Control of root growth and development by cyclin expression

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Root development is plastic, with post-embryonic organogenesis being mediated by meristems[1]. Although cell division is intrinsic to meristem initiation, maintenance and proliferative growth, the role of the cell cycle in regulating growth and development is unclear. To address this question, we examined the expression of *cdc2* and *cyc* genes, which encode the catalytic and regulatory subunits, respectively, of cyclin-dependent protein kinases that control progression through the cell cycle[2]. Unlike *cdc2*, which is expressed not only in apical meristems but also before lateral root initiation[3] in quiescent, pericycle cells arrested in the G2

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phase of the cell cycle[4], *cyc1At* transcripts accumulate specifically in dividing cells immediately before cytokinesis. Ectopic expression of *cyc1At* under the control of the *cdc2aAt* promoter in *Arabidopsis* plants markedly accelerates growth without altering the pattern of lateral root development or inducing neoplasia. Thus cyclin expression is a limiting factor for growth, which in turn drives indeterminate development of the root system.

The levels of *cdc2* messenger RNA and $p34^{cdc2}$ protein did not change markedly after stimulation of lateral-root initiation by the auxin indoleacetic acid (IAA) (Fig. 1*a*). Hence, whereas *cdc2* expression is correlated with the competence to divide, root growth and initiation of lateral roots do not appear to be limited by the abundance of the p34 catalytic subunit of cyclin-dependent protein kinase; moreover, ectopic expression of *cdc2* in transgenic *Arabidopsis* fails to perturb growth or development[5].

In contrast, IAA treatment of *Arabidopsis* roots induced expression of several *cyc* genes over the low basal levels; in particular, *cyc1At* mRNA, which encodes a mitotic cyclin[6], rapidly increased 15–20-fold (Fig. 1*b*). *In situ* hybridization showed that, unlike *cdc2*, *cyc1At* transcripts were not detectable in quiescent pericycle cells, but accumulated in single, cytoplasmically dense cells of incipient lateral root primordia. In the emergent organ, *cyc1At* was expressed exclusively in the meristem (Fig. 2*a–d*). Moreover, crucifer roots consist of long cell files that arise by transverse divisions followed by longitudinal expansion[7], and within such a continuous spatial display of sequential cell-division phases, *cyc1At* transcripts accumulated only in large cells immediately before cytokinesis, declining to background levels in adjacent small daughter cells (Fig. 2*e*, *f*). A similar, stringent spatio–temporal relationship of cyclin expression and mitosis was seen in *Antirrhinum* shoot apical meristems[8].

The close correlation between *cyc1At* expression and cell division during growth of the root apical meristem and the

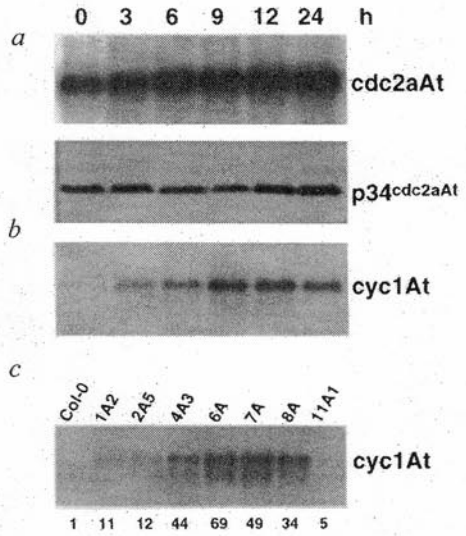


FIG. 1 Steady-state levels of *a*, *cdc2aAt* mRNA and p34 protein; *b*, *cyc1At* mRNA during IAA induction of lateral root meristems; *c*, *cyc1At* mRNA in selected non-induced transgenic lines; normalized transcript levels relative to wild-type are indicated. Col-0, wild type; 1A2, 2A5, 4A3, 11A1: T2 homozygous; 6A, 7A, 8A: T1 heterozygous transgenic lines. *cyc1At* mRNA levels in the lines 4A3, 6A, 7A, 8A, and 3A (not shown) exceed those of IAA-induced wild-type roots.

METHODS. *Arabidopsis* seedlings (ecotype Columbia) were grown in 20 ml MS medium[24]. Plants, 8–10 days old, were transferred to MS medium buffered with 50 mM potassium phosphate, pH 5.5, and initiation of lateral roots was stimulated by addition of IAA to 10 μM (non-dissociated IAA). Roots were collected at the time indicated and total RNA and protein isolated. 500 ng poly(A)$^+$ RNA was separated on 1% formaldehyde gels[25], transferred to Nytran membranes (Schleicher and Schüll) and hybridized to ^{32}P-labelled probes corresponding to nucleotides (nt) 674–1,004 of *cyc1At*[6], or nt 661–1,386 of *Arabidopsis cdc2aAt*[26], followed by hybridization with nt 2,576–2,824 of *Arabidopsis UBQ3*[27] for normalization. Blots were quantified with a Molecular Dynamics Phosphorimager. *cyc1At* is a single-copy gene in *Arabidopsis* (data not shown). Total protein was separated on 12% SDS–PAGE and transferred to PVDF membranes. $p34^{cdc2aAt}$ was detected with serum raised in rabbits against the peptide YFKDLGGMP, corresponding to amino acids 286–294, and visualized by enhanced chemiluminescence (Amersham).

TABLE 1 Comparison of root apical growth rates, cell size and root growth after IAA treatment in wild-type and transgenic *Arabidopsis* lines

(*a*)

Plant line		Root apical growth: Rate (μm h^{-1})	Root apical growth: Per cent of wild type	*n*
Col-0	(–)	254.1	100	57
3A	(+)	341.4*	134.4	20
3A	(–)	259.4	102.1	30
4A3	(+)	291.6*	114.8	47
6A	(+)	354.1*	139.5	20
6A	(–)	252.4	99.3	16
7A	(+)	344.9*	135.7	24
7A	(–)	249.8	98.3	19
8A	(+)	335.4*	131.9	21
8A	(–)	258.6	101.8	31
11A1	(+)	258.8	101.8	45
2A5	(+)	253.1	99.6	56

(*b*)

	Plant line: Col-0 (wild type)		7A (transgenic)		8A (transgenic)	
Cell type	Size (μm)	*n*	Size (μm)	*n*	Size (μm)	*n*
Epidermis	137	37	129	34	158	12
Cortex	159	31	135*	7	160	9
Endodermis	109	23	90*	22	107	11
Pericycle	73	26	67	19	71	57

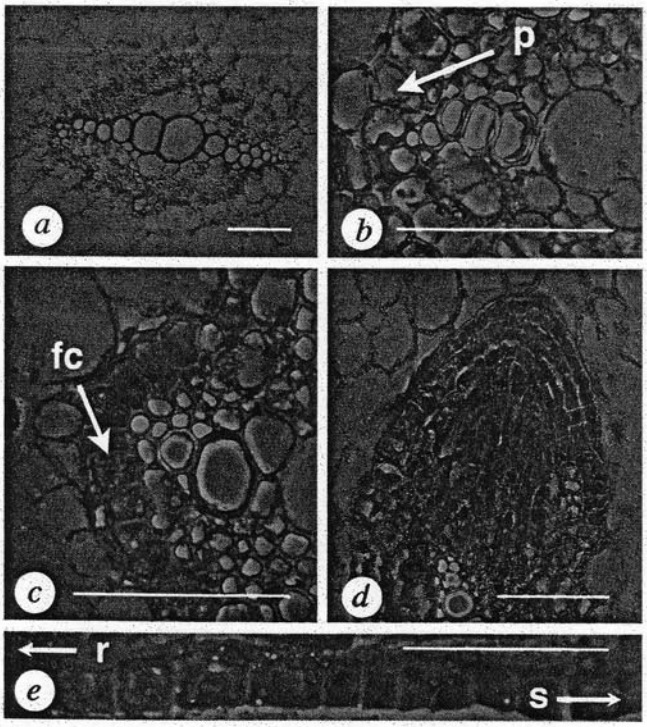
(*c*)

	Growth of seedling root system: Fresh weight (mg)		Dry weight (mg)		DNA per root (μg)	
Plant line	3 d	6 d	3 d	6 d	3 d	6 d
Col-0	11	25	1.7	2.4	5	14
4A3	31	136	4.5	15.4	8	35
6A	30	155	4.2	19.3	10	46
7A	24	156	3.5	16.8	9	38
8A	18	134	2.5	12.8	8	33

Lines 3A, 6A, 7A, 8A are heterozygous T1 populations with more than one introduced transgene; (+) denotes plants with increased *cyc1At* transcript, (–) plants with wild-type levels of *cyc1At* transcripts. The following T2 lines are homozygous for *cdc2aAt::cyc1At*: 2A5, 4A3 and 11A1; constitutive *cyc1At* expression in 4A3, but not in 2A5 and 11A1, exceeds that induced by IAA in wild type (Fig. 1). *n*, Number of plants analysed; asterisk indicates a significant difference from the wild type; for *a*, $P < 0.001$, for *b*, $P < 0.01$. Homozygous or heterozygous seed were plated on MS agar and plants grown in vertical orientation for 7 d with a 16 h day/8 h night schedule at 22 °C. Four images of each plate were acquired with a Speedlight Platinum frame grabber (Lighttools Research) at 24-h intervals and root growth analysed with NIH-Image by measuring the displacement of root apices. After growth analysis, roots from 10 plants of each class were collected and their RNA analysed (not shown). To measure cell sizes, roots were cleared by overnight incubation in saturated chloral hydrate, visualized with Nomarski optics, photographed and analysed with NIH-Image. Statistical analysis (*t*-test with unpaired variances) was performed with MS Excel. Root growth in IAA-treated plants was assessed 3 and 6d after induction by determining the fresh wet weight of roots excised from liquid-grown plants and then the dry weight after lyophilization for 24 h. Total DNA was extracted from dried material[25].

initiation of lateral roots, together with the pattern of *cyc1At* promoter activity deduced from the expression of *cyc1At::uidA* gene fusions in transgenic *Arabidopsis*[9], suggested that cyclin abundance might be a key factor in regulating root growth and development. To test this idea we generated transgenic *Arabidopsis*[10] containing *cyc1At* under the control of the *cdc2aAt* promoter and obtained five transformants in which the level of *cyc1At* mRNA in untreated roots exceeded that in IAA-stimulated roots of wild-type plants (Fig. 1*c*). Investigation of these lines showed that strong expression of the *cdc2aAt::cyc1At* transgene caused a marked increase in the rate of organized root growth (Fig. 3*a*). In heterozygous T2 progeny, increased growth rate, measured by displacement of the apex of the main root in time-lapse photography, strictly co-segregated with transgene expression; individuals lacking the transgene grew at the same rate as wild-type plants (Table 1*a*). The average size of epidermal, cortical, endodermal and pericycle cells was equivalent or slightly reduced in *cdc2aAt::cyc1At* transformants compared to wild-type plants (Table 1*b*), and hence increased growth reflects increased cell number rather than cell size. The pattern of spontaneous lateral root initiation and overall root morphology were indistinguishable in wild-type and transgenic plants (Fig. 3*a*). When treated with 1 μM IAA, which induces well separated lateral root primordia, the frequency of primordia initiated per unit length of the main root was not altered (mean, 1.08 initials mm^{-1}, with a standard deviation of 0.09 in wild type, compared with 1.14 ± 0.07 and 1.09 ± 0.13, respectively, in the two transgenic lines). Growth and development of lateral roots following induction by 10 μM IAA, however, was markedly accelerated in the *cdc2aAt::cyc1At* transformants, giving rise to a much-enlarged root system (Fig. 3*b*). Enhanced root growth in *cdc2aAt::cyc1At* plants following IAA treatment superficially resembles the *alf1* phenotype[11] these plants have increased amounts of *cyc1At* transcripts (not shown), but in contrast to *cdc2aAt::cyc1At* transformants, *alf1* plants initiate supernumerary lateral roots. The several-fold greater gain of fresh weight in IAA-treated *cdc2aAt::cyc1At* plants compared to equivalent wild-type controls was accompanied by marked increases in DNA content and dry weight (Table 1*c*). Confocal microscopy confirmed that the enhanced growth response to IAA, also seen in several lines with weaker *cdc2aAt::cyc1At* expression, did not reflect transgene stimulation of cell vacuolation or elongation. Ectopic cyclin expression thus enhances root growth by stimulation of cell division in meristems, increasing the rate of cell production without altering meristem organization, but it is not known whether these effects are mediated by interaction with cdc2a or with other cyclin-dependent kinases[2].

Our data indicate that *cdc2aAt::cyc1At* expression is sufficient to enhance growth from established root apical meristems, suggesting that the cell cycle regulates meristem activity. But failure to induce gratuitous lateral root primordia by ectopic expression of *cyc1At* under the control of the *cdc2aAt* promoter, which gives strong expression in quiescent pericycle cells[4,12], indicates that there are additional control points in the generation of a new apical meristem—either through post-translational regulation of cyclin-dependent protein kinase activity or the operation of parallel regulatory pathways. In most animal cells, commitment to cell division occurs late in G1 (ref. 13), and cyclin D1 and cyclin E are rate-limiting for progression through G1 in cultured cells[14–16]. Increased cyclin D1 is found in several tumours[17–19], and ectopic expression in transgenic mice promotes hyperplasia and adenocarcinomas[20]. In contrast, ectopic expression of *cyc1At* does not result in neoplasia but stimulates organized growth without altering meristem organization or size, as monitored by confocal microscopy. Root morphology was not altered in our transgenic plants and increased growth was accompanied by accelerated development of the root system. Cyclin expression is thus a limiting upstream factor in an intrinsic regulatory hierarchy governing meristem activity, organized growth and indeterminate development of the root system. This regulatory hierarchy is



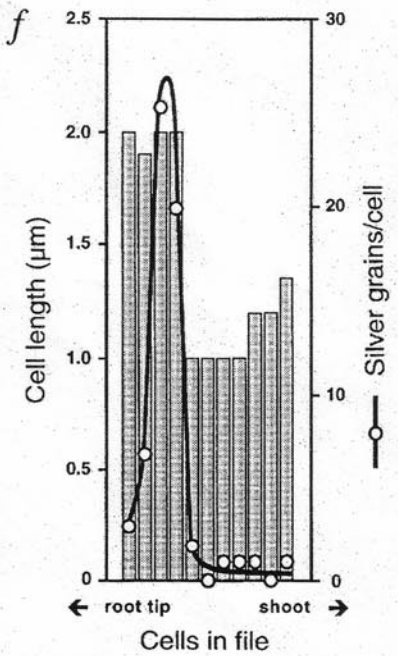


FIG. 2 *In situ* hybridization analysis of *cdc2aAt* and *cyc1At* transcripts in root apices and developing lateral roots. *a–d*, Cross sections of quiescent roots (*a*, *b*) or proliferating cells in primordia (*c*, *d*) were hybridized to *cdc2aAt* (*a*) or *cyc1At* (*b–d*) antisense probes. *e*, *f*, *cyc1At* mRNA abundance in contiguous meristematic cell files in root apices. Transcript accumulation is indicated by silver-grain deposition and visualized by indirect red illumination. Scale bar is 10 μm in *a–d*, 5 μm in *e*. fc, Founder cell accumulating *cyc1At* transcripts; p, pericycle cell layer; r, towards the root apex; s, towards the shoot. METHODS. Tissue samples for *in situ* hybridization were treated with 10 μM IAA (*b–d*; as for Fig. 1). After 8 or 24 h incubation, radish (*Raphanus sativa* var Scarlet Globe) roots were processed as described[28]. Sections (8 μm) were hybridized to a ^{33}P-labelled RNA probe, corresponding to nt 674–1,004 of *cyc1At*[6] (*b–e*) or to a ^{35}S-labelled probe, used in *a*, corresponding to nt 661–1,386 of *cdc2aAt*[26], for 14 h at 48 °C in 50% formamide. After hybridization, final washes were for 1 h at 58 °C in 0.015 M NaCl; slides were then exposed for 3 weeks (*cyc1At*) or 5 days (*cdc2aAt*). After developing, silver grains were illuminated laterally with red light, specimens were visualized by phase contrast and double exposures were taken on FUJI Velvia film. Images were assembled in ADOBE Photoshop. For the analysis summarized in *f*, silver grains were counted and cell size measured in the cell file shown in *e*.

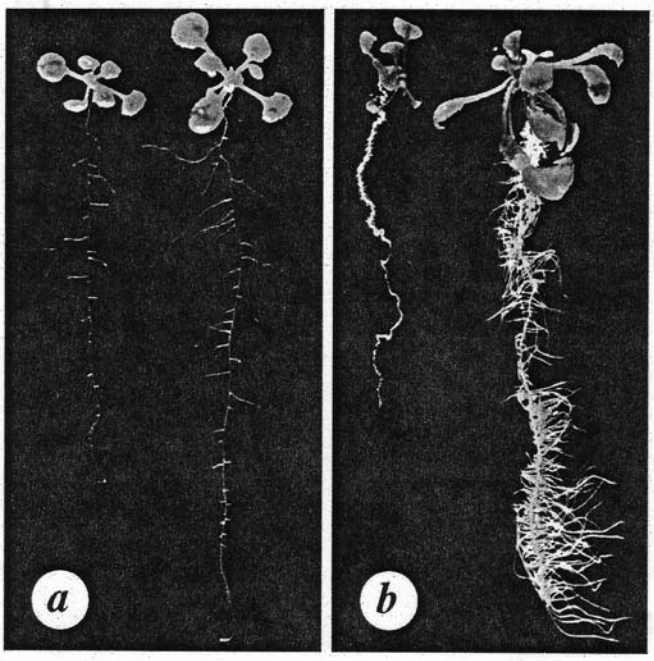


FIG. 3 Increased root-growth rate in *Arabidopsis* ectopically expressing *cyc1At* cyclin. *a*, Wild-type (left) or transgenic line 6A (T1 generation) containing the *cdc2aAt::cyc1At* gene fusion (right). *Arabidopsis* seeds were plated on MS (3% sucrose) agar and grown in a vertical orientation for 7 d. Plants transformed with vector alone or with unrelated promoter::*uidA* constructs or with a *cdc2aAt::cyc1At* fusion, in which the *cdc2aAt* 5′ untranslated leader was interrupted by a DS transposon insertion, did not show this phenotype. *b*, Wild-type (left) or transgenic line 6A (T1 generation) (right) 6 d after IAA induction of lateral roots. One-week-old seedlings grown hydroponically were treated with 10 μM IAA to stimulate lateral root development.

METHODS. An *Nhe*I site was introduced in the third codon of the *cyc1At* cDNA by *in vitro* mutagenesis and this open reading frame ligated to the *cdc2aAt* promoter with an *in vitro*-generated *Xba*I site at codon 3. This fragment was ligated into pBiB-Hyg[29] and transfected into *Agrobacterium tumefaciens* GV3101[30]. *Arabidopsis* (ecotype Columbia) was transformed by vacuum infiltration[10], and transgenic seedlings (T0 generation) were selected on MS plates containing 30 μg ml^{-1} hygromycin. 52 independent transgenic lines were obtained and elevated levels of *cyc1At* mRNA were detected in 9 of the 11 lines analysed. Growth assays were done on heterozygous T1 and homozygous T2 progeny as indicated.

different from that in animals, in which determinate development limits proliferative growth, and is illustrated by the strict morphogenetic control of cell division that occurs during muscle differetiation[21,22]. It may underlie the striking plasticity of root growth and development[23]. We propose that cyclin abundance functions as a 'rheostat' to allow flexible growth control in response to changes in the environment such as nutrient availability. □

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