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# Cyclin D-knockout uncouples developmental progression from sugar availability

Stefan Lorenz, Stefanie Tintelnot, Ralf Reski and Eva L. Decker*
*Freiburg University, Plant Biotechnology, Schaenzlestrasse 1, 79104 Freiburg, Germany (*author for correspondence; e-mail eva.decker@biologie.uni-freiburg.de)*

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**Abstract**

Multicellular organisms need to modulate proliferation and differentiation in response to external conditions. An important role in these processes plays the mitogen-stimulated induction of *cyclin* D (*cyc*D) gene expression. D-type cyclins have been identified as the crucial intracellular sensors for cell-cycle regulation in all eukaryotes. However, *cyc*D deletions have been found to cause specific phenotypic alterations in animals but not yet in plants. An insertional mutation of a so far uncharacterized *Arabidopsis cyc*D gene did not alter the plant phenotype. To gain new insights into CycD function of land plants, we generated targeted *cyc*D gene knockouts in the moss *Physcomitrella patens* and observed a surprisingly limited disruption phenotype. While wild-type plants reacted to exogenous glucose sources with prolonged growth of juvenile stages and retarded differentiation, *cyc*D knockouts exhibited developmental progression independent of sugar supply. On the other hand, growth rate, cell sizes or plant size were not affected. Thus, we conclude that *Physcomitrella* CycD might not be essential for cell-cycle regulation but is important for coupling the developmental progression to nutrient availability.

*Abbreviations:* CDK, cyclin-dependent kinase; CTAB, cetyltrimethylammonium bromide; CycD, cyclin D; EDTA, ethylenediaminetetraacetic acid; EST, expressed sequence tag; G418, Geneticin; PEG, polyethylene glycol; SDS, sodium dodecyl sulfate; Rb, retinoblastoma protein

## Introduction

A highly coordinated progression through the cell cycle in response to environmental conditions is of crucial importance for growth and development of all multicellular organisms. For plants, the influence of certain cell-cycle regulators on differentiation events has been described recently (Riou-Khamlichi *et al.*, 1999; Rossi and Varotto, 2002).

One of the key regulatory points within the eukaryotic cell cycle displays the G1/S transition, which is controlled by the highly conserved retinoblastoma pathway in animals as well as in higher plants (Sherr, 1993; Francis, 1998; Burssens *et al.*, 1998). Here, a binary complex of a D-type cyclin (CycD) and a cyclin-dependent kinase (CDK-a) provides the commitment to the G1/S transition by phosphorylation of the retinoblastoma protein (Rb). Rb phosphorylation subsequently causes the release of active E2F transcription factors, which are required for S-phase progression (den Boer and Murray, 2000).

Within this pathway the D-type cyclins are believed to provide the complex interconnection between sensing of environmental signals and cell-cycle decisions of cellular growth and differentiation, respectively (Meijer and Murray, 2000; Oakenfull *et al.*, 2002). Expression of *cyc*D genes is stimulated by mitogens, i.e. growth factors in mammals (Matsushime *et al.*, 1991; Sherr, 1994) and phytohormones in plants (Riou-Khamlichi *et al.*, 1999; Gaudin *et al.*, 2000; Hu *et al.*, 2000; Oakenfull *et al.*, 2002). In addition, sucrose has been shown to differentially regulate D-type cyclin expression in *Arabidopsis* regarding both kinetics and the rate of induction (Riou-Khamlichi

*et al.*, 2000). Recent analyses of plant cell-cycle genes revealed a high complexity of D-type cyclins in plants. For example, *Arabidopsis* contains seven CycD subclasses with ten different members which are thought to be, at least in part, functionally redundant (Swaminathan *et al.*, 2000; Vandepoele *et al.*, 2002). The existence of several CycD subclasses suggests that distinct proteins are used for fine tuning of cell proliferation and differentiation of specific tissues in response to various environmental and developmental conditions (Oakenfull *et al.*, 2002; Vandepoele *et al.*, 2002).

Although D-type cyclins were found in nearly all classes of eukaryotes, functional characterization of these proteins especially in plants is so far still at the dawn. Over-expression of *cyc*D is connected to enhanced proliferation and dedifferentiation, i.e. tumours in mammals and calluses in plants (Sherr, 1996; Riou-Khamlichi *et al.*, 1999; Cockcroft *et al.*, 2000). In contrast, reports on CycD deficiencies have not given consistent results between animals and plants yet. While in mice targeted disruption of *cyc*D genes resulted in distinct developmental abnormalities, growth retardation, and increased mortality (Sicinski *et al.*, 1995), an insertional mutation of a so far uncharacterized *Arabidopsis cyc*D gene revealed no obvious phenotypic alterations (Swaminathan *et al.*, 2000). This was interpreted by functional redundancy of the CycD family in *Arabidopsis*.

As in the rather complex seed plants correlation of changes in differentiation to a single gene knockout event may be difficult to draw, we attempted to study plant CycD function by use of a lower land plant, the moss *Physcomitrella patens*. *Physcomitrella* combines critical advantages that make it especially feasible as a model system to study cellular differentiation processes. The generation of knockout mutants is facilitated as this moss is unique among land plants in its efficient gene targeting system (Reski, 1998a; Strepp *et al.*, 1998; Schaefer, 2001; Imaizumi *et al.*, 2002). In addition, the average gene family size in *Physcomitrella* is smaller than in *Arabidopsis* (Rensing *et al.*, 2002), enabling the precise establishment of gene/function relationships (Holtorf *et al.*, 2002). Furthermore, the simple development of mosses is well characterized. Early developmental processes rely on a cell lineage, coupling organization of the whole plant to the differentiation of single cells (Cove, 1992; Reski, 1998b). *Physcomitrella* development starts with the growth of a filamentous tissue of apical-dividing so-called chloronema cells, characterized by numerous plastids and cross walls perpendicular to the growth axis. The first differentiation step represents the switch to caulonema cells with fewer plastids and oblique cross walls. On this filamentous juvenile tissue, altogether named protonema, formation of buds marks the next step in development. The buds subsequently bring about the adult tissues (leafy gametophores) which bear the sex organs. The induction of buds is a typical and specific reaction to the plant hormone cytokinin (Cove, 1992; Reski, 1998b). Unfavourable environmental conditions like low temperature and short-day cultivation accelerate the developmental progression terminated by sexual reproduction (Hohe *et al.*, 2002a).

Here we report the identification and targeted disruption of a *cyc*D gene homologue in *Physcomitrella*. Knockout plants exhibited unimpaired developmental progression under growth conditions in which wild-type differentiation is retarded. The CycD mutant phenotype indicates an uncoupling of growth decisions from nutrient availability.

## Materials and methods

### *Plant material and growth conditions*

*Physcomitrella patens* (Hedw.) B.S.G. was grown axenically under standard conditions (agitated liquid Knop medium, 250 mg/l $KH_2PO_4$, 250 mg/l $MgSO_4 \cdot 7H_2O$, 250 mg/l KCl, 1000 mg/l $Ca(NO_3)_2 \cdot 4H_2O$, 12.5 mg/l $FeSO_4 \cdot 7H_2O$, pH 5.8) in a growth chamber (25 $\pm 1$ $^{\circ}$C; light provided from above by two fluorescent tubes, Philips TL-D 36W/25; light flux 55 $\mu$mol $s^{-1}$ $m^{-2}$ outside the flasks, light/dark regime 16/8 h). Plants were sub-cultured at 7-day intervals.

### *DNA isolation and Southern blot analysis*

DNA isolation from plant material was performed as described (Bierfreund *et al.*, 2003). For Southern blot analysis, the digested genomic DNA (7 $\mu$g) was separated on a 0.7% agarose gel and transferred onto Hybond $N^+$ nylon membranes (Amersham Biosciences, Freiburg, Germany) according to standard procedures (Sambrook and Russell, 2001). Probes were radioactively labelled with $^{32}$P-dCTP by use of the Rediprime Random Prime Labelling System (Amersham Biosciences) according to the manufacturer's instructions. The *cycD* probe was located within the cyclin box, the region of highest conservation. Hybridization and

washes were performed at low-stringency conditions (60 °C, 2× SSC wash).

*RNA isolation*

Plants were ground to a fine powder under liquid nitrogen and mixed with 3 ml extraction buffer (0.15 M NaCl, 5 mM EDTA, 50 mM Tris-HCl pH 9.0) and 0.7 ml 10% SDS per gram plant material. The suspension was incubated with 7 ml phenol for 15 min with agitation. After centrifugation at 1600 × *g* for 10 min the supernatant was extracted twice with 1 volume phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform. The supernatant was mixed carefully with 1 volume 5 M LiCl, and RNA was precipitated overnight at 4 °C. After centrifugation at 4500 × *g* the pellet was washed twice with 70% ethanol and re-suspended in $H_2O$.

*Yeast cell culture and transformation*

The yeast strain BF305-15d #21 was grown on YPD medium (1% yeast extract, 2% peptone, 2% glucose, 2% Bactoagar) and YEPGR medium (2% glucose replaced by 1% galactose (<0.01% glucose, Sigma) and 1% raffinose) at 30 °C. Transformation was done as described (Soni *et al.*, 1993). Transformed yeast cells have been selected on His-Leu-Trp drop-out plates.

*Transfection of* Physcomitrella *protoplasts*

For transformation of *Physcomitrella* protoplasts via PEG-mediated DNA uptake both gene-targeting constructs were linearized with *Eco*RI. Protoplasts were isolated and $3 \times 10^5$ cells were transfected with 25 $\mu$g of linearized plasmid DNA and regenerated in regeneration medium (Knop medium, 3% mannitol, 5% glucose, pH 5.8). Selection of transgenic plants was performed against G418 as described (Strepp *et al.*, 1998). Three rounds of selection with the transgenic plants were performed against 50 $\mu$g/ml G418.

*Screening of Physcomitrella transformants*

Transformed moss plants were analysed after three rounds of selection by direct PCR from gametophores as described previously (Schween *et al.*, 2002) with PCR primers that span the 5′ and 3′ integration sites of the knockout constructs, respectively. Primers for 5′ integration were cycD114f (CTGTTGACCGTTTAGATTGG) and RT1 (TGTCGTGCTCCACCATGTT). Primers used for verification of the 3′ integration were RT4 (GTTGAGCATA TAAGAAACCC) and cycD922r (CTAACACCAAG CTTGTGG). Positively tested plants were checked for the absence of the transcript in RT-PCR with the primer combination cycD114f/cycD922r. Superscript II reverse transcriptase (Invitrogen) was used for first-strand synthesis.

*Analysis of protoplast regeneration*

Moss protoplasts were diluted to a final concentration of 50 000/ml in regeneration medium, and samples were taken for analysis every two days. The final concentration of cytokinin (6-($\gamma$, $\gamma$-dimethylallylamino)purine, 2iP) was 5 $\mu$M. For analysis of protoplast regeneration in minimal medium, the protoplasts were pre-cultivated in regeneration medium for four days, centrifuged and re-suspended in Knop medium.

## Results

*Isolation of the* Physcomitrella *cyclinD gene locus*

From a *Physcomitrella* cDNA library one full-length clone, encoding a putative D-type cyclin (PpCycD), was isolated via homology search in a clustered EST database (Rensing *et al.*, 2002). The corresponding genomic locus was isolated by PCR. It spans 2324 bp which contain six exons and five introns comprising a coding sequence of 1083 bp. Detailed screening of public EST data and of our in-house EST database, which is expected to cover at least 95% of the *Physcomitrella* transcriptome (Rensing *et al.*, 2002), did not reveal any other *cyc*D gene expressed in this plant. In order to look for further *cyc*D genes, Southern blot analysis was performed under low-stringency conditions with probes that bind to conserved domains. DNA for *cyc*D analyses was cut with enzymes *Vsp*I, *Eco*RI, and *Hin*dIII. The first two enzymes do not cut within the hybridized *cyc*D sequence, while *Hin*dIII should cut once, yielding two fragments. One fragment has the predicted size of 962 bp (Figure 1A) as a second known *Hin*dIII site is located outside of the hybridized sequence. These results support the indication that *cyc*D might be a single gene.

Expression analysis of protonema tissue by semi-quantitative RT-PCR revealed no clear regulation by sucrose or cytokinin (data not shown). Expression analysis of the low-abundance cell-cycle genes in *Phy-*

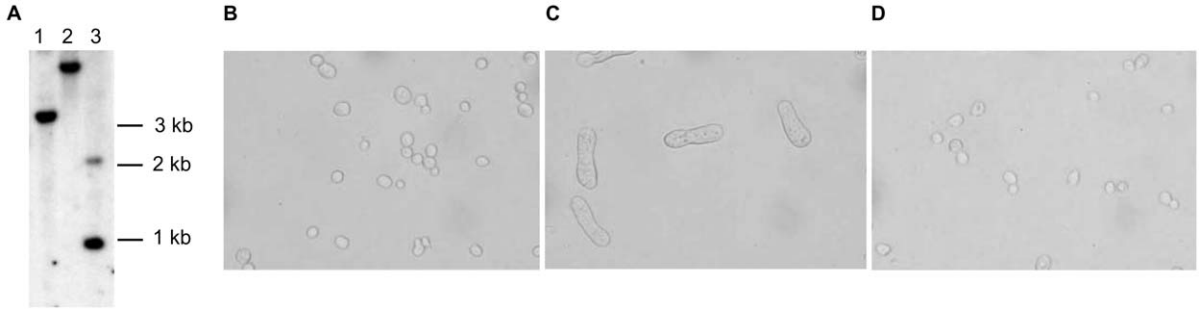


*Figure 1.* Isolation of a functional cyclin D homologue from *Physcomitrella patens*. A. Estimation of the *cyc*D gene number by Southern blot analysis. Genomic *Physcomitrella* DNA was digested with *Vsp*I (lane 1), *Eco*RI (lane 2), and *Hin*dIII (lane 3). The first two enzymes do not cut within the hybridized sequence, while *Hin*dIII cuts once, yielding a predicted fragment of 962 bp. B–D. Complementation of G1 cyclin-deficient yeast cells with *Ppcyc*D cDNA. The strain BF305-15d #21 has a galactose-dependent growth phenotype due to deletion of CLN1 and CLN2 and placement of CLN3 under the control of the galactose-inducible GAL10-gene promoter (Soni *et al.*, 1995). Cells of this strain grow normally on YEPGR medium containing galactose (B) but cease division when transferred to glucose-containing YPD medium (C). For functional analysis, the *Ppcyc*D cDNA was inserted into the yeast expression vector pJR1138 (D). Expression of moss *cyc*D restored normal growth of the yeast mutant.

*scomitrella* is hampered as synchronization attempts of the cultures have not been successful so far.

### *PpCycD complements a yeast cell-cycle mutant*

Phylogenetic classification of the moss sequence within other plant D-type cyclins (data not shown) suggested a cell-cycle regulating role for PpCycD. Functional identification of PpCycD as a cyclin acting in G1 phase of the cell cycle was confirmed by complementation of a yeast strain (Soni *et al.*, 1995; Figure 1B–D). This mutant strain is impeded in cell division under restrictive conditions (Figure 1C). It could be rescued to wild-type growth by ectopic *Ppcyc*D expression (Figure 1D).

### *Targeted disruption of the* cyc*D gene locus*

For a reverse-genetics approach a gene-targeting construct was created by inserting a 35S promoter-driven *npt*II selection cassette into the single *Kpn*I site of a 1073 bp *Ppcyc*D genomic fragment (Figure 2A), thus disrupting the encoded protein just downstream the Rb-binding domain at its very N-terminus. The resulting construct, containing the *npt*II cassette flanked by 347 bp and 704 bp *Ppcyc*D genomic sequence, respectively, was subsequently used for transfection of *Physcomitrella* protoplasts. Upon regeneration transgenic plants were selected for antibiotic resistance against G418 and subsequently screened via direct PCR from moss tissue for targeted gene disruption (Figure 2B). Of 43 independent transgenics, 8 (18.6%) showed proper integration of the knockout construct within the *cyc*D locus. Sequencing of the PCR products revealed perfect matches at the integration site without any base pair additions or deletions (data not shown). Expression analysis of the knockout plants by RT-PCR proved the disruption of the *Ppcyc*D gene (Figure 2C, lanes 3–10). In contrast, *Physcomitrella* wild type did express the gene (Figure 2C, lane 1) as did a control plant in which the knockout cassette had been integrated via illegitimate recombination (Figure 2C, lane 2). In addition to the wild type, this transgenic plant was used as a further control in subsequent experiments. Both plants are hitherto termed control plants.

### *Cell division of* Ppcyc*D-knockout plants is not impaired*

Deficiency of cyclin D anticipates a defect in cell division rates or cell sizes and total plant size, respectively. Therefore, we initially analysed the phenotype of knockout plants grown under standard conditions on solid medium. In any case, cell division was not obviously impaired, as we observed similar growth rates and overall morphology between controls and knockout plants (Figure 3A). Also leaf shape as well as cell sizes and cell numbers of the knockouts appeared to be wild-type-like (Figure 3B).

### *PpCycD is essential for correct onset of differentiation*

As overall growth rates and cell sizes were not obviously affected in the adult knockout plants, consequences of the *cyc*D knockout were analysed at the youngest developmental stages. In order to guarantee

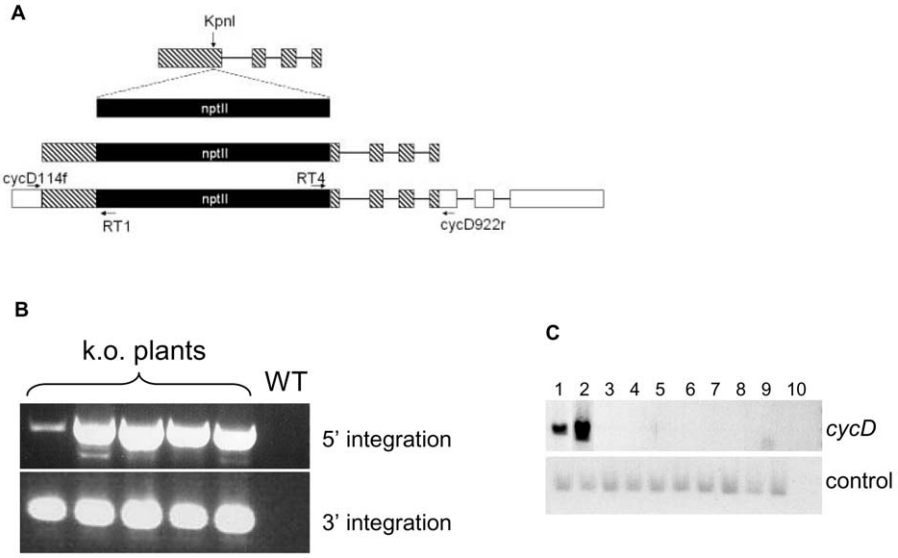


*Figure 2.* Targeted disruption of the *cyc*D locus in *Physcomitrella*. A. Schematic representation of the cloning strategy for *cyc*D knockout generation as well as PCR primer specifications for knockout analysis. A 1073 bp genomic fragment was disrupted by insertion of an *npt*II selection cassette (black bar) into the *Kpn*I site. Exons are shown in hatched bars, introns as lines. Pairs of PCR primers were specific for the 5′ integration site (cycD114f/RT1) and 3′ integration site (RT4/cycD922r), respectively. Dimensions are drawn to scale. B. PCR analysis from gametophore tissue proved correct 5′ and 3′ integration of the knockout construct. C. RT-PCR demonstrated expression of *cyc*D in WT (1) and a transgenic plant generated by illegitimate recombination (2) and absence of *cyc*D mRNA in eight knockout plants (3–10). As an internal control the constitutively expressed mRNA for the ribosomal protein L21 was used (Reski *et al.*, 1998).

a high uniformity of differentiation stages we started from single cells. In mosses, isolated protoplasts regenerate directly to intact fertile plants without any exogenous growth factors. Normal development of *Physcomitrella* will be obtained via standard cultivation in a simple medium of inorganic salts devoid of any additional carbohydrates. However, regeneration of freshly isolated protoplasts requires initial cultivation in a medium containing glucose (5%) und mannitol (3%). Isolated protoplasts from knockout and control plants were cultivated in this regeneration medium for four days. Afterwards, the plants were transferred to standard medium (i.e. without any sugar). Here, all plants, i.e. the different *cyc*D knockouts and the control plants, switched from the early chloronema to the successive caulonema stage in a similar manner earlier than 11 days after protoplast isolation (Figure 3C, D).

In contrast, in regeneration medium the wild type as well as the transgenic control exhibited caulonema formation only at day 19, demonstrating a severely retarding effect of the regeneration medium on *Physcomitrella* development (Figure 4A, B). This retardation could be ascribed to the glucose component which is not present under standard growth conditions. When we examined development of adult tissues (gametophores) in standard medium vs. glucose-containing medium, a dramatic reduction in gametophore numbers by more than 95% could be observed due to glucose. However, knockouts deviated strongly from controls in caulonema formation. All *Ppcyc*D knockouts exhibited caulonema development earlier, the latest at day 11 (Figure 4A, B) like in minimal medium before. Thus, sugar supply did not show any influence on knockout differentiation while it severely affected the control plants.

Furthermore, it is known that the next developmental step, bud formation, is triggered by cytokinin. Exogenously applied cytokinins also lead to enhanced and premature induction of buds, but these quite often do not further develop into adult gametophores (Cove, 1992; Reski, 1998b).

Taken together, cytokinin accelerates developmental progression in *Physcomitrella*. In order to see if cytokinin is able to antagonize the retarding sugar signal, protoplasts were regenerated in sugar-containing medium in the presence of cytokinin (5 $\mu$M). This double treatment again revealed a developmental difference between knockouts and the controls. Bud initiation was first detectable in the wild type and the other control plant only at day 13. In contrast, the

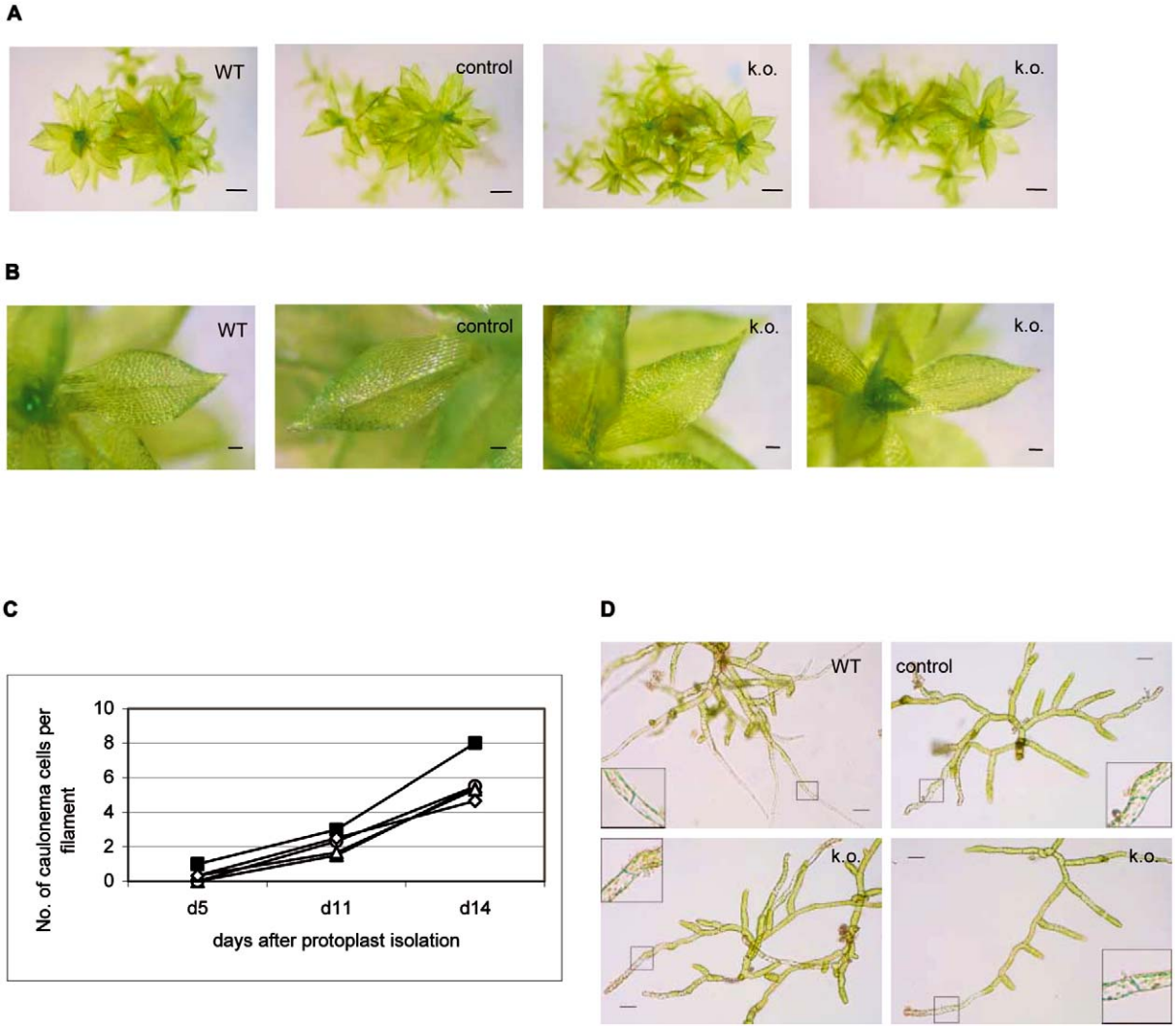


*Figure 3.* Regeneration under standard growth conditions. Wild-type *Physcomitrella* (WT), a transgenic plant generated by illegitimate recombination (control), and two independent *cyc*D knockouts (k.o.) displayed a similar morphology when grown in standard medium (i.e. without sugar). A. Overall shape of adult moss plants, scale bar 0.5 mm. B. Details showing the single cell layer of moss leaves, scale bar 100 $\mu$m. C. Development of caulonema cells within juvenile cell filaments. The average number of caulonema cells is similar between controls and knockouts. ■, WT; ▲, control; ○, ◊, △, different knockout plants. D. Juvenile cell filaments eleven days after protoplast regeneration, scale bar 50 $\mu$m. The rate of differentiation and growth is similar for all plants. Inserts highlight caulonema cells (oblique cross wall).

knockout plants started bud formation two days earlier and produced them in larger numbers (Figure 4C, D).

## Discussion

Cyclin D function is a crucial part of cell-cycle regulatory processes in all higher eukaryotes. Mammals and, especially, higher plants contain several D-type cyclins, a fact that may create difficulties in assigning distinct functions to a single family member. On the other hand, D-type cyclins in lower land plants have not been characterized yet. We chose the model plant *Physcomitrella patens* for a reverse genetics approach. *Physcomitrella* seems to contain smaller gene families than *Arabidopsis* (Rensing *et al.*, 2002). Furthermore, this plant provides a simple body plan and well-characterized differentiation steps. Surprisingly, the data from EST database analysis as well as Southern blot analyses suggested the presence of only one member of D-type cyclins in *Physcomitrella*. In addition, searches for *cyc*A and *cyc*B genes in the EST

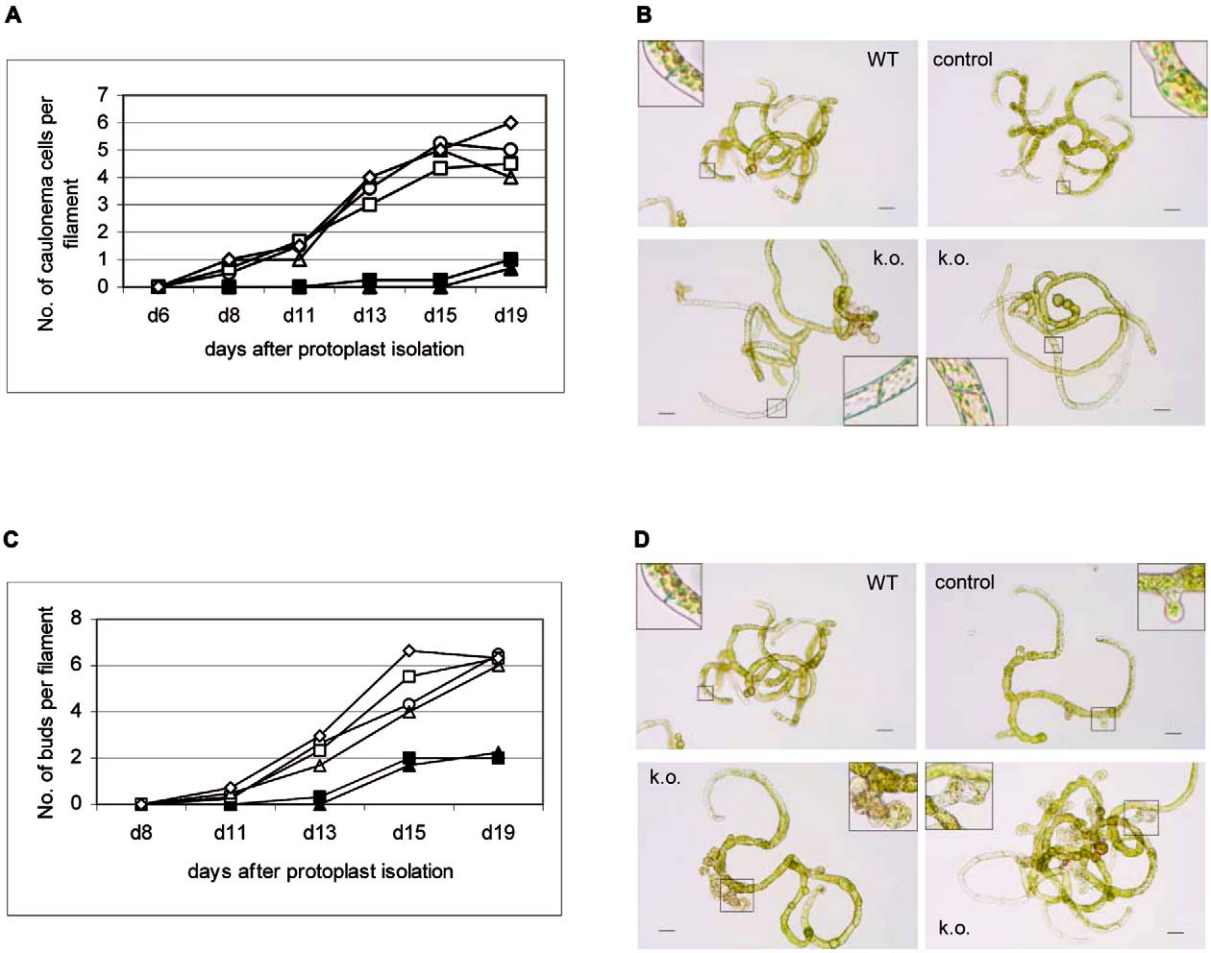


*Figure 4.* Development in glucose-supplemented regeneration medium. Wild type (WT), the transgenic control (control), and *cyc*D knockouts (k.o.) were grown for 19 days after protoplast isolation. A. The average number of caulonema cells per filament shows the accelerated development of the knockouts. ■, WT; ▲, control; ○, □, ◊, △, different knockout plants. B. Development in regeneration medium at day 15; scale bar 50 $\mu$m. Inserts highlight the chloronema cells (perpendicular cross wall) in WT and control, and premature caulonema development (oblique cross wall) in the knockouts. C. Development in glucose-containing medium supplemented with 5 $\mu$M cytokinin. The average number of buds per regenerated filament shows the enhanced and preceded bud production. ■, WT; △, control; ○, □, ◊, △: different knockout plants. D. Cell filaments at day 15; scale bar 50 $\mu$m. Inserts highlighted by a factor of 3 enhanced bud production in the knockout plants.

database yielded also only one member per group (data not shown). A single *cyc*D gene would display a striking difference from higher plants, which are known to contain a much higher complexity of D-type cyclins (Vandepoele *et al.*, 2002). The presence of protein motifs characteristic of D-type cyclins and the successful complementation of a cyclin-deficient yeast mutant together support the designation of the reported *Physcomitrella* protein as a functional CycD.

Since the destruction of the putatively single member of this cell-cycle regulatory factor should result in a significant phenotype, we expected a knockout of the *Ppcyc*D gene to be of particular interest. This was not the case for the only so far reported *cyc*D knockout from *Arabidopsis* (Swaminathan *et al.*, 2000). The lack of a deviating phenotype was interpreted by functional redundancy of different D-type cyclins. Interestingly, the knockout of the *Ppcyc*D gene was viable, showing neither differences in terms of growth rate or shape nor in cell sizes or numbers. However, if the gene was an irreplaceable factor of cell-cycle regulation, the knockout should have shown more pronounced defects. Thus, we suggest that this specific CycD from *Physcomitrella* is not essential for the regulation of cell division and cell growth. As there is so far no indication for a second

*Physcomitrella* D-type cyclin, partial compensation of PpCycD cell-cycle functions by other cyclins may be considered. Some overlap of individual cyclin functions has been observed before (John *et al.*, 2001). For example, in mice a 'knockin' of cyclin E to the *cyc*D1 locus rescued CycD1 cell-cycle functions (Geng *et al.*, 1999). However, in plants no cyclin E homologue has been described so far and D-type cyclins apparently represent the only G1-cyclin family (Vandepoele *et al.*, 2002). Possibly, a loss of CycD function could partially be restored by specific mitotic cyclins. For *Medicago sativa* it has been shown that CycA2 contributed to cell-cycle specific kinase activity not only at the G2/M transition but also at the entry to the S-phase (Roudier *et al.*, 2000). This demonstrates that a so far as G2/M-specific designated cyclin also functions in G1/S transition. Putative homologues of mitotic cyclin genes (*cyc*A and *cyc*B) are expressed in *Physcomitrella* as well and might thus retain cell-cycle activity in the absence of CycD. Alternatively, the restriction point at the G1/S transition might be of minor importance in *Physcomitrella* compared to other eukaryotes, where it is known to be the main control point of the cell cycle. The latter is supported by the finding that in most other eukaryotic species the cell cycle is arrested in G0/G1, while in *Physcomitrella* most of the cells are predominantly in the G2 phase (Schween *et al.*, 2003). This indicates that a major regulation of the *Physcomitrella* cell cycle may occur at the G2/M transition.

Despite the lack of morphological differences of plants grown on standard minimal medium, a clear phenotype could be found when the plants were transferred to sugar-supplemented medium, showing earlier caulonema development and earlier, enhanced bud formation in the presence of cytokinin.

Thus, in wild-type *Physcomitrella* as well as in the other control plant glucose supply led to prolongation of the juvenile stages by delaying differentiation decisions that would lead to adult tissues. In contrast, developmental progression of the *Ppcyc*D knockout plants was in no way affected by the exogenous carbohydrate sources, indicating that here differentiation decisions were uncoupled from nutrient availability.

In mosses such as *Physcomitrella*, the formation of caulonema cells and buds are the first essential differentiation steps towards sexual reproduction (Reski, 1998b). Here, we have demonstrated that the presence of glucose retards this developmental program resulting in a prolongation of juvenile stages. As *Ppcyc*D knockouts continued development from chloronema to caulonema irrespectively of sugar availability, they were obviously not able to sense or integrate the sugar signal. In a similar manner, next developmental step was accelerated after application of cytokinin (i.e. bud formation). All knockout plants integrated this stimulus more effectively than the controls, as they did not sense the retarding and thus opposing signal. Based on our observations, we speculate that in the presence of glucose PpCycD is up-regulated and thus promotes G1/S transition, which in turn leads to enhanced proliferation and retardation of caulonema formation. This interpretation is consistent with observations from *Arabidopsis* where *cyc*D3 was shown to be inducible by sugar (Riou-Khamlichi *et al.*, 2000) and where ectopic expression of this gene resulted in an inhibition of cellular differentiation (Riou-Khamlichi *et al.*, 1999).

Different explanations for the observed phenotype are conceivable. First, it is known that addition of carbon sources accelerates *Physcomitrella* cell proliferation (Hohe *et al.*, 2002b). Therefore, the failure of the mutant to react to sugar supply may be due to an inability of increasing growth rates in response to exogenous sugar. As an indirect consequence, the mutant would proceed straight to caulonema development. This possibility has to be rejected as we found no differences in growth rate between knockouts and control plants in the presence of sugar. Second, the response to sugar might be an indirect signalling via CycD by an alternative developmental pathway that requires CycD activity to allow it to be adopted over the default pathway, which is immediate caulonema formation. Third, CycD may directly act as an intracellular sensor for sugar and therefore nutrient availability in *Physcomitrella*. Hence, we postulate an involvement of PpCycD in sugar sensing that integrates exogenous signals for nutrient availability into the plant developmental program. This hypothesis is supported by similar findings from gametophytic tobacco spore cell cultures, where sugar starvation was reported to be sufficient to induce developmental progression from the gametophytic stage to haploid sporophytes (Vicente *et al.*, 1991). Thus, PpCycD may not be essential in the central regulation of the cell cycle but might play a role in the regulation of developmental progression.

In correlation to earlier findings in mice, the knockout plants showed much more limited effects than expected for a protein that is supposed to be necessary for the onset of cell cycle progression. The described developmental steps were only affected under specific environmental conditions and, additionally,

the effect of CycD seems to be restricted to certain cell types. Also in mice the knockout of *Cyclin* D1 resulted in only limited effects (Sicinski *et al.*, 1995). Cyclin D1-deficient mice were expected to show important defects due to disordered cell proliferation of embryonic cells but surprisingly the knockout animals were viable and exhibited only developmental abnormalities that were restricted to tissues such as the retina, the nervous system and the breast epithelium. This again argues for the relevance of CycD in certain well-defined developmental steps. In contrast, observations from *CycD* over-expression in *Arabidopsis* did not only influence differentiation but also growth rate and the rate of cell cycling (Cockcroft *et al.*, 2000), indicating the relevance of this protein for cell cycle regulation.

Taken together, the function of PpCycD as a cell cycle regulator remains elusive. However, the results discussed here clearly show an involvement of this protein in the integration of environmental, i.e. nutritional, signals into the initiation of well-defined developmental steps.

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