Spatial pattern of *cdc2* expression in relation to meristem activity and cell proliferation during plant development

(*Arabidopsis thaliana*/cell division/*in situ* hybridization/*Raphanus sativus*)

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ABSTRACT The p34 protein kinase encoded by the *cdc2* gene is a key component of the eukaryotic cell cycle required for the G₁- to S-phase transition and entry into mitosis. To study the regulation of plant meristem activity and cell proliferation, we have examined the tissue-specific accumulation of *cdc2* transcripts in *Arabidopsis thaliana* and the related crucifer radish (*Raphanus sativus*) by *in situ* hybridization using *A. thaliana cdc2* cDNA sequences as a probe. *cdc2* transcripts accumulated in leaf primordia and within the vegetative shoot apical meristem. During flower development, high levels of expression were observed in meristems, in the basal regions of developing organs, in the developing vasculature, and associated with rib meristems elaborated late in the development of some floral organs. In root tips, *cdc2* transcripts accumulated in the meristematic region and adjacent daughter cells but were not detected in the quiescent center. There was strong hybridization throughout the pericycle, and a further localized accumulation of *cdc2* transcripts was observed in the initial stages of the activation of a new meristem at sites of lateral root development. We conclude that *cdc2* expression is a critical factor in the regulation of meristem activity and establishment of proliferative competence.

The p34 protein kinase encoded by *cdc2* is a key component of the eukaryotic cell cycle required for the G₁- to S-phase transition and entry into mitosis (1–3). p34 protein kinases become active only after forming a complex with a cyclin, and mutant analysis in *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* have demonstrated the presence of both positive regulators (e.g., *cdc25*⁺ and *nim1*⁺) and negative regulators (e.g., *wee1*⁺) of M-phase p34 protein kinase activity (1–3). p34 protein kinases are highly conserved in evolution and have been found in all eukaryotes analyzed, including several plant species. *cdc2* cDNA clones from *Arabidopsis thaliana* (4, 5), alfalfa (6), pea (7), and maize (8) complement *cdc2* mutants in *Sc. pombe* or mutants in the equivalent gene (*cdc28*) in *S. cerevisiae*.

Molecular cloning of plant *cdc2* sequences provides the basis for mechanistic analysis of the regulation of cell proliferation in relation to the unique stem cell organization of higher plants mediating continuing organogenesis during postembryonic growth. In the present paper, we demonstrate by *in situ* hybridization analysis of the tissue-specific accumulation of *cdc2* transcripts in *A. thaliana* and the related crucifer radish (*Raphanus sativus*) that the spatial and temporal pattern of *cdc2* expression is a critical factor in regulation of meristem activity.

MATERIALS AND METHODS

Plant Material. *A. thaliana* ecotypes Columbia and Landsberg *erecta* were grown in the greenhouse under a 16-h

light/8-h dark regimen. To isolate RNA from roots, plants were grown in liquid MS medium (9). For auxin induction, radish (cv. Scarlet Globe) seeds were surface sterilized and germinated in the dark (10). Roots were cut into 2-cm pieces and incubated in the dark at 25°C in MS medium with 10 μ M indoleacetic acid (IAA). To prepare RNA, samples were pulverized in liquid N₂ and lysed in guanidinium thiocyanate (11).

cDNA Isolation. Two degenerate oligonucleotides were used for amplification of *A. thaliana* (ecotype Columbia) genomic DNA: 5'-CGAATTC(T/C)TCNGGN(G/C)(A/T/C)NC(G/T)(A/G)TACCA-3' and 5'-GGNGA(A/G)GGNACNTA(T/C)GGNGTNGTNTATAA-3'. The polymerase chain reaction (PCR) was carried out at 92°C for 1.5 min, 47°C for 2.5 min, and 72°C for 3 min for 32 cycles. The products were separated on a 1.5% agarose gel, blotted onto a nitrocellulose filter, hybridized with a gel-purified radiolabeled DNA fragment of the *S. cerevisiae CDC28* gene (12), and washed at 50°C in 2 $\times$ SSC (1 $\times$ SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0). The 960-base-pair (bp) fragment to which this probe hybridized was gel-purified, cloned into the *Sma* I restriction site of pIBI24, and sequenced (13). Seven cDNA clones were isolated from a λ gt10 library made from immature floral buds of *A. thaliana* ecotype Landsberg *erecta* (14) using this PCR product as a probe. The largest insert was subcloned into pGEM7 (Promega) and sequenced.

Blot Hybridization. RNA samples were separated on 1% agarose formaldehyde gels (13), transferred onto Nytran membranes (Schleicher & Schuell), and hybridized with a radiolabeled gel-purified DNA fragment in 0.5 M sodium phosphate, pH 7.2/1 mM EDTA/1% bovine serum albumin/7% SDS at 60°C for 18 h (15). Filters were washed twice in 2 $\times$ SSC/1% SDS at 60°C and twice in 0.2 $\times$ SSC/1% SDS at 60°C.

In Situ Hybridization. Tissue preparation and *in situ* hybridization were carried out as described by Drews *et al.* (16). Hybridization was for 12–16 h at 42°C in 50% formamide. After hybridization, the final washes were for 1 h at 57°C in 0.015 M NaCl. Radish specimens were treated identically.

RESULTS

***A. thaliana cdc2*.** Oligonucleotide primers corresponding to the highly conserved peptide motifs GEGTYG and WYRAPE (single-letter code) were used to amplify *cdc2* sequences from *A. thaliana* (ecotype Columbia) genomic DNA by PCR. A 960-bp product of this reaction, which hybridized to a probe derived from the *S. cerevisiae CDC28* gene, was cloned and used to isolate a cDNA clone from a λ gt10 library (14). The

Abbreviation: IAA, indoleacetic acid.

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FIG. 1. *cdc2* expression in *A. thaliana* (ecotype Columbia) and radish. (A) From left to right: 20 μ g of total RNA, isolated from 2-week-old whole plants (lane W), was hybridized with a radiolabeled probe including nucleotides 1–564 of the PCR product. Total RNA (5 μ g), isolated from *A. thaliana* roots treated (lane R +) or untreated (lane R –) with 0.1 μ M IAA for 48 h, hybridized to the same probe. Total RNA (10 μ g), isolated from radish roots (lane rsR), was hybridized to a radiolabeled probe corresponding to nucleotides 670–983 of the cDNA (4). (B) Total RNA (5 μ g), isolated from roots (lane R), bolt (lane S), leaves (lane L), floral buds (lane B), and flowers (lane F), was hybridized with the truncated PCR probe described in A.

nucleotide sequence of the insert was identical to the CDC2a clone described by Hirayama *et al.* (5).

***cdc2* Expression.** RNA was extracted from whole 2-week-old *A. thaliana* plants (ecotype Columbia) grown in liquid medium and analyzed by blot hybridization. At high stringency, a single species of 1.4 kilobases hybridized to a probe derived from the PCR product (Fig. 1A). *cdc2* transcripts were present at moderate to low levels in all the vegetative organs examined from mature plants, whereas flowers contained much higher levels (Fig. 1B). IAA treatment (0.1 μ M) induced *cdc2* mRNA in roots (Fig. 1A), correlated with an increase in the number of lateral initials. Since these tissues exhibit high levels of mitotic activity, our data suggested that *cdc2* expression might be involved in the developmental regulation of cell division. To test this hypothesis we examined the pattern of *cdc2* mRNA accumulation in relation to meristem organization and cell proliferation by *in situ* hybridization analysis with single-stranded RNA probes derived from the *A. thaliana cdc2* cDNA sequence.

Flower Development. Flower development in *A. thaliana* has been divided into 12 stages based on morphological criteria (17). Fig. 2 A and B shows bright- and dark-field

FIG. 2. *cdc2* expression in vegetative and reproductive meristems. Tissue sections of *A. thaliana* (ecotype Landsberg *erecta*) were hybridized with a *cdc2* antisense probe including nucleotides 1387–1 of the cDNA (5). Specimens are shown in paired bright- and dark-field micrographs. (A and B) Longitudinal section through an inflorescence meristem and stage 3 floral primordium. (C and D) Longitudinal section through a stage 3 floral primordium on the incipient pedicel. (E and F) Longitudinal section through a stage 7 bud. (G and H) Longitudinal section through a stage 8 bud on the receptacle. Arrows designated St label the parietal cells that give rise to tapetal and endothelial cells. (I and J) Transverse section through an early stage 10 bud. (K and L) Transverse sections through a stage 13 or 14 bud. (M–P) Longitudinal sections through a stage 13 or 14 bud. (Q and R) Longitudinal section through a vegetative meristem. an, Anthers; fi, filament; Fm, floral meristem; G, gynoecium; IM, inflorescence meristem; L, leaf; Lp, leaf primordium; M, meristem; ov, ovules; ow, ovary wall; Pe, petal; pl, placenta; Pm, procambium; Se, sepal; Sp, stipule; St, stamen; V, vasculature; Vc, vascular cambium. (Bar = 25 μ m, except in I, K, M, and O, where it is 50 μ m.)

FIG. 3. Control *in situ* hybridization with a *cdc2* sense probe including nucleotides 1–1387 of the cDNA (5) with tissue sections of *A. thaliana* ecotype Landsberg *erecta*. (A and B) Longitudinal section through an inflorescence meristem with a stage 3 floral primordium and stage 7 bud. (C and D) Longitudinal section through a stage 8 bud. (E and F) Transverse section through a stage 13 or 14 floral bud. Note that the apparent signal around the periphery of some specimens in B, D, and F is due to refraction at a phase boundary and is not due to accumulation of silver grains. Fp, floral primordium; G, gynoecium; IM, inflorescence meristem; L, leaf; Lp, leaf primordium; M, meristem; Pe, petal; Pm, procambium; Se, sepal; Sp, stipule; St, stamen. (A and E, bar = 50 μ m; C and H, bar = 25 μ m.)

micrographs, respectively, of a longitudinal section of an inflorescence meristem carrying two flower primordia hybridized with the antisense probe to detect *cdc2* transcripts. These primordia were at stage 3, which begins with the appearance of sepal (whorl 1 organ) primordia on their flanks. Hybridization was strongest in the flower primordia, the procambium within the inflorescence axis, and the developing vasculature (Fig. 2B). *cdc2* expression levels in the inflorescence meristem were lower than in the floral primordia but higher than in the pith of the inflorescence axis. At greater magnification, a stage 3 primordium (Fig. 2C) revealed a distinct stratification of the surface cell layers, whereas internal tissues showed no obvious spatial organization. High levels of *cdc2* mRNA were observed in these basal regions and the incipient pedicel, with little expression in the surface layers (Fig. 2D).

During stages 5–7, the organ primordia of whorls 2–4 develop (17). The stamen and petal primordia appear at stage 5, but the latter do not develop further until stage 8. The gynoecium is initiated in stage 6, and in stage 7 its growth accelerates. Fig. 2E shows a longitudinal section of a stage 7 flower, with sepals enclosing distinct staminal and gynoecial primordia. The hybridization signal was highest in the base of the bud (Fig. 2F), with lower signal intensity in the growing organ primordia. Hybridization of a section encompassing stages 1–7 with a sense strand probe as a control showed no signal above background (Fig. 3A and B).

At stage 8, locules develop at the stamen terminus (Fig. 2G). The highest levels of *cdc2* transcript were found in the receptacle, with intermediate levels in the developing locule, predominantly in the primary parietal cells (18), and very low levels in the other cells of the incipient stamens and in the carpels (Fig. 2H). Using the sense strand as a control probe, no background hybridization was detected (Fig. 3C and D). Fig. 2I and J shows an oblique cross-section of an early stage 10 bud in bright and dark fields, respectively. Expression of *cdc2* transcripts in the locule was highest in the tapetal and endothelial cells (18) and was also observed in the gynoecium, the petals, and sepals. At this and later stages of floral development, *cdc2* was expressed in tissues consisting of differentiated cells, in contrast to earlier stages, where expression was predominantly associated with morphologically undifferentiated cells.

Fig. 2K–P shows oblique transverse and longitudinal sections of stage 13 or 14 flowers. Concomitant with anthesis,

further growth of staminal filaments, the pedicel, and the gynoecium occurs. High levels of *cdc2* transcript were observed in these rapidly growing organs (Fig. 2L, N, and P). Within the gynoecium the hybridization signal was highest in the ovary wall (Fig. 2N), with lower levels of expression in the placenta and developing ovules. In the elongating pedicel, strong hybridization was associated with the vascular cambium (Fig. 2O and P). Control hybridization with a sense probe (Fig. 3F) showed no signal above background.

Vegetative Shoot Meristem. To study expression in the vegetative shoot apex, we performed *in situ* hybridization on apices dissected from plants with two or four true leaves. A longitudinal section through the apex showed the meristematic zone, leaf primordia at various stages of development, stipules, mature leaves, procambium, and differentiating vasculature (Figs. 2Q and 3G). High levels of *cdc2* transcripts were observed uniformly distributed throughout the meristematic zone, young leaf primordia, and differentiating vasculature (Fig. 2R). Lower levels were observed in the procambium and stipules, and no expression was seen in maturing leaves. Hybridization with a sense probe (Fig. 3H) gave no signal above background.

Root Meristems. For *in situ* hybridization analysis of roots we used radish seedlings. This closely related crucifer offers the advantage of increased root diameter and cell numbers in each tissue (19), and lateral root development has been extensively studied in radish (10). The *A. thaliana* cDNA strongly hybridizes to the radish transcript (Fig. 1A). The root apical meristem in radish consists of three superimposed cell layers, which supply progenitors of the root cap and epidermis, the cortex, and the vasculature (20). The initiating center for these layers is termed the quiescent center because of its low mitotic activity (21). A longitudinal section of the root tip (Fig. 4A) revealed the stratified organization of the apex. *In situ* hybridization showed that the *cdc2* transcript was present at near background levels in the quiescent center but accumulated to high levels in the meristematic cells giving rise to the various ground tissues (Fig. 4B).

Lateral roots are produced when meristems develop *de novo* from one or a few cells within the pericycle, a differentiated tissue surrounding the vascular cylinder (20). In radish, lateral initiation is radially restricted to the xylem poles (20). *cdc2* transcripts accumulated in the pericycle and stelar parenchyma with little or no hybridization above background in other tissues (Fig. 4D and F). Expression was

FIG. 4. *cdc2* expression in radish roots. *In situ* hybridization of root tissue with *cdc2* antisense (A–L) or sense (M and N) probes including nucleotides 670–983 (4). (A and B) Longitudinal section through a radish root apex. Longitudinal (C and D) and transverse (E and F) sections through a mature main root ≈ 2 cm from the root tip. (G–L) Longitudinal sections through a mature main root with lateral root primordia at successive stages of development. Specimen in I and J was from a root treated with $10 \mu\text{M}$ IAA. Note that the apparent signal around the periphery of the specimen in N is due to refraction at a phase boundary, not to accumulation of silver grains. C, cortex; ci, cortical initials; Cy, calyptra; En, endodermis; Ep, epidermis; Lr, lateral root primordium; Pc, pericycle; px, protoxylem; Qc, quiescent center; ri, root cap and epidermal initials; Spy, stelar parenchyma; vi, vascular initials; Xy, xylem. (Bar = $25 \mu\text{m}$.)

uniform along the pericycle and was not restricted to the xylem poles. Longitudinal sections of root segments with primordia at successive developmental stages showed that *cdc2* transcripts accumulated in the cells of the new meristem from the earliest detectable time point and throughout all subsequent stages of lateral root development (Fig. 4 H, J, and L). As in *A. thaliana*, treatment of roots with $10 \mu\text{M}$ IAA increased the frequency of lateral root initiation, and therefore the amount of transcript, but did not affect the uniform pattern of *cdc2* expression throughout the pericycle and in each new initial (Fig. 4 J). Hybridization with a sense probe (Fig. 4 N) showed no signal above background.

DISCUSSION

We have used *in situ* hybridization to delineate the pattern of accumulation of *cdc2* transcripts in relation to the functional organization of plant meristems and the developmental control of cell proliferation. The probe used in this study corresponds to sequences from the *A. thaliana cdc2* gene that complement temperature-sensitive *cdc2* mutations in *Sc. pombe* and a *cdc28* mutation in *S. cerevisiae* (4, 5). This probe hybridizes to a single RNA species in radish and *A. thaliana* (Fig. 1). Hence, the *in situ* hybridization analysis monitors the specific pattern of accumulation of transcripts encoded by the *A. thaliana* functional homolog of the *Sc. pombe cdc2* gene. In some cases, an apparent signal was observed at the periphery of specimens. However, this represents total reflection at a phase boundary rather than specific hybridization, and in each experiment controls with a sense probe showed only background nonspecific hybridization (Figs. 3 and 4).

Previous RNA blot hybridization studies have shown higher levels of *cdc2* transcripts in juvenile, rapidly growing

tissues compared with slowly growing, mature tissues (4, 6, 8). The present *in situ* hybridization study demonstrated strong, but nonuniform expression of *cdc2* transcripts within plant meristems, such that the spatial pattern of *cdc2* expression delineated the functional architecture of the meristems. This was seen most clearly in the root apex, where there was a striking contrast between the low levels of *cdc2* expression in the quiescent center and the high levels of expression in the adjacent rapidly proliferating cell files. This specific distribution of *cdc2* expression in the root apex exactly parallels the pattern of mitotic activity (22). Likewise, in the vegetative shoot apex, the distribution of *cdc2* transcripts exactly corresponds to the pattern of mitotic activity and [^3H]thymidine incorporation (23–25). The discrete patterns of *cdc2* hybridization observed during flower development argue for a similar relationship between *cdc2* expression and cell proliferation. Previous anatomical studies have localized a file meristem in the incipient receptacle (26), where *cdc2* transcripts accumulated to high levels. These data suggest that selective expression of *cdc2* plays a critical role in establishing the pattern of mitotic activity in specific regions of the root and vegetative shoot apex, although we cannot rule out additional posttranscriptional controls.

The distribution of *cdc2* transcripts in the early stages of flower development (Fig. 2 D–H) exhibits an interesting reciprocal relationship with the expression of the homeotic genes *agamous* (16) and *apetala3* (27). Thus, *agamous*, which functions in the determination of the identity of organs in whorls 3 and 4, is specifically expressed in the stratified apical cell layers of the organ primordia (16) and *apetala3* transcripts are detected in cells that will give rise to petals and stamens (27). In contrast, *cdc2* transcripts were restricted to the basal regions of the primordia. A similar reciprocal relationship may also hold in the root, since dissection and

regeneration experiments indicate that the quiescent center is the site of pattern generation (28), whereas the flanking regions of the meristem are involved in morphogenetic amplification of this pattern. The reciprocal patterns of agamous or *apetala3* transcript accumulation compared with *cdc2* suggest that floral homeotic genes may function as inhibitors of *cdc2* transcription, and likewise substrates of the p34 protein kinase encoded by *cdc2* may modulate homeotic gene expression.

We also observed a strong correlation between *cdc2* expression and high rates of cellular proliferation in posthistogenic floral development, including the generation of nutritive tissues in the tapetum and ovary wall. Overall, these data establish a close relationship between *cdc2* expression and mitotic activity, suggesting that the selective expression of *cdc2* plays a major role in determining the developmental pattern of cell proliferation. However, additional levels of control may also be involved, and the strong expression of *cdc2* in root pericycle and perivascular parenchymal cells may be an example in which *cdc2* expression is involved in determining competence for proliferation but in which additional signals are required for cell division to proceed. Pericycle cells are arrested in G₂ (29) and lateral root initiation involves the stimulation of cell division exclusively opposite the xylem poles of the stele, leading to the development of discrete primordia (10, 20). Selective stimulation of cell division at these sites may reflect the specific accumulation of cyclin transcripts, or alternatively both the cyclin and *cdc2* genes may be coordinately expressed in all pericycle cells, with lateral root initiation resulting from posttranslational regulation of the activity of the p34 protein kinase-cyclin complex. However, the accumulation of *cdc2* transcripts in lateral root primordia from their inception indicates that *cdc2* expression is an early event in the *de novo* activation of meristems.

Although *cdc2* plays a critical role in regulation of eukaryotic cell division, remarkably little is known about the developmental control of *cdc2* expression. While RNA blot hybridization studies have established a general correlation with cellular proliferation in both plants and animals (4, 6, 8, 30, 31), the present *in situ* hybridization analysis has allowed delineation of the pattern of *cdc2* expression at the cellular level in relation to pattern formation and morphogenesis. These studies indicate that *cdc2* expression plays a key role in initiation and organization of meristem activity and the control of cellular proliferation throughout development. Plants with their unique developmental features including plasticity, postembryonic organogenesis mediated by organized meristems, and spatial display of serial developmental events may be particularly well suited for dissection of the fundamental mechanisms that govern the relationship between pattern formation and its morphogenetic amplification.

Note Added in Proof. Similar conclusions regarding transcriptional regulation of *cdc2* expression in mammalian cells have recently been reported (32).

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