LOCATION AND ENZYME ACTIVITY OF THE KEY CELL CYCLE PROTEIN p34^{cd2} IN MAIZE

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ABSTRACT

An antibody specific for maize p34\textsuperscript{cdc2} was generated and used to investigate the abundance and activity of the key cell cycle protein p34\textsuperscript{cdc2} independently of any closely related protein kinases. An assessment of whether p34\textsuperscript{cdc2} localised with the microtubular cytoskeleton in maize root tip cells using the anti-p34\textsuperscript{cdc2} antibody was also performed.

The antibody was raised against a 17 amino acid sequence from the carboxyterminus of a p34\textsuperscript{cdc2} homologue isolated from maize by Colasanti et al. (1991). This region was chosen for its difference between cdk variants which could allow a p34\textsuperscript{cdc2} specific antibody to be generated. Serum from the rabbit, injected with the p34\textsuperscript{cdc2} peptide conjugated to carrier, detected a protein at 34 kDa. The binding of this protein was eliminated when the serum was pre-competited with p34\textsuperscript{cdc2} peptide used as an antigen. To increase the specificity of the antibody for p34\textsuperscript{cdc2} it was purified by protein-A chromatography, DEAE ion chromatography and affinity purification using an antigen column.

Using the affinity purified antibody the capability of the antibody to detect denatured and native p34\textsuperscript{cdc2} protein in total protein, soluble protein and insoluble protein fractions was examined by Western dot blot analysis. The antibody consistently identified both denatured and native p34\textsuperscript{cdc2} protein from meristem and elongation zone tissue. The capacity of the antibody to detect p34\textsuperscript{cdc2} protein in its native form prompted its exploitation for immunorecovery of p34\textsuperscript{cdc2} using antibody bound to protein-A beads or immobilised on CNBr-Sepharose. Recovery was eliminated by pre-competition.

The capacity of the anti-p34\textsuperscript{cdc2} antibody to immunorecover native p34\textsuperscript{cdc2} protein provided the opportunity for the first time to investigate the distribution and activity in plant tissue of p34\textsuperscript{cdc2} independent of other cyclin dependent kinases that may be present. The p34\textsuperscript{cdc2} antibody was used to probe Western blots of maize proteins taken from sequential segments cut along the length of the developing maize leaf. The level of p34\textsuperscript{cdc2} protein was found to be highest at the base of the blade, corresponding to the region of actively dividing cells. The transition of dividing to differentiating cells in the leaf was accompanied by a 16 fold decline in p34\textsuperscript{cdc2} abundance in the total protein fraction suggesting that p34\textsuperscript{cdc2} may have a role in the regulation of cell division.
Likewise the activity of both p34\(^{cd2}\)-like and p34\(^{cd2}\) protein kinases was found to be highest in the region associated with cell division and then to decline steeply. Comparison of p34\(^{cd2}\) activity with total soluble protein kinase activity in regions of the leaf demonstrated that p34\(^{cd2}\) activity is regulated independently of the majority of leaf protein kinases, as while p34\(^{cd2}\) activity declined seven fold in cells leaving the basal 10 mm of the leaf, total soluble protein kinases declined only two fold in the same region. Investigation of p34\(^{cd2}\) abundance and activity in maize root meristem and elongation zone tissue supported the correlation with cell division. In both the developing maize leaf and root a greater amount of p34\(^{cd2}\) in the insoluble protein fractions was observed in dividing rather than non-dividing cells. The greater abundance of p34\(^{cd2}\) in association with the structural fraction of dividing rather than non-dividing cells was confirmed by Western dot blots of denatured and native protein. These data suggested that p34\(^{cd2}\) protein may be associated with the cytoskeleton in meristematic tissue.

Prior to an investigation of the localisations of p34\(^{cd2}\) protein in relation to the microtubular cytoskeleton, the immunofluorescence method was optimised for detection of the p34\(^{cd2}\) protein by the antibody. Consistently the best p34\(^{cd2}\) and microtubule labelling was observed when cells were fixed with 4% paraformaldehyde and the cell walls digested before exposure to a denaturing agent such as saponin, picric acid, NP40 or to pH 4.0, or pH 10.0. When p34\(^{cd2}\) antibody, in conjunction with an anti-\(\beta\) tubulin antibody, was used to label fixed maize root tip cells that had been treated with a denaturing agent after cell wall digestion, the p34\(^{cd2}\) protein was observed to be localised to the nucleus during interphase. Its localisation was still predominantly nuclear in early prophase, however a band of p34\(^{cd2}\) protein that encircled the cell was also detected in late prophase cells characterised by condensed chromatin. When cells were double labelled with p34\(^{cd2}\) and tubulin antibodies this band of p34\(^{cd2}\) protein was seen to exactly correspond to the microtubule pre-prophase band which predicts the site of cell division in plants. Association of p34\(^{cd2}\)-like and p34\(^{cd2}\) protein with the pre-prophase band in plants has been reported previously, and was confirmed in this study, using an independently raised specific maize p34\(^{cd2}\) antibody (Colasanti et al., 1993). It is proposed that the association of p34\(^{cd2}\) with the pre-prophase band in late prophase indicates that p34\(^{cd2}\) may have a role in pre-prophase band disassembly.
Association of p34\textsuperscript{cdc2} to the region of the mitotic spindle was also observed in this study; a location not previously reported in plants. However in animal cells p34\textsuperscript{cdc2} has commonly been observed to associate with the mitotic apparatus leading to proposals that p34\textsuperscript{cdc2} may have a role in the function and regulation of the mitotic apparatus. Additionally, a localisation of p34\textsuperscript{cdc2} to the phragmoplast was also observed, however this may possibly be explained by the diffusion of the inactive soluble protein in late mitosis to an area within the cell which is characterised by a greater accessible volume due to the exclusion of organelles by the phragmoplast microtubules in this region. As calmodulin has also been reported to associate with the pre-prophase band, spindle and phragmoplast the possibility of p34\textsuperscript{cdc2} participating with other proteins in the regulation of both mitosis and cytokinesis in higher plants is discussed.
GENETIC ABBREVIATIONS

I. For all genes (except those deriving from human/mammalian systems) italic characters are used.
   eg, cdc2 is the cell division cycle gene identified in fission yeast. The equivalent gene in budding yeast is CDC28. NOTE: cdc2/CDC28 is named as CDK1 by some authors.
   Mutant forms of these genes are indicated in fission yeast by superscripts of lowercase eg cdc2' signifies a mutation of cdc2. In budding yeast the mutant form is denoted by giving its name in lowercase eg cdc28.

II. Gene products are described as p34<sup>cdc2</sup> (signifying both the gene and the size of the protein in kDa) or Cdc2 (differing from the gene name by the initial capital and non-italic form). The equivalent forms in budding yeast are p34<sup>CDC28</sup> or Cdc28

III. Families of proteins are denoted by uppercase, eg, CDK for cyclin dependent kinase, or CKI for cyclin dependent kinase inhibitor.

IV. Generic descriptions of widely conserved genes are lowercase and non-italicised, eg, cdc2.

ABBREVIATIONS

Standard SI units and abbreviations are used throughout this thesis. Non-standard abbreviations used include the following:

- ATP: adenosine triphosphate
- BCIP: 5-bromo-4-chloro-3-indolyphosphate p-toludine salt
- BSA: bovine serum albumen
- CAK: cdk or p34<sup>cdc2</sup> activating kinase
- CAKAK: CAK- activating kinase
- CaM: calmodulin
- c-AMP: cyclic adenosine monophosphate
- cDNA: copy deoxyribonucleic acid
- cdc: cell division cycle
- CDI: cdk inhibitory protein
- CDK: cyclin dependent kinase
- CKI: cdk inhibitory protein
- CNBr: cyanogen bromide
- CTAC: cetyl trimethyl ammonium chloride
- DAPI: 4, 6-diamidino-2-phenylindole
- DEAE: dimethylaminoethyl
- DIC: differential interference contrast
- DMF: dimethyl formamide
- DMSO: dimethyl sulfoxide
- DNA: deoxyribonucleic acid
- DPM: disintegrations per minute
- DTT: dithiothreitol
- ECL: enhanced chemiluminescent detection
- EDTA: ethylenediaminetetraacetic acid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>EGTA</td>
<td>ethylene glycol bis (β-aminoethylether N, N, N', N'-tetraacetic acid)</td>
</tr>
<tr>
<td>GA</td>
<td>glutaraldehyde</td>
</tr>
<tr>
<td>gfw</td>
<td>gram fresh weight</td>
</tr>
<tr>
<td>GUS</td>
<td>β-glucuronidase analysis</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>HBK</td>
<td>Hepes buffered KCl</td>
</tr>
<tr>
<td>HDW</td>
<td>high detergent wash</td>
</tr>
<tr>
<td>HEPES</td>
<td>N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]</td>
</tr>
<tr>
<td>HMD</td>
<td>buffer containing Hepes, MgCl₂, DTT</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-B-D-thiogalactoside</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>KLH</td>
<td>keyhole limpet haemocyanin</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani medium</td>
</tr>
<tr>
<td>MAP</td>
<td>microtubule-associated protein</td>
</tr>
<tr>
<td>MHS</td>
<td>succinimidyl 6-(N-maleimido)-n-hexanoate</td>
</tr>
<tr>
<td>MPF</td>
<td>maturation or M-phase promotion factor</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>MSH</td>
<td>2-mercaptoethanol</td>
</tr>
<tr>
<td>MT</td>
<td>microtubule</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>NBT</td>
<td>nitroblue tetrazolium chloride</td>
</tr>
<tr>
<td>NDE</td>
<td>no detergent extraction</td>
</tr>
<tr>
<td>NLS</td>
<td>nuclear localisation signal</td>
</tr>
<tr>
<td>NP40</td>
<td>NONIDET P-40, non-ionic detergent</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycol</td>
</tr>
<tr>
<td>PFA</td>
<td>paraformaldehyde</td>
</tr>
<tr>
<td>PMD</td>
<td>PBS buffer supplemented with MgCl₂ and DTT</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PPB</td>
<td>preprophase band</td>
</tr>
<tr>
<td>PPD</td>
<td>p-phenylenediamine</td>
</tr>
<tr>
<td>pRb</td>
<td>retinoblastoma protein</td>
</tr>
<tr>
<td>PSTAIR</td>
<td>EGVPSTAIREISLLKE amino acid residues</td>
</tr>
<tr>
<td>RIPA</td>
<td>multi-component protein extraction buffer</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TBSBT</td>
<td>Tris buffered saline containing BSA and Tween</td>
</tr>
<tr>
<td>TCA</td>
<td>trichloroacetic acid</td>
</tr>
<tr>
<td>TEMED</td>
<td>N, N, N', N'-tetramethyl ethylene diamine</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>Tween 20</td>
<td>polyoxyethylenesorbitan monolaurate</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
</tbody>
</table>
CHEMICALS AND MATERIALS

General laboratory chemicals and materials used in this thesis were supplied by AJAX Chemicals, Sydney, Australia or BDH Chemicals, Victoria, Australia unless otherwise specified below.

Cellulysin (cellulase) was purchased from Calbiochem-Novabiochem, USA.

Chemiluminescent substrate solution was supplied by Amersham, International, UK.

CNBr-Sepharose beads were purchased from Pharmacia-Biotech, Sweden.

DEAE-Sepharose was supplied by Pharmacia-Biotech, Sweden.

Microdye binding assay reagent was purchased from Biorad Laboratories, Australia.

Micro ultrafiltration system was supplied by Amicon, USA.

Protein-A column (Protein-A MemSep 1000 Cartridge) was supplied by Millipore, USA.

Protein-A-Sepharose 4B was supplied by Sigma Chemical Company, USA.

Radioactive isotopes (\(^{32}\)P ATP and \(^{125}\)I anti-rabbit-IgG) were purchased from Bresatec, Australia and Amersham International, UK.
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CHAPTER 1:

GENERAL INTRODUCTION
1.1 Introduction

Recent research on eukaryotes has indicated not only conserved mechanisms in the replication and segregation of genetic material, but also the widespread retention of genes that regulate the cell division cycle (Nurse, 1990; Jacobs, 1992; Norbury and Nurse, 1992; Francis and Halford, 1995). Briefly, the eukaryotic cell cycle consists of four sequential phases: G₁, a prereplicative period, in which growth of the cell and its organelles can be extensive prior to the; S phase, in which DNA replication occurs; G₂, the time preceding nuclear division; and M the mitotic phase when cell division occurs. Regulation of the temporal order of these events ensures that DNA replication and mitosis occur separately and retain the integrity of the genetic information.

Genetic investigation of the cell division cycle in eukaryotes has been performed largely in Schizosaccharomyces pombe (fission yeast) and Saccharomyces cerevisiae (budding yeast). Fission and budding yeasts have the advantages of short generation times and existence of stable haploid and diploid forms, enabling the isolation of cell division cycle (cdc) conditional mutants (Hartwell et al., 1970; 1973; 1974; Hartwell, 1974, 1991; Nurse, 1975; Nurse et al., 1976; Nurse and Thuriaux, 1980; Fantes, 1981; Nasmyth and Nurse, 1981). Two distinct control points during the cell cycle, responsible for coordinating growth and cell division, were identified at the G₁-S and G₂-M transitions (Nurse and Bisset, 1981; Feilotter et al., 1992; Rowley et al., 1992). The late G₁ regulatory step was designated as START (Hartwell, 1974) and one of the early START conditional mutants identified in budding yeast was cdc28-1, which remained capable of growth while unable to enter the division sequence of S, G₂ and M events at the restrictive temperature (Reid and Hartwell, 1977). Furthermore, the CDC28 gene was found to also have a G₂-M regulating function (Piggot et al., 1982). Subsequently many other cdc START mutations were isolated in budding yeast and their involvement in mating factor and the cAMP-mediated signal transduction pathways has underlined the fact that this control point is a major branch point in the life cycle, regulating alternatives of mating or proliferation (Reed, 1980; Bedard et al. 1981; Prendergast et al., 1990 in Rowley et al., 1992). In fission yeast the cdc2 gene was originally defined as causing G₂ arrest when mutated (Nurse and Thuriaux, 1980).
but soon found to also have a G1 role (Nurse and Bisset, 1981). The discovery by Beach et al. (1982) that the cdc2/CDC28 genes were functional homologues, which both functioned at G1-S and G2-M control points suggested that the cdc2/CDC28 gene was a primary regulator of the cell cycle. The long evolutionary separation of the two yeasts pointed dramatically to widely conserved cell cycle mechanisms. This has led to an investigation of the role of cdc2/CDC28 and the mechanisms responsible for its regulation not only in yeast but in diverse eukaryotes including higher plants that still continues today. The present thesis considers the location and activity of the Cdc2 gene product in plant cells and tissues.

1.2 cdc2/CDC28 genes

1.2.1 cdc2/CDC28 genes in yeast

The Cdc2 and Cdc28 protein of 34 kDa was identified as a serine-threonine kinase (Simanis and Nurse, 1986). This p34\textsuperscript{cdc2} protein kinase was found to be regulated by phosphorylation (Simanis and Nurse, 1986; Gould and Nurse, 1989) and to be independent of calcium and cAMP for H1 kinase activity, which peaked at mitosis in the cell cycle (Russell and Nurse, 1987a; Draetta, 1990; Nurse, 1990). In both budding and fission yeast, cdc2 was found to be essential for cell cycle progression through the G1-S and G2-M transitions, as loss of function of cdc2/CDC28 resulted in cell cycle arrest in either G1 or G2 (Nurse and Bisset, 1981; Pringle and Hartwell, 1981; Piggot et al., 1982; Nurse, 1985; Reed and Wittenberg, 1990). Additionally, examination of conditional cdc2 mutants, which accelerated M phase entry, revealed that cdc2 is normally rate limiting (Nurse and Thuriaux, 1980). Budding and fission yeast probably separated in evolution at least 1,000 million years ago, since they possess less than 70% homology of 5S RNA and cytochrome C sequences (Mao et al., 1982; Russell and Hall, 1982). That both yeasts share a key enzyme governing cell cycle progression suggested that control mechanisms were highly conserved. It was considered that if this were indeed the case, then homologues of these cell cycle regulatory genes should also be present in diverse other metazoans.
1.2.2 \textit{cdc2}/\textit{CDC28} homologues

Homologues to the \textit{cdc2}/\textit{CDC28} genes have subsequently been isolated from diverse species including humans (Lee and Nurse, 1987; Draetta et al., 1987), \textit{Xenopus} (Dunphy et al., 1988; Gautier et al., 1988), starfish (Arion et al., 1988; Labbe et al., 1988), mouse (Lee et al., 1988; Morla et al., 1989), rat (Draetta et al., 1988a; Akhurst et al., 1989), sea urchin (Meijer et al., 1989), clam (Draetta et al., 1989), chicken (Krek and Nigg, 1989) and more recently in higher plants, \textit{Zea mays} (Colasanti et al., 1991), \textit{Arabidopsis} (Ferreira et al., 1991), alfalfa (Hirt et al., 1991), \textit{Petunia} (Bergouninoux et al., 1992), mothbean (Hong et al., 1993) and \textit{Glycine max} (Miao et al., 1993). Not only do \textit{cdc2}/\textit{CDC28} homologues contain highly conserved regions in common with other protein kinases that are involved in ATP binding and phosphorylation, but in addition regions specific to the \textit{cdc2}/\textit{CDC28} gene have been identified. The largest of these, the EGVPSTAIREISLLKE (Glu Gly Val Pro Ser Thr Ala Ile Arg Glu Ile Ser Leu Leu Lys Glu) sequence, commonly abbreviated to PSTAIR (Lee and Nurse, 1987), has been found perfectly conserved in all functional homologues sequenced so far. This universal conservation of the \textit{cdc2}/\textit{CDC28} gene has led to a proposal that the p34\textit{cdc2}-based mechanisms of a common ancestral eukaryote were inherited by all eukaryotes (John et al., 1989; Norbury and Nurse, 1992).

1.3 \textbf{Mechanisms that can influence the activity of the Cdc2 gene product}

1.3.1 \textbf{Introduction}

The p34\textit{cdc2} protein kinase is inactive as a monomer and acquisition of enzyme activity by this protein and its close variants is mediated by several mechanisms: 1) physical association with other proteins termed cyclins (because association with cyclins is necessary for their activity, p34\textit{cdc2} and related proteins have been termed \textit{cyclin} dependent \textit{kinases} (CDKs)), 2) posttranslational phosphorylation (reviewed by Nurse, 1990), 3) activity of CDKs can also be influenced by transient association with \textit{cyclin} dependent \textit{kinase} inhibitor proteins (CKI or CDI), which have been found to bind to and inhibit CDK at G1/S phase in animal cells, 4) another class of CDK-binding
protein is p13\textsuperscript{SucI} (or cyclin dependent kinase subunit, CKS) that is necessary for inactivation of p34\textsuperscript{cdc2} at anaphase and for completion of mitosis. p13\textsuperscript{SucI} protein was used in the work described here for affinity purification of p34\textsuperscript{cdc2}.

This introduction will survey: 1) the significance of associations with cyclins, 2) the importance of changes in phosphorylation of p34\textsuperscript{cdc2}, 3) the existence of variants of p34\textsuperscript{cdc2} in higher eukaryotes and their control, in part by CKI proteins and 4) the contribution of CKS proteins will be outlined.

1.3.2 Cyclins

Cyclins were initially isolated as a class of proteins seen to oscillate in level in synchrony with cell division, in the large oocytes of lower animals. The cyclin protein level peaked at G\textsubscript{2}-M and then underwent rapid and complete proteolytic destruction by the end of M-phase (Evans et al., 1983; Swenson et al., 1986; Standart et al., 1987; Hunt, 1989). The following observations led to the suggestion that cyclins may have a role in the periodic activation of MPF (maturation or M-phase promotion factor): 1) cyclin synthesis is essential for mitotic initiation (Minshull et al., 1989), 2) its destruction is necessary for exit from mitosis (Picard et al., 1985; Murray et al., 1989), 3) MPF activation is concurrent with cyclin accumulation, and lastly that 4) cyclin degradation is required for a decline in MPF activity (Picard et al., 1985). Indeed a single cyclin, specifically a cyclin B subunit, and p34\textsuperscript{cdc2} protein kinase were identified as the fundamental components of MPF in both \textit{Xenopus} (Lohka et al., 1988) and starfish (Labbe et al., 1989a, b). The inhibition of MPF activity has been induced by the destruction of cyclin mRNA in \textit{Xenopus} extracts (Minshull et al., 1989; Murray and Kirschner, 1989). Furthermore, Moreno et al. (1989) showed that in fission yeast deletion of the mitotic cyclin gene \textit{cdc13} prevented p34\textsuperscript{cdc2} activation at mitosis. The significance of MPF is demonstrated by the observation that when the complex isolated from metaphase arrested \textit{Xenopus} oocytes is injected into immature oocytes, entry into M-phase is induced (Masui and Markert, 1971). The MPF complex has subsequently been identified in diverse eukaryotes (Francis and Halford, 1995) and its function as the regulator of the G\textsubscript{2}-M transition is widely indicated (Nurse, 1990).
The first cyclins to be identified were those that are commonly termed mitotic cyclins, as they have a role in the G2-M transition. Several families of cyclins have been isolated, and designated as early G1, late G1, mitotic A and mitotic B cyclins. Despite the division of the cyclins into these distinct classes, which have only minimal overall sequence homology to each other (Xiong and Beach 1991; Pines, 1992), all cyclins isolated to date contain a homologous amino acid sequence of approximately 100-200 residues, designated the “cyclin box” (Nugent et al., 1991). It is this region of the cyclin subunit that binds to the CDK partner, so activating its kinase activity. De Bondt et al. (1993) predicted that cyclin binding would cause a change in the conformation of the complexed CDK. Recently Jeffrey et al. (1995) have confirmed these predictions. In addition, all cyclins regardless of their class and differences in structure and function are inactivated and removed from the cell via the same mechanisms, a ubiquitin-dependent proteolysis system (Glotzer et al., 1991; Hershko et al., 1991). That cyclins are a fundamental element required for cell cycle progression is suggested by: the universal presence of both G1 and mitotic cyclins across diverse species and the high degree of conservation of the role they play in regulation of cell division in conjunction with the p34\(^{cd2}\) protein kinase. Indeed it is possible that the temporal order of cell cycle events is maintained by the sequential presence of different classes of cyclin directing their CDK partners to phosphorylate a specific set of substrates (Heichman and Roberts, 1994).

Mitotic A and B cyclins have been isolated in diverse higher eukaryotes (Evans et al., 1983; Lehner and O'Farrell, 1989; Pines and Hunter, 1989), and also in fission yeast (cdc13\(^+\)) (Booher and Beach, 1988) and budding yeast (CLB1-4) (Surana et al., 1991). The mitotic cyclins in metazoans share several functional similarities; addition of either A or B-type cyclin will induce oocyte maturation in Xenopus (Swenson et al., 1986; Westendorf et al., 1989), both will initiate DNA replication in G1 cell extracts (D'Urso et al., 1990) and they also phosphorylate histones H1 and H2B on identical residues (Minshull et al., 1990; Parker et al., 1991). Despite these similarities, cyclin A and B appear to perform distinct functions in the regulation of cell division.

Cyclin A is found in association with both p34\(^{cd2}\) and the variant of p34\(^{cd2}\), p33\(^{CDK2}\), which is found in animal cells as described more fully below (1.3.4) (Clarke et al., 1992; Kobayashi et al., 1992). The cyclin A/Cdc2 complex is activated prior to the
cyclin B/Cdc2 kinase complex, perhaps because of less efficient Tyr$^{15}$ phosphorylation (Clarke et al., 1992). As a result it has been suggested to function as a starter kinase, regulating early mitotic progression and the activation of the cyclin B/Cdc2 complex (Minshull et al., 1990; Buendia et al., 1991). The cyclin A/Cdk2 complex, in contrast, appears to have a role in the progression of S phase. Not only does injection of cyclin A antibodies or antisense plasmids during G$_{1}$ inhibit S phase entry (Girard et al., 1991; Walker and Maller, 1991; Pagano et al., 1992a; Zindy et al., 1992) but the activity of the cyclin A/Cdc2 complex first detected at the G$_{1}$/S transition is observed to increase during S phase and G$_{2}$ then decline at the onset of metaphase (Minshull et al., 1990; Rosenblatt et al., 1992).

Three B-type cyclins, B$_{1}$, B$_{2}$ and B$_{3}$, have been described in multicellular organisms (Minshull et al., 1989; Gallant and Nigg, 1992, 1994; Jackman et al., 1995). All three share a similar pattern of expression during the cell cycle. Initially their levels are very low in the G$_{1}$ phase, increasing in the S and G$_{2}$ phases to a peak at mitosis which is followed by abrupt proteolysis in anaphase (Gallant and Nigg, 1994; Jackman et al., 1995). Despite this uniformity, multiple B cyclins appear not to be redundant and recent histological evidence suggests they associate with different subcellular structures during mitosis in animal cells (Jackman et al., 1995).

In addition to the mitotic A and B cyclins, cyclins involved in the regulation of the cell cycle at the G$_{1}$-S transition have also been isolated. These G$_{1}$ cyclins have been demonstrated to be a rate limiting factor for passage through START in yeast (Reed, 1991) and through the restriction point in eukaryotes, since higher expression levels or reduced proteolysis both lead to shorter G$_{1}$ phase (Ohtsubo and Roberts, 1993; Quelle et al., 1993; Resnitzky et al., 1994). The first G$_{1}$ cyclins to be identified were the three CLN genes in S. cerevisiae (Carter and Sudbery, 1980; Sudbery et al., 1980; Cross, 1988; Nash et al., 1988; Hadwiger et al., 1989b; Richardson et al., 1989). S. cerevisiae also encodes two additional genes, CLB5 and CLB6, that are classed as B type cyclins, yet they appear to have a role in the regulation of S phase (Schwob and Nasmyth, 1993). Neither of these genes appears to be necessary as double mutations are not lethal and merely result in a delay in the start of S phase (Schwob and Nasmyth, 1993). Transcription of the CLB5 and CLB6 genes has been linked to the prior activation of the Cln G$_{1}$ cyclins, so establishing a dependence of the Clb5, Clb6 S phase signal upon
A range of G1 cyclins has also been isolated in higher eukaryotes. These have been designated as cyclin C (Lew et al., 1991), cyclins D1, D2 and D3 (Lew et al., 1991; Matsushime et al., 1991; Motokura et al., 1991; Withers et al., 1991; Xiong et al., 1991), cyclin E (Koff et al., 1991), cyclin G (Tamura et al., 1993) and cyclin I (Nakamura et al., 1995).

A new class of cyclin, merging G1 and mitotic characteristics, has also been identified. The sole current representative is cyclin F, isolated in humans by Bai et al. (1994). It appears most closely related to cyclin A in sequence and expression, peaking in G2 and decreasing prior to cyclin B decline. However cyclin F possesses a PEST-rich C-terminus, a defining characteristic of G1 cyclins. Bai et al. (1994) conclude that cyclin F is most likely to have a role in coordinating events during the S and G2 phases which are not mediated by cyclins A and B.

In mammalian cells many G1 cyclins associate with variants of the p34cdc2 kinase that are active in G1 and S phase, as will be described in section 1.3.4.

1.3.3 Control of p34cdc2 activity by phosphorylation

Modifications necessary to fully activate the Cdc2/cyclin complex include a series of activating and inhibitory phosphorylations. Phosphorylation of Tyr15 in S. pombe p34cdc2 was found to decline at mitosis. Mutation of Tyr15 to a non-phosphorylatable phenylalanine residue was also observed to cause cells to prematurely enter mitosis, suggesting that the phosphorylation of this residue was crucial for keeping p34cdc2 inactive prior to mitosis (Gould and Nurse, 1989). In higher eukaryotes an additional negative regulatory phosphorylation site, Thr14, has been identified (Krek and Nigg, 1991a; Norbury et al., 1991; Solomon et al., 1992); dephosphorylation of both Tyr15 and Thr14 at the G2-M transition is required for full activation of the p34cdc2 protein kinase (Krek and Nigg, 1991a, b; Norbury et al., 1991; Solomon et al., 1992). These inhibitory phosphorylations are controlled by the opposing actions of protein kinase and phosphatase.

Protein kinases specific for p34cdc2 are responsible for the phosphorylation of Tyr15 and in mammals Thr14. The wee1 gene, first identified in fission yeast, acts on p34cdc2 as a dosage and nutrient dependent negative regulator of mitosis (Nurse, 1975;
Nurse and Thuriaux, 1977; Fantes and Nurse, 1978; Fantes, 1979, 1981; Russell and Nurse, 1987). The wee1 gene was identified by a mutation that advanced cells into mitosis early, resulting in “wee” cells half the size of wildtype (Nurse, 1975; Nurse and Thuriaux, 1980). This is consistent with the functional gene acting to restrain mitosis. Congruous with this, overexpression of wee1 inhibits S. pombe and causes arrest in cdc2 cells (Russell and Nurse, 1987b). The wee1+ gene encodes a 107 kDa protein that functions as a dual specificity (tyrosine/serine) kinase (Russell and Nurse, 1987b; Featherstone and Russell, 1991), responsible for the phosphorylation of the Tyr15 residue (Norbury et al., 1991; Parker et al., 1991, 1992; Solomon et al., 1992). Like other regulatory proteins central to cell cycle progression Wee1 has been conserved, as illustrated by the isolation of a human wee1 homologue. WEE1Hu, encoding a 94 kDa protein (McGowan and Russell, 1995), was identified by its ability to rescue fission yeast wee1 mutants (Igarashi et al., 1991). Like p107weel, p94WEE1 also phosphorylates p34cdc2 at Tyr15 (Honda et al., 1992; Parker and Piwnica-Worms, 1992; McGowan and Russell, 1993; Watanabe et al., 1995). Another kinase, Mik1, with high homology to Wee1, is now also known to directly phosphorylate p34cdc2 at Tyr15 (Lee at al, 1994) and was first isolated in a cdc2/wee1 mutant suppression screen (Lundgren et al., 1991). The existence of this gene was suggested by the fact that wee1 mutant cells do not enter mitosis prematurely but retain viability and some phosphorylation of p34cdc2 at Tyr15 (Gould et al., 1990). Cells lacking both wee1/mik1 genes are not viable because they catastrophically enter mitosis prematurely. They also can not phosphorylate p34cdc2 at Tyr15 (Lundgren et al., 1991) suggesting that the wee1+ and mik1+ genes encode partially redundant protein kinases both involved in the phosphorylation of Tyr15 on p34cdc2. The identity of the protein kinase responsible for the phosphorylation of p34cdc2 at Thr14 remains unknown (Sebastian et al., 1993; Hoffman and Karsenti, 1994; McGowan and Russell, 1995; Watanabe et al., 1995).

Wee1 enzyme activity is regulated as part of a cascade by the niml gene, which encodes a 67 kDa protein kinase. The Nim1 kinase has been shown to directly inactivate Wee1 by phosphorylation (Coleman et al., 1993; Wu and Russell, 1993) and is therefore an inducer of mitosis. It was isolated twice, first by its capability to suppress cdc25 mutants when overexpressed (Russell and Nurse, 1987a) and then
isolated again from mutants observed to alter their response to nutrient limitation and named as cdr1 (Young and Fantes, 1987; Feilotter et al., 1991).

Wee1 enzyme is itself regulated by phosphorylation. In fission yeast two genes have been found, when overexpressed, to delay the onset of mitosis by a wee1-dependent mechanism. pyp1 and pyp2 encode protein tyrosine phosphatases and upon disruption of pyp1 mitosis is advanced (Ottolie et al., 1991; Millar et al., 1992). However it appears that these genes have no effect upon Mik1 activity (Millar et al., 1992), so other kinases may influence Mik1 enzyme activity. In human cells a decline in Wee1 activity at mitosis correlates with increased phosphorylation of the enzyme (McGowan and Russell, 1995).

Acting in opposition to the enzymes that phosphorylate the Tyr\textsuperscript{15} (and probably Thr\textsuperscript{14}) residues of p34\textsuperscript{cdc2} is the cdc25 gene. In fission yeast this gene is rate limiting for entry into mitosis since increased expression shortens G\textsubscript{2} phase (Russell and Nurse, 1986; Edgar and O'Farrell, 1989; Ducommun et al., 1990; Moreno et al., 1990). The cdc25 gene was first cloned from Schizosaccharomyces pombe, where it encodes an 80 kDa gene product (Russell and Nurse, 1986). Multiple homologues to the yeast cdc25 gene have been subsequently discovered in a wide range of species including Saccharomyces cerevisiae (MIH) (Russell et al., 1989), Xenopus (Kumagai and Dunphy, 1992), two homologues in Drosophila, string (O'Farrell et al., 1989; Edgar and O'Farrell, 1989) and twine (Jimenez et al., 1990) and in both humans and mice, three homologues CDC25A, B and C (Sahdu et al., 1990; Galaktionov and Beach, 1991; Millar et al., 1991a; Nagata et al., 1991; Kakizuka et al., 1992; Gautier, 1993). Russell and Nurse (1986) observed that wee1 and cdc25 were counterbalanced in that deletion of cdc25 no longer blocked mitosis if wee1 was also inactive. The importance of their interaction in timing mitosis was also indicated by the effect of overexpressing cdc25 together with loss of the wee1 gene, which had an additive effect resulting in catastrophic early initiation of mitosis. Subsequently Cdc25 has been confirmed to act as a protein tyrosine phosphatase, in S. pombe and its homologues, that dephosphorylates Tyr\textsuperscript{15} and probably Thr\textsuperscript{14} (Gould and Nurse, 1989; Ducommun et al., 1990; Dunphy and Kumagai, 1991; Gautier et al., 1991; Millar et al., 1991b; Strausfeld et al., 1991; Izumi and Maller, 1995). Recently, Rime et al. (1994) have confirmed by microinjection that Cdc25 induces in vivo MPF activation.
The primary kinase responsible for the phosphorylation of CDC25C, the closest vertebrate homologue to fission yeast cdc25, is the Cdc2/cyclin B complex (Hoffman et al., 1993). Interestingly other cdc25 homologues are known to be activated by different CDK/cyclin complexes (Hoffman et al., 1994; Izumi and Maller, 1995). The discovery of multiple cdc25 homologues that interact with different CDK/cyclin complexes implies a potential for cdc25 to fulfill diverse roles in cell cycle progression. cdc25 is dephosphorylated and hence inactivated by a type 2A phosphatase (Clarke et al., 1993). Murray (1993) suggested a potential candidate for this may be INH, which encodes a phosphatase that was purified as an inhibitor of MPF activation (Lee et al., 1991).

In addition to Tyr15 and Thr14, two other phosphorylation sites in p34\(^{\text{cdc2}}\) have been identified, Thr\(^{167}/\text{Thr}^{161}\) and Ser\(^{277}\). The Thr\(^{167}\) residue in yeast (Gould et al., 1991) and the homologous site in vertebrates, Thr\(^{161}\) (Krek and Nigg, 1991a), act as a universal positive regulatory site. Mutation of the Thr\(^{167/161}\) site to non-phosphorylatable residues resulted in a loss of capability to rescue temperature sensitive cdc2 mutants; this was due to the formation of enzymatically inactive MPF (Atherton – Fessler et al., 1993). The mutation was also found to interfere with cyclin binding (Ducommun et al., 1991; Gould et al., 1991; Krek et al., 1992; Solomon et al., 1992). This evidence suggested that the phosphorylation of Thr\(^{161/167}\) was required for the activation of p34\(^{\text{cdc2}}\). The specific kinase responsible for the phosphorylation of this residue has been identified as MO15, also known as CAK or Cdk7. The first indications of the presence of such an enzymatic activity were detected by Solomon et al. (1992) in Xenopus egg extracts. They termed it CAK, for cdc2/CDK activating kinase. They observed that CAK induced phosphorylation of Thr\(^{161}\) only in the presence of cyclin, indicating that the process was cyclin dependent. The catalytic subunit of CAK was almost simultaneously discovered by three separate research groups in 1993 (Fesquet et al., 1993; Poon et al., 1993; Solomon et al., 1993). They were unanimous in their identification of the product of the MO15 gene as the catalytic component of CAK. Homologues of p40\(^{\text{MO15}}\) have been identified in a diverse range of species including Xenopus (Shuttleworth et al., 1990; Poon et al., 1993), human (Darbon et al., 1994; Tassan et al., 1994), mouse (Ershler et al., 1993), goldfish (Onoe et al., 1993), starfish (Fesquet et al., 1993), rice (Hata 1991) and possibly budding yeast (Simon et al., 1986). The high degree of conservation of this gene can be attributed to its requirement for the
activation of the p34\textsuperscript{cdc2} protein kinase at mitosis. Furthermore p40\textsuperscript{M015} has been found to regulate the activity of Cdk2 and Cdk4 in a similar manner (Fesquet et al., 1993; Poon et al., 1993; Matsuoka et al., 1994) demonstrating that it functions as a CDK activating kinase. The p40\textsuperscript{M015} protein has been consistently isolated in association with at least one other protein subunit, which it was speculated may function as an activator or regulator of p40\textsuperscript{M015} (Fesquet et al., 1993; Poon et al., 1993; Tassan et al., 1994). This has indeed proven to be the case with the discovery that the accompanying protein is a novel cyclin distantly related to cyclin C (Fisher and Morgan, 1994), now designated cyclin H (Fisher and Morgan, 1994; Mäkelä et al., 1994). Formation of an active kinase requires the association of p40\textsuperscript{M015} and cyclin H. Fisher and Morgan (1994) have proposed that p40\textsuperscript{M015} be renamed as Cdk7, as activation of p40\textsuperscript{M015} requires phosphorylation at Thr\textsuperscript{176} and the presence of cyclin H significantly increases CAK activity \textit{in vitro} (Fisher and Morgan, 1994; Mäkelä et al., 1994). The identity of the kinase responsible for the phosphorylation of p40\textsuperscript{M015} and hence its activation, termed CAKAK (\textbf{CAK}-activating \textbf{kinase}) (Solomon, 1994), remains unknown. Although the phosphorylation catalysed by CAK is essential, there is no evidence that it is utilised in a regulatory mechanism; it seems likely that p34\textsuperscript{cdc2} is phosphorylated by CAK soon after synthesis and remains so in actively proliferating cells.

The final p34\textsuperscript{cdc2} phosphorylation site identified is Ser\textsuperscript{277}. In chicken p34\textsuperscript{cdc2} there is evidence that it becomes phosphorylated during G\textsubscript{1}, the level then decreasing upon entry into S phase (Krek and Nigg, 1991a). This residue has not been recognised as phosphorylated in other systems. Serine phosphorylation of p34\textsuperscript{cdc2} has also been identified in NIH3T3 cells prior to DNA synthesis (Lee et al., 1988; Norbury and Nurse, 1990) suggesting a possible role in G\textsubscript{1}-S progression, however again this remains an isolated report.

\subsection*{1.3.4 CDKs (cyclin dependent kinases)}

In yeast Cdc2/Cdc28 is known to be the only cyclin dependent \textbf{kinase} (CDK), directly involved in initiating S or M phase events (Fitch et al., 1992; Richardson et al., 1992; Nasmyth, 1993). Although recently a yeast homologue of CAK has been identified (Buck et al., 1995) and a cyclin-protein kinase association has been revealed
in the control of phosphatase gene expression (Espinoza et al., 1994; Measday et al., 1994). In contrast, in higher eukaryotes an extensive family of p34\textsuperscript{cdc2}-related proteins that regulate different parts of the cell cycle has been identified from \textit{Drosophila} (Lehner and O'Farrell, 1990) to humans (Meyerson et al., 1992) and possibly higher plants (Colasanti et al., 1991; Hirayama et al., 1991). The CDKs identified so far have been isolated in two ways: either as 1) polymerase chain reaction (PCR) products using p34\textsuperscript{cdc2}-related primers, or 2) clones that can complement the \textit{cdc2} or \textit{CDC28} genes of yeasts (Pines, 1993).

The first CDK to be characterised was \textit{cdc2}/\textit{CDC28}, as described in 1.2.1. This gene is sometimes called CDK1. A variant of \textit{cdc2}, called CDK2, was identified later in a variety of multicellular organisms (Elledge and Spottswood, 1991; Hirai et al., 1992; Koff et al., 1991; Ninomiya-Tsuji et al., 1991; Paris et al., 1991; Tsai et al., 1991; Pagano et al., 1992b; Rosenblatt et al., 1992). Cloning of the cDNA for p33\textsuperscript{CDK2} (Tsai et al., 1991) identified it as the human homologue of the \textit{Xenopus} Eg-1 gene (Paris et al., 1991). Interestingly, human CDK2 has sometimes been claimed to complement budding yeast \textit{cdc28} mutants (Elledge and Spottswood, 1991; Koff et al., 1991; Ninomiya-Tsuji et al., 1991; Meyerson et al., 1992) which would require its participation in G\textsubscript{1}-S and mitotic functions. Subsequently, Paris et al. (1994) have found that \textit{Xenopus} CDK2 was capable of interacting positively with \textit{S. pombe} G\textsubscript{2}-M machinery as it could rescue \textit{cdc2} mutants specifically blocked in G\textsubscript{2}. Other evidence indicating that CDK2 may play a role at a later stage in the cell cycle is that its kinase activity has been detected in G\textsubscript{2} (Rosenblatt et al., 1992), continuing on until early M phase (Pagano et al., 1993). However in animal cells, CDK2 is considered to fulfil a specialised function at S phase since it is found to be active at that time only. (Pagano et al., 1992b; Rosenblatt et al., 1992). Further, it has been demonstrated that S phase initiation is promoted by CDK2 (Chevalier et al., 1995) and that DNA replication in a \textit{Xenopus} cell free system is inhibited by Cdk2 depletion (Fang and Newport, 1991). Microinjection studies utilising anti-Cdk2 antibodies have also demonstrated inhibition of entry into S phase (Pagano et al., 1993). Cdk2 is also found in complexes with transcription factors for genes of DNA synthesis (Lees et al., 1992).

Several other less close variants of p34\textsuperscript{cdc2} that cannot complement \textit{cdc28} mutants in yeast have subsequently been isolated: CDK3; CDK4 (originally identified
as PSK-J3); CDK5 (formerly PSSALRE); and CDK6 (previously PLSTIRE) (Hanks, 1987; Elledge and Spottswood, 1991; Ninomiya-Tsuji et al., 1991; Tsai et al., 1991; Meyerson et al., 1992; Xiong et al., 1992). Cyclin partners have been identified for most of the above CDKs. Specifically, Cdk2 associates with cyclin A (Kobayashi et al., 1991; Tsai et al., 1991) and also with the G1 cyclins D and E (Dulic et al., 1992; Koff et al., 1992; Xiong et al., 1992). Cyclin D is representative of cyclins that may be concerned with initiating growth in response to hormone stimulation rather than by direct involvement in cell cycle events. Some CDKs may also share that function. For example Cdk4, Cdk5 and Cdk6 bind with D-type cyclins that are induced by hormone but do not show cell cycle related oscillations in abundance (Matsushime et al., 1992; Bates et al., 1994; Meyerson and Harlow, 1994). Cyclin D-dependent Cdk4 and Cdk6 activity first becomes evident in mid-G1 phase and continues to increase up until the G1-S boundary (Matsushime et al., 1994; Meyerson and Harlow 1994). Hence it appears that Cdk4 and Cdk6 may, as in the case of Cdk2, have a role in the G1-S stage of cell cycle. In contrast, no essential role in cell cycle progress has been identified for Cdk5, despite its capability to complex to D-type cyclins (van den Heuvel and Harlow, 1993). In fact the only kinase activity associated with Cdk5 has been detected in terminally differentiated neuronal cells (Lew et al., 1992). No cyclin partner has been identified for Cdk3, however recent evidence suggests that cyclin E may also activate it (Harper et al., 1995). The functions of Cdk3 are less well understood than other CDKs, however, a dominant negative CDK3 mutant has been found to cause a G1 block. Cell cycle progression can be subsequently restored upon the inclusion of wild type CDK3, indicating that Cdk3 function is required for G1 progression (van den Heuvel and Harlow, 1993).

1.3.5 CKIs or CDIs (CDK-inhibitory proteins)

Recently several inhibitors of CDKs active in G1 phase have been discovered in animal and yeast cells. These proteins can bind to CDKs and block catalytic activity, indicating that the cell cycle is not solely regulated by positive catalysts (reviewed in Peter and Herskowitz, 1994a; Sherr and Roberts, 1995). In animal cells CKI proteins are believed to contribute two main functions. One is to restrain transit through the late
commitment point (referred to as the restriction point by Pardee, 1989) until hormonal and physiological conditions are fully appropriate; thus CKIs are an important restraint of malignant proliferation. The second function is to be able to arrest progress through S phase until DNA is fully repaired thus avoiding deleterious mutations.

Perhaps the best characterised CKI is p21 (also known as Waf1, Cip1, Sdi1, Cap20) which was initially isolated through its ability to bind to Cdk2/cyclin complexes (Nasmyth and Hunt, 1993). The p21 protein has also been observed to bind directly to p34\(^{cdc2}\) (Cdk1) and Cdk4 (Zhang et al., 1993b) and to act as a strong and conserved inhibitor of CDK activity capable of arresting cell cycle progression (Gu et al., 1993; Harper et al., 1993; Xiong et al., 1993). p21 and other CKIs are present in most normal cycling cells and cyclin/CDK complexes may become active only when they exceed the amount of CKI (Harper et al., 1995).

Another vertebrate CDK inhibitor p27\(^{kip1}\) (Polyak et al., 1994) has been found to have high homology with p21 (Hengst et al., 1994; Toyoshima and Hunter, 1994). Like p21, p27 is also expressed in cycling cells, implying again that cyclin/CDK complexes must exceed an inhibitory threshold to become active. It appears that the mechanism adopted to enable kinase activation is the sequestering of p27 and p21 into complexes with cyclin D-dependent kinases (Toyoshima and Hunter, 1994; Harper et al., 1995). Sherr and Roberts (1995) have proposed that the inhibitory threshold mechanism set by CKIs may establish a dependency of cyclin E/Cdk2 activation on the prior assembly of cyclin D-dependent kinases.

Several other mammalian CKIs have been identified. All inhibit cyclin-D dependent kinase activity inducing cell cycle arrest, however little is known about what their specific cell cycle roles may be. They include p57\(^{kip2}\), which also inhibits cyclin E/Cdk2, E/Cdk3 and A/Cdk2 kinase activities (Lee et al., 1995; Matsuoka et al., 1995), and the Ink4 family, consisting of p16 (also known as Mst1, CdkN2, Cdk4l) (Serrano et al., 1993) and p15, p18 and p19 (Guan et al., 1994; Hannon and Beach, 1994; Chan et al., 1995; Hirai et al., 1995). Two G\(_1\) CKIs have also been identified in yeast, the Far1 (Chang and Herskowitz, 1991; Peter et al., 1993; Tyers and Futcher, 1993; Peter and Herskowitz, 1994b) and p40 proteins (Mendenhall, 1993; Schwob et al., 1994).
Animal cells have not been reported to contain CDK-inhibitory proteins acting on CDKs active during the G2 and M phases of the cell cycle. However in fission yeast Moreno and Nurse, (1994) identified the rum1 gene, which is expressed in G1 phase, potently inhibiting the Cdc2/mitotic cyclin complex and hence preventing premature mitosis. Additionally, Rum1 protein inhibits the Cig2/Cdc2 cyclin complex that is active in G1 phase and by this means is believed to delay START until cell size is adequate (Labib and Moreno, 1996). The existence of Rum1 acting on mitotic kinase could indicate that animal cells contain undiscovered CKI proteins that act at mitosis.

1.3.6 p13suc1 and related proteins

One other class of protein has a role in regulating the activity of the p34cdc2 protein kinase. A 13 kDa protein encoded by the suc1 gene was identified originally in fission yeast as capable of rescuing temperature sensitive cdc2 alleles (Hayles et al., 1986a, b). At high levels p13suc1 has an inhibitory effect upon p34cdc2 activity (Dunphy and Newport, 1989; Hayles et al., 1986a) and overexpression of suc1+ has been shown to cause a delay in mitotic onset (Hayles et al., 1986a; Dunphy and Newport, 1989). However the biochemical basis of p13suc1 action remains unclear and it seems more likely that it has an essential role in the inactivation of p34cdc2 at anaphase, which is required for exit from M-phase, since cells lacking p13suc1 block in mitosis (Moreno et al., 1989; Basi and Draetta, 1995). The p13suc1 protein, like cyclin, has also been found to become physically associated with the p34cdc2 protein kinase (Brizuela et al., 1987; Hayles et al., 1986b; MacNeil et al., 1991). Highly conserved homologues have been identified in diverse species (Endicott et al., 1995) including budding yeast (Hadwiger et al., 1989a), higher vertebrates (Richardson et al., 1990) and in higher plants (John et al., 1991), thus indicating that p13suc1 is a fundamental regulatory protein essential to the cell division cycle although its biochemical role is still unknown.
1.4 Localisation of CDKs, cyclins and their substrates during the cell cycle

A full understanding of cell cycle progress must include knowledge of where within the cell CDK/cyclin complexes are located and the substrate proteins that they act upon. This sort of information can contribute to understanding of the mechanisms driving cell cycle progress. At G1-S it is believed that CDKs must be present in the nucleus where they phosphorylate transcription factor binding proteins, transcription factors and DNA replicating proteins resulting in the expression of genes that promote DNA synthesis. There is also believed to be expression during S phase of genes encoding cyclins that allow activities required later in the cell cycle. At G2-M the key cell cycle events driven by p34\textsuperscript{cdc2} are largely structural rearrangements involving both cytoplasmic, cytoskeleton and nuclear structures such as the chromosomes, nuclear envelope and mitotic spindle. The kinase is therefore required at diverse specific locations.

With respect to G1 controls, much work has been done investigating the role of phosphorylation of the retinoblastoma protein (pRb). pRb in animal cells is known to bind to and negatively regulate transcription factors, like E2F, whose action is required for S phase entry (reviewed in Cobrinik et al., 1992; Sherr, 1993, 1994). It is known that D-type cyclins can bind directly to pRb (Kato et al., 1993). Hinds et al. (1992) have also demonstrated that the expression of cyclins A, E and D1 can rescue cells arrested in G1 by wild type pRb. Nevertheless it still remains unresolved which CDK complexes actually contribute \textit{in vivo} to pRb phosphorylation during G1 (Reed et al., 1994). Other potential G1 CDK substrates include pRb related proteins like p107 and p130 and proteins involved in DNA replication initiation, however, as yet these remain unidentified.

The candidates, during S phase, for CDK substrates involved in unwinding of DNA at replication origins and initiation of DNA replication have been less well investigated. Some, however, have been identified including the SV40 T antigen (Jans et al., 1991), single stranded DNA binding replication protein A (Dutta and Stillman, 1992; Fotedar and Roberts, 1992) and perhaps DNA polymerase α (Nasheuer et al., 1991). The G2-M transition too, has been sparsely investigated in this respect, yet there is some evidence that Cdc25, the G2-M Cdc2/cyclin B activating phosphatase, may
itself be activated by phosphorylation (Galaktionov and Beach, 1991; Kumagai and
Dunphy, 1992).

In contrast, many potential targets for CDK action have been recognised during
M phase, a period characterised by extensive structural reorganisation in the cell.
Firstly, extensive phosphorylation of histone H1 (Reeves, 1992) and several
transcription factors, SW15 (Moll et al., 1991), c-fos (Abate et al., 1991), oct1 (Roberts
et al., 1991) and c-myb (Lüscher and Eisenman, 1992) is known to accompany
chromosome condensation. The phosphorylation at CDK sites is believed to weaken the
interactions of these proteins with DNA. The disassembly of the nuclear lamina is
another process regulated to some degree by CDK action. There is evidence that
Cdc2/cyclin B directly phosphorylates lamins and that this action is at least partially
responsible for causing disassembly (Peter et al., 1990, 1991; Ward and Kirschner,
1990; Dessev et al., 1991; Nigg, 1992). The cyclin B/Cdc2 complex has also been
observed to phosphorylate sites on the cytoplasmic intermediate filament subunits,
vimentin and desmin (Chou et al., 1990; Dessev et al., 1991). Other cytoskeletal
proteins which have been identified as potential in vivo Cdc2/cyclin B substrates include
two associated with the actomyosin system, caldesmon (Yamashiro and Matsumura,
1991; Yamakita et al., 1992), and the regulatory light chain of myosin II (Satterwhite et
al., 1992). Both cyclin A/Cdc2 and cyclin B/Cdc2 kinase are involved in reorganising
the microtubule (MT) network at mitosis (Verde et al., 1992), however the specific
substrates involved remain unidentified. It appears that Cdc2/cyclin B is primarily
responsible for phosphorylation events occurring during M-phase, nevertheless other
kinases are also activated during mitosis, hence Cdc2/cyclin cannot be considered to be
exclusively responsible. A recurrent problem is the identification of which of the many
substrates that may be phosphorylated by a CDK in vitro are truly substrates in the live
cells. Information concerning the location of CDK proteins may help to clarify the
situation.

The hypothesis that progression of cell cycle events is regulated by sequential
associations of different cyclins with their CDK partners (Heichman and Roberts, 1994;
Jackman et al., 1995) is supported by the changing localisations of cyclins within the
cell. To date cyclin D, E and all cyclin A homologues identified are nuclear proteins,
with the exception of Drosophila A-type cyclins (Lehner and O'Farrell, 1989;
Maldonado-Codina and Glover, 1992). Cyclin A is accumulated within the nucleus as soon as it is synthesised (Pines and Hunter, 1991b), its transport there dependent upon complex formation with a CDK (Maridor et al., 1993). The nuclear localisation of these cyclins is consistent with their association with CDKs that phosphorylate transcription factors and other proteins involved in DNA replication (Nigg, 1993).

In contrast cyclins B1 and B3 are initially cytoplasmic in localisation. Upon the onset of prophase they are rapidly translocated into the nucleus (Pines and Hunter, 1991b; Bailly et al., 1992; Gallant and Nigg, 1992; Ookata et al., 1992; Jackman et al., 1995). This process is apparently mediated by possession of an N-terminal specific region (Pines and Hunter, 1994). Cyclin B2, however, does not relocate to the nucleus but remains distributed throughout the cell (Jackman et al., 1995). Furthermore, while cyclin B1 is found to associate with centrosomes, chromosomes and microtubules (Maldonado-Codina and Glover, 1992; Ookata et al., 1992), cyclin B2 colocalises predominantly with the Golgi apparatus (Jackman et al., 1995).

During mitosis \( p34^{cd2} \) appears to have both cytoplasmic and spindle localisations (Rattner et al., 1990). It has been detected in fixed cells in association with kinetochores, centrosomes and spindle microtubules (Bailly et al., 1989; Riabowol et al., 1989; Alfa et al., 1990; Rattner et al., 1990; Tombes et al., 1991; Kubiak et al., 1993; Ookata et al., 1993; Andreassen and Margolis, 1994). There have also been reports of \( p34^{cd2} \) associated with the chromosomes (Ookata et al, 1993) or with vesicles that are localised in the region between the separating chromosomes (Bailly et al, 1989). These observations suggest that in animals \( p34^{cd2} \) could have a role in formation of the mitotic apparatus.

The localisation of \( p13^{suc1} \), which can bind \( p34^{cd2} \) (Brizuela et al., 1987; Moreno et al., 1989; Kusubata et al., 1992), only partially coincides with the reported position of \( p34^{cd2} \). Several studies have examined the localisation of \( p13^{suc1} \) within the cell. Three studies found that \( p13^{suc1} \) was targeted to the nucleus, becoming cytoplasmic at nuclear envelope breakdown (Bailly et al., 1989; Riabowol et al., 1989; Hepler et al., 1994). In addition Bailly et al. (1989) reported \( p13^{suc1} \) localisation to the centrosome but in live unfixed cells Hepler et al. (1994) find no association of \( p13^{suc1} \) to any part of the mitotic apparatus. Neither was there any detected localisation of \( p13^{suc1} \) to the pre-prophase band although antibodies to \( p34^{cd2} \) and \( p34^{cd2} \)-like proteins have indicated
that p34^{cdc2} may be associated with the prophase band (PPB) in plants during mitosis (Mineyuki et al., 1991; Colasanti et al., 1993). The issue of possible localisation of p34^{cdc2} to the PPB remains unresolved and evidence from p13^{suc1} localisation is complicated by the fact that only a small proportion of the p34^{cdc2} (5%) is believed to be bound to p13^{suc1} at any one time (Brizuela et al., 1987).

In higher plants the localisations within the cell of the key cell division cycle proteins has yet to be established. Homologues to cdc2 and other conserved genes have been identified, however to date all studies of p34^{cdc2} location have used anti-PSTAIR antibodies which have the potential to detect variants of p34^{cdc2} (Pines and Hunter, 1991a; Meyerson et al., 1992; Okuda et al., 1992; Graña et al., 1994; Basi and Draetta, 1995), with the exception of that of Colasanti et al. (1993). Hence it has not been possible to categorically determine the location of p34^{cdc2}. This thesis will present data relevant to the localisation of p34^{cdc2} within proliferating cells of maize.

1.5 Differences between cell division processes in plants and animals

CDK proteins in plant cells could have different intracellular locations than in animal cells because the plant cytoskeleton is arranged very differently, being involved with formation of the cell wall during interphase and the new cross wall at cytokinesis. Plants must maintain precise control of the spatial aspects of cell division because cells cannot alter their relative position after formation. Of particular significance is the plane of cell division, changes in which enable alterations in the polarity of growth. A second factor is that the sessile nature of plants requires them to respond to environmental factors without moving. One aspect of this response is that mature, differentiated cells may upon stimulation, such as in wounding, dedifferentiate and reenter the cell cycle (Van’t Hof, 1985). Furthermore, unlike animals, plants are capable of producing new organs throughout their lives. Thirdly, in plants cell division is confined to specialised meristems, again in stark contrast to animal systems in which mitotic activity occurs in many parts of the adult organisms, resupplying specific cell populations.

The last main disparity between plants and animals is in cytoskeletal structures associated with division. The majority of plants lack a centrosome but do have several
unique structures - a preprophase band, phragmosome and a phragmoplast. The preprophase band (PPB) is a cortical band of microtubules that encircles a cell, appearing after the end of S phase and developing throughout G2 (Gunning and Sammut, 1990). The PPB is significant in plant division as it is a predivision indicator of the plane of cell division. Where the PPB is to be positioned in a cell is dependent upon the position of the nucleus (Murata and Wada, 1991). Proximity to the metaphase nucleus and/or spindle has also been associated with the degradation of the PPB (Murata and Wada, 1991). However Zhang et al. (1992) showed that nuclear envelope breakdown and spindle initiation were not required for PPB disassembly in okadaic acid inhibited cells. PPB disassembly was simultaneous with p34^{cdc2}-like kinase activation even if other mitotic events were blocked. Other evidence that a phosphorylation-regulated mitotically-active kinase, possibly p34^{cdc2} or a related enzyme, is associated with the PPB may be found in the work of Katsuta and Shibaoka (1992). They found that the formation of the PPB in tobacco BY-2 cells was inhibited by protein kinase inhibitors, suggesting that protein phosphorylation is associated with PPB assembly.

Following PPB disassembly two structures unique to plants form in the region marked by the PPB. These are the phragmosome and phragmoplast which are both involved in cytokinesis. The phragmosome is a disc of cytoplasm that forms in late prophase in vacuolate cells. It ensures cytoplasmic continuity across the cell lumen at the division site previously determined by the PPB, within which the mitotic apparatus and later the phragmoplast can form. The phragmoplast is an open cylindrical structure of microtubules, within which the developing cell plate assembles.

In view of the different structures in plant cells it is therefore important to establish the subcellular localisation of cell cycle proteins in plant cells although the identity of many of these proteins may resemble those of animals and yeast cells.

1.6 Homologues to central cell division cycle elements in plants

The high degree of conservation of genes essential to the cell division cycle observed in yeasts and diverse animal systems also extends to the plant kingdom. Homologues to p34^{cdc2}, all encoding the conserved "PSTAIR" motif and partially, if not completely, capable of complementing yeast cdc2 mutants, have been isolated from
several different species. These species include *Chlamydomonas* and *Avena* (John et al., 1989), *Arabidopsis* (John et al., 1990; Ferreira et al., 1991; Hirayama et al., 1991), pea (Feiler and Jacobs, 1990), wheat (John et al., 1990), alfalfa (Hirt et al., 1991), carrot (Gorst et al., 1991), *Lilium* (Yamaguchi et al., 1990), maize (Colasanti et al., 1991), *Petunia* (Bergounioux et al., 1992), rice (Hashimoto et al., 1992), mothbean (Hong et al., 1993) and soybean (Miao et al., 1993). Furthermore the detection in several species (Feiler and Jacobs, 1991; Hata, 1991; Hirayama et al., 1991; Imajuku et al., 1992; Hirt et al., 1993; Miao et al., 1993) of more than one homologue to p34cdc2 and p34cdc2-like proteins suggests that a family of CDK proteins also exists in plants. As yet, however, it has not been possible to ascertain if any of the identified plant p34cdc2 homologues or p34cdc2 related proteins are indeed the plant equivalent to Cdk2 or other CDK family members.

The close association of the p34cdc2 protein kinase with cyclins also appears to occur in plants. Plant homologues of cyclins have been isolated from various species including carrot and soybean (Hata et al., 1991), alfalfa (Hirt et al., 1992; Meskiene et al., 1995), pea (Jacobs, 1992), *Arabidopsis* (Hemerly et al., 1992; Ferreira et al., 1994a, Day et al., 1996), *Antirrhinum majus* (Fobert et al., 1994), maize (Renaudin et al., 1994), *Sesbania* and tobacco (in Ferreira et al., 1994b, Setiady et al., 1995) and rice (Sauter et al., 1995). With the exception of one series of cyclin proteins, recently isolated by Soni et al. (1995), all the plant cyclins identified to date have been mitotic-like cyclins. Sequencing has allowed their tentative classification into three classes and indicates that they may be A or B-like types (Renaudin et al., 1994). These “mitotic” cyclins contain an N-terminal destruction box characteristic of B type cyclins (Hata et al., 1991) and microinjection of some has led to oocyte maturation (Hata et al., 1991; Hemerly et al., 1992) indicating a role in mitosis.

The observation that their transcripts are abundant in proliferating cells (Hemerly et al., 1992; Fobert et al., 1994) further supports a role in regulation of plant cell division. In addition to these mitotic cyclins Soni et al. (1995) appear to have isolated several D-type cyclins from *Arabidopsis*. These δ cyclins can rescue yeast *cln* mutants and like the D-type G1 cyclins of vertebrates lack an N-terminal destruction box but do contain a potential PEST sequence.
Several cell cycle proteins may therefore be in common between plants and other eukaryotes, but important questions arise concerning how the unique features of the plant cell cycle may be regulated by the oscillations in CDK-activity.

1.7 Control of Cdc2 expression and activation during organogenesis

As well as underlying specific events in the cell cycle, there is some evidence that the presence and capacity for activation of p34<sup>cdc2</sup> is a factor in shaping the restricted occurrence of cell division in the plant.

In plants the switch from cell proliferation to cessation of division and hence to cell differentiation is critical in plant development. Cells outside the defined meristematically active regions are allowed to develop structures and functions that may require their enlargement to several times the size at which division normally occurs. The equivalence of START cell cycle controls between plants and animals, where p34<sup>cdc2</sup> is known to be a key control element in cell division (Nurse, 1990; Norbury and Nurse, 1992), suggested that p34<sup>cdc2</sup> may have a similar role in plants. Thus suppression of the size stimulation of division (John, 1984; John et al., 1993a) may be part of the control of differentiation. Indeed the localisation of p34<sup>cdc2</sup> reflects and perhaps determines the organisation of plant tissue into meristematic and non-meristematic zones; in that a greater abundance and activity of p34<sup>cdc2</sup> and p34<sup>cdc2</sup>-like protein have been observed in regions of cell proliferation in wheat (John et al., 1990), alfalfa (Hirt et al., 1991), Arabidopsis (Ferreira et al., 1991), carrot (Gorst et al., 1991) and in maize (Colasanti et al., 1991). Specifically, John et al. (1990) found that the level of p34<sup>cdc2</sup>-like protein relative to other proteins declined to one-sixteenth of that in the meristem. A subsequent study determined that the boundary of active cell division in the basal meristem was defined as the region in which p34<sup>cdc2</sup>-like protein was active (John et al., 1993b). Staiger and Doonan (1993) suggest that this correlation may be in part due to operation of a transcriptional control mechanism. Martinez et al. (1992) were able to demonstrate that this was indeed the case in an in situ hybridisation study of Arabidopsis. They found the cdc2 mRNA transcripts were at their highest level in the leaf primordia, the vegetative shoot meristem, at the base of developing floral organs, the pericycle and in the root tip meristem. cdc2 transcript was also detected in non-
dividing cells but at significantly lower levels, leading them to suggest that cdc2 expression was a prerequisite for cell division. Hemerly et al. (1993) using promoter-β-glucuronidase (GUS) analysis came to a similar conclusion. These last two studies however were not able to confirm whether the level of p34\textsuperscript{cdc2} protein follows those of the mRNA transcript. Furthermore earlier studies investigating p34\textsuperscript{cdc2} protein abundance and activity in higher plants (John et al., 1991; Gorst et al., 1991; John et al., 1993a, b) have utilised anti-PSTAIR antibodies or the mitotic protein p13\textsuperscript{suc1} to isolate putative p34\textsuperscript{cdc2}. Either method has the potential to detect not only p34\textsuperscript{cdc2} but p33\textsuperscript{CDK2} or other possible closely related CDKs in addition to p34\textsuperscript{cdc2} (Pines and Hunter, 1991a; Meyerson et al., 1992; Okuda et al., 1992; Graña et al, 1994; Basi and Draetta, 1995), hence it has not been possible to determine specifically what the role of p34\textsuperscript{cdc2} is in the cell division cycle. This thesis will present new evidence concerning the localisation of specifically identified p34\textsuperscript{cdc2} protein and activity with actively dividing cells in tissue.

As p34\textsuperscript{cdc2}-like kinase activity has been observed to peak at mitosis in plant cells (John et al., 1993a), it is therefore timely to investigate whether this rise in activity is associated with PPB assembly or disassembly. Circumstantial evidence for a role in disassembly was noted by Zhang et al. (1992) (discussed above in 1.5) and interaction of p34\textsuperscript{cdc2} with the cytoskeleton has previously been observed in animal cells.

1.8 Interaction and association of Cdc2 and Cdc2-like protein kinases with the cytoskeleton in plants

Microinjection of p34\textsuperscript{cdc2} has been shown to have a profound effect on cellular organisation. In animal cells it induced alterations in the microtubule and actin microfilament components of the cytoskeleton as well as premature chromatin condensation (Lamb et al., 1990). As a consequence of microinjection of MPF into Tradescantia stamen hair cells, recently Hush et al. (1996) have reported disassembly of the PPB, chromosome condensation, nuclear envelope breakdown and initiation of mitosis. The profound effects that microinjection of p34\textsuperscript{cdc2} appears to induce in both plants and animals may be explained by the association of p34\textsuperscript{cdc2} with structural elements of the cell. Several immunofluorescence studies have reported that, in animal cells, p34\textsuperscript{cdc2} consistently localises to the mitotic spindle apparatus, specifically the
microtubular cytoskeleton, centrosome and kinetochores (Bailly et al., 1989; Riabowol et al., 1989; Alfa et al., 1990; Rattner et al., 1990; Tombes et al., 1991; Kubiak et al., 1993; Ookata et al., 1993; Andreassen and Margolis, 1994). It has been suggested that the association of p34\textsuperscript{cdc2} be mediated by MAP (microtubule associated proteins) (Ookata et al., 1993, 1995), homologues of which have recently been cloned from several plant species (Duerr et al., 1993; Jonak et al., 1993; Strafstrom et al., 1993; Mizoguchi et al., 1994).

In plants p34\textsuperscript{cdc2} and many of its regulatory and associated proteins have been identified and appear to function in a similar, if not identical manner, to comparable homologues in animals (Jacob, 1992; Francis and Halford, 1995). This suggests perhaps that p34\textsuperscript{cdc2} may exhibit comparable specific associations to plant cell division structures, for which there is further evidence. Mineyuki et al. (1991), using an antibody raised against the conserved "PSTAIR" sequence, were the first to detect specific localisation of p34\textsuperscript{cdc2} or p34\textsuperscript{cdc2}-like protein to the PPB. Subsequently John et al. (1993a), also using PSTAIR antibody, detected the presence of p34\textsuperscript{cdc2} or a closely related protein in PPB. The advent of this association of a p34\textsuperscript{cdc2} homologue with the PPB in plants, like the association with mitotic centrosomes in animals, suggests a role for p34\textsuperscript{cdc2} in determining the polarity of cell division. These two initial studies were unable to determine whether it was p34\textsuperscript{cdc2} or a closely related protein that was associating to the PPB. However using a specific maize p34\textsuperscript{cdc2} antibody, Colasanti et al. (1993) also found p34\textsuperscript{cdc2} localisation to the PPB. The association was not strong and was furthermore only detectable in 10% of cells possessing a PPB. These cells were characterised by the possession of visibly condensed chromatin, which led Colasanti et al. (1993) to suggest that the association was transient, occurring only at the G\textsubscript{2}-M transition and that it was significant with regard to the mechanism by which cell division site determination is achieved. This thesis will present evidence confirming the localisation of p34\textsuperscript{cdc2} to the PPB and will also report for the first time in higher plants localisation of p34\textsuperscript{cdc2} with other microtubule structures during cell division.
1.9 **Aims of the thesis**

This review of the mechanisms fundamental to the regulation of the cell division cycle in plants and animals illustrates that, while much information has been revealed, many issues are still left unanswered. Work described in this thesis investigates two such issues in plants: 1) what the distribution of p34\(^{cdcl}\) (rather than its possible variants) is, where it is active and whether this activity is associated with meristematic tissue, and 2) whether p34\(^{cdcl}\) protein does localise with any cytoskeletal structures associated with cell division. Maize has been utilised as the model system for analysis. First, a specific maize anti-p34\(^{cdcl}\) antibody was generated and affinity-purified (Chapter 2). Then the capabilities of the anti-p34\(^{cdcl}\) antibody and the immobilisation and accessibility of p34\(^{cdcl}\) in fixed cells was examined using a Western dot blot technique (Chapter 3). The affinity purified maize anti-p34\(^{cdcl}\) antibody was then utilised in an attempt to immunoprecipitate native maize p34\(^{cdcl}\) from maize root tissue (Chapter 4) and also for kinase activity assays in both maize root and leaf tissue (Chapter 5). The last phase of the project employed diverse immunofluorescence techniques to investigate whether p34\(^{cdcl}\) associates specifically with the microtubular cytoskeleton in maize (Chapter 6).
CHAPTER 2:

ISOLATION AND PURIFICATION OF AN ANTIBODY SPECIFIC FOR MAIZE p34<sup>cdc2</sup>
2.1 Introduction

2.1.1 cell division cycle genes in higher plants

The central role of the p34^{cdc2} kinase in the regulation of the cell cycle in yeast and animals is well established (Nurse, 1990, Norbury and Nurse, 1992). In plants homologues to p34^{cdc2} have been identified in a number of species including pea, wheat, Arabidopsis, alfalfa, maize, Chlamydomonas, Petunia and soybean (John et al., 1989; Feiler and Jacobs, 1990; Colasanti et al., 1991; Ferreira et al., 1991; Hirt et al., 1991; Bergounioux et al., 1992; Miao et al., 1993). These plant homologues all contain the 16 amino acid "PSTAIR" motif, a highly conserved sequence, found in all known p34^{cdc2} homologues to date. This suggests that plant cdc2 genes may, like yeast cdc2, function centrally in the regulation of cell division processes. However, as in animals, some plants species are known to contain more than one protein kinase gene with some sequence similarities to p34^{cdc2} (Francis and Halford, 1995), indicating that protein kinases closely related to p34^{cdc2} known as CDKs (cyclin dependent kinases) may be potentially involved in cell division processes. The presence of these closely related kinases currently makes it difficult to assess whether in plants the p34^{cdc2} gene alone is responsible for cell cycle progress. Thus cytologically it is difficult to assess whether a protein detected by anti-PSTAIR antibody is p34^{cdc2} or a CDK variant. The generation of antibodies specifically targeted against p34^{cdc2} therefore presents an opportunity for the more specific investigation of the distribution and activity of p34^{cdc2}.

2.1.2 The generation of antibodies

The present investigation used the strategy of deducing the amino acid sequence encoded by a known p34^{cdc2} homologue of maize (Colasanti et al., 1991) and using a synthetic peptide corresponding to the carboxyterminus to generate an antibody. Several advantages accompany the use of synthetic peptides: 1) anti-peptide antibodies may be generated (from the amino acid sequence of a protein as soon as it is known) without requiring purification of the protein, 2) antibodies may be raised against specific sites such as the carboxyterminal region which is variable between CDKs and capable
of specifically identifying \( p34^{\text{cdc2}} \), 3) most peptides elicit a good antigenic response due to their mode of presentation to the immune system, 4) many anti-peptide antibodies bind well to denatured protein.

How effective the antibody will be in identifying the antigen depends on what region the peptide sequence used to generate the antibody is derived from. Several alternatives have been utilised over the last few decades in an effort to generate the best reaction. Initially, peptides containing hydrophilic amino acids and proline residues were generated, as these were thought to be exposed on the surface of native proteins (Hopp and Woods, 1981, 1983). Subsequently carboxyterminal sequences have been utilised (Walter, 1986) as this region is synthesised last and often folds onto the outside surface of the protein. This reasoning together with the expected specificity is the basis for our selection of a peptide derived from the carboxyterminal sequence of maize \( p34^{\text{cdc2}} \).

It is important for good immune reaction that because of its small size the peptide be linked to a carrier molecule by either the amino or carboxy terminal residue. A variety of carrier proteins can be used for coupling to synthetic peptides intended for use in rabbits. Among those most commonly used are keyhole limpet haemocyanin (KLH), bovine serum albumen (BSA) and thyroglobulin. In this study KLH was used. Several types of coupling reagents can be used in the process of linking peptides to carrier molecules. One is glutaraldehyde, a bifunctional coupling reagent that creates stable linkages between compounds through their amino groups. An alternative coupling reagent is succinimidyl 6-(N-maleimido)-n-hexanoate (MHS). This heterobifunctional reagent links peptide to carrier through cysteines and free amino groups and it allows a more predictable coupling of peptide to carrier, avoiding the potential for mass random coupling possible when using bifunctional reagents. However tests in our laboratory have found for the carboxyterminal sequence of \( p34^{\text{cdc2}} \) that glutaraldehyde coupling gives a higher success rate and may indicate that the synthetic peptide is then presented with greater similarity to its configuration in \( p34^{\text{cdc2}} \). Analysis of serum by Western blotting allowed the time of development of an immune reaction to be established. Antibody was purified from serum to allow maximum specificity of detection of maize \( p34^{\text{cdc2}} \) protein.
2.1.3 Techniques for antibody purification

Purified antibodies have lower background reaction in cell staining, immunoassays and immunoblots. A wide range of techniques for protein fractioning can be used for antibody purification. Three that were used in the present study are described briefly below:

1) DEAE cellulose purification

DEAE cellulose purification is an example of ion exchange chromatography. This technique is useful as antibodies have a more basic isoelectric point than the majority of other serum proteins. Two strategies are commonly used. In the first a pH below the isoelectric point of most of the antibodies is maintained. They therefore do not bind to an anion exchange resin such as DEAE, although other serum proteins will. Alternatively, the pH is raised above the isoelectric point so that antibody molecules will bind to DEAE cellulose. The antibodies can then be eluted with increasing salt concentration.

2) Protein-A column purification

This method uses the presence in IgG molecules of high affinity sites for protein-A. Once bound to protein-A in a column, antibodies can be eluted by lowering the pH of the buffer to partially denature the antibodies.

3) Immunoaffinity purification using an antigen column

This method allows purification of antigen-specific antibodies from polyclonal serum. Pure antigen is bound covalently to a solid support like CNBr-activated agarose then polyclonal serum is run through the column and the antibodies specific to the antigen are allowed to bind. The unbound antibodies are removed by washing and then the specific antibodies eluted by acid or alkaline pH. The method possesses a unique ability to isolate specific antibodies out of a polyclonal pool. Unfortunately the method requires large amounts of serum and elution conditions can lead to loss of antibody activity. It must also be noted that the carrier protein used in the creation of the column must be distinct from that used for immunisation.

This chapter reports the generation of a specific maize antibody against the cell division cycle protein p34\textsuperscript{cdc2} by standard immunological procedures. Once generated
this antibody was purified, using the techniques discussed above, in preparation for its use to investigate the role of p34\textsuperscript{cdc2} in cell division in higher plants.

2.2 Materials and methods

2.2.1 Production of the p34\textsuperscript{cdc2} peptide

The maize peptide sequence used to generate a specific maize anti-p34\textsuperscript{cdc2} antibody was derived from the data of Colasanti et al. (1991) describing the sequence of a maize p34\textsuperscript{cdc2} clone that could complement loss of CDC28 (equivalent to p34\textsuperscript{cdc2}) function in budding yeast. Our antibody was raised against the 17 amino acids at the C terminus of the cdc2ZmA clone. The peptide sequence is ARQALEHEYFKDAL-EVVQ.

The peptide was synthesised using solid phase techniques and then purified by the Biomolecular Resource Unit at the John Curtin Medical School of Research at A.N.U.

2.2.2 Coupling of p34\textsuperscript{cdc2} peptide to a carrier protein by glutaraldehyde

Maize peptide was coupled to keyhole limpet haemocyanin (KLH) by glutaraldehyde, prior to injection.

5 mg of maize p34\textsuperscript{cdc2} peptide was dissolved in 3 ml of 50 mM sodium citrate buffer, pH 6.5, (50 mM sodium citrate + 50 mM citric acid, pH 6.5). Then 188 µl of KLH (in sodium citrate buffer) was added and the solution mixed. Next 100 µl of a 25 % glutaraldehyde solution was added to 2.5 ml of sodium citrate buffer and 0.6 ml added to the peptide/KLH solution. The solution was placed into a 12 ml Falcon tube, sealed with Nescofilm and put onto an end-over-end rotor for 2 h at room temperature. After 2 h 0.375 ml of 1 M glycine, pH 7.2, was added to block unreacted glutaraldehyde and the tube mixed again by rotation then the peptide/KLH/glutaraldehyde solution was dialysed against 2 L of PBS buffer (137 mM NaCl, 2.7 mM KCl, 7 mM Na\textsubscript{2}HPO\textsubscript{4}, 1.5 mM KH\textsubscript{2}PO\textsubscript{4}, pH 7.4) in 6-8,000 MW cut-off tubing for 2 d with several changes of buffer.
2.2.3 Immunisation schedule of p34cdc2 peptide conjugate into rabbit

A series of six subcutaneous injections was carried out, at approximately two week intervals, the first in complete Freud’s adjuvant. With the exception of the last injection all were of a 500 µg amount of antigen (see Table 2.1).

<table>
<thead>
<tr>
<th>Date of Injection</th>
<th>Amount of Antigen</th>
<th>Type of Freud’s Adjuvant</th>
</tr>
</thead>
<tbody>
<tr>
<td>25.01.93</td>
<td>500 µg</td>
<td>complete</td>
</tr>
<tr>
<td>10.02.93</td>
<td>500 µg</td>
<td>incomplete</td>
</tr>
<tr>
<td>24.02.93</td>
<td>500 µg</td>
<td>incomplete</td>
</tr>
<tr>
<td>12.03.93</td>
<td>500 µg</td>
<td>incomplete</td>
</tr>
<tr>
<td>24.03.93</td>
<td>500 µg</td>
<td>incomplete</td>
</tr>
<tr>
<td>06.04.93</td>
<td>750 µg</td>
<td>incomplete</td>
</tr>
</tbody>
</table>

Table 2.1: Immunisation schedule for rabbit injected with maize p34cdc2 peptide

2.2.4 Serum sampling, preparation, storage and analysis

Seven serum samples were taken including one prior to immunisation. Two subsequent samples were used to determine when the antibody reaction was maximal, after which a succession of four substantial serum collections was carried out at approximately weekly intervals (see Table 2.2).

<table>
<thead>
<tr>
<th>Date of Bleed</th>
<th>Nature of Bleed</th>
</tr>
</thead>
<tbody>
<tr>
<td>16.12.92</td>
<td>pre-immune</td>
</tr>
<tr>
<td>03.03.93</td>
<td>1st immune test</td>
</tr>
<tr>
<td>16.04.93</td>
<td>2nd immune test</td>
</tr>
<tr>
<td>16.04.93</td>
<td>I : 40 ml</td>
</tr>
<tr>
<td>23.04.93</td>
<td>II : 40 ml</td>
</tr>
<tr>
<td>30.04.93</td>
<td>III : 40 ml</td>
</tr>
<tr>
<td>07.05.93</td>
<td>IV : 40 ml</td>
</tr>
</tbody>
</table>

Table 2.2: Sampling schedule for bleeds from rabbit injected with p34cdc2 peptide
After collection blood was allowed to clot for 30-60 min at 37°C. The clot was then separated from the sides of the collection vessel and placed at 4°C overnight to allow it to contract. The serum was then removed from the clot and any remaining insoluble material by centrifugation at 10,000 rpm for 10 min at 4°C.

After preparation the serum was aliquoted into 1.5 ml eppendorf tubes and placed into a -70°C freezer.

All serum samples were analysed for detection of maize anti-p34<sup>cdc2</sup> presence by the standard Western immunoblotting method (see 2.2.10).

### 2.2.5 Purification of maize anti-p34<sup>cdc2</sup> antibody using a protein-A column

Whole serum was dialysed against 1 L of load buffer (0.05 M Tris/HCl, 0.1 M NaCl, pH 8.0) overnight at 4°C prior to passage through the protein-A column. Particulate material was removed from the dialysed antibody by centrifugation for 2 min at 14,000 rpm then passed through a 0.2 μm Sartorius filter. The cleared antibody was diluted to 25% with load buffer then loaded onto the protein-A column (Protein-A MemSep 1000 Cartridge, Millipore, USA) which had been equilibrated by the passage of 25 ml of equilibration buffer (0.05 M Tris/HCl, 0.1 M NaCl, pH 8.0) through the device. The antibody was passed through the column at least twice then the column was rinsed with wash buffer (0.05 M Tris/HCl, 0.1 M NaCl, pH 8.0). 15 drop fractions were collected and their absorbance monitored at 280 nm until a baseline value was reached. A sample of the eluate was retained to test for possible unbound antibody.

The protein was desorbed by passing 0.1 M glycine/HCl, pH 3.0, through the cartridge. Ten fractions of 15 drops each were collected into tubes containing 2 drops of 0.5 M PO<sub>4</sub>, pH 8.0. After elution of the proteins, the protein-A cartridge was washed with 25 ml of 0.1 M Tris/HCl/0.02% NaN<sub>3</sub>, pH 8.0, buffer then recapped and stored at 4°C.

Absorbance at 280 nm was monitored in the fractions eluted from the column and those with the highest O.D. were stabilised by the addition of 1% BSA. These fractions were dialysed against TBS buffer (10 mM Tris/HCl, 0.15 M NaCl, pH 7.4)/0.05% NaN<sub>3</sub> in 6-8,000 MW cut-off tubing overnight at 4°C, then analysed by Western blotting (2.2.10) at a dilution of 1/500. The fractions found to identify p34<sup>cdc2</sup>
protein were pooled and stored at 4°C. At a later date the purified antibody was concentrated using a Micro Ultrafiltration System (Amicon, USA).

2.2.6 DEAE - Sephacel purification of maize anti-p34<sup>cd2</sup> antibody

The procedure used was a combination of batch and column DEAE ion chromatography methods, accomplishing initially a quick bulk purification of the antibody followed by a more precise purification by utilising an increasing ionic strength gradient to elute the antibody from the column.

For batch chromatography 5 ml of whole serum was dialysed against three changes of 5 mM sodium phosphate, pH 6.5, overnight at 4°C prior to mixing with 10 ml of DEAE-Sepharose (Pharmacia Biotech, Sweden) that had been washed extensively with 0.5 N HCl, 0.5 N NaOH and finally 5 mM sodium phosphate, pH 6.5, until the pH was 6.5. The dialysed antibody and DEAE-Sepharose were mixed at room temperature for 1 h on a rotating wheel. After 1 h the DEAE-Sepharose was removed by centrifuging the solution at 3,500 rpm for 5 min. The supernatant containing the bulk of the IgG was removed and retained. The Sepharose was resuspended in 5 ml of sodium phosphate, pH 6.5, and the wash repeated. The two supernatants were pooled and dialysed against three changes of 2 L of 10 mM Tris/HCl, pH 8.5, in 12,000 MW cut-off tubing over a 24 h period.

Prior to DEAE-Sepharose column purification of the anti-p34<sup>cd2</sup> antibody 10 ml of the washed DEAE-Sepharose was rinsed with 10 mM Tris, pH 8.5, until the pH had equilibrated then the Sepharose was packed into a 10 ml column. The batch purified antibody was passed through the column, then the column was washed with 10 mM Tris, pH 8.5. Samples of the antibody before and after passage through the column were collected to test for completeness of antibody binding to the column.

The proteins were sequentially eluted by increasing the NaCl concentration linearly from 0-0.5 M in 10 mM Tris, pH 8.5, buffer. Samples of 25 drops (approximate volume 1.5-1.75 ml) were collected and absorbance at 280 nm was monitored. Eluted proteins were stabilised by the addition of 1% BSA in TBS/0.05% azide. Analysis by Western blotting (2.2.10) and staining of electrophoretically separated proteins (2.2.8) by Coomassie Brilliant Blue (2.2.9) was used to assess which fractions contained the
purified antibody. The selected fractions were dialysed against 2 L of PBS/0.05% azide overnight then stored at 4°C.

2.2.7 Immunoaffinity purification of maize anti-p34\textsuperscript{cdc2} antibody using an antigen column

Antigen column preparation by coupling peptide to carrier protein by succinimidyl 6-(N-maleimido)-n-hexanoate

Because KLH was used as the carrier in the immunisations antibodies specific for KLH were avoided and antibodies specific for p34\textsuperscript{cdc2} were selected for by complexing peptide to a different carrier protein, thyroglobulin. 10 mg of thyroglobulin was dissolved in 2 ml of 0.1 M PO\textsubscript{4}, pH 8.5, and 5 mg of succinimidyl 6-(N-maleimido)-n-hexanoate (MHS) in dimethyl formamide (DMF). These two solutions were then combined together drop by drop and left to mix in a scintillation vial for 1 h at room temperature. To separate the conjugate from excess reagent, the solution was run through a 50 ml G25 column that had been washed with 200 ml of 0.1 PO\textsubscript{4}, pH 8.5. The conjugate was eluted with the same buffer, and 1 ml fractions collected, until slightly greater than one bed volume had been passed. The absorbance at 280 nm of these fractions was measured in order to determine if a protein peak at 0.4 bed volumes (Vo) followed by a broader peak rising to 1 bed volume (Vt), consisting of the excess reagents, were present. The fractions within the first peak were then pooled and the rest discarded.

The MHS/thyroglobulin conjugate was now reacted with the terminal cysteine that had been added to the aminoterminus of the p34\textsuperscript{cdc2} peptide. The peptide was first made fully reactive by reduction with borohydride. Approximately 1 mg of p34\textsuperscript{cdc2} maize peptide was washed three times in 0.1 M borate, pH 9.1, sonicated, then the peptide was reduced by the addition of 100 \mu l of 0.1 M NaBH\textsubscript{4}. After mixing for 5 min excess NaBH\textsubscript{4} was removed by lowering the pH to 1.0, with 1 M HCl and then raising it to pH 6.0, with 1 M NaOH. The reduced peptide was then added to the MHS/thyroglobulin solution. This combined solution was then stirred, for 1 h at room temperature, to allow a reaction to occur. After 1 h, 3 \mu l of mercaptoethanol was added
in order to block any unreacted MHS and left to mix for another 30 min. The peptide/thyroglobulin conjugate was then separated on the original 50 ml G25 column which had been washed with 0.1 M NaHCO₃, pH 8.3. The column was eluted with this same buffer and fractions collected and monitored as before. The early fractions of the first peak were retained and the latter fractions of the first peak were discarded as they may have contained free peptide. Retained fractions were pooled and NaN₃, added to 0.01% prior to storage at 4°C.

**Coupling of peptide-carrier protein complex to CNBr-Sepharose**

The peptide/carrier complex was coupled to CNBr-Sepharose beads (Pharmacia Biotech, Sweden) which had been swollen for 15 min in 1 mM HCl, then washed in a vacuum flask with 1 mM HCl and 0.1 M PO₄, pH 7.5. Approximately 32 ml of 0.5 mg/ml peptide/carrier, which had been dialysed against 2 L of 0.1 M PO₄, pH 7.5, in 3,500 MW cut-off tubing overnight at 4°C, was added to 2 ml of (50% v/v) CNBr-Sepharose beads and placed onto a rotating wheel at room temperature for 2 h. After 2 h the peptide/thyroglobulin/CNBr-Sepharose complex was spun in a bench centrifuge for 2 min at 7,000 rpm. The gel was retained, as well as a 50 µl sample of the supernatant used later for analysis of unbound peptide. The retained gel was resuspended in 10 ml of 0.5 M PO₄, pH 7.5, then spun as before, again all liquid was discarded and all gel retained. The 0.5 M PO₄, pH 7.5, wash was repeated once more, after which the gel was washed in 10 ml of 1 M NaCl/50 mM PO₄, pH 7.5, following the same procedure. To block any unreacted Br groups the gel was then resuspended in 10 ml of 1 M ethanolamine, pH 8.0, for 1-2 h at room temperature on a rotating wheel. The coupled beads were then washed with 10 ml of 0.1 M acetate buffer/0.5 M NaCl, pH 4.0, as before, after which the gel was washed with 10 ml of coupling buffer (0.1 M PO₄, pH 7.5). These two washes were repeated three more times, followed by two washes with 10 ml of PBS buffer then the gel was suspended in an equal volume of PBS containing 0.1% merthiolate and stored at 4°C.

A series of SDS-PAGE gels (7%, 15%, 5-20% gradient) (2.2.8) was run to compare samples taken before and after coupling, of the peptide/MHS/thyroglobulin complex to the CNBr-Sepharose, to determine if the coupling was successful. These
gels were transferred to nitrocellulose and probed as Western blots (2.2.10) with pre-immune serum and whole serum anti-p34\textsuperscript{cdc2} antibody diluted to 1/500.

**Immunoadfinity purification of maize p34\textsuperscript{cdc2} antibody using an antigen column**

The peptide/MRS/thyroglobulin coupled CNBr-Sepharose beads were packed into a 1.7 ml column (10 mm x 50 mm), which was washed extensively with 200 ml of 10 mM Tris, pH 7.5, followed by 200 ml of 100 mM glycine, pH 2.5, then 200 ml of 10 mM Tris, pH 8.8, and 200 ml of 100 mM triethylamine and lastly 10 mM Tris, pH 7.5, till the pH was 7.5, to clear it of any loose or unbound proteins. Maize anti-p34\textsuperscript{cdc2} antibody (DEAE purified IgG or whole serum), which had been dialysed against TBS overnight at 4°C then spun at 14,000 rpm for 10 min, was loaded directly onto the column. The unbound fraction was retained to check for possible unbound antibody. Then the column was washed with 6 ml (4 bed volumes) of 10 mM Tris, pH 7.5, the unbound fraction was again retained for analysis of the unbound p34\textsuperscript{cdc2} antibody remaining after passage through the column. The column was then washed with approximately 50 ml of 10 mM Tris, pH 7.5, followed by 60 ml of TBS to remove impurities.

Bound antibody was eluted from the column by the passage of 6 ml of 100 mM glycine pH 2.5. Then after washing of the column with 10 mM Tris, pH 8.8, proteins bound but not dislodged by acid pH were eluted by 6 ml of 100 mM triethylamine, pH 11.5. The column was then washed with 10 mM Tris, pH 7.5, until the pH reached 7.5, at which point merthiolate to 0.01% was added the column plugged and stored at 4°C. The proteins eluted by the acid and alkali were collected in 20 ml scintillation vials containing 300 µl of 1 M Tris, pH 8.0, solid BSA was added to 1% and they were dialysed against TBS/0.01% NaN\textsubscript{3} in 6-8,000 MW cut-off tubing overnight at 4°C.

The activity and specificity of the immunoaffinity purified antibody was tested by Western blotting (2.2.10). Fractions eluted from the column by acid and alkali were tested at dilutions of 1/8, 1/10, 1/25, 1/50, 1/100 and undiluted and compared to anti-PSTAIR and whole serum maize anti-p34\textsuperscript{cdc2} antibodies diluted to 1/500.

Variations of the standard elution conditions described above were employed when DEAE purified p34\textsuperscript{cdc2} antibody was processed through the antigen column. On
one occasion more extreme conditions of 100 mM glycine, pH 2.0, and 100 mM triethylamine, pH 12.0, were used to elute bound antibody from the column. Additionally the effect of 4 M urea rather than extreme pH was tested as an eluant.

2.2.8 SDS-polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE was conducted using the system of Laemmli (1970). The standard gels used in this study contained 12% (w/v) acrylamide, occasionally depending on the size of the proteins to be resolved, several gels of different concentrations (7%, 15%, 5-20%) were run. All gels were topped by a 4% acrylamide stacking gel and run in an electrophoresis buffer that consisted of 25 mM Tris/HCl, pH 8.3, 193 mM glycine and 0.1% SDS. The current was held at 25 mA per gel during stacking and increased to 45 mA per gel once the dye front had entered the separating gel. Electrophoresis was halted when the dye was within 5-10 mm of the bottom of the gel. Protein samples were mixed prior to loading with an equal volume of Sx2 (SDS) electrophoresis sample buffer, which contained 0.125 M Tris/HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 2% (v/v) 2-mercaptoethanol and 0.002% bromophenol blue. Pharmacia low molecular weight standards were used, comprising α-lactalbumin (14.4 kDa), trypsin inhibitor (20.1 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), albumin (67 kDa) and phosphorylase B (94 kDa).

2.2.9 Coomassie dye staining of gels

Two methods of staining proteins in gels, with Coomassie Brilliant Blue, were used in this study. For the "fast" method acrylamide gels were placed into Coomassie stain (0.2% Coomassie Brilliant Blue a250, 40% methanol AR and 10% glacial acetic acid) then heated in a microwave until the solution just began to boil (around 5 min), after which the gels, still in the stain, were placed onto a shaker to cool for 15 min. The stain was then poured off and fast destain solution (45% methanol AR, 10% glacial acetic acid) added, for 30 min, with shaking. After 30 min, if destaining was not sufficient new fast destain solution was added and the gels left to shake for another 30 min. When gels were partially destained but retained significant blue background the
fast destain was replaced with slow destain solution (5% methanol AR, 7.5% glacial acetic acid) and the gels left to shake overnight, during which the dye concentrated onto the protein bands. The “slow” method involved placing the gels into Coomassie stain and shaking for a minimum of 2 h after which the stain was poured off and fast destain solution added. Gels remained in fast destain for two changes of 1 h or overnight. Once the gels were sufficiently destained the fast destain was replaced with slow destain solution, for a minimum of 12 h, with shaking.

2.2.10 Western blotting and immunodetection in blots

Western blotting and immunodetection in blots was conducted following the conventional methods outlined in Towbin et al. (1979).

Gels and nitrocellulose were immersed in transfer buffer (25 mM Tris/HCl, pH 8.3, 192 mM glycine, 20% methanol AR) for 5 min. Transfer of proteins of median size range 20-60 kDa from gels to 0.45 micron nitrocellulose was optimal at 8 h at 130 mA. After transfer the nitrocellulose was immersed in Ponceau S solution (0.4% Ponceau S, 3% trichloroacetic acid (TCA)) for 15-30 min. Excess stain was washed off with 0.1% acetic acid until the background of the nitrocellulose was white. The nitrocellulose was then photographed using Kodak Tech Pan film (100 ASA) with a green filter. After photography, excess nitrocellulose and the protein size standards were removed in preparation for blocking. Whole nitrocellulose, or strips of nitrocellulose were placed into blocking solution (10 mM Tris/HCl, 0.15M NaCl and 5% skim milk powder) for a minimum of 1 h at room temperature (or overnight at 4°C) with shaking. They were then rinsed, with shaking, in three changes of TBS/0.05% Tween 20 for a total of 15 min.

Blocked nitrocellulose blots were either dried on 3MM paper and stored until they were required or probed immediately. The primary antibodies used to probe blots were anti-PSTAIR (dilution 1/500), whole serum anti-p34\(^{cd2}\) (1/500), affinity purified anti-p34\(^{cd2}\) antibody (1/50) and the IgG fraction of affinity purified anti-p34\(^{cd2}\) antibody (1/20). They were diluted in TBSBT buffer (10 mM Tris/HCl, pH 7.4, 0.15 M NaCl, 0.05% Tween 20, 1% BSA, 0.02% NaN\(_3\)). The anti-p34\(^{cd2}\) antibody was also competed with maize p34\(^{cd2}\) peptide at a concentration of 1 µM, incubated for 90 min
at 25°C. Standard primary antibody incubation on the blot was for 1-2 h at room temperature. Unbound primary antibody was rinsed off by two washes in TBS/0.05% Tween 20 and one in TBS/0.2% Tween 20, each of 10 min duration. After washing, the nitrocellulose was placed upon new Nescofilm and secondary antibody applied for 1 h at room temperature. The secondary antibody was usually anti-rabbit IgG (F(Ab) fragment) produced in goat, conjugated with alkaline phosphatase (Tago Inc., USA), diluted to 1/4000 with TBSBT. The nitrocellulose was then washed twice, with shaking, in TBS/0.2% Tween 20, then once with TBS for 10 min each. Prior to detection the nitrocellulose was washed, for 5 min, in alkaline phosphatase substrate buffer (0.1 M Tris/HCl, pH 9.5, 0.1 M NaCl and 50 mM MgCl2·6H2O). Detection of labelled proteins was with either the alkaline phosphatase substrate NBT (nitrobue tetrazolium chloride)/BCIP (5-bromo-4-chloro-3-indolylphosphate p-toluidine salt) (100 mg of NBT was dissolved in 1.3 ml 70% dimethyl formamide (DMF), 100 mg of BCIP in 2 ml 100% DMF; for 5 ml of substrate solution 22 µl of NBT and 16 µl of BCIP were added to 5 ml of distilled water) or Fast Blue/Naphthol AS-MX Phosphate (100 mg of Fast Blue was dissolved in 10 ml of 70% DMF, 100 mg of Naphthol Phosphate in 10 ml of 100% DMF; for 5 ml of substrate solution 150 µl of Fast Blue and 100 µl of Naphthol AS-MX Phosphate were added to 4.75 ml of distilled water). The reactions were halted after 10-15 min by rinsing the blots in distilled water.

2.3 Results

2.3.1 Testing serum for development of maize anti-p34^cdc2 antigen

Serum from the rabbit injected with conjugated maize p34^cdc2 peptide was screened by Western analysis to determine if there had been any development of antibody to the peptide. The second serum sample, removed one week after the third injection (Table 2.2), identified a protein at 34 kDa (Figure 2.1, lane 2) which was visible when the serum was diluted to 1/500 and aligned with p34^cdc2 detected by the anti-PSTAIR antibody (Figure 2.1, lane 1). This suggested that the protein detected by the rabbit serum at 34 kDa was p34^cdc2. This was confirmed by the elimination of the band at 34 kDa when serum pre-competed with maize p34^cdc2 peptide was used to probe
the blot (Figure 2.2, lane 2). Approximately two weeks after the last injection of p34\textsuperscript{cdc2} peptide the titre of antibody was judged to be optimal and large quantities of serum were collected to provide a pool of antibody that could be purified. Figure 2.3 shows the Western blot analysis of the third and fourth collections at dilutions up to 1/5000.

2.3.2 Protein-A column purification of maize anti-p34\textsuperscript{cdc2} antibody

Protein-A column chromatography was utilised to purify an IgG fraction from whole serum containing anti-p34\textsuperscript{cdc2} antibody (Figure 2.4). An IgG purified antibody fraction that identified maize p34\textsuperscript{cdc2} was clearly isolated (Figure 2.4, lanes 1-7). A small though distinct decrease in the level of p34\textsuperscript{cdc2} in samples taken before (lane 8) and after (lane 9) passage through the column indicates that the column was binding p34\textsuperscript{cdc2}.

2.3.3 DEAE-Sephacel purification of maize whole serum anti-p34\textsuperscript{cdc2} antibody

As a consequence of problems encountered with the serum purified with a protein-A column, new serum was purified by a DEAE-Sephacel purification procedure. After initial batch purification, which removed mass impurities, maize whole serum p34\textsuperscript{cdc2} antibody was loaded onto a DEAE-Sephacel column. The elution profile (Figure 2.5) shows several substantial protein peaks one of which (*) contained the purified antibody. Selected fractions were run on a gel, transferred, then stained for proteins using Ponceau S (Figure 2.6). The putative IgG was seen as protein of 50-60 kDa (Figure 2.6, lanes 4-9). This band aligns with standard IgG immunoglobulin (lane 10). Fractions were tested on Western blots to determine their capacity to detect p34\textsuperscript{cdc2}. (Figure 2.7). In all selected fractions detection of a band at 34 kDa was distinctly evident even at an antibody dilution of 1/100. The band aligned with that detected by the PSTAIR (lane 1) and whole serum anti-p34\textsuperscript{cdc2} (lane 2) antibody tracks (Figure 2.7) indicating that the p34\textsuperscript{cdc2} antibody was being recovered. The strength of the 34 kDa band when purified fractions were used to probe the blot compares well to the p34\textsuperscript{cdc2} protein detected by unpurified anti-p34\textsuperscript{cdc2} antibody suggesting that the
column was working efficiently at purifying anti-p34\textsuperscript{cdc2} antibody from the whole serum.

### 2.3.4 Immunoaffinity purification of maize anti-p34\textsuperscript{cdc2} antibody using an antigen column

#### 2.3.4.1 Construction of an antigen column

To further purify the p34\textsuperscript{cdc2} antibody which had been obtained by DEAE-Sephacel purification an antigen column was constructed by binding p34\textsuperscript{cdc2} peptide to a carrier then to CNBr-Sepharose. The initial step was to couple the carrier protein, thyroglobulin, to MHS, for later reaction with peptide. Excess MHS reagents were removed using a G25 column. Two protein peaks were observed (Figure 2.8a) the first of these (V\textsubscript{o}) containing the coupled MHS/thyroglobulin was retained. Prepared peptide was then added to the pooled V\textsubscript{o} fractions. Unbound peptide was removed by passing the coupled peptide/MHS/thyroglobulin complex through a G25 column. One broad peak was recovered (Figure 2.8b), which contained the peptide/MHS/thyroglobulin complex, the latter fractions of which were discarded as containing a high proportion of free peptide.

Samples of the peptide/MHS/thyroglobulin complex were taken both before and after coupling and analysed by Western blotting. The detection of peptide coupled to CNBr-Sepharose beads (Figure 2.9, lane 4) by anti-p34\textsuperscript{cdc2} antibody confirms that the peptide complex had indeed coupled to the CNBr beads. The specificity of the reaction between anti-p34\textsuperscript{cdc2} antibody and the coupled maize peptide is indicated by the failure of pre-immune serum to react (Figure 2.9, lane 2).

#### 2.3.4.2 Immunoaffinity purification of maize anti-p34\textsuperscript{cdc2} antibody

Initial attempts to recover antibody after binding to peptide/thyroglobulin immobilised on CNBr-Sepharose were unsuccessful whether the eluant had an acid pH as low as 2.0, or an alkaline pH as high as 12.0, and similarly unsuccessful if 4 M urea was used as eluant. Absence of any capacity to detect p34\textsuperscript{cdc2} in the column eluate
(Figure 2.10, lanes 3-5) indicated complete binding of antibody. The column was found to have a considerable capacity for anti-p34\textsubscript{cdc2} antibody even though its bed volume was only 1.7 ml since it was never saturated even by 5 ml samples of whole serum. The capacity was so great that initially very little antibody could be recovered from the column but eventually, after loading a total of about 10 ml of serum, saturation of the highest affinity sites was approached and antibody could be recovered at both pH 2.5, (Figure 2.10, lanes 6, 8 and 10) and also after the acid elutions by a switch to pH 11.5, (Figure 2.10, lanes 7, 9 and 11).

2.3.4.3 Protein-A column purification of immunoaffinity purified whole serum maize anti-p34\textsubscript{cdc2} antibody

As some protein impurities remained in the sera, even after immunoaffinity purification (Figures 2.10), it was decided to run some affinity purified anti-p34\textsubscript{cdc2} through the protein-A column. Three fractions of immunoaffinity purified maize anti-p34\textsubscript{cdc2} antibody were passed through a protein-A column, all three gave basically the same result (Figure 2.11). A distinct band at 34 kDa could be seen, (Figure 2.11, lanes 5-7) with little background evident.

2.4 Discussion

The aim of the work described in this chapter was to generate an antibody specific for maize p34\textsubscript{cdc2}. An antibody was successfully raised and then purified using protein-A column, DEAE ion chromatography and immunoaffinity purification methods.

2.4.1 Generation of an antibody specific for maize p34\textsubscript{cdc2}

A carboxyterminal sequence was used for antibody generation as this region is generally believed to be one consistently capable of acting as an antibody binding site as it is synthesised last and is thought to be often left exposed on the protein surface. Furthermore significant species specific variation has been identified in the tail of the
carboxyterminal region, in contrast to the high homology exhibited by most of the internal p34\textsuperscript{cdc2} sequence between diverse plant species (Feiler and Jacobs, 1990; Colasanti et al., 1991; Hirt et al., 1991; Hong et al., 1993; Miao et al., 1993), suggesting that perhaps a truly p34\textsuperscript{cdc2} specific p34\textsuperscript{cdc2} antibody could be generated by using this region.

It was necessary to couple a large carrier molecule to the peptide in order to generate an immunogenic response as the peptide was effectively too small to engender a successful response on its own. Maize p34\textsuperscript{cdc2} peptide was coupled to KLH by glutaraldehyde. Glutaraldehyde was chosen for the simplicity of the coupling method and the stability of the cross linking, and KLH was chosen because it is a large alien immunogenic molecule successfully used in antibody generation. An antibody to the injected coupled peptide in maize was successfully generated (Figures 2.1-2.3).

As part of the purification process p34\textsuperscript{cdc2} peptide was again coupled to a carrier to create an antigen column. It was essential to use a different carrier molecule, in this case thyroglobulin, otherwise many antibodies to KLH would have been recovered as contaminants. MHS was used as the coupling reagent with thyroglobulin as it allowed predictable coupling of peptide to carrier. The stability of MHS has also been shown to be excellent in comparison to other coupling reagents (Peeters et al. 1989).

A hyperimmunisation strategy was followed, with large dose injections of peptide occurring approximately every two weeks (Table 2.1) in an attempt to generate a maize specific anti-p34\textsuperscript{cdc2} antibody. This approach was successful, and the progression of p34\textsuperscript{cdc2} antibody development was monitored by Western blot analysis of sera (Figures 2.1-2.3).

2.4.2 Purification of the anti-p34\textsuperscript{cdc2} antibody

Purification of antibody was performed to increase specificity, particularly as p34\textsuperscript{cdc2} is a rare protein believed to represent no more than 0.05% of the total protein (Draetta et al., 1988b; John et al., 1989). Several purification methods were utilised, some in conjunction with each other, in an effort to obtain pure, concentrated antibody that would be useful for an investigation of the distribution and activity of maize p34\textsuperscript{cdc2} protein.
Maize anti-p34\textsuperscript{cdc2} antibody was purified by protein-A column chromatography (Figure 2.4), DEAE ion chromatography (Figure 2.7), immunoaffinity purification using an antigen column (Figures 2.10), and lastly protein-A column purification of immunoaffinity purified whole serum (Figure 2.11). With respect to the antigen column it appeared that once all the high affinity sites had been filled, it was possible to elute purified antibody from low affinity sites. These sites appeared to bind and retain p34\textsuperscript{cdc2} when whole serum was passed through the column, but in contrast to the high affinity sites the p34\textsuperscript{cdc2} could be eluted from these low affinity sites. In all cases a band of protein at 34 kDa, located at the same level as the band of p34\textsuperscript{cdc2} protein seen in anti-PSTAIR and whole serum anti-p34\textsuperscript{cdc2} antibody probed tracks, was detected by the purified fractions eluted from the columns by acid and alkali.

Lastly it was observed that all purification methods achieved a decrease in the background caused by reaction with non-p34\textsuperscript{cdc2} proteins. This demonstrated that the columns were largely effective in their purification of the p34\textsuperscript{cdc2} antibody from among the many proteins present in whole serum.

2.4.3 Conclusions

In conclusion a polyclonal antibody specific to p34\textsuperscript{cdc2} for maize was successfully generated by standard immunological techniques. Several techniques were employed to purify the antibody from whole serum including protein-A column, DEAE ion chromatography and immunoaffinity purification. The capacity of this antibody to detect and immunorecover native p34\textsuperscript{cdc2} protein is investigated in work described in Chapters 3 and 4. It is formally possible that the antibody might recognise other plant CDKs, however, the variability of carboxyterminal regions of known cdcs mitigates against this possibility, which cannot in practise be tested until plant CDKs have been isolated and sequenced. Such cross-reactivity of antibodies to carboxytermini of CDKs and cdcs from animal and fungal systems has not proved to be a problem.
Figure 2.1
Detection of p34\textsuperscript{cd2} protein in a Western blot of maize proteins using specific maize anti-p34\textsuperscript{cd2} antibody.
Soluble proteins were extracted from maize root tissue in RIPA buffer (see 3.2.2, p 46). The proteins were separated on 12% SDS-PAGE and then transferred to nitrocellulose. The blot was probed using anti-PSTAIR and anti-p34\textsuperscript{cd2} antibody, an alkaline phosphatase conjugated secondary antibody (Tago Inc., USA) was used for detection of labelled proteins.
Lane 1: p34\textsuperscript{cd2}-like proteins detected by anti-PSTAIR antibody
Lane 2: proteins detected by anti-p34\textsuperscript{cd2} antibody (1/500), a band of protein at 34 kDa aligns with the p34\textsuperscript{cd2} protein detected by the anti-PSTAIR antibody (arrow)

Figure 2.2
Detection of p34\textsuperscript{cd2} protein in a Western analysis of serum after the final injection of maize p34\textsuperscript{cd2} peptide, which was eliminated when the serum was pre-competeted with p34\textsuperscript{cd2} peptide.
Proteins extracted from maize root tissue in RIPA buffer were run on a 12% acrylamide gel then blotted onto nitrocellulose.
Lane 1 was probed with anti-p34\textsuperscript{cd2} antibody, lane 2 with anti-p34\textsuperscript{cd2} antibody competed to 1 \textmu M with p34\textsuperscript{cd2} peptide at 25°C for 90 min and lane 3 with anti-PSTAIR antibody. The secondary antibody used was alkaline phosphatase conjugated (Tago Inc., USA).
Lane 1: p34\textsuperscript{cd2} protein detected at 34 kDa (arrow) with anti-p34\textsuperscript{cd2} antibody
Lane 2: elimination of reaction with p34\textsuperscript{cd2} protein when p34\textsuperscript{cd2} antibody was pre-competed
Lane 3: p34\textsuperscript{cd2}-like and p34\textsuperscript{cd2} protein detected with anti-PSTAIR antibody

Figure 2.3
Western blot analysis of capacity of serum to detect p34\textsuperscript{cd2} protein in representative bleeds.
Maize root protein extract was electrophoresed (12% SDS-PAGE) and the separated proteins transferred to nitrocellulose. Bulk collections of serum from rabbit injected with p34\textsuperscript{cd2} peptide were used to probe for p34\textsuperscript{cd2} protein. Labelled proteins were detected with an alkaline phosphatase conjugated secondary antibody (Tago Inc., USA).
Lane 1: p34\textsuperscript{cd2} protein (arrow) detected by the anti-PSTAIR antibody, that aligns with p34\textsuperscript{cd2} detected at 34 kDa by the anti-p34\textsuperscript{cd2} antibody
Lanes 2-5: p34\textsuperscript{cd2} protein detected at 34 kDa by anti-p34\textsuperscript{cd2} antibody (third substantial serum collection) diluted by 1/500 (lane 2), 1/1000 (lane 3), 1/2000 (lane 4), 1/5000 (lane 5)
Lanes 6-9: p34\textsuperscript{cd2} protein detected at 34 kDa by anti-p34\textsuperscript{cd2} antibody (fourth substantial serum collection) diluted by 1/500 (lane 6), 1/1000 (lane 7), 1/2000 (lane 8), 1/5000 (lane 9)
**Figure 2.4**

Western blot showing detection of p34\(^{cdc2}\) by whole serum anti-p34\(^{cdc2}\) antibody after purification with a protein-A column.

Soluble proteins were isolated from maize root tissue in RIPA buffer (see 3.2.2, p 46), separated on 12% SDS-PAGE, then transferred to nitrocellulose. Whole serum anti-p34\(^{cdc2}\) antibody was purified by passage through a protein-A column, the bound IgG proteins were eluted at pH 3.5, and 3.0. The purified serum was used to probe the blot, labelled proteins were detected using an alkaline phosphatase conjugated secondary antibody (Tago Inc., USA).

Lanes 1 and 2: p34\(^{cdc2}\) protein (arrow) detected at 34 kDa by purified anti-p34\(^{cdc2}\) antibody eluted from the column at pH 3.5

Lanes 3-7: p34\(^{cdc2}\) protein detected at 34 kDa by purified anti-p34\(^{cdc2}\) antibody eluted from the column at pH 3.0

Lane 8: whole serum anti-p34\(^{cdc2}\) antibody before passage through protein-A column

Lane 9: whole serum anti-p34\(^{cdc2}\) antibody after passage through protein-A column

Lane 10: p34\(^{cdc2}\) and p34\(^{cdc2}\)-like protein detected by anti-PSTAIR antibody
Figure 2.5
Profile of protein concentration in eluate from DEAE-SephaGel column.
Whole serum anti-p34\textsuperscript{cdc2} antibody was first purified by batch DEAE ion chromatography then loaded onto a DEAE-SephaGel column, and the bound proteins were eluted by an increasing salt gradient. The absorbance of fractions was monitored at 280 nm. The large central peak (*) was determined by Coomassie Brilliant Blue staining of proteins to contain IgG and by Western blot analysis to contain purified anti-p34\textsuperscript{cdc2} antibody.
Fractions eluted from DEAE column by NaCl gradient
Figure 2.6
Protein retained after DEAE batch purification (lanes 2, 3) and passage through DEAE-Sephacel column (lanes 4-9) shown by Ponceau S staining after blotting. Whole serum anti-p34\(^{cdc2}\) antibody was purified by batch DEAE chromatography then the purified fraction was passed through a DEAE-Sephacel column. The bound proteins were eluted by an increasing NaCl gradient. Proteins in fractions of high A\(_{280}\) were separated on 12% SDS-PAGE then transferred to nitrocellulose which was stained with Ponceau S.

Lane 1: proteins in whole serum anti-p34\(^{cdc2}\) antibody after dialysis against 5 mM NaPO\(_4\), pH 6.5
Lanes 2 and 3: proteins in batch DEAE purified anti-p34\(^{cdc2}\) antibody, before (lane 2) and after (lane 3) dialysis against 10 mM Tris, pH 8.5
Lanes 4-9: protein in fractions of the central peak (*) (Figure 2.5) that were eluted from DEAE-Sephacel column with increasing NaCl gradient
Lane 10: IgG protein at \(~55\) kDa that aligns with the proteins recovered after DEAE purification

Figure 2.7
Western blot analysis showing detection of p34\(^{cdc2}\) protein by DEAE purified anti-p34\(^{cdc2}\) antibody.
Whole serum maize anti-p34\(^{cdc2}\) antibody was purified by batch and column DEAE ion chromatography. Fractions containing the purified antibody were used to probe maize root tissue proteins for the presence of p34\(^{cdc2}\) protein. The labelled protein was detected with an alkaline phosphatase conjugated secondary antibody (Tago Inc., USA).
Lane 1: p34\(^{cdc2}\) protein detected at 34 kDa (arrow) by anti-PSTAIR antibody, is observed to align with protein at 34 kDa detected by purified anti-p34\(^{cdc2}\) antibody
Lane 2: p34\(^{cdc2}\) protein detected by whole serum anti-p34\(^{cdc2}\) antibody
Lane 3-6: p34\(^{cdc2}\) protein detected by fraction 23 eluted from DEAE column containing purified anti-p34\(^{cdc2}\) antibody, undiluted (lane 3), diluted to 1/100 (lane 4), 1/50 (lane 5), 1/25 (lane 6)
Lanes 7-10: p34\(^{cdc2}\) protein detected by fraction 24 eluted from DEAE column containing purified anti-p34\(^{cdc2}\) antibody, undiluted (lane 7), diluted to 1/25 (lane 8), 1/50 (lane 9), 1/100 (lane 10)
Lanes 11-14: p34\(^{cdc2}\) protein detected by fraction 25 eluted from DEAE column containing purified anti-p34\(^{cdc2}\) antibody, undiluted (lane 11), diluted to 1/25 (lane 12), 1/50 (lane 13), 1/100 (lane 14)
Figure 2.8
Profile of proteins when thyroglobulin/MHS complex (a) and p34\textsuperscript{cd2} peptide/MHS/thyroglobulin complex (b) were eluted from G25 column.

To create an antigen column a carrier protein, thyroglobulin, was coupled first to MHS then to p34\textsuperscript{cd2} peptide finally the peptide/MHS/thyroglobulin complex was coupled to CNBr-Sepharose. The two stages of coupled reagents were cleansed of excess uncoupled reagents by separation on G25 columns. When thyroglobulin/MHS was passed through a G25 column (a) the first peak (Vo) contained the coupled complex, the fractions from this peak were then mixed with p34\textsuperscript{cd2} peptide and passed through a G25 column, the broad peak seen in (b) contained the coupled peptide/MHS/thyroglobulin.
8. (a)

Protein fractions eluted from G25 column by 0.1 M PO$_4$

8. (b)

Protein fractions eluted from G25 column by 0.1 M NaCO$_3$, pH 8.3
Figure 2.9
Western blot showing detection of p34<sup>cdc2</sup> peptide/MHS/thyroglobulin complex by anti-p34<sup>cdc2</sup> antibody (lane 4), which was not recognised by pre-immune serum (lane 2). p34<sup>cdc2</sup> peptide was coupled to thyroglobulin by MHS as previously described (methods 2.2.7). Peptide before and after coupling to thyroglobulin was run on 5-20% SDS-PAGE, then transferred to nitrocellulose. Pre-immune and anti-p34<sup>cdc2</sup> antibody were used to probe the blot, labelled protein was detected with an alkaline phosphatase conjugated secondary antibody (Tago Inc., USA).
Lane 1: MHS/thyroglobulin probed on the blot with pre-immune serum
Lane 2: p34<sup>cdc2</sup> peptide/MHS/thyroglobulin probed on the blot with pre-immune serum
Lane 3: MHS/thyroglobulin probed on the blot with anti-p34<sup>cdc2</sup> antibody
Lane 4: p34<sup>cdc2</sup> peptide/MHS/thyroglobulin probed on the blot with anti-p34<sup>cdc2</sup> antibody. The p34<sup>cdc2</sup> peptide/MHS/thyroglobulin complex can be seen as a diffuse band of high molecular weight at the top of the gel

Figure 2.10
Western blot showing detection of p34<sup>cdc2</sup> protein (lanes 6-11) by immunoaffinity purified anti-p34<sup>cdc2</sup> antibody derived from whole serum. Whole serum anti-p34<sup>cdc2</sup> antibody, that had been dialysed and spun, was loaded onto the antigen column. Proteins bound to the column were eluted by washing at pH 2.5. The column was then brought to a neutral pH before an alkaline elution was performed at pH 11.5. The fractions eluted from the column were used to probe a blot of maize proteins, which had been electrophoresed (12% SDS-PAGE) then blotted onto nitrocellulose. Labelled proteins were detected with an alkaline phosphatase conjugated secondary antibody (Tago Inc., USA).
Lane 1: p34<sup>cdc2</sup> protein detected at 34 kDa (arrow) by anti-PSTAIR antibody
Lane 2: p34<sup>cdc2</sup> protein detected by whole serum anti-p34<sup>cdc2</sup> antibody
Lanes 3-5: proteins detected by whole serum anti-p34<sup>cdc2</sup> antibody after passage through antigen column in three successive experiments (lanes 3-5 respectively). Note absence of p34<sup>cdc2</sup> at 34 kDa indicating retention of anti-p34<sup>cdc2</sup> antibody by the column
Lanes 6 and 7: p34<sup>cdc2</sup> detected by antibodies eluted at pH 2.5 and pH 11.5, respectively from the first experiment. Antibody tested at 1/100
Lanes 8 and 9: as in lanes 6 and 7 but from second experiment
Lanes 10 and 11: as in lanes 6 and 7 but from third experiment and antibody tested at 1/50
Figure 2.11

Immunoblot showing detection of p34\textsuperscript{cdc2} protein (lanes 5-7) by antibody purified first with antigen then with protein-A.

Affinity purified anti-p34\textsuperscript{cdc2} antibody eluted by pH change from antigen column, that had been dialysed, spun and filtered, was loaded onto the protein-A column, which was then washed with 4 ml of buffer. Proteins bound to the column were eluted at pH 3.0. The fractions eluted from the protein-A column were used to probe a blot of maize proteins, which had been separated on a 12% acrylamide gel, then transferred to nitrocellulose. Labelled proteins were detected with an alkaline phosphatase conjugated secondary antibody (Tago Inc., USA).

Lane 1: p34\textsuperscript{cdc2} protein detected at 34 kDa (arrow) by anti-PSTAIR antibody
Lane 2: p34\textsuperscript{cdc2} protein detected by whole serum anti-p34\textsuperscript{cdc2} antibody
Lane 3: p34\textsuperscript{cdc2} protein detected by affinity purified anti-p34\textsuperscript{cdc2} antibody before passage through protein-A column
Lane 4: proteins detected by affinity purified anti-p34\textsuperscript{cdc2} antibody after passage through protein-A column, note absence of p34\textsuperscript{cdc2}
Lane 5: p34\textsuperscript{cdc2} detected by antibody eluted from protein-A column at pH 3.0, diluted to 1/20 (first experiment)
Lane 6: as in lane 5 but from second experiment and antibody diluted to 1/30
Lane 7: as in lane 5 but from third experiment
11.

(kDa)

-43

-30

1 2 3 4 5 6 7
CHAPTER 3:

CHARACTERISATION OF MAIZE ANTI- p34^{cdc2} ANTIBODY -
ASSESSMENT OF CAPACITY TO DETECT NATIVE AND
DENATURED FORMS OF p34^{cdc2} USING DOT BLOTS
3.1 Introduction

The experiments described in this chapter were designed to complement the immunofluorescence studies described in Chapter 6. Dot blot assays were used to assess the effect of procedures used during preparation of specimens for immunofluorescence, upon the interaction of \( p34^{\text{cdc2}} \) with the specific anti-\( p34^{\text{cdc2}} \) antibody. The objectives were: 1) to determine whether the antibody that had been found to detect \( p34^{\text{cdc2}} \) protein, that had been unfolded by SDS in Western blotting (Chapter 2), could also detect \( p34^{\text{cdc2}} \) that had been immobilised by paraformaldehyde (PFA) cross-linking, 2) to determine whether \( p34^{\text{cdc2}} \) enzyme neither fixed nor denatured could be recognised, and 3) to assess the extent to which \( p34^{\text{cdc2}} \) could be retained during processing of fixed cells for immunostaining and also to assess the possibility that the epitope targeted by the antibody could be made more accessible by unfolding the \( p34^{\text{cdc2}} \) with SDS at some point prior to antibody reaction.

3.1.1 Diverse antigenic nature of polyclonal antibodies

Polyclonal antibodies are characterised by the presence of antibodies with different sites of recognition (epitopes) on the antigen. Presumably the polyclonal maize anti-\( p34^{\text{cdc2}} \) antibody serum that was generated in work described in Chapter 2 conforms to this generalisation. The capacity for polyclonal antibodies to identify a variety of epitope recognition sites on a single antigen potentially allows polyclonal serum to identify the target protein in both its denatured and native conformations. The size of the sub-populations of individual polyclonal sera antibodies varies and so the potential antibody/antigen interaction of each new antibody is unknown. The maize anti-\( p34^{\text{cdc2}} \) antibody described in Chapter 2 was repeatedly shown there to identify the denatured form(s) of maize \( p34^{\text{cdc2}} \). However its potential to identify the native form of maize \( p34^{\text{cdc2}} \) remains unknown. The creation of a specific higher plant \( p34^{\text{cdc2}} \) antibody opens opportunities for an investigation of \( p34^{\text{cdc2}} \) separate from the closely related CDKs (cyclin dependent protein kinases). Furthermore an antibody capable of identifying native protein and thus useable for immunoprecipitation, immunorecovery
and immunocytobiology techniques could be a valuable reagent for cytological and biochemical studies of this key protein.

3.1.2 Epitope recognition by antibodies generated from peptide

Many antibodies raised against peptides exhibit good identification of the denatured antigenic protein but often will not recognise the native form of the protein (Harlow and Lane, 1988). Recognition of the native protein by antibody is dependent upon the conformation of the peptide sequence displayed on the surface of the native molecule being similar to that of the peptide/carrier conjugate. Success therefore depends on the location and conformation in the target protein of the peptide used as the antigen. A carboxylterminal sequence was selected for the generation of a p34<sup>cdc2</sup> antibody in this study in the hope that this region, which is often exposed on the outside of the protein, would generate antibody with the capacity to identify both the denatured and native protein forms. To identify p34<sup>cdc2</sup> protein, native or denatured, the anti-p34<sup>cdc2</sup> antibody was used as a probe in a modified Western blot analysis method.

3.1.3 Dot blot assay of antigen retention and antibody interaction

In the dot blot technique, protein samples are dotted directly onto dry nitrocellulose strips. The proteins bind immediately to the nitrocellulose which may then be blocked and processed as for a Western blot.

There is an advantage in avoiding electrophoresis because fixing tissue cross links proteins with the result that they do not run freely in electrophoresis and also undenatured proteins will not predictably dissociate from bound subunit proteins, nor have a dependable negative charge in the absence of SDS, therefore again the electrophoretic mobility is unpredictable. Use of dot blot therefore allows study of fixed proteins and undenatured proteins in a way not possible by conventional SDS-PAGE and Western blot analysis. For the dot blot method 2 µl volumes of maize protein were found to give areas of equal size when applied to nitrocellulose. Quantification of the amounts of p34<sup>cdc2</sup> protein identified by antibody was then made possible by the use of an ¹²⁵I-labelled secondary antibody.
3.2 Materials and methods

3.2.1 Plant material

Large quantities of *Zea mays* (cv. miracle) seed were planted in 260 x 320 mm plastic boxes onto a 60 mm deep layer of moistened vermiculite then overlaid with 10-20 mm of vermiculite. Large sheets of foil were placed over the boxes to prevent excess moisture loss yet still allow oxygen exchange. The boxes were then placed into a darkened 26°C constant temperature room for approximately 72 h. After 72 h the majority of seeds had germinated and the average root length was approximately 50 mm. The seedlings were harvested and washed to remove any excess vermiculite. The tip 10 mm was cut from each root using a scalpel blade. This region was separated into an apical 3 mm portion containing the root meristem and a 7 mm portion containing the elongation zone. These meristem and elongation zone sections were placed separately in 1.5 ml eppendorf tubes, frozen with liquid nitrogen and stored at -70°C. An equal proportion of root tips was processed as above but in addition was fixed in 4% PFA (paraformaldehyde) in 50 mM potassium phosphate/ 5 mM EGTA buffer, pH 7.0, for 1-2 h then washed in water three times for a total of 30 min prior to freezing with liquid nitrogen.

3.2.2 Protein extraction from maize root cells

Unfixed and fixed maize root meristem and elongation zone tissue was ground to powder in liquid nitrogen using a mortar and pestle. Protein was extracted from the ground tissue by vigorously mixing it with RIPA buffer, pH 7.4, in eppendorf tubes, using a vortex mixer, three times over 1 min at 0°C. RIPA buffer consisted of 20 mM Tris/HCl, pH 7.4, 5 mM EDTA, 100 mM NaCl, 0.1% Tween 20, 1 μM dithiothreitol (DTT), 10 μM pepstatin A, 10 μM leupeptin, 1 mM NaF, 1 mM EGTA, 1 mM sodium pyrophosphate and 12 μM β-glycerophosphate. Immediately prior to use 1 mM sodium orthovanadate and 200 μM phenylmethylsulfonyl fluoride (PMSF) were added to complete the buffer. The protein extract, after mixing, was centrifuged at 14,000 rpm
for 10 min. The supernatant containing the soluble extracted protein was retained avoiding any particles.

For electrophoresis (2.2.8) equal volumes of protein extract and Sx2 SDS Laemmli electrophoresis buffer (containing 125 M Tris/HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 2% (v/v) 2-mercaptoethanol and 0.002% bromophenol blue) were mixed and boiled for 2 min.

Quantification of protein in the extracts was done by both the Coomassie Brilliant Blue (3.2.3.1) and Ponceau S stain (3.2.3.2) assays.

### 3.2.3 Quantification of protein concentration in maize root extracts

#### 3.2.3.1 Coomassie Brilliant Blue assay

Protein concentration was quantified by an assay at 595 nm (Spector, 1978) in which 200 µl of sample was added to 2 ml of dye reagent. The dye reagent was made by dissolving 0.05 g of Coomassie Brilliant Blue G250 in 25 ml 95% ethanol and mixing thoroughly, after which 50 ml 85% phosphoric acid was added and the solution made up to 500 ml with water. Ovalbumin was used as a standard.

#### 3.2.3.2 Ponceau S stain assay

The method outlined in detail in Zhang et al. (1996) operates by the gel electrophoresis (2.2.8) then transfer to nitrocellulose of 20 µl protein samples (including standard markers of known weight) which are then stained with Ponceau S stain (2.2.10). The protein-bound stain was then eluted from the nitrocellulose in eluting buffer (50 mM Tris, pH 9.3) and assayed at 518 nm.
3.2.4 Maize Western dot blot experiments

3.2.4.1 Detection of p34\textsuperscript{cd2} protein by the maize anti-p34\textsuperscript{cd2} antibody in unfixed and fixed maize root tissue

This method was used to compare p34\textsuperscript{cd2} protein detected in unfixed maize meristem and elongation zone tissue to that detected in the same tissues after fixation.

Soluble proteins were extracted into RIPA buffer by vortexing. Typically 0.05 g of meristem tissue was extracted into 150 µl of RIPA, or 0.1 g of ground up elongation zone tissue into 75 µl of RIPA buffer. The supernatant was removed by spinning the tissue samples for 10 min at 14,000 rpm at 4°C in an Eppendorf bench centrifuge. The retained supernatant contained all the immediately soluble proteins in the tissue. The pellet that was left after the removal of the supernatant was also retained, it was resuspended in an amount of RIPA buffer equal to the volume of supernatant removed. The pellet sample represented the protein remaining after most of the soluble protein was removed. These pelleted proteins approximate to the residual proteins in cells that have been permeabilised for antibody labelling. One final set of fixed and unfixed meristem and elongation zone tissue samples were vigorously vortexed in RIPA buffer but not centrifuged. These were retained in this slurry state as representative of the total protein. All samples were kept on ice, while strips of 0.45 µm nitrocellulose (70 x 20 mm) were prepared and labelled, then the protein samples were prepared for application as dots onto the nitrocellulose.

Preparation of the protein fractions for dotting involved first the readjustment of protein quantity between the fractions so that they were approximately equal and second the exposure of the protein extracts to a protein denaturing agent. To equalise the protein contents of the soluble, total slurry and pellet protein fractions from fixed and unfixed maize meristem and elongation zone tissue the following protocol was used; 9 µl of extracts derived from meristem tissue were diluted by the addition of 11 µl of RIPA buffer. After dilution of the meristem samples, all samples (~ 20 µl in volume), whether derived from fixed or unfixed meristem or elongation zone tissue, were divided into two. To one set of samples 10 µl of modified Sx2 electrophoresis buffer (0.125 M Tris, pH 6.8, 0.5% SDS, 20% glycerol, 2% 2-mercaptoethanol, 0.002% bromophenol
blue) was added. The remaining 10 µl of undenatured protein extract was retained to represent the protein in its native state. This was also diluted by 50% with 0.125 M Tris buffer, pH 6.8.

2 µl of each sample was applied to nitrocellulose laid on Nescofilm, then the protein was left to dry for 10 min at room temperature. The nitrocellulose strips were stained with Ponceau S, blocked and analysed by standard (2.2.10) and 125I (3.2.5) Western immunodetection methods. Whole serum (1/500) and affinity purified maize anti-p34<sup>cdc2</sup> antibodies (at 1/50) were used to probe the blots. Duplicate samples were probed with affinity purified p34<sup>cdc2</sup> antibody pre-competed with maize p34<sup>cdc2</sup> peptide to a concentration of 1 µM.

### 3.2.4.2 Effect of denaturation with SDS and presence of protease inhibitors on the retention of p34<sup>cdc2</sup> and its detection by antibody in the particulate fraction from root meristem

The persistence of detectable p34<sup>cdc2</sup> protein was investigated at different stages during processing of fixed meristem tissue for immunostaining (see 6.2.2.1) when protein was either denatured after fixation but before cell wall digestion (protocol A) or else denatured after both fixation and wall digestion (protocol B in diagram below).

First the effect of denaturing protein with SDS after fixation but before cell wall digestion was tested together with the effects of including protease inhibitors during cell wall digestion (protocol A). Soluble protein was extracted from 0.05 g of ground fixed meristem tissue into 300 µl of RIPA buffer by vortexing. The supernatant was removed by spinning the tissue at 14,000 rpm for 10 min at 4°C. The pellet was retained, resuspended in 300 µl of RIPA and divided in two. The supernatant containing the soluble proteins was discarded in these experiments. Half of the resuspended pellet sample (of approximately 150 µl volume) was mixed with an equal volume of modified Sx2 (SDS) electrophoresis buffer, to test the effect of SDS on antigenicity, and 2 µl dotted onto nitrocellulose (20 x 125 mm). The sample with SDS was then spun for 2 min at 14,000 rpm and 2 µl of the supernatant was then dotted to assess material solubilised from the pellet by SDS. The retained pellet was then resuspended by vortexing in 300 µl of cold TBS buffer. Then 2 µl of the resuspended pellet in TBS was
dotted onto the nitrocellulose strip to measure the residual p34\text{cdc2} after SDS extraction. The remaining pellet sample still in TBS was then divided and the two samples separately spun down at 14,000 rpm for 2 min. The cell wall material in one of the retained pellets was then digested with 1% cellulysin (Calbiochem-Novabiochem, USA) in 50 mM potassium phosphate/5 mM EGTA buffer, pH 7.0, and the second with the same enzyme with protease inhibitors 4 µg/ml aprotinin, 2 µg/ml pepstatin A, 1 µg/ml leupeptin, 0.1 µM benzamidine, 0.02 mg/ml soybean trypsin inhibitor and 1 mM PMSF, for 20 min at room temperature. Samples were taken to test survival of p34\text{cdc2} during digestion. The pellet sample that had been digested in the presence of protease inhibitors was further investigated to determine whether the cellulysin enzyme mixture had solubilised the peptide, by centrifugation and sampling the supernatant and the pellet after it was suspended in TBS. The effect of washing the pellet was then investigated by washing twice with PBS buffer. After the final wash 2 µl of the resuspended pellet sample was dotted for the last time onto the nitrocellulose. The dotted protein was then left to dry at room temperature.

A parallel experiment summarised in protocol B in the diagram below investigated the effect of denaturing protein after both fixation and cell wall digestion had been completed. The method was as described above except SDS was not added to the pellet first obtained from RIPA extract but was added after treatment with cellulysin in the absence of protease inhibitors.

The protein samples that had been dotted onto nitrocellulose, after air drying, were placed directly into blocking solution, with shaking, at 4°C overnight. After blocking the strips were processed for immunodetection of p34\text{cdc2} protein using as the primary antibody affinity purified anti-p34\text{cdc2} antibody at a dilution of 1/50. The blots were processed by standard (2.2.10) and \textsuperscript{125}I Western immunodetection procedures (3.2.5).
resuspended pellet split into two equal halves

(A)  

equal volume S×2 (SDS) added  
2 µl dotted  
↓  
spin, remove supernatant  
2 µl dot of supernatant  
↓  
resuspend pellet in TBS  
2 µl dotted  
↓  
divide resuspended pellet into two  

spin, resuspend pellet in enzyme + protease inhibitors, 20 min  
2 µl dotted  
↓  
spin, remove supernatant  
2 µl dotted  
↓  
resuspend pellet in TBS  
2 µl dotted  
↓  
spin, resuspend pellet in PBS, repeat wash once  
2 µl dotted  

(B)  

2 µl dotted  
↓  
spin, resuspend pellet in enzyme, 20 min  
2 µl dotted  
↓  
spin, remove supernatant  
2 µl dot of supernatant  
↓  
resuspend pellet in RIPA  
2 µl dotted  
↓  
equal volume S×2 (SDS) added  
2 µl dotted  
↓  
spin, remove supernatant  
2 µl dotted  
↓  
resuspend pellet in TBS  
2 µl dotted  
↓  
spin, resuspend pellet in PBS, repeat wash once  
2 µl dotted
3.2.5 ¹²⁵I Western blotting and immunodetection

The method was identical to that described for Western blotting (2.2.10) until the application of the secondary antibody (anti-rabbit Ig, ¹²⁵I-labelled species specific whole antibody (from donkey) (Amersham International, UK) diluted to 0.5 µCi/ ml with TBS, 1% BSA, 0.2% Tween, 0.1% NaN₃) which was laid over the nitrocellulose blots in a shallow box with a lid. The blots were incubated for 1 h at room temperature in a fume hood with shaking. After 1 h the secondary antibody was removed and the blots washed three times with TBS/0.2% Tween for a total of 30 min. After the final wash the nitrocellulose blots were placed onto 3MM paper to dry at room temperature for 30 min. When the blots were dry they were stuck down onto 3MM paper, covered with cling film that was stretched smooth and taped into place, then exposed in a Phosphorimage cassette for one day at room temperature. The image was scanned using a Molecular Dynamics Phosphorimager Model 400B and the dots analysed using the Image Quant and Microsoft Excel programs.

3.3 Results

3.3.1 Detection of p34cdc2 immobilisation by cross linkage due to paraformaldehyde fixation and retention of antibody recognition

One of the intended uses of the antibody characterised in work described in the preceding chapter was to probe fixed cells to determine the location of p34cdc2. It was therefore desirable to investigate the extent to which the soluble p34cdc2 enzyme could be immobilised by the PFA fixation procedure applied to plant tissue and therefore may be retained in its in vivo location. Additionally information was sought concerning whether the fixed enzyme could still be detected by the antibody.

To assess the cross linkage of p34cdc2 to other proteins the electrophoretic mobility of the enzyme was assessed after fixation since cross linkage should increase its size and reduce penetration into the gel. Also the amount of p34cdc2 protein that could be recovered in the 14,000 rpm supernatant could be reduced if cross-linked to cytoskeletal or other structures attached to membranes.
Figure 3.1 shows that the soluble extract of unfixed tissue from either meristem or elongation zone regions (lanes 1, 2) contains p34\textsubscript{cdc2} that is entirely free to move after SDS dissociation into the electrophoretic gel. Approximately equal loading of protein was compared in lanes 1 and 2 and also in lanes 3 and 4 based upon Coomassie and Ponceau S protein assays (methods 3.2.3). In contrast with the solubility of p34\textsubscript{cdc2} seen in extract of unfixed cells the total protein suspension (slurry) derived from fixed tissue showed a preponderance of proteins trapped at the top of the gel due to cross linkage into multi-molecular complexes that could not be broken even by boiling with SDS in preparation of the electrophoretic samples. p34\textsubscript{cdc2} was also largely cross linked in this way from evidence of the considerable reduction in the amount at 34 kDa. Additional evidence of p34\textsubscript{cdc2} cross linkage was seen in the almost complete transfer of the protein into the 14,000 rpm sedimented (pellet) fraction and its elimination from the soluble supernatant (lanes 5, 6).

Since much of the p34\textsubscript{cdc2} in extract from fixed cells becomes immobilised at the top of the gel it was considered that electrophoresis based upon Western blotting was inappropriate for study of the enzyme from fixed cells.

3.3.2 Antibody detection of p34\textsubscript{cdc2} in extracts of unfixed and fixed cells and increased detection by denaturation with SDS

Dot blotting was adopted to investigate whether antibody can detect: 1) p34\textsubscript{cdc2} protein when unfixed and undenatured, as when freshly extracted from plant cells, 2) fixed and undenatured as in some potential immunostaining methods for use on cells, and also 3) whether fixed p34\textsubscript{cdc2} can be made more accessible to antibody by denaturation.

Roots were selected as a source of meristematic and differentiating cell tissue and the reaction of antibody with p34\textsubscript{cdc2} from these tissues was investigated with and without fixation. The effect of fixation in cross-linking and immobilising p34\textsubscript{cdc2} was further assessed by examination of soluble, total and pelleted protein fraction. The latter fraction was considered a possible parallel to fixed cells permeabilised for antibody probing.
Figure 3.2 (quantified in Figure 3.3 and 3.4) shows that throughout the signal is essentially due to specific retention of p34\textsuperscript{cdc2}, as the p34\textsuperscript{cdc2} signal was eliminated by pre-competition with 1 µM peptide (in Figures 3.2 and 3.3 compare Ap with A, Bp with B, Cp with C, Dp with D, Ep with E and Fp with F) and quantification of Figure 3.3 (see Figure 3.4) shows that the decrease in signal is tenfold. Previous experiments have tested the ability of the antibody to bind p34\textsuperscript{cdc2} after it has been unfolded with SDS and this experiment shows that the antibody can also detect p34\textsuperscript{cdc2} never treated with detergent or fixative (Figures 3.2 and 3.3, treatments 1 and 3 in panels B, D and F). Detection was not dependent upon any effect of acidic Ponceau S protein stain since omission of the stain did not affect the signal. Furthermore the PFA fixed but not otherwise modified p34\textsuperscript{cdc2} is also detected by the antibody (treatments 1 and 3 in panels A, C and E).

To test the possibility that p34\textsuperscript{cdc2} prevented from unfolding by cross-linking or blocked by associated proteins, particularly in the pelleted fraction from fixed cells, might be made more accessible to the antibody by denaturation the effect of applying 0.5% SDS and 2% 2-mercaptoethanol (Sx2 modified electrophoresis buffer) was tested. SDS-containing samples were of the same 2 µl volume but spread over a much larger area. Tests showed that binding to nitrocellulose was not reduced provided the concentration of SDS was not above 0.5%. The intensity of antibody reaction was increased three fold by SDS treatments of the pelleted fraction from both fixed and unfixed cells (Figure 3.2 compare E1 sample without SDS to E2 sample treated with SDS from fixed tissue and F1 with F2 from unfixed tissue). No increase of the antibody reaction due to SDS was seen with soluble proteins (e.g. Figure 3.2, compare A1 with A2) indicating that it is associated proteins that can be dislodged by SDS that partially mask p34\textsuperscript{cdc2} in the pelleted fraction.

This analysis also provided information on the abundance of p34\textsuperscript{cdc2} relative to other proteins in the meristem and elongation zone. The p34\textsuperscript{cdc2} protein was seen at a higher level in the meristem region, comparing equal amounts of extracted protein whether assayed without SDS treatment (compare in Figure 3.4b, 1A with 3A) or with SDS treatment (compare in Figure 3.4b, 2A with 4A). The ratios in these cases are 2 fold and 1.2 fold greater amounts of p34\textsuperscript{cdc2} in the meristem. This differential accumulation of p34\textsuperscript{cdc2} is considered in more detail in Chapter 5.
The observation that SDS can increase the antibody capability to detected p34\textsuperscript{cdc2} particularly in the pellet fraction was of interest since the protein in this material may be in a state that approximates to that in permeabilised fixed cells.

### 3.3.3 Investigation of SDS denaturation as a component of an immunostaining procedure

The previous experiment proved that SDS denaturation increased the access of antibody to p34\textsuperscript{cdc2}, particularly in the pelleted fraction of plant extracts from fixed tissue. Since this fraction approximates to that surviving in cells prepared for antibody probing the possible value of SDS in such a procedure was assessed. In particular the qualitative retention of p34\textsuperscript{cdc2} was investigated when SDS was used either before or after cell wall digestion. The sequence of treatments when the pelleted protein fraction was denatured before cell wall digestion is shown in the diagram below.

- resuspend pellet
- ↓
- add equal volume Sx2 (SDS)
- sample 1
- ↓
- spin, remove supernatant
- sample 2
- ↓
- resuspend pellet in TBS
- sample 3
- ↓
- divide resuspended pellet into two
  - spin, resuspend
  - pellet in enzyme + protease inhibitors,
  - 20 min
  - ↓
  - sample 5
  - ↓
  - spin, remove supernatant
  - sample 6
When the protein fraction was denatured after fixation but before cell wall digestion (Figure 3.5, panel A) the p34\textsuperscript{cdk2} protein level declined extensively by leaching from the pellet fraction into the soluble fraction on first application of 0.5% SDS and 2% MSH (compare samples 2 and 3 in Figure 3.5, panel A). Cell wall digestion increased access of the antibody to the p34\textsuperscript{cdk2} protein (Figure 3.5, panel A samples 3 and 4), and the increase was greater if protease inhibitors were included (Figure 3.5, panel A compare 3 and 5). However the p34\textsuperscript{cdk2} was removed almost completely in later washes (Figure 3.5, panel A, 6-8). In contrast when the initial protein sample was digested by cell wall degrading enzymes first and then denatured by mixing with modified Sx2 electrophoresis buffer containing 0.5% SDS and 2% MSH (see diagram below for the sequence of treatments) the levels of detected p34\textsuperscript{cdk2} protein persisted during processing. In fact the level of detectable p34\textsuperscript{cdk2} appeared to increase during the process, particularly after addition of SDS and MSH suggesting that this was enabling better access for the antibody. It must be noted, though, that very high levels of p34\textsuperscript{cdk2} appeared in the supernatant (Figure 3.5, panel B sample 6) that was to be discarded after exposure to SDS and MSH indicating a substantial solubilisation of p34\textsuperscript{cdk2} on first exposure to SDS. The residual p34\textsuperscript{cdk2} protein in the insoluble fraction fell further during washing (Figure 3.5, panel B samples 7 and 8). This pattern of accessibility and persistence of p34\textsuperscript{cdk2} was confirmed quantitatively using an \textsuperscript{125}I secondary antibody (Figure 3.6a, b). This conclusively demonstrates that denaturation after fixation and cell wall digestion does preserve more p34\textsuperscript{cdk2}, with almost twice as much being retained as compared to when tissue was denatured prior to cell wall digestion (although the level rapidly declined in both).
3.4 Discussion

An aim of the work in this chapter was to assess the capability of the maize p34\textsuperscript{cdc2} antibody to detect the denatured and native forms of the p34\textsuperscript{cdc2} protein in fixed and unfixed meristem and elongation zone tissue of the maize root tip. In addition, the possibility of increasing access of the antibody to p34\textsuperscript{cdc2} that is present in the particulate fraction of plant material was studied with a view to refining procedures for permeabilisation of cells prior to antibody probing.
3.4.1 Detection of p34\textsuperscript{cdc2} in unfixed and fixed root protein extracts using the anti-p34\textsuperscript{cdc2} antibody and investigation of the effect of denaturation with SDS

The antibody identified p34\textsuperscript{cdc2} protein in extracts of all tissues including unfixed, undenatured tissue indicating that the anti-p34\textsuperscript{cdc2} antibody or a portion of the antibody is capable of identifying native p34\textsuperscript{cdc2}. Identification of the native p34\textsuperscript{cdc2} protein by the antibody opens avenues for research into p34\textsuperscript{cdc2} distribution and activity in higher plants. The addition of 0.5% SDS and 2% MSH increased p34\textsuperscript{cdc2} detection substantially, indicating that without these agents perhaps only a small fraction of the native maize p34\textsuperscript{cdc2} is detected. The fact that the increase due to SDS and MSH was considerably greater for particulate material indicates that the main effect may have been due to detachment of associated proteins with which the p34\textsuperscript{cdc2} associates \textit{in vivo}, so allowing greater access of antibody. Nevertheless as the antibody detects p34\textsuperscript{cdc2} not only in unfixed, undenatured tissue protein fractions, but in all fixed protein tissue fractions as well, the antibody should be capable of locating any p34\textsuperscript{cdc2} associations to the maize cell cytoskeleton when it is used to immunostain fixed material.

The quantitative analysis of the p34\textsuperscript{cdc2} protein detected by the specific anti-p34\textsuperscript{cdc2} antibody allows some conclusions to be drawn about where in the maize root the p34\textsuperscript{cdc2} is and how it may act. These data suggest that, in general, slightly higher levels of p34\textsuperscript{cdc2} are present in the meristem than in elongation zone tissue fractions in the maize root. This conforms with previous studies on higher plants (John et al., 1990; Gorst et al., 1991) that have shown that the level of p34\textsuperscript{cdc2} is at its highest when associated with actively dividing cells.

3.4.2 Investigation of the effect of SDS denaturation on survival of maize p34\textsuperscript{cdc2} during processing of fixed meristem tissue for immunostaining

The standard immunostaining protocol (6.2.2.1) that has been used extensively in trialling the immunodetection capabilities of the maize anti-p34\textsuperscript{cdc2} antibody in Chapter 6, was used here to assess the survival of p34\textsuperscript{cdc2} in a form that remained capable of reacting with the antibody. The effects of initially adding a denaturing agent,
then digesting with or without protease inhibitors as compared to completion of cell wall digestion first then addition of a denaturing agent were monitored.

The addition of 0.5% SDS and 2% MSH to the pellet fraction increased antibody detection substantially, particularly when applied after completion of both the fixation and cell wall digestion steps, presumably by dislodging bound proteins so that access of the antibody to its epitope is improved. Interestingly, digestion of the protein slurry with a cell wall degrading enzyme also increased the p34\textsuperscript{cdc2} signal suggesting that digestion results in greater access of the antibody to the epitope. Inclusion of protease inhibitors appeared to increase p34\textsuperscript{cdc2} detection in the standard Western dot blot indicating that proteases were active during the processing of the tissue and that their activity alters the antibody epitope sites. Quantification of the p34\textsuperscript{cdc2} protein detected using an 125\textsuperscript{I} secondary antibody confirmed that digestion of the protein increased the p34\textsuperscript{cdc2} signal but only by a minimal amount. The inclusion of protease inhibitors appeared to have little quantifiable effect.

Lastly, significant losses of p34\textsuperscript{cdc2} protein were observed in both methods when the tissue samples were washed with TBS/PBS buffers at the end of the protocol, indicating that the remaining p34\textsuperscript{cdc2} was not tightly bound in the tissue. This gives cause for concern about the validity of immunofluorescence localisations.

At the end of processing the level of p34\textsuperscript{cdc2} detected when the denaturation step was introduced after the completion of cell wall digestion was close to twice that observed when the tissue was denatured before cell wall digestion. This suggests that the later the denaturation of protein occurs the more p34\textsuperscript{cdc2} that is retained in the tissue, perhaps a result of less opportunity for protein solubilisation. This difference could prove to be crucial when using the antibody for immunolabelling of maize tissue.

### 3.4.3 Conclusions

The capability of the specific maize anti-p34\textsuperscript{cdc2} antibody, generated in Chapter 2, to detect denatured and native forms of the p34\textsuperscript{cdc2} protein was assessed by utilisation of a Western dot blot technique. A distinct p34\textsuperscript{cdc2} signal was identified in unfixed, undenatured tissue indicating that the antibody was capable of detecting native p34\textsuperscript{cdc2} protein. The anti-p34\textsuperscript{cdc2} antibody also identified p34\textsuperscript{cdc2} protein in fixed tissue. When
fixed tissue was processed in preparation for immunostaining, denaturation of the protein after completion of cell wall digestion was found to lead to a higher retention of the p34\textsuperscript{cdc2} protein in the tissue, than when protein was denatured before cell wall digestion. These data suggest that the p34\textsuperscript{cdc2} antibody can be used for the immunorecovery of the p34\textsuperscript{cdc2} and also to investigate the localisations of the p34\textsuperscript{cdc2} protein in fixed cell material.
Figure 3.1
Western blot showing the detection of p34<sup>cdc2</sup> in soluble (lanes 1, 2, 5, and 6), total slurry (lanes 3 and 4) protein fractions from unfixed (lanes 1 and 2) and fixed (lanes 3-6) maize root meristem and elongation zone tissue using the maize anti-p34<sup>cdc2</sup> antibody.

0.5 g of unfixed and fixed maize root meristem tissue was thawed in RIPA buffer, vortexed, then the soluble protein fraction was removed by centrifugation. Total protein slurry from meristem tissue was prepared by thawing tissue in RIPA buffer and vortexing and retaining the whole sample. Identical fractions were prepared from elongation zone tissue. Proteins extracted from meristem tissue were adjusted for equal protein content to the elongation zone based on quantification of protein in these fractions by the Coomassie Brilliant Blue assay. The extracted proteins were separated on 12% SDS-PAGE, transferred to nitrocellulose then probed with anti-p34<sup>cdc2</sup> antibody at a dilution of 1/500. The labelled proteins were detected with an alkaline phosphatase conjugated secondary antibody (Tago Inc., USA).

Lane 1: p34<sup>cdc2</sup> protein in the soluble protein fraction extracted from unfixed meristem tissue
Lane 2: p34<sup>cdc2</sup> protein in the soluble protein fraction extracted from unfixed elongation zone tissue
Lane 3: p34<sup>cdc2</sup> protein in the total slurry fraction extracted from fixed meristem tissue
Lane 4: p34<sup>cdc2</sup> protein in the total slurry fraction extracted from fixed elongation zone tissue
Lane 5: p34<sup>cdc2</sup> protein in the soluble protein fraction extracted from fixed meristem tissue
Lane 6: p34<sup>cdc2</sup> protein in the soluble protein fraction extracted from fixed elongation zone tissue

The detection of p34<sup>cdc2</sup> protein by the antibody at 34 kDa (arrow) is distinct in all lanes except that containing the fixed meristem soluble protein fraction.
Figure 3.2
Dot blot analysis of p34^cdc2 protein detection in fractions from maize root containing soluble proteins (panels A and B), total protein slurry (panels C and D) and pellet proteins (panels E and F). Within each category, protein was derived from either meristem tissue (1 and 2 throughout) or elongation zone tissue (3 and 4 throughout). Samples 2 and 4 throughout were treated with 0.5% SDS and 2% MSH before dotting onto nitrocellulose. Samples from tissue fixed with 4% PFA before grinding and extraction are shown in panels A, C and E and from tissue not fixed before extraction in panels B, D and F. Panels designated (p) were treated with antibody pre-competited with 1 µM antigen peptide for 90 min at 25°C.
The meristem protein fractions were adjusted to approximately equal protein content with the elongation zone tissue. All samples were divided equally, 10 µl of modified Sx2 electrophoresis buffer (containing 0.5% SDS and 2% MSH) was added to one half, 10 µl of 0.125 M Tris buffer to the other. 2 µl of each sample was dotted onto nitrocellulose strips. The labelled proteins were detected with an alkaline phosphatase conjugated secondary antibody (Tago Inc., USA).

Dot 1: p34^cdc2 protein in meristem tissue
Dot 2: p34^cdc2 protein in denatured meristem tissue
Dot 3: p34^cdc2 protein in elongation zone tissue
Dot 4: p34^cdc2 protein in denatured elongation zone tissue
Panels A and B: p34^cdc2 protein detected in soluble protein fractions of fixed (panel A) and unfixed (panel B) tissue
Panels Ap and Bp: protein detected in soluble protein fractions of fixed (panel Ap) and unfixed (panel Bp) tissue when competed anti-p34^cdc2 antibody was used to probe the blot
Panels C and D: p34^cdc2 protein detected in total slurry protein fractions of fixed (panel C) and unfixed (panel D) tissue
Panels Cp and Dp: protein detected in total slurry protein fractions of fixed (panel Cp) and unfixed (panel Dp) tissue when competed anti-p34^cdc2 antibody was used to probe the blot
Panels E and F: p34^cdc2 protein detected in pellet protein fractions of fixed (panel E) and unfixed (panel F) tissue
Panels Ep and Fp: protein detected in pellet protein fractions of fixed (panel Ep) and unfixed (panel Fp) tissue when competed anti-p34^cdc2 antibody was used to probe the blot
Figure 3.3
Phosphorimage of dot blot analysis of p34\(^{cd2}\) protein detection in fractions from maize root containing soluble proteins (panels A and B), total protein slurry (panels C and D) and pellet proteins (panels E and F). Within each category, protein was derived from either meristem tissue (1 and 2 throughout) or elongation zone tissue (3 and 4 throughout). Samples 2 and 4 throughout were treated with 0.5% SDS and 2% MSH before dotting onto nitrocellulose. Samples from tissue fixed with 4% PFA before grinding and extraction are shown in panels A, C and E and from tissue not fixed before extraction in panels B, D and F.

Identical soluble, total slurry and pellet protein fractions from fixed and unfixed maize root meristem and elongation zone tissue (to those depicted in Figure 3.2) were prepared and dotted onto nitrocellulose as described there. Panels designated (p) were treated with antibody pre-competed with p34\(^{cd2}\) peptide to 1 \(\mu\)M for 90 min at 25\(^\circ\)C. The labelled proteins were detected with a \(^{125}\)I labelled anti-rabbit Ig whole secondary antibody (Amersham Int., UK).

Dots 1-4: carry the same samples as Dots (1)-(4) respectively in Figure 3.2
Panels A-F: carry the same samples as Panels (A)-(F) respectively in Figure 3.2
Panels Ap-Fp carry the same samples as Panels (Ap)-(Fp) respectively in Figure 3.2
3.

A  B

Ap  Bp

C  D

Cp  Dp

E  F

Ep  Fp
Figure 3.4

Analysis of levels of $p34^{cdc2}$ in maize root meristem and elongation zone tissue by quantification of bound isotope in Phosphorimage of $p34^{cdc2}$ protein detection in soluble, total slurry and pellet protein fractions (Figure 3.3).

(a) $p34^{cdc2}$ protein in soluble protein fractions

<table>
<thead>
<tr>
<th>Data set 1</th>
<th>meristem tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Data set 2</td>
<td>denatured meristem tissue</td>
</tr>
<tr>
<td>Data set 3</td>
<td>elongation zone tissue</td>
</tr>
<tr>
<td>Data set 4</td>
<td>denatured elongation zone tissue</td>
</tr>
</tbody>
</table>

Data set A: $p34^{cdc2}$ protein detected in unfixed tissue

Data set B: protein detected in unfixed tissue when pre-competed anti-$p34^{cdc2}$ antibody was used to probe the blot

Data set C: $p34^{cdc2}$ protein detected in fixed tissue

Data set D: protein detected in fixed tissue when pre-competed anti-$p34^{cdc2}$ antibody was used to probe the blot

The lanes are itemised below:

1A: $p34^{cdc2}$ protein in soluble protein fraction extracted from unfixed meristem
1B: protein detected when soluble protein fraction extracted from unfixed meristem was probed with competed anti-$p34^{cdc2}$ antibody
1C: $p34^{cdc2}$ protein in soluble protein fraction extracted from fixed meristem
1D: protein detected when soluble protein fraction extracted from fixed meristem was probed with competed anti-$p34^{cdc2}$ antibody

2A: $p34^{cdc2}$ protein in denatured soluble protein fraction extracted from unfixed meristem
2B: protein detected when denatured soluble protein fraction extracted from unfixed meristem was probed with competed anti-$p34^{cdc2}$ antibody
2C: $p34^{cdc2}$ protein in denatured soluble protein fraction extracted from fixed meristem
2D: protein detected when denatured soluble protein fraction extracted from fixed meristem was probed with competed anti-$p34^{cdc2}$ antibody

3A: $p34^{cdc2}$ protein in soluble protein fraction extracted from unfixed elongation zone
3B: protein detected when soluble protein fraction extracted from unfixed elongation zone was probed with competed anti-$p34^{cdc2}$ antibody
3C: $p34^{cdc2}$ protein in soluble protein fraction extracted from fixed elongation zone
3D: protein detected when soluble protein fraction extracted from fixed elongation zone was probed with competed anti-$p34^{cdc2}$ antibody

4A: $p34^{cdc2}$ protein in denatured soluble protein fraction extracted from unfixed elongation zone
4B: protein detected when denatured soluble protein fraction extracted from unfixed elongation zone was probed with competed anti-$p34^{cdc2}$ antibody
4C: $p34^{cdc2}$ protein in denatured soluble protein fraction extracted from fixed elongation zone
4D: protein detected when denatured soluble protein fraction extracted from fixed elongation zone was probed with competed anti-$p34^{cdc2}$ antibody

(b) $p34^{cdc2}$ protein in total slurry protein fractions

Data set 1-4: carry the same samples as Data set (1)-(4) in Figure 3.4a

Data set A-D: carry the same samples as Data set (A)-(D) in Figure 3.4a

(c) $p34^{cdc2}$ protein pellet protein fraction

Data set 1-4: carry the same samples as Data set (1)-(4) in Figure 3.4a, b

Data set A-D: carry the same samples as Data set (A)-(D) in Figure 3.4a, b
4. (a) $p34^{cd2}$ in soluble fractions

4. (b) $p34^{cd2}$ in total slurry fractions

4. (c) $p34^{cd2}$ in pellet fractions
Figure 3.5
Dot blot analysis of the effect of denaturation with SDS before cell wall digestion, with and without protease inhibitors (panel A), and after cell wall digestion, without protease inhibitors (panel B), on the retention of p34\textsuperscript{cdc2} and its detection by anti-p34\textsuperscript{cdc2} antibody in the pellet protein fraction of fixed root tip meristem.

Pellet protein was extracted from fixed maize root by centrifugation and resuspended in an equal amount of RIPA buffer. The resuspended pellet was divided equally, one half of the tissue was denatured immediately by the addition of modified Sx2 (0.5% SDS + 2% MSH) electrophoresis buffer, the other half after the tissue had undergone cell wall digestion. Both tissue samples were processed as for immunostaining (methods 6.2.2.1). At various steps during processing 2 µl of the tissue samples were dotted onto nitrocellulose. The two nitrocellulose blots were probed with affinity purified maize anti-p34\textsuperscript{cdc2} antibody and detected with alkaline phosphatase conjugated secondary antibody (Tago Inc., USA).

Panel A, Dot 1: resuspended pellet plus Sx2 (0.5% SDS, 2% MSH) electrophoresis buffer
Dot 2: supernatant discarded after SDS addition
Dot 3: pellet resuspended in TBS buffer
Dot 4: cell wall digestion of half of pellet sample without protease inhibitors
Dot 5: cell wall digestion of half of pellet sample with protease inhibitors
Dot 6: supernatant discarded after cell wall digestion with protease inhibitors
Dot 7: pellet resuspended in TBS buffer
Dot 8: pellet resuspended after PBS buffer washes

Panel B, Dot 1: pellet resuspended in RIPA buffer
Dot 2: cell wall digestion of pellet
Dot 3: supernatant discarded after cell wall digestion of pellet
Dot 4: pellet resuspended in RIPA buffer
Dot 5: resuspended pellet plus Sx2 (0.5% SDS, 2% MSH) electrophoresis buffer
Dot 6: supernatant discarded after SDS addition
Dot 7: pellet resuspended in TBS buffer
Dot 8: pellet resuspended after PBS buffer washes

Figure 3.6
Phosphorimage (a) and analysis (b) of dot blot showing effect of denaturation with SDS and cell wall digestion, with and without protease inhibitors, on retention of p34\textsuperscript{cdc2} and its detection by anti-p34\textsuperscript{cdc2} antibody in the particulate protein fraction from fixed root meristem shown in Figure 3.5.

An identical pellet protein fraction was extracted from fixed maize root tissue, prepared and dotted as described for Figure 3.5. The nitrocellulose strips were probed with affinity purified maize anti-p34\textsuperscript{cdc2} antibody and protein detected with a \textsuperscript{125}I labelled anti-rabbit Ig whole secondary antibody (Amersham Int., UK) (a). Analysis of the bound isotope in (a) produced the distributions shown in (b).

Panel A; Dots 1-8: carry the same samples as Panel (A); Dots (1)-(8) respectively in Figure 3.5
Panel B; Dots 1-8: carry the same samples as Panel (B); Dots (1)-(8) respectively in Figure 3.5
5.

6. (a)

6. (b)

p34\textsuperscript{cd} protein detected using \textsuperscript{125}I labelled secondary antibody: Phosphorimage counts (x10\textsuperscript{5})
CHAPTER 4:

ASSESSMENT OF THE CAPACITY OF ANTI-p34\textsuperscript{cdc2} ANTIBODY TO RECOVER NATIVE MAIZE p34\textsuperscript{cdc2} PROTEIN
4.1 Introduction

4.1.1 Reaction of polyclonal anti-C-terminal antibody with native maize p34\textsuperscript{cdc2}

Antibodies in a polyclonal serum can bind to multiple sites on an antigen. Experimental investigation is required to determine whether polyclonal antibodies raised against a chemically synthesised peptide that has been conjugated with a heterologous protein to become antigenic will recognise the native protein, of which the peptide forms a small part. Anti-peptide antibodies, such as the maize anti-p34\textsuperscript{cdc2} antibody used in the present work, often bind well to denatured proteins but may not recognise the native protein, if the peptide is not exposed at the surface of the molecule (Harlow and Lane, 1988). In the present study the peptide used was at the carboxyterminus and this, being synthesised last, often lies at the outside of a protein that will have begun to fold while being synthesised. Furthermore the Western dot blot experiments described in Chapter 3 appear to indicate that our antibody, or specifically a fraction of our antibody, is indeed capable of detecting maize p34\textsuperscript{cdc2} in its native form. The implications of this result are far-reaching, as it provides the opportunity of immunoprecipitation and assessment of the distribution of p34\textsuperscript{cdc2} independent of other CDK protein kinases that may be present in plants. It also provides the opportunity for specific immunofluorescence staining.

4.1.2 Immunoprecipitation

Immunoprecipitation when used in conjunction with SDS-PAGE is capable of assessing the quantity and relative molecular weight of the antigen (Harlow and Lane, 1988). Polyclonal antibodies have advantages for precipitation because they bind to multiple sites on the antigen. Further, if the immune complexes are collected on a solid phase, such as protein-A beads, the availability of multiple binding sites enables the formation of particularly stable multimeric antigen-antibody-protein-A complexes.

Two major factors must be taken into consideration when utilising polyclonal antibodies for immunoprecipitation. Firstly, the concentration of the antigen in the original extract and secondly, the affinity of the antibody for the antigen. When
immunoprecipitation is coupled with SDS-PAGE it becomes possible to detect rare cellular proteins. This method is capable of concentrating proteins to the order of 100,000 to 1,000,000 fold. Higher plant p34\textsuperscript{cdc2} can be categorised as a rare protein, as it has been shown to be present in plants, in similar concentrations to its counterparts in other eukaryotes (John et al., 1989) for which the levels of p34\textsuperscript{cdc2} are 0.02-0.05% (Draetta et al., 1988b).

The second factor affecting success in immunoprecipitation is the affinity of the antibody for the antigen, as the immune complex must form when the antigen is low in concentration. Purity of the recovered protein may be reduced by binding of non-specific proteins during the formation of immune complexes. Fortunately this effect may be minimised by offering the smallest amount of antibody essential for recovery of the antigen. In addition, as the immune complex is stable, non-specific background problems may often be reduced by stringent washing.

In the present study minimum amounts of antibody were used and washing procedures were optimised and in particular antigen pre-competition was used to determine the fraction of recovered protein that was due to specific antibody reaction. The protocol that was developed took account of the following factors. Cells were broken by grinding in liquid nitrogen to minimise the effects of proteases that could degrade the antigen. Soluble protein was then extracted from the ground tissue using buffer containing a cocktail of protease inhibitors (RIPA buffer see 3.2.2) that included pepstatin A, leupeptin, and PMSF. Additionally, the purity of the product was increased by pre-clearing of the lysate with pre-immune serum and protein-A beads.

The Western dot blot experiments described in Chapter 3 indicated that our specific anti-p34\textsuperscript{cdc2} antibody can detect native maize p34\textsuperscript{cdc2}, which suggested that the antibody might immunoprecipitate native p34\textsuperscript{cdc2} in extracts from maize tissue, particularly as the immunoprecipitation method has the potential to detect rare proteins. This chapter reports the results of this investigation.
4.2 Materials and methods

4.2.1 Plant material, growth and protein extraction

*Zea mays* (cv. miracle) was planted and germinated exactly as previously described in 3.2.1 However, for this series of immunoprecipitation experiments no distinction between meristem and elongation zones was made. To derive a large amount of tissue that contained many dividing cells the apical 5 mm of maize roots was excised using a scalpel blade, frozen in liquid nitrogen and ground to a powder in a mortar and pestle prior to storage at -70°C until proteins were extracted into RIP A buffer (3.2.2).

4.2.2 Antibody preparations

The specific maize anti-p34\(^{cd2}\) antibody that we had raised was used in two purified forms; “affinity-purified” denotes antibody that had been affinity purified using a column containing carboxyterminal peptide coupled to thyroglobulin with MHS and conjugated to CNBr-Sepharose (2.2.7). The second form was “affinity-purified IgG” which denotes the IgG fraction purified from affinity purified antibody by passage through a protein-A column (2.2.5).

4.2.3 Recovery of p34\(^{cd2}\)-immune complex with protein-A beads

A method was devised that minimised non-specific recovery by pre-clearing extract with pre-immune serum that was then removed with protein-A beads, then a specifically bound fraction recovered by immune antibodies was determined as protein displaced from binding by pre-competition of the antibody with peptide antigen.

Soluble proteins were extracted into cold RIP A buffer by vortexing. Typically 0.4 g of ground tissue was extracted into 1,200 µl of RIPA (as two lots of 0.2 g each in 600 µl). A supernatant was recovered by spinning the tissue for 10 min at 14,000 rpm at 4°C in a Eppendorf bench centrifuge and was held briefly on ice until pre-cleared.

Pre-clearing was performed by adding 10 µl of pre-immune serum from the individual rabbit that yielded the immune serum and incubating it in ice for 60 min.
The pre-immune serum was removed by reaction for 1 h, with gentle rotation at 4°C, with 100 µl of 50% (v/v) protein-A Sepharose 4B (Sigma Chemical Company, USA). The protein-A Sepharose was washed twice by suspension in RIPA before use and was recovered by centrifugation at 14,000 rpm for 10 min.

Protein in the pre-cleared extract that reacted specifically with antibody was identified as being present in the fraction recovered with affinity-purified antibody, but not in control treatments with the same antibody after pre-competition with p34\textsuperscript{cdc2} peptide. Pre-cleared extract was divided into two 600 µl aliquots one of which received antibody that had been pre-competited (50 µl of affinity-purified antibody incubated for 1 h at 25°C with 0.5 µM peptide), while the other received an equal amount of antibody incubated with buffer before use. They were then mixed gently and placed on ice for 1 h. After 1 h, 20 µl of prepared protein-A Sepharose (50% v/v in RIPA buffer) was added to each and both were placed on a rotating wheel for 1 h at 4°C. The beads were recovered at 7,000 rpm for 5 min then resuspended in 1 ml of cold TBS, spun again as before and the supernatant discarded. This wash was repeated and the final pellets were suspended in 160 µl Sx1 buffer (Sx2 electrophoresis buffer diluted by 50% with RIPA buffer), boiled for 2 min, and then spun down at 14,000 rpm for 10 min. From each treatment 150 µl of supernatant was collected and the pellets discarded. The final supernatant samples together with a sample of whole maize extract as a positive control were then run on a 12% acrylamide gel as described in 2.2.8 and then transferred to nitrocellulose and Western blotted as described in 2.2.10. The blots were probed with affinity-purified maize anti-p34\textsuperscript{cdc2} antibody at a dilution of 1/50, in TBS/Tween/NaN\textsubscript{3}. Duplicate samples were also probed with anti-p34\textsuperscript{cdc2} antibody which had been pre-competited with maize p34\textsuperscript{cdc2} peptide at 1 µM.

Experiments compared (a) affinity purified anti-p34\textsuperscript{cdc2} antibody with the IgG fraction derived from affinity purified anti-p34\textsuperscript{cdc2} antibody, (b) the effect of decreasing the antibody to antigen ratio and (c) the level of non-specific background generated by affinity-purified compared with the IgG fraction from affinity purified p34\textsuperscript{cdc2} antibody.
4.2.4 Western blot development using enhanced chemiluminescent detection

For enhanced chemiluminescent (ECL) detection of western blots, proteins were transferred to MSI nitrocellulose (Micron Separations Inc. Nitrobind 0.45 microns), stained with Ponceau S, for 20 min, then excess stain was rinsed off with 0.1% acetic acid (2.2.10). The membrane was then blocked in 5% skim milk powder in TBS, for 1 h at room temperature, washed three times in TBS/0.05% Tween for a total of 15 min. Then the nitrocellulose membrane was incubated with anti-p34\textsuperscript{cdc2} antibody, diluted using TBS/BSA/0.01% merthiolate, for 90 min at room temperature. The blot was rinsed free of the primary antibody by two washes with TBS/0.05% Tween followed by one with TBS/0.2% Tween for a total of 30 min. The secondary antibody used was an affinity isolated F(ab') goat anti-rabbit peroxidase conjugate (Tago, Incorporated, USA) diluted to 1/8000 with TBS/BSA/merthiolate. The blot was incubated in this secondary antibody for 60 min at room temperature then washed twice with TBS/0.2% Tween, then once with detergent-free TBS. After washing, the membrane was blotted dry between several sheets of filter paper for 1-2 min before being placed onto fresh Nescofilm and chemiluminescence substrate solution (Amersham International, UK Cat. No. RPN 3000) applied for 1 min. After 1 min the membrane was again blotted dry then placed between two clear plastic sheets and secured by tape in an Amersham Hypercassette cartridge with Hyperfilm-ECL (Amersham International, UK) or X-ray film. Exposure of film to the actively luminescing blot was for 20 sec to 2 min and was optimised by trial exposure and development. Development was by standard X-ray development procedures.

4.2.5 Immunorecovery of p34\textsuperscript{cdc2} using an antibody column

Purified antibody was coupled to 1 ml CNBr-Sepharose (Pharmacia Biotech, Sweden), as previously described (2.2.7), using 5 ml of purified antibody at 1 mg/ml, dialysed against phosphate buffer, overnight at 4°C, prior to coupling. 0.8 ml of coupled beads was packed into a column (10 mm x 50 mm), washed to remove unbound protein by pumping at 400 ml h\textsuperscript{-1} approximately 20 ml of Tris, pH 7.5, then 10 ml of 100 mM glycine, pH 2.5, followed by approximately 20-30 ml of Tris pH 8.8, till the
pH was 8.8. Then 10 ml of fresh 100 mM triethylamine, pH 11.5, was run through the column. Finally the column was washed with Tris, pH 7.5, until the pH reached 7.5, then 0.01 % merthiolate was added prior to storage at 4°C.

Extract containing p34cdc2 for recovery was obtained by extracting five 0.2 g aliquots of frozen ground maize root meristem each with 600 µl of RIPA buffer, vortexing twice for 20 sec and then spinning down at 14,000 rpm for 5 min at 4°C. 600 µl of supernatant was collected from each, avoiding all pellet and pooled as three 1 ml volumes. To pre-clear the extracts 10 µl of pre-immune serum was added to each, they were then left on ice to react for 1 h. During this incubation protein-A beads were prepared. 0.05 g of dry protein-A Sepharose 4B was suspended in 333 µl of TBS/azide to give 50 % v/v and then washed twice in RIPA buffer. After washing the beads were resuspended in an equal volume of RIPA and 84 µl of protein-A beads 50 % v/v in RIPA were added to each 1 ml treatment to remove the pre-immune serum by reaction on a slowly rotating wheel for 1 h at 4°C. The samples were spun down at 14,000 rpm for 10 min and the supernatants recovered as 3 ml of pre-cleared maize extract for loading into the antibody column. The column was washed with 20 ml of 10 mM Tris pH 7.5, then 10 ml of fresh RIPA buffer before use. As extract was loaded into the column the first 20 drops containing the void volume were discarded, the remaining eluate was collected and re-passed through the column twice more. The final eluate was retained to test for possible unbound antigen. The column was then washed to remove unbound proteins with 3.5 ml of TBS buffer. The bound proteins were recovered by passing 3 ml of fresh 100 mM glycine pH 2.5, through the column by gravity. Twelve 10 drops fractions (each approximately 500 µl) were collected into eppendorf tubes containing 25 µl of 1 M Tris pH 8.0. The column was washed then with 2 ml of TBS, and left for 10 min at room temperature. Then proteins that could be eluted at alkaline but not acid pH were eluted by passing 3 ml of fresh 100 mM triethylamine, pH 11.5, through the column and fractions collected as described for acid elution above. After the alkaline elution the column was washed with 30 ml of 10 mM Tris pH 7.5, pumped at 400 ml h⁻¹. Then 10 ml of the same buffer supplemented with 0.01 % merthiolate was run through and the column plugged and stored at 4°C.

Absorbance at 280 nm was monitored in the eluted fractions and those with the highest O.D. were dialysed against 25 mM Tris, pH 7.0, in 6-8,000 MW cut-off tubing,
at 4°C overnight. The dialysed samples were run on three duplicate 12% acrylamide gels (2.2.8) and analysed by standard (2.2.10) and ECL Western blotting (4.2.4) and by Coomassie (2.2.9) and silver (4.2.6) gel staining. The primary antibody used for both the standard and ECL Westerns was affinity purified maize anti-p34<sup>cdc2</sup> (glycine pH 2.5, eluted fraction) freshly diluted to 1/50 in antibody dilution buffer (10 mM Tris, 0.15 M NaCl, 0.05% Tween, 1% BSA, 0.05% NaN<sub>3</sub>). The third gel was first Coomassie stained then destained and restained with silver.

Three variations to the standard method described above were employed in this study. The first two to investigate respectively (a) the effect of reducing the amount of antigen introduced to the column and (b) to compare the proteins recovered from a column that had been pre-competed with maize p34<sup>cdc2</sup> peptide at 100 nM. The third variation incorporated a p13<sup>sucl</sup> affinity purification of p34<sup>cdc2</sup>-like proteins from the maize extract, prior to their application to the anti-p34<sup>cdc2</sup> antibody column to test whether the column could retain the p34<sup>cdc2</sup>-like protein recovered by specific binding to the mitotic protein. Purification by p13<sup>sucl</sup> affinity binding used yeast p13<sup>sucl</sup> protein coupled to CNBr-Sepharose (p13 beads) held in a column.

To construct a p13 bead column 2 ml of beads coupled to p13, prepared by the method described in 2.2.7, were added to 10 ml of NDE (no detergent extraction) buffer (20 mM Hepes, pH 7.4, 100 mM NaCl, 15 mM DTT, 3 µg/ml leupeptin, 20 mM EGTA, pH 7.4, 80 mM β-glycerophosphate, pH 7.4, 1 mM orthovanadate, 30 mM p-nitrophenylphosphate di Na salt, 50 mM NaF, 15 mM MgCl<sub>2</sub>, 0.2 mM ammonium molybdate and 0.5 mM PMSF). The suspended beads were then spun down for 2 min at 7,000 rpm, the supernatant discarded and the wash in NDE repeated. The beads were then resuspended in an equal volume of NDE to give a 50% (v/v), transferred to a small column (7mm x 40 mm) and left to settle overnight at 4°C.

Extract for purification in the p13 column was derived from 1 g of frozen ground maize root meristem divided as five 0.2 g aliquots and each extracted in 600 µl of NDE buffer following the same method described earlier in this section for the standard column immunorecovery. The resulting 3 ml of supernatant was then passed through the p13 column three times by gravity and the unbound fraction was retained to check for removal of p34<sup>cdc2</sup>. The column was then quickly washed using 1.5 ml of HDW (high detergent wash) buffer (2 mM EDTA, pH 7.4, 150 mM NaCl, 1% NP4O, 5 µg/ml
leupeptin, 0.1 mM orthovanadate, 50 mM NaF and 10 mM NaPO₄, pH 7.0) followed by 1.5 ml of PMD buffer (PBS with 10 mM MgCl₂ and 1 mM DTT). Next the p34cdc2 bound to p13sucl immobilised in the column was eluted by exchange with free p13sucl that had previously been dialysed overnight against HMD buffer (25 mM Hepes, 10 mM MgCl₂, 1 mM DTT, pH 7.4). Free p13 at 1 mg/ml in HMD was flowed through the column and eight fractions with an approximate volume of 500 µl each were collected and their absorbances read at 280 nm. The peak fractions were then pooled to be applied to the antibody bead column which was washed and eluted as described earlier in this section.

4.2.6 Silver staining of SDS-PAGE gels

This staining method is based on that of Morrissey (1981). Gels were placed into pre-fix A (50 % methanol, 10 % glacial acetic acid in double distilled water) for a minimum of 1 h, with shaking. They were then transferred into pre-fix B (5.4% methanol, 7.6% glacial acetic acid in double distilled water) for a minimum of 1 h, with shaking. After shaking in pre-fix B the gels were washed three times in double distilled water for a minimum of 1 h each, with shaking. Next gels were washed in a DTT solution (0.75 mg DTT in 100 ml double distilled water) for 1-2 h with gentle shaking. The gels were then transferred to a silver solution (0.1 g AgNO₃ in 100 ml double distilled water) 1-2 h with gentle shaking, after which the gels were rinsed twice, quickly (about 30 sec each), with double distilled water. The stain-developing solution (13.5 g NaCO₃, 510 µl 16 % paraformaldehyde added to 450 ml of double distilled water) was then poured over the gels in three aliquots. The first one-third was swirled around and discarded when it went brown. Then the next third was added and swirled around until it started to go brown. It was then discarded and the remaining developing solution poured over the gels. The gels were rocked gently in this solution until bands became visible (1-2 min). At that point the stop solution was added before the background could get too high. This was accomplished by adding 5 g of citric acid directly to the gels still in developing solution. The gels were then left in the “stopped” developing solution, gently shaking for 30 min, after which they were rinsed in double distilled water for 1-2 h and photographed.
4.3 Results

4.3.1 Immunorecovery of p34\textsuperscript{cdc2} with protein-A beads

4.3.1.1 Affinity purified compared with IgG affinity purified antibody

Preliminary experiments were designed to check whether the p34\textsuperscript{cdc2} antibody that had been generated (Chapter 2) was capable of immunorecovering p34\textsuperscript{cdc2} protein from extracts of maize root tips.

An initial assessment involved comparison of affinity purified antibody, with the IgG fraction that could be purified from this antibody. Affinity purified antibody recovered a distinct band at 34 kDa (Figure 4.1, panel A; lanes 2, 3). This band is not present in the comparable sample which had been pre-competed during the immunoprecipitation (Figure 4.1, panel B; lanes 5, 6) nor was it visible in the uncompeted track in the duplicate blot that was probed with competed antibody (Figure 4.1 b, panel B; lanes 2, 3). These results suggest that the band at 34 kDa represents maize p34\textsuperscript{cdc2}, which has been immunorecovered. Unfortunately the band is only a little denser than background. The experiment was repeated using an IgG fraction from the affinity purified antibody and in addition, the amount of antibody used to precipitate the p34\textsuperscript{cdc2} was also decreased (Figure 4.2a). As in the initial experiment, a band at 34 kDa is visible (Figure 4.2a, panel A; lanes 2, 3) and is not present in any fractions that have been competed with maize peptide (Figure 4.2a, panel A; lanes 5, 6). From this we may conclude that this band at 34 kDa again represents immunorecovered native maize p34\textsuperscript{cdc2}. A slight reduction in background was evident with the use of the purified IgG. The background is characterised by a very strong band at approximately 50 kDa and a fainter signal from 28-50 kDa which causes the greatest interference with the band at 34 kDa. As this background is undiminished or unaltered by competition we can conclude that it represents non-specifically bound proteins.

Probing the Western blot with an \textsuperscript{125}I-labelled secondary antibody (Figure 4.2b) did not improve detection of p34\textsuperscript{cdc2} relative to background.
4.3.1.2 Testing whether excess antibody contributed to non-specific background

Three decreasing amounts of affinity purified IgG were separately added to identical maize protein extracts and the immunoprecipitation results analysed by standard (Figure 4.3a) and enhanced chemiluminescent (Figure 4.3b) Western blotting. The same pattern is basically evident from both, a faint distinct band can be seen at 34 kDa in the treatment with the greatest amount of antibody (80 µl) (Figure 4.3a, b, lane 2). It is fainter in the 40 µl antibody treatment (Figure 4.3a, b, lane 5) and lost in the 20 µl treatment (Figure 4.3a, b, lane 8). All three antibody treatments were also competed and the 34 kDa band was fully displaced (Figure 4.3a, lanes 3, 6, 9) with particular clarity in the ECL developed Western (Figure 4.3b, lanes 3, 6, 9), so we may conclude that it represents maize p34cdc2 immunorecovered by the antibody. The fact that the recovered amount of maize p34cdc2 decreases with reducing antibody indicates that the amount of antibody was limiting yield and background could not be reduced by reducing the amount of antibody present during blotting.

4.3.1.3 Source of background; rabbit protein contaminating the antibody

The persistent recovery of proteins of broad molecular weight distribution in immunoprecipitates suggested that either: 1) large numbers of maize proteins were binding non-specifically to the antibody during precipitation and then binding again during development of the Western blot, or else 2) that the antibody itself contained contaminating proteins (of a range of sizes) that were detected on the Western blot by the second antibody against rabbit serum proteins. The second possibility could be tested by performing the immunoprecipitation procedure in the absence of maize protein. The resulting recovered protein-A-antibody complex was analysed by Western blotting, using second antibody conjugated with alkaline phosphatase (Figure 4.4a) or tagged with 125I (Figure 4.4b). Both unequivocally show the high background levels previously observed in all protein-A immunoprecipitations, therefore indicating that the antibody itself must be responsible. There was an approximate halving of background when using the IgG fraction purified from the affinity purified antibody (Figure 4.4a, panels A and B, lane 2 and 4 respectively).
In contrast, in the $^{125}$I probed Western there was no detectable difference between the IgG and whole affinity purified antibodies. There was a distinct lowering of background detected by the $^{125}$I conjugated antibody when first antibody was omitted (Figure 4.4b, panel B) indicating that non-specific binding of this antibody to serum proteins contributes some background. Absence of any effect of peptide competition (Figure 4.4b, panels A and B, lanes 3, 5) confirms that none of the binding is specific for p34$^{cd2}$. Clearly most of the background signal experienced with the method of protein-A bead recovery of p34$^{cd2}$ immune complex results from proteins introduced with the antibody and not discarded prior to Western blotting. To allow the possibility of preventing these proteins entering the recovered fraction a method was developed for immobilising antibody in a column. Theoretically this method would immobilise all antibody proteins and enable washing of immuno-complexes before elution of p34$^{cd2}$ from the column for analysis.

4.3.2 Column immunorecovery

A procedure was developed using a p34$^{cd2}$ antibody column from which bound antigens were eluted by changing the pH.

Antibody was coupled to CNBr-Sepharose and care was taken to avoid overcoupling that can reduce antibody effectiveness by reducing flexibility of the tethered protein. Persistence of some free antibody after coupling (Figure 4.6a, lanes 1, 2) indicated that most coupled antibody molecules were linked at only one place and were therefore expected to retain antigen binding properties. The resulting antibody beads were packed into a column. Among proteins bound by the column a faint band at 34 kDa was seen in several fractions that were eluted (Figure 4.5a(i), lanes 5-8). This band is located at the same position as one at 34 kDa in the whole maize extract (Figure 4.5a(i), lanes 3, 9) run in parallel, however there was little difference between the level of p34$^{cd2}$ protein in the fractions taken before and after the passage of the extract through the column (Figure 4.5a(i), lanes 1, 2). This suggested that only a small proportion of the p34$^{cd2}$ in the extract is bound by the column, perhaps because only a proportion of the polyclonal antibody, even when affinity purified, is capable of binding native maize p34$^{cd2}$ and/or only a small proportion of maize p34$^{cd2}$ exposes the
carboxyterminal region to which the antibody is targeted. A silver stained gel of these fractions and of the fraction eluted from the column with the strongest protein signal (Figure 4.5a(ii)) also shows that the degree of depletion caused by passage of the extract through the column is small (Figure 4.5a(ii), lanes 1, 2). The eluted fraction however, has been immensely purified, only a few protein bands are visible, although none is located at 34 kDa (Figure 4.5a(ii), lane 3).

Using the same column another experiment in which the amount of maize protein was reduced tenfold was attempted. A large reduction in the amount of $p34^{cd2}$ in the extract after passage through the column was noticeable (Figure 4.5b). This supports the conclusion that only a small proportion of our $p34^{cd2}$ antibody binds native $p34^{cd2}$ rather than the alternative possibility, that only a small proportion of $p34^{cd2}$ is in antigenic form.

To test whether the band at 34 kDa, that was eluted (Figure 4.5a(i)) was $p34^{cd2}$, by the criterion of specific antibody reaction on Western blots, duplicates of fractions taken of the extract before and after passage through the column together with the eluted fraction with the strongest 34 kDa signal were probed with competed primary antibody (Figure 4.5c, lanes 4, 5) and uncompeted antibody (Figure 4.5c, lanes 1-3). Complete competition of the 34 kDa signal demonstrated that the protein eluted is indeed $p34^{cd2}$ protein so some native maize $p34^{cd2}$ protein was immunorecovered.

4.3.2.1 Test of specific immunorecovery by antigen pre-competition

To test more thoroughly the specificity of immunorecovery of $p34^{cd2}$ two columns were run in parallel and one of these, was pre-competed with the maize $p34^{cd2}$ peptide. The uncompeted column recovered a distinct protein band at 34 kDa in fractions that had been eluted from the column by acid or alkali (Figure 4.6a). This band, as in the initial column immunorecovery experiment, is located at the same position as the $p34^{cd2}$ band in the whole maize extract run in parallel (Figure 4.6a, lanes 5, 10), suggesting immunorecovery of $p34^{cd2}$. There is some background evident in fractions that have been eluted from the column (Figure 4.6, lanes 6-9, 11-13) but it is largely restricted to the 45-70 kDa range and therefore does not interfere with the discrimination of the band at 34 kDa. The difference in $p34^{cd2}$ concentration between
maize extract before and after passage through the column is consistent with significant retention by the column of p34\textsuperscript{cdc2} protein (Figure 4.6a, lanes 3, 4).

A duplicate blot developed using enhanced chemiluminescence (Figure 4.6b) enables even better visualisation of the results. In another gel of these samples (Figure 4.6c) silver staining showed that there was a considerable purification of protein in the fractions eluted from the column, particularly if these are compared with the fractions representing the protein content of the maize extract, before and after passage through the column. Several protein bands were still visible in the eluted fractions, more in the acid then in the alkaline. Some are located between 30 and 40 kDa and a band in two of the acid eluted fractions (lanes 8, 9) could be p34\textsuperscript{cdc2} since it coincides with the Western blot signal (Figure 4.6a, b, lanes 8, 9).

When the specificity with which the column was binding the 34 kDa protein was tested, by blocking the antibody in the column with the p34\textsuperscript{cdc2} peptide before loading the maize extract (peptide was also added to the extract and to the first column wash), it was evident that the 34 kDa protein previously retained by the uncompeteted column (Figure 4.6a, b, lanes 8, 9, 11, 12), was no longer retained (Figure 4.7a, lanes 5-7, 9-10). This indicated that it was retained by specific reaction with anti-p34\textsuperscript{cdc2} antibody. The greater contrast in the enhanced chemiluminescent Western (Figure 4.7b) decisively backs up this observation. Another gel of duplicate samples was also silver stained (Figure 4.7c). The 34 kDa region is also weakly stained, but it is difficult to be confident that this signifies detection of the p34\textsuperscript{cdc2} protein in the eluate from the uncompeteted column because staining is a little weaker overall in the gel from the competed column.

In summary the comparison of these recoveries (illustrated in Figures 4.6 and 4.7) allowed us to conclude that the 34 kDa protein we have consistently immunorecovered is indeed native p34\textsuperscript{cdc2}. As a final test p34\textsuperscript{cdc2} was partially purified, by affinity for the yeast mitotic protein p13\textsuperscript{suc1}, before offering to the column.
4.3.3 p13\textsuperscript{sucl} purified p34\textsuperscript{cd2} recovered by antibody column

Purification of maize p34\textsuperscript{cd2} from whole maize protein extract was conducted using the yeast mitotic protein p13\textsuperscript{sucl} (Moreno et al., 1989) immobilised on CNBr-Sepharose as a p34\textsuperscript{cd2} affinity column. Efficiency of maize p34\textsuperscript{cd2} binding to the column was indicated by the low level in extract that had passed through (Figures 4.8a, b, lane 2). The maize p34\textsuperscript{cd2} eluted from the column, by exchange with p13\textsuperscript{sucl} free in solution, was then run onto the antibody column. The reduction in p34\textsuperscript{cd2}, as a consequence of passage through the antibody column (Figures 4.8a, b, lane 5) indicated that the column was binding p34\textsuperscript{cd2} previously purified by p13\textsuperscript{sucl}. The bound p34\textsuperscript{cd2} protein could be eluted at alkaline pH (Figures 4.8a, b, lane 8). Several non-specifically bound proteins of 45-70 kDa present in the eluted fractions have been decreased in strength by the prior p13 column purification. Of particular note here though is that one strong band seen at approximately 55 kDa in the eluate was not evident in the purified maize p34\textsuperscript{cd2} applied to the column (Figures 4.8a, b, lane 4). This suggests that some of the background we have observed is caused by bleeding of antibody bound to the column when it is exposed to extremes of pH, in spite of the washing at acid and alkaline pHs before use (4.2.5). Duplicate samples of those probed as Westerns were also silver stained (Figure 4.8c), showing the protein purification that the p13 column attained (contrast lanes 3 and 4). However, the protein p34\textsuperscript{cd2}, which was detected by sensitive Western blotting (Figures 4.8a, b), could not be directly detected by silver staining (Figure 4.8c).

4.4 Discussion

The aim of the work described in this chapter was to determine if it was possible to immunorecover native maize p34\textsuperscript{cd2} using our affinity purified maize anti-p34\textsuperscript{cd2} antibody. Success was achieved with both immunoprecipitation, using protein-A beads to recover the immune complex, and also with antibody immobilised in a column for immunorecovery.
4.4.1 Immunorecovery of p34\textsuperscript{cdc2} with protein-A beads

Protein-A beads were successfully used to recover p34\textsuperscript{cdc2} protein in immunocomplex from the maize extract. The recovered p34\textsuperscript{cdc2} however was observed in the presence of a background of proteins that reacted in the Western blot. The Western blots were probed with a primary antibody, rabbit anti-p34\textsuperscript{cdc2}, this antibody was also used in the immunoprecipitation. The secondary antibody used in the Western blots was necessarily anti-rabbit IgG. This caused a difficulty that the secondary antibody used with the Western blots could detect antibody molecules added for immunoprecipitation, as well as antibody molecules that may have been bound during probing of the Western blots.

Virtually all of the background signal was revealed, by analysis of the reagents, in the absence of maize proteins (Figures 4.4a, b) to derive from the antibody used in the initial preparative immunoreaction. It was to be expected that IgG molecules in this antibody might still be recognised by the secondary antibody during subsequent qualitative Western blot analysis. However, it was expected that all the IgG molecules would be between 40-65 kDa in size and therefore would not interfere with detection of the 34 kDa p34\textsuperscript{cdc2} protein. Unfortunately a minor proportion of the antibody was comprised of smaller fragments of 32-36 kDa, which caused significant background in the region of interest. These fragments may have derived from the IgG molecules that in the live rabbit are in the process of protein turnover. The fragments are a very small proportion of the total IgG, but if protein-A is used to recover all IgG molecules together with any proteins that may be binding to them, then the IgG fragments are present in the final recovered fraction. Their presence causes significant background because of the sensitivity of the secondary antibody detection system in Western blotting.

4.4.2 Column immunorecovery of p34\textsuperscript{cdc2}

Immobilising the antibody that was used for the recovery of the p34\textsuperscript{cdc2} immune complex offered two advantages. First the antibody was not part of the recovered fraction as it was bound to Sepharose beads held in a column and was intended to
remain there while bound antigen was eluted by changing the pH. Second, the immune complex in the column could be washed free of non-specifically bound or absorbed proteins by the passage of buffer through the column. The antigen could then be dislodged from the specific antibody complex by pH change.

The p34\(^{\text{cdc2}}\) antibody immobilised in a column clearly retained a 34 kDa protein that was observed against a lower background (Figure 4.5a(i)) than the background that was seen when protein-A beads were used in recovery. The retention of this protein was determined to be a consequence of a specific reaction with the anti-p34\(^{\text{cdc2}}\) antibody, since pre-competition of the antibody with the p34\(^{\text{cdc2}}\) peptide, eliminated the 34 kDa protein from the fractions eluted from the column (Figure 4.7a, b) indicating that it was p34\(^{\text{cdc2}}\). This conclusion was supported by the capacity of the column to retain (Figure 4.8a, b) the 34 kDa protein that was purified by binding to p13\(^{\text{suc1}}\), which forms a physical association with p34\(^{\text{cdc2}}\) (Brizuela et al., 1987; Hayles et al., 1986b; MacNeil et al., 1991).

Protein impurities persisted in the recovered fraction. Comparison with proteins offered to the column showed that the major contaminants at 55 kDa (Figure 4.8) and 65 kDa (Figure 4.8b, c) do not derive from maize extract but are detached from the column by the extreme pHs used for elution. The column had been washed at these pHs prior to use (methods 4.2.5) but presumably the washing was incomplete. However more extensive washing at extreme pH could have led to denaturation and inactivation of the antibody. This risk, and the relatively large amounts of antibody consumed in attempted preparative recoveries, prevented the testing of more extensive or harsher washing of the antibody column before use.

Although Western blotting (and later enzyme activity assays, Chapter 5) indicated the presence of p34\(^{\text{cdc2}}\) in immunorecovered fractions the protein could not be unequivocally detected by silver staining (Figures 4.6c and 4.8c). This can be attributed to a requirement for more than 10 nanograms of protein for detection (Wray et al., 1981), compared with the low level of p34\(^{\text{cdc2}}\) protein even in dividing cells where it is at most about 0.05% of total protein (Draetta et al., 1988b; John et al., 1989), together with incomplete retention of p34\(^{\text{cdc2}}\) by the antibody column (Figures 4.5b and 4.6b).
4.4.3 Conclusions

In conclusion a column immunorecovery method has been developed that provides the most effective means of recovering native maize p34\textsuperscript{cdc2} with the currently available technology. Earlier attempts by Colasanti et al. were reported as unsuccessful in this respect (Colasanti et al., 1993). An opportunity is therefore available to obtain new data concerning the distribution and activity of p34\textsuperscript{cdc2} in parts of the maize plant, additionally the intracellular distribution of p34\textsuperscript{cdc2} can be probed with antibody now known to be capable of binding maize p34\textsuperscript{cdc2}. These matters are taken up in Chapters 5 and 6.
Figure 4.1

Western blot probed with affinity purified antibody showing recovery of native maize p34cdc2 (lanes 2, 3), which was eliminated from the immunocomplex when antibody was pre-competed (lanes 5, 6) and was not detected when antibody was pre-competed before probing the blot (lanes 7-12; panel B).

Extract of root tip meristem was reacted with affinity purified maize anti-p34cdc2 peptide antibody and the immunocomplexed proteins were recovered with protein-A beads, then separated on 12% SDS-PAGE and blotted onto nitrocellulose.

Panel A; lanes 1-6, were probed on the blot with uncompeted affinity purified maize anti-p34cdc2 antibody and detected with alkaline phosphatase conjugated secondary antibody (Tago Inc., USA).

Lanes 1 and 4: whole extract, in which the 34 kDa p34cdc2 band (arrow) aligns with the recovered 34 kDa protein
Lane 2: immunoprecipitated proteins; Lane 3: half dilution of (2)
Lane 5: fraction recovered using competed antibody for precipitation; Lane 6: half dilution of (5)

Panel B; lanes 7-12, were probed on Western blot with competed first antibody.
Lanes 7-12: carry the same samples as 1-6 respectively.

Pre-competition before Western blotting was by incubation of affinity purified p34cdc2 antibody at 1/50 dilution, with 1 µM peptide at 25°C for 1 h. For pre-competition before immunoprecipitation, affinity purified p34cdc2 antibody was incubated at 25°C for 1 h with ~450 µM peptide.

Figure 4.2(a)

Immunoblot analysis using the IgG fraction from affinity purified antibody to probe native p34cdc2 (lanes 2, 3), which was eliminated from the immunocomplex when antibody was pre-competed (lanes 5, 6) and was not detected when antibody was pre-competed before probing the blots (lanes 7-12; panel B).

Root tip meristem extract was reacted with the IgG fraction of affinity purified maize anti-p34cdc2 antibody and the immunocomplexed proteins were recovered with protein-A beads, then separated on 12% SDS-PAGE and blotted onto nitrocellulose.

Panel A; lanes 1-6, were probed on the blot with affinity purified maize anti-p34cdc2 IgG and detected with alkaline phosphatase conjugated secondary antibody.
Lanes 1, and 4: whole extract, the p34cdc2 band at 34 kDa (arrow) is observed to align with the recovered 34 kDa protein
Lane 2: immunoprecipitated proteins; Lane 3: half dilution of (2)
Lane 5: fraction recovered using competed antibody for immunoprecipitation; Lane 6: half dilution of (5)

Panel B; lanes 7-12, were probed on the blot with competed primary antibody.
Lanes 7-12: carry the same samples as (1)-(6) respectively.

For pre-competition before immunoprecipitation, affinity purified anti-p34cdc2 IgG at 1/20 dilution was incubated at 25°C for 90 min with 100 µM peptide. Pre-competition before Western blotting was by incubation of affinity purified anti-p34cdc2 IgG (1/20) with 1 µM peptide at 25°C for 1 h.

(b)

Western blot using affinity purified first antibody and 125I-tagged second antibody showing the recovery of native p34cdc2 (lanes 2, 3) which was eliminated from the immunocomplex when antibody was pre-competed (lanes 5, 6) and was not detected when antibody was pre-competed before probing the blot (lanes 7-12; panel B).

Extract of root tip meristem was reacted with affinity purified maize anti-p34cdc2 IgG and the immunocomplexed proteins were recovered with protein-A beads, then separated on 12% SDS-PAGE and blotted onto nitrocellulose.

Panel A; lanes 1-6, were probed on the blot with affinity purified maize anti-p34cdc2 IgG and detected with a 125I labelled anti-rabbit Ig whole second antibody (Amersham Int., UK).
Lanes 1-6: carry the same samples respectively as in Figure 4.2a; panel A
Panel B; lanes 7-12, were probed on the blot with competed first antibody.
Lanes 7-12: carry the same samples respectively as in Figure 4.2a; panel B
Figure 4.3(a)
Effect of decreasing amounts of antibody on recovery of p34\textsuperscript{cdc2}. Western blot analysis of native p34\textsuperscript{cdc2} protein (lanes 2, 5, 8), which was displaced from the recovered fraction when the antibody was pre-competed (lanes 3, 6, 9).
Extract of meristematic root tip tissue was reacted with decreasing amounts of affinity purified maize anti-p34\textsuperscript{cdc2} IgG and the immunocomplexed proteins were recovered with protein-A beads, then run on 12% SDS-PAGE and transferred to nitrocellulose.
The blot was probed with affinity purified maize anti-p34\textsuperscript{cdc2} IgG and detected with alkaline phosphatase conjugated secondary antibody.
Lanes 1, 4, 7: whole extract, in which the p34\textsuperscript{cdc2} protein at 34 kDa (arrow) runs level with the recovered 34 kDa protein
Lanes 2, 5, 8: immunoprecipitated protein derived from the use of 80 µl, 40 µl and 20 µl of primary antibody respectively in the immunoprecipitation
Lanes 3, 6, 9: fractions recovered using 80 µl, 40 µl and 20 µl respectively of competed antibody for precipitation
Pre-competition before immunoprecipitation was by incubation of affinity purified anti-p34\textsuperscript{cdc2} IgG with 1 µM peptide at 25°C for 90 min.

(b)
ECL developed Western blot showing recovery of native p34\textsuperscript{cdc2} protein (lanes 2, 5, 8), which was displaced when antibody was pre-competed before use in immunoprecipitation (lanes 3, 6, 9).
Root tip meristem extract was reacted with decreasing amounts of affinity purified maize anti-p34\textsuperscript{cdc2} IgG and the immunocomplexed proteins were recovered with protein-A beads, electrophoresed using 12% SDS-PAGE and blotted onto nitrocellulose.
The blot was probed with affinity purified anti-p34\textsuperscript{cdc2} IgG and the secondary antibody used was an affinity isolated goat anti-rabbit peroxidase conjugate (Tago, Inc., USA). The image was developed using a chemiluminescent substrate (Amersham Int., UK).
Lanes 1-9: carry the same samples respectively as in Figure 4.3a
3. (a)

3. (b)
Figure 4.4(a)
Western blot analysis of the source of background observed in immunoblots in which the immunocomplexed proteins were recovered with protein-A beads.
Affinity purified maize anti-p34\textsuperscript{cdc2} antibody and affinity purified maize anti-p34\textsuperscript{cdc2} IgG were reacted with protein-A beads. The bound proteins were recovered then separated on 12\% SDS-PAGE and transferred to nitrocellulose.
Panel A; lanes 1 and 2, were probed with affinity purified anti-p34\textsuperscript{cdc2} IgG and detected with alkaline phosphatase conjugated second antibody (Tago Inc., USA).
Lane 1: proteins from affinity purified anti-p34\textsuperscript{cdc2} antibody that bound to protein-A beads
Lane 2: proteins from affinity purified anti-p34\textsuperscript{cdc2} IgG that bound to protein-A beads
Panel B; Lanes 3 and 4, were probed only with the secondary antibody.
Lane 3: carries the same sample as (1)
Lane 4: carries the same sample as (2)

(b)
Analysis of the source of background observed in blots in which immunocomplexed proteins were recovered with protein-A beads using \textsuperscript{125}I-tagged second antibody.
Affinity purified maize anti-p34\textsuperscript{cdc2} antibody and affinity purified anti-p34\textsuperscript{cdc2} IgG were reacted with protein-A beads. The proteins that bound to the beads were recovered then run on a 12\% acrylamide gel and blotted onto nitrocellulose.
Panel A; lanes 1-5, were probed with affinity purified anti-p34\textsuperscript{cdc2} IgG and detected with a \textsuperscript{125}I labelled anti-rabbit Ig whole secondary antibody (Amersham Int., UK).
Lane 1: whole extract, in which the p34\textsuperscript{cdc2} band at 34 kDa can be seen (arrow)
Lane 2: proteins from affinity purified maize anti-p34\textsuperscript{cdc2} antibody that bound to protein-A beads
Lane 3: proteins recovered when competed affinity purified maize anti-p34\textsuperscript{cdc2} antibody was reacted with protein-A beads
Lane 4: proteins from affinity purified maize anti-p34\textsuperscript{cdc2} IgG that bound to protein-A beads
Lane 5: proteins recovered when competed affinity purified anti-p34\textsuperscript{cdc2} IgG was reacted with protein-A beads
Panel B; lanes 6-10, were probed only with the \textsuperscript{125}I tagged secondary antibody.
Lanes 6-10: carry the same samples as lanes 1-5 respectively in panel A
4. (a)

4. (b)
Western blot showing the recovery of native maize \( p^{34}_{\text{cdc}2} \) (lanes 4-8) in fractions eluted by pH from a \( p^{34}_{\text{cdc}2} \) antibody column. Root tip meristem extract was reacted with affinity purified anti-\( p^{34}_{\text{cdc}2} \) antibody immobilized by coupling to CNBr-Sepharose beads in a column. Unbound proteins were washed from the \( p^{34}_{\text{cdc}2} \) antibody column and the immunocomplexed proteins recovered by pH change, then separated on 12% SDS-PAGE and blotted onto nitrocellulose.

Lanes 1-9, were probed on the blot with affinity purified maize anti-\( p^{34}_{\text{cdc}2} \) antibody and detected with alkaline phosphatase conjugated secondary antibody (Tago Inc., USA).

Lanes 1, 3 and 9: whole extract before passage through the column, in which the 34 kDa \( p^{34}_{\text{cdc}2} \) band is visible (arrow) and aligns with recovered \( p^{34}_{\text{cdc}2} \)

Lane 2: whole extract after passage through the column

Lanes 4-8: fractions recovered from the column by pH change (pH 2.5), a band at 34 kDa, equivalent in mobility to the \( p^{34}_{\text{cdc}2} \) band in whole extract is visible in lanes 5-8

(aii)
Silver stained 12% SDS gel showing degree of protein purification by the \( p^{34}_{\text{cdc}2} \) antibody column.

Immunocomplexed proteins were recovered from the column by pH change, run on a 12% acrylamide gel, then the separated proteins were stained with silver.

Lane 1: whole extract before passage through the column
Lane 2: whole extract after passage through the column
Lane 3: fraction recovered from the column by pH change; carries the same sample as Figure 4.5(ai); lane 7

(b)
Western blot analysis of efficiency of \( p^{34}_{\text{cdc}2} \) protein binding by the \( p^{34}_{\text{cdc}2} \) antibody column by reducing amount of plant extract.

One-tenth of normal amount of maize root tip meristem extract was reacted with affinity purified maize anti-\( p^{34}_{\text{cdc}2} \) antibody immobilized by binding to Sepharose beads in a column. Samples of whole extract before and after passage through the column were electrophoresed and then transferred to nitrocellulose.

The blot was probed with affinity purified maize anti-\( p^{34}_{\text{cdc}2} \) antibody and developed with alkaline phosphatase conjugated secondary antibody (Tago Inc., USA).

Lane 1: one-tenth whole extract before passage through the column, the \( p^{34}_{\text{cdc}2} \) band at 34 kDa is clearly visible (arrow)

Lane 2: one-tenth whole extract after passage through the column, a significant reduction in the amount of \( p^{34}_{\text{cdc}2} \) protein left in the whole extract after passing through the column indicates that the column is effective in binding the \( p^{34}_{\text{cdc}2} \) protein

(c)
Western blot showing recovery of native \( p^{34}_{\text{cdc}2} \) (lane 3), which was eliminated from the immunocomplex when antibody was pre-competed before probing the blot (lane 5).

Maize root tip meristem extract was reacted with affinity purified maize anti-\( p^{34}_{\text{cdc}2} \) antibody, immobilized by coupling to CNBr-Sepharose beads in a column. The immunocomplexed proteins were recovered by pH change, then run on a 12% acrylamide gel and blotted on nitrocellulose.

Lanes 1-3, were probed with un compéted affinity purified maize anti-\( p^{34}_{\text{cdc}2} \) antibody and detected with alkaline phosphatase conjugated second antibody (Tago Inc., USA).

Lane 1: whole extract before passage through the column, the 34 kDa \( p^{34}_{\text{cdc}2} \) band (arrow) aligns with the recovered 34 kDa protein (lane 3)

Lane 2: whole extract after passage through the column
Lane 3: fraction recovered from the column by pH change, a faint band at 34 kDa is visible

Lanes 4 and 5 were probed with competed affinity purified maize anti-\( p^{34}_{\text{cdc}2} \) antibody.

Lane 4: carries the same sample as (1)
Lane 5: carries the same sample as (3)

Pre-competition before Western blotting was by incubation, of affinity purified maize anti-\( p^{34}_{\text{cdc}2} \) antibody at 1/50 dilution, with 1 µM peptide at 25°C for 90 min.
Figure 4.6
Western blot and silver stain analysis of recovery of native p34\textsuperscript{cdc2} from p34\textsuperscript{cdc2} antibody column by acid pH 2.5 (lanes 6-9), and alkaline pH 11.5 (lanes 11-13). Extract of root tip meristem was reacted with affinity purified maize anti-p34\textsuperscript{cdc2} antibody bound to Sepharose beads in a column. The immunocomplexed proteins were recovered by pH change and then separated on 12% SDS-PAGE. Figures 4.6(a), (b), the proteins were transferred to nitrocellulose.

(a)
Lanes 1-13, were probed with affinity purified maize anti-p34\textsuperscript{cdc2} antibody and detected with alkaline phosphatase conjugated secondary antibody (Tago Inc., USA).
Lane 1: affinity purified maize anti-p34\textsuperscript{cdc2} antibody before coupling to Sepharose beads
Lane 2: affinity purified maize anti-p34\textsuperscript{cdc2} antibody after coupling to Sepharose beads
Lane 3: whole extract before passage through the column
Lane 4: whole extract after passage through the column
Lanes 5 and 10: whole extract (one-third dilution of (3)), in which the 34 kDa p34\textsuperscript{cdc2} band (arrow) aligns with the recovered 34 kDa protein
Lanes 6-9: fractions recovered from the column by pH 2.5
Lanes 11-13: fractions recovered from the column by pH 11.5

(b)
Lanes 1-13, were probed with affinity purified maize anti-p34\textsuperscript{cdc2} antibody, the second antibody was an affinity isolated goat anti-rabbit peroxidase conjugate (Tago, Inc., USA). The blot was developed using a chemiluminescent substrate (Amersham Int., UK).
Lanes carry the same samples as (1)-(13) in Figure 4.6(a)

(c)
Lanes 1-13, the separated proteins were stained with silver.
Lanes carry the same samples as (1)-(13) in Figures 4.6(a), (b)
Figure 4.7
Procedure used in Figure 4.6 modified by pre-competition of the antibody column before introduction of plant extract showing the elimination of native p34\textsuperscript{cdc2} recovery from the immunocomplex (lanes 5-7, 9-11).
Affinity purified maize anti-p34\textsuperscript{cdc2} antibody was coupled to CNBr-Sepharose in a column. The immobilised antibody was pre-competeted with maize p34\textsuperscript{cdc2} peptide at 100 nM for 1 h at 25°C. Maize root tip meristem extract was reacted with the competed antibody beads. 100 nM peptide was also added to the pre-cleared whole extract and first column wash. The immunocomplexed proteins were recovered by pH change and then separated on 12% SDS-PAGE. Figures 4.7(a), (b), the separated proteins were transferred to nitrocellulose.

(a)
Lanes 1-11, were probed on the blot with affinity purified maize anti-p34\textsuperscript{cdc2} antibody and detected with alkaline phosphatase conjugated secondary antibody (Tago Inc., USA).
Lane 1: whole extract before passage through the column, note p34\textsuperscript{cdc2} protein at 34 kDa (arrow)
Lane 2: whole extract with p34\textsuperscript{cdc2} peptide before passage through the column
Lane 3: whole extract with p34\textsuperscript{cdc2} peptide after passage through the column
Lanes 4 and 8: whole extract one-third dilution of (1)
Lanes 5-7: fractions recovered from the competed column by pH 2.5
Lanes 9-11: fractions recovered from the competed column by pH 11.5

(b)
Lanes 1-11, were probed on the blot with affinity purified maize anti-p34\textsuperscript{cdc2} antibody, the second antibody was an affinity isolated goat anti-rabbit peroxidase conjugate (Tago, Inc., USA). The blot was developed using a chemiluminescent substrate (Amersham Int., UK).
Lanes carry the same samples respectively as (1)-(11) in Figure 4.7(a)

(c)
Lanes 1-11, separated proteins stained with silver.
Lanes carry the same samples respectively as (1)-(11) in Figures 4.7(a), (b)
Figure 4.8
Western blot and silver staining analysis of recovery of native p34\textsuperscript{cdc2} by pH change (lane 8) after purification of p34\textsuperscript{cdc2} with p13\textsuperscript{suc1} then passage of the purified p34\textsuperscript{cdc2} immunocomplex through p34\textsuperscript{cdc2} antibody column.
Extract of maize root tip meristem was reacted with p13\textsuperscript{suc1} bound to CNBr-Sepharose beads in a column. The purified p34\textsuperscript{cdc2} protein was recovered by exchange with free p13. The recovered protein was then reacted with affinity purified maize anti-p34\textsuperscript{cdc2} antibody coupled to Sepharose in a column. The immunocomplexed proteins were recovered by pH change, then separated on 12% SDS-PAGE. Figure 4.8(a), (b), the separated proteins were blotted onto nitrocellulose.

(a)
Lanes 1-10, were probed on the blot with affinity purified maize anti-p34\textsuperscript{cdc2} antibody and detected with alkaline phophatase conjugated secondary antibody (Tago Inc., USA).
Lane 1: whole extract before passage through the p13 column
Lane 2: whole extract after passage through the p13 column
Lanes 3 and 10: whole extract one-third dilution of (1), in which 34 kDa p34\textsuperscript{cdc2} protein (arrow) aligns to recovered 34 kDa protein
Lane 4: pooled fractions containing purified p34\textsuperscript{cdc2} recovered from p13 column before passage through p34\textsuperscript{cdc2} column
Lane 5: pooled fraction containing purified p34\textsuperscript{cdc2} recovered from p13 column after passage through p34\textsuperscript{cdc2} column
Lanes 6 and 7: fractions recovered from the antibody column by pH 2.5
Lanes 8 and 9: fractions recovered from the antibody column by pH 11.5

(b)
Lanes 1-10, were probed on the blot with affinity purified maize anti-p34\textsuperscript{cdc2} antibody, the second antibody was an affinity purified goat anti-rabbit peroxidase conjugate (Tago, Inc., USA). The blot was developed using a chemiluminescent substrate (Amersham Int., UK).
Lanes carry the same samples respectively as (1)-(10) in Figure 4.8(a)

(c)
Lanes 1-10, the separated proteins were silver stained.
Lanes carry the same samples respectively as (1)-(10) in Figures 4.8(a), (b)
CHAPTER 5:

DISTRIBUTION IN DEVELOPING LEAF AND ROOT TISSUES
OF p34<sup>cd2</sup> PROTEIN KINASE ACTIVITY, STUDIED BY p13<sup>suc1</sup>

AFFINITY PURIFICATION AND IMMUNORECOVERY
5.1 Introduction

5.1.1 Cell division in plant development

A property of plant development that distinguishes it from animal is the greater persistence of embryonic meristematic zones in plants. In animals embryonic growth ceases early in development, though cell division persists in many tissues. In plants the apical meristems of the root and shoot are sources of new, undifferentiated cells throughout the life of the plant. In dicotyledonous plants there can be additional development of secondary regions of cell division, which allows secondary thickening of stems and roots, and proliferation of cells, in response to wounding. Monocotyledonous plants, such as maize, do not share the capacity for resuming cell proliferation in most tissues, hence their lack of secondary thickening. Their response to wounding is most commonly the lignification of adjacent cells rather than callus formation. However all plants share the persistence of cell division in specialised meristems that are without precise counterpart in animals.

The persistence of cell division in meristems means that a continuing element in plant development is a switch from cell proliferation to cessation of division while cell growth continues, producing cells of specialised shape, composition and metabolic function. This switch from proliferation to differentiation probably requires specialised controls because it seems likely from evidence in unicellular yeasts (Jagadish et al., 1977), plants (Donnan and John, 1983) and from cultured cells (Smith and Martin, 1974; Brooks et al., 1980) that increasing cell mass triggers division. Therefore in the development of plant tissues containing differentiated cells that are often many times larger than those in meristems, the division processes must be suppressed as cells enlarge.

Suppression of division assumes additional significance in monocotyledonous plants since their differentiated cells do not usually resume division and, in conformity with this, in culture they do not respond to hormones that can stimulate division in isolated dicotyledonous cells. A consequence of this unresponsiveness of monocotyledonous cells is that they are difficult subjects for transgenic manipulation.
5.1.2 p34\textsuperscript{cdc2} and p34\textsuperscript{cdc2}-like protein levels in plants

John et al. (1990) in a study of the seedling wheat leaf identified a decline in the level of p34\textsuperscript{cdc2}-like protein relative to other proteins to one sixteenth of that in meristem cells and noted a correlation of the decline with inability to divide in culture. A subsequent study identified the boundary of active cell division in the basal meristem as being defined by the region in which p34\textsuperscript{cdc2}-like protein was catalytically active as a protein kinase (John et al., 1993b). The small region immediately outside the active meristem contained p34\textsuperscript{cdc2}-like protein at levels above basal but not catalytically active. These data suggest that regions in which p34\textsuperscript{cdc2}-like protein is abundant and enzymatically active are significant in plant development (John et al., 1993a). The principle has also been suggested by Martinez et al. (1992) on the basis of in situ detection of cdc2 mRNA in root meristems and elaborated by Hemerly et al. (1993) who used GUS as a reporter for the promoter of the Arabidopsis cdc2A gene. These last two studies did not confirm that levels of p34\textsuperscript{cdc2} followed those of the mRNA or mRNA transcription. The earlier study of p34\textsuperscript{cdc2} level (John et al., 1990) used an antibody which is now recognised as potentially capable of identifying variants of p34\textsuperscript{cdc2} that share the conserved 16 amino acid PSTAIR sequence. It has been argued that this potential lack of specificity is not significant since the variants of p34\textsuperscript{cdc2} are not abundant and the close variant p33\textsuperscript{CDK2}, which has a perfect PSTAIR sequence, is also directly involved in cell cycle progress and therefore is equally significant (John et al., 1993a). Earlier studies of p34\textsuperscript{cdc2} activity were made using the mitotic protein p13\textsuperscript{SUC} as an affinity purification tool to recover p34\textsuperscript{cdc2} (Hindley et al., 1987, Arion et al., 1988, Dunphy et al., 1988, John et al., 1991, 1993a). The p13\textsuperscript{SUC} protein was utilised as it associates physically with p34\textsuperscript{cdc2} (Hayles et al., 1986b, Brizuela et al., 1987, MacNeil et al., 1991). However a consequence of its use is that again the possible presence of close variants of p34\textsuperscript{cdc2} in the recovered fraction cannot be eliminated, since p33\textsuperscript{CDK2} too has been found to bind p13\textsuperscript{SUC} (Pines and Hunter, 1991a). Thus technical difficulties have in some respects compromised the specificity of earlier estimates of p34\textsuperscript{cdc2} protein level and activity, or else study of the mRNA only has been conducted.
The maize leaf provides an opportunity for unambiguous measurement of the level and enzyme activity specifically of p34\textsuperscript{cdc2} by making use of an antibody raised against the carboxyterminal region of the p34\textsuperscript{cdc2} protein from maize. Colasanti et al. (1991) isolated and characterised two cDNA clones in maize. They demonstrated that one of these, cdc2Zma, was a functional homologue of p34\textsuperscript{cdc2} as it was able to complement a cdc28 mutation in budding yeast. We used the sequence of the cdc2Zma clone in this study to generate an antibody against the p34\textsuperscript{cdc2} protein in maize. A suitable antibody (as described in earlier chapters) can be used for quantitative probing of Western blots to measure level, and in principle may be used for specific immunoprecipitation of the p34\textsuperscript{cdc2} protein to recover activity. The p34\textsuperscript{cdc2} protein from humans has been found catalytically active in complex with antibody bound to the carboxyterminal region thus the plant enzyme could be similarly active. Although Colasanti et al. (1993) reported that their antibody raised against the carboxyterminal sequence of maize was ineffective in immunoprecipitating the enzyme, the antibody developed here did specifically precipitate p34\textsuperscript{cdc2} (Chapter 4). This chapter therefore reports specific estimates of p34\textsuperscript{cdc2} protein and activity for the first time for plant tissue.

5.2 Materials and methods

5.2.1 Plant material

\textit{Zea mays} (cv. miracle) was germinated in vermiculite as previously described in 3.2.1. After approximately 72 h the seedlings were lifted and washed to remove that remaining. Root tissue was then harvested either as: 1) a 5 mm tip section, 2) a 3 mm meristem then a 7 mm elongation zone section, 3) a series of eleven sections, the first six from the tip back of size 1 mm, the next five following of 2 mm size, 4) a 4 mm tip region was removed and these seedlings allowed to grow on for 4 days when the next 6 mm of root was removed. All root sections once harvested were frozen in liquid nitrogen then ground to powder and stored at -70\textdegree C until used. The root “de-tipped” seedlings, as well as those which were to grow on to provide maize leaf material were grown by immersing the roots in 25% Hoagland’s nutrient solution (100% Hoagland’s contained 2 mM potassium nitrate, 5 mM calcium nitrate, 2 mM magnesium sulfate,
1 mM potassium dihydrogen phosphate, 46 µM boric acid, 9.1 µM manganese chloride, 0.77 µM zinc sulphate, 0.32 µM copper sulphate, 0.11 µM molybdic acid, 90 µM iron-EDTA complex and dH₂O to 1 litre). The roots were suspended in culture medium by passing them through a perforated plastic platform, each holding approximately 100 seedlings, the platforms were then placed into open plastic boxes of dimensions 220 x 125 x 75 mm with 1 L of medium. The top of the boxes was then covered with cling wrap to prevent excess evaporation, leaving open the area immediately above the growing seedlings. The seedling boxes were then placed into a growth cabinet set to a 12 h day cycle with 21°C day and 18°C night temperatures, a humidity of 90% and light intensity of 1000 microEinsteins. Plant growth was checked daily and the medium topped up, with additional medium, whenever necessary.

Once the dynamics of maize leaf growth, under these conditions had been established, maize leaf material was harvested at day 12 of liquid culture. Leaf 4, which was then approximately 100 mm long was separated out and then cut into twenty 5 mm sections. These sections were collected separately into eppendorf tubes, frozen in liquid nitrogen, ground to a powder using a mortar and pestle, and then stored at -70°C. Leaf 5, which was at day 12 approximately 10 mm in length, was also collected as a pure meristematic sample and processed by freezing and grinding as described above.

5.2.2 Cell size and division activity in developing leaf and root

5.2.2.1 Leaf growth dynamics

We wished to study p34cdc2 protein kinase activity in maize leaf tissue which contained a transition from meristematic to non-meristematic regions. However, it was unknown under our growth conditions when cell division was most active in particular leaves. Hence leaf growth was measured in seedlings that had been germinated in vermiculite and transferred to Hoagland's medium, by daily dissecting two seedlings of average size over a period of 40 d. To investigate the histology, tissue was embedded in polyethylene glycol (PEG), sectioned and DNA was stained to reveal mitotic activity.
5.2.2.2 PEG embedding and sectioning of leaf tissue

The process of PEG embedding was loosely based upon that of Gao (1993). Prior to embedding, the tissue was fixed in 4% paraformaldehyde in 50 mM potassium phosphate/5 mM EGTA, pH 7.0, buffer for 2 h, then segments were rinsed free of the fixative with three changes of buffer over a period of 45 min. The embedding process involved immersion of segments for 3-4 h or overnight in increasing concentrations of aqueous 400 M.W. PEG (10%, 20%, 30%, 40%, 50%, 60%, 70%). Immersion carried out in 80%, 90% and 100% PEG was then left for 7 h or overnight, after which the segments were incubated in 100% PEG (1000 M.W.) at 45°C for 7 h. This incubation was repeated three times overnight with fresh PEG. Finally the tissue segments were processed in higher molecular weight PEG through three overnight changes in 100% PEG (1450 M.W.) at 45°C. At this point the segments in PEG were removed from the oven and transferred individually to square plastic moulds. Fresh viscous 1450 M.W. PEG (45°C) was poured over them and they were left to set at room temperature, then stored at 4°C.

Prior to sectioning it was necessary to trim the excess PEG from around the sections so that a transverse face of the segment was exposed. The tissue blocks were then mounted individually onto square wooden blocks by melting the PEG of the blocks and sealing it securely to the wood with excess melted PEG. Once set hard the blocks could now be sectioned using a standard Leitz rotary microtome. A range of section thicknesses from 15 to 100 µm was tested and 15 µm was identified as best for providing a clear representation of cell structure. Sections of 15 µm were cut from all twenty tissue blocks comprising the entire fourth seedling leaf of day 12 old plants.

For viewing, sections were placed onto clean glass slides that had been coated with a solution of 0.5% gelatine and 0.05% chromic potassium sulphate and then allowed to dry. Minimal water was then added to the slides to dissolve the PEG from around and within the sections. The water and dissolved PEG was removed carefully, so as not to dislodge the sections, by using strips of filter paper. This wash procedure was repeated until all the PEG had been removed. Sections were then dried down onto the slides, over low-medium heat on a slide warmer. After the slides had cooled, DNA in the sections was stained by covering them with 0.5 µg/ml DAPI for 10 min then
removing the stain carefully using filter paper strips. Mounting medium (moviol plus 2% \textit{p}-\textit{phénylenediamine} (PPD)) was added and a coverglass lowered on, then excess mounting medium removed from the edges before the slides were left to set overnight at 4°C.

The slides were viewed using a Zeiss Axioplan Fluorescent Light Microscope under: 1) UV excitation to reveal DNA-DAPI fluorescence and allow identification and counting of mitotic nuclei, and 2) using Nomarski optics to observe cell size and structure. Measurements of cell dimensions over the length of leaf 4 were made using an ocular scale and converted to µm by calibrating the ocular using a haemocytometer.

5.2.2.3 De-tipping of seedling roots

Seedling roots were de-tipped to investigate the effect of effectively removing the sole active meristematic zone of the root. Maize seeds were planted in vermiculite and the seeds germinated as described in 3.2.1. Then the seedlings were removed from the vermiculite and cleaned. The tip 4 mm of root in half the seedlings was then cut off with a razor blade. Once de-tipped the seedlings were then set up to grow further, together with controls still tipped in liquid medium as described in 5.2.1. After 4 days, all seedlings, de-tipped and tipped were removed from the liquid medium and the terminal 6 mm of the de-tipped roots removed, collected and frozen with liquid nitrogen. The tipped roots, at this time had the equivalent 4 mm tip and next 6 mm section of root removed, collected and frozen with liquid nitrogen as well. All frozen root tissue samples were then ground to powder using a mortar and pestle and then stored at -70°C.

5.2.3 Protein extraction for Western blotting from maize leaf and root tissue

Tissue was ground in liquid nitrogen and aliquots were weighed while still frozen. Three protein fractions were extracted from the tissue; soluble protein in RIPA buffer, total protein in Sx2 (SDS) electrophoresis buffer and insoluble proteins, left in the pellet after soluble proteins have been removed, in an equal volume of Sx2 buffer and RIPA buffer.
To obtain soluble proteins, routinely 0.02 g of ground tissue was vortexed in 20 µl of cold RIPA buffer for two periods of 30 sec, cooling on ice for 1 min after each. The insoluble pellet was discarded after spinning the sample down for 5 min at 14,000 rpm and the supernatant fraction retained. A sample was frozen for a subsequent protein assay and the bulk was mixed with an equal volume of Sx2 (SDS) electrophoresis buffer (Laemmli, 1970, described in 3.2.2) for Western blot analysis. This soluble protein fraction allows purification of p34\(^{cdc2}\) however the possibility was considered that a significant proportion of the p34\(^{cdc2}\) might have been in the insoluble fraction perhaps bound to membrane or other structural components. Total proteins, including the insoluble fraction could be detected by Western blotting after solubilisation with SDS by mixing 0.02 g of frozen ground tissue with 20 µl of Sx2 buffer then 440 µl of Sx2 diluted to half (Sx1) and vortexing twice for 20 sec. The sample was then spun down at 14,000 rpm for 5 min and the supernatant removed and retained as representative of the total protein in the tissue. To directly examine the insoluble protein fraction in non-detergent buffer extracts the pellet left after supernatant removal was resuspended in an approximately equal volume of Sx2 buffer (20 µl). The pellet was dispersed and then boiled briefly after which the total volume was made up to 260 µl with equal volumes of RIPA buffer to Sx2 buffer.

All samples prior to being loaded onto a gel were analysed using the micro dye binding protein assay (5.2.4) to quantify the protein concentrations of individual samples. The gel loadings were then adjusted so that all fractions contained approximately equal protein. For all maize leaf and root samples, soluble, total and insoluble protein fractions were run on separate 12% SDS-polyacrylamide gels (as described in 2.2.8). The subsequent transferred gels were then blotted and probed as Western blots with both histochemical (2.2.10) and \(^{125}\)I detection (3.2.5) of first antibody binding.

5.2.4 Quantification of protein concentration by micro dye binding assay

Dye reagent, containing phosphoric acid and methanol (Biorad Laboratories, Australia), was diluted 1:4 with water and reacted in ELISA microtitre wells with known amounts of BSA in the range 0.5 to 8 µg and with unknown amounts of root or
leaf protein within this range. 200 µl of dye reagent was mixed with 5 µl and 15 µl of plant extract that had been diluted to 1/10 with water and the standard protein was dissolved in extract buffer diluted to 1/10. Thus unknown and calibration assays contained the same buffer and were all made to the same final volume of 215 µl. Absorbance of the samples was then determined at 600 nm using a Titertek Multiscan Plus MKII.

5.2.5 p13 overexpression and purification of the p13sucl protein

Induction of p13sucl by IPTG

The bacterial strain BL21(DE3), which contains a plasmid in which expression of the sucl DNA fragment from S. pombe is driven by the T7 promoter, was used according to the strategy of Studier and Moffat (1986) to overexpress p13sucl following induction of the T7 RNA polymerase with isopropyl-B-D-thiogalactoside (IPTG) essentially as described by Dunphy et al. (1988).

The bacterium was grown in small scale LB medium culture containing 100 µg/ml of ampicillin overnight and then transferred to a larger culture also containing 100 µg/ml of ampicillin. IPTG was added when the O.D. 600 of the culture was 0.4-0.5 to induce expression of the p13sucl protein.

After the induction with IPTG for 3 h the cells were harvested by spinning for 5 min at 6,000 rpm. The cells were resuspended in cold 20 mM Tris/HCl, pH 7.5. Glycerol was added to this cell suspension giving a 15% final concentration. The cell suspension was then ready for storage by freezing or for purification of p13sucl protein.

Purification of p13sucl protein

The method was essentially as described by Brizuela et al. (1987) with the addition of a phenyl Sepharose chromatography step.

The cell suspension was supplemented with PMSF to 1 mM and cells were broken with a French press at 0°C. The p13sucl protein in the supernatant was concentrated by precipitation at 70% saturated ammonium sulphate. 44.2 g ammonium
sulphate was dissolved in 100 ml of supernatant, kept in ice for 1 h and then centrifuged at 10,000 rpm in a JA 20 rotor for 15 min. The protein pellets were dissolved in less than 10 ml of 50 mM Tris/HCl, pH 7.5, and spun again at 10,000 rpm for 10 min to sediment any undissolved particles. The p13<sub>sucI</sub> containing supernatant was loaded onto a S200 acrylamide gel beads column that had been equilibrated with at least one bed volume of 50 mM Tris/HCl, pH 7.5. The column was eluted by one bed volume of the same buffer. The fractions containing p13<sub>sucI</sub> were located as a peak of U.V. absorbing material in mid eluate and were identified by electrophoresis then pooled.

For phenyl Sepharose chromatography ammonium sulphate was added to 0.85 M by adding 11.3 g ammonium sulphate per 100 ml of p13<sub>sucI</sub> solution and the protein was loaded onto a phenyl Sepharose column that had been equilibrated with 0.85 M ammonium sulphate. The column was eluted by a declining concentration from 0.85 M ammonium sulphate in 50 mM Tris/HCl, pH 7.5, to zero ammonium sulphate in the same buffer. The fractions which contained the prominent p13<sub>sucI</sub> protein were identified by reading their absorbance at 280 nm and confirmed by 15% acrylamide gel electrophoresis (2.2.8). The protein was recovered by precipitation with 80% ammonium sulphate (52.3 g of ammonium sulphate added to 100 ml of p13<sub>sucI</sub>) at 4°C for 10 min. The precipitated protein was pelleted by centrifugation at 10,000 rpm and then dissolved in 5 ml of PBS buffer. The resuspended p13<sub>sucI</sub> protein was dialysed (M.W. cut-off 3,500) against 2 L of 25 mM Hepes, pH 7.4, 10 mM MgC<sub>2</sub>, 1 mM DTT buffer at 4°C overnight.

The protein concentration of p13<sub>sucI</sub> was determined by absorbance at 280 nm and more accurately by the Coomassie Brilliant blue assay (3.2.3.1). The purity of p13<sub>sucI</sub> was determined by electrophoresis on a 15% acrylamide gel (2.2.8) and purified p13<sub>sucI</sub> was also used to make p13<sub>sucI</sub>-Sepharose beads for purification of p34<sup>cdcl2</sup>-like protein kinase.

5.2.6 Purification of p34<sup>cdcl2</sup>-like protein by p13<sub>sucI</sub> binding

p13<sub>sucI</sub> covalently coupled to CNBr-Sepharose (p13 beads) was prepared. 8 mg of p13<sub>sucI</sub> protein purified, as above (5.2.5), at a concentration of 1 mg/ml, was coupled to 1 ml of prepared CNBr-Sepharose by the method recommended by the manufacturers.
and as described in 2.2.7. The resulting beads were then used for the purification of p34\textsuperscript{cdc2}-like protein.

Samples of tissue ground in liquid nitrogen were mixed with 150 µl of cold NDE per 0.05 g tissue sample. NDE buffer contained 20 mM Hepes, pH 7.4, 100 mM NaCl, 15 mM DTT, 3 µg/ml leupeptin, 20 mM EGTA, pH 7.4, 80 mM β-glycerophosphate, pH 7.4, 1 mM orthovanadate, 30 mM p-nitrophenylphosphate di Na salt, 50 mM NaF, 15 mM MgCl\textsubscript{2}, 0.2 mM ammonium molybdate and 0.5 mM PMSF. The samples were then vortexed vigorously for 20 sec, then placed on ice for 1 min, then mixed again for 20 sec after which they were spun for 5 min, at 14,000 rpm, at 4°C. A clear supernatant of 130 µl was removed from all samples into new tubes on ice. Any remaining supernatant was collected for use in a protein quantification assay.

The supernatant was then pre-cleared of potential agarose binding proteins by adding 40 µl of agarose bead suspension previously washed in NDE (containing 20 µl of gel). The eppendorf tubes were placed onto a rotating wheel for 1 h at 4°C, then agarose was sedimented and discarded by spinning for 5 min at 14,000 rpm, at 4°C. 130 µl of supernatant was removed from each sample to a new tube in ice and received 40 µl of the prepared p13 beads in NDE (containing 20 µl of beads), and the tubes placed onto the rotating wheel, for 2 h at 4°C, to allow the p34\textsuperscript{cdc2} to bind.

To recover the bound p34\textsuperscript{cdc2}-like protein samples were spun for 2 min at 7,000 rpm and each pellet was then suspended in 1 ml of cold HDW buffer and transferred to a new tube on ice. HDW buffer contained 2 mM EDTA, pH 7.4, 150 mM NaCl, 1% NP40, 5 µg/ml leupeptin, 0.1 mM orthovanadate, 50 mM NaF, pH 7.4, 10 mM NaPO\textsubscript{4}, pH 7.0, and dH\textsubscript{2}O to 20 ml. The gel was recovered by centrifugation and immediately resuspended in HDW buffer and then spun as before. The third wash was by suspension in 400 µl of HBK buffer (supplemented with 1 mM DTT) and it was then followed immediately by spinning for 2 min at 7,000 rpm. The supernatants were discarded and the gel pellets were retained. HBK buffer contained 25 mM Hepes, pH 7.4, 1 mM EGTA, pH 7.4, 5 mM MgCl\textsubscript{2}, 160 mM KCl, dH\textsubscript{2}O to 20 ml and 1 mM DTT.

The p34\textsuperscript{cdc2} was now eluted by the addition of 50 µl of 0.5 mg/ml free p13 in HMD buffer (25 mM Hepes, 10 mM MgCl\textsubscript{2}, and 1 mM DTT added just before use, pH 7.4, in 1 L) to all samples. The sample tubes were then placed onto the rotating wheel for 10 min at 4°C, spun for 5 min, at 14,000 rpm, and the 45 µl supernatants
removed to new tubes in ice. From these, 20 µl samples were routinely assayed for kinase enzyme activity the remaining 25 µl was aliquoted and frozen at -70°C.

5.2.7 \(34^{\text{cd2}}\) protein kinase assay

Assay of \(34^{\text{cd2}}\)-like protein kinase was based on the transfer of \(^{32}\text{P}\) from \(^{32}\text{P}\)ATP to H1 histone substrate.

The reaction mixture for the \(34^{\text{cd2}}\)-like protein kinase assay contained 25 mM \(\beta\)-glycerophosphate, pH 7.3, 10 mM EGTA, 10 mM MgCl\(_2\), 1 mM DTT, 25 mM Hepes, pH 7.3, and per 50 µg of H1 histone 33 pmol ATP and 0.25 µCi\(^{32}\text{P}\) (7.6 µCi/nmol). The assay was modified for use with antibody precipitates by use of 10 pmol ATP per assay, giving a three fold increase in specific activity to 23 µCi/nmol.

The reaction was begun, after pre-warming the assay tubes for 3 min at 30°C, by adding ATP. After 5 min the reaction was stopped by addition of 10 µl of 1% (w/v) acetic acid mixing briefly and placing on ice.

Once all the samples had been assayed, 24 µl of each was individually spotted onto separate strips of P81 paper (Whatman) 10 x 20 mm, that were laid out upon Nescofilm. 30 seconds was allowed for the histone to bind to the P81 paper, then using forceps all strips were transferred to a beaker of 75 mM phosphoric acid. The strips were rinsed in ~200 ml of phosphoric acid, using a slowly rotating flea, to circulate the liquid, for 5 min then the acid carefully poured off. This wash was repeated three more times. The strips were lifted using washed forceps out onto Nescofilm and quickly blotted before being placed into individual labelled eppendorf tubes whose hinges had been removed. 1.4 ml of scintillation fluid (Packard "Emulsifier Safe") was added to each, the tubes inverted to mix and then each sample placed into separate glass scintillation vials and counted for 5 min using a Beckman Liquid Scintillation Counter Model LS 3801. The samples were passed through the counter more than once.

The remaining 36 µl of each assay contained histone that could be examined electrophoretically after neutralising the acetic acid with 7.4 µl of 174 mM NaOH. The pH was then stabilised by addition of 10 µl of 0.5 M Tris, pH 7.8, and 50 µl of Sx2 buffer was added to each sample, and they were all boiled for 2 min. They were then stored in a shielded container at -20°C overnight until electrophoresed.
The samples were run on 12% SDS-polyacrylamide gels (2.2.8) then transferred to nitrocellulose (2.2.10). Once the transfer of proteins was completed, the nitrocellulose was left to dry on 3MM paper. After drying it was taped to a slightly larger square of 3MM paper covered with cling wrap that was stretched, smoothed and secured so that no wrinkles or folds were evident over the nitrocellulose and exposed in a clean Phosphorimage cassette for two to three days. The Phosphorimage cassette was scanned using a Molecular Dynamics Phosphorimager Model 400B. The resulting image was analysed quantitatively using the Image Quant program in conjunction with Microsoft Excel.

5.2.8 Purification of p34<sub>cd2</sub> protein with antibody beads and assay of its activity

Maize anti-p34<sub>cd2</sub> carboxyterminal antibody was coupled to CNBr-Sepharose as described for p13<sub>suc1</sub> coupling (5.2.6).

Tissue that had been ground in liquid nitrogen was weighed while still frozen and samples of 0.05 g were thawed in 150 µl NDE buffer, vigorously vortexed twice for 20 sec and then spun for 5 min at 14,000 rpm at 4°C. A clear supernatant of 130 µl was removed from all samples into new tubes on ice. Extracts were depleted of agar-binding proteins, as in the early stages of purification with p13 beads, by incubation with agarose beads for 1 h at 4°C. After 1 h the agarose gel was removed by spinning the samples for 5 min at 14,000 rpm at 4°C. The supernatant (130 µl) was removed, from each sample, to a new tube in ice and 40 µl of the prepared p34<sub>cd2</sub> antibody beads in NDE (containing 20 µl of beads) were added to each. The eppendorf tubes were then placed onto a rotating wheel for 2 h, at 4°C, to allow the maize p34<sub>cd2</sub> in the extract to bind to the antibody coupled to the beads.

After approximately 2 h the samples were removed from the wheel and then spun for 2 min at 7,000 rpm. The supernatants were discarded. In the final optimised procedure each pellet was resuspended first in 1 ml of TBS/0.02% Tween and transferred to a new tube on ice. Immediately, the samples were spun again and all gel retained. The pellets were then washed with 1 ml of 0.02% NP40 and finally the pellets were each resuspended in 1 ml of modified assay buffer. Modified assay buffer contained 25 mM β-glycerophosphate, pH 7.3, 10 mM EGTA, 10 mM MgCl₂, 1 mM
DTT, and 25 mM Hepes, pH 7.3. This assay buffer wash was repeated once more, removing as much supernatant as possible without substantial bead loss. All samples were then kept on ice until it was possible to begin the p34\textsuperscript{cdc2} protein kinase assay with ATP at 23 µCi/nmol (5.2.7).

5.2.8.1 p34\textsuperscript{cdc2} purification by anti-p34\textsuperscript{cdc2} antibody beads with use of pre-competition and enhanced washing

Pre-competition of the anti-p34\textsuperscript{cdc2} antibody on beads, with peptide corresponding to maize p34\textsuperscript{cdc2} carboxyterminal region, was used to test the extent of binding that was due to specific antibody affinity for this epitope. For pre-competition antibody beads in NDE were incubated in 1 µM maize terminal peptide at room temperature for approximately 90 min. The stock solution of peptide was 10 µM in TBS buffer. The maize protein extract was also supplemented to 1 µM just prior to the addition of the competed antibody beads. The first wash after the incubation of extract and antibody beads was made with TBS/0.2% Tween containing 100 nM peptide. With these exceptions competed sample treatment was identical to that of uncompetited samples.

Denaturing agents were used to try to dislodge proteins that may have become non-specifically bound to the p34\textsuperscript{cdc2} antibody on the beads. The denaturants tested were urea at 3.5 M, 4.0 M, 4.5 M, 5.0 M and 5.5 M; SDS at 0.5%; NP40 at 0.2%, 0.4% and 0.8%; 1.5 M NaCl and buffer at pH 4.0, and pH 10.0. The solids and detergents were dissolved in NDE buffer, while for the pH treatments, NDE with altered pH was used. The denaturants were used after a preliminary TBS/0.2% Tween wash. Beads were treated for 5 min on ice and then all the samples spun for 2 min at 7,000 rpm. Supernatant was removed without disturbing the beads then the beads were resuspended in 1 ml of modified assay buffer for one wash, after which 25 µl of complete assay buffer was added to each sample and they were ready to be used for a p34\textsuperscript{cdc2} protein kinase assay.
5.2.9 Assay of total H1 histone protein kinase in the unpurified soluble protein fraction

This method was developed to gain an approximate estimate of total histone kinase activity in extracts from different regions of maize leaf.

Samples containing 0.02 g of tissue ground in liquid nitrogen were thawed in 60 µl of cold NDE that had 1-2 min earlier received PMSF. All samples were vortexed for 20 sec, placed on ice for 1 min, vortexed again for 20 sec then spun at 14,000 rpm for 5 min at 4°C. Supernatant (60 µl), avoiding sediment, was then collected into new tubes and placed on ice.

Before the assay the extracts were diluted 1/50 and immediately after they were assayed for H1 histone kinase activity as described in 5.2.7.

5.3 Results

5.3.1 Maize leaf growth

Initial studies were aimed at detecting when an active meristem might be present in seedling maize leaves. Daily measurements of leaf dimension were taken of maize seedlings, after placing their roots in liquid medium. Figure 5.1 shows the growth pattern of leaf 4 over a 40 day period. It can be seen here that growth of the blade is slow over the first 5 days, but then a period of rapid growth follows. It was during this period, at day 12, that leaf 4 was collected for experimental use. At this stage leaf 4 was still juvenile, not yet having developed auricles or ligule that are seen at the position of blade and sheath conjunction (see Figure 5.2) in mature leaves. It is also interesting to note that blade growth and expansion in leaf 4 was almost fully completed by the time that petiole growth began. The blade reached its maximum size by day 25 and the petiole by day 35.
5.3.1.1 Cell size and division activity in the actively growing fourth leaf

Leaf 4 was collected on the twelfth day after the seedlings were placed to grow in liquid medium when the average leaf length was approximately 100 mm (Figure 5.1). Twenty 5 mm segments were cut, providing samples from base to tip of the blade. Sections were infiltrated with PEG (5.2.2.2) and then sections cut for light microscopy. The sections were stained with DAPI to reveal DNA and mitotic activity was found to be greatest in the basal 5 mm (Figure 5.3a). Division rapidly decreased over the next 10 mm, then residual activity declined to zero from 20 mm from the base. The smaller size of actively cycling cells is reflected in the greater frequency of nuclei (Figure 5.3b).

Dimensions were also directly measured in mesophyll cells (Figures 5.4a, b). The initial width of mesophyll cells, measured in the direction from leaf margin to margin, was 7.1 µm in the basal 5 mm of leaf. Cell width then increased to a maximum size of 21 µm by 40 mm from the leaf base (Figure 5.4a). The average length of mesophyll cells (from abaxial to adaxial surfaces) followed a very similar pattern although less increase was necessary to reach full cell length which was obtained by 30 mm from the base. Structural changes accompanied the increases in cell dimensions (Figure 5.5). In the meristematic region the cells were small and the nucleus occupied much of the cell while further up the leaf thickening of cell walls was evident.

Protein concentration (mg per gfw) for each section was highest at the leaf base, at 21.6 mg/gfw of extractable soluble protein, and then rapidly declined over the next 30 mm to a basal level of approximately 5 mg/gfw which was maintained along the remaining length of the leaf (Figure 5.6).

The average protein concentration in leaf segments when assessed in conjunction with the mesophyll cell dimensions (Figure 5.4a, b) allowed an approximate estimate of the amount of protein present per mesophyll cell, making the assumption that other cell types are comparable in size to mesophyll cells and/or are present in negligible amounts. Assuming that mesophyll cells are cylindrical in nature, the approximate mean volume of the cells increases from 5 mm to 35-40 mm from the base at which point the volume size stabilises at about 8000 µm$^3$ and is maintained to near the tip of the leaf when cell volume decreases slightly (Figure 5.7). The approximate number of cells per ml (per gfw) of tissue (still assuming they are cylindrical and that non-mesophyll cells and
intercellular spaces are negligible) is highest in the basal 5 mm and rapidly declines in the next 5 mm, so that by 20-25 mm from the base a constant low number of cells, per gfw, is reached (Figure 5.8). The approximate protein per cell then for cylindrical mesophyll cells is at its lowest in the small cells of the basal 5 mm of the leaf at a mean of 1.2 fg per cell and this level rapidly increased over the next 5 mm, then more steadily over the next 30 mm when an approximate mean of 4 fg/cell is reached. This level of protein is maintained till just near the tip of the leaf when it decreases to a little below 3 fg/cell (Figure 5.9).

An alternative assumption that cell shape was ellipsoid not cylindrical was also used in parallel calculations of approximate mean cell volumes (Figure 5.10) numbers of cells per ml of tissue (Figure 5.11) and amounts of protein per cell (Figure 5.12) in leaf regions. The resulting data were almost identical to that described above when cell shape was assumed to be cylindrical, thus assumption of a cylinder may be acceptable although errors from cells of different types and from intercellular spaces have not been evaluated. The data do indicate a broad picture of increasing cell size and protein content outside the 5 mm basal meristem without accompanying cell division, although increase in cell size may drive the initiation of successive divisions in the meristem region.

5.3.2 p34\textsuperscript{cdc2} in total, soluble and insoluble maize leaf protein fractions

To investigate the proportion of p34\textsuperscript{cdc2} relative to total protein, soluble and insoluble pellet protein fractions were adjusted to similar protein content, separated on acrylamide gels and transferred to nitrocellulose. After blocking the blots were probed using p34\textsuperscript{cdc2} antibody and \textsuperscript{125}I secondary antibody (Figure 5.13). In the total protein fraction (Figure 5.13a) the level of p34\textsuperscript{cdc2} was highest in the first 5 mm and then rapidly decreased over the next segment and declined gradually to the tip. In the soluble protein (Figure 5.13b) the p34\textsuperscript{cdc2} followed a similar pattern, although the amount of p34\textsuperscript{cdc2} recovered here is only half of that observed in the total fraction and the decline between 5 and 10 mm was a little less than that seen in the total protein fraction. In contrast, the amount of p34\textsuperscript{cdc2} detected in the insoluble pellet protein fraction (Figure 5.13c) declined more extensively outside the meristem, thus the presence of p34\textsuperscript{cdc2} in
the particulate fraction may be a characteristic of dividing cells, perhaps reflecting associations with chromosomes or the cytoskeleton.

5.3.3 p34\textsuperscript{cdc2}-like protein kinase activity recovered using p13\textsuperscript{sucl} beads

To allow valid comparison of p34\textsuperscript{cdc2}-like enzyme content in different samples it was necessary to establish what amounts of tissue allowed full recovery of the enzyme on p13 beads. Increasing amounts of ground meristem tissue in the range 0.02 to 0.1 g were extracted and reacted with 20 µl of packed p13 beads and the recovered enzyme was assayed (Figure 5.14a, b). It was deduced that 0.05 g could be used routinely for comparing different segments of leaf.

p34\textsuperscript{cdc2}-like protein purified from extracts of different leaf segments using p13 beads was assayed for histone H1 protein kinase activity (Figure 5.15a, b). The p34\textsuperscript{cdc2}-like activity was very high over the first 10 mm at the base of the leaf blade and dropped to a low basal level maintained along the length of the leaf.

5.3.4 p34\textsuperscript{cdc2} protein kinase activity recovered using p34\textsuperscript{cdc2} antibody beads

The linearity of recovery of p34\textsuperscript{cdc2} enzyme with increasing amounts of leaf meristem protein was investigated, with recovered enzyme being assayed for histone H1 kinase activity (Figure 5.16a, b). It was then possible to compare the level of active enzyme from different regions of leaf (Figure 5.17a, b). As with p13-recovered enzyme the activity in the first and second basal segments (0-10 mm) was very high corresponding to the meristem region then declining to low basal levels elsewhere. The Phosphorimage shows that in addition to phosphorylation of H1 histone other proteins have been labelled, providing an indication that maize proteins other than p34\textsuperscript{cdc2} have been recovered.

The specific activity, relative to amount of crude protein in the initial extract, was calculated for both the p13 bead and p34\textsuperscript{cdc2} antibody bead recovered enzyme (Figure 5.18a, b respectively). For the p13 purified enzyme the highest activity was 0.06 pmol/mg of protein per min in the basal 5-10 mm. This level declined steeply over the next 15 mm to 0.01 pmol/mg protein per min. For the p34\textsuperscript{cdc2} antibody recovered
enzyme the highest activity was only 0.006 pmol/mg of protein per min, an approximately 10 fold lower recovery of activity than obtained with p13 beads. This level detected in the first 5 mm declined steadily and rapidly, till at 55 mm from the base no significant activity remained. The antibody may therefore not be as effective in recovering all of the p34\textsuperscript{cdc2} or else the bound enzyme is not fully active when in immune complex. However, it may be more specific in recovering p34\textsuperscript{cdc2} and not other CDK enzymes and therefore the activity profile along the leaf is of interest in demonstrating the relative (if not absolute) levels of p34\textsuperscript{cdc2} in different regions.

5.3.4.1 **Effect of dissociants and surfactants on the recovery of p34\textsuperscript{cdc2} with antibody**

A selection of different denaturants and detergents was included in washes of the enzyme on p34\textsuperscript{cdc2} antibody beads to attempt to dislodge non-specifically bound proteins. Several agents completely abolished any detectable protein kinase activity. These included a range of urea concentrations from 4 to 5.5 M, 0.5 % SDS and 1.5 M NaCl (data not shown).

In these experiments the recovery of authentic p34\textsuperscript{cdc2} enzyme, due to specific binding, was assessed by the decline in yield when the antibody beads were pre-competed with p34\textsuperscript{cdc2} peptide at a concentration of 1 µM. By this criterion 0.2% NP40 detergent allowed the best retention of p34\textsuperscript{cdc2} protein. In comparison washing with NDE at pH 10.0, reduced approximately by half the recovery of p34\textsuperscript{cdc2}. Greater losses occurred with 0.4 and 0.8% NP40 and greater still with 3.5 M urea and NDE at pH 4.0 (Figure 5.19). Evidence of residual non-specifically bound proteins after these washes came from the labelling of proteins other than H1 histone when protein kinase activity was measured (Figure 5.19b). Only 3.5 M urea removed all alternative substrates but at the cost of discarding much of the p34\textsuperscript{cdc2} activity.

The specific activity was calculated, relative to protein in the original crude soluble extract, for enzyme recovered after washing with denaturing agents and detergents (Figure 5.20). It is again apparent that the NP40 washes retain the most p34\textsuperscript{cdc2} activity. Also, pH 4.0 NDE and 3.5 M urea recover much less total activity and pH 10.0, in comparison recovers by far the greatest total activity, but only a very small
amount of p34\textsuperscript{cd2} specific activity judging by the small reduction in activity when 
authentic p34\textsuperscript{cd2} binding was blocked by pre-competition of the antibody (Figure 5.20).

5.3.4.2 Activity of p34\textsuperscript{cd2} protein kinase seen by optimised immunorecovery from 
regions of leaf

To obtain a more accurate estimate of authentic p34\textsuperscript{cd2} H1 histone kinase 
activity, in regions of the leaf, proteins bound to antibody beads were washed with 0.2% 
NP40 for optimum retention of p34\textsuperscript{cd2} activity while discarding extraneous proteins, 
and authentic p34\textsuperscript{cd2} activity was detected as the activity displaced when the antibody 
had previously been blocked with p34\textsuperscript{cd2} carboxyterminal peptide. Activity of p34\textsuperscript{cd2} 
was at its highest in the first 5 mm at the base of the leaf blade. A substantial reduction 
in specific activity relative to total extracted soluble protein is evident over the next 
5 mm and by 15 mm the p34\textsuperscript{cd2} activity appears to have reached a basal level 
(Figure 5.21). There appears also to be a reduction in the amount of extraneous proteins 
that have been carried over into the assay since the majority of protein labelling is 
restricted to the histone H1 band (Figure 5.21b).

5.3.5 Total soluble protein kinase activity in the leaf

The activity of the total soluble protein kinase in maize leaf tissue was assayed 
after dilution of whole extract to 1/50 to avoid exhaustion of ATP during the assay. 
This dilution factor was derived by a comparison of activity detected with different 
amounts of leaf protein, which showed linearity of activity up to 33 µg total soluble 
protein in the assay (Figure 5.22). At a dilution of 1/50 all samples of leaf tissue 
contributed protein amounts to the kinase assay on the linear portion of the curve. The 
activity detected (Figure 5.23) showed that the cells outside the meristem, in which 
p34\textsuperscript{cd2} kinase activity has fallen essentially to zero, retain other active kinases that are 
presumably involved in the metabolic functions of differentiated cells. The significance 
of this study is two fold: 1) the metabolic protein kinases did not penetrate the purified 
p34\textsuperscript{cd2} fractions (Figure 5.17a, Figure 5.21a), 2) differential control of p34\textsuperscript{cd2} kinase 
occurs in development.
5.3.6 Investigation of p34\textsuperscript{cd2}-like and p34\textsuperscript{cd2} protein and activity in the maize root

The root provides an additional example of cells switching from proliferation to expansion and differentiation as they leave the zone of cell division at the tip meristem. The active zone of cell division in the maize root starts at approximately 0.75 mm back from the tip and continues to 2 - 2.5 mm, a transition between active division and cell elongation then occurs and the elongation zone begins. The elongation zone continues to 8-10 mm from the tip at which point growth ceases (Figure 5.24a).

Erickson and Sax (1956) found in their definitive study of the maize root that cell division was at its highest at 1.25 mm back from the tip, this level rapidly decreased so that by 3 mm from the tip no more actively dividing nuclei were observed (Figure 5.24b). These dimensions were used as the guidelines in this study for the comparison of p34\textsuperscript{cd2} abundance and activity in the meristem and elongation zone regions of the maize root.

5.3.6.1 p34\textsuperscript{cd2} in total, soluble and insoluble maize root protein fractions

To investigate the relative proportions of p34\textsuperscript{cd2} and other proteins in different regions of the root, extracted protein from root meristem and elongation zone regions were fractionated into total, soluble and insoluble pellet protein. These fractions were adjusted to approximately equal protein content and subject to Western blot analysis using anti-p34\textsuperscript{cd2} antibody (Figure 5.25). The level of p34\textsuperscript{cd2} in the total protein fraction of meristem and elongation zone tissue appears to be very similar. In the soluble protein fraction the level of p34\textsuperscript{cd2} was greater in the meristem. In the insoluble pellet protein fraction, this difference is even more evident. There is thus a disparity between levels in total protein and the two subfractions of soluble and insoluble proteins. In the maize leaf when equivalent protein fractions were isolated (5.3.2) the soluble and insoluble fractions summed together approximately equalled the p34\textsuperscript{cd2} detected in the total protein fraction. In contrast the p34\textsuperscript{cd2} detected in the soluble and insoluble protein fractions obtained from the maize root do not sum to the p34\textsuperscript{cd2} recovered in the total fraction indicating that there is a source of inaccuracy within these data.
5.3.6.2 Maize root \( p34^{cd2} \) protein kinase activity recovered with \( p13^{mcl} \)-beads

To enable comparison of \( p34^{cd2} \)-like enzyme activity recovered from different maize root tissue samples it was necessary to first determine what amount of tissue allowed full recovery of the enzyme. As in the leaf 0.05 g of meristem tissue was found to fall within the zone of linear recovery of activity (Figure 5.26a, b) and thus was utilised for comparison of the different maize root regions (Figure 5.27a, b). The activity of the \( p34^{cd2} \)-like protein purified using \( p13 \) beads from maize root tissue was greatest in the first 2 mm of the root. The highest level recovered overall was detected in the 1-2 mm region. Over the next 1 mm, the \( p34^{cd2} \)-like activity dropped steeply, the level then declined more gradually until 5 mm from the tip, at which point the activity leveled out (Figure 5.27a). The Phosphorimaged histone H1 confirms that the only significant \( p34^{cd2} \)-like activity was located in the first 5 mm (Figure 5.27b).

5.3.6.3 Maize root \( p34^{cd2} \) protein kinase activity recovered with antibody

The recovery of \( p34^{cd2} \) enzyme activity was investigated using \( p34^{cd2} \) antibody beads and 0.01 to 0.04 g amounts of root meristem tissue (Figure 5.28a, b). Linear recovery of activity was found when tissue between 0 and 0.03 g was used, therefore 0.02 g of tissue was used to compare the activity from different regions of the root. \( p34^{cd2} \) protein was purified using \( p34^{cd2} \) antibody beads and duplicate samples of each were competed with \( p34^{cd2} \) peptide at 1 µM (Figure 5.29a, b). The total immunorecovered activity from the meristem tissue was approximately a third greater than that from the elongation zone (compare root meristem with root elongation zone in Figure 5.29). However the specifically recovered \( p34^{cd2} \) activity from the meristem sample, indicated by the amount of activity lost when antibody was pre-competed with \( p34^{cd2} \) peptide, was extremely small, only 26,000 counts from 520,000 counts total activity recovered. Nevertheless, this amount is greater than for the elongation zone tissue, in which no authentic \( p34^{cd2} \) protein kinase activity appears to have been recovered.

The recovery of \( p34^{cd2} \) enzyme activity was also investigated in maize seedlings which had their roots detipped of the apical 4 mm. After 4 days the “detipped” roots
showed signs of lateral root primordia extending into the apical region. Equivalent aged seedlings which had not had the apical 4 mm of their roots removed, designated as “tipped” produced lateral primordia only in the mature distal regions of root. After 4 days growth the apical 4 mm and the next 6 mm of root tissue were collected from the “tipped” plants and the next 6 mm of root tissue remaining from the “detipped” plants. These tissue fractions were then assayed for p34\textsuperscript{cdc2} activity using uncompeteted and pre-competed antibody beads (Figure 5.30a, b, c). The 4 mm meristem region from “tipped” plants showed the greatest total activity. Interestingly, the total activity recovered from “detipped” plant elongation zone tissue was greater than that for the “tipped” plants, coincident with the induction of primordia in this region. However specific p34\textsuperscript{cdc2} activity displaced when the antibody beads were pre-competed with p34\textsuperscript{cdc2} peptide was only small (Figure 5.30c). In intact roots the p34\textsuperscript{cdc2} activity was higher in the meristem, almost twice as much as that detected in the elongation zone tissue. Interestingly there was a dramatic increase in the level of p34\textsuperscript{cdc2} activity in the elongation zone tissue from “detipped” plants suggesting that de-tipping the roots has had some effect upon the regulation of p34\textsuperscript{cdc2} activity which correlated with induction of primordia.

5.4 Discussion

In this chapter the specific maize anti-p34\textsuperscript{cdc2} antibody, which was demonstrated to successfully immunorecover native maize p34\textsuperscript{cdc2} protein (Chapter 4), was used to investigate the abundance and activity of p34\textsuperscript{cdc2} in maize leaf and root tissue. This report is notable for the specificity with which p34\textsuperscript{cdc2} is recovered and assayed. Previous studies, which have utilised the mitotic protein p13\textsuperscript{huc1} to affinity purify p34\textsuperscript{cdc2}, may have included closely related variants of p34\textsuperscript{cdc2} in the recovered fraction.

5.4.1 Abundance and activity of p34\textsuperscript{cdc2} and p34\textsuperscript{cdc2}-like protein in the maize leaf

The fourth leaf from maize seedlings harvested 12 days after germination was selected as the most likely to possess an active meristematic region, as well as a zone of differentiated non-dividing cells. Tissue including a transition from division to
differentiation was sought to allow further investigation into an apparent correlation of raised level and activity of p34^{cdk} with regions of active cell division in higher plants (John et al., 1990; Gorst et al., 1991; Martinez et al., 1992). The present study aimed at testing this with a more specific reagent.

When the mitotic index (Figure 5.3a) was determined in 12 day-old fourth leaf it became apparent that actively dividing cells were restricted to the base of the blade. These data agree with previous work showing that maize, like wheat (Kemp, 1980), is possessed of a basal meristem (Sharman, 1942; Poethig, 1984). It must be noted, however, that Sylvester et al. (1990) dispute the existence of a basal meristem in the maize leaf. They described development in terms of division becoming restricted to the base of the leaf, only late in development, usually long after differentiation of the blade is complete. The apparent difference in the findings of Sylvester et al. probably derive from their convention of describing the meristem region as a percentage of overall leaf length. In absolute terms, the meristem occupied a relatively constant 25 mm region from the base of the leaf, and this contributed a progressively decreasing percentage of the growing leaf, although in reality it was not shrinking.

Concurrent with scoring for mitotically active cells, the cell dimensions of width (Figure 5.4a) and length (Figure 5.4b) were measured for mesophyll cells along the length of the fourth leaf. Both cell width and length were smallest at the base, then gradually increased. This progression in cell growth supports the mitotic activity data, as meristematic regions are characterised by the presence of many small cells, then as the frequency of divisions decreases and cells begin to differentiate, cell size increases and the structure of the cells becomes more rigid and defined (Esau, 1977). Taking into consideration these data and making the assumption that mesophyll cells are approximately cylindrical in shape (and that non-mesophyll cells and intercellular spaces are negligible) approximate estimates were made of cell volume (Figure 5.7), cell number (Figure 5.8) and amount of protein per cell (Figure 5.9). These data fit the picture of an active basal meristem, then a transitional zone in which cells expand, and then a region of fully expanded differentiated cells. Confirmation was gained by calculating cell volume, cell number and amount of protein per cell given the assumption that cell shape is ellipsoid rather than cylindrical (Figures 5.10-5.12), and the results obtained were a near perfect match to those based on an assumed cylindrical
cell shape. The close correlation of the two calculations suggests that they give a useful
guide to regions of cell division and growth in the maize leaf.

To investigate whether a change in level of p34\textsuperscript{cdc2} relative to other proteins
coincided with the change in cell division activity the abundance of the p34\textsuperscript{cdc2} protein
was investigated. The level of p34\textsuperscript{cdc2} protein relative to others was consistently highest
at the base of the leaf. Overall, a total decline in the range of 13-16 fold in the level of
p34\textsuperscript{cdc2} was observed in the total and insoluble pellet fractions (Figures 5.13a, c). In the
soluble fraction the decline of p34\textsuperscript{cdc2} was eight fold from base to tip (Figure 5.13b).
Comparison with the average amount of total soluble protein per gfw (Figure 5.6),
which declines three-fold over the length of the leaf, demonstrated that the decline of
p34\textsuperscript{cdc2} protein over the same range is substantially greater and indicates specific
regulation of levels of the protein.

The fact that the p34\textsuperscript{cdc2} protein is more abundant in the region that has been
identified as mitotically active and then declines steadily outside this region suggests an
active role in cell division in the maize leaf. It has been well established that p34\textsuperscript{cdc2}
plays a key role in cell cycle control (reviewed by Lee and Nurse, 1988; Nurse, 1990).
More recently in higher plants this involvement has been indicated by greater levels of
p34\textsuperscript{cdc2}/p34\textsuperscript{cdc2}-like abundance and activity consistently associated with actively
dividing tissue (John et al., 1990; Gorst et al., 1991; Martinez et al., 1992; Hemerly et
al., 1993). Association of p34\textsuperscript{cdc2} with structural elements of the cell is suggested by the
recovery of significant amounts of p34\textsuperscript{cdc2} in the insoluble protein fraction. The specific
decline in p34\textsuperscript{cdc2} confirms a finding made earlier in wheat leaf with less specific
antibody (John et al., 1990). Possible associations of p34\textsuperscript{cdc2} with cytoskeletal elements
have indeed been reported (Alfa et al., 1990; Mineyuki et al., 1991; Ookata et al., 1993)
including in maize (Colasanti et al., 1993).

The abundance of the p34\textsuperscript{cdc2} protein in the region of actively dividing cells
however does not indicate whether the p34\textsuperscript{cdc2} protein kinase activity is also higher in
this region. In the past the activity of p34\textsuperscript{cdc2}-like protein kinases has been determined
using p13 beads (Hindley et al., 1987; Arion et al., 1988; Dunphy et al., 1988; John et
al., 1990). However, the use of p13\textsuperscript{suc2} could allow presence of other CDKs in the
purified fraction as p13 has been shown to bind Cdk2 (Pines and Hunter, 1991a) and
also weakly to Cdk3 (Meyerson et al., 1992) but not to PCTAIRE1 or PCTAIRE3
(Okuda et al., 1992). As the existence of a multiple CDK family in plants has also been indicated (Hirayama et al., 1991; Hashimoto et al., 1992; Hirt et al., 1993) this becomes a consideration that must be taken into account in work on plant systems. Colasanti et al. (1993) concluded that it was impossible to interpret, without ambiguity, the results of any studies that had used antibodies that had not been raised against a conserved region specific to the p34^cdc2 protein. In this study a p34^cdc2 specific maize antibody has been raised that is capable of immunoprecipitating maize p34^cdc2 (Chapter 4) suggesting that the antibody should also be capable of specifically recovering p34^cdc2 activity.

Coupling of anti-maize p34^cdc2 antibody to Sepharose beads enabled the purification of p34^cdc2 from leaf tissue extract, and the bound protein proved to be enzymatically active (Figure 5.17), however other proteins were present as contaminants. In an effort to detach these non-specifically bound proteins but still retain the p34^cdc2 protein, the beads were exposed to several different dissociants and surfactants. The most efficient treatment was determined to be washing of the beads prior to assaying with 0.2% NP40 (Figure 5.19).

The patterns of activity observed for p34^cdc2-like and p34^cdc2 protein kinases in the maize leaf (Figures 5.15, 5.17) recovered respectively by p13 and antibody beads were very similar. This is consistent with p34^cdc2 being the most abundant CDK recovered with p13^suc1 beads. Activity was highest at the very base coinciding with the region of highest mitotic activity. In older cells both p34^cdc2 activity and mitotic activity exhibited a steep decline over the basal 5-15 mm of the leaf. The strong correlation of p34^cdc2 activity with mitotic activity suggests an active role for p34^cdc2 in the control of cell division in the maize leaf. Further support for this proposal comes from the observation that cell volume (Figures 5.7, 5.10) increased in the region where mitotic activity ceased and correlated with the decline in p34^cdc2 activity. Cell size is known to be a critical element in the determination of microbial cell capacity to divide (Nurse and Fantes, 1981; John et al., 1993a, b) including unicellular plants (Donnan and John, 1983). Shutting off activity of the key p34^cdc2 protein kinase may allow cessation of division in cells that are enlarging and differentiating.

The specific activity was calculated for the recovered p34^cdc2-like (Figure 5.18a) and p34^cdc2 protein kinase (Figures 5.18b, 5.20) to assess possible changes in the cell cycle kinase relative to the total population of other enzymes. In both cases the patterns
of specific activity are highest at the base corresponding to the peak mitotic activity. These data again indicate a specific role for p34\(^{\text{cdc2}}\) in cell division. Comparison of the specific activity of p34\(^{\text{cdc2}}\)-like protein kinase and p34\(^{\text{cdc2}}\) protein kinase, after washing with 0.2% NP40 to remove non-specifically bound proteins, indicates that antibody recovered activity is 100 fold lower than activity retained and eluted from p13 beads. This raises the issue of whether the p34\(^{\text{cdc2}}\) activity recovered by the p34\(^{\text{cdc2}}\) antibody beads is an accurate representation of p34\(^{\text{cdc2}}\) abundance in the leaf. The protein may be difficult to recover by antibody because of low levels. Draetta et al. (1988b) found p34\(^{\text{cdc2}}\) to be 0.05% of protein in HeLa and Xenopus cells and only 0.025% in mouse 3T3 cells. However they state that the level is “highly variable in other cell types”. As higher plants have been shown to contain similar amounts of p34\(^{\text{cdc2}}\) to other eukaryotes, including HeLa (John et al., 1989) it seems reasonable to conclude that p34\(^{\text{cdc2}}\) in the maize leaf is also within the established range and as such represents only a very small proportion of the total protein. The capabilities of the antibody must also be taken into account. It has been demonstrated that our p34\(^{\text{cdc2}}\) antibody while capable of detecting native p34\(^{\text{cdc2}}\) also detects denatured p34\(^{\text{cdc2}}\) (Chapter 3) suggesting that only a fraction of the polyclonal antibody can identify the native conformation of p34\(^{\text{cdc2}}\). The retention of only a small amount of p34\(^{\text{cdc2}}\), present in whole extract, loaded onto p34\(^{\text{cdc2}}\) antibody columns (Chapter 4) supports this conclusion. Additionally, antibody-bound enzyme may be catalytically less active than that obtained free with p13\(^{\text{sucl}}\). Therefore, taking into consideration that p34\(^{\text{cdc2}}\) is a rare protein and that the antibody is recovering only a small proportion that may also be hampered in activity, the recovered enzyme can be considered authentic p34\(^{\text{cdc2}}\) and its distribution of significance.

Comparison of the recovery of p34\(^{\text{cdc2}}\) protein kinase activity to the total soluble protein kinases (Figure 5.23) illustrates the rarity of the protein and also demonstrates that the p34\(^{\text{cdc2}}\) activity is regulated independently from the majority of leaf protein kinases. As p34\(^{\text{cdc2}}\) activity decreases seven fold, in the basal 10 mm of the leaf, total soluble protein kinases in contrast exhibit only a two fold decline.
5.4.2 Abundance and activity of the p34<sup>cdc2</sup> and p34<sup>cdc2</sup>-like protein in the maize root

The abundance and activity of p34<sup>cdc2</sup> in the maize root was also investigated in this study. The approximate boundaries observed by Erickson and Sax (1956) for the meristem and elongation zone regions of the maize root were confirmed and used for comparison of p34<sup>cdc2</sup> between these regions. The level of p34<sup>cdc2</sup> protein was consistently higher in the meristem region in soluble, particulate and total protein fractions (Figure 5.25). The amount of p34<sup>cdc2</sup> recovered in the meristem total protein fraction however was less than that detected in the meristem from soluble and insoluble protein fractions suggesting some error in p34<sup>cdc2</sup> detection. The greater abundance of p34<sup>cdc2</sup> protein in the root meristem compared with the elongation zone region was more significant in the insoluble fraction than in others. In the insoluble fraction the content of p34<sup>cdc2</sup> in the meristem was 1.6 fold higher than that in the elongation zone region (Figure 5.25). This observation correlates with the finding in the leaf meristem that the proportion of p34<sup>cdc2</sup> protein in the insoluble fraction is higher in dividing cells. Comparing the meristem region with the adjacent region of non-dividing cells at 12.5 mm average distance up the leaf the level of p34<sup>cdc2</sup> in the meristem was 3.5 fold higher in the insoluble fraction but only 1.3 fold higher in the soluble fraction (Figure 5.13(bi), (ci)). There may therefore be an increased association of p34<sup>cdc2</sup> with structural components in dividing cells. One possibility is an association with the cytoskeleton since protein(s) reacting with anti-PSTAIR and anti-maize p34<sup>cdc2</sup> antibodies have been reported at the PPB (Mineyuki et al., 1991; Colasanti et al., 1993). This possibility is investigated in Chapter 6.

The greater abundance of the p34<sup>cdc2</sup> protein in the meristem tissue of the root suggests that perhaps the protein is more catalytically active in this region. However when activity of specifically-recovered p34<sup>cdc2</sup> was assayed in the meristem of maize root the recovery of p34<sup>cdc2</sup> was minimal (Figure 5.29). Nonetheless recovery of p34<sup>cdc2</sup>-like protein kinase with p13-beads was more successful (Figure 5.27) and was highest in the apical 2 mm of the root, this region being the most mitotically active (Erickson and Sax, 1956). A similar correlation with cell division was observed when the apical 4 mm of roots containing the meristem was removed and lateral root
primordia were induced. In this circumstance antibody beads detected an increase in extractable p34\(^{\text{cdc2}}\) kinase activity (Figure 5.30). The elevation of p34\(^{\text{cdc2}}\) activity in these cells suggests that p34\(^{\text{cdc2}}\) may have a crucial role in this process.

5.4.3 Conclusions

The abundance and activity of p34\(^{\text{cdc2}}\) and p34\(^{\text{cdc2}}\)-like protein kinases were investigated respectively by the \(^{125}\text{I}\) Western blot detection and histone H1 assay techniques in the maize leaf and root. p34\(^{\text{cdc2}}\) protein was highest in the region of active cell division. The activity of the p34\(^{\text{cdc2}}\) protein kinase was likewise highest in meristem regions and resulted from controls exerted on this protein that were not in common with total protein or total protein kinase, neither of which declined to the same extent. The specific independent association of p34\(^{\text{cdc2}}\) with the meristematic region of the maize leaf and root suggests that it may have a role in regulation of cell division. The enzyme was more abundant in the insoluble protein fraction of dividing rather than non-dividing cells. This observation correlates with earlier reports of the association of p34\(^{\text{cdc2}}\)-like (Mineyuki et al., 1991; John et al., 1993a) and p34\(^{\text{cdc2}}\) protein (Colasanti et al., 1993) with cytoskeletal structures involved in cell division.
Figure 5.1
Growth of the fourth maize leaf; changing dimensions of blade (open squares) and sheath (closed diamonds) over a period of 40 days. Leaf 4 was harvested at day 12 after germination (arrow) at which stage the sheath had not yet begun to differentiate. Maximum blade growth was reached by day 25, and sheath growth for analysis of division and p34cdc2 activity by day 35.

Figure 5.2
Structural features of the adult maize leaf. An adaxial view of the mature leaf near the ligule. Note that the auricles are narrow at the midrib and expand marginally to form a wedge (adapted from Freeling and Lane, 1994).
1. 

![Graph showing growth over time](image)

**Time in liquid medium (day)**

Length (mm)

2. 

![Diagram of leaf structures](image)

- intermediate veins
- lateral veins
- midvein
- blade
- blade surface
- midrib
- midrib surface
- intermediate vein
- auricle / ligule surface
- sheath surface
- sheath
Figure 5.3
Frequency distribution of cells in mitosis, in 100 mm long juvenile leaf 4 of maize taken 12 days after germination under conditions described previously (methods 5.2.1). Sequential 5 mm segments were taken from the leaf base to tip. The segments were embedded in PEG, and 15 µm sections were cut and stained with DAPI.

(a)
The mitotic index was determined by counts of cells in the late prophase, metaphase and telophase stages of the cell division cycle. The basal region of the blade was characterised by a high incidence of cells undergoing division suggesting the existence of a basal meristem.

(b)
DAPI staining of cell nuclei showing their distribution in segments cut along the length of the maize leaf at 5 mm intervals. A: 0-5 mm B: 5-10 mm C: 10-15 mm D: 15-20 mm E: 20-25 mm F: 25-30 mm G: 30-50 mm H: 50-75 mm I: 75-100 mm. Actively dividing nuclei are visible only in the basal segments of the blade (arrows).
3. (a)

% cells in prophase, metaphase, telophase

Distance from base in leaf 4 (mm)

3. (b)

A  B  C
D  E  F
G  H  I
Figure 5.4
Dimensions of mesophyll cells, in 100 mm long leaf 4 of juvenile maize seedling harvested 12 days after germination under conditions described previously (methods 5.2.1).
Consecutive 5 mm segments were cut along the length of the blade from base to tip. The segments were embedded in PEG, 15 µm sections cut and stained with DAPI.

(a) Average mesophyll cell width, measured in the direction parallel with the upper leaf surface from margin to margin of the leaf.

(b) Average mesophyll cell length, measured in the direction from abaxial to adaxial surfaces of the leaf.
4. (a) Mesophyll cell width

Distance from base in leaf 4 (mm)

Mesophyll cell width, margin to margin (μm)

4. (b) Mesophyll cell length

Distance from base in leaf 4 (mm)

Mesophyll cell length, abaxial to adaxial surfaces (μm)
Figure 5.5
Series of DIC images showing the changes in cell structure along the length of the fourth leaf of maize seedlings from base to tip. The nuclei (arrows) are densely packed in small cells in the basal 5 mm of the leaf, and distally cell size rapidly increases. In the differentiated tissue of the tip the cell walls have thickened and organelles are sparsely distributed. The images are the bright field equivalent of the DAPI stained cells presented in Figure 5.3b. A: 0-5 mm B: 5-10 mm C: 10-15 mm D: 15-20 mm E: 20-25 mm F: 25-30 mm G: 30-50 mm H: 50-75 mm I: 75-100 mm.

Figure 5.6
Changes in the amount of average soluble protein per fresh weight during leaf cell development (mg protein/gfw).
Sequential 5 mm segments were cut along the length of 100 mm long leaf 4 from juvenile maize seedlings harvested 12 days after germination. The segments were frozen in liquid nitrogen and then ground to powder. The soluble protein was extracted from the ground tissue using RIPA buffer as described previously (methods 5.2.3) and assayed (methods 5.2.4).
Figure 5.7
Approximate volume of average mesophyll cell assuming that mesophyll cells are approximately cylindrical in shape.
Volume per cell was calculated using the mesophyll cell width and length dimensions presented in Figure 5.4, for cells in 5 mm segments cut along the length of a juvenile maize leaf collected 12 days after germination.

Figure 5.8
Approximate number of mesophyll cells along the length of maize leaf 4 assuming that mesophyll cells are approximately cylindrical in shape.
Number of cells per cubic ml of tissue was calculated by division of an estimate of the volume of a 10 mm$^3$ region of tissue (1x10$^{12}$ µm) by the cell volumes calculated in Figure 5.7.

Figure 5.9
Approximate amount of protein per cell for mesophyll cells along the length of a juvenile maize leaf assuming mesophyll cells are approximately cylindrical in shape.
The amount of protein per cell (fg) was calculated by division of the soluble protein concentration (mg protein/gfw) shown in Figure 5.6 by the approximate number of mesophyll cells per ml of tissue calculated in Figure 5.8.
7. Mesophyll cell volume

8. Number of mesophyll cells

9. Amount of protein
Figure 5.10
Approximate volume of average mesophyll cells assuming that mesophyll cells are approximately ellipsoid in shape.
Volume per cell was calculated using the average mesophyll width and length dimensions described previously (Figure 5.4) for cells in 5 mm sequential segments cut from a 100 mm long juvenile maize leaf taken 12 days after germination.

Figure 5.11
Approximate number of mesophyll cells along the length of maize leaf 4 assuming that mesophyll cells are approximately ellipsoid in shape.
The number of cells per cubic ml of tissue was calculated by division of an estimate of the volume of a 10 mm$^3$ region of tissue (1x10$^{12}$ µm) by the cell volumes calculated in Figure 5.10.

Figure 5.12
Approximate amount of protein per cell for mesophyll cells along the length of a juvenile maize leaf assuming that mesophyll cells are approximately ellipsoid in shape.
The amount of protein per cell (fg) was calculated by division of the soluble protein concentration (mg protein/gfw) shown in Figure 5.6 by the approximate number of mesophyll cells per ml of tissue calculated in Figure 5.11.
10. Mesophyll cell volume

Distance from base in leaf 4 (mm)

11. Number of mesophyll cells

Distance from base in leaf 4 (mm)

12. Amount of protein

Distance from base in leaf 4 (mm)
Figure 5.13

Level of $p34^{cd2}$ protein relative to other proteins from base to tip of maize leaf 4. Sequential 5 mm segments were cut from 100 mm long fourth leaf of juvenile leaf of maize seedlings taken 12 days after germination. The segments were frozen in liquid nitrogen and ground to powder. Three protein fractions were extracted from the tissue segments; (a) total protein was extracted from 0.02 g of frozen cell powder in Sx2 (SDS) electrophoresis buffer; (b) soluble protein was extracted from 0.02 g of ground tissue in RIPA buffer; (c) insoluble protein was extracted from 0.02 g of frozen tissue in equal volumes of Sx2 electrophoresis buffer and RIPA buffer. Equal loadings of protein were separated on 12% SDS-PAGE and the separated proteins transferred to nitrocellulose. The blots were probed with affinity purified maize anti-$p34^{cd2}$ antibody and detected with $^{125}$I anti-rabbit Ig second antibody (Tago, Inc., USA). The images shown in 5.13(ai), (bi), (ci) were obtained by exposure of the blot in a Phosphorimager. Analysis of the image was performed to determine the levels of $p34^{cd2}$ protein as shown in 5.13(ai), (bi), (ci).
13. (ai) $p^{34 \text{cdc}2}$ in total protein fraction of leaf

![Graph showing $p^{34 \text{cdc}2}$ in total protein fraction of leaf.](graph1)

13. (a(ii))

![Image showing protein bands.](image1)

13. (bi) $p^{34 \text{cdc}2}$ in soluble protein fraction of leaf

![Graph showing $p^{34 \text{cdc}2}$ in soluble protein fraction of leaf.](graph2)

13. (b(ii))

![Image showing protein bands.](image2)

13. (ci) $p^{34 \text{cdc}2}$ in insoluble protein fraction of leaf

![Graph showing $p^{34 \text{cdc}2}$ in insoluble protein fraction of leaf.](graph3)

13. (c(ii))

![Image showing protein bands.](image3)
Figure 5.14
Recovery of $p34^{cd2}$-like protein kinase activity with p13 beads from different amounts of leaf meristem tissue.
Proteins were extracted in NDE buffer from the frozen ground tissue of the fifth leaf of a juvenile maize seedling taken 12 days after germination. The $p34^{cd2}$-like protein kinase was purified from the proteins by 20 µl of p13 sucl beads and eluted with 50 µl of 0.5 mg/ml of free p13 sucl. (a) The activity of the kinase was measured using histone H1 as a substrate at 30°C for 5 min. (b) Phosphorimaged histone H1 was obtained by separating 30 µl of reaction mixture on 12% SDS-PAGE, transfer to nitrocellulose and exposure in a Phosphorimager.
Lane 1: histone H1 labelled by enzyme from 0.02 g leaf meristem
Lane 2: histone H1 labelled by enzyme from 0.04 g leaf meristem
Lane 3: histone H1 labelled by enzyme from 0.06 g leaf meristem
Lane 4: histone H1 labelled by enzyme from 0.1 g leaf meristem
Quantification of the image in (b) produced the figure shown in (a). The activity detected was linear between 0 and 0.1 g of tissue indicating that enzyme was fully recovered within this range.

Figure 5.15
$p34^{cd2}$-like protein kinase activity along the length of maize leaf 4 recovered with p13 beads.
Sequential 5 mm segments were cut from the 100 mm long fourth juvenile maize leaf harvested 12 days after germination. The segments were frozen and ground into powder. Proteins were extracted from the frozen tissue in NDE buffer. The $p34^{cd2}$-like protein was purified with p13 sucl beads. The activity of the $p34^{cd2}$-like protein was assayed using histone H1 as a substrate at 30°C for 5 min. The histone H1 image shown in (b) was obtained by separating 30 µl of reaction mixture on 12% acrylamide gel, transfer to nitrocellulose then exposure in a Phosphorimager. Quantification of the enzymatically attached phosphate produced the distribution shown in (a).
14. (a)

[p34cdc2-like activity recovered with p13]

([32P]PO4 transferred to H1 histone; Phosphorimager counts x10^5)

Weight maize leaf meristem tissue (g)

14. (b)

[Image of gel]

15. (a)

[p34cdc2-like activity recovered with p13]

([32P]PO4 transferred to H1 histone; Phosphorimager counts/fresh weight tissue x10^5)

Distance from base in leaf 4 (mm)

15. (b)

[Image of gel]
Figure 5.16
Recovery of p34<sup>cdc2</sup> protein kinase activity from maize leaf meristem by antibody beads. Proteins were extracted in NDE buffer from different amounts of frozen ground tissue of the fifth leaf of maize seedlings collected 12 days after germination. The p34<sup>cdc2</sup> protein kinase was purified by binding to 20 µl of p34<sup>cdc2</sup> antibody beads. The activity of the kinase bound to the beads was measured using histone H1 as a substrate at 30°C for 5 min. The amount of 32P transferred to H1 histone was measured by placing 20 µl of reaction mixture onto P81 phosphocellulose paper, washing with 75 mM phosphoric acid and counting in a scintillation counter (a). Phosphorimaged histone H1 was obtained by separating 30 µl of reaction mixture on 12% SDS-PAGE, transfer onto nitrocellulose and exposure in a Phosphorimagier (b).
Lane 1: histone H1 labelled by enzyme recovered from 0.02 g leaf meristem
Lane 2: histone H1 labelled by enzyme recovered from 0.04 g leaf meristem
Lane 3: histone H1 labelled by enzyme recovered from 0.06 g leaf meristem
Lane 4: histone H1 labelled by enzyme recovered from 0.1 g leaf meristem
The recovered activity was linear with amounts of tissue between 0 and 0.1 g indicating that the enzyme was fully recovered within this range.

Figure 5.17
p34<sup>cdc2</sup> protein kinase activity from base to tip of the fourth leaf of maize recovered by antibody beads. Consecutive 5 mm segments were cut from the 100 mm length of leaf 4 from juvenile maize seedlings 12 days after germination. The segments were frozen in liquid nitrogen and ground to powder. Proteins were extracted from the ground tissue in NDE buffer. The p34<sup>cdc2</sup> protein was purified by binding to p34<sup>cdc2</sup> antibody beads. The activity of the protein was assayed using histone H1 as a substrate at 30°C for 5 min. The Phosphorimage shown in (b) was obtained by running 30 µl of reaction mixture on a 12% acrylamide gel, the separated proteins were then transferred to nitrocellulose and exposed in a Phosphorimagier. Quantification of the image produced the distribution shown in (a).
16. (a)

![Graph showing p34cdc2 activity recovered with antibody (pmol [32P]PO4 transferred to H1 histone) vs. weight maize leaf meristem tissue (g).]

16. (b)

![Image of autoradiogram with lanes labeled 1 through 4.]

17. (a)

![Graph showing p34cdc2 activity recovered with antibody (pmol [32P]PO4 transferred to H1 histone) vs. distance from base in leaf 4 (mm).]

17. (b)

![Image of autoradiogram with lanes labeled.]

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p34cdc2 activity recovered with antibody (pmol [32P]PO4 transferred to H1 histone) vs. weight maize leaf meristem tissue (g).

Phosphorimager counts/fresh weight tissue x 10^5 vs. distance from base in leaf 4 (mm).
Specific activity relative to total extracted soluble proteins of (a) p34\textsuperscript{cdc2}-like protein kinase recovered by p13 beads and (b) p34\textsuperscript{cdc2} protein kinase recovered by antibody beads. Note that the ordinate scale in (b) is eleven times smaller than in (a).

Serial 5 mm segments were cut from the 100 mm long fourth leaf of maize seedlings 12 days after germination. The segments were frozen and then ground to powder. Proteins were extracted from the frozen tissue in NDE buffer. Affinity purification with p13\textsuperscript{suc1} beads purified the p34\textsuperscript{cdc2}-like proteins as described previously (methods 5.2.6). The p34\textsuperscript{cdc2} proteins were purified by binding to p34\textsuperscript{cdc2} antibody beads. The activity of the p34\textsuperscript{cdc2}-like protein eluted from p13 beads by p13\textsuperscript{suc1} and p34\textsuperscript{cdc2} proteins still bound to antibody beads was measured using histone H1 as a substrate at 30°C for 5 min. The amount of $^{32}$P transferred to H1 histone was measured by aliquotting 20 µl of reaction mixture onto P81 phosphocellulose paper, washing with 75 mM phosphoric acid and counting in a scintillation counter. Specific activity was calculated by division of histone phosphorylation (Figures 5.15, 5.17) by the amount of soluble protein detected in the corresponding leaf segment (Figure 5.6).
18. (a)

p34cdc2-like activity recovered with p13
(pmol [\( {^{32}P} \)PO\(_4 \)] transferred to H1 histone/mg protein/min at 30°C)

Distance from base in leaf 4 (mm)

18. (b)

p44/62 activity recovered with antibody
(pmol [\( {^{32}P} \)PO\(_4 \)] transferred to H1 histone/mg protein/min at 30°C)

Distance from base in leaf 4 (mm)
**Figure 5.19**
Effect of dissociants and surfactants present during the purification procedure on the recovery of p34\(^{cdc2}\) protein kinase activity from maize leaf meristem tissue using antibody beads.

Proteins were extracted in NDE buffer from the frozen ground tissue of leaf 5 from maize seedlings 12 days after germination. The p34\(^{cdc2}\) protein was purified by binding to p34\(^{cdc2}\) antibody beads. To remove non-specifically bound proteins the beads were washed prior to the assay in various dissociant and surfactant solutions. (a) The activity of the bound proteins was measured using histone H1 as a substrate at 30\(^{\circ}\)C for 5 min. After the assay 30 µl of reaction mixture was run on 12% SDS-PAGE, the separated proteins were transferred to nitrocellulose and then quantified in a Phosphorimager. (b) Phosphorimage of labelled H1 histone after assay.

Panel A shows the phosphate recovered when antibody beads were washed with:
- Lane 1: 0.2% NP40
- Lane 2: 0.4% NP40
- Lane 3: 0.8% NP40
- Lane 4: pH 4.0
- Lane 5: pH 10.0
- Lane 6: 3.5 M urea

Panel B shows the phosphate detected when antibody beads were pre-competed with 1 µM p34\(^{cdc2}\) peptide prior to protein binding. Lanes 1-6 contain the same washes respectively as in Panel A.

Quantification of the enzymatically attached phosphate shown in (b) Panel A is shown in (a).

**Figure 5.20**
Specific activity relative to total extracted soluble proteins of the p34\(^{cdc2}\) protein kinase recovered from maize leaf meristem after inclusion of various dissociants and surfactants during the purification procedure.

The best recovery of p34\(^{cdc2}\) protein kinase activity was observed when NP40 at 0.2%, 0.4% and 0.8%, pH at 4.0, and 10.0, and 3.5 M urea were used to wash the antibody beads after binding p34\(^{cdc2}\). The difference between the equivalent uncompeted and competed beads for each treatment signifies the exact amount of p34\(^{cdc2}\) recovered.

Proteins were extracted in NDE buffer from the frozen ground tissue of leaf 5 from maize seedlings harvested 12 days after germination. The p34\(^{cdc2}\) protein was purified by binding to uncompeted and competed p34\(^{cdc2}\) antibody beads. Antibody beads were pre-competed with 1 µM p34\(^{cdc2}\) peptide prior to protein binding for 90 min at 25\(^{\circ}\)C. Non-specifically bound proteins were removed from the beads by washes prior to the assay of the beads in various dissociants and surfactants. The activity of the bound proteins was assayed using histone H1 as a substrate at 30\(^{\circ}\)C for 5 min. The amount of \(^{32}\)P transferred to H1 histone was measured by placing 20 µl of reaction mixture onto P81 phosphocellulose paper, washing with 75 mM phosphoric acid and counting in a scintillation counter.
19. (a)

- **p34<sup>cdcl2</sup>** activity recovered with antibody
- ([<sup>32</sup>P]PO<sub>4</sub> transferred to HI histone; Phosphorimager counts/fresh weight tissue x10<sup>5</sup>)
- **Treatments**

- 0.2% NP40, 0.4% NP40, 0.8% NP40, pH 4.0, pH 10.0, 3.5 M urea

19. (b)

- **A**
- **B**

20.

- **p34<sup>cdcl2</sup>** activity recovered with antibody
- ([<sup>32</sup>P]PO<sub>4</sub> transferred to HI histone; pmol/mg protein/min at 30°C)
- **Treatments**

- 0.2% NP40, 0.4% NP40, 0.8% NP40, pH 4.0, pH 10.0, 3.5 M urea
Figure 5.21
Distribution within the maize leaf of authentic p34\textsuperscript{cdc2} protein kinase activity that competed with p34\textsuperscript{cdc2} peptide for binding to p34\textsuperscript{cdc2} antibody beads.
Consecutive 5 mm segments were cut from base to tip of the fourth leaf of 100 mm long juvenile maize seedlings harvested 12 days after germination. The segments were frozen and then ground to powder. Proteins were extracted from the ground tissue in NDE buffer. The p34\textsuperscript{cdc2} proteins were purified by binding to un-competed and competed p34\textsuperscript{cdc2} antibody beads. Pre-competed beads were incubated with 1 µM p34\textsuperscript{cdc2} peptide for 90 min at 25°C prior to protein binding. Non-specifically bound proteins were removed from the beads by washing in 0.2% NP40 prior to the assay. The activity of the p34\textsuperscript{cdc2} protein was measured using histone H1 as a substrate at 30°C for 5 min.
(b) The Phosphorimage shown was obtained by running 30 µl of reaction mixture on a 12% acrylamide gel, the separated proteins were then blotted onto nitrocellulose and exposed in a Phosphorimager. Quantification of the image produced the distribution shown in (a).
21. (a)

Authentic P\textsuperscript{32} activity recovered with antibody

(\textsuperscript{32}P)PO\textsubscript{4} transferred to H\textsubscript{1} histone;

Phosphorimagery counts/fresh weight tissue x 10\textsuperscript{6}

21. (b)
Figure 5.22
Catalytic activity of the total soluble protein kinases detected in assays of increasing amounts of extract from maize leaf meristem.
The basal 5 mm of the fourth leaf of maize seedlings collected 12 days after germination was frozen and then ground into powder. Proteins were extracted from different amounts of the ground tissue in NDE buffer. The supernatant containing the total soluble proteins in the extract was diluted to 1/50 and 20 µl assayed. The activity of the proteins was measured using histone H1 as a substrate at 30°C for 5 min. The amount of $^{32}$P transferred to H1 histone was assessed by placing 20 µl of reaction mixture onto P81 phosphocellulose paper, washing with 75 mM phosphoric acid and counting in a scintillation counter (a). Phosphorimaged H1 histone was obtained by separating 30 µl of reaction mixture on 12% SDS-PAGE, transferring to nitrocellulose then exposure in a Phosphorimager (b).
Lane 1: H1 histone labelled by enzyme in extract from 33 µg of leaf meristem protein
Lane 2: H1 histone labelled by enzyme in extract from 11.1 µg of leaf meristem protein
Lane 3: H1 histone labelled by enzyme in extract from 3.7 µg of leaf meristem protein
Lane 4: H1 histone labelled by enzyme in extract from 1.2 µg of leaf meristem protein
Lane 5: H1 histone labelled by enzyme in extract from 0.41 µg of leaf meristem protein
Lane 6: H1 histone labelled by enzyme in extract from 0.14 µg of leaf meristem protein
The recovery of the total protein kinase activity was linear between 0 and 33 µg indicating that the ATP was not exhausted during assay of enzyme from amounts of tissue in this range.

Figure 5.23
Detection of total soluble protein kinase activity along the length of the maize leaf. Sequential 5 mm segments were cut from the length of the fourth leaf of maize seedlings 12 days after germination. The segments were frozen in liquid nitrogen and then ground to powder. Proteins were extracted from the ground tissue in NDE buffer. The proteins were diluted 1/50, then 20 µl containing proteins from 0.02 g of tissue was assayed. The activity of the unpurified proteins was assayed using histone H1 as a substrate at 30°C for 5 min. After the reaction, proteins were electrophoretically separated, transferred to nitrocellulose and exposed in a Phosphorimager (b). Quantification of (b) to determine total labelled proteins is shown in (a).
22. (a) Total soluble protein kinase activity (pmol [32P]PO₄ transferred to H1 histone/mg protein/min at 30°C x 10⁵) vs. Soluble leaf meristem protein (μg).

22. (b) Image showing a gel with bands.

23. (a) Total soluble protein kinase activity ([32P]PO₄ transferred to H1 histone; phosphorimager counts/fresh weight tissue x 10⁶) vs. Distance from base in leaf 4 (mm).

23. (b) Image showing a gel with bands.
Figure 5.24
Zones of regional cell specialisation within the maize root (a) and distribution of cells in the mitotic phase within the region of actively dividing cells (b).
(a) The maize root tip is characterised by four regions of cell specialisation; first is the root cap; the second is where the meristem resides (stippled); the third is the cell elongation zone in which cell division has ceased and the fourth in which cells have differentiated and growth ceases. The region of active cell division is within the apical 2.5 mm of the root tip.
(b) The mitotic index was calculated by maceration of cells from segments of the meristem and scoring of cells judged to be in the prophase, metaphase, anaphase and telophase phases of the cell cycle. [(a) adapted from Luxova, 1980; (b) redrawn from (Erickson and Sax, 1956)].

Figure 5.25
Level of p34\textsuperscript{cdc2} protein in maize root meristem (M) and elongation zone (E) tissue. The apical 3 mm of root tip was collected for the meristem tissue, the next 7 mm of the root containing the elongation zone was also collected. This tissue was frozen in liquid nitrogen then ground to powder. Three different protein fractions were extracted from the meristem and elongation zone tissues. Total protein (T) was extracted from 0.02 g of ground tissue in Sx2 (SDS) electrophoresis buffer. Soluble protein (S) from 0.02 g of ground tissue was extracted in RIP A buffer and remained in the supernatant after 5 min at 14,000 rpm. Insoluble protein (I) was extracted from the pellet remaining after soluble proteins were removed, in equal volumes of Sx2 electrophoresis buffer and RIPA buffer. Equal loadings of protein were separated on 12% SDS-PAGE and the separated proteins blotted onto nitrocellulose. The blot was probed with affinity purified maize anti-p34\textsuperscript{cdc2} antibody and detected with \textsuperscript{125}I anti-rabbit Ig second antibody (Tago, Inc., USA). The three meristem protein fractions are grouped together; TM: total protein, SM: soluble protein, IM: insoluble protein. Similarly elongation zone protein fractions are grouped together; TE: total protein, SE: soluble protein, IE: insoluble protein.
24. (a)

![Diagram showing root and cell zones.]

24. (b)

![Bar graph showing mitotic index across distances from root tip.]

25.

![Bar graph showing protein detection across maize root meristem and elongation zone protein fractions.]

Maize root meristem (M) and elongation zone (E) protein fractions
**Figure 5.26**
Recovery of p34\(^{\text{cdc2}}\)-like protein kinase activity with p13 beads from maize root meristem tissue.

Different amounts of frozen ground maize root meristem between 0 and 0.1 g were thawed in NDE buffer and the proteins extracted. The p34\(^{\text{cdc2}}\)-like protein kinase was purified by binding to 20 µl of p13\(^{\text{suc1}}\) beads and eluted with 50 µl of 0.5 mg/ml of p13\(^{\text{suc1}}\). The activity of the kinase was assayed using histone H1 as a substrate at 30°C for 5 min. A Phosphorimage of the histone H1 was obtained by separating 30 µl of the reaction mix on a 12% acrylamide gel, transferring to nitrocellulose and exposure in a Phosphorimager (b).

Lane 1: histone H1 labelled by enzyme recovered from 0.02 g of root meristem
Lane 2: histone H1 labelled by enzyme recovered from 0.04 g of root meristem
Lane 3: histone H1 labelled by enzyme recovered from 0.06 g of root meristem
Lane 4: histone H1 labelled by enzyme recovered from 0.1 g of root meristem

Quantification of the enzymatically attached phosphate is shown in (a). Linear recovery of activity was detected between 0 and 0.1 g indicating the enzyme was fully recovered from tissue weights within this range.

---

**Figure 5.27**
Distribution of p34\(^{\text{cdc2}}\)-like protein kinase activity recovered by p13 beads in the basal 15 mm of the maize root.

Six 1 mm segments then five 2 mm segments were cut sequentially back from the apical tip of the root. The sections were frozen in liquid nitrogen and then ground to powder. Protein was extracted from the ground tissue in NDE buffer. The p34\(^{\text{cdc2}}\)-like protein kinase was recovered by affinity purification with p13\(^{\text{suc1}}\) beads and elution with free p13. The activity of the kinase was measured using histone H1 as a substrate at 30°C for 5 min. The histone H1 image shown in (b) was obtained by separating 30 µl of the reaction mixture on 12% SDS-PAGE, blotting to nitrocellulose then exposure in a Phosphorimager. Quantification of the enzymatically attached phosphate is shown in (a).
p34\textsuperscript{cdc2}-like activity recovered with p13
\((\text{I}^3P_3)\text{PO}_4\) transferred to H1 histone; Phosphorimager counts/fresh weight tissue x10\(^5\)

Weight maize root meristem tissue (g)

p34\textsuperscript{cdc2}-like activity recovered with p13\((\text{I}^3P_3)\text{PO}_4\) transferred to H1 histone; Phosphorimager counts/fresh weight tissue x10\(^5\)

Distance from root tip (mm)
Figure 5.28
Recovery of p34^{cd2} protein kinase catalytic activity with antibody beads from maize root meristem tissue.
Different amounts of frozen ground maize root meristem between 0 and 0.04 g were thawed in NDE buffer and the proteins extracted. The p34^{cd2} protein kinase was purified by binding to 20 µl of p34^{cd2} antibody beads. Non-specifically bound proteins were removed from the beads by washing in 0.2% NP40 prior to the assay. The activity of the p34^{cd2} kinase was measured using histone H1 as a substrate at 30°C for 5 min. Phosphorimaged histone H1 was obtained by separating 30 µl of the reaction mixture on a 12% acrylamide gel, transferring to nitrocellulose and exposure in a Phosphorimag (b).
Lane 1: histone H1 labelled by enzyme recovered from 0.01 g of maize root meristem
Lane 2: histone H1 labelled by enzyme recovered from 0.02 g of maize root meristem
Lane 3: histone H1 labelled by enzyme recovered from 0.03 g of maize root meristem
Lane 4: histone H1 labelled by enzyme recovered from 0.04 g of maize root meristem
The recovery of activity was linear between 0 and 0.03 g indicating that the antibody on the 20 µl of beads assayed was not saturated within this range.
Quantification of H1 histone labelling seen in (b) produced the distribution shown in (a).

Figure 5.29
Level of p34^{cd2} protein kinase activity recovered with antibody beads from meristem and elongation zone tissue of the maize root.
The apical 3 mm of the root tip was collected as the meristem sample, then next 7 mm of root tissue containing the elongation zone was excised and collected. This tissue was frozen and ground to powder. Protein was extracted from the meristem and elongation zone tissue in NDE buffer. The p34^{cd2} protein was purified by binding parallel samples to 20 µl of uncompeted and pre-competed p34^{cd2} antibody beads. Antibody beads were pre-competed with 1 µM p34^{cd2} peptide at 25°C for 90 min prior to protein binding. Non-specifically bound proteins were removed from the beads by washing in 0.2% NP40 prior to the assay. The activity of the p34^{cd2} kinase was measured using histone H1 as a substrate at 30°C for 5 min. Histone H1 labelled by enzyme recovered on uncompeted and competed beads was separated by electrophoresis of 30 µl of reaction mixture, transfer to nitrocellulose then exposed in a Phosphorimag (b).
RM: p34^{cd2} activity recovered from meristem tissue using uncompeted antibody beads
CRM: p34^{cd2} activity recovered from meristem tissue using competed antibody beads
RE: p34^{cd2} activity recovered from elongation zone tissue using uncompeted antibody beads
CRE: p34^{cd2} activity recovered from elongation zone tissue using competed antibody beads.
Quantification of the histone H1 image in (b) is shown in (a).
28. (a)

![Graph showing p34cdc2 activity recovered with antibody against [32P]PO₄ transferred to H1 histone; Phosphorimager counts/fresh weight tissue x10⁶ vs. Weight maize root meristem (g).]

28. (b)

![Image showing gel electrophoresis lanes 1 to 4.]

29. (a)

![Bar chart showing p34cdc2 activity recovered with antibody against [32P]PO₄ transferred to H1 histone; Phosphorimager counts/fresh weight tissue x10⁶ for RM, CRM, RE, and CRE root tissue and treatments.]

29. (b)

![Image showing gel electrophoresis for RM, CRM, RE, and CRE.]

Figure 5.30

Level of p34^cdc2 protein kinase activity recovered with antibody beads in maize root meristem and elongation zone tissue from "detipped" roots in which the meristem was removed and "tipped" roots which retained their meristem region.

After germination the apical 4 mm of root tip was removed from half the seedlings designated de-tipped. The "tipped" and "detipped" seedlings were grown for 4 days with their root ends immersed in liquid medium, after which the end 6 mm of the "detipped" roots was removed. The equivalent 6 mm segment of root was collected from "tipped" plants. The tissue segments were frozen and ground to powder. Protein was extracted from the ground tissue in NDE buffer. The p34^cdc2 protein was purified from samples treated in parallel with 20 µl of uncompe ted/pre-competed p34^cdc2 antibody beads. Antibody beads were pre-competed using 1 µM p34^cdc2 peptide at 25°C for 90 min prior to protein binding. Non-specifically bound proteins were removed from the beads by washing in 0.2% NP40 prior to the assay.

(a) The activity of the p34^cdc2 kinase was measured using histone H1 as a substrate at 30°C for 5 min.

(b) Phosphorimaged histone H1 was obtained by separating 30 µl of reaction mixture on 12% SDS-PAGE, blotting onto nitrocellulose then exposure in a Phosphorimager.

Lane 1: TRM - histone H1 labelled by enzyme recovered from meristem of "tipped" maize seedlings using uncompe ted antibody beads
Lane 2: CTRM - histone H1 labelled by enzyme recovered from meristem of "tipped" maize seedlings using competed antibody beads
Lane 3: TRE - histone H1 labelled by enzyme recovered from elongation zone of "tipped" maize seedlings using uncompe ted antibody beads
Lane 4: CTRE - histone H1 labelled by enzyme recovered from elongation zone of "tipped" maize seedlings using competed antibody beads
Lane 5: DTRE - histone H1 labelled by enzyme recovered from elongation zone of "detipped" maize seedlings using uncompe ted antibody beads
Lane 6: CDTRE - histone H1 labelled by enzyme recovered from elongation zone of "detipped" maize seedlings using competed antibody beads

Quantification of the enzymatically transferred phosphate on H1 histone is shown in (a).

(c) The recovery of authentic p34^cdc2 enzyme due to specific binding assessed by the decline in yield when antibody beads were competed with p34^cdc2 peptide.
30. (a) 

Total activity recovered with antibody (\([^{32}P] PO_4\) transferred to H1 histone/fresh weight tissue; Phosphorimager counts x10^5)

Root tissue and treatments

30. (b) 

30. (c) 

Authentic p34cyc activity recovered with antibody (\([^{32}P] PO_4\) transferred to H1 histone; Phosphorimager counts/fresh weight tissue x10^5)
CHAPTER 6:

DEVELOPMENT OF PROCEDURES FOR INDIRECT IMMUNOFLUORESCENCE STAINING OF p34cde2 AND INVESTIGATION OF ITS ASSOCIATION WITH THE MICROTUBULAR CYTOSKELETON
6.1 Introduction

6.1.1 Associations of p34cdc2 with the microtubular cytoskeleton

In animals cells p34cdc2 has been identified in association with division specific structures including centrosomes, chromosomes, kinetochores, microtubule fibres and the mitotic spindle apparatus (Bailly et al., 1989; Riabowol et al., 1989; Alfa et al., 1990; Rattner et al., 1990; Tombes et al., 1991; Kubiak et al., 1993; Ookata et al., 1993; Andreassen and Margolis, 1994). Given then that p34cdc2 seems to fulfil a similar key position in the regulation of the cell division cycle in plants (reviewed in Nurse, 1990; Jacobs, 1992; Staiger and Doonan, 1993; Francis and Halford, 1995), it is reasonable to expect a comparable association of p34cdc2 with division specific elements in plants. This does indeed appear to be the case, with the first evidence of such an association reported by Mineyuki et al. (1991). They observed co-localisation of a p34cdc2-like protein with the PPB, using a monoclonal anti-PSTAIR antibody. John et al. (1993a) subsequently confirmed this observation using an independently developed polyclonal anti-PSTAIR antibody. However the use of anti-PSTAIR antibodies in these studies only indicated the possible involvement of p34cdc2. Colasanti et al. (1993) were the first to observe any localisation using a specific anti-p34cdc2 antibody. As in the two previous studies, p34cdc2 was found associated with the PPB only. From the low incidence of detection it was speculated that this association was only transient but, the authors suggest, significant. They propose that the p34cdc2 protein kinase may have a role in the determination of the division site. Thus although the expectation was that p34cdc2 would be associated with division-specific structures in common between plants and animals, the published results to date show only an association with a division-specific structure that is not found in animals, namely the PPB.

Knowledge of the activity and localisation of p34cdc2 in higher plant cells is limited. In this thesis we presented new evidence (Chapter 5) concerning the specific activity of p34cdc2 in dividing compared with differentiating cells and intend in this chapter to address the question of p34cdc2 localisation in the cell. Some general considerations of the use of immunological procedures are relevant, in view of difficulties encountered in the course of attempts to preserve and visualise p34cdc2.
6.1.2 Indirect immunofluorescence detection of plant antigens

Several steps are involved in the process of immunolabelling antigens. The first is sample preparation, followed by fixation, antibody binding and finally detection. Fixation of the sample is particularly crucial as ideally it should result in authentic maintenance of cellular (ultra)structure, while also preserving the antigens. Unfortunately use of common aldehydes, such as paraformaldehyde and glutaraldehyde, to fix samples does carry some risk of reduced antigenicity, and many epitopes may be masked or altered as a consequence of the fixation process. It becomes necessary then to determine the optimal fixation conditions for each new specimen and antigen association (Geiger et al., 1981; Brandtzaeg, 1982; Grzanna, 1982; Wick and Duniec, 1986). Fixation conditions, together with the use of effective denaturing and protease inhibiting agents, have been designed to ensure the best possible access of antibodies to antigen epitopes. A final consideration when using aldehyde fixatives is that they may not give the antibody access to key sites in the tissue, hence application of an organic solvent or nonionic detergent after fixation is often required for permeabilisation of the sample.

An additional consideration specific to immunocytological investigations of plants is that the cell wall may form an impenetrable barrier between externally-applied antibodies and their targets inside the cell. The cell wall can be digested enzymatically, but this process has its own hazards including antigen damage as a result of proteolytic activity from contaminants of commercially available cell wall digesting enzymes (Wick, 1993). Protease inhibitors may help to reduce this effect.

The type of antibody used also has an impact on the success of antigen detection. A polyclonal serum, such as the one we have raised against maize p34<sup>cdc2</sup>, contains multiple antibodies directed against diverse epitopes on the antigen. This characteristic may confer an advantage as some of the antibody species may be targeting denaturation-resistant epitopes or epitopes that have had their conformation altered from the native state. One disadvantage inherent in the use of a polyclonal antibody is that not only are specific antibodies to the target antigen present but potentially so too are non-specific antibodies that, if not removed, may result in high levels of background labelling. This
constraint may be diminished by immunoaffinity purification of the desired antigen-specific antibodies.

Successful antigen detection is also reliant upon the retention of the antigen during processing of the tissue prior to immunolabelling. Melan and Sluder (1992) correctly state that an unsaid assumption of all immunofluorescence studies is that the conditions used to prepare cells for antibody labelling preserve accurately the \textit{in vivo} distribution of the antigen(s) under investigation. They demonstrated in their study that this assumption is not necessarily valid as they observed loss and artifactual precipitation of soluble proteins as a consequence of permeabilisation and fixation of tissue. These effects were reduced when the tissue was fixed prior to permeabilisation. The loss of the \( p34^{\text{cdc2}} \) protein in maize root tip tissue during immunolabelling processing was reported in Chapter 3. In the present chapter the effect of different fixatives, detergents and denaturing agents was investigated with the aim of optimising the retention of \( p34^{\text{cdc2}} \). The optimised procedure was then used in an investigation of the association of \( p34^{\text{cdc2}} \) with the microtubular cytoskeleton.

\section*{6.2 Materials and methods}

The methods described in detail below are aimed at localising maize \( p34^{\text{cdc2}} \) in meristematic cells of maize root tips. As conventional processing did not quite give clear-cut results, it was necessary to investigate a wide variety of protocols, in all cases using microtubule immunofluorescence as a check, (microtubules) MTs being well characterised structures with which the \( p34^{\text{cdc2}} \) protein associates in certain circumstances.

\subsection*{6.2.1 Plant material}

Three different \textit{Zea mays} cultivars (miracle, honeysweet and iochief improved) were used for this series of indirect immunofluorescence experiments. Two different methods were employed to germinate the seed; the first is exactly as described in 3.2.1. The second method, used when only a small amount of germinated seed was required, involved distributing seed evenly onto folded moistened tissues in a square clear plastic
box, approximately 120 x 120 x 20 mm in size. The box was then closed with a clear 
loose fitting lid, to allow air and water to exchange and propped at an angle of 45 to 60° 
in a rectangular plastic box which contained water to a depth of 20 mm approximately. 
A piece of clear plastic wrap was then loosely draped over the whole and the box placed 
in the dark at a constant temperature of 24-26°C for two days or until the majority of the 
seeds had germinated and had roots that were at least 10 mm in length.

For these indirect immunofluorescence experiments only the apical 1-2 mm of 
the root tip containing the meristematic tissue was required. The tips were removed 
using a scalpel blade and immediately placed into the fixative to be used.

6.2.2 Development of procedures for indirect immunofluorescence staining of 
maize root tip meristem tissue

Many variations of a basic protocol (see 6.2.2.1) were employed in an effort to 
optimise the immunolabelling of our maize anti-p34cdc2 antibody and its detection of 
p34cdc2 via indirect immunofluorescence techniques. These included a diversity of 
fixatives, protease inhibitors and denaturing agents.

6.2.2.1 Standard immunofluorescence method

The apical 1-2 mm of maize root tips was fixed (4% paraformaldehyde (PFA) in 
50 mM potassium phosphate/5 mM EGTA buffer, pH 7.0) for 1 h at room temperature. 
These root tips were then washed in three changes of wash buffer (50 mM potassium 
phosphate/5 mM EGTA, pH 7.0) for a total of 15 min. After washing, the root tips were 
transferred to a cell wall digestion solution (1% cellulysin (Calbiochem-Novabiochem, 
USA) in 50 mM potassium phosphate/5 mM EGTA, 0.2% Triton X-100) for 40 min at 
room temperature. Upon completion of digestion the root tips were washed as before 
but with only two changes of buffer. Next the tips were treated with pre-cooled 
methanol (-20°C) for 10 min at -20°C and then rinsed once in wash buffer. After this 
last wash the root tips were squashed between two slides to release the cells. The cells 
from (generally) two tips per slide were then dispersed using fine tweezers. The spread 
cells were then left to dry down, at room temperature, onto the slides. For
immunolabelling of the cells the slides were first rinsed briefly six times in PBS buffer (10 mM Na$_2$PO$_4$, 140 mM NaCl, pH 7.4) and then the cells blocked with 1% BSA in PBS buffer for 15 min. After removal of the blocking solution the primary antibodies (anti-β tubulin (Amersham International, UK) diluted to 1/500, our affinity-purified maize p34$^\text{cdc2}$ antibody diluted to 1/50 and an affinity-purified maize p34$^\text{cdc2}$ antibody donated by Dr J. Colasanti diluted to 1/100) were applied and the slides incubated at 37°C for 45-60 min. For pre-competition experiments, the 17 amino acid maize peptide, dissolved in PBS to a concentration of 1 mM, was added to the primary antibody at a final concentration of 1 µM and incubated as described above. The slides were then rinsed for a total of 12 min with several changes of PBS. The secondary antibodies (for tubulin: anti-mouse Ig F(ab') fragment, FITC-conjugated (from sheep) (Silenus Laboratories, Australia), diluted to 1/30 or anti-mouse Ig, Texas Red-linked whole antibody (from sheep) (Amersham International, UK), diluted to 1/40 and for p34$^\text{cdc2}$ anti-rabbit Ig, fluorescein-linked whole antibody (from donkey) (Amersham International, UK), diluted to 1/40) were then applied and the slides incubated as described above. After this incubation was completed the slides were washed once with PBS for 5 min and the slides blotted dry. Then 0.5 mg/ml of 4, 6-diamidino-2-phenylindole (DAPI) in PBS was overlayed onto the cells. After 10 min the slides were washed again in PBS as before and then dipped into distilled water, blotted dry, and mounted in Moviol containing 0.2% p-phenylenediamine (PPD). The finished slides were then stored at 4°C overnight to allow the mounting medium to set.

Successful double labelling of squashed maize root tip cells was accomplished by a protocol in which mouse monoclonal anti-β tubulin antibody and one of the rabbit anti-p34$^\text{cdc2}$ antibodies was added simultaneously to the cells. After incubation at 37°C the slides were rinsed and the two different fluorochromes necessary to discriminate between bound anti-β tubulin and anti-p34$^\text{cdc2}$ were added together. The lack of cross contamination between the two signals is demonstrated in section 6.3.3.

All fluorescence microscopy and photography was performed with a Zeiss Photomicroscope using a x100 oil-immersion objective lens and the following combination of filters: excitation filter 365, dichroic mirror 395 and barrier filter 420 (for UV), excitation filter 450-490, dichroic mirror 510 and barrier filters 515-565 (for
111

FITC), and excitation filter 546, dichroic mirror 580 and barrier filters 590 (for Texas Red). Images were recorded on Kodak Tmax 400 ASA film.

6.2.2.2 Variations to the standard immunofluorescence method

Several variations to the standard fixative (4% PFA in 50 mM potassium phosphate/5 mM EGTA, pH 7.0) were trialled in this study. They were: dilutions in the strength of the standard fixative, inclusion of 0.2% saponin into the standard fixative, inclusion of 0.2% saponin and 0.2% glutaraldehyde into the standard fixative and fixation with methanol only.

Variations to the standard cell wall digestion solution were also trialled, including the addition of 0.5 mM PMSF and 1 mg/ml leupeptin to the standard digestion mix. In the absence of 0.2% Triton X-100 from the standard cell wall digestion solution, the effect of the addition of 0.5 mM PMSF and 1 mg/ml leupeptin was examined as was the addition of 1% BSA to these protease inhibitors. When Triton X-100 was excluded from the digestion solution the addition of a protease inhibitor cocktail (Stock solution x100: 400 µg/ml aprotinin, 200 µg/ml pepstatin A, 100 µg/ml leupeptin, 10 µM benzamidine, 2 mg/ml soybean trypsin inhibitor and 100 mM PMSF) was investigated.

Lastly the introduction of different agents intended to alter the conformation of the p34\(^{\text{cdc2}}\) protein, aimed at making the potential epitopes more accessible to the antibody, were trialled. These denaturation agents were introduced either after completion of the fixation step and before cell wall digestion or after the completion of both the fixation and cell wall digestion steps. A range of agents with protein denaturing or extraction qualities, at different concentrations, was utilised to test whether it was necessary to remove proteins that may be bound to p34\(^{\text{cdc2}}\) or to unravel the p34\(^{\text{cdc2}}\) to allow access by the antibody to the carboxyterminal region. Agents that were tested included several detergents (saponin, SDS, NP40), protein dissociating chemicals (urea, getyl trimethyl ammonium chloride (CTAC) together with 2-mercaptoethanol (MSH)), picric acid, and the protease trypsin. Alterations in pH and temperature were also tested.

The table below details all the various combinations of alternate fixatives, cell wall digestion solutions and denaturation agents that were investigated:
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<th>Method number</th>
<th>Fixative</th>
<th>Cell wall digestion</th>
<th>Denaturation when?</th>
<th>Denaturing agent</th>
<th>Treatment code in text</th>
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<td>None</td>
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<td>After fixation, before digestion</td>
<td>4M urea 6M urea 8M urea 0.2% picric acid 0.2% saponin 0.8% saponin 3% saponin 0.2% CTAC/ 0.1% MSH 1% CTAC/ 0.5% MSH 4% CTAC/2% MSH pH 1.5 pH 2.0 pH 2.5 pH 9.0 pH 10.0 pH 11.0 1% SDS 5% SDS for 10 min</td>
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<td>After fixation, before digestion</td>
<td>0.2% saponin 0.8% saponin 1% CTAC/0.5% MSH 0.2% picric acid 1% SDS for 10 min</td>
<td>3A 3B 3C 3D 3E</td>
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<td>1% cellulysin + 0.2% Triton X-100 in 50 mM potassium phosphate/5 mM EGTA, pH 7.0</td>
<td>After fixation, before digestion</td>
<td>0.2% saponin</td>
<td>5A</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>0.8% saponin</td>
<td>5B</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1% CTAC/0.5% MSH</td>
<td>5C</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>MSH</td>
<td>5D</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.2% picric acid</td>
<td>5E</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1% SDS</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>for 10 min</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>4% PFA + 0.2% saponin in 50 mM potassium phosphate/5 mM EGTA, pH 7.0</td>
<td>1% cellulysin + 0.2% Triton X-100 in 50 mM potassium phosphate/5 mM EGTA, pH 7.0</td>
<td>Included in fixation</td>
<td>0.2% saponin</td>
<td>6A</td>
</tr>
<tr>
<td>Method Number</td>
<td>Fixative</td>
<td>Cell wall digestion</td>
<td>Denaturation when?</td>
<td>Denaturing agent</td>
<td>Treatment code in text</td>
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<tr>
<td>7</td>
<td>0.4% PFA + 0.2% saponin in 50 mM potassium phosphate/5 mM EGTA, pH 7.0</td>
<td>1% cellulysin + 0.2% Triton X-100 in 50 mM potassium phosphate/5 mM EGTA, pH 7.0</td>
<td>Included in fixation</td>
<td>0.2% saponin</td>
<td>7A</td>
</tr>
<tr>
<td>8</td>
<td>4% PFA + 0.2% glutaraldehyde + 0.2% saponin in 50 mM potassium phosphate/5 mM EGTA, pH 7.0</td>
<td>1% cellulysin + 0.2% Triton X-100 in 50 mM potassium phosphate/5 mM EGTA, pH 7.0</td>
<td>Included in fixation</td>
<td>0.2% saponin</td>
<td>8A</td>
</tr>
<tr>
<td>9</td>
<td>0.4% PFA + 0.2% glutaraldehyde + 0.2% saponin in 50 mM potassium phosphate/5 mM EGTA, pH 7.0</td>
<td>1% cellulysin + 0.2% Triton X-100 in 50 mM potassium phosphate/5 mM EGTA, pH 7.0</td>
<td>Included in fixation</td>
<td>0.2% saponin</td>
<td>9A</td>
</tr>
<tr>
<td>10</td>
<td>4% PFA in 50 mM potassium phosphate/5 mM EGTA, pH 7.0</td>
<td>1% cellulysin + 0.2% Triton + 0.5 mM PMSF + 1 mg/ml leupeptin in 50 mM potassium phosphate/5 mM EGTA, pH 7.0</td>
<td>0.2% saponin after fixation and before digestion; 1 mg/ml trypsin after fixation and digestion</td>
<td>0.2% saponin then 1 mg/ml trypsin for 10 min each</td>
<td>10A</td>
</tr>
<tr>
<td>11</td>
<td>4% PFA in 50 mM potassium phosphate/5 mM EGTA, pH 7.0</td>
<td>1% cellulysin + 0.5 mM PMSF + 1 mg/ml leupeptin in 50 mM potassium phosphate/5 mM EGTA, pH 7.0</td>
<td>After fixation, after digestion</td>
<td>0.0625 mg/ml, 0.125 mg/ml, 0.25 mg/ml, 0.5 mg/ml, and 1 mg/ml trypsin for 10 min</td>
<td>11A, 11B, 11C, 11D, 11E</td>
</tr>
<tr>
<td>12</td>
<td>0.4% PFA in 50 mM potassium phosphate/5 mM EGTA, pH 7.0</td>
<td>1% cellulysin + 0.5 mM PMSF + 1 mg/ml leupeptin in 50 mM potassium phosphate/5 mM EGTA, pH 7.0</td>
<td>After fixation, after digestion</td>
<td>0.0625 mg/ml, 0.125 mg/ml, 0.25 mg/ml, 0.5 mg/ml, and 1 mg/ml trypsin for 10 min</td>
<td>12A, 12B, 12C, 12D, 12E</td>
</tr>
<tr>
<td>Method Number</td>
<td>Fixative</td>
<td>Cell wall digestion</td>
<td>Denaturation when?</td>
<td>Denaturing agent</td>
<td>Treatment code in text</td>
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</tr>
<tr>
<td>13</td>
<td>4% PFA in 50 mM potassium phosphate/5 mM EGTA, pH 7.0</td>
<td>1% cellulysin in 50 mM potassium phosphate/5 mM EGTA, pH 7.0</td>
<td>After fixation, before digestion</td>
<td>0.2% saponin for 10 min</td>
<td>13A</td>
</tr>
<tr>
<td>14</td>
<td>4% PFA in 50 mM potassium phosphate/5 mM EGTA, pH 7.0</td>
<td>1% cellulysin + 0.5 mM PMSF + 1 mg/ml leupeptin + 1% BSA in 50 mM potassium phosphate/5 mM EGTA, pH 7.0</td>
<td>After fixation, before digestion</td>
<td>0.2% saponin for 10 min</td>
<td>14A</td>
</tr>
<tr>
<td>15</td>
<td>0.4% PFA in 50 mM potassium phosphate/5 mM EGTA, pH 7.0</td>
<td>1% cellulysin in 50 mM potassium phosphate/5 mM EGTA, pH 7.0</td>
<td>After fixation, before digestion</td>
<td>0.2% saponin for 10 min</td>
<td>15A</td>
</tr>
<tr>
<td>16</td>
<td>0.4% PFA in 50 mM potassium phosphate/5 mM EGTA, pH 7.0</td>
<td>1% cellulysin + 0.5 mM PMSF + 1 mg/ml leupeptin + 1% BSA in 50 mM potassium phosphate/5 mM EGTA, pH 7.0</td>
<td>After fixation, before digestion</td>
<td>0.2% saponin for 10 min</td>
<td>16A</td>
</tr>
<tr>
<td>17</td>
<td>4% PFA in 50 mM potassium phosphate/5 mM EGTA, pH 7.0</td>
<td>1% cellulysin + protease inhibitor cocktail in 50 mM potassium phosphate/5 mM EGTA, pH 7.0</td>
<td>After fixation, after digestion</td>
<td>0.2% saponin 0.2% NP4O 0.2% picric acid 1% SDS pH 4.0 pH 10.0 for 10 min</td>
<td>17A 17B 17C 17D 17E 17F</td>
</tr>
<tr>
<td>18</td>
<td>4% PFA in 50 mM potassium phosphate/5 mM EGTA, pH 7.0</td>
<td>1% cellulysin in 50 mM potassium phosphate/5 mM EGTA, pH 7.0</td>
<td>Before fixation 50°C 60°C 100°C microwave on HIGH for 1 min</td>
<td>18A 18B 18C 18D</td>
<td></td>
</tr>
</tbody>
</table>
### Table 6.1: Variations to standard immunofluorescence method trialled to optimise immunolabelling of maize anti-p34\(^{\text{cdc2}}\)

<table>
<thead>
<tr>
<th>Method Number</th>
<th>Fixative</th>
<th>Cell wall digestion</th>
<th>Denaturation when?</th>
<th>Denaturing agent</th>
<th>Treatment code in text</th>
</tr>
</thead>
<tbody>
<tr>
<td>19</td>
<td>3.5% PFA + 0.2% saponin + 0.2% picric acid in 50 mM potassium phosphate/5 mM EGTA, pH 7.0</td>
<td>1% cellulysin in 50 mM potassium phosphate/5 mM EGTA, pH 7.0</td>
<td>Included in fixation</td>
<td>0.2% saponin, 0.2% picric acid</td>
<td>19A</td>
</tr>
<tr>
<td>20</td>
<td>100% methanol</td>
<td>1% cellulysin + 0.2% Triton X-100 in 50 mM potassium phosphate/5 mM EGTA, pH 7.0</td>
<td>After fixation, before digestion</td>
<td>0.8% saponin, 1% SDS, 5% SDS for 10 min</td>
<td>20A 20B 20C</td>
</tr>
</tbody>
</table>

6.3 Results

6.3.1 Fixatives and denaturants used to optimise immunolabelling of maize p34\(^{\text{cdc2}}\) and microtubules

A wide variety of protocols based upon the standard immunofluorescence method (6.2.2.1) was investigated in an effort to optimise the localisation of maize p34\(^{\text{cdc2}}\) in actively dividing root tip cells of maize. The microtubule network was also labelled in parallel with p34\(^{\text{cdc2}}\) in all experiments. MTs are well characterised structures with which the p34\(^{\text{cdc2}}\) protein has been observed to associate during cell division. Observation of the MT network allowed an assessment of cell structure and general antigen preservation after processing of tissue for immunolabelling. These data enabled conclusions to be drawn about which conditions were optimal for the immunolabelling of p34\(^{\text{cdc2}}\). Numerous preparations were examined in the course of the survey and, for each preparation, numerous cells. The accompanying plates show selected images representing some of the main trends in the results.
6.3.1.1 Fixatives

A range of alternative fixatives was trialled with the aim of determining if any resulted in better preservation of the cell cytoskeleton and antigen retention or exposure to antibody. Paraformaldehyde was used at the standard concentration of 4% and, to check whether this relatively high concentration caused deleterious cross-linking, also at 0.6% [treatments 3A-3E Table 6.1], 0.4% [treatments 4A-4S, 7A, 9A, 12A-12E, 15A] and 0.3% [5A-5E]. The preservation of MTs was consistently better in tissue fixed with 4% PFA in all trials in which the concentration of PFA was varied. The interphase (Figure 6.1) and prophase (Figure 6.2) microtubule arrays were clearly evident in cells after preparation by the standard immunofluorescence method (6.2.2.1) [1A]. In these cells only the larger MT fibres were distinct. The inclusion of 0.2% saponin in the standard 4% PFA fixative [6A] did not appear to improve discrimination of the finer MT fibre network (Figures 6.3, 6.4). Addition of 0.2% glutaraldehyde [8A] was not helpful and in fact added to background fluorescence (Figure 6.5). Two additional fixatives were tested, the first was taken from Colasanti et al. (1993) and incorporated not only saponin but picric acid into a basic PFA fixative [19A], and secondly fixation in methanol only [20A]. The former resulted in good preservation of the MT network but was accompanied by high background fluorescence (Figure 6.6) and the latter in discrimination of the larger MT fibres only (Figure 6.7). From these data one may conclude that the best preservation of microtubules was given by fixation in 4% PFA only.

6.3.1.2 Inclusion of denaturing agents or protease in post-fix treatments and protease inhibitors in the digestion mix

All the denaturing agents that were trialled to investigate their effect on tissue preservation, prior to immunolabelling with anti-p34^cdc2, were tested at a range of concentrations. Consistently the best MT preservation resulted when the most dilute conditions were used.

The introduction of 0.2% saponin, a detergent previously used in immunofluorescence to permeabilise membranes and to extract the soluble components
of the cytoplasm (Wick and Duniec, 1986), after fixation but before cell wall digestion produced consistently good preservation and labelling of MTs at interphase (Figure 6.8) and prophase (Figure 6.9) [2E, 13A]. Addition of protease inhibitors to the cell wall digestion solution [14A, 17A] when saponin was utilised had no visible effect on MT preservation (Figures 6.10, 6.11). However the best preservation of the finer MT fibres at both interphase (Figure 6.12) and prophase (Figure 6.13) was seen when saponin was used after completion of cell wall digestion [17A] rather than prior to digestion [2E, 13A, 14A].

The exposure of cells to trypsin protease at 0.06-1.0 mg/ml [11A-11E] produced consistently strong labelling of the larger MT fibres only (Figures 6.14, 6.15). In contrast the inclusion of 0.2% picric acid, either before enzymatic cell wall digestion [2D] or after digestion [17C] (Figures 6.16, 6.17) resulted in preservation of highly detailed MT arrays. MT labelling was consistently stronger when picric acid was introduced after digestion.

When SDS (sodium dodecyl sulfate), a detergent used to denature proteins prior to gel electrophoresis, and NP40, another detergent used in immunofluorescence to extract cytoplasm (Wick and Duniec, 1986), were applied to cells [2Q, 2R, 17B, 17D] the best MT preservation was obtained when these agents were introduced after the completion of cell wall digestion (Figures 6.18-6.21) [17B, 17D]. Both preserved the finer MT fibres under these conditions, however the labelling of tubulin was noticeably better when NP40 was used (Figures 6.20, 6.21).

Root tip tissue was also treated with urea in concentrations from 4M to 8M [2A-2C] and with CTAC/MSH [2H-2J]. Faint extensive MT arrays were recovered when the most dilute concentrations of these agents were used (Figures 6.22-6.25), this recovery of MTs deteriorated when higher concentrations were utilised.

Alterations in pH and temperature were the final denaturing factors trialled. The preservation of MTs during both interphase and prophase was best when pH was neither extremely acidic (Figures 6.26, 6.27) or alkaline (Figures 6.28, 6.29). Furthermore the discrimination of MTs was improved when the pH treatment was applied after cell wall digestion [17E, 17F] rather then before digestion [2K-2P]. Finally when raised temperature was used prior to fixation [18A-18D], the only MT fibres preserved were
6.3.1.3 Summary of microtubule immunofluorescence

The state of MT preservation and imaging in both interphase and prophase cells with a PPB allowed an assessment of the large number of protocols that were trialled. All the detergents, particularly saponin, picric acid and some pH treatments appear to lead to some improvement of MT visualisation. The best results were obtained with the least concentrated of each of these. Furthermore it appeared that introduction of the various denaturing treatments after completion of both fixation and cell wall digestion led to better MT resolution than if introduced after fixation but before cell wall digestion. This extensive work on MTs provides a useful baseline for consideration of attempts to localise p34<sup>cdc2</sup> in the same tissue.

6.3.2 Localisation of p34<sup>cdc2</sup> protein in maize root tip cells

This section describes attempts to localise p34<sup>cdc2</sup> using the specific anti-p34<sup>cdc2</sup> antibody which we had found capable of precipitating native p34<sup>cdc2</sup> (see previous chapters). Many protocols were used (Table 6.1), all of which were checked in parallel localisations of MTs in similar tissue samples.

6.3.2.1 During interphase

When interphase cells were labelled with our anti-p34<sup>cdc2</sup> antibody, the p34<sup>cdc2</sup> protein was observed to be predominantly concentrated in the nucleus (Figures 6.31-6.36). This pattern was distinct regardless of the method of tissue preparation. It is also identical to that observed by Colasanti et al. (1993) using a specific p34<sup>cdc2</sup> antibody. Further we confirmed that their antibody, under our preparatory conditions, also reproduces the localisation they previously reported and that we obtained independently with our antibody (Figures 6.37-6.42).
6.3.2.2 During prophase

Cells in prophase were identified by observing the state of their chromatin using differential interference contrast microscopy. In prophase cells that had been labelled with anti-p34\textsuperscript{cdc2} antibody, p34\textsuperscript{cdc2} protein was primarily concentrated in the nucleus but some diffuse cytoplasmic fluorescence was also evident. We also consistently identified a band of p34\textsuperscript{cdc2} protein that encircled the nucleus, as the PPB characteristically does, in prophase cells in which the nuclear material had begun to condense. Often the band was visible in both surface and midplanes of view (Figures 6.44-6.49). However it must be noted that not every prophase cell, with condensed chromatin or otherwise, showed this localisation pattern. Furthermore this band appears only tenuous, or is not visible at all, in tissue prepared by the standard method (Figure 6.43) and when denaturing agents such as detergents were introduced after fixation and before cell wall digestion. The discrimination of this band increased significantly when the detergents, picric acid and pH treatments were applied after completion of both the fixation and cell wall digestion steps (Figures 6.44-6.49). This finding of a circumferential band in a small proportion of prophase cells suggests that the p34\textsuperscript{cdc2} protein identified by our antibody does occur in the PPB.

To confirm this a series of double labelling studies was undertaken, using an anti-tubulin antibody and our specific anti-p34\textsuperscript{cdc2} antibody. In cells prepared by the standard method again it is difficult to identify a p34\textsuperscript{cdc2} band (Figure 6.50). However, when tissue was treated with 0.2% NP40, 0.2% picric acid and pH 10.0, after the completion of fixation and cell wall digestion steps, a significant improvement in p34\textsuperscript{cdc2} band visualisation was evident. In these cells a p34\textsuperscript{cdc2} band is seen to correspond exactly to the PPB image (Figures 6.51-6.53).

I also tested another specific p34\textsuperscript{cdc2} antibody, under our conditions, that had been previously found to detect p34\textsuperscript{cdc2} protein in association with the PPB (Colasanti et al., 1993). This antibody was also found to detect a distinct p34\textsuperscript{cdc2} band in prophase cells with condensed chromatin (Figures 6.54-6.58). The discrimination of this band was again faint after the standard preparation method (Figure 6.54). As observed previously detection of p34\textsuperscript{cdc2} markedly improved after inclusion of different detergents or...
0.2% picric acid or pH treatments after the completion of the fixation and cell wall digestion steps (Figures 6.55-6.58).

**6.3.2.3 During metaphase**

In several animal systems p34\(^{cdc2}\) has been localised in the mitotic spindle (Bailly et al., 1989; Riabowol et al., 1989; Alfa et al., 1990; Rattner et al., 1990; Ookata et al., 1993; Andreassen and Margolis, 1994). Given the conservation of p34\(^{cdc2}\) function in eukaryotes an association of p34\(^{cdc2}\) protein with plant mitotic spindles could be expected. Observation of metaphase cells in this study labelled with our p34\(^{cdc2}\) specific antibody identified just such an association.

In metaphase cells p34\(^{cdc2}\) protein was consistently detected, spread faintly throughout the cytoplasm but also in a halo around the condensed chromosomes (Figures 6.59-6.64). In tissue prepared by the standard method (Figure 6.59) this pattern was distinct but fainter than that observed when a variety of detergents or picric acid or pH agents were introduced after the completion of both fixation and cell wall digestion steps (Figures 6.60-6.64). Double labelling studies utilising anti-\(\beta\) tubulin and anti-p34\(^{cdc2}\) antibodies together demonstrated that this halo around the metaphase plate of chromosomes corresponded to the spindle region (Figures 6.65-6.67). The association was consistent and distinct under all conditions. The localisation we observed with our specific anti-p34\(^{cdc2}\) antibody was duplicated when the other independently-raised (Colasanti) maize anti-p34\(^{cdc2}\) antibody was used to label cells (Figure 6.68-6.72). With both antibodies the p34\(^{cdc2}\) protein appeared to associate not distinctly to individual spindle fibres but was spread diffusely in a barrel shape around the chromosomes.

**6.3.2.4 During telophase**

In this study an apparent localisation of p34\(^{cdc2}\) protein to the phragmoplast was consistently observed. The p34\(^{cdc2}\) protein was, as in metaphase cells, spread diffusely through the cytoplasm but also localised as two distinct discs one on either side of the developing cell plate. A distinct exclusion of the protein from the condensed nuclei was also evident (Figures 6.73-6.78). This association was present when tissue was prepared
by the standard method (Figure 6.73). A distinct improvement in its imaging however was obvious when denaturing treatments such as detergents, picric acid and pH alterations were included into the standard method after fixation and cell wall digestion step completion (Figures 6.74-6.78). When root tip cells were double-labelled, with both our specific anti-p34cdc2 antibody and an anti-tubulin antibody, the p34cdc2 protein localisation was observed to correspond exactly to the MT phragmoplast (Figures 6.79-6.82). This same distinct pattern of association of p34cdc2 protein with the phragmoplast was also observed when cells were labelled with the independently-raised (Colasanti) specific p34cdc2 antibody (Figures 6.83-6.87).

6.3.3 Experimental controls for localisation of p34cdc2 protein

The consistent detection of p34cdc2 localised distinctly to the PPB during prophase, to the spindle during metaphase, to the phragmoplast during telophase and in the nucleus of interphase cells with not only our specific anti-p34cdc2 antibody, but another that has been independently raised, suggests that p34cdc2 during higher plant division associates closely with division-specific cytoskeletal structures. When a number of cells (over 1000) were stained with uncompetted p34cdc2 antibody a small percentage approximately 5-10% of those in prophase with condensed chromatin exhibited signs of p34cdc2 localisation to the PPB (as shown in 6.3.2.2). In contrast when an equal number of cells were stained with pre-competted anti-p34cdc2 antibody the consistent p34cdc2 band observed in late prophase cells disappeared (Figure 6.88). This effect was also observed for the other mitotic stages, for example, the localisation to the phragmoplast in telophase (Figure 6.89) indicating that the protein localisations observed in this study are indeed those of the p34cdc2 protein. Double labelling presents other possibilites for misinterpretation as the observed p34cdc2 bands may be the result of the Texas Red labelled PPB bleeding through into the FITC channel hence creating an illusory p34cdc2 band. Also possible is the cross reaction of primary and secondary antibodies, for example, if the anti-rabbit FITC customarily used to label the anti-p34cdc2 antibody was actually labelling the anti-mouse tubulin antibody instead. Several tests demonstrated that these potential problems were not actually occuring. Texas Red labelled MTs were not visible on the FITC channel (Figures 6.90, 6.91) nor was p34cdc2
labelled with FITC visible on the Texas Red channel (Figures 6.92-6.93). Furthermore, even in the presence of anti-rabbit FITC secondary antibody (Figures 6.94-6.96), only anti-mouse Texas Red secondary antibody detected anti-mouse tubulin primary antibody. No interaction at all was detected when anti-mouse tubulin was subsequently followed only by an anti-rabbit FITC secondary antibody (Figures 6.97-6.99). These results were applicable to any of the associations of the p34\(^{cdc2}\) protein with the MT cytoskeleton previously noted.

6.4 Discussion

6.4.1 Trialling of variations to the standard immunofluorescence method to optimise labelling of p34\(^{cdc2}\) and microtubules

Alterations to the fixation and cell wall digestion solutions were investigated to see what effect they had upon the preservation and immunoreactivity of the p34\(^{cdc2}\) protein. The introduction of a range of denaturing and extraction agents to the standard method was also studied. In addition MTs were labelled with an anti-tubulin monoclonal antibody in parallel with p34\(^{cdc2}\) labelling. Monitoring of the state of preservation of microtubules (with which p34\(^{cdc2}\) has been observed to associate) allowed an assessment of ultrastructure preservation after the different processing methods.

Fixation with 4% PFA was found consistently to result in the best preservation and imaging of p34\(^{cdc2}\) protein and MTs. This finding corresponds to the majority of previous immunofluorescence studies of meristematic plant cells which have reported good immunofluorescence results and low autofluorescence levels when using this fixative (e.g. Wick et al., 1981; Wick and Duniec, 1983, 1986). The cross-linking of proteins when 4% PFA was used appeared to preserve the intracellular structure and also strengthen the cells enabling them to survive handling during preparation for immunolabelling. Both of these attributes were affected detrimentally when lower concentrations of PFA were used. Addition of glutaraldehyde (GA), another cross-linking aldehyde which has been extensively used to study tubulin localisation in plants (Bajer et al., 1982; De Mey et al., 1982; Schmidt et al., 1983; Wick and Duniec, 1986),
to the PFA fixative was also detrimental in maize root tip cells because it diminished the
discrimination of MT details by raising background fluorescence. Wick and Duniec
(1986), who experienced the same problem in their study, state that the background is a
consequence of autofluorescence and non-specific antibody binding after GA fixation.

Two other fixatives investigated in this study incorporated extraction and/or
precipitation agents (saponin, picric acid) into the PFA fixative, the last fixative trialled
used -20°C methanol as the sole fixation agent. In all cases the finer MTs could no
longer be discriminated suggesting that these fixatives either did not cross-link the
protein structure together strongly enough to withstand the handling of cells during
processing or that the denaturation/extraction agents were dismantling the microtubules
faster than the fixative could cross-link them.

In addition to investigating the effects of alternative fixatives on the preservation
and immunolabelling of p34\textsuperscript{cdc2} and MTs, the effect was also examined of including
protease inhibitors into the cell wall digestion solution and introducing a range of
denaturing agents, including several detergents - saponin, SDS and NP40, picric acid a
non-metallic precipitant, urea, CTAC/MSH, different pH and temperature solutions in a
range of concentrations, at different points in the processing of root tissue.

Of these treatments consistently the best immunolabelling of p34\textsuperscript{cdc2} and MTs
was observed when either 0.2% saponin, 0.2% NP40, 0.2% picric acid or the pH 4.0 and
10.0 treatments were utilised. The improvement of p34\textsuperscript{cdc2} and MT immunolabelling
was further optimised when these agents were introduced after the completion of both
fixation and cell wall digestion steps rather than after fixation and prior to cell wall
digestion. This observation supports experimental data presented in Chapter 3 of this
thesis, where it was found that a higher level of p34\textsuperscript{cdc2} protein was retained when a
denaturation agent was introduced after fixation and completion of cell wall digestion
during the standard method. These data also indicated that inclusion of protease
inhibitors into the cell wall digestion solution gave a slight improvement in the retention
of p34\textsuperscript{cdc2}, however no such improvement was apparent in the immunolabelling of
p34\textsuperscript{cdc2} or MTs when protease inhibitors were included, perhaps because the gain was
too slight to be noticeable by immunolabelling. Correlation of the immunolabelling
data presented here with the quantitative Western blot data from Chapter 3 suggests that
that state of MT preservation does indeed reflect the immunolabelling of p34\textsuperscript{cdc2} after
the different preparatory methods. Having established the optimal immunofluorescence method for immunolabelling of p34\textsuperscript{cdc2}, the next step was to investigate the localisation of p34\textsuperscript{cdc2} during the cell cycle in maize root tip cells.

6.4.2 Localisation of p34\textsuperscript{cdc2} in maize root tip cells

The specific maize anti-p34\textsuperscript{cdc2} antibody (Chapter 2) and another antibody raised by Colasanti et al. (1993) gave identical pictures of p34\textsuperscript{cdc2} localisation when different stages of the cell cycle in maize root tip cells were examined by indirect immunofluorescence microscopy. During interphase the p34\textsuperscript{cdc2} protein was concentrated in the nucleus. This observation is in accord with the work of Colasanti et al. (1993) in maize and with several studies on p34\textsuperscript{cdc2} protein localisation in yeast (Booher et al., 1989) and animal cells (Bailly et al., 1989; Riabowol et al., 1990). Colasanti et al. (1993) observed a transfer of p34\textsuperscript{cdc2} protein from the nucleus to the cytoplasm during prophase, a similar transfer was detected in this study. Furthermore in a small proportion of cells in late prophase, those with visibly condensed chromatin, a distinct band of p34\textsuperscript{cdc2} protein that girdled the nucleus was detected. When cells were double labelled, with anti-p34\textsuperscript{cdc2} and anti-tubulin antibodies, this band was seen to correspond exactly in position and form to that of the PPB that also characterised these cells. In their earlier study Colasanti et al. (1993) also found this co-localisation of p34\textsuperscript{cdc2} to the PPB in less than 10% of prophase cells and suggested that the association was only a transient one. They also determined, through oryzalin disruption of the MTs, that detection of the p34\textsuperscript{cdc2} band was dependent upon the presence of the PPB.

Other work has indicated that a protein kinase is involved in the disassembly of the PPB. Katsuta and Shibaoka (1992) demonstrated by the use of protein kinase inhibitors that protein kinase activity was essential for PPB breakdown. Furthermore, Zhang et al. (1992) found that okadaic acid disrupted the dependence of PPB disassembly on the formation of the mitotic apparatus, indicating that the normal signal for disassembly involved a phosphorylation step. They later proposed that the p34\textsuperscript{cdc2} kinase drives PPB disassembly as they observed the continuation of p34\textsuperscript{cdc2} activation and PPB disassembly, but not other mitotic events, in the presence of okadaic acid (John et al, 1993a, b). Immunofluorescence studies using the PSTAIR antibody, which is
raised against the conserved sequence present within all cdc2 homologues, have identified association of p34cdc2 or p34cdc2-like protein with the PPB (Mineyuki et al., 1991; John et al., 1993a). This suggested that p34cdc2 kinase could be well placed to act on the PPB. However no conclusions about the identities of the p34cdc2-like enzyme(s) could be drawn from this work as the PSTAIR antibody has been demonstrated to cross-react with kinases that contain closely related yet different motifs to that of PSTAIR (Meyerson et al., 1992; Okuda et al., 1992; Graña et al., 1994), hence the protein(s) identified to localise to the PPB in these studies may not be p34cdc2. The study by Colasanti et al. (1993) and the work presented in this thesis demonstrate that p34cdc2 protein can occur in association with the PPB. The p34cdc2 band was seen in mature PPBs only, supporting the idea that it has a role in PPB disassembly.

Localisation of p34cdc2 to MT structures associated with cell division has been reported in several studies (Bailly et al., 1989; Riabowol et al., 1989; Alfa et al., 1990; Rattner et al., 1990; Tombes et al., 1991; Kubiak et al., 1993; Ookata et al., 1993; Andreassen and Margolis, 1994). The consistency of the localisation of p34cdc2 to mitosis-associated structures in animals and plants suggests that p34cdc2 may play a role in regulating these structures during division. Dramatic alterations in the organisation of the interphase MTs and in chromatin structure have in fact been observed in cell-free Xenopus extracts after the addition of p34cdc2 kinase (Verde et al., 1990). Microinjection of p34cdc2 into mammalian fibroblasts also causes alterations that mimic early mitosis events (Lamb et al., 1990). Recently Hush et al. (1996) have reported the first direct evidence in plants of active MPF. In their study microinjection of a plant MPF into live plant cells was observed to result in disassembly of the PPB, chromosome condensation, nuclear envelope breakdown and initiation of mitosis. Exactly how the interaction of active p34cdc2 with those structures is mediated is unknown; Ookata et al. (1993) found that the Cdc2/cyclin B complex was associated with MTs via microtubule-associated proteins (MAPs). Phosphorylation of MAPs is believed to destabilise MTs (Jameson et al., 1980). Vandre et al. (1991) have determined by use of an MPM-2 antibody that MAP4 in HeLa cells is phosphorylated at mitosis. MAPs have also been identified in higher plants (Cyr, 1991a; Schellenbaum et al., 1992; Goddard et al., 1994) where they have also been observed to alter the morphology and stability of MT in vitro (Cyr and Palevitz, 1989). It may therefore be
that p34\textsuperscript{cdc2} kinase regulates MT dynamics by phosphorylation of MAP proteins. Recently Ookata et al. (1995) have provided evidence to support this theory, finding that p34\textsuperscript{cdc2} associates with MTs by the binding of cyclin B to a proline rich region of MAP4. Furthermore the MAP4 that was associated with the p34\textsuperscript{cdc2} kinase was phosphorylated by the kinase and this level of phosphorylation was observed to enhance MT instability. More recently McNally (1996) has proposed that MAP4 may regulate not only the effects of the p34\textsuperscript{cdc2} protein kinase on the MT network at nuclear envelope breakdown, but in addition mediate the activation of p34\textsuperscript{cdc2} at the onset of anaphase. MAP4 however cannot be solely responsible for regulation of MT dynamics during cell cycle as it induces \textit{in vivo} a rate of MT turnover that does not equal that observed in interphase when MT turnover is at its lowest level (McNally, 1996). Hence other MAP candidates may also have roles to play; as yet the precise details remain unknown.

Association of p34\textsuperscript{cdc2} protein with the spindle and phragmoplast has also been observed in this study. Consistent patterns were detected with both specific p34\textsuperscript{cdc2} antibodies used here. Colasanti et al. (1993) in their previous study detected no associations of p34\textsuperscript{cdc2} with the phragmoplast and only a tenuous association with the perichromosomal region during metaphase. Yet their antibody under our processing conditions did detect very distinctive localisations of p34\textsuperscript{cdc2} during metaphase and telophase. Furthermore, the Colasanti p34\textsuperscript{cdc2} antibody was often observed to be stronger in its detection of p34\textsuperscript{cdc2} protein than the specific anti-p34\textsuperscript{cdc2} antibody we had generated. Perhaps differences in tissue preparation were responsible for the detection by the Colasanti antibody of p34\textsuperscript{cdc2} localisations that they had not observed. Dibbayawan et al. (1995) have suggested that differences between antibodies raised against the same peptide could stem from differences in the aldehyde linkages and carrier molecules used during coupling. These differences during coupling could cause subtle alterations in peptide conformation leading to differences in the binding specificites of the generated antibodies. This may explain the differences observed between our anti-p34\textsuperscript{cdc2} antibody and the Colasanti anti-p34\textsuperscript{cdc2} antibody, with respect to immunolabelling, and also why our antibody has been demonstrated to be capable of immunoprecipitating native p34\textsuperscript{cdc2} protein (Chapter 4) while Colasanti et al. (1993) were unable to do this with their antibody.
Association of p34<sup>cdc2</sup> protein with the spindle and phragmoplast has not previously been observed in plants. However, there have been many reports of p34<sup>cdc2</sup> localisation to the mitotic apparatus in animal studies (Bailly et al., 1989; Riabowol et al., 1989; Alfa et al., 1990; Rattner et al., 1990; Ookata et al., 1993; Andreassen and Margolis, 1994) which have led to proposals that p34<sup>cdc2</sup> may have a role in the function and regulation of the mitotic apparatus (Pines and Hunter, 1991b; Alfa et al., 1990; Kubiak et al., 1993). Rattner et al. (1990) also noticed that the p34<sup>cdc2</sup> pattern that they identified within the mitotic apparatus was identical to that found for calmodulin (CaM). Calmodulin is a highly conserved ubiquitous calcium binding protein (Fisher and Cyr, 1993) which has been consistently observed to localise to the PPB, mitotic apparatus and phragmoplast (Gunning and Wick, 1985; Vantard et al., 1985; Wick, 1985; Wick et al., 1985). CaM inhibitor studies (Wick, 1988) have shown that CaM has a likely role in regulation of mitosis and cytokinesis in meristematic cells. Furthermore, the interaction of CaM with MTs has been determined to be mediated via MAP proteins (Schliwa et al., 1981; Cyr, 1991b; Durso and Cyr, 1994) just as has been proposed for the p34<sup>cdc2</sup> kinase. The associations of p34<sup>cdc2</sup> with spindle and phragmoplast we have identified in this study appear identical in appearance to those reported for calmodulin. However it must be noted that Hepler et al. (1994) observed a greater accumulation of p13<sup>suc1</sup> and BSA independently to the phragmoplast after microinjection, which they attributed to the existence of greater accessible free volume in the phragmoplast due to exclusion of organelles by the palisade of MTs. Furthermore as the p34<sup>cdc2</sup> protein kinase is inactive at telophase (Nurse, 1990) it is logical that this soluble protein is distributed randomly throughout the cell and hence its apparent localisation to the phragmoplast has no significance with respect to regulation of the cell division cycle. Nevertheless, given the central role the p34<sup>cdc2</sup> kinase has in cell division, these localisations suggest that in higher plants p34<sup>cdc2</sup> together with CaM may be members of a protein subset involved in coordination of mitosis and cytokinesis (Rattner et al., 1990).

The final consideration in this study is the assumption that the methods used to prepare tissue for immunolabelling do indeed preserve the in vivo distribution of proteins accurately. Melan and Sluder (1992) have demonstrated that soluble proteins may be artifactually lost or precipitated during specimen processing. The effects
however were minimised in tissue that had been fixed before detergent permeabilisation. Nevertheless the present study indicates that caution is indeed warranted when interpreting immunolocalisation results for soluble proteins. Several lines of evidence support the $p34^{\text{cdc2}}$ localisations identified in this study. Firstly, competition of the $p34^{\text{cdc2}}$ antibodies by the $p34^{\text{cdc2}}$ peptide in our study and that of Colasanti et al. (1993) have shown that the localisations are valid. Second, two independently-raised anti-$p34^{\text{cdc2}}$ antibodies under a variety of tissue preparation conditions consistently identify the same localisations of $p34^{\text{cdc2}}$ during interphase and prophase. Third, the many reports of $p34^{\text{cdc2}}$ localisation to the spindle and MTs in animals (Bailly et al., 1989; Riabowol et al., 1989; Alfa et al., 1990; Rattner et al., 1990; Ookata et al., 1993; Andreassen and Margolis, 1994) and of calmodulin to spindle and phragmoplast in plants (Gunning and Wick, 1985; Vantard et al., 1985; Wick, 1985; Wick et al., 1985) suggest that a similar localisation of $p34^{\text{cdc2}}$ in plants would not be unexpected. Fourth, microinjection of $p34^{\text{cdc2}}$ into live cells in animals (Lamb et al., 1990) and plants (Hush et al., 1996) has demonstrated that the $p34^{\text{cdc2}}$ kinase has a real effect upon MTs and structures such as the PPB that are related to the regulation of cell division. Lastly, joint immunolabelling of similar sized proteins calmodulin and centrin (Harper, J.D.I. pers. comm.), in plants, has determined that while calmodulin is seen to localise to MT structures, centrin does not. This indicates that the localisations observed for soluble proteins with MT structures need not mean that these proteins have been trapped within or precipitated there and could mean that the localisation to the MTs is an accurate reflection of the in vivo distributions.

### 6.4.3 Conclusions

Stages of the cell cycle were investigated, by indirect immunofluorescence microscopy, to characterise spatial associations between $p34^{\text{cdc2}}$ and the MT cytoskeleton. By use of two independently generated specific anti-$p34^{\text{cdc2}}$ maize antibodies we found that $p34^{\text{cdc2}}$ protein was primarily accumulated in the nucleus during interphase. The distribution changed during prophase, becoming cytoplasmic and nuclear. Late prophase cells were also characterised by a band of $p34^{\text{cdc2}}$ protein that in double labelling was seen to coincide exactly with the MT PPB. Furthermore,
during metaphase and telophase both antibodies also detected consistent localisation of p34\textsuperscript{cdc2} in the mitotic apparatus and phragmoplast. The localisations identified here suggest that the p34\textsuperscript{cdc2} protein kinase may have a role in the regulation of events during mitosis and cytokinesis in higher plants.
Figures 6.1-6.7
Immunofluorescent staining of maize root tip cells in interphase and prophase (Bar 10 µm).

**Figure 6.1.** Cortical MTs in cell fixed with PFA [1A].
**Figure 6.2.** Pre-prophase band of MTs in cell fixed with PFA [1A] a. surface view of cell; b. midplane view of same cell.
**Figure 6.3.** Interphase MT array in cell fixed with PFA/saponin [6A].
**Figure 6.4.** Broad PPB in cell fixed with PFA/saponin [6A]. a. surface view; b. midplane view of same cell.
**Figure 6.5.** Cortical MTs in cell fixed with PFA/GA/saponin [8A].
**Figure 6.6.** MT array in interphase cell fixed with PFA/saponin/picric acid (Colasanti et al., 1993) [19A].
**Figure 6.7.** Interphase MTs in cell fixed with methanol [20A].
Figures 6.8-6.15
Tubulin immunofluorescence in maize root tip cells in interphase and prophase (Bar 10 µm).

Figure 6.8. Interphase MT array in cells fixed with PFA and treated with saponin, after fixation but before cell wall digestion [2E].

Figure 6.9. PPB of MTs in cell fixed with PFA and treated with saponin, after fixation but before cell wall digestion [2E]. a. surface view; b. midplane view of same cell.

Figure 6.10. Cortical MTs in cell fixed with PFA, protease inhibitors have been included in cell wall digestion and cell was also treated with saponin after fixation but before cell wall digestion [14A].

Figure 6.11. PPB in cell that was fixed with PFA, treated with saponin after fixation but prior to cell wall digestion, the cell wall digestion solution also included protease inhibitors [14A]. a. surface view; b. midplane view of same cell.

Figure 6.12. Interphase MTs in cell fixed with PFA and treated with saponin after fixation and completion of cell wall digestion steps [17A].

Figure 6.13. PPB in cell fixed with PFA and treated with saponin after fixation and completion of cell wall digestion [17A]. a. surface view; b. midplane view of same cell.

Figure 6.14. MTs in interphase cell fixed with PFA and exposed to trypsin after fixation and cell wall digestion steps [11A].

Figure 6.15. PPB of MTs in cell fixed with PFA and treated with trypsin after completion of fixation and cell wall digestion [11A]. a. surface view; b. midplane view of same cell.
Figures 6.16-6.23
Maize root tip cells in interphase and prophase stained with anti-tubulin antibody (Bar 10 µm).

Figure 6.16. Cortical MTs in cells fixed with PFA and treated with picric acid after completion of cell wall digestion [17C].
Figure 6.17. PPB in cell fixed with PFA and treated with picric acid after fixation and completion of cell wall digestion [17C]. a. surface view; b. midplane view of same cell.
Figure 6.18. Interphase MT array in cell fixed with PFA and treated with SDS, after fixation and completion of cell wall digestion [17D].
Figure 6.19. PPB of MTs in cell fixed with PFA and treated with SDS, after fixation and cell wall digestion [17D]. a. surface view; b. midplane view of same cell.
Figure 6.20. MTs in interphase cell fixed with PFA and treated with NP40 after completion of cell wall digestion [17B].
Figure 6.21. PPB in cell fixed with PFA and treated with NP40 after fixation and completion of cell wall digestion [17B]. a. surface view; b. midplane view of same cell.
Figure 6.22. Interphase MTs in cell fixed with PFA and treated with urea prior to cell wall digestion [2A].
Figure 6.23. PPB of MTs in cell fixed with PFA and treated with urea, after fixation but before cell wall digestion [2A]. a. surface view; b. midplane view of same cell.
Figures 6.24-6.30
Tubulin immunofluorescence in maize root tip cells in interphase and prophase (Bar 10 µm).

Figure 6.24. MT array in interphase cell fixed with PFA and treated with CTAC/MSH before cell wall digestion [2H].

Figure 6.25. PPB in cell fixed with PFA and treated with CTAC/MSH after fixation but prior to cell wall digestion [2H] a. surface view; b. midplane view of same cell.

Figure 6.26. Cortical MTs in cell fixed with PFA and exposed to pH 4.0 after completion of cell digestion [17E].

Figure 6.27. PPB of MTs in cell fixed with PFA and treated with pH 4.0 after fixation and cell wall digestion [17E] a. surface view; b. midplane view of same cell.

Figure 6.28. Interphase MTs in cell fixed with PFA and treated with pH 10.0 after fixation and completion of cell wall digestion [17E].

Figure 6.29. PPB in cell fixed with PFA and exposed to pH 10.0 after completion of cell wall digestion [17F] a. surface view; b. midplane view of same cell.

Figure 6.30. MTs in interphase cell that was exposed to 100°C prior to fixation with PFA [18C].
Figures 6.31-6.36
Immunofluorescent staining of maize root tip cells in interphase with our maize p34\textsuperscript{cdc2} antibody that have been fixed in PFA and then treated with a denaturing agent after completion of cell wall digestion (Bar 10 µm). The p34\textsuperscript{cdc2} protein is primarily concentrated in the nucleus (n) of the cells although it is excluded from the nucleolus (*).

Figure 6.31. Standard method [1A].
Figure 6.32. Cell treated with saponin [17A].
Figure 6.33. Cell treated with SDS [17D].
Figure 6.34. Cell treated with picric acid [17C].
Figure 6.35. Cell treated with pH 4.0 [17E].
Figure 6.36. Cell treated with pH 10.0 [17F].

Figures 6.37-6.42
Immunofluorescence staining of interphase maize root tip cells with Colasanti p34\textsuperscript{cdc2} antibody that have been fixed with PFA and then treated with a denaturing agent after completion of cell wall digestion (Bar 10 µm). The p34\textsuperscript{cdc2} protein is concentrated in the nucleus (n) but excluded from the nucleolus (*).

Figure 6.37. Standard method [1A].
Figure 6.38. Cell treated with saponin [17A].
Figure 6.39. Cell treated with NP40 [17B].
Figure 6.40. Cell treated with picric acid [17C].
Figure 6.41. Cell treated with pH 4.0 [17E].
Figure 6.42. Cell treated with pH 10.0 [17F].
Figures 6.43-6.46
Immunofluorescently labelled maize root tip cells in prophase stained with our maize p34cdc2 antibody. The cells have been fixed in PFA then treated with a denaturing agent after completion of cell wall digestion (Bar 10 µm). Note the band of p34cdc2 (arrows) that crosses the nucleus under all experimental conditions except when the tissue was prepared by the standard method. The same root tip cell is shown in (a), (b) and (c) for each figure.

Figure 6.43. Standard method [1A]. a. surface view; b. midplane view; c. DNA stained with DAPI.
Figure 6.44. Cell treated with saponin [17A]. a. surface view; b. midplane view; c. DAPI stained DNA.
Figure 6.45. Cell treated with SDS [17D]. a. surface view; b. midplane view; c. DAPI.
Figure 6.46. Cell treated with NP40 [17B]. a. surface view; b. midplane view; c. DNA stained with DAPI.
Figures 6.47-6.49
Immunofluorescence staining of maize root tip cells in prophase stained with our maize p34<sup>cdc2</sup> antibody. The cells have been fixed in PFA then treated with a denaturing agent after cell wall digestion (Bar 10 µm). A band of p34<sup>cdc2</sup> protein (arrows) that crosses the nucleus can be seen in all experimental conditions. The same root tip cell is shown in (a), (b) and (c) for each figure.

**Figure 6.47.** Cell treated with picric acid [17C]. a. surface view; b. midplane view; c. DAPI.

**Figure 6.48.** Cell treated with pH 4.0 [17E]. a. surface view; b. midplane view; c. DNA stained with DAPI.

**Figure 6.49.** Cell treated with pH 10.0 [17F]. a. surface view; b. midplane view; c. DAPI stained DNA.
Figures 6.50 and 6.51
Immunostaining of maize root tip cells in prophase (Bar 10 µm).

Figure 6.50a-e. The same cell prepared by standard immunofluorescence method [1A] stained with a. tubulin antibody, surface view of cell; b. p34^{cdc2} antibody, surface view; c. DAPI; d. tubulin antibody, midplane view of cell; e. p34^{cdc2} antibody, midplane view of cell.

Figure 6.51a-e. The same cell treated with NP40 during preparation [17B] stained with a. tubulin antibody, surface view of cell; b. p34^{cdc2} antibody, surface view. A band of p34^{cdc2} protein that corresponds exactly in position to the MT PPB is visible (arrows). c. DAPI; d. tubulin antibody, midplane view of cell; e. p34^{cdc2} antibody, midplane view of cell. Arrows indicate the cross-sected p34^{cdc2} band.
Figures 6.52 and 6.53
Immunofluorescence staining of prophase maize root tip cells (Bar 10 μm).

Figure 6.52a-e  The same cell fixed with PFA and then treated with picric acid after cell wall digestion [17C] stained with a. tubulin antibody, surface view of cell; b. p34^{cdc2} antibody, surface view. A band of p34^{cdc2} protein (closed arrows) that corresponds exactly in position to the MT PPB (open arrows) is visible. c. DAPI; d. tubulin antibody, midplane view of cell; e. p34^{cdc2} antibody, midplane view of cell. The p34^{cdc2} protein (closed arrows) appears to localise in the same pattern as the MTs (open arrows).

Figure 6.53a-e. The same cell exposed to pH 10.0 after fixation and cell wall digestion [17F] stained with a. tubulin antibody, surface view of cell; b. p34^{cdc2} antibody, surface view; c. DAPI; d. tubulin antibody, midplane view of cell; e. p34^{cdc2} antibody, midplane view. The arrows in (b) and (e) indicate the position of a p34^{cdc2} band that corresponds exactly to the MT PPB.
Figures 6.54-6.58
Immunofluorescently labelled maize root tip cells in prophase stained with Colasanti p34cdc2 antibody. The cells have been fixed in PFA then treated with a denaturing agent after completion of cell wall digestion (Bar 10 µm). Note the distinct band of p34cdc2 (arrows) that crosses the nucleus under all experimental conditions except when the tissue was prepared by the standard method. The same root tip cell is shown in (a), (b) and (c) for each figure.

Figure 6.54. Standard method [1A]. a. surface view; b. midplane view; c. DNA stained with DAPI.
Figure 6.55. Cell treated with saponin [17A]. a. surface view; b. midplane view; c. DAPI
Figure 6.56. Cell treated with SDS [17D]. a. surface view; b. midplane view; c. DAPI
Figure 6.57. Cell treated with picric acid [17C]. a. surface view; b. midplane view; c. DNA stained with DAPI
Figure 6.58. Cell treated with pH 4.0 [17E]. a. surface view; b. midplane view; c. DAPI
Figures 6.59-6.64
Immunofluorescence staining of maize root tip cells in metaphase with the p34\textsuperscript{cdc2} antibody (Bar 10 µm). The p34\textsuperscript{cdc2} protein is primarily concentrated in a halo around the metaphase plate of chromosomes (arrows) from which it is excluded (*). The same root tip cell in metaphase is shown in (a) and (b) for each figure.

Figure 6.59. Cell prepared by standard method [1A]. a. surface view of cell; b. DAPI.
Figure 6.60. Cell treated with saponin [17A]. a. surface view of cell; b. DAPI.
Figure 6.61. Cell treated with SDS [17D]. a. surface view; b. DAPI.
Figure 6.62. Cell treated with NP40 [17B]. a. surface view of cell; b. DAPI.
Figure 6.63. Cell treated with picric acid [17C]. a. surface view; b. DAPI.
Figure 6.64. Cell treated with pH 4.0 [17E]. a. surface view of cell; b. DAPI.
Figures 6.65-6.67
Immunostaining of maize root tip cells in metaphase (Bar 10 µm). The cells have been labelled with both anti-tubulin and p34cdc2 antibodies. The halo of p34cdc2 protein (open arrows) appears to correspond closely to the dimensions of the mitotic spindle (closed arrows) that surrounds the chromosomes. The same cell is shown in (a), (b) and (c) for each figure.

**Figure 6.65.** Cell prepared by standard method [1A]. a. tubulin antibody; b. p34cdc2 antibody; c. DAPI.

**Figure 6.66.** Cell treated with saponin [17A]. a. tubulin antibody; b. p34cdc2 antibody; c. DAPI.

**Figure 6.67.** Cell treated with NP40 [17B]. a. tubulin antibody; b. p34cdc2 antibody; c. DAPI.
Figures 6.68-6.72

Immunofluorescence staining of maize root tip cells in metaphase with the Colasanti p34<sup>cdc2</sup> antibody (Bar 10 µm). The p34<sup>cdc2</sup> protein is localised primarily in a halo around the metaphase plate of chromosomes (arrows) from which it is excluded (*). The same root tip cell in metaphase is shown in (a) and (b) for each figure.

**Figure 6.68.** Cell prepared by standard method [1A]. a. surface view; b. DAPI.

**Figure 6.69.** Cell treated with saponin [17A]. a. surface view; b. DAPI.

**Figure 6.70.** Cell treated with NP40 [17B]. a. surface view; b. DAPI.

**Figure 6.71.** Cell treated with picric acid [17C]. a. surface view; b. DAPI.

**Figure 6.72.** Cell treated with pH 10.0 [17F]. a. surface view; b. DAPI.
Figures 6.73-6.78

Immunofluorescently labelled maize root tip cells in telophase (Bar 10 µm). The cells have been stained with p34\textsuperscript{cdc2} antibody. The p34\textsuperscript{cdc2} protein appears primarily to localise to a region in the cell between the two recently divided nuclei that corresponds to where the MT phragmoplast would be located (arrows). The same cell is shown in (a) and (b) for each figure.

**Figure 6.73.** Cell prepared by standard method [1A]. a. p34\textsuperscript{cdc2} antibody; b. DAPI.
**Figure 6.74.** Cell treated with saponin [17A]. a. p34\textsuperscript{cdc2} antibody; b. DAPI.
**Figure 6.75.** Cell treated with SDS [17D]. a. p34\textsuperscript{cdc2} antibody; b. DAPI.
**Figure 6.76.** Cell treated with NP40 [17B]. a. p34\textsuperscript{cdc2} antibody; b. DAPI.
**Figure 6.77.** Cell treated with picric acid [17C]. a. p34\textsuperscript{cdc2} antibody; b. DAPI.
**Figure 6.78.** Cell treated with pH 4.0 [17E]. a. p34\textsuperscript{cdc2} antibody; b. DAPI.
**Figures 6.79-6.82**

Immunofluorescence staining of root tip cells in telophase (Bar 10 µm). The cells have been labelled with both the tubulin and p34^cdc2^ antibodies. The concentration of p34^cdc2^ protein in the cell is strongest (open arrows) between the two new daughter nuclei coinciding exactly with the MT phragmoplast (closed arrows). The same cell is shown in (a), (b) and (c) in each figure.

**Figure 6.79.** Cell prepared by the standard method [1A]. a. tubulin antibody; b. p34^cdc2^ antibody; c. DAPI.

**Figure 6.80.** Cell treated with saponin [17A]. a. tubulin antibody; b. p34^cdc2^ antibody; c. DAPI.

**Figure 6.81.** Cell treated with SDS [17D]. a. tubulin antibody; b. p34^cdc2^ antibody; c. DAPI.

**Figure 6.82.** Cell treated with NP40 [17B]. a. tubulin antibody; b. p34^cdc2^ antibody; c. DAPI.
Figures 6.83-6.87
Immunofluorescently labelled maize root tip cells in telophase (Bar 10 µm). The cells have been stained with the Colasanti p34\(^{\text{cdc2}}\) antibody. The p34\(^{\text{cdc2}}\) protein is concentrated primarily to a region in the cell between the two recently divided nuclei that corresponds to where the MT phragmoplast would be located (arrows). The same cell is shown in (a) and (b) for each figure.

**Figure 6.83.** Cell prepared by standard method [1A]. a. surface view; b. DAPI.
**Figure 6.84.** Cell treated with saponin [17A]. a. surface view of cell; b. DAPI.
**Figure 6.85.** Cell treated with SDS [17D]. a. surface view; b. DAPI.
**Figure 6.86.** Cell treated with NP40 [17B]. a. surface view of cell; b. DAPI.
**Figure 6.87.** Cell treated with pH 10.0 [17F]. a. surface view; b. DAPI.
Figures 6.88 and 6.89
Pre-competition block of p$_{34}^{\text{cdc2}}$ staining of root tip cells (Bar 10 µm).

**Figure 6.88.** Immunofluorescence staining of prophase cell, after pre-incubation with the maize p$_{34}^{\text{cdc2}}$ peptide. **a.** p$_{34}^{\text{cdc2}}$ antibody; **b.** DAPI.

**Figure 6.89.** Immunofluorescence staining of telophase cell, after pre-competition with maize p$_{34}^{\text{cdc2}}$ peptide. **a.** p$_{34}^{\text{cdc2}}$ antibody; **b.** DAPI.
Figures 6.90-6.99 present controls for the double immunofluorescence staining reactions shown in Figures 6.50-6.53, 6.65-6.67 and 6.79-6.82.

Figures 6.90 and 6.91
Immunostaining of maize root tip cells in prophase and telophase with mouse anti-tubulin antibody and anti-mouse Texas Red secondary antibody, viewed with rhodamine and FITC filters (Bar 10 µm) to check that there is no “bleed-through” of fluorescence.

**Figure 6.90a-e.** The same prophase cell treated with saponin during preparation [15A] stained with tubulin antibody. 
- **a.** surface view of cell showing MT PPB (arrows) viewed using rhodamine filter; 
- **b.** same plane of focus using FITC filter. Note absence of fluorescence at the PPB in (b); 
- **c.** DAPI; 
- **d.** midplane view of cell showing cross-section of PPB (arrows) using rhodamine filter; 
- **e.** same view of cell using FITC filter.

**Figure 6.91.** The same telophase cell treated with saponin during preparation [15A] stained with tubulin antibody. 
- **a.** surface view of cell showing MT phragmoplast (arrows) using rhodamine filter; 
- **b.** same view of cell but using FITC filter; 
- **c.** DAPI.
**Figures 6.92 and 6.93**

Immunofluorescently labelled maize root tip cells in metaphase and telophase (Bar 10 μm). The cells have been stained with p34\textsuperscript{cdc2} antibody and an anti-rabbit FITC secondary antibody then viewed with the FITC and rhodamine filters to check that there is no “bleed-through” of fluorescence (converse control to Figures 6.90 and 6.91).

**Figure 6.92.** The same metaphase cell treated with saponin during preparation [15A] stained with p34\textsuperscript{cdc2} antibody. 

- a. surface view of cell showing p34\textsuperscript{cdc2} (arrows) concentrated around metaphase plate of chromosomes (*), FITC filter; 
- b. same view of cell using rhodamine filter. Note disappearance of p34\textsuperscript{cdc2} signal; 
- c. DAPI.

**Figure 6.93.** The same telophase cell treated with saponin during preparation [15A] stained with p34\textsuperscript{cdc2} antibody. 

- a. surface view of cell showing p34\textsuperscript{cdc2} localising to phragmoplast region (arrows), FITC filter; 
- b. same view of cell using rhodamine filter; 
- c. DAPI.
Figures 6.94-6.96
Immunofluorescence staining of maize root tip cells in prophase, metaphase and telophase with anti-mouse tubulin antibody and by both anti-mouse Texas Red and anti-rabbit FITC secondary antibodies, then viewed using rhodamine and FITC filters (Bar 10 µm).

Figure 6.94a-e. The same prophase cell treated with saponin during preparation [15A] stained with tubulin antibody. a. surface view of cell showing MT PPB (arrows), rhodamine filter; b. same view of cell, FITC filter; c. DAPI; d. midplane view of cell showing cross-section of PPB (arrows), rhodamine filter; e. same view as (d) using FITC filter.

Figure 6.95. The same metaphase cell treated with saponin during preparation [15A] stained with tubulin antibody. a. surface view of cell showing MT spindle (arrows), rhodamine filter; b. same view of cell as shown in (a) but using FITC filter, note absence of MT signal; c. DAPI.

Figure 6.96. The same telophase cell treated with saponin during preparation [15A] stained with tubulin antibody. a. surface view of cell showing MT phragmoplast (arrows), rhodamine filter; b. same view of cell as shown in (a) but using FITC filter, note absence of MT signal; c. DAPI.
Figures 6.97-6.99
Immunostaining of maize root tip cells with anti-mouse tubulin and anti-rabbit FITC secondary antibody viewed using FITC filter (Bar 10 µm).

Figure 6.97. Prophase cell treated with saponin during processing [15A]. a. surface view of cell; b. DAPI. Note absence of any MT signal.

Figure 6.98. Metaphase cell treated with saponin during preparation [15A]. a. surface view of cell. No sign of MT spindle is evident; b. DAPI.

Figure 6.99. Telophase cell treated with saponin during processing [15A]. a. surface view of cell. Note absence of MT phragmoplast; b. DAPI.
CHAPTER 7:

FINAL DISCUSSION

Several studies on higher plant have shown that the levels of the p34^cyc-like protein and activity are associated with cell division. Tsuda et al. (1983), Tsuda et al. (1991), Meurer et al. (1973), and Goto et al. (1991) have investigated p34^cyc-like activity in various stages of the cell cycle using various methodologies. The activity of p34^cyc-like protein is usually at a peak during the S phase, and is lower during the G1 and G2 phases (Meurer et al. 1973; Goto et al. 1991). Although several studies have been conducted to determine the levels of the p34^cyc-like protein, there has been no significant evidence to date indicating that the levels of p34^cyc-like protein are significantly elevated during the S phase of the cell cycle. Furthermore, the relationship between the levels of p34^cyc-like protein and cell division has not been determined. Therefore, it is not possible to determine the levels of p34^cyc-like protein and cell division solely based on the activity of p34^cyc-like protein.
7.1 cdc2 in the plant cell division cycle

The p34cdc2 protein has been identified as a key element in regulation of the cell division cycle (Nurse, 1990; Norbury and Nurse, 1992; Francis and Halford, 1995). The significance of the p34cdc2 protein kinase is reflected by its conservation in a variety of diverse taxa including yeast (Reid and Hartwell, 1977; Beach et al., 1982), sea urchin (Meijer et al., 1989), mouse (Lee et al., 1988), chicken (Krek and Nigg, 1989), humans (Lee and Nurse, 1987; Draetta et al., 1987) and its recent detection in higher plants, Zea mays (Colasanti et al., 1991), Arabidopsis (Ferreira et al., 1991), alfalfa (Hirt et al., 1991), Petunia (Bergouninoux et al., 1992), mothbean (Hong et al., 1993) and Glycine max (Miao et al., 1993).

Several studies on higher plants have indicated that greater levels of p34cdc2 (or p34cdc2-like) protein and activity are associated with actively dividing tissue (John et al., 1990; Gorst et al., 1991; Martinez et al., 1992; Hemerly et al., 1993). The proposal that plants use the level of cell cycle proteins to control division is supported by the capacity of the level of p34cdc2-like protein to change relative to other proteins in wheat and carrot tissue (John et al., 1990; Gorst et al., 1991). However, to date all studies that have investigated p34cdc2 abundance and activity have utilised anti-PSTAIR antibodies or the mitotic protein p13suc1. The use of these two methods has led to problems in identification of the protein kinases detected, because p13suc1 is known to bind to closely related variants of p34cdc2 as well as to p34cdc2 (Pines and Hunter, 1991a; Meyerson et al., 1992; Hirt et al., 1993). Additionally, antibodies raised against the PSTAIR peptide are known to cross react with p34cdc2 variants that share the sequence (Fang and Newport, 1991; Meyerson et al., 1992; Okuda et al., 1992; Graña et al., 1994). Hence it has not been possible to determine the level and activity solely of the p34cdc2 protein. This thesis has presented evidence for the first time of the association of specific p34cdc2 protein and activity with actively dividing tissue by use of an antibody raised against the carboxyterminal region of the p34cdc2 protein from maize. Given the central role of p34cdc2 protein in the cell cycle the depression of level and activity of p34cdc2 in cells leaving the meristem provides an adequate explanation of their capacity to continue growing yet abstain from cell division.
7.2 Investigation of maize Cdc2 distribution and enzyme activity using a specific maize anti-Cdc2 antibody

Colasanti et al. (1991) isolated and characterised two p34\(^{\text{cdc2}}\)-like cDNA clones in maize, one of these, cdc2Zma was found to be a functional homologue of p34\(^{\text{cdc2}}\) as it complemented a cdc28 mutation in budding yeast. The sequence of the cdc2Zma clone was used in this study to generate a specific antibody against the p34\(^{\text{cdc2}}\) protein in maize. The carboxyterminal region of the sequence was used for two reasons: as 1) this region, being synthesised last, often folds to the outside of the protein, and 2) it tends to be very variable compared to the high sequence conservation seen in most other regions of CDKs. Thus the carboxyterminal region could create an antibody that would effectively react with p34\(^{\text{cdc2}}\) specifically and not other CDKs.

An antibody raised against the 17 amino acid sequence at the C-terminus was observed to identify a 34 kDa protein consistently, of the same electrophoretic mobility as PSTAIR protein, which was eliminated when the p34\(^{\text{cdc2}}\) antibody was pre-competed with p34\(^{\text{cdc2}}\) peptide (Chapter 2), indicating that an antibody had been successfully generated which was capable of identifying denatured p34\(^{\text{cdc2}}\) protein. However, as polyclonal antibodies can bind to multiple sites on an antigen, it was also possible that this antibody would be able to identify native p34\(^{\text{cdc2}}\) protein. To investigate the capabilities of the antibody a Western dot blot technique was utilised (Chapter 3). A distinct p34\(^{\text{cdc2}}\) signal was identified when probing unfixed, undenatured tissue, indicating that the antibody was capable of detecting native p34\(^{\text{cdc2}}\) protein. However, only a small proportion of the antibody may identify the native protein since a three fold increase in p34\(^{\text{cdc2}}\) protein was detected after the protein was denatured by the addition of 0.5% SDS and 2% MSH.

The dot blot technique allowed the analysis of known amounts of starting material. It was also possible to quantify losses of antigen, and altered access of antibody to antigen. Using this method the p34\(^{\text{cdc2}}\) protein was observed to be susceptible to loss by leaching even from fixed cells. This loss was more evident from non-dividing cells of the elongation zone than from the meristem, suggesting that the p34\(^{\text{cdc2}}\) in the meristem was perhaps stably associating with the cytoskeleton. Associations of p34\(^{\text{cdc2}}\)-like and p34\(^{\text{cdc2}}\) protein with the MT PPB have indeed been
reported (Mineyuki et al., 1991; Colasanti et al., 1993; John et al., 1993a) and new evidence was presented in Chapter 6 of this thesis concerning p34^{cdc2} localisation with division-specific MT structures. Solubilisation and loss of p34^{cdc2} protein from fixed tissue was significantly increased by denaturing agents but this loss was reduced if denaturation was imposed only after cell wall digestion. This information was successfully used to optimise an immunostaining procedure (Chapter 6) and imaging of p34^{cdc2} was improved by denaturing the tissue after the completion of cell wall digestion.

These data are significant: as 1) detectable p34^{cdc2} protein remains in fixed tissue after processing hence the antibody can be used to investigate the intracellular localisation of p34^{cdc2} and 2) the ability of the antibody to bind native p34^{cdc2} protein provides the first opportunity to obtain data concerning the abundance and enzyme activity of p34^{cdc2} during plant organogenesis.

Native catalytically active maize p34^{cdc2} enzyme was successfully immuno-recovered with affinity purified maize anti-p34^{cdc2} antibody by immunoprecipitation using protein-A beads and by immobilising the p34^{cdc2} antibody in a column (Chapter 4). The ability of the anti-p34^{cdc2} antibody to immunorecover p34^{cdc2} meant that it could be used to determine the specific enzyme activity of p34^{cdc2}. Chapter 5 describes the level of p34^{cdc2} protein and its activity along developmental gradients in maize leaves and roots. Previous studies of p34^{cdc2}-like protein abundance and activity have consistently reported that p34^{cdc2}-like protein is highest in meristematic regions (John et al., 1990; Gorst et al., 1991; John et al., 1993a, b). John et al. (1990) observed in wheat a sixteen fold decline of p34^{cdc2}-like protein relative to other proteins, the decline in p34^{cdc2}-like protein correlating with inability of cells to divide. Subsequently John et al. (1993b) determined that the region in which p34^{cdc2}-like protein was catalytically active corresponded to that which contained actively dividing cells. These data suggested that changes in the level of p34^{cdc2} have a role in the switch between division and differentiation. This possibility was more rigourously tested in the present study using both maize leaf and root tissue. The level of p34^{cdc2}-like and significantly of p34^{cdc2} protein was observed to be highest where cells were actively dividing, then to decline steeply at the margin of leaf meristems. In the leaf p34^{cdc2} protein in the soluble protein fraction declined eight fold relative to others, and the decline was more...
extensive in the total and insoluble protein fractions where up to a 16 fold difference was seen. This suggests an active role for p34\textsuperscript{cdc2} in cell division. However, the abundance of the protein does not necessarily indicate whether p34\textsuperscript{cdc2} activity is also higher because John et al. (1993a) have shown that catalytically inactive p34\textsuperscript{cdc2}-like protein was present at higher than basal levels in the region just outside the wheat leaf meristem. Using the specific anti-p34\textsuperscript{cdc2} antibody it was possible to determine for the first time where p34\textsuperscript{cdc2}, free of other CDK enzymes, was most active. In the leaf, the highest activity was observed associated with the actively dividing cells and the decline in activity corresponded closely with the decline in mitotic activity. Comparison of p34\textsuperscript{cdc2} activity to the total soluble protein kinases demonstrated that p34\textsuperscript{cdc2} activity is independently regulated because, while p34\textsuperscript{cdc2} activity declined seven fold in the basal 10 mm, total soluble protein kinases in contrast declined only two fold in activity. In the maize root the data were less distinct, nevertheless it was also apparent that p34\textsuperscript{cdc2} activity was highest when associated with the meristem. These data then serve to complement and confirm in plant development the central role that has been proposed for p34\textsuperscript{cdc2} in the regulation of cell division.

7.3 \textbf{Investigation of localisation of Cdc2 with the microtubular cytoskeleton using the maize anti-Cdc2 antibody}

In fixed animal cells p34\textsuperscript{cdc2} has been located by antibody probes as localised within the mitotic apparatus, specifically with the kinetochores, centrosomes, chromosomes, microtubules, and the mitotic spindle (Bailly et al., 1989; Riabowol et al., 1989; Alfa et al., 1990; Rattner et al., 1990; Tombes et al., 1991; Kubiak et al., 1993; Ookata et al., 1993; Andreassen and Margolis, 1994). However, there are clearly structural and developmental differences between plants and animals (reviewed in detail in the Introduction) therefore it is possible that p34\textsuperscript{cdc2} may act upon other proteins or in different locations than in animal cells (discussed by Francis and Halford, 1995). To date the only cytoskeletal localisation proposed for p34\textsuperscript{cdc2}-like and p34\textsuperscript{cdc2} protein has been to the PPB, a division-specific structure unique to plants (Gunning, 1982; Mineyuki et al., 1991; Colasanti et al., 1993; John et al., 1993a).
In the work described here the specific anti-\( p^{34}_{\text{cdc2}} \) antibody (see Chapter 2) and an equivalent antibody independently raised by Colasanti et al. (1993) were used to investigate the possible association of \( p^{34}_{\text{cdc2}} \) with the microtubular cytoskeleton. During interphase \( p^{34}_{\text{cdc2}} \) protein was seen in all cells to be concentrated in the nucleus. In prophase while the \( p^{34}_{\text{cdc2}} \) protein was still primarily nuclear some diffuse cytoplasmic fluorescence was evident. Furthermore, in some late prophase cells with condensed chromatin a distinct band of \( p^{34}_{\text{cdc2}} \) protein was identified that corresponded exactly to the MT PPB. Colasanti et al. (1993) had previously observed this co-localisation of \( p^{34}_{\text{cdc2}} \) to the PPB and suggested that the association was transient, as it was only observed in approximately 10% of prophase cells. Its appearance in late prophase cells supports the idea that \( p^{34}_{\text{cdc2}} \) has a role in PPB disassembly (Katsuta and Shibaoka, 1992; Zhang et al., 1992; Hush et al., 1996). Localisation of \( p^{34}_{\text{cdc2}} \) to the metaphase spindle and phragmoplast was also observed in the present work. This type of association has not previously been observed in plants, but it must be noted that the association with the phragmoplast may not indicate a positive localisation; a recent study by Hepler et al. (1994) has shown by microinjection of fluorescent BSA into live cells that soluble proteins can easily diffuse into the available space between the phragmoplast microtubules. However, the association with the PPB and spindle is probably more significant and is reminiscent of the distribution reported for calmodulin (Gunning and Wick, 1985; Vantard et al., 1985; Wick, 1985, 1988; Wick et al., 1985).

That cell cycle progress involves biosynthetic events at precise locations in both cytoplasm and nucleus is well established (Nurse, 1990; Norbury and Nurse, 1992; Heichman and Roberts, 1994; Francis and Halford, 1995; Jackman et al., 1995) and is clearly demonstrated by the changes in \( p^{34}_{\text{cdc2}} \) distribution observed here. However, the mechanisms by which cell cycle catalysts may be directed to either compartment are largely not understood. Nuclear localisation signals (NLS) composed of short amino acid sequences have been identified that mediate the nuclear import of proteins (Garcia-Bustos et al., 1991; Osbourne and Silver, 1993; Boulikas, 1996). Boulikas (1996) identified weak NLSs in all CDKs examined and proposed that possession of a weak NLS indicates a role in both cytoplasmic and nuclear compartments. However, the small amount of information for cell cycle proteins suggests that the mechanism determining location is more complex, perhaps because reversible movement between
compartments is required. For example, cyclin B is predominantly cytoplasmic but moves into the nucleus at prophase. A cytoplasmic retention signal has been identified in cyclin B and its effectiveness demonstrated by its ability to block nuclear import of cyclin A, if fused to cyclin A (Pines and Hunter, 1994b). However, the cytoplasmic retention signal must be in some way inactivated or masked when cyclin B/Cdc2, which has first been activated in the cytoplasm, is then translocated into the nucleus where it acts on membrane and chromosome proteins (Ookata et al., 1992). Furthermore, the issue of how cyclin B is transported to the nucleus remains unresolved as no NLS has been identified for it (Boulikas, 1996), leading to suggestions that perhaps cyclin B is imported into the nucleus via its close association with p34\textsuperscript{cdc2} or other nuclear proteins. It is clear that much remains to be learned about the positioning of cell cycle proteins and the determining factors of that positioning.

Nonetheless, to date it has been established that p34\textsuperscript{cdc2} is a key element involved with cell cycle control in all eukaryotes (Nurse, 1990; Norbury and Nurse, 1992; Francis and Halford, 1995). The research reported in this thesis has defined for the first time in higher plants the distribution and activity of p34\textsuperscript{cdc2}, independent of other CDKs, and has confirmed its close association with cell division and its significance in regulation of the transition from active cell division to cell differentiation. Furthermore, the localisation of p34\textsuperscript{cdc2} with the MT PPB and spindle observed here suggest that the p34\textsuperscript{cdc2} protein kinase may in animals and plants have a role in controlling the assembly of MT structures required for mitosis.

7.4 Future research

The generation of a specific maize anti-p34\textsuperscript{cdc2} antibody has allowed investigation of higher plant p34\textsuperscript{cdc2} separate from any closely related CDKs. While it is not possible to alter the capacity of the antibody to detect native maize p34\textsuperscript{cdc2} it might be possible to improve the recovery by further trialling of dissociants and surfactants and optimisation of the p34\textsuperscript{cdc2} antibody bead method used in the kinase assays. The maize root clearly requires further investigation. Particularly intriguing was the finding that p34\textsuperscript{cdc2} activity in roots lacking a meristem significantly increased coincident with the induction of primordia. Repeat testing of this result is necessary and could be
followed by an investigation of the effects of hormones, such as auxin and cytokinin, which have previously been demonstrated to effect $p34^{cdc2}$ synthesis and the stability of the PSTAIR protein in other tissues (Gorst et al., 1991; John et al., 1993a, b).

In this thesis the anti-$p34^{cdc2}$ antibody was also used to investigate localisations of $p34^{cdc2}$ in relation to the microtubular cytoskeleton, finding that it was associated with the MT PPB, spindle and possibly phragmoplast. These localisations of $p34^{cdc2}$ to division-specific MT structures have only been observed in fixed material. A logical progression is to try to examine $p34^{cdc2}$ protein localisation in living cells. This can be attempted by microinjection of the anti-$p34^{cdc2}$ antibody labelled with FITC for detection. Such examination of $p34^{cdc2}$ in living cells could overcome the possible losses of antigen in processing by conventional fixation/digestion/extraction methods (Melan and Sluder, 1992). However, there are possible artefacts from using live cells. For example presence of antibody could interfere with activity of the antigen and disturb cell cycle progression. Similarly if a fluorescent tag is attached directly to a $p34^{cdc2}$ protein the behaviour of a tagged protein may also be altered. Therefore evidence from immunostaining of fixed cells provides a valuable form of evidence for comparison with live cells. It is reassuring that comparison of MT imaging from microinjection (Cleary et al., 1992; Zhang et al., 1993a; Hush et al., 1994) to conventional light microscope immunofluorescence studies (Gunning, 1982; Wick, 1985; Palevitz, 1988) demonstrates that there is a close correspondence between these studies of MT localisation. However this has yet to be determined for soluble proteins like the $p34^{cdc2}$ protein kinase which are subject to loss during permeabilisation of cells for antibody probing.

To examine the relationship between MTs and $p34^{cdc2}$ the microinjection into living cells of MT inhibitors such as oryzalin and taxol or protein kinase/phosphatase inhibitors could be employed. Colasanti et al. (1993) used oryzalin to disrupt the PPB prior to fixing cells and found after this treatment there was no localisation of $p34^{cdc2}$ to the potential PPB region. They concluded that formation of the PPB was required for localisation of $p34^{cdc2}$ to that site. Whether in living cells the same response will occur remains to be seen. The relationship of actin to MTs and $p34^{cdc2}$ dynamics could also be investigated in fixed tissue and living cells. Actin microfilaments are an essential and abundant component of the cytoskeleton (Seagull, 1989; Lambert et al., 1991; Staiger and Lloyd, 1991; Wick, 1991) and they have been observed in association with the PPB
and phragmoplast. Eleftheriou and Palevitz (1992) found that disruption of actin by the inhibitor cytochalasin D prevented narrowing of the PPB and caused re-broadening of PPBs already condensed, indicating that actin is required for bundling and stabilisation of MTs. Therefore it would be interesting to not only examine what effect disruption of actin microfilaments would have on the relationship of MTs and p34\textsuperscript{cdc2}, and hence regulation of the cell cycle but also to ascertain whether there is some direct relationship between actin and the p34\textsuperscript{cdc2} protein kinase.

### 7.4 Conclusions

In this thesis the abundance, activity and cellular localisations of maize p34\textsuperscript{cdc2}, a key cell cycle protein, were investigated using a specific maize anti-p34\textsuperscript{cdc2} antibody. The antibody generated by standard immunological procedures was found to detect authentic p34\textsuperscript{cdc2} (Chapter 2). The antibody was affinity purified and analysed by a Western dot blot technique (Chapter 3). The discovery that the antibody was capable of identifying native p34\textsuperscript{cdc2} protein suggested that it could be used to determine for the first time the level and activity specifically of p34\textsuperscript{cdc2}. Successful immunorecovery of p34\textsuperscript{cdc2} protein by the antibody (Chapter 4) supported this idea. In developing maize leaf and root, abundance and activity of p34\textsuperscript{cdc2} was investigated by \textsuperscript{125}I Western blot analysis of the amount of protein and assay of enzyme activity after purification with p13\textsuperscript{sucl} or specific antibody. p34\textsuperscript{cdc2} protein and activity defined the boundaries of actively dividing tissue in developing leaves and roots. Comparison with total soluble protein level and activity indicated specific regulation of the p34\textsuperscript{cdc2} protein, independently of the majority of other proteins (Chapter 5). The specific association of p34\textsuperscript{cdc2} with the meristem suggests that it may have a role in regulation of cell division. How p34\textsuperscript{cdc2} functions in division processes in plant cells remains unknown, however p34\textsuperscript{cdc2} has previously been observed to co-localise with division-specific MT structures in plants and animals. This study confirmed the association of p34\textsuperscript{cdc2} with the MT PPB and also detected localisation, using the anti-p34\textsuperscript{cdc2} antibody, to the mitotic spindle and phragmoplast (Chapter 6). These data suggest that the p34\textsuperscript{cdc2} protein kinase may have a role in the regulation of events during mitosis in higher plants.
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