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RAPID COMMUNICATION

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Transition of G1 to early S phase may be required for zinnia mesophyll cells to trans-differentiate to tracheary elements

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Abstract We have used the *Zinnia elegans* mesophyll cell system, in which single isolated leaf mesophyll cells can be induced to trans-differentiate into tracheary elements in vitro, to study the relationship between the cell division cycle and cell differentiation. Almost all cells go through several rounds of division before characteristic features of tracheary element formation are observed. The addition of aphidicolin, a DNA synthesis inhibitor, blocks cell division but not cell differentiation in the zinnia system. Low concentrations of aphidicolin, which possibly delay cells in the early S phase, can significantly enhance levels of tracheary element formation. In contrast, roscovitine, an inhibitor of cyclin-dependent kinase activity, decelerates the cell division cycle and inhibits tracheary element formation with similar dose responses. Cells blocked in S phase and then transferred to roscovitine-containing medium can divide once, indicating that roscovitine may target the G1/S transition, but do not differentiate. Cells inhibited in G1/S in roscovitine-containing medium that are subsequently blocked in S phase by transfer to aphidicolin-containing medium, do not divide but do differentiate. Taken together, our results indicate that cells may be required to transit the G1/S checkpoint and enter early S phase to acquire competence to trans-differentiate to tracheary elements.

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Abbreviations BAP: 6-Benzylaminopurine, cytokinin · cdc: Cell division cycle · DAPI: 4′, 6′-Diamidino-2-phenylindole · DMSO: Dimethyl sulfoxide · NAA: α-Naphthalene acetic acid, auxin

Introduction

The coordination of cell proliferation and differentiation is essential for the normal growth and development of organisms. In plants, disruption of the cell cycle regulatory machinery does not necessarily affect the execution of the developmental programme. Thus, an Arabidopsis dominant negative mutation of the key cell cycle regulator *cdc2* reduced division rates but did not alter organ formation or normal development in transgenic tobacco plants (Hemerly et al. 1995). Over-expression of cyclin B in Arabidopsis (Doerner et al. 1996) or cyclin D in tobacco (Cockcroft et al. 2000) accelerated cell division and differentiation but did not severely affect morphogenesis. However, evidence for causal links between cell cycle and differentiation elements also exists. Dominant negative mutants of *cdc2* caused severe developmental effects on the cotyledons of Arabidopsis embryos and similar morphological defects occurred when *cdc2* activity was inhibited by ICK1 (Wang et al. 2000). Also, constitutive over-expression of D cyclin prolongs cell proliferation and inhibits leaf cell differentiation in Arabidopsis (Dewitte et al. 2002). The Arabidopsis HOBBIT gene, which encodes a cdc27 homolog, was found to be involved in both cell division and differentiation in meristems, indicating that the molecular machinery of division and differentiation may indeed be coupled (Blilou et al. 2002).

We investigated the relationship between the cell cycle and differentiation of the water-conducting cells of the xylem, tracheary elements. The developmental program of xylogenesis can be reprised in vitro using the zinnia mesophyll cell system. Addition of auxin and cytokinin to

mesophyll cells freshly isolated from *Zinnia elegans* cv. Envy leaves in liquid culture (Fukuda and Komamine 1980a) induces synchronous differentiation of the cells to form tracheary elements. Previous cytological studies reported that 60% of differentiated cells were "single", in other words, had not previously undergone division in the zinnia system (Fukuda and Komamine 1980b). Analysis of DNA content by both cell sorting and microspectrophotometry also showed that only part of the initial cell population goes through the cell cycle before differentiation (Fukuda and Komamine 1981a). It was therefore concluded that tracheary element formation occurs independently of cell division and the cell cycle (Fukuda and Komamine 1980b, 1981a).

We used a time-course modified from the original system (Fukuda and Komamine 1980a) in which growth factors are added 48 h after cell isolation in liquid culture (Milioni et al. 2001). Both in this new time-course and in the original time-course, nearly 100% of zinnia cells have divided by 96 h of liquid culture, using ultraviolet (UV) epifluorescence to count 4′,6′-diamidino-2-phenylindole (DAPI)-stained nuclei rather than bright-field microscopy to count cross-walls. To investigate whether cell cycle progression is required for tracheary element formation, we used two cell cycle inhibitors, aphidicolin and roscovitine. Using aphidicolin to block S phase and thus cell cycle progression in the culture, we found that cell differentiation was not inhibited. Therefore, we conclude that progression of the cell cycle after the S phase, and indeed cell division, is not required for tracheary element formation. Furthermore, delay in early S phase enhanced differentiation levels. However, roscovitine, an inhibitor of cyclin-dependent kinases, inhibited cell division and cell differentiation with very similar dose responses, indicating a relationship between the two processes. We found that tracheary element formation was blocked by the same concentration of roscovitine that inhibits cell cycle progression through G1/S in culture. We hypothesize that cell cycle entry, and progression until the early S phase, potentiates trans-differentiation in the zinnia system.

Materials and methods

Plant material and cell culture

Mesophyll cells of 14-day-old leaves from *Z. elegans* cv. Envy plants were isolated in medium with no growth factors, and cultured as described in Domingo et al. (1998), then transferred to medium containing 1.0 mg l^{-1} BAP (6-benzylamino-purine, cytokinin) and 1.0 mg l^{-1} NAA (α-naphthalene acetic acid, auxin) after 48 h of culture, as described in Milioni et al. (2001).

Addition of cell cycle inhibitors

Roscovitine and aphidicolin were obtained in powder form from Sigma (St. Louis, Mo.), diluted in dimethyl sulfoxide (DMSO, Sigma) and added to the cultures in final concentrations of 0.8, 4, 20, 50 and 100 μM for roscovitine and 5, 10, 20, 50 and 100 μM for aphidicolin. As a control, the same volume of DMSO was added. To remove an inhibitor over the course of the incubation, cells were washed in "conditioned medium" (obtained from a sample of the culture to which no inhibitor was added, incubated in parallel with the inhibitor-treated cells) three times with centrifugation at 800 rpm for 2 min in an MSE Mistral 2000 centrifuge.

Light microscopy—scoring of cell division and tracheary element formation

Cells were harvested from 0.5 ml culture by quick centrifugation and fixed for 10 min with 4% formaldehyde in phosphate buffered saline on multi-well slides (ICN Biomedicals, Irvine, Calif.), previously coated with poly-L-lysine (Sigma). Cells were subsequently stained with 0.03 μg/ml DAPI (Sigma) and 0.1% w/v Calcofluor (Calcofluor white, Sigma), mounted in Citifluor (Agar Scientific, Stansted, UK), and imaged with UV epifluorescence using a Nikon eclipse E-800 microscope. The scoring of tracheary element formation was based on the appearance of secondary cell wall thickenings, by 120 h of culture, using a Zeiss inverted light microscope. One differentiation event was scored whether the initial cell had previously divided in culture and whether all or some of the daughter cells had formed tracheary elements. For every value of division or tracheary element formation, at least 200 cells were scored in each independent experiment.

Results and discussion

Aphidicolin can uncouple cell division from tracheary element differentiation

From the time of cell isolation (Fig. 1a) until the addition of auxin and cytokinin at 48 h, no morphological changes are observed by light microscopy. DAPI staining reveals no cell divisions at 48 h of culture (Fig. 1b). By 72 h, some cells have elongated, although cell division is not visible using the light microscope (Fig. 1c). However, DAPI and Calcofuor staining reveal the first cell divisions at 72 h (Fig. 1d), which were scored at an average of 47% of living cells. Overt signs of tracheary element formation, in the form of secondary wall thickenings, are usually visible at about 96 h and completed by 120 h (Fig. 1e). Cell divisions can be seen in differentiated cells as cross walls that often interrupt the pattern of wall thickenings. Just before differentiation occurs, at 96 h, a great number of divisions make DAPI-stained cells appear as multi-cellular clusters (Fig. 1f). At the end-point of tracheary element formation, at 120 h, cells have autolysed their cell contents. An average of 97% of the initial cell population had divided

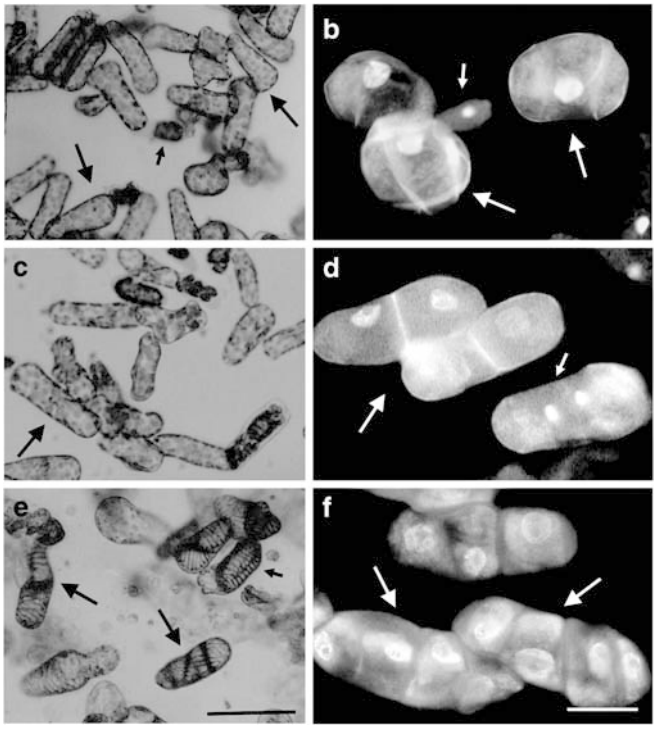

Fig. 1a–f Cell division and tracheary element formation in the zinnia system. **a** Freshly isolated mesophyll cells, consisting of living cells (*large arrows*) and dark green necrotic cells (*small arrow*) are placed in culture medium at 0 h. **b** Staining with 4′,6′-diamidino-2-phenylindole (DAPI) and Calcofluor shows only single cells (*large arrows*) between isolation and 48 h of culture. Dark cells (*small arrow*) are dead cells. **c** At 72 h, few morphological changes, apart from some signs of cell elongation (arrow), are visible. **d** DAPI and Calcofuor staining show cells having undergone (*large arrows*) or still undergoing (*small arrow*) their first division at 72 h. **e** Cell differentiation is usually initiated at 96 h and completed by 120 h in culture. At the final stage, tracheary elements display easily visible secondary wall thickenings (*arrows*). Division planes, either transverse (*large arrows*) or longitudinal (*small arrow*) are also visible in differentiated cells. **f** By 96 h, cells have divided several times and multiple DAPI-stained nuclei (arrows) are observed by UV epifluorescence. *Bars* **a**, **c**, **e** 100 μm; **b**, **d**, **f** 50 μm

(several times) by 96 h. Tracheary element formation, initiated at about 96 h, was scored at 120 h at an average of 33%. We therefore conclude that almost all cells go through cell division before differentiating to tracheary elements. If auxin and cytokinin are added upon initial cell isolation, as described in Fukuda and Komamine (1980a), then 95% of cells divide by 96 h. Fukuda and Komamine (1980b), using simple visual inspection to assay cell division, reported that only 30% of tracheary elements had previously divided in the culture. Using bright-field microscopy to score division in a similar way, on the basis of visible cross walls between daughter cells, we recorded only 52% of cells as divided by 96 h. This discrepancy is explained by the observation that some of the cross walls (stained with Calcofluor white) were not visible with bright-field microscopy and it is therefore likely that division levels were previously under-estimated.

To address whether cell division may be a prerequisite for tracheary element formation, we used the inhibitor aphidicolin to block S phase progression (Glab et al. 1994; Planchais et al. 1997), and consequently cell division in the zinnia system (Fukuda and Komamine 1981b). Aphidicolin was diluted in DMSO, and added to the culture at 48 h at final concentrations of 0, 5, 10, 20, 50 and 100 μM, together with auxin and cytokinin. Cell division, scored at 96 h, showed a dose-dependent response to aphidicolin and was inhibited at concentrations of 50 μM and above (Fig. 2a). Cell differentiation was observed even at a concentration of 50 μM aphidicolin, at which cell division was entirely blocked. We conclude that completion of the cell cycle after the early S phase, at which point aphidicolin arrests, is not required for tracheary element formation. In fucoid zygotes (Corellou et al. 2000) or Xenopus embryos (Rollins and Andrews 1991), early morphogenetic events can also occur when the cell cycle has been arrested by aphidicolin.

At concentrations of aphidicolin between 5 and 20 μM, the percentage of tracheary element formation was significantly enhanced, whilst division was only partially inhibited. It is possible that, at low concentrations, aphidicolin only prolongs S phase progression (Reichheld et al. 1995) and that this delay is associated with enhanced levels of tracheary element differentiation.

Roscovitine inhibits tracheary element formation and decelerates the cell division cycle

To investigate the possibility that the early cell cycle, before S phase, plays a role in tracheary element differentiation, we used a second cell cycle inhibitor, roscovitine (Meijer et al. 1997), which can inhibit both the G1/S and the G2/M transitions in plant cultures (Planchais et al. 1997; Trehin et al. 1998). Zinnia cells at 48 h are induced to enter the cell cycle and, with the simultaneous addition of roscovitine, we expected to block the first G1/S transition. Cell differentiation was entirely blocked at 50 μM roscovitine (Fig. 2b) or higher, although the ability of cells to divide by 96 h was not entirely blocked even at the highest concentration of roscovitine (Fig. 2b). However, cell division at 72 h, when about one-half of the cells have divided only once, decreased in a similar dose-dependent pattern to the levels of tracheary element formation (Fig. 2c). A closer look at the DAPI-stained cells at 96 h revealed that the number of divisions decreased in a dose-dependent manner, implying that roscovitine delayed rather than blocked cell cycle progression. In 0.8 μM roscovitine, more than 50% of cells had undergone between two and four rounds of division, whereas in 20 μM roscovitine, only 28% of cells had divided more than once by 96 h, although dividing ability appears similar in these two concentrations of roscovitine (Fig. 2b). In the presence of 50 μM roscovitine, 61% of cells had divided by 96 h

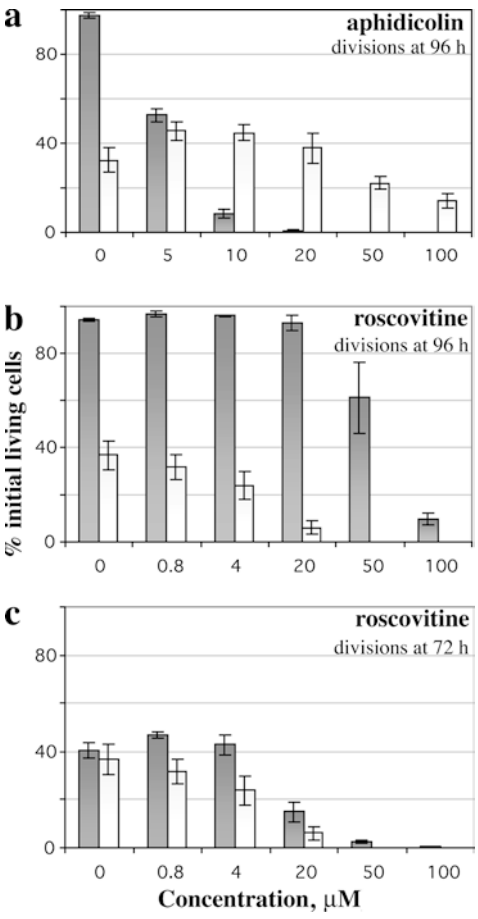

Fig. 2a–c The effect of aphidicolin and roscovitine on cell division and tracheary element formation. *Dark grey columns* Percentages of dividing cells, *pale grey columns* percentages of tracheary element formation in each panel. **a** Percentages of cell division (scored at 96 h) and differentiation (scored at 120 h) vs concentration of aphidicolin. At concentrations of between 5 and 20 μM aphidicolin, cell cycle progression is inhibited but not entirely blocked, whereas tracheary element formation is recorded at higher levels than the control. Between 50 and 100 μM, no cell division occurs, but tracheary elements are still formed. The mean values of four independent experiments are shown together with error limits. **b** Percentages of division (scored at 96 h) and differentiation (scored at 120 h) vs concentration of roscovitine. Although there is a clear dose-dependent effect on tracheary element formation, with a complete block at 50 μM, the ability of cells to divide remains high up to a concentration of 100 μM roscovitine. **c** Percentages of cell division scored at 72 h—when most cells have only divided once—and differentiation scored at 120 h vs roscovitine concentration. Cell division levels scored at 72 h show a similar dose-response to roscovitine as tracheary element formation. The mean values from three independent experiments are shown together with error limits

and only once, and in 100 μM roscovitine, only 10% of cells had divided and only once.

Roscovitine inhibits progression through the cell cycle

To test whether roscovitine targets the G1/S or the G2/M transition in culture, we mapped the timing of inhibition by roscovitine onto the S phase arrest by aphidicolin. First, 50 μM aphidicolin was added between 48 h and 72 h of culture, after which the cells were washed in conditioned medium and then 50 μM roscovitine was added. At 96 h, 63% of cells had divided once, i.e., they were blocked by roscovitine at the first G1/S after release from S phase, and 37% had not divided, i.e., they were blocked by roscovitine at G2/M. None of these cells subsequently form tracheary elements. In the reciprocal experiment, roscovitine was added in culture at 48 h, and, at 72 h, cells were transferred to conditioned medium to which aphidicolin was added. We scored no divided cells at 96 h, suggesting that roscovitine had blocked cells entirely in G1/S transition before the S-phase block by aphidicolin. After release from the G1/S block, cells arrested in S phase can differentiate to form tracheary elements.

From these two experiments, it appears that 50 μM roscovitine can inhibit the cell cycle at both the G1/S and the G2/M transitions, when added after an S phase arrest, but impedes G1/S transition when added to quiescent zinnia cells at 48 h. The preferential inhibition of G1/S phase can be explained by a stronger roscovitine affinity to "cognate" cyclin-dependent kinases, which function at the G1/S transition, than the "variant" cyclin-dependent kinases, members of which are only present at G2/M (Magyar et al. 1997; Porceddu et al. 2001).

There are several models to explain the effects of roscovitine on the zinnia culture. The simplest is that roscovitine blocks differentiation through inhibition of cell cycle progression. Roscovitine may affect one or more cyclin-dependent kinases that are involved in both the G1/S transition of the cell cycle and the differentiation process in the zinnia system. Factors affecting initiation of DNA replication can also be involved in controlling development; for example geminin in Xenopus has dual roles in S-phase and neuronal development (Kroll et al. 1998). Other developmental processes, such as the transition to flowering, require cells to be within the cell cycle, suggesting that this may be a common mechanism (Dewitte and Murray 2003).

A second hypothesis is that cell cycle progression may provide windows of competence to differentiation factors. In animal systems, HL-60 cells (Hatse et al. 1999) and osteoclasts (Meiyanto et al. 2001) specifically differentiate at the S phase whereas Drosophila neural precursors acquire competence after G2 arrest (Negre et al. 2003). Delamination of premigratory neural crest cells occurs specifically during S phase and is blocked by inhibition of the G1/S transition (Burstyn-Cohen and Kalcheim 2002). If we hypothesize that zinnia cells at the early S phase are most "competent" to respond to differentiation-inducing signals, then we can explain why delaying cells in S phase with aphidicolin treatment enhances tracheary element formation. Roscovitine inhibits G1/S transition and perturbs progression into S phase, and is also a potent inhibitor of cell differentiation. Both of these models are supported by the similarity in the dose response curves of the two processes to roscovitine inhibition, the apparent requirement for at

least cell cycle entry, and, indirectly, by the restoration of the competence of cells to differentiate once they are released from a roscovitine-induced block at the G1/S transition and subsequently blocked in S phase.

Finally, it is very likely that roscovitine may have multiple targets as plants contain a large family of cyclin-dependent kinases and similar proteins, few of which have been well characterised (Dewitte and Murray 2003). Whether part of the cell cycle machinery or not, it will be important to identify the target molecules for roscovitine in order to understand the molecular basis of differentiation.

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