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Localization of the Functional p34^{cdc2} Homolog of Maize in Root Tip and Stomatal Complex Cells: Association with Predicted Division Sites

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We have used an antibody against the functional homolog of the cdc2 kinase of maize to localize the p34^{cdc2} protein within dividing cells of the root apex and the stomatal complex of leaf epidermis. The microtubule cytoskeletal structure of plant cells was visualized concomitantly with a monoclonal antibody specific for α-tubulin. We found that the cdc2 protein is localized mainly to the nucleus in plant cells at interphase and early prophase. This finding contrasts markedly with the predominantly cytoplasmic staining obtained using antibody to the PSTAIRE motif, which is common to cdc2 and numerous cdc2-like proteins. In a subpopulation of root cells at early prophase, the p34^{cdc2} protein is also distributed in a band bisecting the nucleus. Double labeling with the maize p34^{cdc2}Zm antibody and tubulin antibody revealed that this band colocalizes with the preprophase band (PPB) of microtubules, which predicts the future division site. Root cells in which microtubules had been disrupted with oryzalin did not contain this band of p34^{cdc2} protein, suggesting that formation of the microtubule PPB is necessary for localization of the p34^{cdc2} kinase to the plane of the PPB. The p34^{cdc2} protein is also localized to the nucleus and PPB in cells that give rise to the stomatal complex, including those cells preparing for the highly asymmetrical divisions that produce subsidiary cells. Association of the p34^{cdc2} protein with the PPB suggests that the cdc2 kinase has a role in establishing the division site of plant cells and, therefore, a role in plant morphogenesis.

INTRODUCTION

A plant develops from a single-celled zygote to a complex, multicellular organism through a series of coordinated cell divisions (Steeves and Sussex, 1989; Lyndon, 1990). In higher plants, cellular differentiation and cell division are closely linked because precise spatial and temporal control of cell proliferation is required for the formation of complex structures. Although the plant and animal cell division cycles share many features, there are fundamental differences between them that may reflect peculiarities of their developmental programs. Many of these differences are apparent at the level of cytoskeletal organization. Plant cells lack discernible centrosomes or spindle pole bodies; at mitosis, no centrioles or asters are visible. There are, however, two microtubule-containing structures found only in cells of higher plants, the preprophase band (PPB) and the phragmoplast, that are important to the process of cell division.

Plant cells form a PPB, a dense array of microtubules that aggregate in the cell cortex at the position in which the future cell wall will join parent cell walls at cytokinesis (Pickett-Heaps and Northcote, 1966; Wick, 1991). The PPB normally forms before, or as, chromosome condensation begins and before nuclear envelope breakdown is apparent and disappears before the appearance of the metaphase spindle. In many plant species, the PPB forms when cells are still in interphase but persists and reaches its maturity close to the onset of mitosis. The position of the PPB is somehow imprinted in cellular memory because its location marks where the new cell wall will form at cytokinesis, when the second microtubule-containing structure, the phragmoplast, forms between the separated daughter nuclei in the central body of the cell (Gunning, 1982). This structure, which is aligned in the plane or curved surface of the cell previously outlined by the PPB, is believed to coordinate the movement of vesicles containing cell wall components to be deposited at the phragmoplast equator for the formation of the cross wall that separates the daughter nuclei. The mechanism that determines where and when the PPB and phragmoplast will form is not known at present. Because there is no cell migration in developing plants, the planes of cell division are extremely important to plant morphogenesis (reviewed by Lyndon and Cunninghame, 1986), and organ initiation is often accompanied by a change in the plane of cell division (Green and Poethig, 1982).

Recent discoveries have revealed that all eukaryotic organisms share a common mechanism for the regulation of cell

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division. A central component of this regulatory mechanism is a 34-kD protein encoded by the cell division cycle gene *cdc2*. The $p34^{cdc2}$ protein is a serine/threonine protein kinase that, in association with other proteins, mediates cell division and DNA replication (reviewed in Doree, 1990; Nurse, 1990). Through the phosphorylation of specific substrates, $p34^{cdc2}$ kinase mediates events such as chromosome condensation, nuclear envelope breakdown, and spindle formation (reviewed in Lewin, 1990). Immunofluorescence microscopy studies in animal cells (Bailly et al., 1989; Riabowol et al., 1990; Krek and Nigg, 1991) and yeast cells (Booher et al., 1989) have shown that the localization of $p34^{cdc2}$ within interphase cells is primarily nuclear, with a fraction of the protein associated with the centrosome or the spindle pole body (Alfa et al., 1990). In these studies, it was also found that most of the $p34^{cdc2}$ kinase is associated with the spindle poles in mitotic cells. Association of the $p34^{cdc2}$ protein with the spindle poles is consistent with other evidence implicating the cdc2 kinase in setting up the mitotic spindle by the phosphorylation of substrates that have not yet been determined (Verde et al., 1990). The highly conserved nature of the cell cycle machinery makes it possible to analyze cell cycle components of demonstrated importance in simpler, single-celled organisms (such as the yeasts) and to determine their potential, analogous function in complex, multicellular organisms.

Although control of cell division by the $p34^{cdc2}$ kinase seems to be common to higher eukaryotes (Lee and Nurse, 1987; Krek and Nigg, 1990; Lehner and O'Farrell, 1990; Colasanti et al., 1991), the way in which each organism uses this function will likely vary between different organisms, depending on their specific developmental requirements. Our laboratory and others have confirmed that higher plant cells also contain a functional $p34^{cdc2}$ kinase homolog (Colasanti et al., 1991, Ferreira et al., 1991; Hirt et al., 1991). Due to the fundamental role played by the cdc2 kinase in reorganizing intracellular structures for mitosis, it is of great interest to study the localization of the $p34^{cdc2}$ kinase within dividing plant cells. Mineyuki et al. (1991) have demonstrated staining of the cytoplasm and the PPB in dividing onion root cells using antibodies directed against a conserved epitope present in cdc2 and cdc2-related proteins. Here, we use immunofluorescence microscopy and antibodies specific to a functional $p34^{cdc2}$ homolog from maize to examine its intracellular location within dividing cells of the root apex and the leaf cells that produce stomatal complexes.

RESULTS

Localization of $p34^{cdc2}$ Protein in Dividing Cells of the Maize Root Tip

Maize root tip cells were examined by immunofluorescence microscopy to determine the intracellular location of the $p34^{cdc2}$ protein throughout the cell cycle. Root tips provide many actively dividing cells that are easily accessible for immunofluorescence studies. The *cdc2* transcript is found in abundance in young roots, as expected for tissues containing actively dividing cells (Colasanti et al., 1991; J. Colasanti and V. Sundaresan, unpublished results). A polyclonal antiserum was raised against a 17-amino acid C-terminal peptide predicted from the sequence of the maize *cdc2* gene that we had previously demonstrated to be functional by complementation of a yeast mutant defective in the G2/M transition (Colasanti et al., 1991). This antibody was used to localize the maize $p34^{cdc2}$ protein within cells. Affinity purification enriched for antibody that is specific for the $p34^{cdc2}$ 34-kD protein, as shown in the protein gel blots of maize root and leaf tissue in the left lanes of Figure 1.

Staining with 4,6-diamidino-2-phenylindole (DAPI) revealed that greater than 90% of the cells in root tip preparations were in interphase, i.e., with decondensed chromatin and intact nucleoli. Staining of interphase cells with antibody specific for the maize $p34^{cdc2}$ protein showed it to be localized mainly in the nucleus of the cell, but excluded from the nucleolus, as seen in Figures 2B and 3B. The $p34^{cdc2}$ protein did not appear to be concentrated within a specific region of the nucleus or the nuclear membrane, and it was not associated with any particular structure. We cannot rule out the possibility that some of the protein is present in the cytoplasm in interphase cells; however, the highest concentration of the $p34^{cdc2}$ kinase is in the nucleus at this stage of the cell cycle.

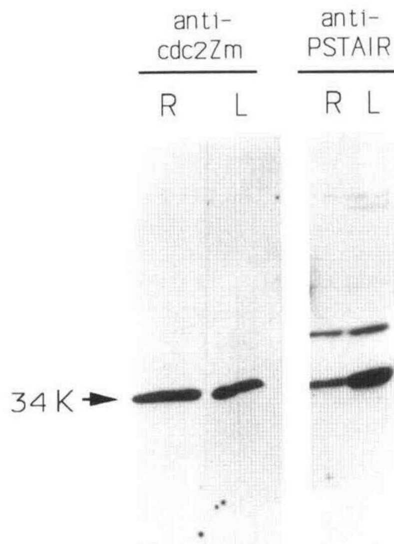

Figure 1. Immunoblot of Total Protein from Maize Root Tips and Immature Leaf Tissue.

Each lane contains ~50 μg of protein extract electrophoresed on a 10% SDS-polyacrylamide gel and electroblotted onto nitrocellulose paper. The gel at left shows a blot stained with affinity-purified maize $p34^{cdc2}$Zm antibody; at right is a blot stained with monoclonal PSTAIRE antibody. R, root; L, leaf; 34K, position of the $p34^{cdc2}$ protein.

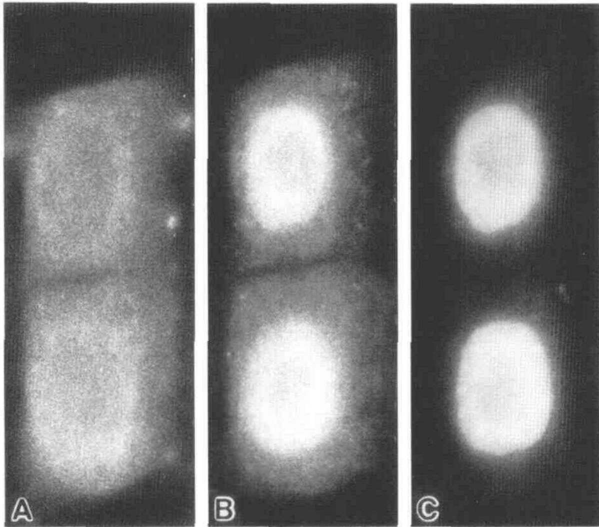

Figure 2. Immunofluorescence Staining of Maize Root Tip Cells in Interphase.

The same root tip cells are shown in **(A)**, **(B)**, and **(C)**.
(A) Cells stained with monoclonal PSTAIRE antibody.
(B) Cells stained with maize p34^{cdc2}Zm antibody.
(C) Cells stained with DAPI.
Magnification ×1050.

We also used a monoclonal antibody specific for the PSTAIRE epitope that is shared by all cdc2 proteins and most cdc2-like proteins from all species studied to date (Lee and Nurse, 1987; Meyerson et al., 1992). Gel blotting shows that the PSTAIRE antibody recognizes a 34-kD band, as well as other bands, including a band of ~40 kD (Figure 1, right lanes). At very long exposures, the antibody to the C terminus of maize p34^{cdc2} protein also showed weak cross-reactivity to a band of ~40 kD (data not shown), but we do not know if this is the same band that is recognized by the PSTAIRE antibody. In general, root cell staining with this monoclonal antibody shows that proteins with the PSTAIRE motif are present in abundance in the cell but are not concentrated in the nucleus, in contrast to what we found with the p34^{cdc2}Zm antibody, as shown in Figure 2. Figure 2A shows staining of entire interphase root cells with the PSTAIRE antibody, with a greater amount of staining in the cytoplasm than in the nucleus. This result is in agreement with that of Mineyuki et al. (1991) who used a PSTAIRE monoclonal antibody and noted predominantly cytoplasmic rather than nuclear staining of interphase cells.

Prior to mitosis, before chromosome condensation is visible by DAPI staining, a PPB of microtubules forms—first as a diffuse band distinguishable among the cortical microtubules and, later, as a tight band or ring-shaped structure, as seen in Figures 3A and 4A, respectively. As demonstrated by the tubulin antibody–stained root tip cells in Figures 4A and 5A, this band usually bisects the nucleus. All meristematic cells of flowering plants are believed to form PPBs prior to mitosis (reviewed in Wick, 1991a). In late interphase and early prophase cells, the p34^{cdc2} protein remains most prominent in the nucleus (Figures 3B and 3C). However, in ~10% of several hundred root cells that had PPBs, the p34^{cdc2} protein was found in a plane of the cell that bisected the nucleus, as well as in the nucleus itself. In these cells, which were stained simultaneously with tubulin antibodies and p34^{cdc2}Zm antibodies, the band of p34^{cdc2} staining always colocalized with microtubules of the PPB (Figures 4A to 4C). The "p34^{cdc2} PPBs" often appeared thinner than the tubulin PPBs, suggesting that the p34^{cdc2} protein is localized to a narrower region than that occupied by microtubules within the PPB structure.

When only the p34^{cdc2}Zm antibody and DAPI were used to stain cells, as in Figures 4D and 4E, respectively, a subpopulation of the protein was associated with a band that bisected the nucleus in ~10% of several hundred cells that had visibly condensed chromatin. This demonstrates that the observed staining of the PPB with antibodies to p34^{cdc2} is not a result of cross-reactivity with the anti-tubulin antibodies used in the double labeling immunofluorescence procedure or "bleed through" of fluorescence from Texas Red–labeled microtubules onto the image of fluorescein-labeled p34^{cdc2} protein. In fact, the p34^{cdc2} bands in single-stained preparations were often more intense than their double-stained counterparts, suggesting that antibody binding to tubulin may interfere with antibody binding to p34^{cdc2} protein. Similar observations of immunofluorescence double staining being less efficient than single staining have been noted with other antibody pairs (S. Wick, unpublished results).

In double-labeling experiments, we have never observed the p34^{cdc2} PPB in the absence of a microtubule PPB, although a "p34^{cdc2} PPB" is frequently absent where a well-defined microtubule PPB is present. The cdc2 PPB is only found in

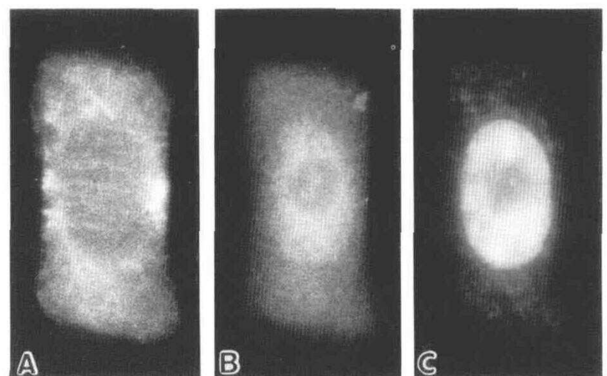

Figure 3. Immunofluorescence Staining of a Maize Root Tip Cell in Late Interphase.

The same root tip cell is shown in **(A)**, **(B)**, and **(C)**.
(A) Cell stained with tubulin antibody.
(B) Cell stained with maize p34^{cdc2}Zm antibody.
(C) Cell stained with DAPI.
Magnification ×1050.

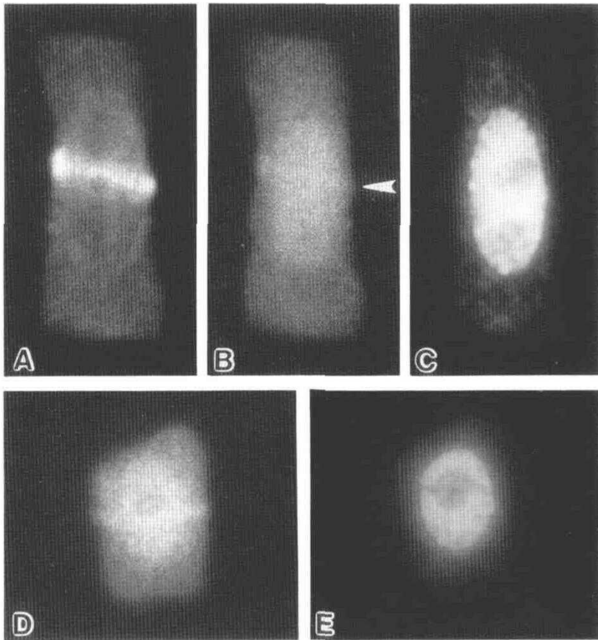

Figure 4. Immunofluorescence Staining of Maize Root Tip Cells in Early Prophase.

(A) to **(C)** The same cell stained with tubulin antibody, maize $p34^{cdc2}Zm$ antibody, and DAPI, respectively. The arrowhead in **(B)** indicates the position of the "$p34^{cdc2}$ PPB."

(D) and **(E)** Immunostaining of a root tip cell with maize $p34^{cdc2}Zm$ antibody only **(D)** and DAPI **(E)**. DAPI staining shows the condensed chromatin indicative of a cell in early prophase.
Magnification ×1050.

cells that are more advanced in G2/M and have mature microtubule PPBs (compare Figures 3A and 3B with 4A and 4B). The lack of consistent colocalization of the $p34^{cdc2}$ protein with the microtubule PPB may be the result of variable staining, or, more interestingly, it may reflect a transient appearance of the cdc2 kinase in this region of the cell (see Discussion).

As a cell progresses into mitosis, the microtubule PPB disappears and a mitotic spindle forms. Figure 6 shows that $p34^{cdc2}$ staining in metaphase and anaphase cells is very diffuse and appears to occupy most of the cell, except for condensed chromatin. It does not appear to be associated with any particular part of the spindle, although it may be tenuously associated with a "perichromosomal" region (Figures 6B and 6E).

In telophase, another microtubule-containing structure, the phragmoplast, forms between the newly separated chromosomes, as depicted in Figures 7A, 7C, 7D, and 7E. The phragmoblast, which appears as the mitotic spindle disassembles, contains a double ring of microtubules at the margins of the newly forming cell wall that expands centrifugally to the walls of the parent cell to achieve cytokinesis (Gunning, 1982). The cells in Figures 7A, 7B, 7D, and 7E show that, unlike the case with the PPB, the $p34^{cdc2}$ protein is not associated with the phragmoplast. As the phragmoplast expands and the chromatin of the daughter nuclei begins to decondense, the $p34^{cdc2}$ protein appears to be sequestered into the newly formed nuclei and is less concentrated in the cytoplasm, as shown in the telophase cells in Figures 7D to 7F.

The specificity of the maize $p34^{cdc2}Zm$ antibody was demonstrated by the ability of the 17-amino acid C-terminal peptide derived from the functional $p34^{cdc2}$ protein to specifically block the staining pattern. Figure 5B shows that coincubation of the peptide with the $p34^{cdc2}Zm$ antibody completely abrogates $p34^{cdc2}$ staining, whereas coincubation of peptide with tubulin antibody has no effect on PPB and other microtubule staining in the same cell (Figure 5A). Some perinuclear staining was observed following peptide preincubation (Figure 5B), but this staining pattern was also observed in controls in which only secondary antibody was used (data not shown).

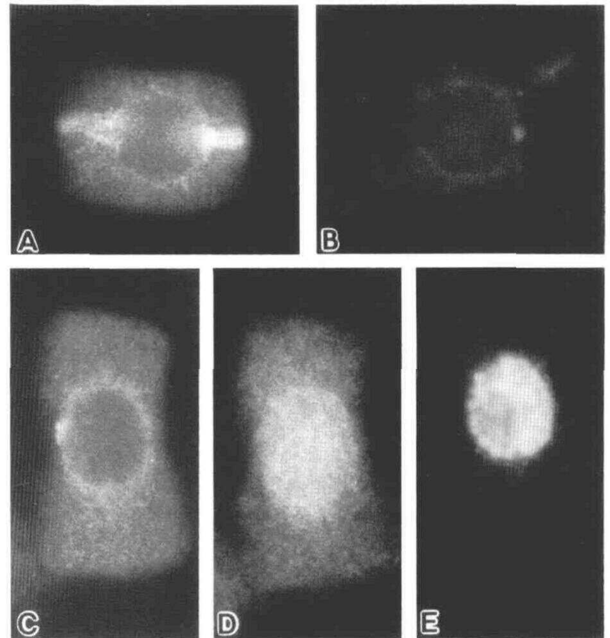

Figure 5. Peptide Block of $p34^{cdc2}Zm$ Staining and Oryzalin Treatment of Root Tip Cells.

(A) and **(B)** Immunofluorescence staining of root tip cells with tubulin antibody **(A)** and $p34^{cdc2}Zm$ antibody **(B)** after preincubation with the 17-amino acid maize cdc2 peptide.

(C) to **(E)** Immunofluorescence staining of root tip cells treated with oryzalin to disrupt microtubule structure and then stained with tubulin antibody **(C)**, maize $p34^{cdc2}Zm$ antibody **(D)**, and DAPI **(E)**.
Magnification ×1050.

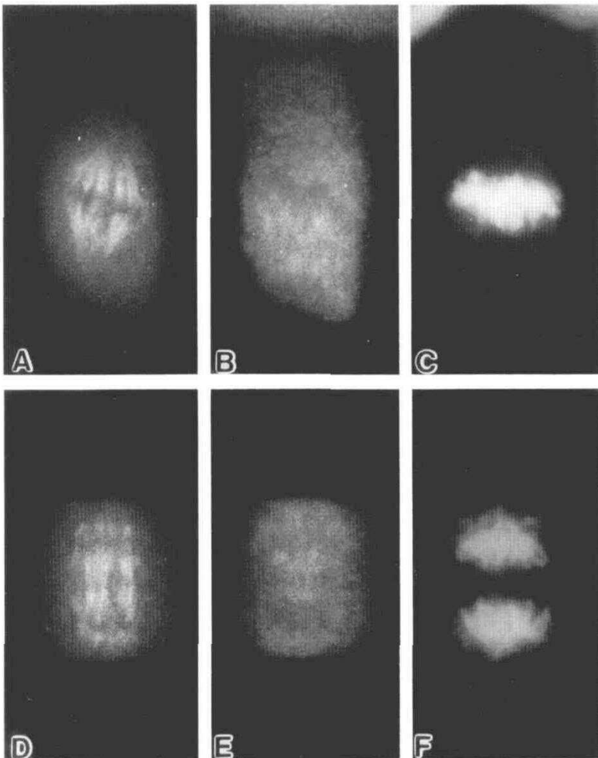

Figure 6. Immunofluorescence Staining of Root Tip Cells in Metaphase and Anaphase.

The same root tip cell in metaphase is shown in **(A)**, **(B)**, and **(C)**. The same root tip cell in anaphase is shown in **(D)**, **(E)**, and **(F)**.
(A) and **(D)** Cells stained with tubulin antibody.
(B) and **(E)** Cells stained with maize $p34^{cdc2}$Zm antibody.
(C) and **(F)** Cells stained with DAPI.
Magnification ×1050.

Microtubule Disruption and $p34^{cdc2}$ Localization

The dinitroaniline herbicide oryzalin was used to disrupt microtubule structures within the cell to determine the effect on $p34^{cdc2}$ localization. Oryzalin has been shown to specifically inhibit plant microtubule formation and cause the depolymerization of plant tubulin (Hugdahl and Morejohn, 1993). Root tip cells exposed to 20 μM oryzalin for 4 hr contained no microtubule structures; that is, interphase arrays, PPBs, spindles, and phragmoplasts were not observed by tubulin antibody staining. Exposure to this level of oryzalin was not lethal to the cells, as the roots resumed normal growth when transferred to oryzalin-free medium. To determine its effect on $p34^{cdc2}$ PPB formation, several hundred cells in early prophase, as determined by DAPI stain of condensing chromosomes, were examined. None of these cells had $p34^{cdc2}$ distributed as a PPB-like structure. Figure 5C shows an oryzalin-treated cell in early prophase that has no visible microtubule structures when stained with tubulin antibody; the brightly fluorescing cytoplasm reflects staining of depolymerized microtubules. The $p34^{cdc2}$Zm antibody staining of the same cell shows that $p34^{cdc2}$ protein is present in a region larger than, but including, the nucleus (Figure 5D). This contrasts with the exclusively nuclear localization of $p34^{cdc2}$ in interphase cells (Figures 2B and 3B) and the nuclear plus PPB distribution of $p34^{cdc2}$ in untreated early prophase cells (Figures 4B and 4D). Exposure to oryzalin effectively eliminated the microtubule PPB in cells about to divide. Therefore, an intact microtubule PPB seems to be necessary for the $p34^{cdc2}$ protein to localize in this region.

Oryzalin-treated cells that were not in interphase or early prophase often exhibited intracellular aberrations: condensed chromosomes were scattered throughout the cells, and the nuclei of telophase cells were often displaced against the sides of the cell instead of centrally positioned. The $p34^{cdc2}$ protein in these cells appeared to be distributed throughout the cytoplasm, similar to its distribution in metaphase and anaphase cells that were not treated with oryzalin (data not shown).

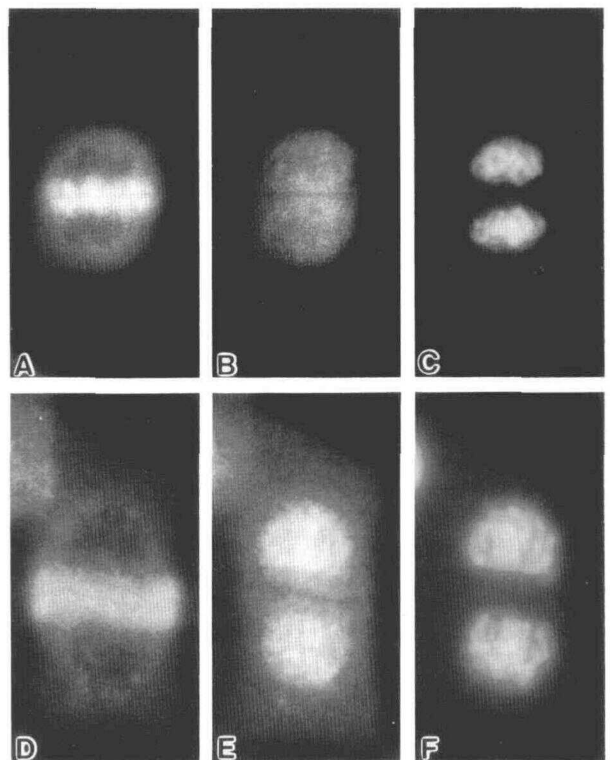

Figure 7. Immunofluorescence Staining of Root Tip Cells in Late Anaphase and Telophase.

The same root tip cell in late anahase is shown in **(A)**, **(B)**, and **(C)**. The same root tip cell in telophase is shown in **(D)**, **(E)**, and **(F)**.
(A) and **(D)** Cells stained with tubulin antibody.
(B) and **(E)** Cells stained with maize $p34^{cdc2}$Zm antibody.
(C) and **(F)** Cells stained with DAPI.
Magnification ×1050.

Localization of p34^{cdc2} Protein within Progenitor Cells of the Stomatal Complex

Immunofluorescence microscopy was also used to localize the p34^{cdc2} protein within the cells that give rise to maize leaf stomatal complexes. In grasses, the components of the stomatal complex arise from longitudinal and asymmetric divisions of progenitor cells: namely, the guard cell mother cell (GMC) and the subsidiary cell mother cell (SMC) (Stebbins and Jain, 1960; Stebbins and Shah, 1960). The GMC divides anticlinally along the longitudinal axis of the leaf and gives rise to guard cells; the stomatal pore forms between these cells. The SMCs on each side of the GMC divide asymmetrically to yield small, lens-shaped subsidiary cells that flank the guard cell (Stebbins and Jain, 1960; Stebbins and Shah, 1960; Cho and Wick, 1989). As in root tip cells, the position of new cell walls is predicted by PPBs of microtubules. However, unlike the situation in root cells in which the PPB might originate from a "bunching up" of transverse interphase microtubules (Pickett-Heaps, 1969; Wick and Duniec, 1983; Lloyd and Traas, 1988; Flanders et al. 1990), in SMCs, it appears that interphase microtubules disassemble and that the periclinal portions of the PPB are formed de novo from points along the anticlinal lateral SMC walls (Cho and Wick, 1989). Initially, these microtubules appear as fanned arrays that eventually fuse to form a curved PPB. Double staining of a few hundred developing stomatal complexes that contained cells with PPBs showed colocalization of the p34^{cdc2} protein with microtubule PPBs in some of the cells examined. As in the case of root tip cells, colocalization of p34^{cdc2} with the PPB appeared to be more frequent in cells that have mature PPBs, i.e., later in G2/M.

Figures 8 and 9 show examples of stomatal complexes in which colocalization is seen in dividing SMCs. Figure 8A shows a SMC (left) and a GMC (center) of a maize stomatal complex stained with tubulin antibody. The SMC shows a PPB, and the GMC has a band of interphase microtubules (Busby and Gunning, 1980). The p34^{cdc2} protein does not colocalize with the interphase microtubule band of the GMC but does colocalize with the PPB of the SMC (Figures 8A and 8B). Figure 9 shows a more complex arrangement in which two files of developing stomatal complexes are adjacent to each other; similar results were observed with localization of p34^{cdc2} in the PPBs of the SMCs but not the interphase band of the GMCs (Figures 9A and 9B).

Developing stomatal complexes of rye leaf epidermis were likewise examined via single and double immunofluorescence labeling with the antibody to maize p34^{cdc2} protein. The antibody to the C terminus of maize p34^{cdc2} recognizes a 34-kD protein in rye, suggesting that it is able to cross-react with the p34^{cdc2} homolog of rye. As was found with maize epidermal cells, a fraction of the rye cells with condensing chromatin exhibited a band of p34^{cdc2} protein, and, in double-label preparations, this band colocalized with the PPB of microtubules. The interphase microtubule band of rye guard mother cells was not associated with a corresponding band of p34^{cdc2} (data not shown).

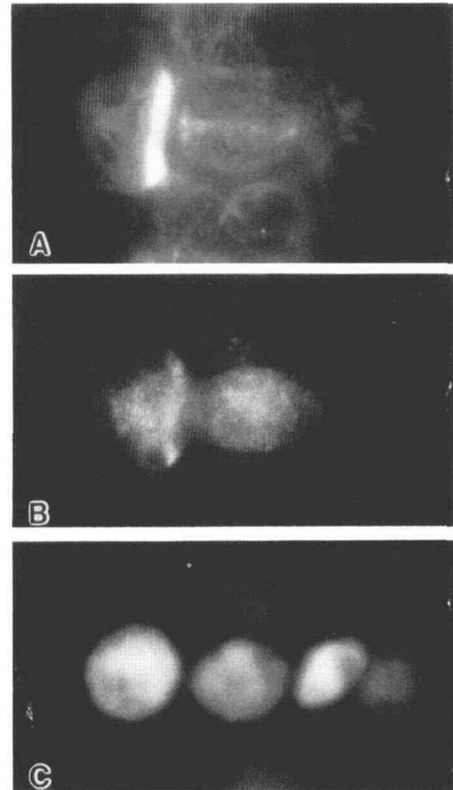

Figure 8. Immunofluorescence Staining of Leaf Epidermis Showing a Developing Stomatal Complex.

The same stomatal complex cells are shown in **(A)**, **(B)**, and **(C)**.
(A) Cells stained with tubulin antibody, which reveals the tranverse interphase band in the GMC and the more brightly stained PPB in the SMC, on the left.
(B) Cells stained with maize p34^{cdc2}Zm antibody.
(C) Cells stained with DAPI.
Magnification ×1600.

DISCUSSION

We have generated antibodies specific to a maize homolog of the cdc2 kinase that we have previously demonstrated to be functional by its ability to complement a yeast mutant defective in the G2/M transition (Colasanti et al., 1991) and used this antibody to localize the p34^{cdc2} protein within plant cells at different stages of the cell cycle by indirect immunofluorescence microscopy. In interphase cells, the cdc2 protein appears to be localized primarily within the nucleus, although it is excluded from the nucleolus. This is similar to the results from studies on cdc2 localization in animal cells (Bailly et al., 1989; Riabowol et al., 1990; Krek and Nigg, 1991) and yeast cells (Booher et al., 1989), except that, in these cells at interphase, a fraction of the cdc2 kinase is associated with the centrosome (Bailly et al. 1989). We did not observe this highly localized

distribution of the cdc2 kinase in our study, possibly because plant cells lack a structural equivalent of the animal cell centrosome. The nuclear localization of the plant cdc2 protein continues in cells approaching the G2/M transition, which is manifested by the appearance of a PPB of microtubules and some chromatin condensation. We have found that in a fraction of such cells, in addition to the nuclear localization, the $p34^{cdc2}$ protein also colocalizes with the PPB. The fact that not all cells with PPBs showed colocalization of cdc2 kinase with the PPB suggests that this association may be transient.

The colocalization of $p34^{cdc2}$ with the microtubule PPB could be explained by two possible mechanisms. The microtubule PPB may be sequestering the $p34^{cdc2}$ protein and directing it to substrates at the cell cortex or, alternatively, because cdc2 plays a role in microtubule nucleation (Verde et al., 1990), the band of cdc2 kinase may be involved in setting up the PPB of microtubules. To distinguish between these possibilities, we disrupted the microtubule PPB by treating cells with oryzalin. We found that there was no PPB-like distribution of $p34^{cdc2}$ in cells that were entering M phase. Therefore, it is likely that formation of the microtubule PPB is required for localization of $p34^{cdc2}$ within the plane of the future division site. This conclusion is consistent with our observation that colocalization of $p34^{cdc2}$ with the PPB seems to occur later in the G2/M transition, in cells with mature PPBs. Furthermore, early prophase cells treated with oryzalin show $p34^{cdc2}$ staining in a region containing the nucleus but larger than the size of the nucleus (Figures 5D and 5E), suggesting that $p34^{cdc2}$ can leave the nucleus but is not directed to the future division site in the absence of the microtubules of the PPB.

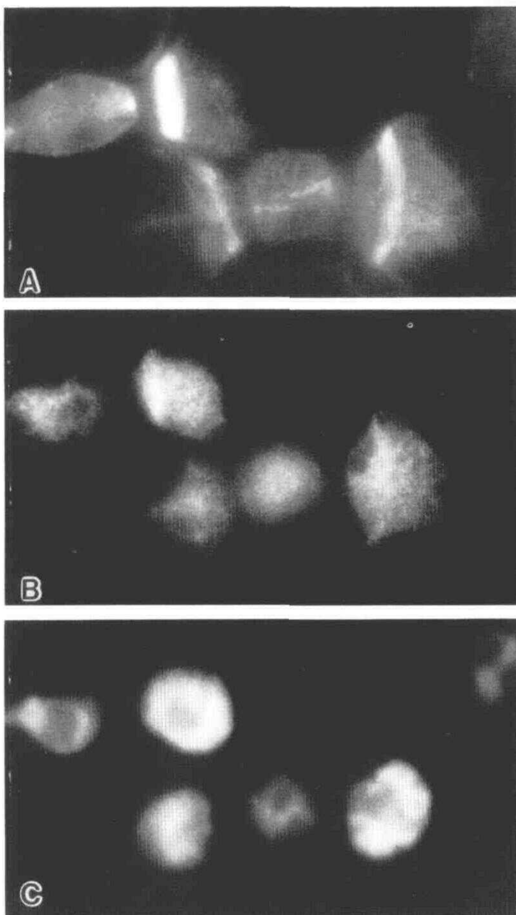

Figure 9. Immunofluorescence Staining of a Region of the Leaf Epidermis with Two Adjacent Files of Developing Stomatal Complexes.

In the upper complex, one SMC is visible on the right side of the GMC, while both SMCs are seen in the lower one.
(A) Cells stained with tubulin antibody.
(B) Cells stained with maize $p34^{cdc2}$Zm antibody.
(C) Cells stained with DAPI.
Magnification ×1600.

In the stages between prometaphase and anaphase, the intracellular distribution of $p34^{cdc2}$ appears diffuse and generally uniform. We observed exclusion of $p34^{cdc2}$ from the region occupied by the chromosomes, although there seemed to be some increased staining in the region immediately surrounding the chromosomes. There was no specific association of $p34^{cdc2}$ with either the mitotic spindle or the spindle poles. The latter observation contrasts with the studies on animal and yeast cells in which specific association of $p34^{cdc2}$ with spindle poles was observed in M phase. However, we note that flowering plant cells, unlike animal and yeast cells, do not have sharply defined centrioles, asters, or spindle pole bodies that are detectable with tubulin staining. Finally, at telophase we did not observe any association of $p34^{cdc2}$ with the phragmoplast and, late in telophase, the $p34^{cdc2}$ was observed to be concentrated within the nascent daughter nuclei.

The immunolocalization studies were repeated with dividing cells that produce the stomatal complex in maize leaf epidermis. The formation of a functional stomate is dependent upon changes in the planes of cell division of the epidermal cells. In contrast to most root apical cells and leaf epidermal cells (both of which commonly divide symmetrically and in a plane transverse to the long axis of the organ), cells dividing to give rise to the stomatal complex in maize undergo highly asymmetric divisions and divisions longitudinal to the organ axis (Cho and Wick, 1989, 1990). With respect to $p34^{cdc2}$ protein localization, we find similar results with progenitor cells of stomatal complexes and with root apical cells. The distribution of $p34^{cdc2}$ is nuclear in interphase cells, and further, colocalization of $p34^{cdc2}$ with the curved PPB of subsidiary mother cells in early prophase could be observed (Figures 8 and 9). The $p34^{cdc2}$ protein does not colocalize with the interphase microtubule band of guard mother cells, again pointing to a specific association of $p34^{cdc2}$ with the morphogenetically important PPB structure rather than indiscriminate association with compact microtubule structures. This result is further strengthened by our studies with

rye leaf epidermis cells in which the results of $p34^{cdc2}$ localization are the same as in maize.

While this work was in progress, a paper on the localization of $p34^{cdc2}$ within dividing onion cells was published; in this study, the association of $p34^{cdc2}$ with the PPB was reported (Mineyuki et al., 1991). Our study differs from this report in two significant respects: (1) the authors used an antibody that is directed against a conserved peptide sequence that contains the PSTAIRE motif found in all cdc2 homologs; and (2) they observed that the interphase localization of $p34^{cdc2}$ in plant cells is predominantly cytoplasmic, rather than the nuclear localization that we observed. In addition, in this paper, we have shown that formation of the PPB is required for localization of $p34^{cdc2}$ to the PPB, and that colocalization of $p34^{cdc2}$ and the PPB also occurs in the progenitor cells of the stomatal complex, which undergo highly asymmetric divisions.

Studies with animal systems have found that there are a number of $p34^{cdc2}$-like proteins that have sequences closely related to or identical to the functional cdc2 kinase in the PSTAIRE region, but that these proteins are not functional homologs (reviewed by Hunter and Pines, 1991). A study in Arabidopsis has suggested that a similar situation may also be true in higher plants (Hirayama et al., 1991). In yeast, there is a closely related 34-kD protein, encoded by the *PHO85* gene, which appears to have no role in cell division, although it contains the PSTAIRE motif (Toh-e et al., 1988). Further, a recent study in human cells showed that there are a number of kinases with closely related motifs such as PCTAIRE and PSSALRE that cross-react with antibodies to the PSTAIRE peptide, some of which have sizes close to that of $p34^{cdc2}$ (Meyerson et al., 1992). Only antibodies that were directed against the nonconserved C terminus of $p34^{cdc2}$ were found to be specific for $p34^{cdc2}$ (Meyerson et al., 1992). Therefore, it is not possible to interpret, without ambiguity, the results of studies using antibodies directed against conserved regions of the $p34^{cdc2}$ kinase family.

The observation by Mineyuki et al. (1991) that $p34^{cdc2}$ is primarily cytoplasmic in interphase cells might be explained as a consequence of the use of the anti-PSTAIRE antibody rather than a specific antibody to a functional cdc2 homolog. We note that both Mineyuki et al. (1991) and our laboratory find that anti-PSTAIRE antibody recognizes multiple bands on protein gel blots (Figure 1, right lanes); by contrast, the C-terminal–specific antibody recognizes predominantly a single band (Figure 1, left lanes). When we used the monoclonal antibody to the PSTAIRE peptide for immunolocalization, we found extensive cytoplasmic staining and relatively weak nuclear staining (Figure 2A), similar to the results of Mineyuki et al. (1991). Under the same conditions, staining with the antibody directed against the C terminus of maize $p34^{cdc2}$ was predominantly nuclear (Figure 2B). We conclude that, as has been found with animal cells (Meyerson et al., 1992), it may not be possible to obtain reliable information about localization of $p34^{cdc2}$ in plant cells using anti-PSTAIRE antibodies.

Studies in animal and yeast cells have demonstrated that the cellular distribution of $p34^{cdc2}$ at the G2/M transition is regulated, with translocation of cdc2 kinase to the intracellular locations where it conducts its mitotic functions (Bailly et al., 1989; Alfa et al., 1990; Pines and Hunter, 1991). The association of $p34^{cdc2}$ with the PPB at the G2/M transition is therefore significant. It is possible that this distribution of $p34^{cdc2}$ in plant cells may be involved in an important cellular function, i.e., the imprinting of the plane of cell division. The plane of cell division is accurately determined by the PPB, because the new cell plate formed by the expanding phragmoplast contacts the cortex at the same site at which the microtubules of the PPB existed prior to mitosis. The connection between the PPB and the plane of cell division, i.e., the mechanism by which the site of PPB formation is "imprinted" before mitosis and remains in cellular memory after mitosis, is still unknown.

Studies on localization of actin within mitotic plant cells have found that, in some cells, a band of F-actin is associated with the PPB and have led to models in which division plane imprinting is carried out by F-actin (Palevitz, 1987; Traas et al., 1987; Lloyd and Traas, 1988). However, the findings of other workers that cortical F-actin in some cells assumes other conformations during preprophase and is not associated with the PPB suggests that previous observations on localization of F-actin may not apply to all cell types (reviewed in Wick, 1991b). In particular, no F-actin was found to be associated with the PPB in the highly asymmetric divisions of stomatal complex progenitor cells in the leaf epidermis of grasses (Cho and Wick, 1990).

In contrast to the results with F-actin localization, we observed that association of $p34^{cdc2}$ with the PPB does occur in these cells. These observations suggest an alternative model for the mechanism of imprinting the plane of cell division. A number of studies have found that the site previously occupied by the PPB appears to exert an attractive force on the edges of the growing phragmoplast (Ota, 1961; Galatis et al., 1984; Gunning and Wick, 1985; Apostolakos and Galatis, 1987). The formation of this "zone of attraction" may be the result of changes occurring at the division site generated by the PPB (Mineyuki and Gunning, 1990). A major function of the PPB may be to direct the $p34^{cdc2}$ kinase to specific sites on the cell cortex, where it can modify the PPB contact point by phosphorylation of various substrates. Actin may be required for the narrowing of the PPB, as suggested by drug studies (Mineyuki and Palevitz, 1990; Eleftheriou and Palevitz, 1992). At this stage of the cell cycle, the G2/M transition, cdc2 kinase activity is expected to be near its maximum to carry out different mitotic functions, although a direct measurement of this activity was not possible using our antibody (see below). In this model, modification by cdc2 of the cortex at the site of contact with the PPB would be responsible for guiding the expanding phragmoplast in cytokinesis to the sites predetermined by the PPB.

In animal cells, it has been shown that localization of $p34^{cdc2}$ is determined by its association with a specific mitotic cyclin; specifically, the cyclin A–cdc2 complex exhibits a different subcellular localization from that of the cyclin B1–cdc2

complex (Pines and Hunter, 1991). We have preliminary evidence for multiple mitotic cyclins in maize (J.-P. Renaudin, J. Colasanti, H. Rime, Z. Yuan, and V. Sundaresan, unpublished results), and it will be of interest to verify whether the fraction of the cdc2 protein in the PPB is associated with a particular cyclin that determines this localization. We have not been able to directly measure the kinase activity of cdc2 immunoprecipitated with our antibody because the C-terminal epitope of the maize cdc2 protein complex in cell extracts is inaccessible to the antibody directed against that epitope. Immunoprecipitation of maize cdc2 from cell extracts was possible only under conditions of mild denaturation that abolished all p13^{suc1}-associated H1 kinase activity (J. Colasanti and V. Sundaresan, unpublished results). However, using antibodies directed against individual cyclins, it should be possible to use immunoprecipitation to determine the kinase activity of the associated cdc2 fraction (Pagano et al., 1992).

One problem for further investigation is the mechanism by which the p34^{cdc2}–cyclin complex is brought to the division site by the microtubules of the PPB; our data indicate that the microtubule PPB is not necessary for p34^{cdc2} to exit the nucleus but is required for the distribution of p34^{cdc2} in a band that contacts the division site. Another question for future studies is the identity of the proposed cortical substrates of p34^{cdc2} kinase involved in imprinting the memory of the division site.

The determination of polarity in cell division is achieved through different mechanisms in different organisms and, in many cases, begins at the cell cortex (reviewed in Strome, 1993). For example, dorsal–ventral polarity in the frog embryo is established by the point of sperm entry (reviewed in Gurdon, 1992). In the budding yeast, the products of the *BUD* genes appear to determine the selection of bud sites (reviewed in Drubin, 1991), and in bacteria, the selection of the site of cell division is accomplished through the *ftsZ* gene product, which forms a ringlike structure at the future division site (Bi and Lutkenhaus, 1991). Our study suggests that establishment of the sites of cell division in the mitotic cells of higher plants may be mediated by the p34^{cdc2} kinase, which is directed to the division site at the cell cortex by the PPB of microtubules.

METHODS

Production of Affinity-Purified Antibody to Maize p34^{cdc2}

From the sequence of *cdc2ZmA*, the gene encoding the functional homolog of cdc2 of maize, a peptide was synthesized based on the 17 amino acids at the C terminus of this deduced protein (Colasanti et al., 1991). The peptide was coupled to keyhole limpet hemocyanin with *m*-maleimidobenzoyl sulfosuccinimide ester and injected into rabbits. Some of the peptide was coupled to BSA by the same method and bound to cyanogen bromide–activated Sepharose (Pharmacia, Piscataway, NJ) for affinity purification of the antibody. All immunological methods were performed essentially as described by Lane and Harlow (1988). After two injection boosts of peptide-coupled keyhole limpet hemocyanin antigen, 5 mL of serum was mixed with 9 volumes of PBS, pH 7.2, and passed through the affinity column three times. The column was washed extensively with 10 mM Tris-HCl, pH 7.2, and the bound antibody was eluted with 100 mM glycine, pH 2.5. After dialysis against a large volume of PBS, the antibody was concentrated by Centricon filtration (Amicon, Beverly, MA) and stored at 4°C.

Protein Blot Analysis

Total protein was prepared from immature maize B73 leaves and root tips by freezing tissues in liquid nitrogen, grinding to a fine powder, and suspending in extraction buffer (100 mM Tris-HCl, pH 7.2, 40 mM β-mercaptoethanol, 5 mM EDTA, 5 mM EGTA, 10% sucrose, 2 mM phenylmethylsulfonyl fluoride). The suspension was filtered through 100-μm nylon mesh (Tetko Inc., Elmsford, NY) to remove large pieces of tissue and then centrifuged for 20 min at 12,000*g* at 4°C to pellet cellular debris. For protein blots, 50 μg of supernatant protein extract was used for each lane of a 10% SDS–polyacrylamide gel. Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell) by semidry electroblotting (Millipore Corp., Bedford, MA) and stained with Ponceau S. Membranes were blocked with 5% nonfat dry milk solution in Tris-buffered saline, pH 7.2, and immunoblotted with a 1/3000 dilution of affinity-purified anti-maize p34^{cdc2} antibody, or a 1/3000 dilution of anti-PSTAIRE monoclonal antibody (Upstate Biotechnology Inc., Lake Placid, NY). Bands were visualized with a chemiluminescent detection system (Amersham Corp.) and exposure to Kodak XAR5 film.

Fixation and Immunostaining of Maize Cells

Root tips were excised from 2- or 3-day-old maize B73 seedlings that had germinated from dark-grown kernels in distilled water. They were immediately immersed in fixative (3.5% paraformaldehyde, 0.2% saponin, 0.2% picric acid, 5 mM EGTA, 50 mM potassium phosphate, pH 7.0) at room temperature for 1 hr. After fixation, the root tips were washed in several changes of wash buffer (50 mM potassium phosphate/5 mM EGTA, pH 7.0) for a total of 45 min. The washed root tips were placed in digestion solution (5% cellulysin/2% macerase [Calbiochem] in 0.4 M mannitol/5 mM EGTA) and incubated at room temperature for 30 min. The digestion solution was removed, and the root tips were washed as before. After the last wash, root cells were released by squashing one or two tips between two poly-L-lysine-coated masked slides (Polysciences Inc., Warrington, PA). Large pieces of tissue were removed and the slides were immediately immersed in 100% methanol at −20°C for 7 to 8 min and then quickly immersed in PBS.

For immunofluorescence microscopy of root tip cells, excess PBS was blotted from the slides and antibody solutions were overlayed on the cells in the wells. Monoclonal antibody specific for the α-tubulin protein (Amersham) was diluted 1/500 in PBS, affinity-purified maize p34^{cdc2} antibody at a concentration of 2 mg/mL was diluted 1/100 in PBS, and anti-PSTAIRE monoclonal antibody was diluted 1/100 in PBS. Several dilutions of maize p34^{cdc2} antibody ranging from 1/50 to 1/500 were tested. At the highest dilution (1/500), cdc2 staining was barely detectable, and at the lowest dilution (1/50), background staining increased. Slides were incubated for 30 min at 37°C and then washed for 20 min with several changes of PBS. Secondary antibodies for cdc2 staining consisted of a 1/200 dilution of biotinylated goat anti-rabbit antibody followed by a 1/100 dilution of fluorescein-conjugated streptavidin (Vector Laboratories, Burlingame, CA). For peptide blocking

experiments, the 17-amino acid maize cdc2 C-terminal peptide was dissolved in PBS, added to the primary antibody mixture at a final concentration of 10 mg/mL, and incubated as described above. For anti-tubulin and anti-PSTAIRE staining, cells were incubated with a 1/150 dilution of Texas Red-conjugated horse anti-mouse antibody (Vector Laboratories) at 37°C for 30 min. After the final antibody incubations, the slides were washed for 20 min in PBS at room temperature and then briefly immersed in distilled water. Excess water was removed by blotting, and 50 ng/mL 4,6-diamidino-2-phenylindole (DAPI; Sigma) solution was added to each well. After ~30 sec in DAPI, the slides were rinsed briefly in distilled water, air dried, and mounted in Fluor-Save (Calbiochem).

Immunofluorescence procedures for leaf epidermal sheets are essentially the same as described earlier (Cho and Wick, 1989; Wick et al., 1989). To stain these tissues, maize $p34^{cdc2}Zm$ antibody was diluted 1/100 and anti-tubulin monoclonal antibody (from the Czechoslovak Academy of Sciences) was diluted 1/500. Secondary antibody for tubulin staining was Texas Red-conjugated goat anti-mouse diluted 1/50.

Fluorescence microscopy and photography of root tip cells was performed with a Nikon microscope with a 60× oil immersion lens. Leaf epidermal cells were examined with a Zeiss Photomicroscope III. Slides were viewed by epifluorescence under standard wavelengths for fluorochrome visualization and photographed with Tmax 400 ASA film (Kodak).

Oryzalin Treatment of Root Tips

Stocks of oryzalin herbicide were brought to 100 mM in DMSO and diluted in water. Seedlings that had germinated in the dark for 2 or 3 days were treated by immersing their root tips in a 20-μM solution of oryzalin for 4 hr. This treatment completely disrupted the microtubule structures of the root cells. After oryzalin treatment, root tips were excised and prepared as described above.

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