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Cyclin D1 and p22^{ack1} play opposite roles in plant growth and development

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Abstract

The plant cell division cycle, a highly coordinated process, is continually regulated during the growth and development of plants. In this report, we demonstrate how two cell-cycle regulators act together to control cell proliferation in transgenic *Arabidopsis*. To identify potential cyclin dependent kinase regulators from *Arabidopsis*, we employed an two-hybrid screening system to isolate genes encoding G1 specific cyclin-interacting proteins. One of these, p22^{ack1}, which encodes a novel 22 kDa protein, binds to cyclin D1. Overexpression of p22^{ack1} in transgenic *Arabidopsis* resulted in growth retardation due to a strong inhibition of cell division in the leaf primordial and meristematic tissue. The leaf shape of p22^{ack1} transgenic *Arabidopsis* was altered from oval in wild-type to dentate. Wild-type phenotype was successfully restored in F1 hybrids by cross-hybridizing the p22^{ackl} *Arabidopsis* mutants with cyclin D1. Taken together, these results suggest that p22^{ack1} and cyclin D1, which act antagonistically, are major rate-limiting factors for cell division in the leaf meristem.

Keywords: *Arabidopsis*; CDK; Cyclin D1; Plant growth; ICK; p22^{ack1}

Plant growth and development is controlled via an intricate network of extracellular and intracellular signaling pathways, which involve both negative, and positive regulatory signals [1–4]. Regulation of the cell cycle during developmental processes involves differential expression of some cell-cycle genes in specific tissues in response to plant growth regulators [5]. Cell division is controlled by the activity of cyclin dependent kinases (CDKs), which are regulated by the presence of cyclins [6,7]. CDKs associate with specific cyclins for activation and the timing of CDK activation depends on the kinetics and localization of cyclin expression [8–10]. CDK complexes composed of cyclin and catalytic CDK subunits are activated in a periodic manner to promote cell-cycle transitions or arrest the cell cycle at different phases [11–13]. Morgan [14] has demonstrated that CDKs have been implicated in the control of gene transcription in plants.

CDK activity may be regulated at various levels, such as gene expression, phosphorylation, differential subcellular localization, and interaction with regulatory proteins. Plant CDK inhibitors such as ICK1 and ICK2 show distinct distributions in different tissues and have specific roles in development [15–17]. The cellular events associated with leaf development and the down-regulation of homobox genes involved in the formation of leaf have been reported [18–20]. A knowledge of the timing, sequence, and localization of CDK activation and inactivation is key for understanding developmental processes. In this report, we have identified a cyclin D1 interacting protein (p22^{ack1}) that can inhibit the kinase activity of CDK and reverse the phenotypic effects of cyclin D1 overexpression, suggesting that p22^{ack1} and cyclin Dl act in concert to control the early stage of leaf formation.

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Materials and methods

Plant material and growth conditions. Arabidopsis thaliana ecotype Columbia was grown on a mixture of soil, vermiculite, and perlite (4:2:1) with a 16 h light/8 h dark cycle in a temperature-controlled growth room (22 °C), unless indicated otherwise.

Molecular cloning and plant transformation. The cyclin D1 cDNA was cloned following a yeast two-hybrid screening method [21].

The binary vector was introduced into *Agrobacterium tumefaciens* strain LBA4404 and used to transform *Arabidopsis* plants by vacuum infiltration [22]. Histochemical analyses of β-glucuronidase (GUS) expression were performed as described previously [23].

Kinase activity. Recombinant cyclin D1 affinity columns were used to purify the corresponding kinase domain(s) from the lysate of *Arabidopsis* suspension cultured cells. Purified $p22^{ack1}$ protein was added to the kinase reaction mixture (50 mM Tris–HCl, pH 7.4, 250 mM NaCl, 50 mM NaF, 5 mM EDTA, 100 μM ATP, and 5.0 zμCi [γ-^{32}P]ATP) and ^{32}P-labeled histone H1 was detected by autoradiography.

RNA extraction and RT-PCR. Total RNA was extracted from leave tissues using TRI reagent (Sigma) in accordance with the manufacturer's instructions. Reverse transcription reactions were carried out using RT-PCR kit (Takara, Japan) with 2 μg of total RNA for 5 min at 55 °C. We then used 1 μl of the reverse transcription reaction as a template in a 20 μl PCR with 5 U of *Taq* DNA polymerase. The PCR consisted of 24 cycles of 30 s at 90 °C, 30 s at 50 °C, and 90 s at 72 °C. The primers (ACK1) used were: 5′ GAGAAAAGACTT-GTTC GATGGTTCTCATA 3′ and 5′ TGAATTGCTTCTTCTTATCGTC T-TGACTC 3′.

In situ hybridization with dioxigenin-labeled RNA probes. Ten micrometer tissue sections were fixed to dimethylsilane-treated microscope slides in 3.7% formaldehyde, 5% acetic acid, and 50% ethanol by overnight incubation at 45 °C, cleared in Histo-clear, and embedded in paraffin. To remove paraffin, the slides were immersed twice in 100% xylene for 10 min and twice transferred into 100% ethanol for 5 min to remove xylene. Slides were then dehydrated in with the following ethanol series [100%–95% 85%–70%–50%–30%–15% PBS (50 mM phosphate buffer, pH 7.0, 130 mM NaCl in DEPC treated water)] for 5 min at each step. The slides were then incubated in l00 μg/ml protease K solution in 100 mM Tris–HCl, pH 7.5, 50 mM EDTA at 37 °C for 20 min, and washed in PBS twice, 5 min each. The slides were immersed immediately in fresh 1% triethanolamine, pH 8.0, 0.5% acetic anhydride mixture. After 10 min, the slides were washed in PBS twice, 5 min each. The slides were then dehydrated in the ethanol series (15%–30%–50%–85%–95%–100%), washed once with 100% ethanol, and vacuum-dried.

Comparable tissue sections were hybridized with different probes ($p22^{ack1}$, cyclin D1). Dioxigenin (DIG)-labeled RNA probes were made by in vitro transcription in accordance with manufacturer's instructions. Hybridized slides were washed for 1 h at room temperature in 2× SSC and 0.01 M DTT and for 1 h at 48 °C in 0.1× SSC, 0.01 M DTT, and 50% formamide. Slides were then dipped into photographic emulsion and exposed for 3–5 days before development.

Results and discussion

Molecular characterization of $p22^{ack1}$

Cell division in plant cells is regulated by a family of CDKs that is in turn controlled by cyclins or CDK inhibitors. CDK-activating kinases (CAKs) may act to regulate CDK activities in meristems, resulting in alterations in cell proliferation rate [24]. To isolate new G1 cyclin-interacting proteins, we used a yeast two-hybrid screening process to search for proteins that interact with cyclin D1. We identified 12 clones interacting with the cyclin D1 bait with inserts ranging from 0.7 to 1.2 kb. One of them, $p22^{ack1}$, contained a 0.9 kb insert (GenBank Accession No. AF106705) encoding a protein of 196 amino acids with a predicted molecular mass of 22 kDa. The 22 kDa protein was identified from synchronized *Arabidopsis* G1 suspension cultured cells. In vitro association of $p22^{ack1}$ with cyclin D1 was confirmed in total cell extracts of *Arabidopsis* (Fig. 1). This result clearly shows that cyclin D1 interacts directly with $p22^{ack1}$. Next, we tested whether $p22^{ack1}$ functions as an inhibitor of CDK activity. $p22^{ack1}$ was used to test for inhibitory activity in CDK kinase assays with H1 histone as a substrate. The $p22^{ack1}$ inhibited activity of the cyclin D1 in *Arabidopsis* suspension culture cells, in a dose-dependent manner (Fig. 1B). To determine where the $p22^{ack1}$ and cyclin D1 genes were expressed, we isolated the 5′-upstream fragments of cyclin D1 and $p22^{ack1}$, and analyzed 10 transgenic *Arabidopsis* for the expression of each promoter::GUS reporter fusion gene (Figs. 1C and D). Cyclin D1(p)::GUS *Arabidopsis* showed a strong GUS signal in the leaf primordia that remained active for a short period during early leaf development (Fig. 1C). $p22^{ack1}$ was detected mainly at the edges of the newly formed leaves and at the boundaries along the leaf veins, petioles, and primary root (Fig. 1D). Analyses of the activities of the cyclin D1 and $p22^{ack1}$ promoters suggest that cyclin D1 and $p22^{ack1}$ act in different regions of the leaf to influence growth and development.

Overexpression of $p22^{ack1}$ in *Arabidopsis* caused a serious defect in leaf formation and a retardation in overall growth, with smaller plants and slower growth rates than wild-type (Fig. 2A). Additionally, numerous growth defects were observed due to overexpression of the $p22^{ack1}$. Epidermal cells were abnormally large and chloroplasts were distributed irregularly in the leaves of the $p22^{ack1}$ transgenic *Arabidopsis* (Fig. 2B). In the marginal region of the leaf where cyclin D1 is normally expressed, cells were distributed irregularly, thus contributing to poor leaf architecture (Fig. 2C). The most distinct phenotypic change was the appearance of deep serrations, suggesting that $p22^{ack1}$ is induced by the inhibitory front that spreads down from the leaf tip. The diameter/length ratio of apical, median, and basal parts of the $p22^{ack1}$ transgenic *Arabidopsis* decreased about 20% compared to wild-type (Fig. 2D). As shown in Fig. 2E, the depth of serrations increased more than 50% in the $p22^{ack1}$ transgenic *Arabidopsis*. The angle of the leaf apex was also decreased by more than 30%, resulting in a narrower shape of the transgenic leaves. From these results, we suggest that the emerging leaves of the $p22^{ack1}$ transgenic *Arabidopsis* cannot form a sufficient number of cells to build the normal leaf structure,

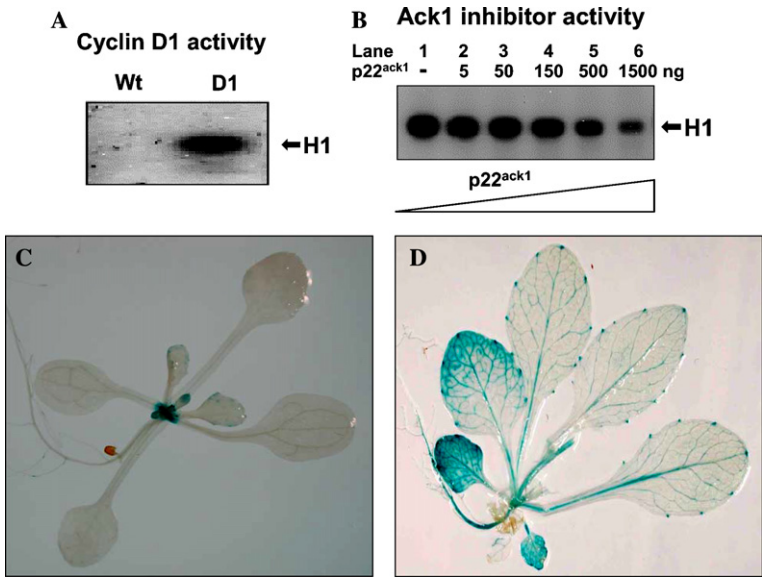

Fig. 1. The inhibitory effects of $p22^{ack1}$ on the cyclin Dl kinase activity and GUS localization of cyclin D1 and $p22^{ack1}$. Cyclin Dl kinase and $p22^{ack1}$inhibitory activity (A,B). Lane 1 indicates a positive control for each kinase without addition of expressed $p22^{ack1}$. Suppression of histone H1 kinase activities was estimated in the presence of increasing amounts of $p22^{ack1}$ (lanes 2–6). (C) Promoter activity of cyclin D1 was shown mainly in newly formed organs such as the shoot apex. (D) Strong signals were seen in the cotyledon, primary root, stipe, and marginal region of the leaves in the $p22^{ack1}$ promoter transgenic plant.

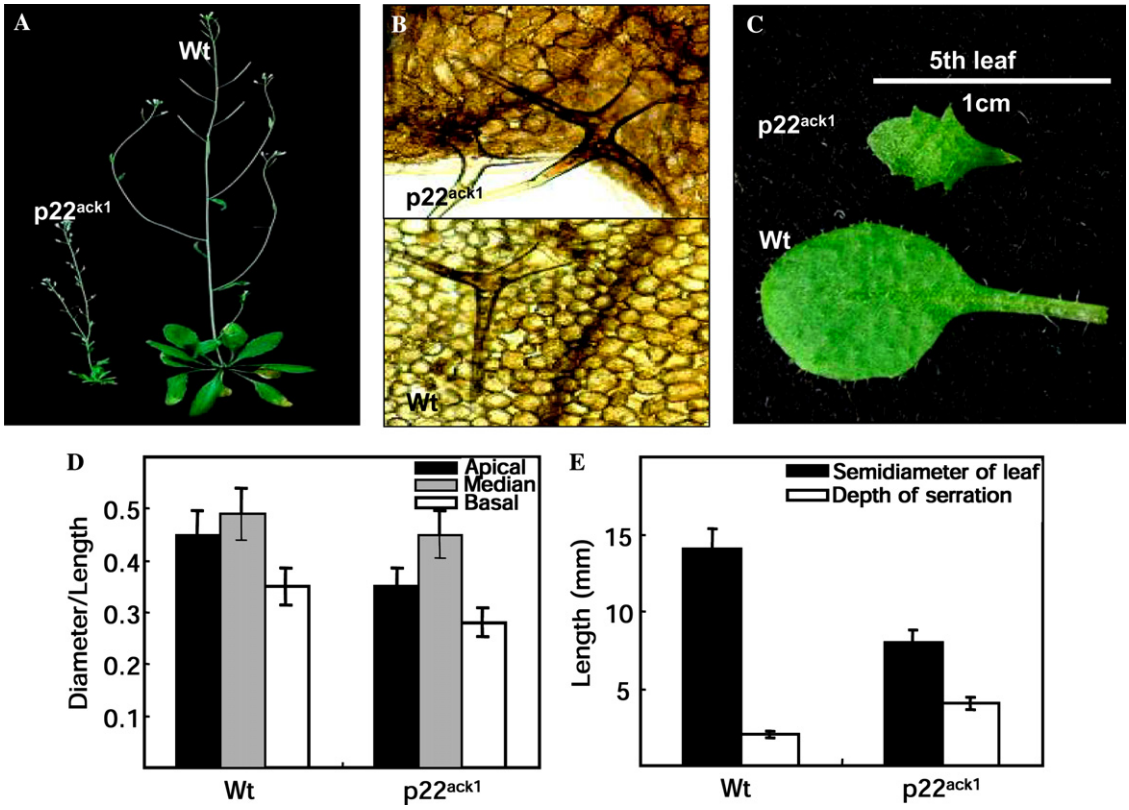

Fig. 2. The role of $p22^{ack1}$ in vivo. (A) Phenotypes of severely distorted $p22^{ack1}$ plants (left) and wild-type (right). (B) The morphological shape of leaves between $p22^{ack1}$ transgenic (top) and wild-type (bottom) showed that the size of the transgenic cells is larger than that of wild-type (100×). (C) The leaf shape changed from the oblanceolate (wild-type) to dentate ($p22^{ack1}$) transgenic. (D) Diameter/length ratio of apical, median, and basal parts of the $p22^{ack1}$ transgenic plants decreased by about 20%. (E) The depth of serrations increased more than 50% in the $p22^{ack1}$ transgenic.

as in the wild-type. These results are similar to the highly lobed leaves, severe dwarf effect seen in plants overexpressing ICK1, a closely related protein known to inhibit cell division [13]. A study of ICK1 done by Wang et al. [16] found that ICK1 binds both CDKA and CYCD3 in yeast two-hybrid assays, as well as inhibiting CDK activity in vitro. This suggests that ICK1 could have roles in the G1 phase of cell division and may act to inhibit both assembly and activity of complexes.

Restoration of Arabidopsis growth in the p22^{ack1} overexpression mutant by cyclin D1

Cyclin Dl overexpression accelerated *Arabidopsis* growth in comparison to wild-type (Fig. 3A). To test whether cyclin D1 overexpression could complement the growth retardation caused by the overexpression of p22^{ack1}, we crossed a p22^{ack1} with a cyclin D1 overexpressing transgenic plant. The phenotype of the F1 hybrids was similar to that of wild-type (Fig. 3B). The expression level of p22^{ack1} in each *Arabidopsis* was visualized by Western immunoblotting. The amount of p22^{ack1} produced generally reflected the severity in growth retardation (Fig. 3C, upper) but the p22^{ack1}/cyclin Dl *Arabidopsis* hybrid retained a higher level of p22^{ack1} without such severe growth defects. RT-PCR analysis showed a higher level of cyclin D1 transcript in the p22^{ack1}/cyclin Dl hybrid plants (Fig. 3C, bottom). It would appear that a certain threshold of cyclin D1 is sufficient to offset the negative effect caused by p22^{ack1}. The phenotypic features displayed by the p22^{ack1} and cyclin D1 transgenic *Arabidopsis* are illustrated in Fig. 3D. We found that the p22^{ack1} is expressed in the young leaf primordial and meristem, suggesting that p22^{ack1} and cyclin Dl play opposite role in the early stage of leaf formation.

The ability of excess cyclin D1 to rescue the defect in leaf morphology caused by the overexpression of p22^{ack1} clearly supports the idea that these two proteins act antagonistically to regulate cell proliferation during leaf growth and development. Ectopic expression of cyclin Dl did not result in any phenotypic abnormality, but stimulated organized growth without altering meristem organization.

Additional specific genetic studies of overexpression transgenic lines were performed to verify the localization of cyclin D1 and p22^{ack1} expression (Fig. 4). Furthermore, in situ hybridization and Western immunoblotting showed the levels of p22^{ack1} and cyclin D1 proteins determine in fully or partially rescued p22^{ack1} plants. These in vivo results suggest a model in which p22^{ack1} plays an attenuating or inhibitory role in the cell-cycle progression; the attenuation/inhibition is then released by the action of cyclin D1, specifically during leaf organogenesis.

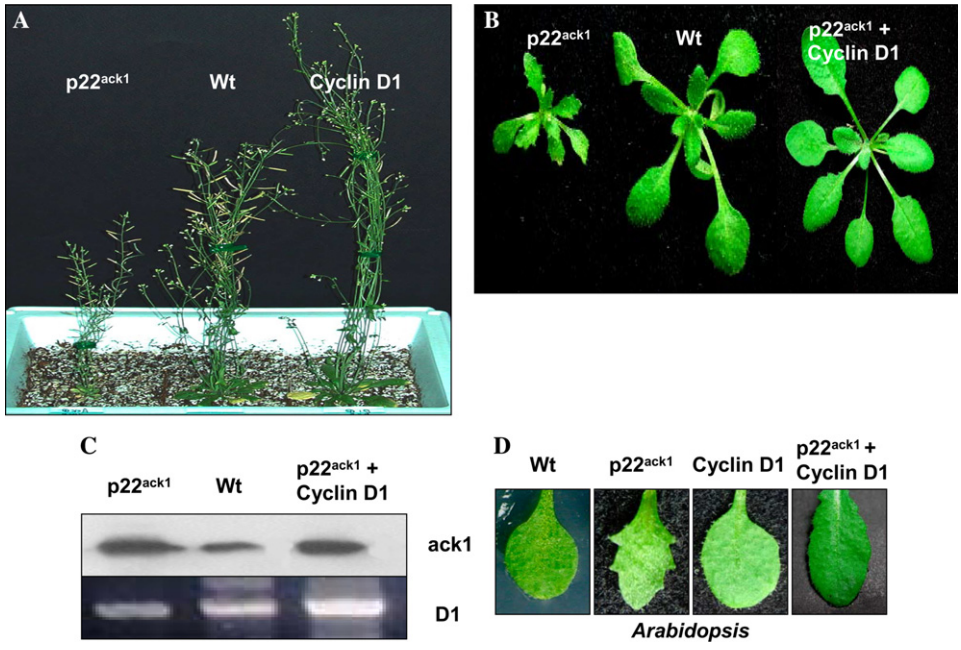

Fig. 3. The phenotype and morphological structure of p22^{ack1}/cyclin D1 hybrid plant. (A) Cyclin D1 transgenic plant showed accelerated plant growth, while p22^{ack1} plant showed growth retardation compared to the wild-type. (B) Phenotypes of p22^{ack1} transgenic (left), wild-type (middle), and p22^{ack1}/cyclin D1 hybrid plant (right). The p22^{ack1} transgenic plants restored the original phenotype of wild-type plant after cross-pollinated with cyclin D1 overexpressing transgenic plants. (C) Expression level of p22^{ack1} and cylin D1 in each plant was visualized by Western immunoblotting using a monospecific polyclonal antibody (upper) and RT-PCR (bottom). The p22^{ack1}-cyclin D1 hybrid plant retains a higher level of p22^{ack1}. (D) Each leaf represents wild-type, p22^{ack1} transgenic, cyclin D1 transgenic, and p22^{ack1}-cyclin D1 hybrid transgenic plant, respectively.

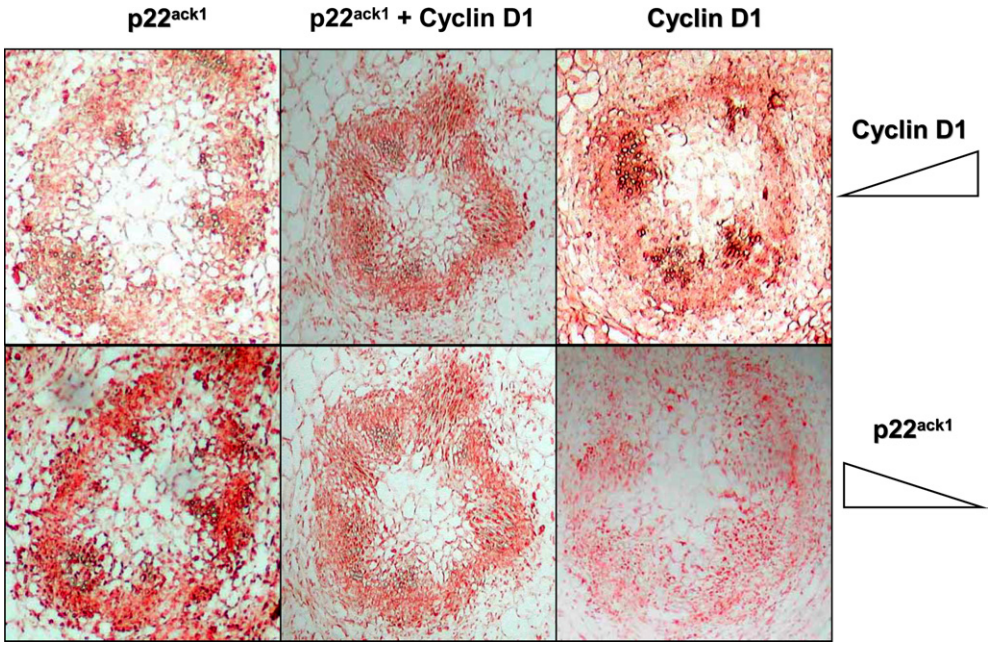

Fig. 4. Immunohistochemical analysis of the accumulation of $p22^{ack1}$, $p22^{ack1}$/cyclin D1 hybrid, and cyclin D1 transgenic plants. Specimens of cross-sections were taken from the central region of 15-day-old seedlings.

We propose that cyclin D1 and its counterpart, the CDK inhibitor, $p22^{ack1}$, are involved in regulating plant leaf development in a spatial and dose dependent manner. Clearly, $p22^{ack1}$ is capable of inhibiting G1 phase specific CDKs and it can be assumed it plays a pivotal role in the G1–S phase transition. Thus, the $p22^{ack1}$ protein could serve to dampen kinase activity during cyclin accumulation until excess cyclin-kinase complexes are produced. The cyclin-kinase complex could thereby act as an internal timing device and thus coordinate cell-cycle transitions and checkpoint controls.

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