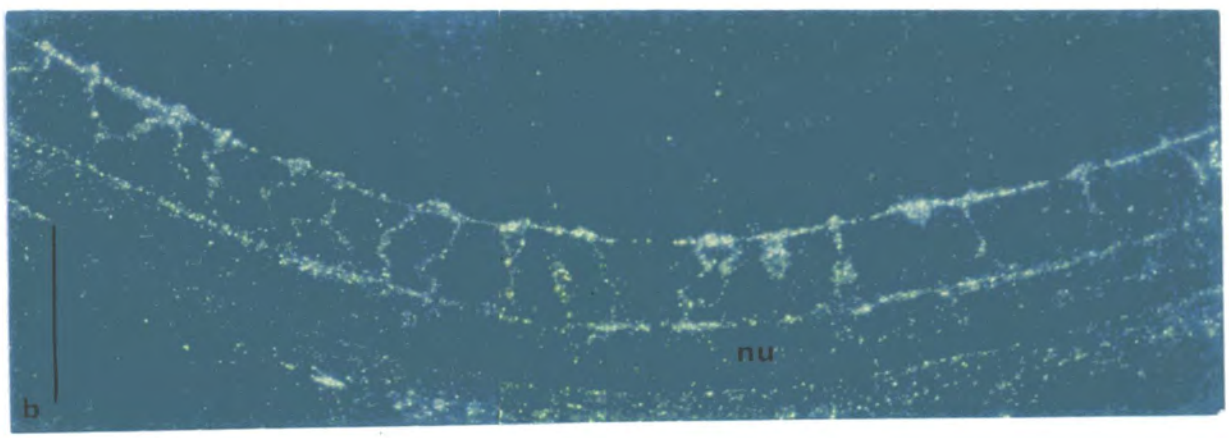
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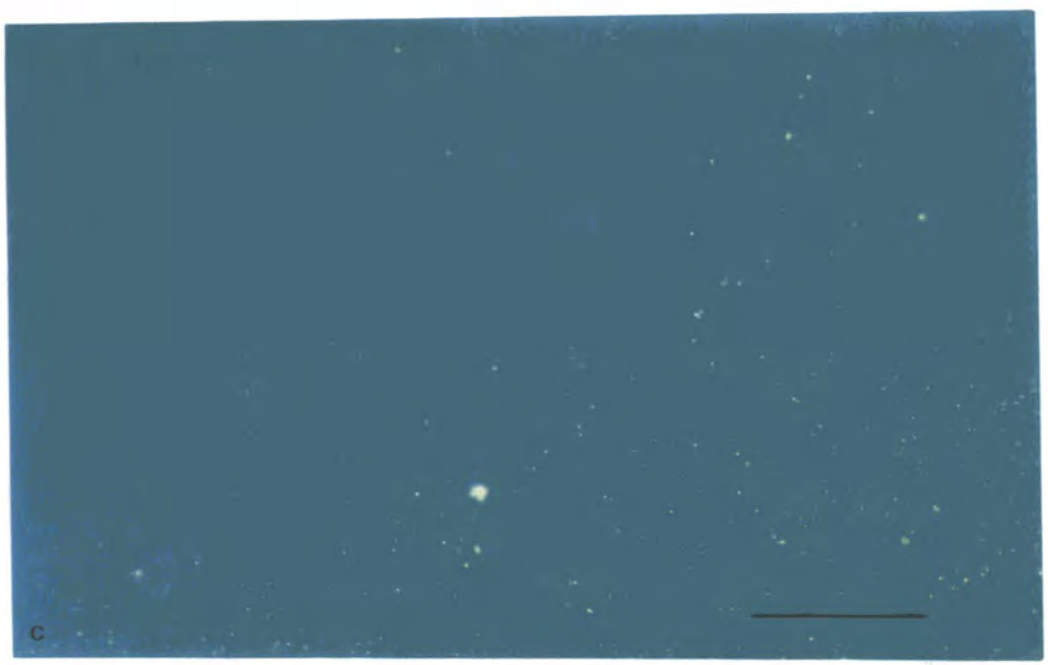

c

3.1.2.3 Discussion

3.1.2.3.1 Localisation of the JIM13 antibody

Labelling with JIM13 was found to be localised to the area of the first anticlinal walls at the start of the cellularisation stage, to cell surfaces within the crease region and to the nucellar boundary. Nuclei and cytoplasmic aggregates within the cellularising endosperm were also immunolabelled. Interestingly, no labelling of any of the cell layers within the caryopsis was observed in sections of the earlier syncytial stage. The JIM13 epitope maintained binding to anticlinal wall areas, nucellar layers, crease region and nuclei during development of the first periclinal walls and also localised to the initial periclinal wall areas. It was not possible to determine whether the JIM13 binding was maintained throughout the rest of the cellularisation stage due to the poor structural integrity of endosperm sections obtained at each of these substages.

The JIM13 antibody recognises carbohydrate epitopes of AGP 1 and AGP 2 and has been found to be associated with plasma membranes rather than cell walls (Knox *et al.*, 1991). Electron microscopy immunolocalisation procedures would be required to discover whether the epitopes in barley caryopsis are associated with the cell wall or plasma membrane.

AGPs are common in plants and a number of functional roles have been suggested. These include cell-cell signalling (Knox *et al.*, 1991), cell fate determination (Knox *et al.*, 1989; Knox *et al.*, 1991; Pennell *et al.*, 1991), cell growth and division (Serpe and Nothnagel, 1994), cell elongation (Willats and Knox, 1996; Zhu *et al.*, 1993), and cell adhesion (Jauh and Lord, 1996). AGPs have not been associated with cell

differentiation although they are linked to cell fate determination and are found during early development. Any and all of the functions listed above would be required during development of the endosperm and the other tissues of the caryopsis. The distribution of JIM13 in early endosperm, nucellar and crease region cells indicates that AGPs may have one or more roles.

Within the developing endosperm, localisation of the JIM13 epitope to the first cell layer may be significant in terms of a possible function in cell-cell signalling and determination of cell fate; these newly formed cells later become aleurone cells. Arabinogalactan proteins have been implicated in having a possible role as markers of cell fate in root meristems (Knox *et al.*, 1991), and floral meristems (Pennell *et al.*, 1991). In these systems there is a sequential change in the spatial and temporal localisation of a number of AGP epitopes which correlates with positioning of particular cell groups within the tissues during different stages of development. For example Knox *et al.* (1989) and Knox *et al.* (1991) found that antibodies JIM4, JIM13 and JIM15 showed modulation during carrot root development. From immunolocalisation experiments on serial sections of carrot roots, it was observed that JIM4 was reactive with the two small diametrically opposed groups of cells that represent the emerging pericycle and indicate the orientation of the future xylem (Knox *et al.*, 1989). The second observation of Knox *et al.*, (1989, 1991) was that JIM13 developed reactivity with the cells lying between the JIM4 reactive cells which represent the future xylem band. Thirdly, JIM15 became unreactive with cells in the central region of the future xylem band at approximately 200μm from the meristem. JIM4, JIM13 and JIM15 were also found to be developmentally regulated in relation to the carrot root epidermis. JIM13 maintained reactivity with the

epidermis as it developed from initials common to the root cap but as it developed further JIM15 lost reactivity and at a later stage it became reactive with JIM4 (in the proximal region where the root cap no longer persisted) (Knox *et al.*, 1991). A further observation was that JIM13 had a variable intensity of staining such that there was a gradual increase in intensity of binding distally from the meristem into the root cap and also binding to cells forming the future xylem band was weakest closest to the meristem (Knox *et al.*, 1991).

Knox *et al.* (1991) state that the binding specificites of these three antibodies reflect the spatial and temporal distinction of cells within the developing meristem and may be indicative of a pre-pattern of cell distinctions that emerge with the establishment of the pattern of cell shapes. They also suggest that the differential expression of AGP epitopes may reflect a role for AGPs in some form of cell-cell signalling which is required in the establishment of cell-type position prior to differentiation. A similar role may be possible for the modulation of the JIM13 epitope in barley endosperm in that its expression may define the position of the future aleurone cells. Further support for this idea comes from work by Anderson *et al.* (1977). They found that the ß-glucosyl derivative of Yariv reagent, which selectively binds to and precipitates AGPs, revealed intense staining in aleurone cells in sections of mature endosperm, and did not show any reactivity with cells of the starchy endosperm. They also suggest that staining was perhaps associated with the plasma membrane which, along with the observation by Knox *et al.* (1991) that JIM13 is localised to the plasma membrane of suspension culture carrot cells, indicates that JIM13 may be associated with the plasma membrane of barley endosperm cells at the early stages of development and also with many other components of the cells of developing caryopses. Cytoplasm and nuclei appeared to be reactive and staining was evident in

many cell types. During the development of barley endosperm, control and regulation of cell growth and mitosis are important in the formation of cell and tissue structure. During the cellularisation phase cells are formed which continue to grow, expand and divide. Division is highly regulated with cells situated at the periphery of the endosperm tissue continuing to divide until 21 DPA whilst in the central region mitotic activity ceases at around 14DPA. From the point at which cells cease to divide, expansion growth continues due to the accumulation of storage products during the grain-filling phase. There is strong evidence which suggests that AGPs have roles, via either a direct route or a complex indirect route, in the modulation of cell growth, expansion and division (Willats and Knox, 1996 and references therein).

From studies of the inhibitory effects of β-GlcY on AGPs Willats and Knox (1996) suggested that reduced root growth was a consequence of a reduction in cell elongation during the post-proliferation phase of elongation at the root apex and was associated with radial expansion of root epidermal cells. Their work also focused on the effects of Yariv reagents on AGPs in relation to cell expansion in carrot cell cultures that had been induced to elongate rather than proliferate and showed that β-GlcY completely inhibited cell elongation. Inhibition of cell expansion following addition of β-GlcY to NaCl-adapted cultured tobacco cells has been demonstrated by Zhu *et al.* (1993), again suggesting that AGPs have a role in some aspects of cell growth regulation. Further evidence for AGPs having a role in the regulation of cell growth and division comes from work by Serpe and Nothnagel (1994). They found that addition of β-GlcY to *Rosa* cell suspensions caused a reduction in cell proliferation. As cell growth and division are related, this reduction in mitotic activity may be related, indirectly, to effects of inhibition of AGP

involvement in cell growth and expansion.

For AGPs to have similar function in control or regulation of cell growth during barley endosperm development it may be expected that AGP expression would be modulated throughout the cellularisation phase and continue to be modulated through to the point at which cells stop expanding due to deposition of storage products at the end of the grain filling phase. If AGPs are involved with regulation of cell division it may again be expected that modulation of their expression would correlate with the time points at which mitotic activity ceases within the central endosperm at 14 DPA and at the periphery at 21 DPA. It is possible that different sets of AGPs may operate either directly or indirectly in aspects of the control of cell growth and division throughout endosperm formation. The differential expression of JIM13 found during the early phases of endosperm development may be the first indication of a role for AGPs in these aspects of barley endosperm formation.
JIM13 labelling was however, not found exclusively on the endosperm, but also stained both nucellar and crease region cells, all of which, although determined, are under go further growth and development during grain-filling.

Recently Schindler *et al.* (1995) tested the hypothesis that AGPs were involved with extension growth by monitoring modulation of AGPs by auxin during maize coleoptile growth. They came to the conclusion that the lack of correlation between extension growth and the spatial and temporal occurrence of AGPs in maize coleoptile indicated that AGPs were not involved with extension growth and that AGP expression was not under the control of auxin. However they did suggest that AGPs are markers of programmed cell death as JIM13 and JIM14 epitopes showed

expression in young tissues, such as those which occur in those regions of the vascular bundles which are composed of dead cells in the mature tissue. Cell death is a major factor in development of the nucellus, xylem and some of the inner cells of the nucellar projection. AGPs may have a role in identifying those cells at an early stage of development which are programmed to die at maturity. It is, however, unclear whether death of the nucellus is a pre-programmed event, as might be inferred by the presence of the JIM labelling, or merely a consequence of crushing as a result of endosperm expansion. With the exception of the aleurone, cells of the endosperm also die by grain maturation.

Another possible role for AGPs during barley endosperm development could be in cell wall formation. AGPs have been implicated as having adhesive, structural and nutritive roles during pollen tube growth. Many glycoproteins are involved with pollen tube germination and growth (Mascarenhas, 1975; Li *et al.*, 1983, 1986; Zhang, 1984). A number of AGP antibodies (including JIM13) have now been used to localise AGP epitopes in pollen tubes. Experiments have shown that they localise to the inner callosic sheath of the pollen tube wall or the plasma membrane, in a periodic ring-like pattern, and not to the outer microfibrillar pectic coating or central cellulosic layers (Li *et al.*, 1992; Jauh and Lord, 1996). The association of AGPs with the callose sheath suggests that AGPs, in this instance, may have a structural role and along with callose aid in providing the pollen tube with radial reinforcement (Heslop-Harrison, 1987; Li *et al.*, 1992). The initial anticlinal walls formed at the earliest phase of barley endosperm cellularisation are composed largely of callose (Brown *et al.*, 1994). As the JIM13 epitope localises to the region of newly forming anticlinal walls it is possible that JIM13 epitope has an association with some aspect

of callose such as synthesis or deposition, or it may play a reinforcing role. Further work on AGPs in pollen tubes suggest they have an adhesive role in interactions between pollen tubes and the stylar transmitting tract. Juah and Lord (1996) propose that AGPs of the pollen tube interact with pectins in cells of the stylar transmitting tract such that adhesion occurs. They also propose a second model of adhesion such that AGPs secreted by the stylar transmitting tract act as a 'glue' between pectins of the pollen tube and the stylar transmitting tract.

3.1.2.3.2 Lack of reactivity of antibodies

The lack of reactivity obtained with MAC207, JIM4, JIM14, JIM15 and JIM16 could have arisen for a number of reasons. Factors which can affect antigenicity and ability to localise antigens include masking or changes to antigen recognition sites, due to fixation and embedding reagents or procedures, and also other tissue structures. Leaching during the fixation, embedding and immunolocalisation procedures can also influence levels of staining as can the specificity and quality of the antibodies, which can be influenced by (poor) storage conditions and/or too many freeze thaw cycles. Finally, lack of reactivity may be a result of there being too little antigen in the tissue for generation of a signal.

As the series of monoclonal antibodies was also being used within the laboratory with success in concurrent experiments it seems unlikely that the antibodies were of a poor quality but that the tissue either contained very little or none of each antigen. In the case of the antigen being present in such small quantities, the methods of immunolocalisation used here may not have been sensitive enough to pick them up. The use of electron microscopy may have proven more successful in localising the

antigens if they were present in small quantities. Electron microscopy with immunogold localisation has been used successfully by Knox *et al.* (1991) with JIM13 and JIM14, Pennell *et al.* (1991) with JIM 8, and Pennell *et al.* (1989) with MAC207. Antibodies JIM 8 (Pennell *et al.,* 1991) and MAC207 (Pennell et *al.,* 1989) were both found to bind to plasma membrane associated epitopes. JIM 13 was found to associate with plasma membrane epitopes whilst JIM 14 was found to be associated with plasma membrane and cell wall epitopes, (Knox *et al.,* 1991). Each of the antibodies have, however, been localised successfully at the LM level in glutaraldehyde or formaldehyde-fixed, resin-embedded tissue except for JIM 16. Reactivity of the antibodies JIM 4, 13, 14 and 15 was obtainable on sections of formaldehyde-fixed, resin-embedded carrot root meristems (without pretreatment with pectinase). JIM 16 showed no reactivity on resin-embedded formaldehyde-fixed tissues but did show reactivity on formaldehyde-fixed cryosections of carrot root tip meristems (Knox *et al.,* 1991), suggesting that this epitope, at least, was masked by the embedding medium or procedure. Pennell *et al.* (1989) localised MAC207 to plasma membranes of cells of sections of formaldehyde-fixed frozen root tips and also living sugar beet and carrot protoplasts. It is possible that aspects of the fixation and embedding procedures used within this project have affected the epitopes such that they were not recognised by the antibodies. Indeed Knox *et al.* (1991), Pennell *et al.* (1989) and Pennell *et al.* (1991) used cold curing to harden the resin whereas hot curing was used here. Use of alternative methods of fixation and embedding may have allowed the localisation of each of the unreactive antibodies. The use of a proteinase or pectinase pretreatment to permeabalise cells may have improved reactivity, although this approach is not used widely for tissue blocks. Knox *et al.* (1991) used a pectinase pretreatment to permeablise, unfixed cells of

plasmolysed suspension culture cells to obtain reactivity of antibodies JIM4, 13, 14, 15 and 16. JIM14 also showed reactivity on untreated plasmolysed suspension culture cells.

An alternative to using antibodies to locate specific AGP epitopes in the tissues would be to carry out a histochemical test with Yariv reagents. This would help determine the presence, and allow an idea of the quantity of AGPs occurring in each stage of endosperm development to be assessed. Yariv reagents are synthetic phenyl glycosides which have specific interactions with AGP molecules (Yariv *et al.*, 1962,1967). They are coloured multivalent molecules which bind to and precipitate AGPs (Yariv *et al.*, 1962, 1967). β-glucosyl Yariv reagent (βGlcY) is commonly used. Yariv reagents have been used to purify AGPs (Jerym and Yeow, 1975; Komalavilas *et al.*,1991), as specific histochemical probes to localise subcellular distribution of AGPs (Clarke *et al.*, 1978; Schopfer 1990; Anderson *et al.*, 1977), as stains to visualise AGPs following electrophoresis (Du *et al.*, 1994; Knox *et al.*, 1991) and more recently in experimental approaches to assess possible functions of AGPs in plant cell cultures and whole plants (Serpe and Nothnagel, 1994; Willats and Knox, 1996). Also, with the recent cloning of some AGP protein cores (eg. Du *et al.*, 1995; Chen *et al.*, 1994; Mau *et al.*, 1995) it is now possible to use molecular biological techniques to study AGP form and function and isolate and characterise AGPs from tissues at the molecular level.

3.2 Construction and screening of a cDNA library

3.2.1 Construction of a cDNA library from whole caryopses of barley aged 1-10 DPA

Total RNA from caryopses aged between 1 and 10 DPA (combined in one extraction) was extracted using a modified version of the Logemann *et al.* (1987) RNA extraction method (2.2.3.1). Extraction with this method yielded large amounts of good quality total RNA as assessed by electrophoretic separation on denaturing agarose gels (2.2.5). An example of electrophoresed total RNA obtained is shown in Figure 5. Due to the band of more intense staining seen at the bottom of each lane in Figure 5, the RNA may be slightly degraded; alternatively, this staining could represent 5S RNA species. The results obtained indicate that the RNA was slightly degraded, as can be seen from the more intense staining at the bottom of each lane in Figure 5. Apart from this indication of slight RNA degradation, the RNA appeared to be of a high quality; the 28S and 18S ribosomal bands were intense, the 28S band being larger than the 18S band and smearing between, above and below was less intense and limited. The staining in the well region is indicative of contaminating DNA. Contaminating DNA was removed from the samples using RNAse-free DNAse. Quality and quantity of the RNA was also assessed by UV spectrophotometry at 260nm and 280nm (2.2.4.1).

RNA enriched in poly $(A)^+$ mRNA was prepared using the Qiagen Oligo d(tex) mRNA Isolation Kit (2.2.3.2). Quality of mRNA obtained was again assessed by electrophoresis on denaturing agarose gels (2.2.5). Figure 6 shows an example of mRNA obtained. The quality and purity of mRNA obtained was judged to be adequate for production of a cDNA library.

Isolated mRNA was cloned into the Lambda Uni-ZAP XR vector (Stratagene) using the Lambda Uni-ZAP XR II cloning kit. The Lambda Uni-ZAP XR vector is an insertion vector capable of taking inserts of up to 10kb in size. The vector supplied with the Lambda Uni-ZAP XR cDNA cloning system is predigested with *EcoRI/Xho1* and purified such that the two Lambda ZAP arms are available, with no intervening fragments. The arms have incompatible ends and hence cannot self ligate, which enables higher cloning efficiencies. The kit also utilises *XhoI* and *EcoRI* oligonucleotide adaptors which are ligated to either end of the synthesised cDNA such that unidirectional cloning is facilitated. Hence cloned cDNA should be in the three forward reading frames, as opposed to the six (three forward and three reverse) reading frames obtained in bidirectional cloning. The advantage of this system is that the orientation of the cDNA clones can be easily predicted and the coding and non-coding strands identified without sequencing. The clones from these vectors can be *in-vivo* subcloned to the pBluescript II SK phagemid (Stratagene) using the ExAssist/SOLR helper phage system. In addition to these advantages, both the T3 and T7 promoters are located on the opposite flanking regions of the multiple cloning site and allow easy production of both sense and anti-sense RNA strands, which can be used in *in-situ* hybridisation techniques. The ZAP-cDNA synthesis kit utilises a hybrid oligo(dT) linker primer, containing an *XhoI* site, which primes first strand synthesis of cDNA. First strand cDNA is transcribed using StrataScript RNase H^- reverse transcriptase, which is a novel Moloney murine leukemia virus reverse-transcriptase (MMLV-RT), and 5-methyl dCTP. Use of 5-methyl dCTP in the production of first strand cDNA, hemimethylates the cDNA, thus protecting it from certain restriction endonucleases used in subsequent stages of

the cDNA synthesis process. Second strand synthesis is carried out with RNase H, which nicks the RNA annealed to the first strand cDNA and provides multiple primer sites for *E. coli* DNA polymerase I, which "nick-translates" the RNA fragments into second strand cDNA. The uneven termini of the cDNA are then made flush using Klenow fragment and *EcoRI* adaptors are ligated to the blunt ends. An asymmetrical cDNA is then produced by digestion with *XhoI*, which removes the *EcoRI* adaptor from the 3' end of the cDNA and allows unidirectional cloning in the Uni-ZAP XR vector arms. A schematic representation of cDNA synthesis is given in Figure 7.

Throughout production of the cDNA, controls utilising radio-labelled tracers were used to monitor size and quality of first and second strand cDNA. Examples of first and second strand cDNA used for cloning are given in Figure 8.

The cDNA was size fractionated (as per the Stratagene manual from the cDNA Synthesis Kit) prior to ligation into the vector arms. The two largest size fractions were used for ligation.

Following ligation of the cDNA into the vector it was packaged using the Gigapack II Packaging Extract (Stratagene) and then the titre was estimated by infecting a liquid culture of XL1-Blue MRF' cells and plating (2.2.10.2) out serial dilutions. The titre was estimated to be $1X10^9$ pfu/ml. The efficiency of cDNA ligation into the vector was assessed by blue/white colour production from LacZ β-galactosidase α-complementation in phage plated in XL1-Blue MRF' cells on plates containing IPTG and X-gal. Blue coloured colonies were non-recombinant and white colonies were recombinant. The ratio of blue to white colonies was 11:480. Indicating

~97% of plaques contained inserts. Following titration, the library was amplified and stored at 4°C until required for screening.

3.2.2 Differential Screening

Differential screening was chosen as the method to screen for clones expressed solely or in higher quantities, at 3 DPA as compared to 10 DPA and 6 DPA.

The library was plated (2.2.10.2) at a density of 40000 plaques per 22X22cm plate and transferred to duplicate nylon membranes using the duplicate plaque lift method (2.2.10.3). DNA was denatured and fixed onto the membranes. Radio-labelled ([α^{32}P] dATP) cDNA probes made from 3 and 10 DPA total RNA, utilising oligo (dT) as a primer for synthesis (2.2.11), were made. These were hybridised one to each duplicate plaque lift and autoradiographs taken. The autoradiographs were aligned with one another and plaques showing unique or increased expression on the 3 DPA as compared to 10 DPA autoradiographs were marked. Plugs of agar containing plaques from the equivalent positions on the plate were picked into SM buffer. As the plugs from this primary screen contained numerous plaques a secondary screen is required at a low density to isolate individual plaques. Secondary screening was initially carried out in a similar manner, with freshly prepared radio labelled probes and plating of phage at a density sufficient to allow individual plaques to be recognised. Unfortunately this secondary screen produced no spots of hybridisation on any of the autoradiographs of hybridised membranes. A number of possibilities as to why this could have happened exist. Firstly, mis-alignment of the primary screen autoradiographs with the plates might have occurred. This was checked and found not to be the case. Secondly, aspects of the plaque lift, hybridisation and/or cDNA labelling methods may have been wrong.

The procedure was repeated and again produced no results. A control, to analyse whether the plaque lift or hybridisation procedure was wrong, was carried out using the normal method of plaque lift and hybridisation with randomly primed Maize Histone 1 and 2 DNA probes. These probes hybridised, with spots of hybridisation to plaques recognisable on the autoradiographs (see Figure 10 for example of hybridisation result). It was deemed that both the plaque lift method and hybridisation technique were sufficient. Aspects which could affect cDNA synthesis include degradation of the template RNA, the occurrence of secondary structure and contamination of the samples with protein or carbohydrates. Possible contaminants were removed from the RNA samples by phenol/chloroform extraction and reprecipitation using 4M lithium chloride. Degradation of total RNA was checked by separation on denaturing agarose gels by electrophoresis and ethidium bromide staining. From comparison of the intensity of ribosomal bands and the smear above, below and between these, little degradation of samples was noted. The RNA was also tested for ability to produce products in a cell-free *in vitro* translation system (Promega Wheat Germ Extract *In-vitro* translation system)(2.2.6). Products were labelled with ^{35}S-methionine and separated by electrophoresis on SDS-polyacrylamide gels (2.2.7). This gave an indication of mRNA quality in the sample. Good banding patterns were obtained following autoradiography of the dried SDS-PAGE gels and hence the sample RNA was deemed to be of a good/reasonable quality (Figure 9). This meant that there were problems in the cDNA synthesis and labelling procedure. A number of different methods for producing labelled cDNA probes (2.2.11) were tried but none gave very high incorporation of radio label as determined by a hand-held Geiger counter. Counts in the order of greater than 1000 per minute were expected but only 50-150 counts per minute were obtained. From

these probes only very little hybridisation of the probes to the filters was obtained as determined from hand-held Geiger counter and autoradiography. Due to time constraints secondary screening was abandoned and a new method worked out. The new method entailed *in vivo* subcloning of a number of plaques to the pBluescript II SK phagemid, excision of the inserts and screening for differentially expressed clones against Northern blotted total RNA (2.2.8) samples of 1, 3, 6, 10, root and leaf total RNA samples. Phagemid from each plaque plug were excised and recovered by plating on selective media (as per the Stratagene manual from the cDNA Synthesis and Cloning Kits). Individual colonies were then picked and subjected to maxi-plasmid preparations (2.2.18) to recover the plasmid DNA. Inserts were excised using enzyme digests, followed by electrophoresis to separate the fragments (Figure 11). Insert fragments were recovered from agarose gels using Gene Clean II Kit. The Prime It II Random Priming Kit was then used to label inserts with α ^{32}P dATP. Labelled inserts were hybridised against Northern blots of electrophoretically separated samples of 1, 3, 6 and 10 DPA total RNA. To speed up the process of identifying clones which had differential expression, hybridisation of digoxigenin 11-UTP labelled inserts (2.3.1) to dot-spotted total RNA samples (2.3.2) (equally loaded) was carried out. From these two methods a number of clones were identified which were putatively differentially expressed. The inserts from a number of these were partially sequenced either manually, using the SequenaseVersion 2.0 Sequencing Kit (2.2.14.1) or by automated sequencing at Durham University (2.2.14.4). Sequences were analysed for identity to data base sequence entries using the Wisconsin GCG package (Genetics Computer Group, 1991).

Results from the sequence analysis indicated that at least 4 clone insert sequences showed identity to those in the data bases. Clone 3B showed high identity (95%) to

sequences representing sucrose synthase in a number of plant species. Clone 27B showed high identity with ketol acid reductoisomerase (KARI) and acetohydroxy acid reductoisomerase (AHARI) sequences within the data base. A third clone, 16B showed some identity to blue copper-binding proteins and a fourth clone, 16D, showed identity to Caffeoyl CoA-O-3-methyltransferase (CCoAOMT). Further details and the hybridisation patterns of each of these clones is given below. Four other clones, 2A, 2C, 14A and 14B, which were sequenced showed no significant identity to entries in the data bases. Figures 26-32 show the forward and/or reverse primed nucleotide sequence readings for each clone and the derived amino acid sequences for a number of possible open reading frames (ORF). *In-situ* hybridisation of riboprobes made from clone inserts was carried out next.

Clone 3B

Clone 3B has an insert size of 5kb. Sequencing using the forward primer allowed 449 nucleotide bases to be sequenced and using the reverse primers allowed a further 575 nucleotides to be identified. Figures 12 and 13 show the forward and reverse nucleotide sequence readings and the derived amino acid sequence for each. The derived amino acid sequences (read from sequences primed in both directions) of both readings showed high identity to a number of sucrose synthase amino acid sequences from a range of plant species, including barley, rice, maize, pea, *Arabidopsis*, and soybean. Figures 14-15 show the prettybox picture of the pileup alignment of clone 3B amino acid sequence to a number of sucrose synthase sequences, Sus1, Sus2 and Susy. Unfortunately it is difficult to tell, from the sequence analysis data, which of these three forms of sucrose synthase the clone corresponds to. The clone is not a full length cDNA; comparison of its length and

sequence obtained for sucrose synthase sequences in the data base indicate that other sucrose synthase sequences have considerably more sequence upstream of the point at which the 3B clone begins. Hybridisation of labelled insert from clone 3B to a Northern blot of total RNA from 1, 3, 6, and 10 DPA caryopses and leaf and root total RNA samples is shown in Figure 16 along with the ethidium bromide stained agarose gel from which the Northern blot was formed. The lane containing total RNA from 6 DPA caryopses is slightly overloaded in comparison to the other total RNA samples. It was not possible in the time available to repeat this experiment, however, the results indicate that clone 3B may show a slight increase in expression at 10 DPA as compared to 1 and 3 DPA. It is likely that there is also an increase in expression at 6 DPA. This, however, would need to be clarified by carrying out further Northern blotting experiments using blots with equally loaded RNA. There was very little hybridisation of clone 3B to leaf and root total RNA samples.

Sucrose synthase catalyses the reversible conversion of sucrose and UDP to UDP-glucose and fructose has a significant role in energy metabolism and is involved with the mobilisation of sucrose into diverse pathways important for the metabolic, structural and storage functions of the cells. Its activity has been correlated with sugar import (Sung *et al.,* 1989), starch synthesis (Chourey and Nelson, 1976), cell wall synthesis (Chourey *et al.*, 1991), and sink strength (Sun *et al.,* 1992). A number of different sucrose synthase genes have been identified and characterised, including Ss1 and Sh2 from maize. cDNAs for barley Sus1 and Sus2 have been characterised along with their tissue specific expression (Sanchez *et al.,* 1992; Martinez de Ilarduya *et al.,*1993). Although sucrose synthase is found in all plant tissues its different forms, Sus1, Sus2 and SuSy all show differential expression in

tissues (Martinez de Ilarduya *et al.*,1993; Amor *et al.*, 1995; Hesse and Willmitzer, 1996). Martinez de Ilarduya *et al.* (1993) have studied differential expression of Sus1 and Sus2 in barley and indicate that Sus1 is not expressed in large amounts in the young endosperm (5 DAP), coleoptiles and 20 DAP aleurone but is expressed in large amounts in 20 DAP endosperm and in young roots. Sus2 was shown to have strong expression in 20 DAP endosperm, low expression in aleurone (20 DAP) and no expression in coleoptiles or roots. From this information, and as clone 3B cDNA showed little expression in leaf and root northern blot hybridisation experiments, it is possible that clone 3B corresponds to Sus2 gene. Further work would have to be carried out to assess this possibility.

Sucrose synthase is obviously an important enzyme during early endosperm development as metabolic rates will be high due to the need for components, especially during the cellularisation stage. It should also be noted that the synthesis of starch begins within the early developmental stages of endosperm formation.

Clone 16B

Clone 16B had an insert size of 600bp. Sequencing using the M13 reverse primer gave 320bp reading. Figure 17 shows the sequence data and the derived amino acid sequence. Analysis of the derived amino acid sequence suggests that the open reading frame (ORF) has significant identity with blue copper proteins (49% and 45.6%). FASTA analysis aligns the derived ORF of 16B with cucumber seedling basic blue copper protein (cucasyanin) and Japanese Laquer tree blue copper protein sequences (stelacyanin). Figure 18 shows the prettybox picture of the pileup alignment of 16B with cucasyanin (babl) and stelacyanin (stel) protein sequences. As 16B aligns with both stelacyanin and cucasyanin proteins from the start of the

protein sequence available for each of them, and no transcription start sequence occurs in either stelacyanin or cucasyanin sequence, it is possible that stelacyanin and cucasyanin could have sequence which would align with the 16B overlap. Presuming that 16B is a full length cDNA it is possible that the methionine residue marked in Figure 18, represents the N terminal translation start point. Further sequencing would need to be carried out to determine whether the 'end' would further match blue copper binding protein sequences in the data bases.

Hybridisation of digoxigenin-UTP labelled 16B to dot spotted total RNA of 1, 3, 6 and 10 DPA caryopsis, leaf and root showed equal levels of hybridisation to 1, 3 and 6 DPA, with less hybridisation to 10 DPA samples. Hybridisation to the leaf samples was similar to that for 1 and 3 DPA and root samples showed no hybridisation.

Plants and bacteria contain small copper binding proteins. These proteins are termed cupredoxins and are involved in electron transfer reactions (Adman, 1991a; Adman, 1991b; Sykes, 1996). The cupredoxins have been divided into two groups based upon their amino acid sequences (Ryden and Hunt, 1993). The first group contains the bacterial and plastidal cupredoxins (eg plastocyanin and azurin) whilst the second group contains the plant cupredoxins which occur in non-photosynthetic tissues (eg stellacyanin [Bergman *et al.*, 1977] and cucumber basic blue copper binding protein [Murata *et al.*, 1982; Guss *et al.*, 1988]). Recently, the discovery of proteins which have similarity to the classical second group members, but differ in that they have two domains, one of which is not involved with copper binding, has led to the sub-division of the second group into single domain cupredoxins and two domain cupredoxins (Mann *et al.*, 1996). The second domain in this two domain

group resembles plant cell wall proteins in having numerous hydroxyprolines (Mann *et al.*, 1992; Van Gysel *et al.*, 1993). From the patterns of hybridisation of digoxigenin labelled clone 16B insert (a putative copper binding protein), and the limited information available on copper binding proteins, it is difficult to come to any conclusion regarding clone 16B without carrying out further sequencing and analysis. Further sequencing and data base search analysis would help to establish the true identity of clone 16B, whilst analysis such as Northern hybridisation against RNA samples from photosynthetic and non-photosynthetic tissues and *in-situ* hybridisation would help to establish its cellular distribution in different tissues. From the data available all we can say is that it is a putative blue copper binding protein. Its function during barley grain formation, in-particularly endosperm formation, cannot be established without further work. One enigma is that blue copper proteins, of the type to which the clone 16B insert shows most similarity to have been indicated to be associated with non-photosynthetic tissues (Bergman *et al.*, 1977). 16B , however, shows hybridisation to both photosynthetic and non-photosynthetic tissues.

Clone 16D

Clone 16D contained an insert of approximately 3500bp size. Sequencing using the M13 reverse primer gave a sequence reading of 395 bases. The sequence reading and the derived amino acid sequence are shown in Figure 19. Analysis using the FASTA program (Genetics Computer Group, 1991) indicates clone 16D has high identity to caffeoyl-CoA 3-O-methyltransferase (CCoAOMT) sequences within the data bases. Figure 20 shows a pileup prettybox alignment of some of the caffeoyl-CoA 3-O-methyltransferase sequences to which clone 16D shows similarity. Clone

16B is a putative caffeoyl-CoA-O-3-methyltransferase (CCoAOMT). O-methyltransferases (OMT) are involved with the methylation of monolignols in the lignin biosynthetic pathway. Several isoforms of OMT have been identified in higher plants (Herman *et al.,* 1987), each differs in terms of substrate specificity and their immunochemical properties. The cDNA encoding CCoAOMT has been characterised in parsley (Schmitt *et al.*, 1991) and no extended homology was found between the sequence of this cDNA and other different OMT cDNAs, confirming that they encode very different proteins. CCoAOMT is potentially specific to the generation of feruyl-CoA and guaiacyl lignin subunits. Recently, Ye *et al.* (1994) isolated a *Zinnia* CCoAOMT which exhibits 93% amino acid similarity with the parsley CCoAOMT (Schmitt *et al.*, 1991). Expression of *Zinnia* CCoAOMT is induced during tracheary element differentiation from mesophyl cells and tissue print hybridisation has shown that the expression of the gene is associated with the timing of lignification in both xylem and phloem fibres in *Zinnia* organs (Ye *et al.*, 1994). Ye *et al.* (1994) propose that CCoAOMT is involved in an alternative pathway in lignin biosynthesis which is predominant in *Zinnia* differentiating tracheids. For further details of lignin biosynthesis the reader is referred to Boudet *et al.* (1995).

Clone 27B

Clone 27B had an insert size of approximately 2000bp. Sequencing using the forward primer gave a sequence reading of 392 bases and using the reverse primer a reading of 195 bases. Figures 21 and 22 show the sequence readings and derived amino acid sequences for clone 27B. Sequence readings from both directions showed high identity to ketol acid reductoisomerase (KARI) amino acid sequences

of spinach and *Arabidopsis* within the Swissprot data base using the FASTA sequence analysis program (Genetics Computer Group, 1991). Figure 23 shows the prettybox display of pileup alignment of the derived amino acid sequence of clone 27B with KARI sequences for spinach and *Arabidopsis*. Hybridisation of [α ^{32}P] dATP labelled insert from clone 27B to a Northern blot of total RNA from 1, 3, 6, and 10 DPA caryopses and leaf and root total RNA samples is shown in Figure 24, along with the ethidium bromide stained agarose gel from which the Northern blot was formed. It can be seen that the agarose gel was evenly loaded with total RNA samples. Clone 27B shows higher hybridisation to 1, 3 and 6 DPA samples than 10 DPA samples. It also shows lower hybridisation to total RNA samples from leaf and root as compared to 1, 3 and 6 DPA samples. These results suggest that clone 27B is expressed at higher levels at least up until 6 DPA and that expression has been reduced by 10 DPA.

Ketol acid reductoisomerase (KARI), also known as acetohydroxy acid reductoisomerase (AHARI), functions as the second enzyme in the biosynthetic pathway of the branched chain amino acids leucine and valine (Bryan, 1980). KARI catalyses a two step reaction in which the substrate, either 2-acetolactate or 2-aceto-2-hydroxybutyrate, is converted via an alkyl migration and a NADPH-dependent reduction to 2,3-dihydroxy-3-methylbutyrate or 2-3-dihydroxy-3-methylvalerate respectively (Dumas *et al.,* 1993). The DNA sequence encoding KARI has been characterised from only a few higher plants, namely *Spinacia oleracea* (spinach) and *Arabidopsis thaliana.* A number of prokaryote and lower eukaryote KARI genes have, however, been isolated (Blazey and Burns, 1984; Petersen and Holmberg,1986; Wek and Hatfield, 1986; Aguilar and Grasso, 1991; Godon *et al.,*

1992; Rieble and Beale, 1992; Sista and Bowman, 1992). KARI is a nuclear-encoded enzyme which is exported to the chloroplasts in plants (and to the mitochondria of fungi) (Dumas, 1993). From this information we might expect KARI to be localised within those cell types of the barley caryopsis which contain chlorophyll.

Clone 16C

Clone 16C contained an insert of approximately 1500bp size. Sequencing using the M13 reverse primer gave a sequence of 320 bp. From sequence analysis using the GCG package (Genetics Computer Group, 1991), the sequence does not appear to contain a single open reading frame. Three possible open reading frames have been derived but none go through to the end of the sequence without a stop codon (Figure 25). There must, therefore, be ambiguities in the sequence obtained during the sequencing and reading procedures. Analysis using FASTA sequence identity program can be used to help clear ambiguities if matches are found. This, however, was not possible as there were no matches to sequences in the data bases. However, FASTA suggests that the sequence is arginine rich as it aligns two of the possible ORF of 16C with a number of arginine rich amino acid sequences (Figure 25). Open reading frames b and c contain high percentages of arginine residues. The first 35 amino acids of ORF b contains 12 arginine residues (23% arginine) and ORF c contains 31% arginine throughout its 75 amino acid length.

Hybridisation of digoxigenin-labelled *in-vitro* transcribed RNA from clone 16C to dot spots of total RNA from 1, 3, 6 and 10 DPA caryopsis, leaf and root shows that hybridisation to 1,3, and 10 DPA caryopsis and root samples to the same degree, with hybridisation at 6 DPA and leaf samples being higher.

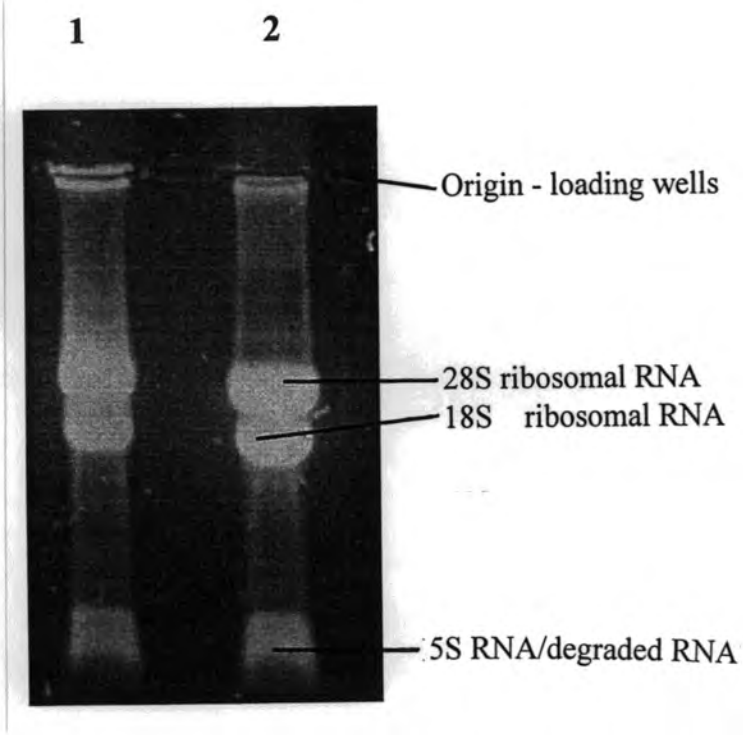


Figure 5. Total RNA.
Lanes 1 and 2 show total RNA from caryopes aged between 1-10 DPA, separated by electrophoresis on a denaturing agarose gel.

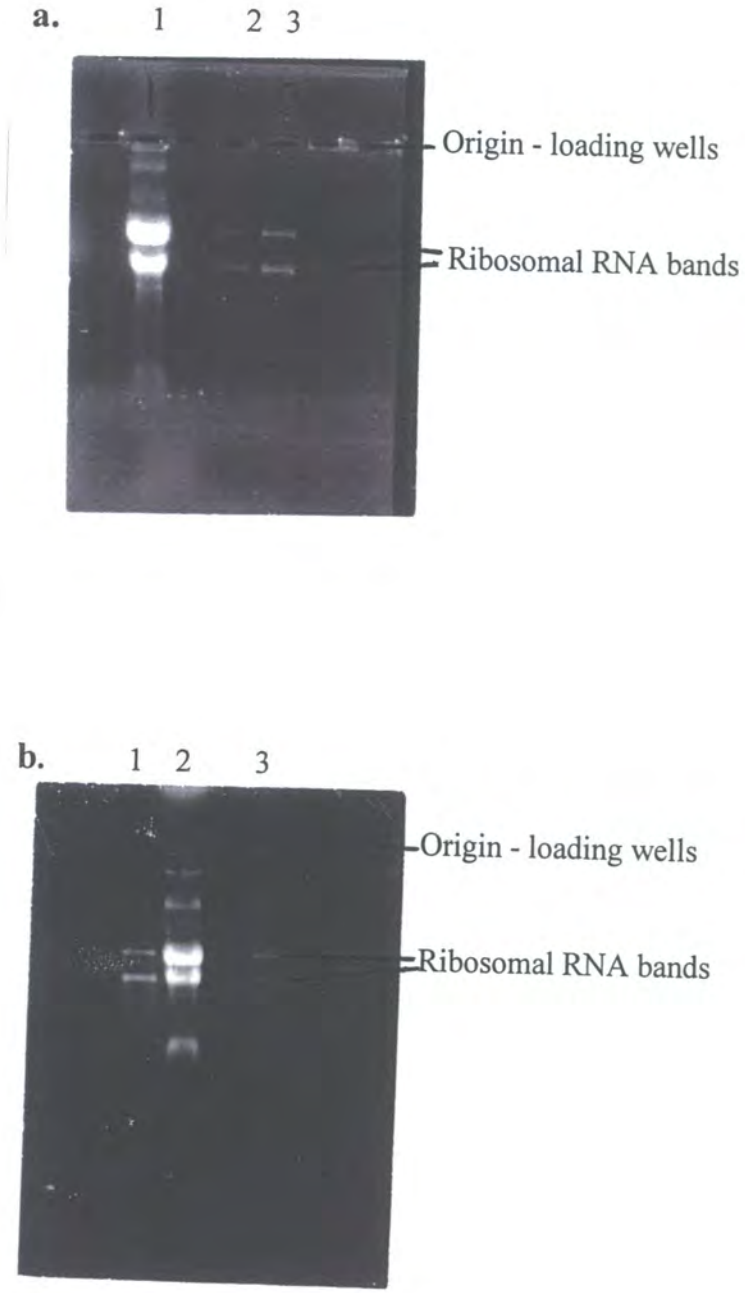


Figure 6. Examples of total and messenger RNA from barley caryopses aged between 1 and 10 DPA. mRNA was used in library construction and screening.

a. 1. total RNA
2. mRNA
3. mRNA

b. 1. mRNA
2. total RNA
3. mRNA

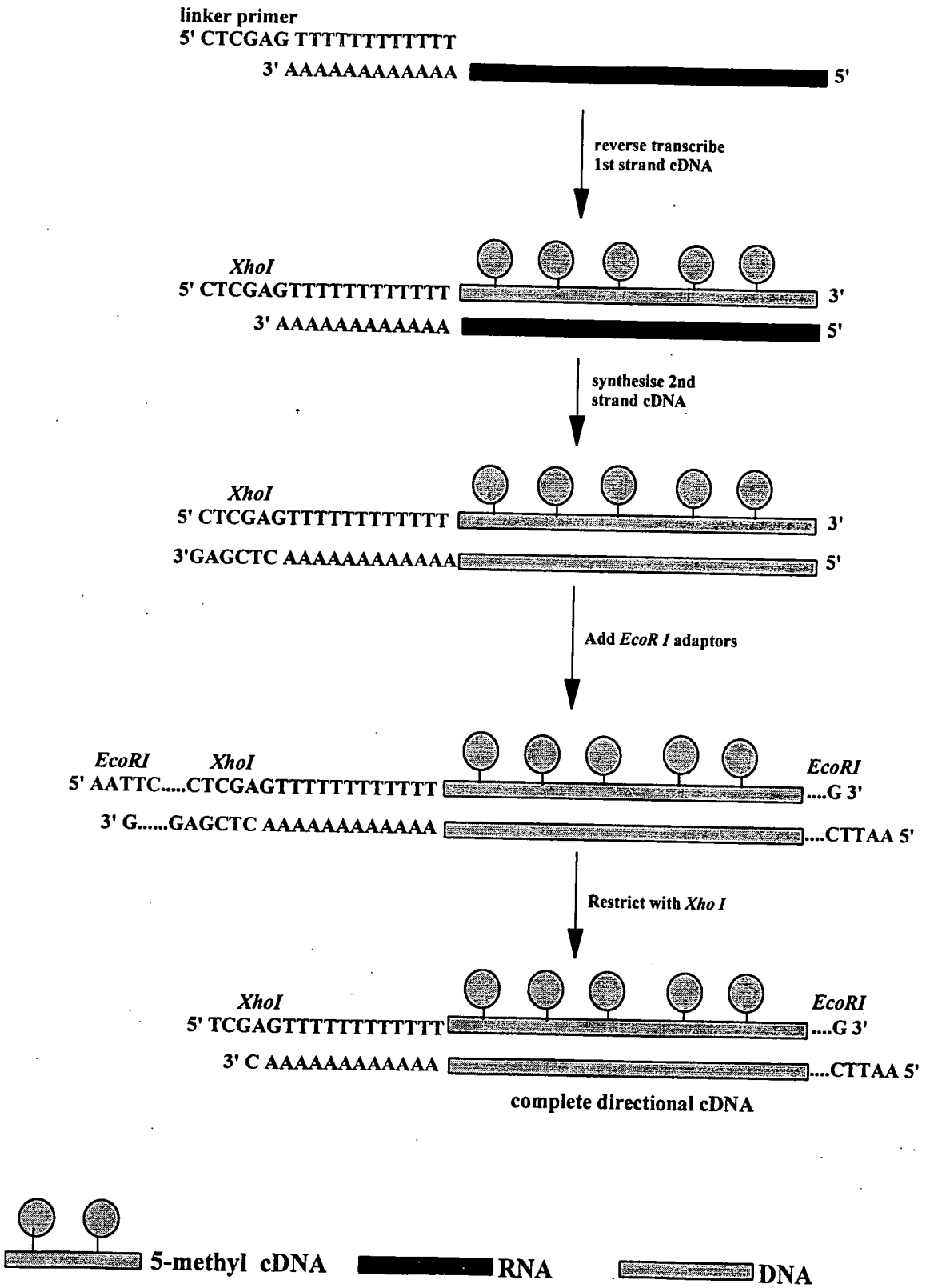


Figure 7. Schematic representation of Stratagene Lambda Uni-ZAP XR cDNA Cloning Kit methodology. Adapted from Stratagene Lambda Uni-ZAP XR cDNA Cloning Kit manual.

Figure 8. **Autoradiograph of 1st and 2nd strand, [α ^{32}P] dATP labelled control (tracer), synthesis products following electrophoresis on an agarose alkaline denaturing gel.**

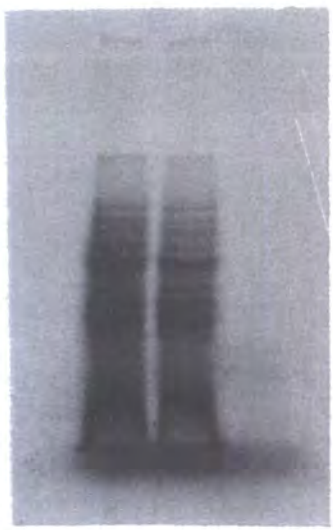


Figure 9. **Example of *in-vitro* translated total RNA.**
Lanes 1 and 2 show autoradiograph of electrophoresed ^{35}S methionine-labelled *in-vitro* translation products from RNA isolated from caryopses aged 1-10 DPA, separated on an SDS-polyacrylamide gel.

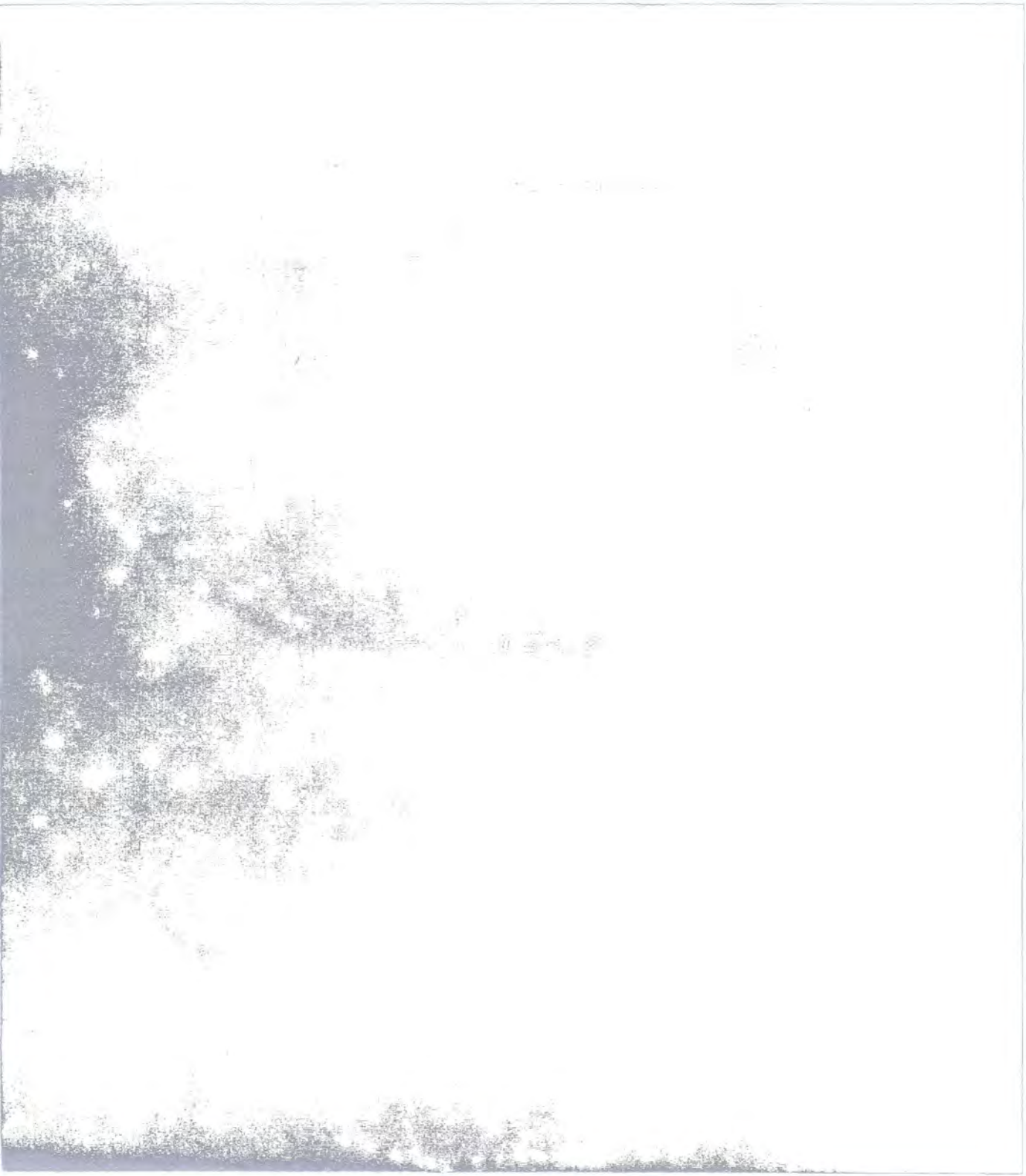


Figure 10. Example of hybridization of Maize Histone 1 clone, labeled with [α-^{32}P] dATP, to a plaque lift of the barley caryopsis library. Arrows point to examples of hybridization to plaques.

Figure 11. Excised inserts from pBluescript subclones.

A.

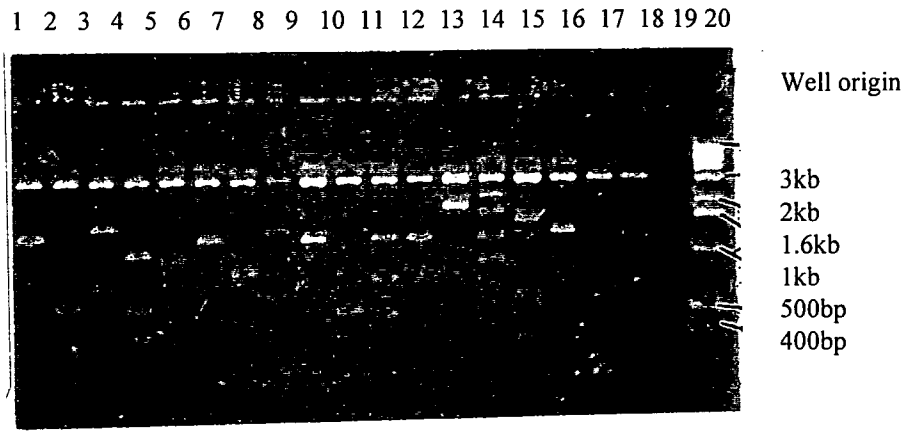
Lane	subclone	Lane	Subclone	Lane	Subclone
1	1A	7	2A	13	3A
2	1B	8	2B	14	3B
3	1C	9	2C	15	3C
4	1D	10	2D	16	3D
5	1E	11	2E	18	3E
6	1F	12	2F	19	3F

Lane 20 Lambda *Hind III* DNA Ladder

B.

Lane	
1	Lambda *Hind III* DNA Ladder
2	clone 14A
3	clone 14B
4	clone 27A
5	clone 27B
6	clone 29
7	clone 30
8	Lambda *Hind III* DNA Ladder

A.



B.

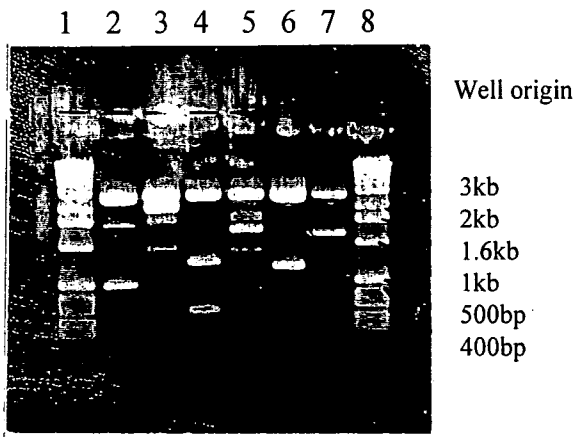


Figure 11. Excised inserts from pBluescript II SK subclones.
Ethidium bromide stained agarose gel of electrophoretically separated inserts of pBluescript II SK subclones following excision by restriction enzyme digestion.

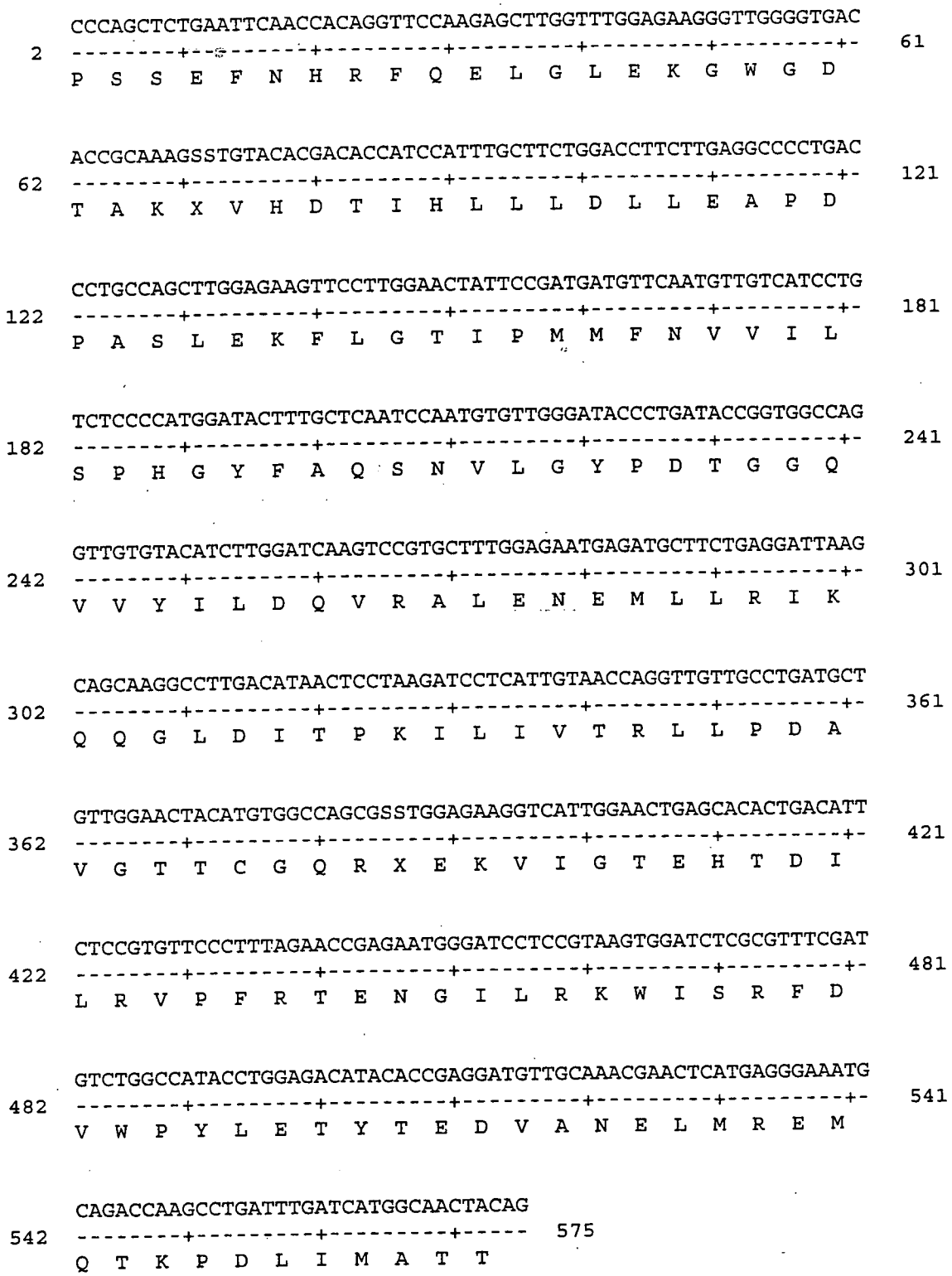


Figure 12. Publish diagram of sequence from clone 3B (reverse primed reading), showing sequence data and the derived amino acid sequence (Genetics Computer Group, 1991).

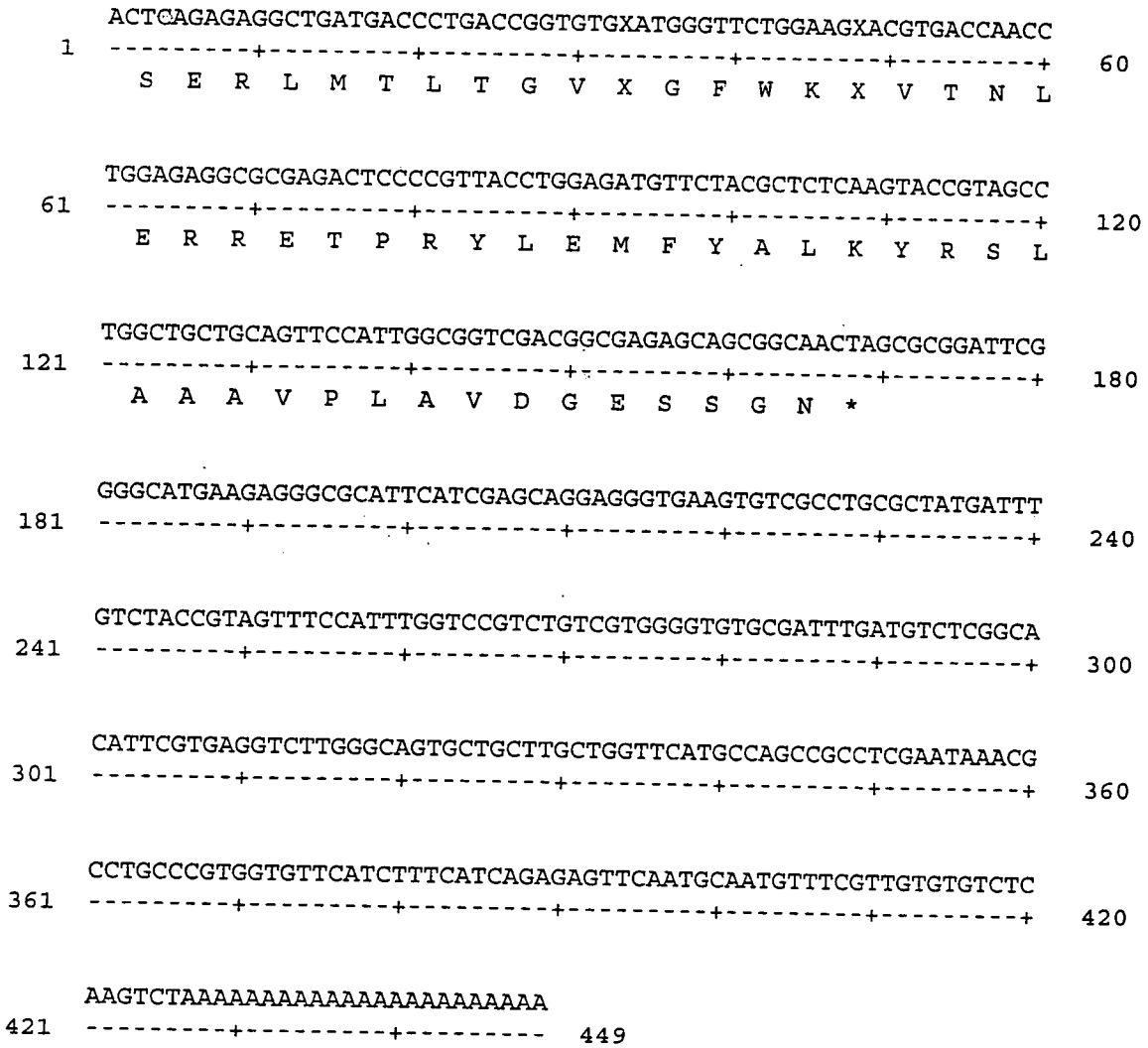


Figure 13. **Publish diagram of sequence from clone 3B (forward primed), showing sequence data and the derived amino acid sequence (Genetics Computer Group, 1991).**

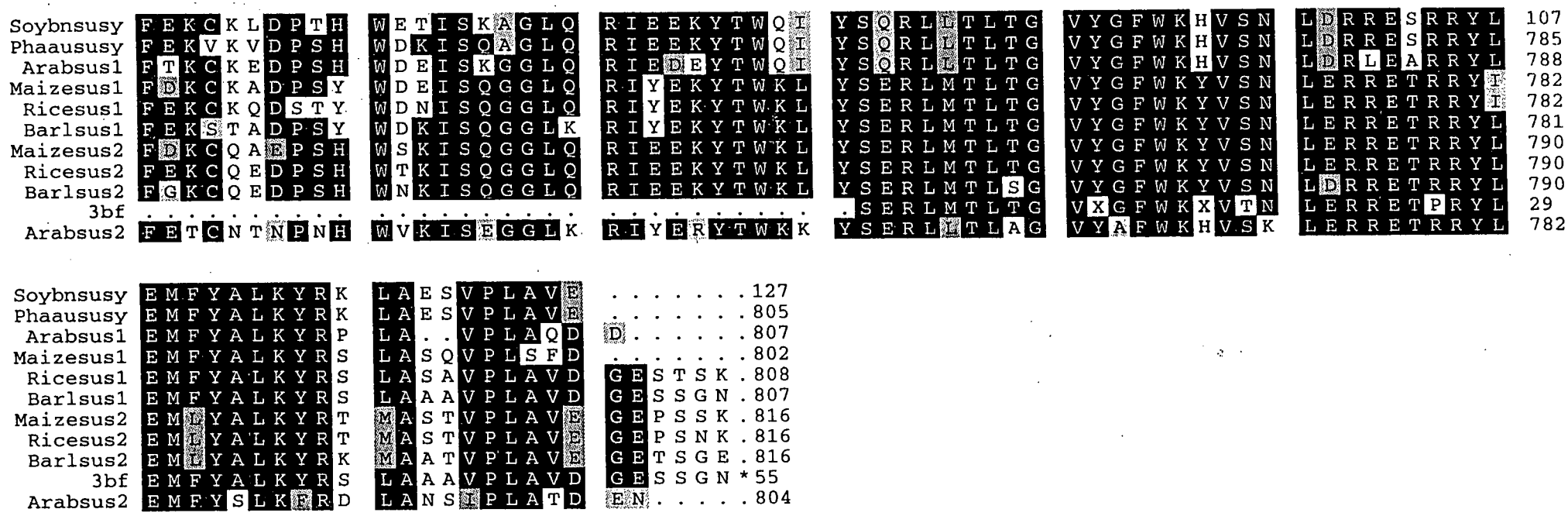


Figure 14. **Pretty box diagram of the pileup alignment of the derived amino acid sequence of clone 3B (forward primed), 3bf, to sucrose synthase amino acid sequences from barley (Barlsus 1, Barlsus2), rice (Ricesus1, Ricesus2), pea (Phaaususy), maize (Maizesus1, Maizesus2), soybean (Soybnsusy) and *Arabidopsis thaliana* (Arabsus1) (Genetics Computer Group, 1991).**

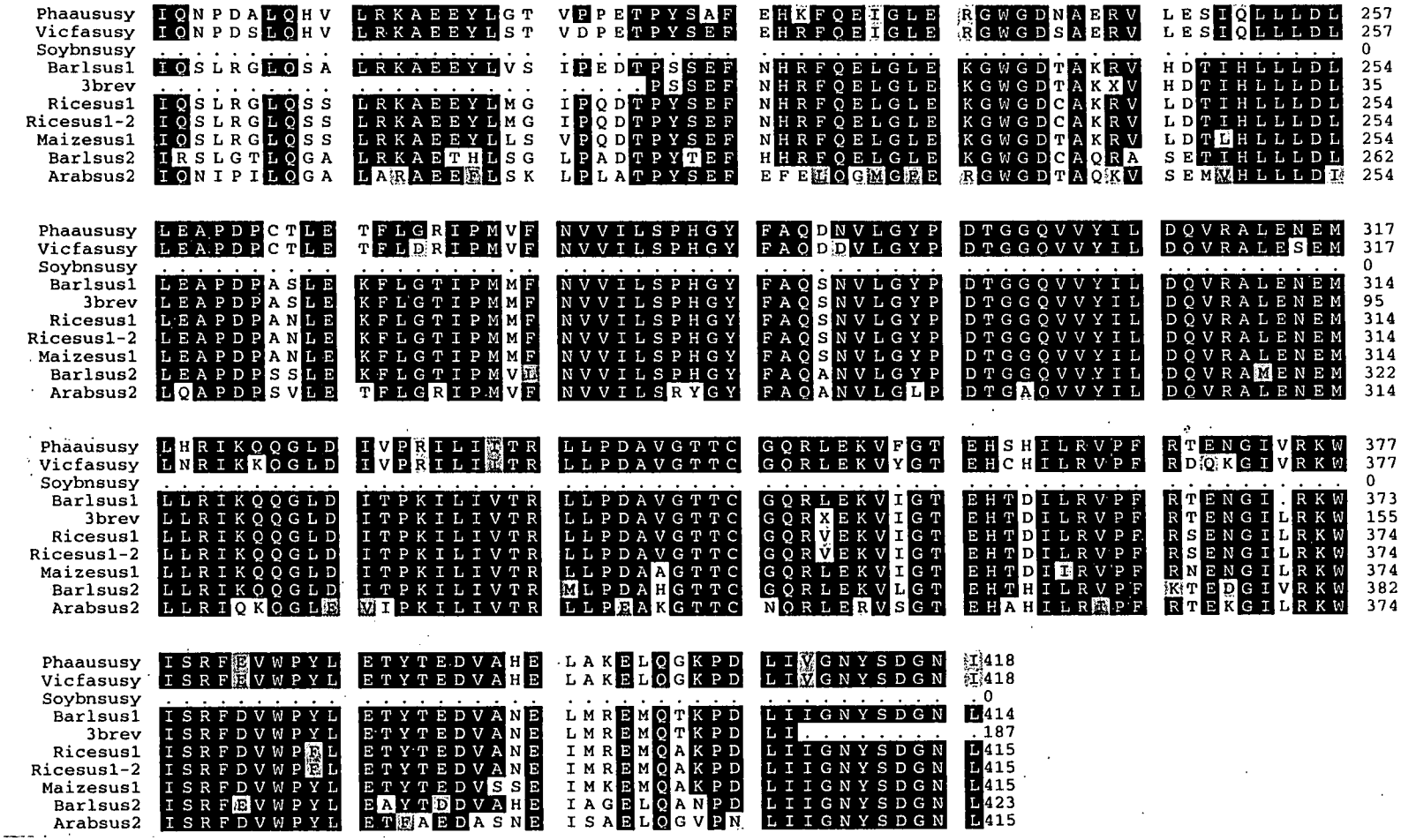


Figure 15. **Pretty box diagram of the pileup alignment of the derived amino acid sequence of clone 3B (reverse primed), 3brev, to sucrose synthase amino acid sequences from barley (Barlsus 1, Barlsus2), rice (Ricesus1, Ricesus2), pea (Phaaususy), maize (Maizesus1, Maizesus2), soybean (Soybnsusy) and *Arabidopsis thaliana* (Arabsus1)) (Genetics Computer Group, 1991).**

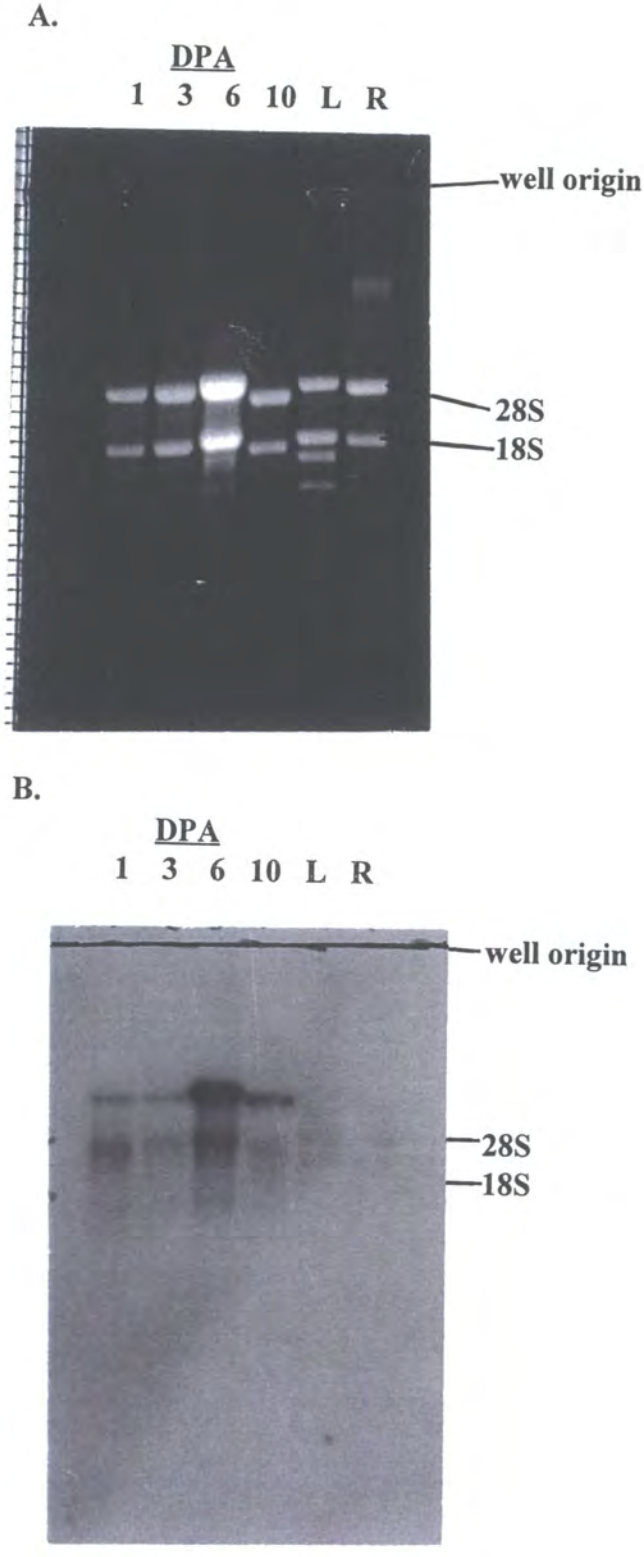


Figure 16. **Hybridisation of [α ^{32}P] dATP labelled insert of clone 3B to Northern blotted total RNA from caryopses aged 1, 3, 6 and 10 DPA and leaf (L) and root (R) total RNA.**

A. agarose gel of ethidium bromide stained, electrophoretically separated total RNA.

B. hybridisation of [α ^{32}P] dATP labelled insert from clone 3B to Northern blotted RNA from the gel in A.

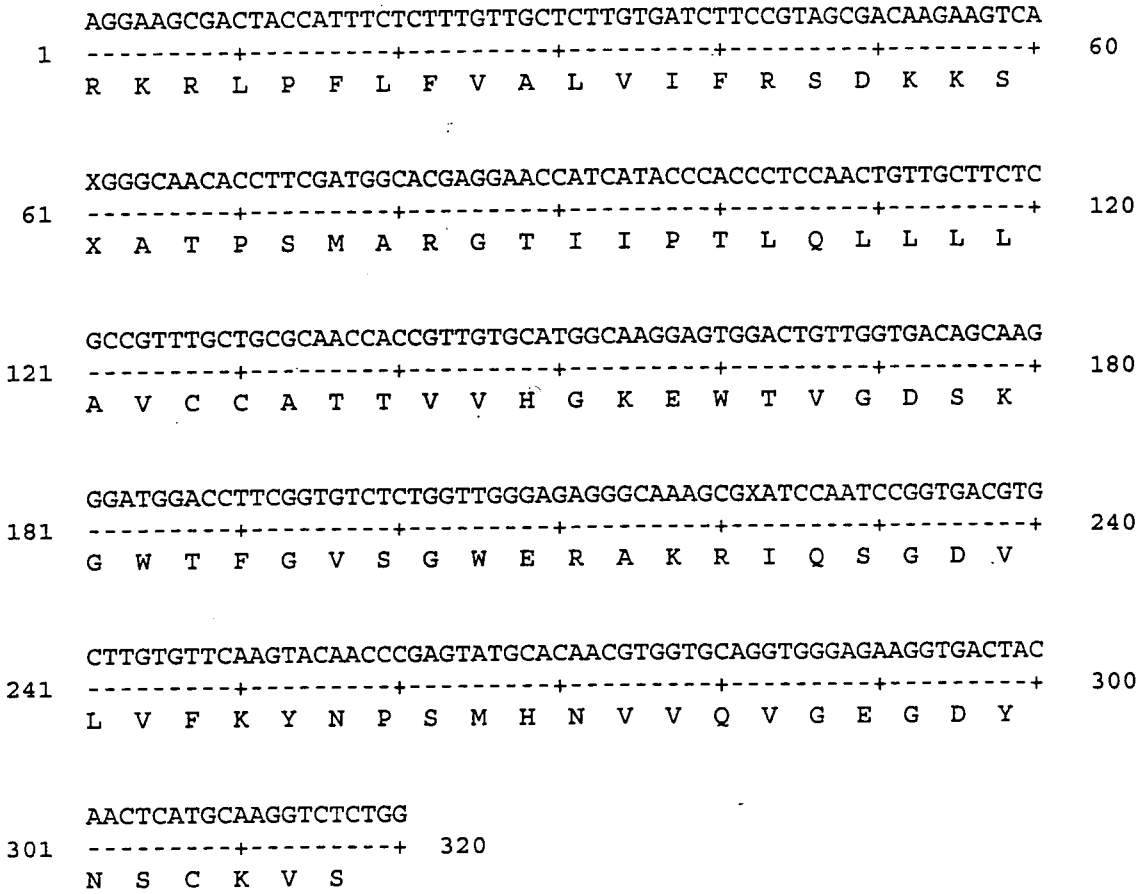


Figure 17. Publish diagram of sequence from clone 16B, showing sequence data and the derived amino acid sequence (Genetics Computer Group, 1991).

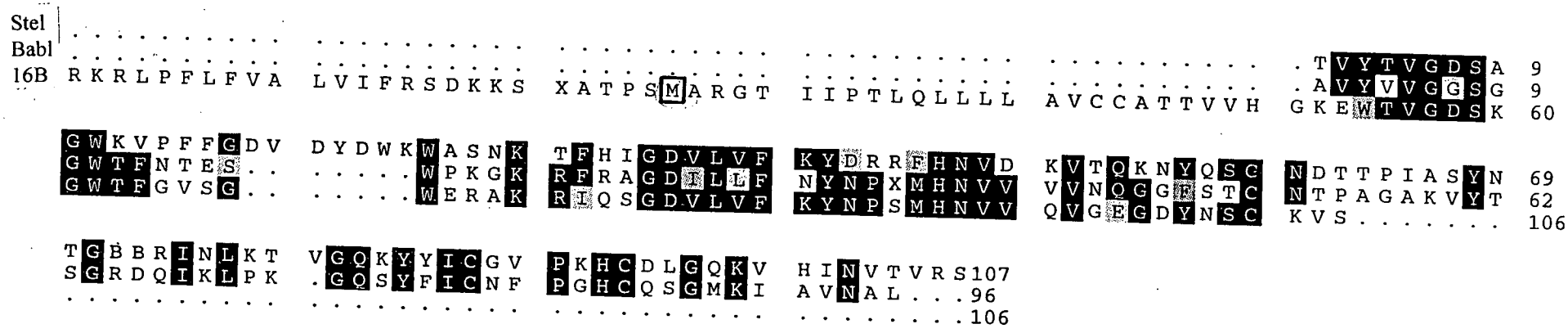


Figure 18. **Prettybox diagram of the pileup alignment of the derived amino acid sequence of clone 16B with cucumber basic blue copper protein (cusacynin) (Babl) and Japanese laquer tree blue copper protein (stelacynin)(Stel) amino acid seqeuences.**

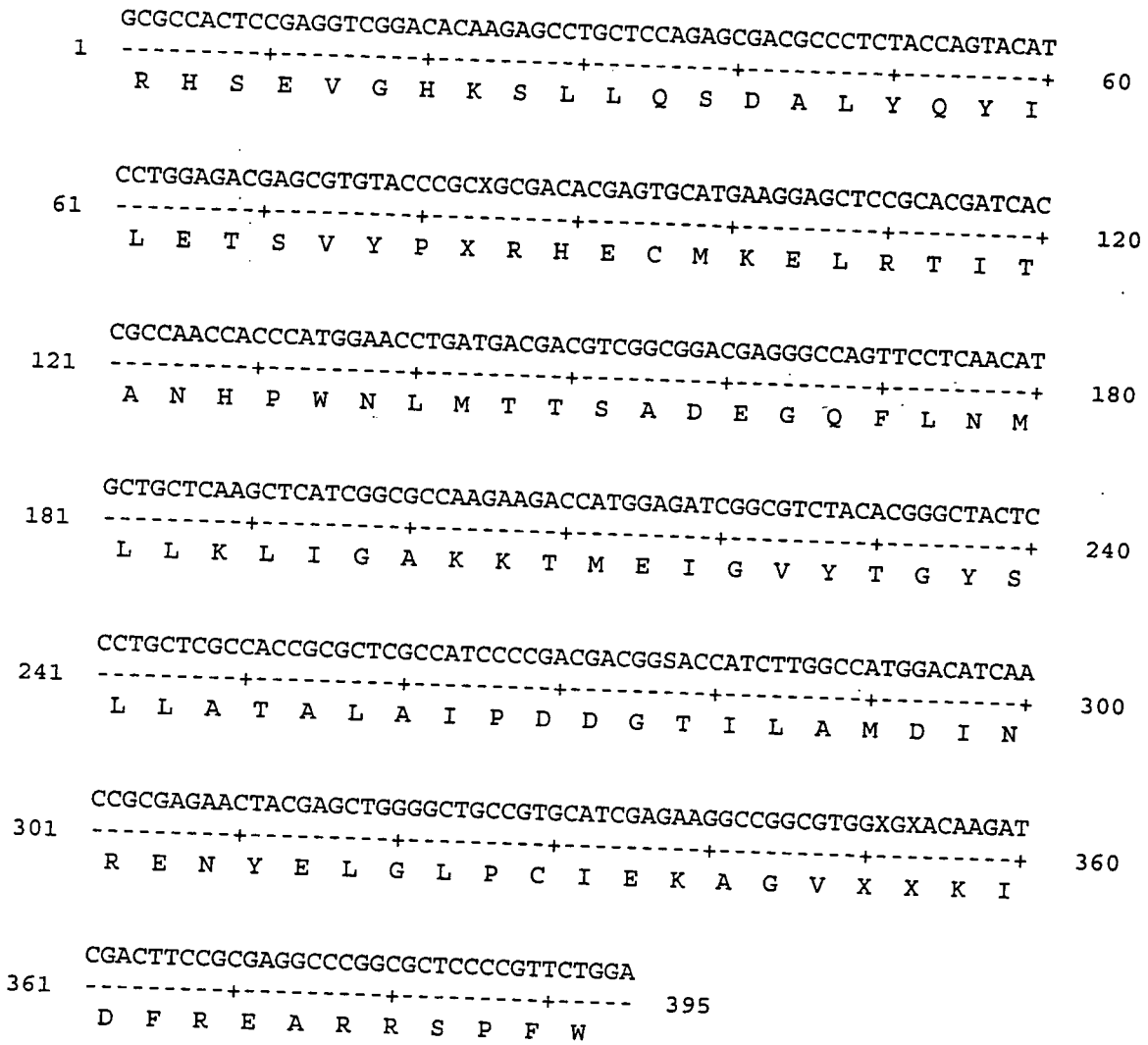


Figure 19. Publish diagram of (reverse primed sequence reading) from clone 16D showing nucleotide sequence data and the derived amino acid sequence(Genetics Computer Group, 1991).

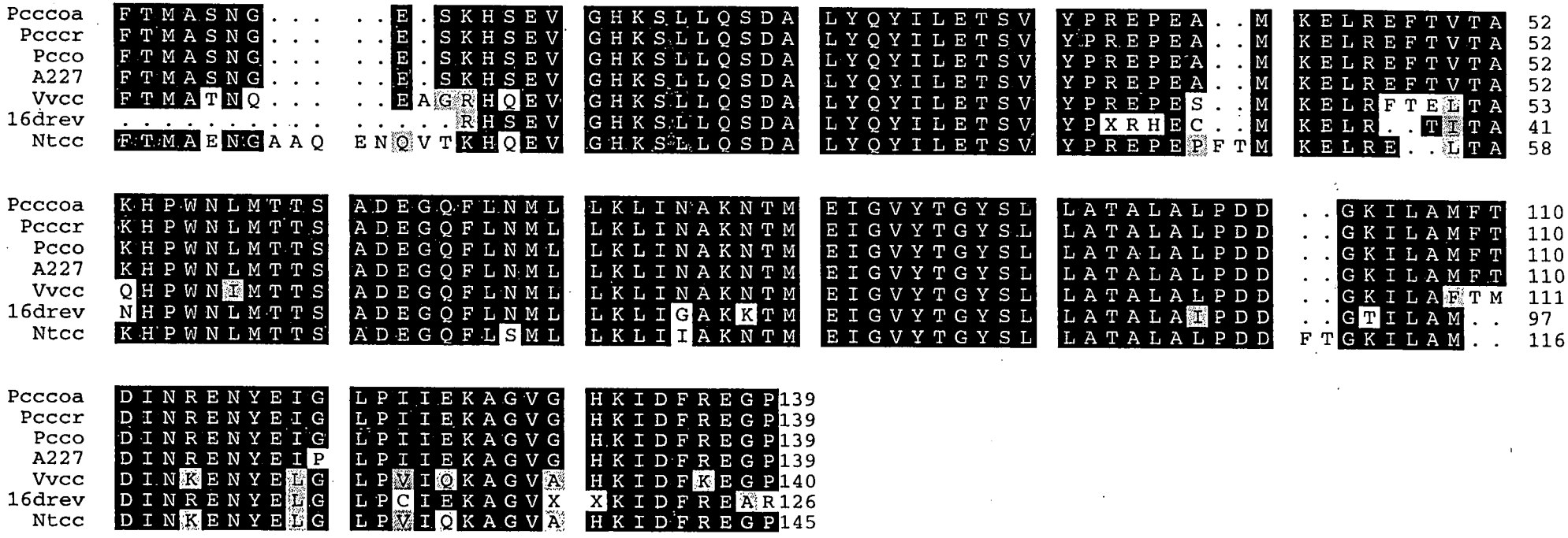


Figure 20. Prettybox diagram of the pileup alignment of the derived amino acid sequence of clone 16d (reverse primed reading) with caffeoyl CoA- 3-O-methyltransferase from *Petroselinum crispum* (Pcccoa, Pcco), *Vicia vinifera* (Vvcc), *Nicotiana tabacum* (Ntaa) and A227 amino acid seqeuences and *P. crispum* (Pcccr) trans-caffeoyl methyltransferase amino acid sequence (Genetics Computer Group, 1991).

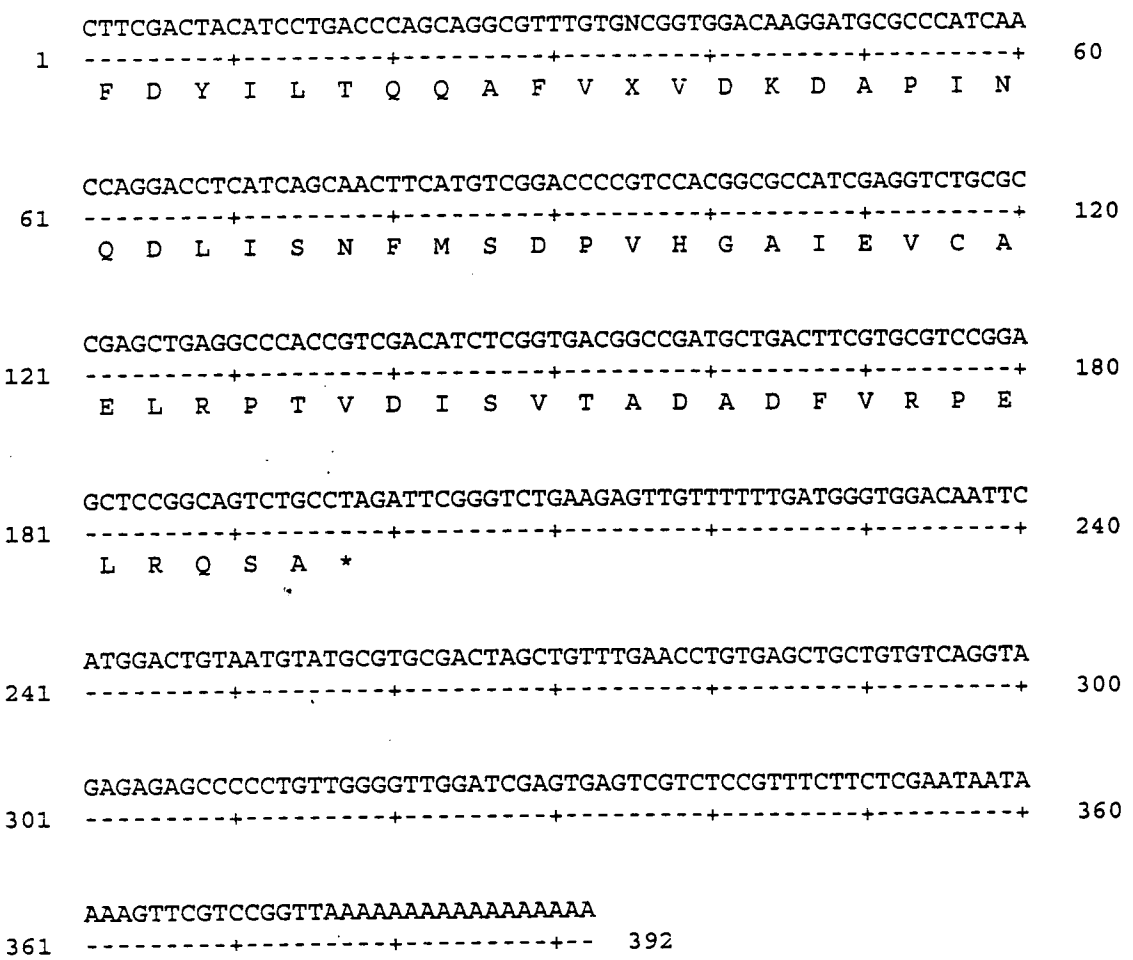


Figure 21. Publish diagram of forward primed sequence from clone 27B showing sequence data and the derived amino acid sequence from the poly A+ tail region(Genetics Computer Group, 1991).

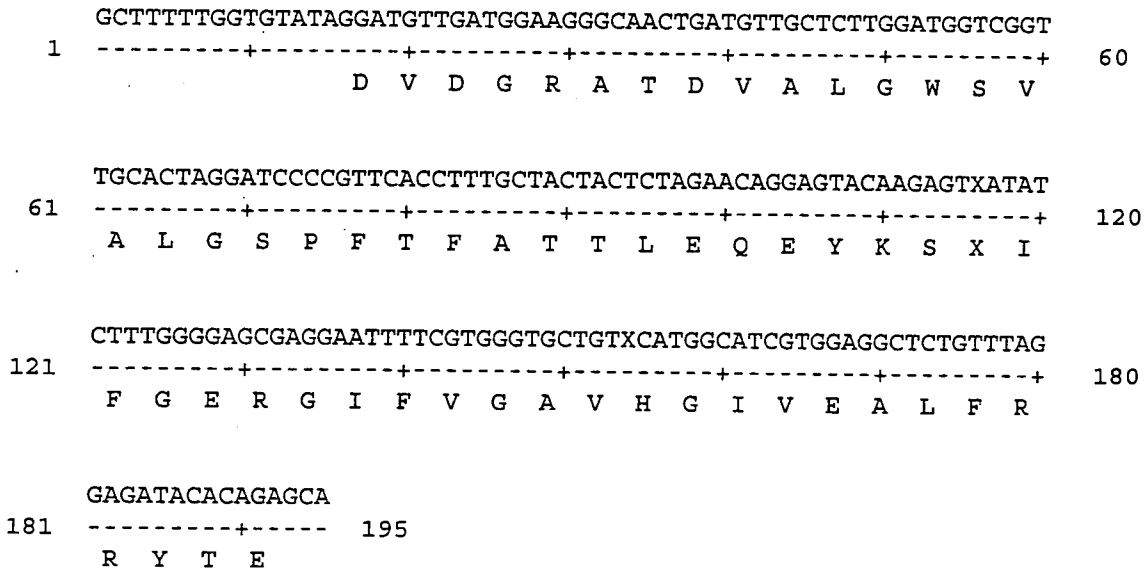
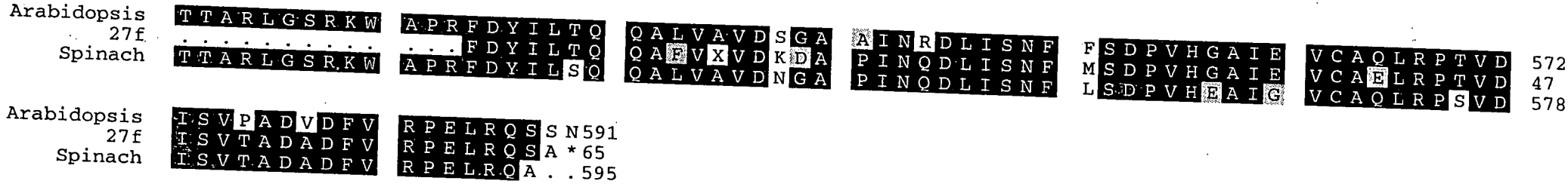


Figure 22. Publish diagram of sequence from clone 27B (reverse primed), showing sequence data and the derived amino acid sequence (Genetics Computer Group, 1991).

a. forward primed sequence reading



b. reverse primed sequence reading

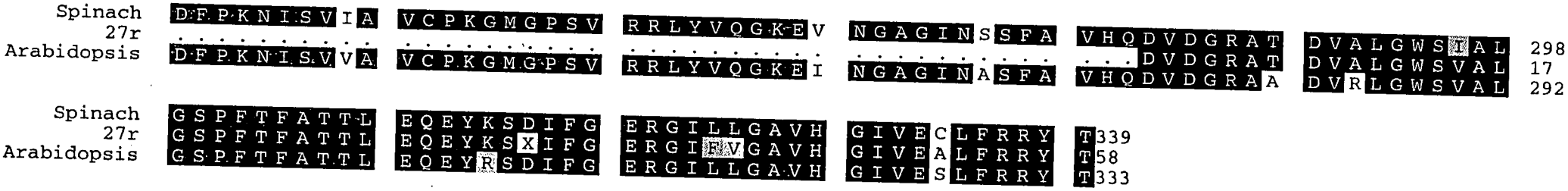


Figure 23. Prettybox diagram of the pileup alignment of the derived amino acid sequence of clone 27B,(a.) forward primed reading (27f) (b.) reverse primed reading (27r) with *Arabidopsis thaliana* and *O. oleracea* (spinach) ketol acid reductoisomerase (KARI) amino acid seqeuences (Genetics Computer Group, 1991).

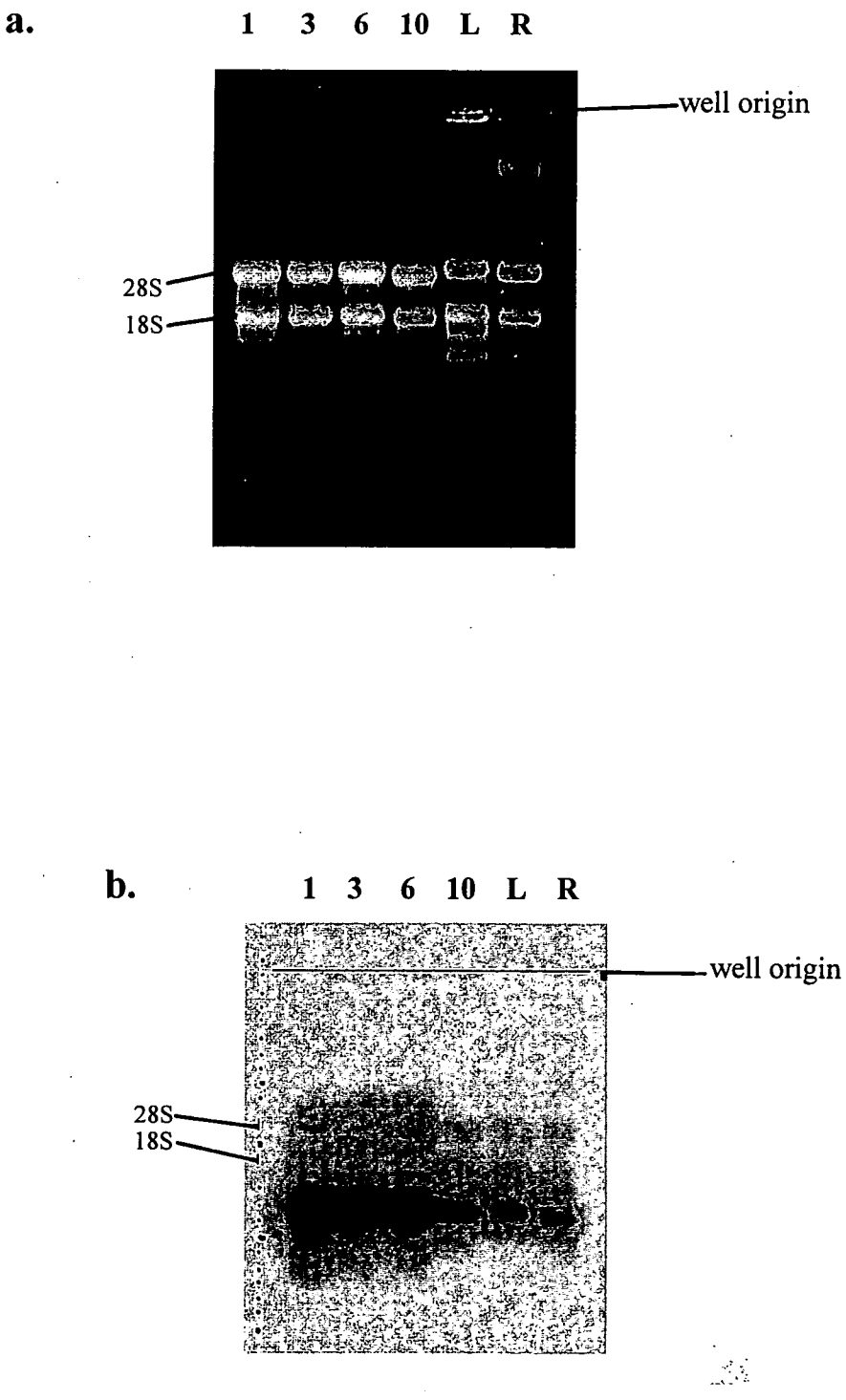


Figure 24. **Hybridisation of [α ^{32}P] dATP labelled clone 27B to Northern blotted total RNA.**

a. **Agarose gel showing electrophoresed total RNA from 1, 3, 6, and 10 DPA caryopses, leaf (L) and root (R) before transfer to Hybond N membrane.**

b. **Autoradiograph showing hybridisation of [α ^{32}P] dATP labelled insert from clone 27B to Northern blotted total RNA as shown in a.**

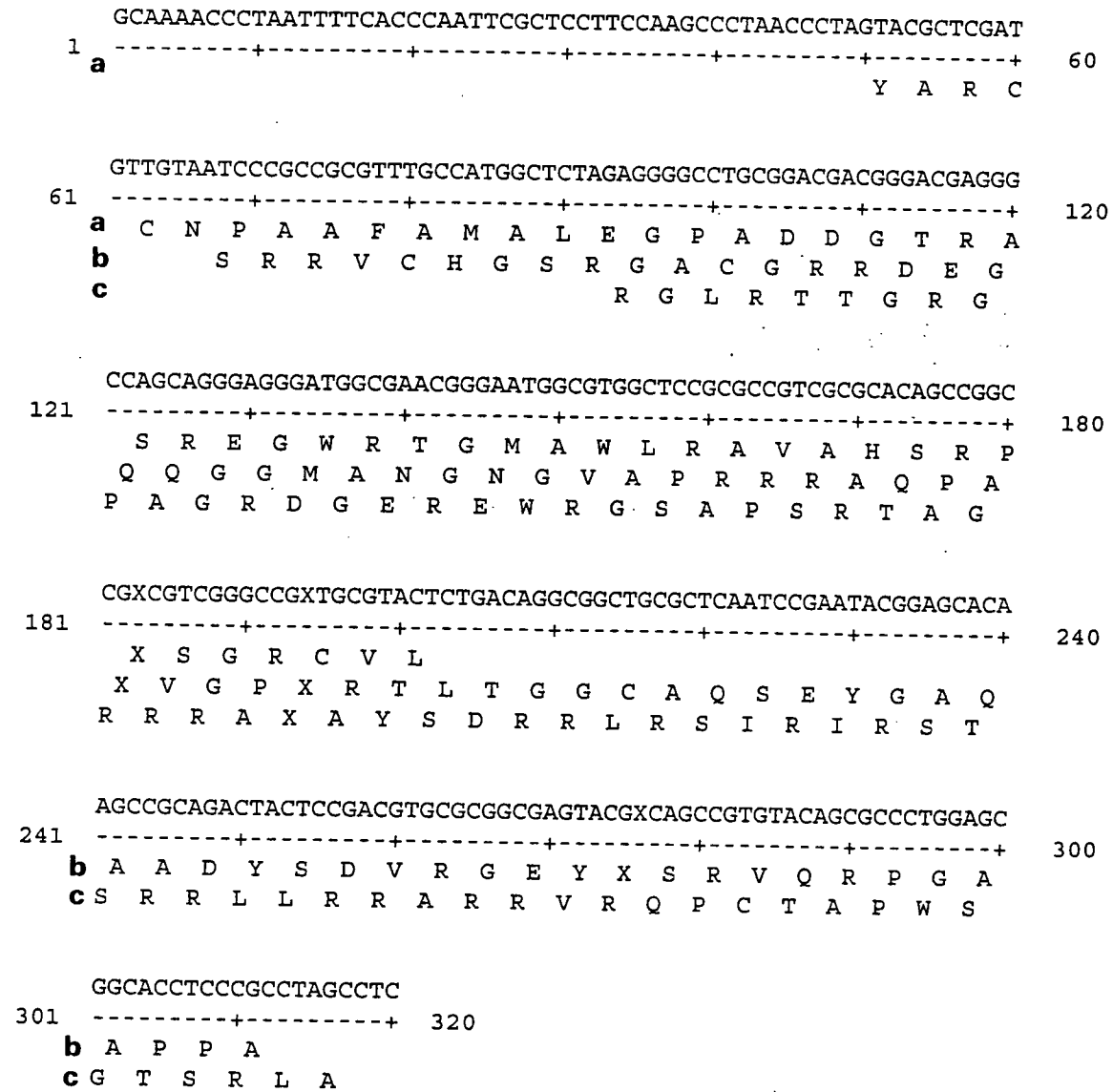


Figure 25. **Publish alignment of clone 16C nucleotide sequence with the derived amino acid sequences of three possible open reading frames - a, b, c (Genetics Computing Group, 1991).**

Figure 26. **Publish diagram of clone 2A (reverse primed sequence reading) showing the nucleotide sequence and the derived amino acid sequence of 4 possible open reading frames a, b, c and d.**

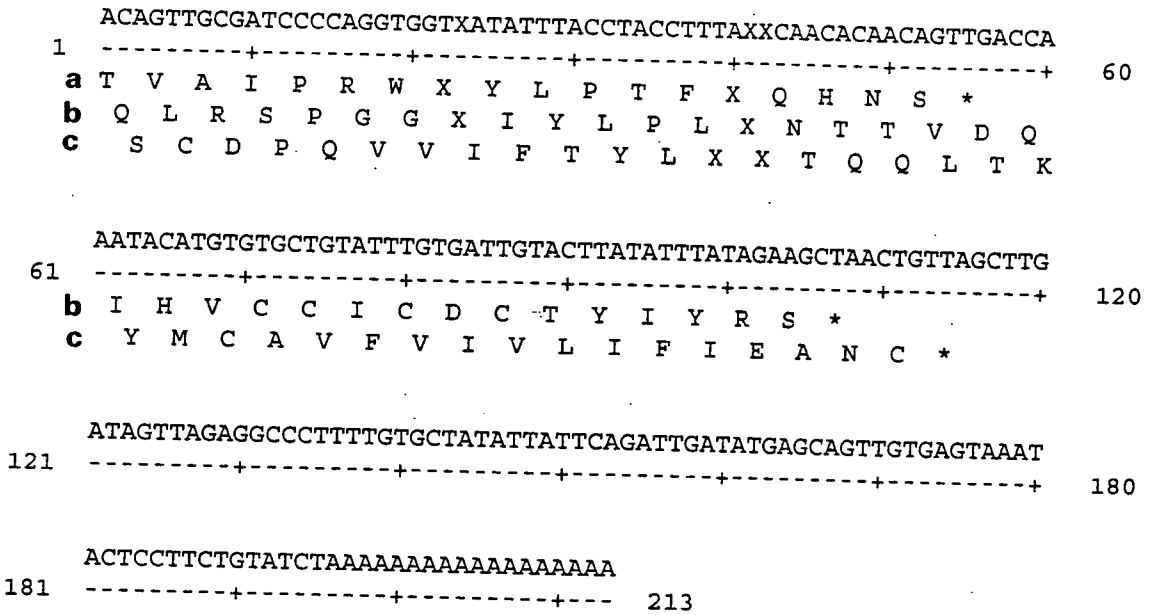


Figure 27. **Publish diagram of clone 2A (forward primed sequence reading) showing the nucleotide sequence and the derived amino acid sequence of 3 possible open reading frames a, b, and c.**

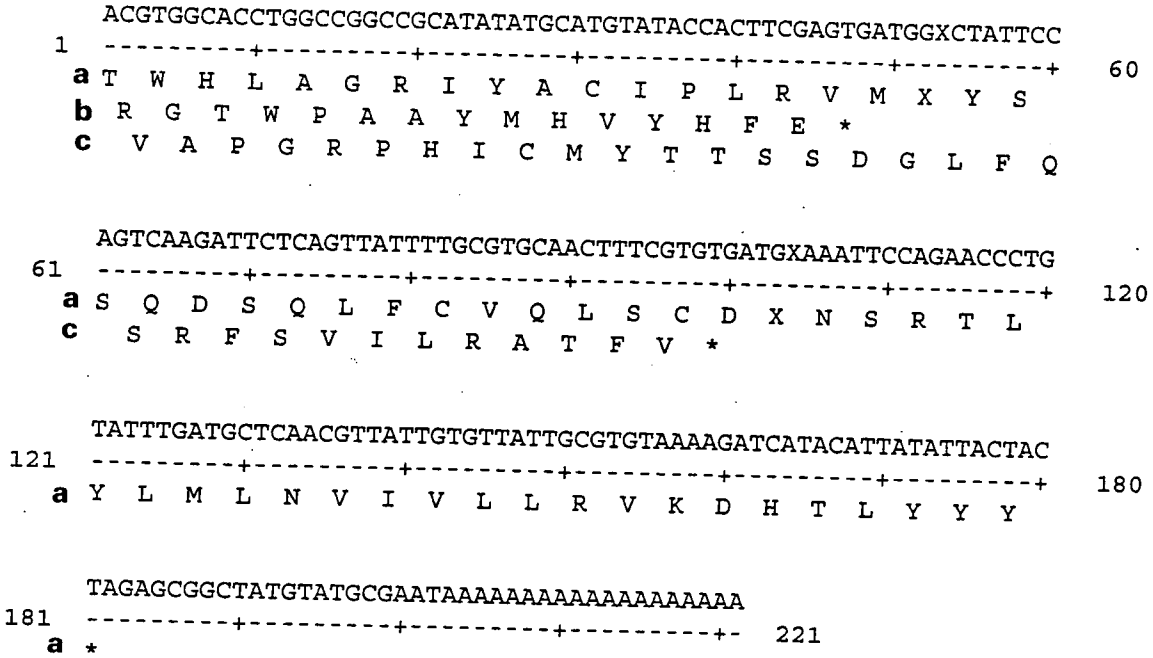


Figure 28. Publish diagram of (forward primed sequence) from clone 2C showing nucleotide sequence data and the derived amino acid sequences(Genetics Computer Group, 1991).

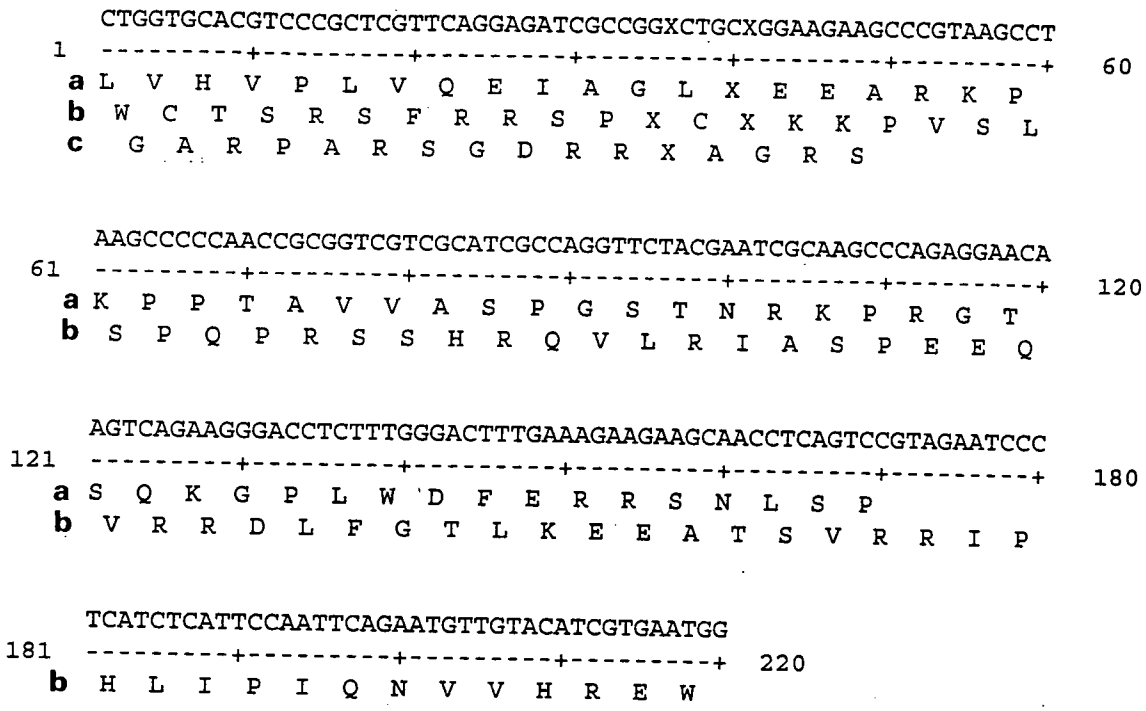


Figure 29. Publish diagram of (reverse primed sequence reading) from clone 14A showing nucleotide sequence data and the derived amino acid sequences of three possible open reading frames (Genetics Computer Group, 1991).

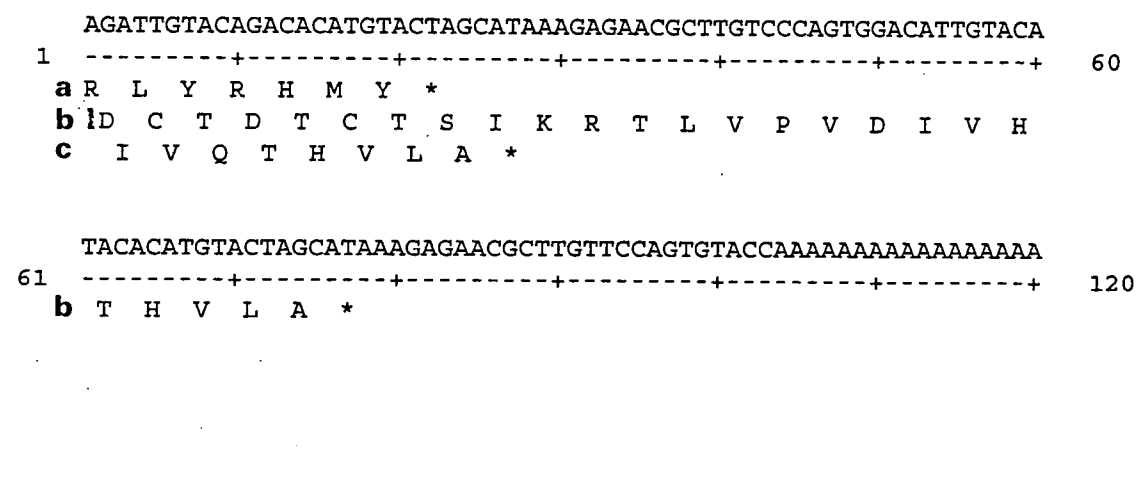


Figure 30. Publish diagram of (forward primed sequence reading) from clone 14A showing nucleotide sequence data and the derived amino acid sequences of three possible open reading frames (Genetics Computer Group, 1991).

```
    GTTGGGGGCTTAGGCTTGACGGGCTTCTTCGXGCAGCCCGGCGATCTCCTGAACGAGCGG
 1  ---------+---------+---------+---------+---------+---------+   60
  a V  G  G  L  G  L  T  G  F  F  X  Q  P  G  D  L  L  N  E  R
  b  W  G  L  R  L  D  G  L  L  R  A  A  R  R  S  P  E  R  A  G

    GACGGCACCAGCACCGCCCTCCACGCC
61  ---------+---------+-------  87
  a D  G  T  S  T  A  L  H  A
  b  R  H  Q  H  R  P  P  R
```

Figure 31. Publish diagram of (reverse primed sequence reading) from clone 14B showing nucleotide sequence data and the derived amino acid sequences of two possible open reading frames (Genetics Computer Group, 1991).

```
    CACAACNGCGCTTTCAACATCAANTGACAAACCTGTATGTTGGNTCATCTCCTCCAGGTT
 1  ---------+---------+---------+---------+---------+---------+   60
  a H  N  X  A  F  N  I  X  *
  b T  T  A  L  S  T  S  X  D  K  P  V  C  W  X  I  S  S  R  L
  c  Q  X  R  F  Q  H  Q  X  T  N  L  Y  V  G  S  S  P  P  G  Y

    ATGTCATAGTGNACATATATACATTGTTGGGCGTAGAACCGATATATGTAGATTGGACGG
61  ---------+---------+---------+---------+---------+---------+  120
  b C  H  S  X  H  I  Y  I  V  G  R  R  T  D  I  C  R  L  D  G
  c  V  I  V  X  I  Y  T  L  L  G  V  E  P  I  Y  V  D  W  T  G

    GTNGTAGTTTGTTTGTTGACACTAGTGACCCGCATCTGTGTTGCCCTAACCACCATCATA
121 ---------+---------+---------+---------+---------+---------+  180
  b X  *
  c  X  S  L  F  V  D  T  S  D  P  H  L  C  C  P  N  H  H  H  N

    ATATGCTTGATTAAAAAAAAAAAAAAAAAA
181 ---------+---------+---------+  210
  c  M  L  D  *
```

Figure 32. Publish diagram of (forward primed sequence reading) from clone 14B showing nucleotide sequence data and the derived amino acid sequences of three possible open reading frames (Genetics Computer Group, 1991).

3.3 *In-situ* Hybridisation

3.3.1 *In-situ* hybridisation of clone inserts to sections.

In-situ hybridisation was carried out to determine whether or not any clones were expressed in the endosperm tissues and to define their temporal expression in terms of developmental stage specificity. Both sense and anti-sense digoxigenin-labelled riboprobes were made utilising the T7 and T3 primer sites which flank the multiple cloning site of pBluescript II SK vector. Riboprobes were hybridised to sections with endosperm at the syncytial stage, early phases of cellularisation stage and those which showed complete cellularisation. Grains of 1, 3, 6 and 10 DPA were used as these were at similar stages of development as the grains from which RNA samples were extracted. Grains of these ages had endosperm at the developmental stages required. Visualisation of localisation was carried out using anti-digoxigenin fab fragments conjugated to either alkaline phosphatase or 5nm colloidal gold. Localisation of colloidal gold conjugated antibodies was visualised following silver enhancement. It was found that alkaline phosphatase gave a signal which was difficult to interpret and hence colloidal gold conjugated antibodies were used in subsequent *in-situ* hybridisation experiments. RNA retention within sections of the caryopsies used in *in-situ* hybridisation experiments was shown to be good by staining with acridine orange (results not shown).

Clones 3B, 14B, 16B, 16C, 16D, and 27B were used in *in-situ* hybridisation experiments. None was found to be localised uniquely to the endosperm tissue at any stage of development analysed. Details of the patterns of hybridisation of each clone to sections of barley caryopsies at different stages of development are given below. It should be noted that sets of

data for each clone are incomplete due to problems with retention of sections on slides. There are no results for *in-situ* hybridisation for any of the clones to sections of leaf material as they did not remain attached to the poly-L-lysine coated slides in any instance.

Plate 15 shows *in-situ* hybridisation of antisense riboprobe from clone 27B to sections of 3, 6 and 10 DPA caryopsies and sense riboprobe to a section from a 6 DPA caryopsis. It can be seen that staining is associated with specific cell layers on each of the sections of 3 and 6 DPA samples (plate 15, figures a and b). In contrast, the 10 DPA section (plate 15, figure c) shows heavy labelling on only one layer. Staining is high on 3 and 6 DPA sections over the areas corresponding to the nucellus, testa and cross cells and also on the endosperm tissues. However, staining on the 10 DPA section differs in that none occurs on the nucellus and testa but appears especially high over the cross cell layers and at a lower intensity over the endosperm tissue. The level of staining appears to be more intense in 6 and 10 DPA sections, however it is difficult to quantify the amount of labelling on each section. The sense riboprobe which acts as a control, had no staining on sections from caryopses with endosperm at any stage of development studied (Plate 15d). The cDNA from Clone 27B is a putative KARI enzyme which, in plants, is targeted to the chloroplasts. Hence it might be expected that localisation would be to those cells of the barley caryopsis which contain chloroplasts. This is obviously not the case, as hybridisation of the probe is also observed on starchy endosperm cell types which contain amyloplasts rather than chloroplasts. In addition, the hybridisation of an RNA probe from clone 27B to Northern blotted total RNA from root, suggests that, it is possible that the clone does not contain a putative KARI cDNA, but something with some complementary sequence. An alternatively explanation is that it may be a form of KARI which is different to the chloroplast-targeted clone. KARI from fungi is targeted to the mitochondria. It may be that the putative KARI obtained here is also targeted

to the mitochodria.

The results obtained from the *in-situ* hybridisation of riboprobes from clone 16D, a putative CCoAOMT cDNA, are again not what might be expected. The antisense riboprobe hybridised to the endosperm tissues and nucellar layer (which are non-lignified) as well as to the cross cells of the pericarp region (of which only the xylem cells are lignified) in sections of all ages analysed. Localisation of the antisense and sense 16D riboprobes on sections aged 10 DPA is shown in Plate 16. If the cDNA from clone 16D is a CCoAOMT cDNA then we would not expect it to be localised in the endosperm tissues, unless the coenzyme is associated with another biosynthetic pathway(s).

Riboprobes synthesised from Clone 3B, a putative sucrose synthase cDNA, hybridised to all areas of sections of 3, 6, and 10 DPA caryopses. The intensity of staining was however, faint. An example of localisation of the antisense riboprobe to 3DPA caryopsis section and the sense (control) riboprobe to 10 DPA caryopis section are given in Plate 17. The pattern of localisation of clone 3B is what might be expected of sucrose synthase, in that all cells express sucrose synthase genes to some degree.

Antisense riboprobe synthesised from clone 14B, which showed no similarity to any data base entries, again showed strong hybridisation to the nucellar, testa, cross cell and pericarp layers as well as the endosperm tissue. Levels of stain intensity were similar on sections of all ages of caryopses studied, although it did appear to be slightly more intense over endosperm tissue of 3 DPA sections than 6 DPA sections (see Plate 18).

The antisense riboprobe synthesised from clone 16C, which also showed no similarity to

entries in the sequence data bases, hybridised to all areas of sections of 3 and 10 DPA caryopses (see Plate 19). Hybridisation to a section of a 10 DPA caryopsis showed a gradual decline in the level of stain intensity from the outer edge of the starchy endosperm to the inner area. This suggests that clone 16C has differential expression per unit area of section within the endosperm of 10 DPA caryopsis. There was very little staining on the nucellar layer, however, the cross cell layer and the pericarp had a small amount of staining. Sense riboprobe from clone 16C showed no staining on any age of caryopsies section.

In-situ hybridisation of antisense riboprobes from clone 16B, a putative blue copper binding protein, showed intense staining over the crease region, nucellar, testa and cross cell layers and over the endosperm tissue of sections from each age of caryopsis studied. Sense riboprobes showed less intent staining over the same areas. Plates 20 and 21 show details of the hybridisation patterns obtained for sense and antisense ribprobes to sections from caryopses aged 3, 6 and 10 DPA.

3.3.2 Dot blot analysis of Riboprobes

Hybridisation analyses of riboprobes from a number of clones to dot spotted total RNA from 1, 3, 6, and 10 DPA caryopses, leaf and root was carried out to help determine whether any of the clones was differentially expressed. Although less accurate than using Northern blot hybridisation experiments the procedure was faster. The results for a number of the experiments are shown in Table 11. The quantity of signal was used as an indication of level of expression. Note that actual dot blot results are not available as they were misplaced. Each of the riboprobes from clones listed in Table 11 showed indications of being differentially expressed. However, all of the patterns observed, did not match patterns in

levels of signal obtained for the same clone in the *in-situ* hybridisation experiments.

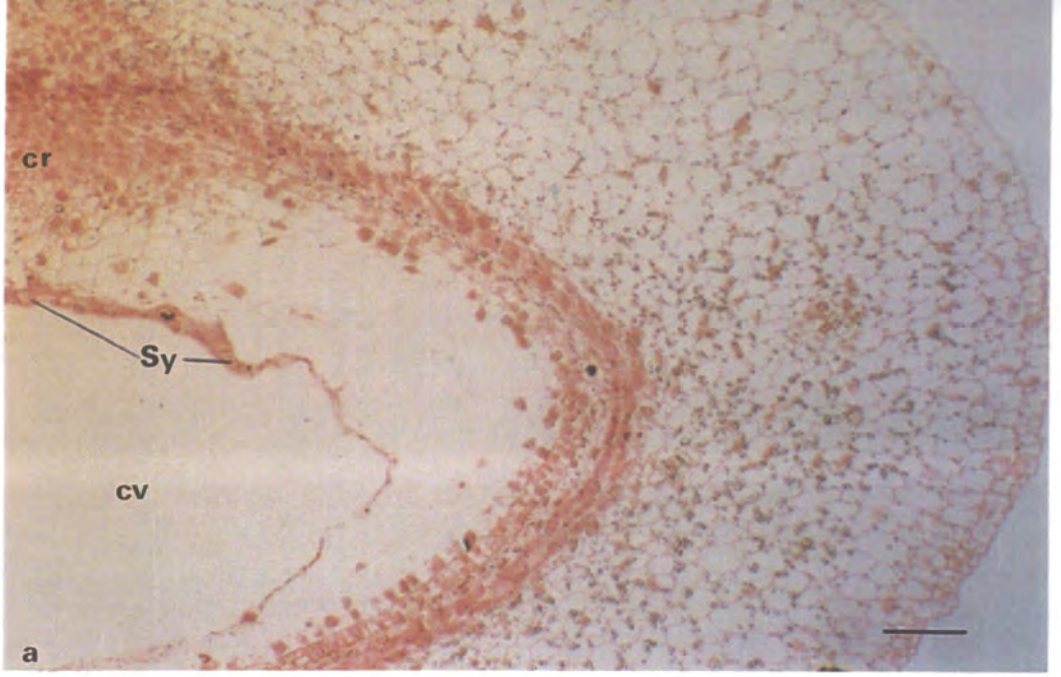
Table 11. Levels of hybridisation (staining) of a number of digoxigenin labelled antisense riboprobes to dot blotted total RNA from 1,3, 6 and 10 DPA barley caryopses, leaf and root.

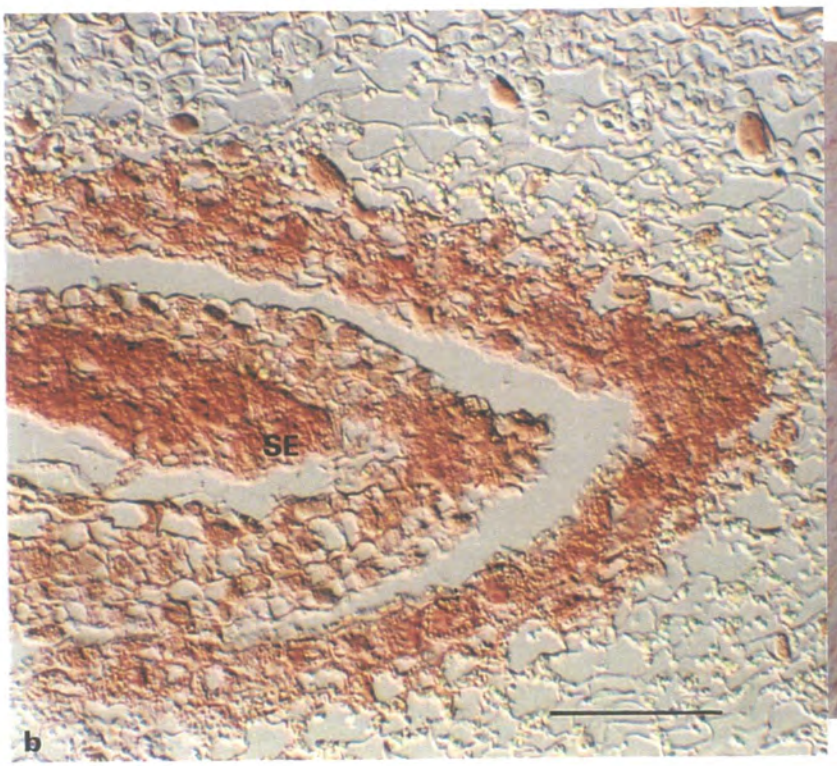
Probe	1 DPA	3 DPA	6DPA	10 DPA	LEAF	ROOT
1F	+	+	+	-	-	-
2C	+	++	+++	++	+++	+
3B	+	+	+++	++	-	-
14A	+	++	+++	++++	+	+
16B	++	++	++	+	++	-
16C	+	+	++	+	++	+
27B	-	+	++	-	-	
30	++	++	+	+	+	+

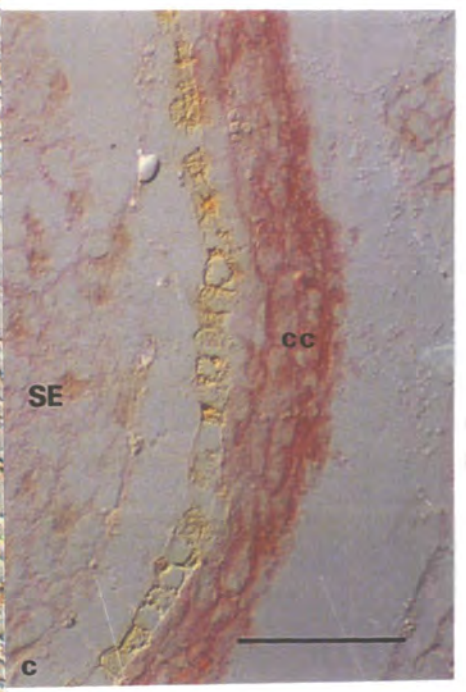
(+ = signal presence and indication of strength; - = no signal present)

It should be noted that obtaining equal loading of total RNA on dot blots is difficult and not necessarly accurate, and this may account for the differences in signal presence and intensity in each sample and age group of sections between dot blot, Northern blot and *in-situ* hybridisation experimental results.

Plate 15. ***In-situ*** **hybridisation of digoxigenin UTP-11 labelled antisense riboprobe from clone 27B (putative KARI cDNA clone) to sections from caryopses aged (a) 3 DPA, (b) 6 DPA and (c) 10 DPA and (d) of digoxigenin UTP-11 labelled sense riboprobe from clone 27B to a section from a caryopsis aged 6 DPA. Each visualised using anti-alkaline phosphatase enzyme substrate precipitation.**


cr
Sy
cv
a


SE
b


cc
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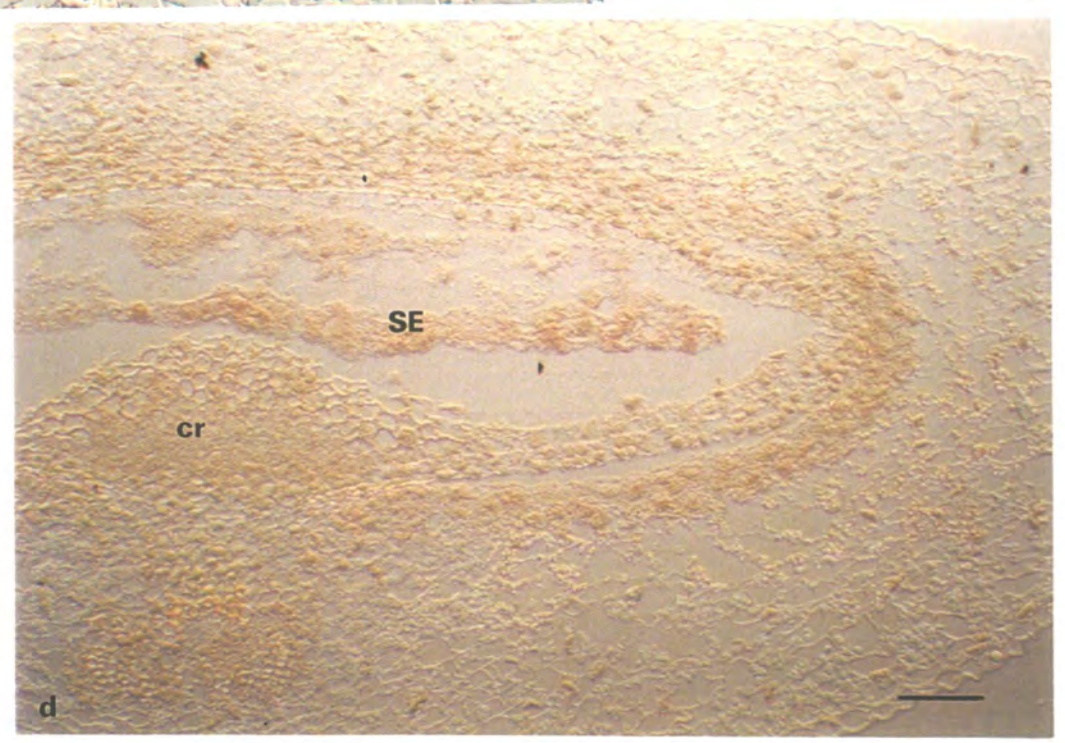
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d

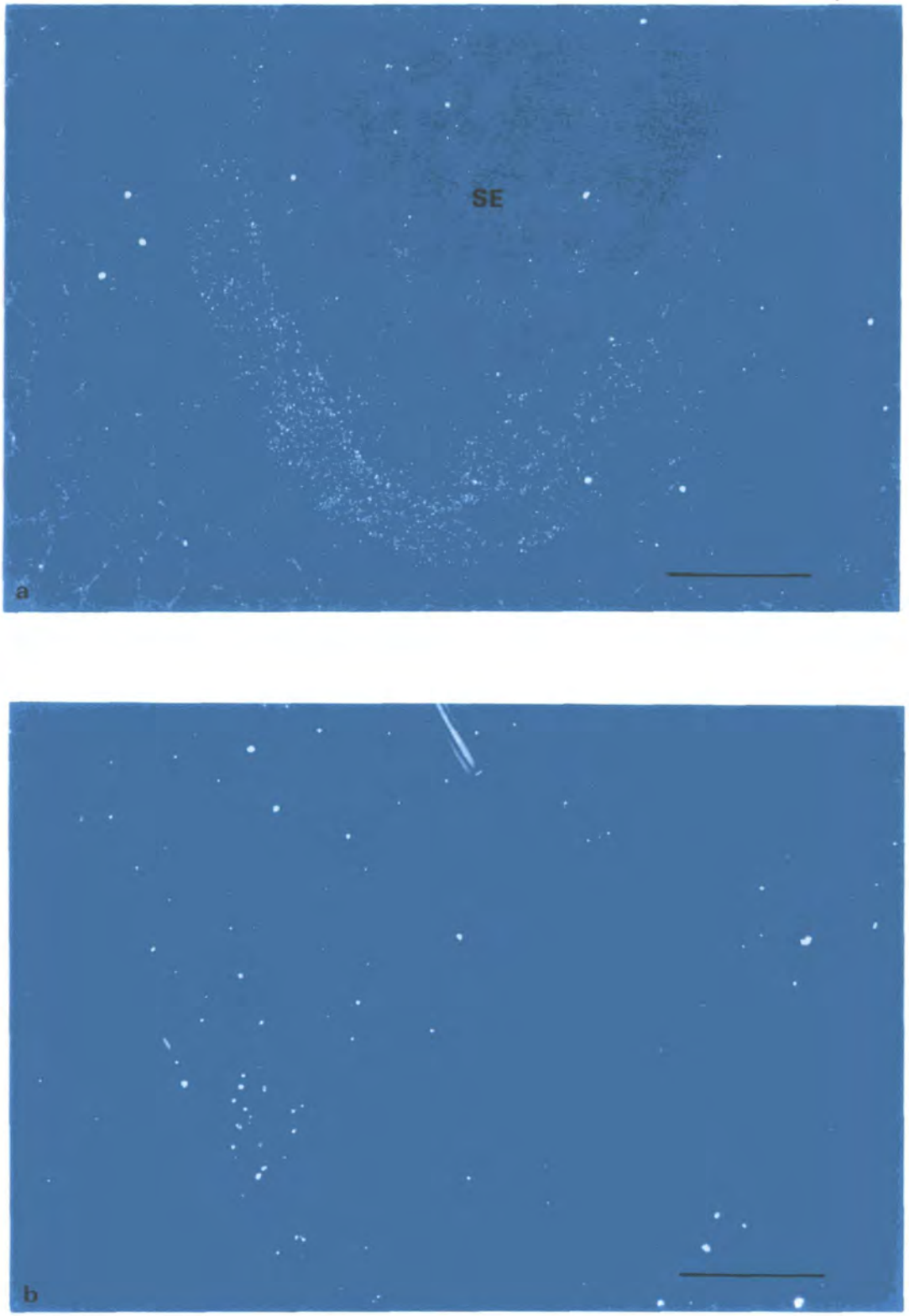


Plate 16. ***In-situ*** **hybridisation of digoxigenin UTP-11 labelled antisense riboprobe (a) and sense riboprobe (b) from clone 16D (putative CCoAOMT cDNA) to sections from a 10 DPA caryopsis, showing the distribution of hybridisation visualised with immunogold and silver enhancement.**

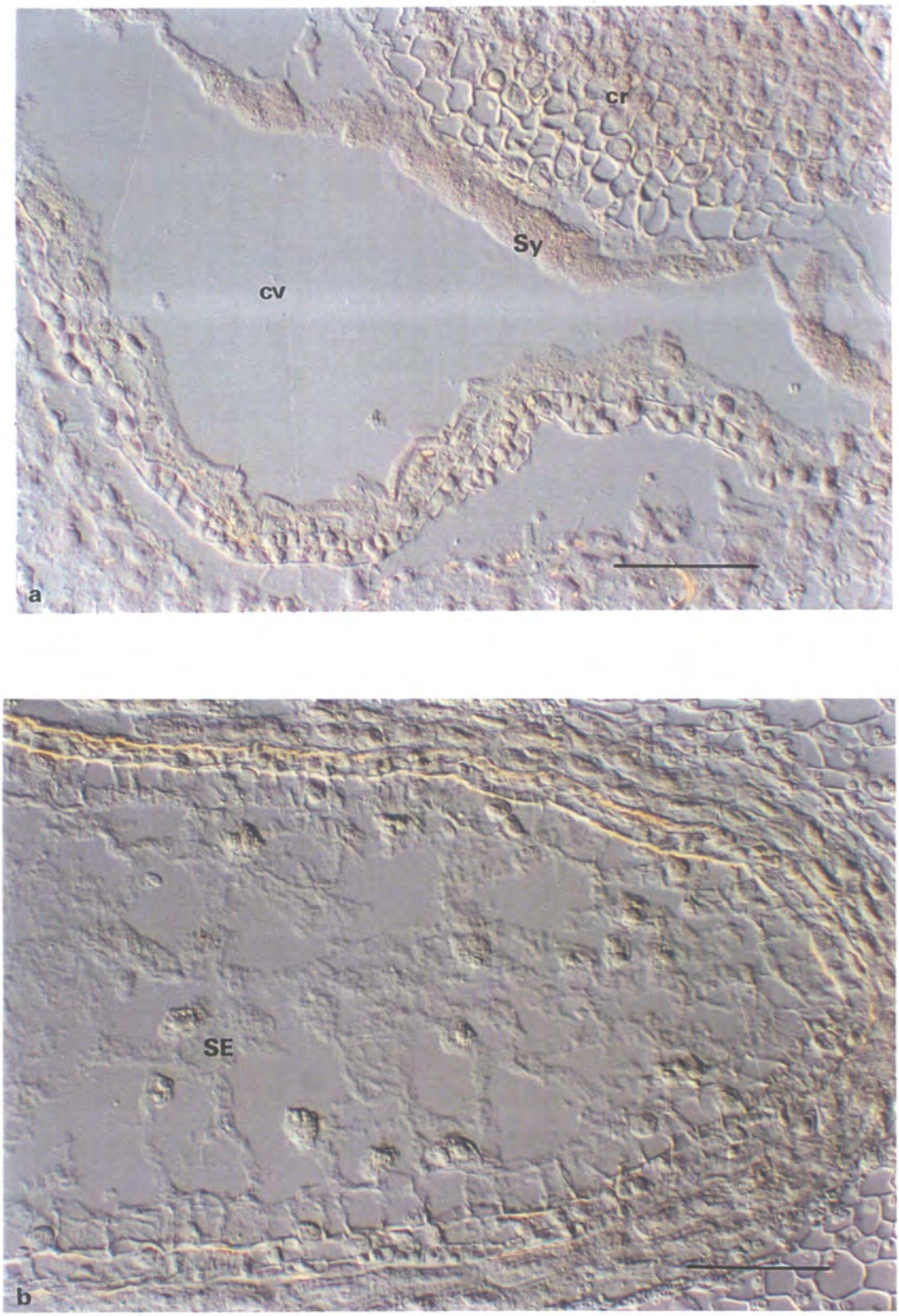
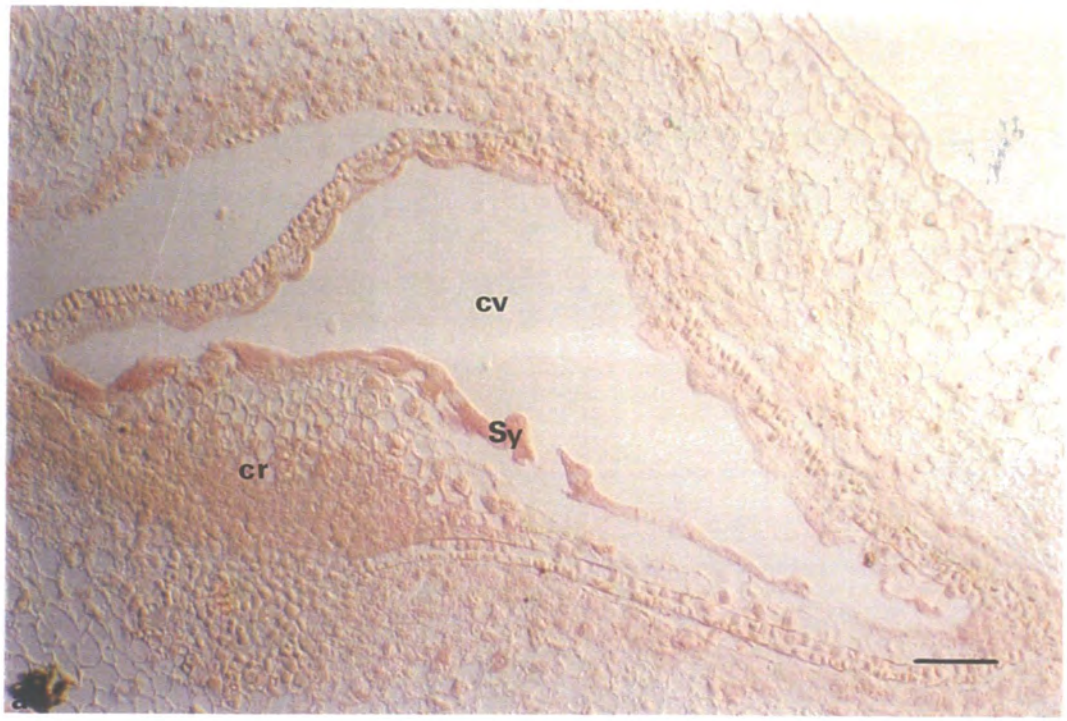


Plate 17. ***In-situ*** **hybridisation of digoxigenin UTP-11 labelled sense and antisense riboprobes from clone 3B (putative sucrose synthase cDNA) to sections of 3 and 10 DPA visualised using alkaline phosphatase enzymatic substrate precipitation.**

a. Antisense riboprobe hybridisation to 3 DPA caryopsis section.

b. Sense riboprobe hybridisation to 10 DPA caryopsis section.



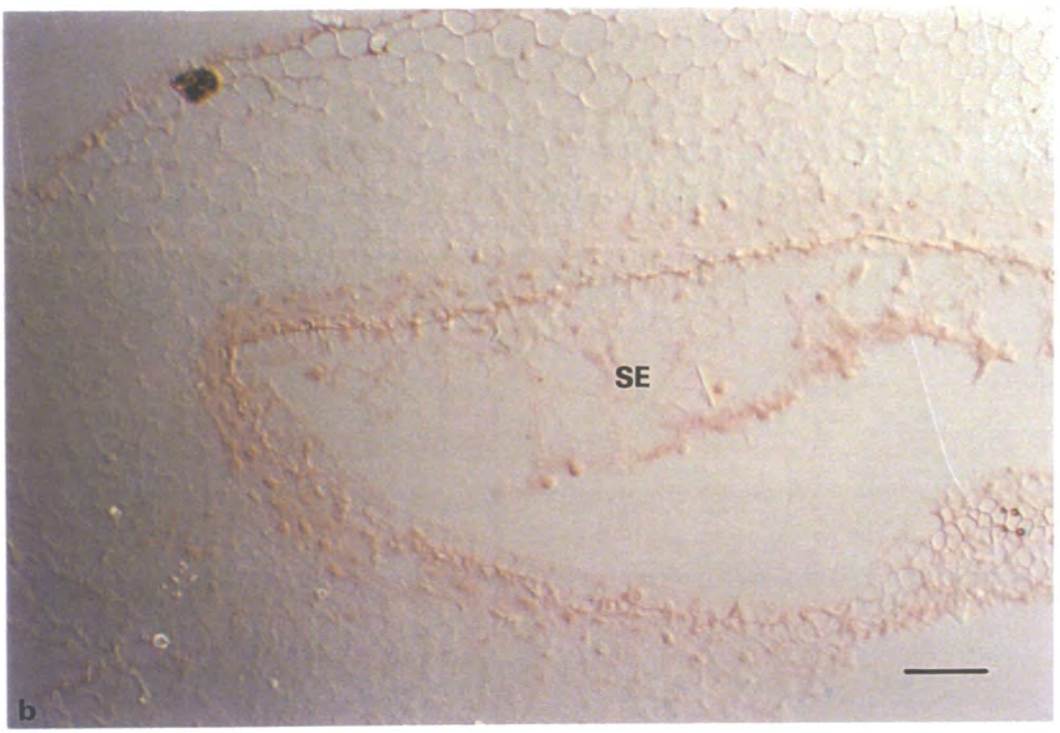


Plate 18. ***In-situ*** **hybridisation of digoxigenin UTP-11 labelled antisense riboprobe from clone 14B to sections of barley caryopses aged (a) 3 DPA and (b) 6 DPA, visualised using alkaline phosphatase enzymatic substrate precipitation.**

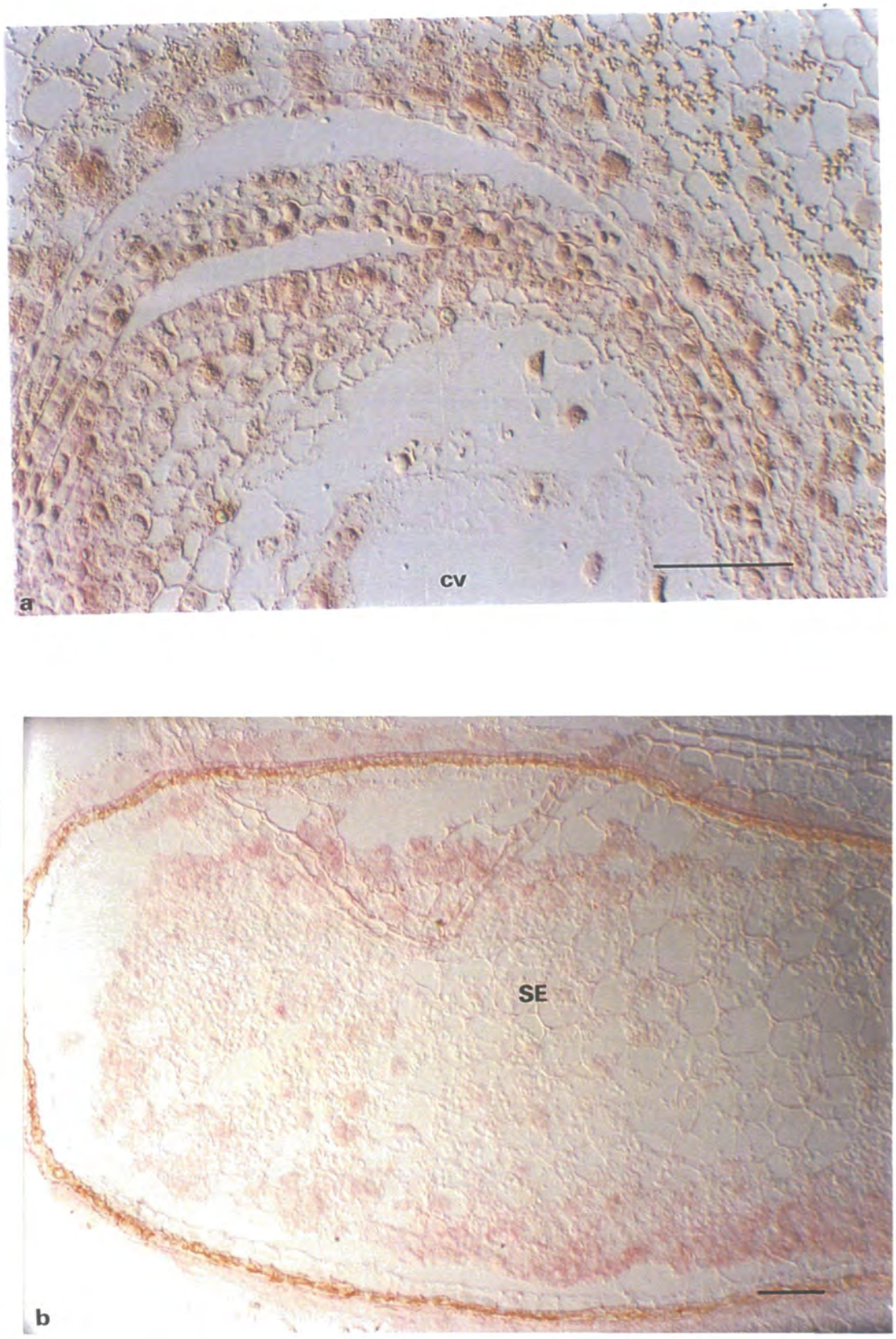
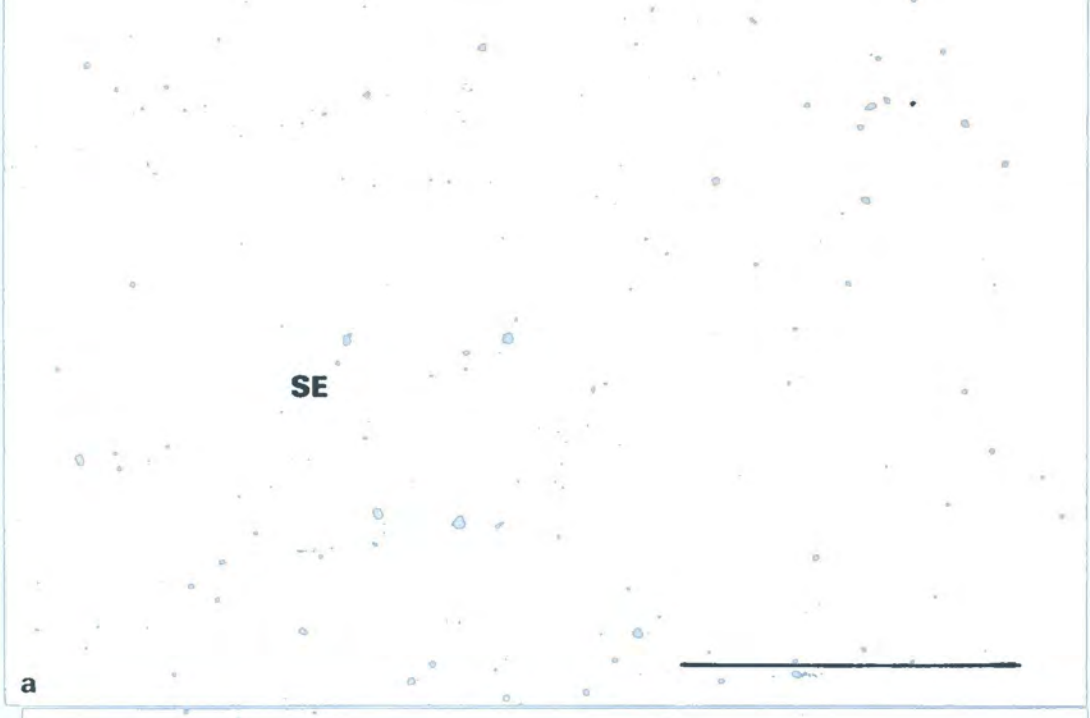


Plate 19. ***In-situ*** **hybridisation of digoxigenin UTP-11 labelled antisense riboprobe from clone 16C to sections of barley caryopses aged (a) 3 DPA and (b) 10 DPA, visualised using alkaline phosphatase enzymatic substrate precipitation.**

Plate 20. ***In-situ*** **hybridisation of digoxigenin UTP-11 labelled antisense riboprobe from clone 16B (putative blue copper binding protein cDNA) to sections of barley caryopses aged 6 DPA (a) wing region (b) crease region (c) wing-crease region, visualised by imunnogold and silver enhancement.**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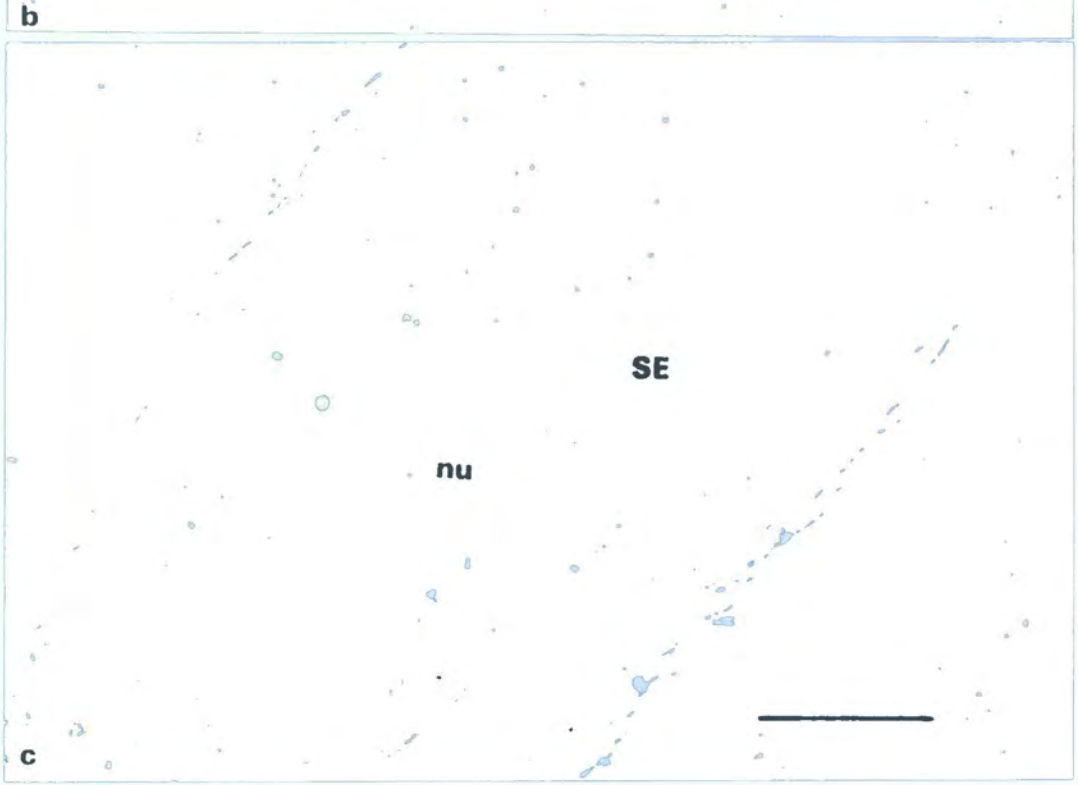
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Plate 21. ***In-situ*** **hybridisation of digoxigenin UTP-11 labelled antisense and sense riboprobes from clone 16B (putative blue copper binding protein cDNA) to sections of barley caryopses.**

a. Antisense riboprobe hybridisation to a 3 DPA caryopsis section (crease region)

b. Sense riboprobe hybridisation to a 6 DPA caryopsis section (crease region)

c. Sense riboprobe hybridisation to a 10 DPA caryopsis section (wing region)

b
c

Chapter 4

Final Discussion and Conclusions

The barley grain is an important commercial crop. To utilise the full potential of barley as a food source and in modern technological industries, including the malting and pharmaceutical industries, we must first have an understanding of the biological processes which occur during the grains' formation, especially the endosperm tissues.

The aim of this project was to investigate aspects of early endosperm development, which have previously been neglected, at the structural and molecular biological levels.

A study of the structural changes which take place during the early stages of endosperm formation was begun prior to the publications by Bosnes *et al.* (1992), Duffus and Cochrane (1993) and Olsen *et al.* (1995). The observations made here were similar to those recorded in these three papers and act as further evidence of the structural changes which occur during endosperm formation.

Over the past decade, a group of glycoproteins termed arabinogalactan proteins have been implicated in having important roles during development of a number of plant systems. Many of these roles, including, cell fate determination (Knox *et al.*, 1989, 1991; Stacey *et al.*, 1990; Pennell *et al.*, 1991; Smallwood *et al.*, 1994;), intercellular signalling (Knox *et al.*, 1989, 1991; Pennell and Roberts, 1990; Pennell *et al.*, 1991),

cell growth (Zhu *et al.,* 1993), cell expansion (Willats and Knox, 1996) and cell division (Serpe and Nothnagel, 1994) can theoretically be applied to aspects of endosperm development. In order to determine the possible presence AGPs and their patterns of expression during the early phases of barley endosperm development a set of monoclonal antibodies which recognise the carbohydrate epitope of AGPs were used in immunolocalisation experiments with sections of barley grains with endosperm at prominent stages of early development. From this it was found that at least one AGP epitope recognised by JIM13 was present in barley caryopses and was differentially expressed such that it was first observed at the cellularisation stage, localising to the first anticlinal cell walls and subsequently to the all the cell walls of the first endosperm cell plate and also to the nucellar/endosperm boundary, crease region cells, nuclei in the endosperm and to cytoplasmic aggregates. There was no indication of its presence during the syncytial stage of development. Due to time constraints and difficulty in experimental procedures it was not possible to determine the patterns of JIM13 localisation at subsequent stages of development. Further work has to be carried out to determine the full expression pattern of JIM 13 epitope during endosperm formation. Also the exact location of the JIM13 epitope at the cell surface should be determined using TEM (transmission electron microscopy) immunolocalisation experiments. The AGP epitopes recognised by the rest of the monoclonal antibodies used in immunolocation studies appeared not to be present during the syncytial and cellularisation stages of endosperm development at the light microscopical level. However, if they are present in small amounts it may be possible to detect them using TEM approaches. The work presented here on AGP expression during early endosperm development acts as an opening for further work in that it has shown

AGPs are present in endosperm tissues and that they are differentially expressed. The need now arises for work to be carried out to identify other AGP molecules which may be present and elucidate their patterns of expression and possible functions during endosperm formation. Further immunolocation studies could be carried out using a different set of AGP carbohydrate-epitope-recognising antibodies. Molecular biological methods (including *in-situ* hybridisation) could be employed, possibly using synthetic oligonucleotide probes designed from the recently cloned AGP protein cores (Du *et al.*, 1995 ; Chen *et al.*, 1994).

The second aim of the work presented here was to isolate from barley grains cDNAs which showed differential expression during the early phases of endosperm formation. Differential screening of a cDNA library synthesised from mRNA of whole caryopses ranging in age from 1-10 DPA, coupled with Northern blot hybridisation and dot blot experiments led to the isolation of a number of cDNA clones. Partial sequencing of selected clones resulted in identification of four cDNAs which showed identity to database entries at the protein sequence level using the FASTA computer program (Genetics Computer Group, 1991). A further four clones did not show identity to sequence database entries. Of those showing sequence similarity clone 3B was shown to be a putative sucrose synthase, 16D a putative CCoAOMT, 16B a putative blue copper binding proteins and 27B a putative KARI. Had time permitted further sequencing of these clones could have been carried out to obtain full length sequences and help solve ambiguities in some sequence readings. With additional time other clones could also have been sequenced. *In-situ* hybridisation carried out using the four clones detailed above and a further group which did not show database similarities revealed that none of the cDNAs sequenced

were uniquely expressed in the endosperm tissues. However, *in-situ* hybridisation experiments indicate that the expression of some of the genes represented by the cDNA clones might vary during the course of development of the caryopsis. Such suggestion is based on the variation in level of *in-situ* hybridisation signal at different developmental stages. These results must however be interpreted with caution since *in-situ* hybridisation is not a quantitative technique. Further work could be carried out to identify cDNAs in the library which are specific to the endosperm at various stages of development by isolating RNA from the endosperm tissues alone and utilising it in differential screening or differential display technique procedures. The delicate milky state of the central cell during the first 4-6 DPA made it extremely difficult to isolate intact tissues and obtain RNA in an undegraded state. PCR-reverse transcription methods would be required to make and amplify cDNA corresponding to mRNA expressed during these early stages of endosperm formation. Alternatively, synthetic oligonucleotide probes designed from known sequences of genes which are likely to be expressed differentially during endosperm formation (for example genes involved with cell cycle control, wall assembly and microtubule dynamics) could be used to probe the library and any interesting clones obtained subsequently used in *in-situ* hybridisation experiments.

Overall the work presented here shows that a combined structural and molecular biological approach can be used to study early endosperm development. However, problems encountered with the development of techniques throughout this period of post-graduate research led to time constraints and limited the extent of analysis which could be made.

The techniques used are still not routine, although the power of the approach suggests that they will become routine in due course, the required optimisations will ensure that the results will never be obtained easily.

Overall, the work presented here shows that the approaches used to study early endosperm development at the structural and molecular level worked well. However, problems encountered throughout led to time constraints and hence a more complete analysis could not be made.

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Appendix A

Buffers and Solutions

Blotto X25	5% Marvel in 0.02% sodium azide in water. Store at 4°C.
Denaturing Buffer (solution)	0.5M NaOH, 1.5M NaCl
Denhardts Reagent X50	1% (w/v) BSA 1% (w/v) ficoll 1% (w/v) PVP
DEPC water	add DEPC to a concentration of 0.1%. Shake vigorously and allow to stand for 8 hours at room temperature before autoclaving.
DNA Hybridisation Buffer	1X Blotto, 6X SSC, 1% Nonidet P40
DNA Loading Buffer X6	0.25% Bromophenol Blue 0.25% xylene cyanol FF 30 % Glycerol in water
Dot Blot Buffer for RNA	DEPC water/20XSSC/formaldehyde (37%) in the ratio 5/3/2
GuHCl Buffer (RNA extraction Buffer)	8M GuHCl, 20mM MES, 20mM EDTA, 50mM mercaptoethanol. Adjust to pH7.0 with NaOH.
Buffer 1	150mM NaCl, 100mM Tris pH7.5.
Buffer 2	100mM NaCl, 100mM Tris, 50mM $MgCl_2$, pH9.0. Note: pH Tris to pH9.0 before adding NaCl and $MgCl_2$
Luria Broth (per liter)	10g NaCl 10g tryptone 5g yeast extract Adjust to pH7.5 with 5N NaOH, add water to 1 liter and autoclave.
Luria Broth Agar plates (per liter)	same as luria broth plus 20g bactoagar.

MOPS X10	0.2M MOPS, 0.8M sodium acetate, 0.15M EDTA. Adjust to pH 7.0 with NaOH. Filter sterilise. Do not autoclave.
Neutralising Buffer	1.5M NaCl, 0.5M Tris HCl pH8.0
Northern Blot Transfer Buffer	10mM Na P, 1mM EDTA
Northern prehybridisation/ hybridisation Buffer	0.5M phosphate buffer (pH7.0), 7% SDS, 1mM EDTA
NZY Agar (per liter)	add 15g of agar to NZY broth, add water to 1 liter and autoclave
NZY Broth (per liter)	5g NaCl 2g $MgSO_4.7H_2O$ 5g yeast extract 10g caseine hydrolysate Adjust to pH7.5 with NaOH, add water to 1 liter and autoclave.
NZY Top Agarose (per liter)	same as NZY Broth plus 0.7% (w/v) agarose
PBS (Phosphate Buffered Saline)	120mM NaCl, 7mM Na_2HPO_4, 3mM NaH_2PO_4, 2.7 mM KCl
Phosphate Buffer (pH7.0)	Add 57.7ml 1M Na_2HPO_4 to 42.3ml 1M NaH_2PO_4
Plaque Lift Prewash Buffer	5xSSC, 0.5% SDS, 1mM EDTA
Proteinase K Buffer	100mM Tris, 5mM EDTA
SB Buffer (RNA loading Buffer)	12µl ethidium bromide 300µl 10X MOPS 80µl formaldehyde (37%) 300µl formamide
20% SDS	dissolve 20g SDS in 100ml distilled water
2X SDS-gel loading buffer	100mM tris.HCl pH 6.8, 200mM DTT, 4% SDS, 20% glycerol, 0.2% bromophenol blue. Add DTT just prior to use.

SM Buffer (per liter)	50mM Tris HCl pH7.5, 100mM NaCl, 8mM $MgSO_4$, 0.01% (w/v) gelatin
Solution I	50mM glucose, 25mM Tris pH8.0, 10mM EDTA
Solution II	0.2M NaOH, 1% SDS
Solution III	5M KOAc, 2M acetic acid
SSC X20 (Standard Saline Citrate Buffer)	3M NaCl, 0.3M sodium citrate
SSPE	3M NaCl, 0.2M NaH_2PO_4, 0.02M EDTA. Adjust to pH7.4 with NaOH. Autoclave.
TBE X10	0.9M tris-borate, 0.02M EDTA
TBS X10	1M tris/HCl, pH8.0, 1.5M NaCl pH with HCl
TE Buffer	10mM tris HCl pH8.0, 1mM EDTA
Tris-glycine electrophoresis buffer	25mM tris base, 250mM glycine pH 8.3, 0.1% SDS

Growth conditions for bacterial strains.

Bacterial strain	**plates for bacterial streak**	**media for glycerol stock**	**media for bacterial cultures for titering phage (final concentration)**
XL1-BlueMRF'	LB tetracycline 12.5ug/ml	LB tetracycline 12.5ug/ml	LB with 0.2% (v/v), maltose 10mM $MgSO_4$
SOLR strain	LB kanamycin 50ug/ml	LB Kanamycin 50ug/ml	LB without a supplement

Appendix B

List of Suppliers

Agar Scientific Ltd
66a Cambridge Road
Standsted
Essex
CM24 8DA

Amersham International plc
Amersham Place
Little Chalfont
Buckinghamshire
England
HP7 9NA

BDH-Merck Ltd.
Hunter Boulevard
Magna Park
Lutterworth
Leics
LE17 4XN

Boehringer Mannheim UK
Diagnostics and Biochemicals Ltd.
Bell Lane
Lewes
East Sussex
BN7 1LG

NBL-Gene Sciences Ltd.
South Nelson Road
Cramlington
Northumberland
NE23 9WF

Promega
Delta House
Enterprise Road
Chilworth Research Center
Southampton
S016 7NS

Qiagen Ltd.
Boundary Court
Gatwick Road
Crawly
West Sussex
RH10 2AX

Sigma-Aldrich Company Ltd.
Fancy Road
Poole
Dorset
BH12 4QH

Stratagene Ltd.
Cambridge Innovation Center
Cambridge Science Park
Milton Road
Cambridge
CB4 4GF

Gibco BRL
Life Technologies Ltd
3 Fountain Drive
Inchinnan Business Park
Paisley
PA4 9RF