−34.5 ± 1.3, z = −24 ± 2.3). Coordinates for these regions were comparable to previous studies (*1*).

15. Path coefficients for Early and Late were estimated with a maximum likelihood function implemented in MatLab (Mathworks, Natick, MA). The operational equations have been reported elsewhere (*22*). The covariance matrices used were calculated on the basis of observations during ENC for Early and Late (Fig. 1A). Two stacked models were compared to assess the significance of changes over time: a free model in which all paths other than the path from PP to ITp were forced to be equal for Early and Late, and a restricted model in which all path coefficients were constrained to be the same for Early and Late. This allowed us to test for differences in fit as a function of one path coefficient (PP to ITp). We analyzed the group as a whole and each participant individually. In the context of multivariate normally distributed variables, the minimum of the maximum likelihood function times the number of observations minus 1 has a χ^2 distribution with $(q/2) \times (q + 1) - p$ degrees of freedom [K. A. Bollen, *Structural Equations with Latent Variables* (Wiley, New York, 1989)], where p is the number of free parameters and q is the number of observed variables. The significance of the difference between models is expressed by the difference in the χ^2 goodness of fit. This χ^2 statistic has n degrees of freedom, where n is the difference in the degrees of freedom between the null model and the one in question.
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19. Mean changes in path coefficients for all three sessions between Early and Late for participants 1 to 6 were calculated using a correlation matrix based on 96 observations during ENC for Early and 96 observations during ENC for Late: 0.27*, 0.21, 0.37*, 0.24*, 0.19, 0.31* (*$P < 0.05$). The group effect was tested by stacking these 96 observations for all six participants, leading to 576 observations for each correlation matrix. Confounding participant and session effects were partialled out by least squares. The group analysis for the left hemisphere showed only a trend [$\chi^2_{diff}(1) = 2.3$, $P = 0.13$]. To account for autocorrelation in the observations, we used the effective degrees of freedom in the calculation of all fit indices [K. J. Worsley and K. J. Friston, *Neuroimage* **2**, 173 (1995)].
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21. The length of Early was varied in seven steps including the first 32, 64, 96, 128, 160, 192, and 224 out of a total of 256 scans for each session (Fig. 1A). The difference for the path coefficient for PP to ITp was calculated for each cut-off in all three sessions for all participants. The peak of the difference [that is, the cutoff (1 to 7)] that maximized the difference between Early and Late is an index of the temporal pattern of changes in effective connectivity. The learning speed parameter k (range 0 to 1), obtained from fitting $1 - e^{-kx}$ to the behavioral data (the percent of correct responses) was regressed on peak cut-off. Between-participant and between-session effects were modeled separately and both reached significance [t (15) = 3, t (15) = 2.1, $P < 0.05$]. Intuitively this result indicates that the temporal pattern of changes in effective connectivity not only predicted a given participant's performance but also differences in learning between sessions for an individual participant.
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23. The interpretation of changes over time in learning experiments can be difficult due to nonspecific time effects (that is, habituation, motivation, and arousal). We dissociated learning-related effects from nonspecific time effects by using three sequential learning sessions. Nonspecific time effects unrelated to learning are expressed over the duration of the whole "experimental time" (that is, over all three sessions). Conversely, learning-related effects should follow a similar pattern, but within each learning session (Figs. 1A and 2C).
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25. We thank the departmental radiographers and the Functional Imaging Laboratory physics group for help with fMRI scanning, O. Josephs for the development of the sound pickup system in fMRI, and A. Kleinschmidt, I. Johnsrude, R. Frackowiak, and R. Henson for invaluable discussions. C.B., J.T.C., and K.J.F. were supported by the Wellcome Trust.

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Cytokinin Activation of *Arabidopsis* Cell Division Through a D-Type Cyclin

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Cytokinins are plant hormones that regulate plant cell division. The D-type cyclin CycD3 was found to be elevated in a mutant of *Arabidopsis* with a high level of cytokinin and to be rapidly induced by cytokinin application in both cell cultures and whole plants. Constitutive expression of CycD3 in transgenic plants allowed induction and maintenance of cell division in the absence of exogenous cytokinin. Results suggest that cytokinin activates *Arabidopsis* cell division through induction of CycD3 at the G_1-S cell cycle phase transition.

Cytokinins are purine derivatives that promote and maintain plant cell division in cultures (*1–8*) and are also involved in various differentiation processes including shoot formation, photomorphogenesis, and senescence (*2*, *3*). In promoting cell division, cytokinins act synergistically with auxins (*3*, *4*).

Because restimulation of quiescent vertebrate cells into division by mitogenic signals requires the transcriptional up-regulation of D-type cyclins (*9*), the existence of related proteins in plants (*10*, *11*) suggests plant D-type cyclins (CycD) as potential mediators of plant mitogenic signals. A possible role for cytokinin in regulating the expression of the *Arabidopsis CycD3* gene in callus material (*10*) was investigated in a dispersed suspension culture of *Arabidopsis* cells (*12*, *13*).

Cells incubated for 24 hours without hormones (*14*) accumulated CycD3 transcripts in response to cytokinin supplied at concentrations down to 10^{-3} μM (Fig. 1, A and B), starting within 1 hour (Fig. 1C). Zeatin, the most abundant cytokinin in *Arabidopsis* (*15*), and 6-benzylaminopurine (BAP) generated the strongest response, whereas adenine, a purine lacking cytokinin properties, showed no effect (Fig. 1A).

CycD3 induction could be a direct response to cytokinin or an indirect response caused by cells reaching a certain cell cycle position under the influence of cytokinin. Cycloheximide (Chx) blocks both G_1 phase progression (*13*) and protein synthesis in these cells (*14*) and did not inhibit CycD3 induction by cytokinin (Fig. 1D), showing that induction is independent of progression through G_1 and involves signal transduction by proteins already present in stationary phase cells. Inhibition of CycD3 induction by the protein phosphatase inhibitor okadaic acid and gratuitous induction by the protein kinase inhibitor staurosporine in the absence of cytokinin (Fig. 1, E and F) implicate regulatory phosphorylations in this process.

Whole plant responses to hormones are complex (*2*, *3*) and do not necessarily reflect

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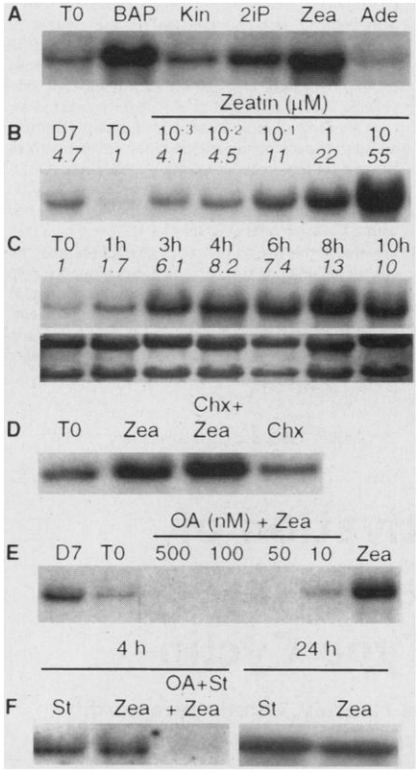

Fig. 1. CycD3 RNA induction by cytokinin in *Arabidopsis* cell suspension cultures (*14*). Northern blots (*28*) were probed with CycD3. Early stationary cells [day 7 (D7)], incubated in the absence of auxin and cytokinin for 24 hours, were sampled before induction [$T = 0$ (T0)] and after the treatments indicated. Quantitation of CycD3 mRNA relative to $T = 0$ is shown in italics. (**A**) Differential induction by cytokinins (1 μM) 4 hours after addition to cells at $T = 0$. Kin, kinetin; 2iP, 2-isopentenyladenine; Zea, zeatin; Ade, adenine (1 mM). (**B**) Concentration dependence. (**C**) Time course and quantitation of CycD3 induction by 1 μM zeatin (top) and loading control (bottom). h, hours. (**D**) Induction is not blocked by Chx. Cells pretreated for 1 hour with Chx were incubated an additional 4 hours with 1 μM zeatin (Chx + Zea) or with Chx alone (Chx). Control cells were incubated with zeatin alone (Zea). (**E**) Induction by 1 μM zeatin is inhibited by okadaic acid (OA). (**F**) Addition of 1 μM staurosporine (St) mimics induction by 1 μM zeatin after 4 and 24 hours, but OA (0.5 μM) blocks both inducers (OA + St + Zea).

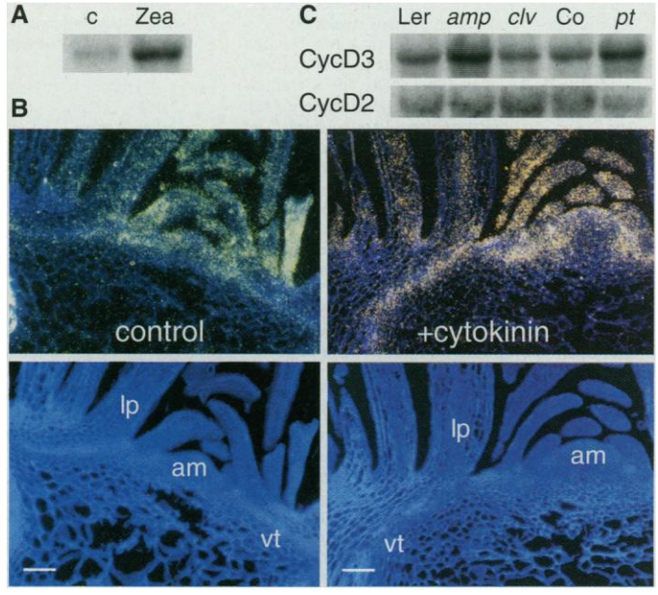

Fig. 2. CycD3 expression is induced by cytokinin in *Arabidopsis* plants and is increased in mutants with high levels of cytokinin. (**A**) Day 8 *Arabidopsis* wild-type seedlings were grown in liquid MS medium and treated for 24 hours with MS alone (c) or MS + 1 μM zeatin (Zea). (**B**) In situ hybridization of control and cytokinin-treated vegetative apices of *Arabidopsis*. (Top) Antisense CycD3 probe. (Bottom) Same section viewed by calcofluor fluorescence. Sense CycD3 probes gave no signal above background (*22*). Scale bar, 100 μm; am, apical meristem; lp, leaf primordium; vt, vascular traces. (**C**) Expression of CycD3 and CycD2 in soil-grown *A. thaliana* L. *erecta* (Ler), *amp1*, *clavata1-2* (*clv*), *A. thaliana* Columbia (Co), and *pt* plants.

those produced in culture, but treatment of *Arabidopsis* seedlings with zeatin caused increased CycD3 mRNA levels (Fig. 2A) (*16*). In situ hybridization (*17*) showed that CycD3 expression is found in the proliferating tissues of the shoot meristem, young leaf primordia, axillary buds, procambium, and vascular tissues of the maturing leaves (Fig. 2B, left). Treatment with cytokinin increased the CycD3 signal without promoting its general accumulation in all cells (Fig. 2B, right), so we conclude that cytokinin can modulate CycD3 levels in responsive plant tissues. However, because the treatment of plants with exogenous hormones may not reflect the endogenous modulation of hormone levels, we also examined CycD3 levels in the *Arabidopsis* mutant *amp1* and its allele *primordia timing* (*pt*). These mutants have an enlarged apical meristem, increased leaf number, and delayed senescence associated with a sixfold higher cytokinin level than that of wild-type plants (*15*). Compared with those of wild-type plants, CycD3 mRNA levels were two- to threefold higher in *amp1* and *pt* plants (Fig. 2C) but were normal in *clavata1* (*clv1*), another mutant with an enlarged apical meristem (*18*). The levels of CycD2, a different D-type cyclin that does not respond to cytokinin (*10*), were similar in *amp1*, *clv1*, and wild-type plants. Cytokinin therefore modulates CycD3 expression at physiological concentrations in *Arabidopsis* plants.

When explanted into culture, many plant tissues dedifferentiate and resume division to form proliferating calli. Induction of division and subsequent callus formation normally require the simultaneous presence of both auxin and cytokinin (*1–3*). If CycD3 mediates cytokinin presence, cell cycle control, or differentiation, we would predict that constitutive expression of CycD3 might lead to altered cytokinin requirement or responses. Transgenic *Arabidopsis* calli were generated (*19*) expressing CycD3 under the constitutive cauliflower mosaic virus (CaMV) 35*S* promoter, but these calli failed to regenerate shoots. To circumvent this problem and produce transgenic plants constitutively expressing CycD3, we used the yeast site-specific recombinase FLP to activate CycD3 expression in the progeny of transformed *Arabidopsis* plants. The *CycD3* gene was originally separated from a 35*S* promoter by the *GUS* (β-glucuronidase) gene flanked by target sites for the FLP site-specific recombinase (Fig. 3A). Such plants were crossed to *Arabidopsis* lines carrying a heat-shock promoter (hsp)–driven *FLP* gene (*20*), so that FLP-mediated recombination excises the *GUS* gene and activates CycD3 expression from the 35*S* promoter (*21*). Three CycD3-expressing lines established from independent original single-copy transformants all showed extensive leaf curling, disorganized meristems, increased leaf number, late flowering, and delayed senescence (*22*).

Leaf explants taken from CycD3-expressing lines and wild-type control plants were tested for their ability to form calli on media containing or lacking cytokinin (*23*). Wild-type leaves produced calli efficiently when exogenous cytokinin and auxin were present, but in the absence of cytokinin, only a few slow growing calli were formed, which failed to become green and degenerated after a period in culture. In contrast, CycD3 transgenics produced healthy green calli with equal efficiency in the presence or absence of exogenous cytokinin (Fig. 3B). These calli could be maintained for extended periods of subculturing in either condition (Fig. 3C). The cytokinin-independent induction of growth of CycD3 calli was not due to an inherently higher cytokinin level in CycD3-expressing plants, as there was no difference in the content of isoprenoid-type cytokinins between CycD3 transgenics and wild-type controls (Fig. 3D).

To confirm the role of CycD3 in the induction of cell division, we monitored expression of S phase–associated histone H4 in wild-type and CycD3-expressing leaf explants. By 5 days after induction, histone H4 was only detectable in wild-type explants on medium containing

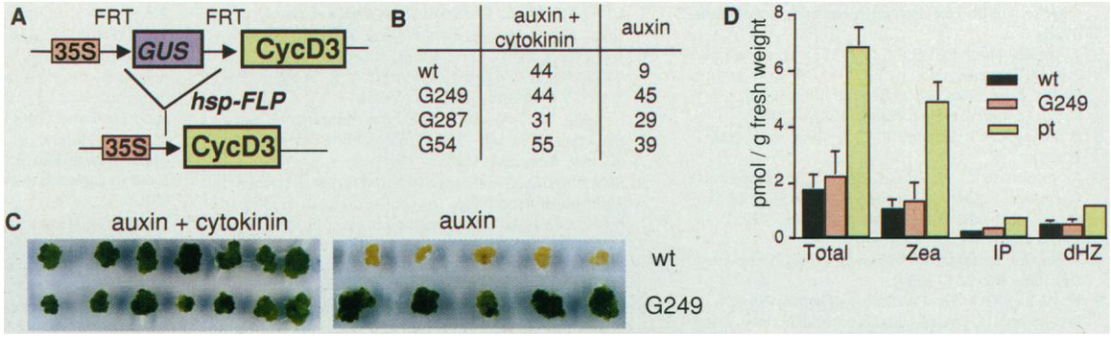

Fig. 3. Callus initiation and maintenance on cytokinin-free medium by CycD3-expressing transgenic leaf explants. (**A**) Strategy to produce transgenic *Arabidopsis* expressing CycD3 under a CaMV 35S promoter (35S) by FLP-mediated excision of a *GUS* gene lying between two FLP recognition targets (FRT) (*21*). (**B**) Numbers of calli induced on wild-type (wt) and CycD3-expressing leaf explants of three independent lines (G249, G287, and G54) in presence of auxin alone or auxin and cytokinin. All visible calli initiated on 10 explants were counted. Wild-type explants lacking cytokinin produced infrequent yellow calli. Equivalent results were obtained in two further repeats. (**C**) Maintenance of wild-type and G249 calli on control (auxin and cytokinin, left) or auxin-only (right) plates 29 days after induction; calli were moved twice to fresh plates, at 7 and 17 days. All calli were trimmed at 17 days except wild-type calli on auxin alone. (**D**) Tissue cytokinin content (*29*) of total isoprenoid cytokinins (Total) and zeatin-type, isopentenyladenine (IP)-type, and dihydrozeatin (dHZ)-type cytokinins in CycD3-expressing (G249) seedlings and in control seedlings of wild-type L. *erecta* and *pt*. *pt* is an allele of *amp1*, which has a fourfold to fivefold higher cytokinin level than that of wild-type plants, and was used to confirm assays relative to published data (*15*). Bars show SEM.

both auxin and cytokinin (Fig. 4A). In contrast, CycD3-expressing calli expressed histone H4 regardless of the presence of exogenous cytokinin. We conclude that constitutive CycD3 expression can replace the requirement for exogenous cytokinin in the induction and proliferation of *Arabidopsis* callus cells. Consistent with the involvement of CycD3 in a pathway of cytokinin response, overexpression of a putative cytokinin receptor also gives cytokinin-independent growth (*24*).

To investigate the cell cycle timing of CycD3 induction and hence the likely point of cytokinin requirement during reentry into the *Arabidopsis* cell cycle, we monitored expression of CycD3 and histone H4 mRNA levels together with DNA synthesis during synchronous activation of quiescent *Arabidopsis* cells into division (Fig. 4B). CycD3 was induced 1 to 2 hours before S phase onset, suggesting that it may be required for the G_1-S phase transition. We predict that cytokinin is therefore normally necessary at this point to induce CycD3 expression. Indeed, regenerating leaf mesophyll protoplasts (*4*, *7*) and tobacco pith explants (*5*) have all been shown to have an absolute cytokinin requirement for DNA synthesis to occur, consistent with cytokinin acting to induce CycD3 at or just before S phase entry. Other studies examining CycD3 expression timing in response to various synchronization protocols in *Arabidopsis* (*10*, *13*), alfalfa (*11*), and tobacco cells (*25*) have also suggested that CycD3 is induced shortly before the G_1-S transition in cells activated from a quiescent or resting state. These experiments all examined CycD3 mRNA levels, and associated kinase activities have not to date been determined.

Our results appear to be inconsistent with a number of reports in which cytokinin-requiring cell suspension cultures deprived of cytokinin are found to arrest variously in G_2, at multiple cell cycle stages, or with no apparent specificity (*5*, *8*, *26*). A peak in zeatin levels in tobacco BY-2 cells at G_2-M is required for cell cycle progression (*27*), and suspension-cultured *Nicotiana plumbaginifolia* cells will arrest at G_2-M on cytokinin removal with cyclin-dependent kinase complexes that can be activated in vitro by the protein phosphatase Cdc25 (*5*). Possibly, this is a feature associated with certain suspension cultures selected for continuous proliferation, because cytokinin appears to be required for efficient S phase entry in previously quiescent plant tissues, explants, cells, or protoplasts (*4*, *5*, *7*). The results presented for *Arabidopsis* and previous reports for tobacco and alfalfa cells show CycD3 activation before the first S phase in stimulated cells. Therefore, from our consistent observations in cells, plants, and mutants, we propose that cytokinin activation of the *Arabidopsis* cell cycle is primarily mediated by transcriptional regulation of CycD3 at the G_1-S boundary.

In addition to their role in cell division, cytokinins are also involved in diverse differentiation processes including shoot initiation and growth, photomorphogenesis, and senescence (*2*, *3*). Whereas wild-type calli could be induced to form shoots, CycD3 calli did not produce shoots on media either containing or lacking cytokinin (*22*), consistent with our original failure to regenerate CycD3-overexpressing transformed calli. Taken together with the greening of CycD3 calli without exogenous cytokinin and the abnormal meristem and leaf phenotypes and delayed senescence of CycD3-expressing transgenics, wider roles for CycD3 in cytokinin-mediated processes may be suggested. In mammals, D-type cyclins are involved in developmentally determined differentiation pathways (*9*). Plants have the unique attribute of modulating their development with the prevailing environmental conditions, and as plant hormones form one link in this response mechanism, D-type cyclins may have evolved novel roles in the integration of environmental signals into plant cell division and development.

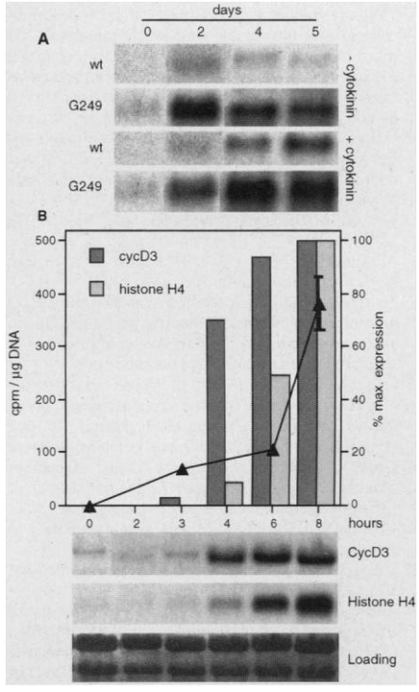

Fig. 4. (**A**) Northern blots of histone H4 expression in wild-type and CycD3-expressing (G249) leaf explants placed (on day 0) in auxin-containing liquid MS medium in the absence (−cytokinin) and presence (+cytokinin) of cytokinin. Histone H4 expression is a marker for S phase (*10*, *11*, *13*, *25*, *30*). (**B**) CycD3 expression shortly precedes S phase in *Arabidopsis* cells reactivated from quiescence by sucrose addition at 0 hours (*31*). At times indicated, [^{3}H]thymidine incorporation into DNA was measured (triangles; left axis) and plotted together with bars showing quantitation of CycD3 and histone H4 levels (bars; right axis), as measured in the Northern blots shown below.

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14. *Arabidopsis thaliana* Landsberg *erecta* suspension culture was maintained by weekly 20-fold dilution (*12*, *13*). Early stationary phases (day 7 after previous subculture) were filtered and washed five times with 200 ml of Murashige and Skoog (MS) medium (containing 3% sucrose, but lacking auxin and cytokinin) and maintained in this medium for 24 hours before addition of inducing agents at time $T = 0$ and sampling at times indicated. Identical results were obtained with exponential (day 3) cells similarly treated. Chx (100 μM) was added 1 hour before other inducers and effectively prevents protein synthesis in *Arabidopsis* [S. Abel, M. D. Nguyen, A. Theologis, *J. Mol. Biol.* **251**, 533 (1995); (*22*)].
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16. Transgenic *Arabidopsis* seedlings carrying the CycD3 promoter linked to a *GUS* reporter gene treated with zeatin also showed twofold to sevenfold induction of GUS but no change in its spatial distribution.
17. Longitudinal sections (8 μm) of 8-week-old vegetative *A. thaliana* Columbia grown under short days, hybridized as described [G. Segers *et al.*, *Plant J.* **10**, 601 (1996)] to 800-nucleotide antisense or full-length sense CycD3 ^{35}S-uridine triphosphate–labeled riboprobes (Promega), were viewed in dark field (autoradiographic signal) or fluorescence (calcofluor staining). For cytokinin induction, 50 μM BAP (Sigma) was applied daily to the apical bud for five successive days.
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22. C. Riou-Khamlichi, A. Jacqmard, J. A. H. Murray, data not shown.
23. Explants cut from rosette leaves of 3-week-old F3 CycD3-expressing and wild-type plants were placed on MS medium with 0.45 μM 2,4-dichlorophenoxyacetic acid (2,4-D) or 2,4-D and 0.44 μM BAP (control). Calli were propagated and trimmed every 10 days; trimming was unnecessary for wild-type calli on medium lacking cytokinin.
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28. RNA blots on GeneScreen Plus (NEN) were stained with methylene blue to confirm equal loading, hybridized at 42°C, and washed (CycD3 blots: 0.1% standard saline citrate and 0.1% SDS at 42°C for 20 min and at 65°C for 10 min; *Arabidopsis* histone H4 blots: 42°C for 10 min and 55°C for 10 min).
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32. We thank colleagues for helpful suggestions; N. Kilby, G. Davies, and A. Sessions for advice on the FLP activation system; L. Dehon for in situ hybridizations; D. Hanke for advice and assistance with cytokinin assays; I. Furner for *Arabidopsis* mutants; and A. Inskip for technical assistance. Support of Biotechnology and Biological Sciences Research Council grant P05114 and Pôles d'attraction interuniversitaires belges (Service du Premier Ministre, Services fédéraux des Affaires scientifiques, techniques et culturelles) P4/15 is acknowledged.

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Increased Insulin Sensitivity and Obesity Resistance in Mice Lacking the Protein Tyrosine Phosphatase-1B Gene

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Protein tyrosine phosphatase–1B (PTP-1B) has been implicated in the negative regulation of insulin signaling. Disruption of the mouse homolog of the gene encoding PTP-1B yielded healthy mice that, in the fed state, had blood glucose concentrations that were slightly lower and concentrations of circulating insulin that were one-half those of their PTP-1B$^{+/+}$ littermates. The enhanced insulin sensitivity of the PTP-1B$^{-/-}$ mice was also evident in glucose and insulin tolerance tests. The PTP-1B$^{-/-}$ mice showed increased phosphorylation of the insulin receptor in liver and muscle tissue after insulin injection in comparison to PTP-1B$^{+/+}$ mice. On a high-fat diet, the PTP-1B$^{-/-}$ and PTP-1B$^{+/-}$ mice were resistant to weight gain and remained insulin sensitive, whereas the PTP-1B$^{+/+}$ mice rapidly gained weight and became insulin resistant. These results demonstrate that PTP-1B has a major role in modulating both insulin sensitivity and fuel metabolism, thereby establishing it as a potential therapeutic target in the treatment of type 2 diabetes and obesity.

PTP-1B is implicated in the attenuation of the insulin signal (*1*). Mice deficient in the heterotrimeric GTP–binding protein subunit $G_{i\alpha 2}$ exhibit a phenotype of insulin resistance characteristic of type 2 diabetes that correlates with the increased expression of PTP-1B (*2*). PTP-1B directly interacts with the activated insulin receptor (*3*), and vanadate, a potent nonselective PTP inhibitor, can function as an insulin mimetic both in vitro and in vivo (*4*). However, PTPs other than PTP-1B can also dephosphorylate the activated insulin receptor (*5*). To clarify the role of PTP-1B in insulin action, we generated mice in which the mouse homolog of PTP-1B was disrupted.

The murine gene encoding PTP-1B was cloned from a 129/Sv mouse genomic library and shown to consist of at least nine exons

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