Manipulation of leaf shape by modulation of cell division

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SUMMARY

The role of cell division as a causal element in plant morphogenesis is debatable, with accumulating evidence supporting the action of cell division-independent mechanisms. To directly test the morphogenic function of cell division, we have utilised a microinduction technique to locally and transiently manipulate the expression in transgenic plants of two genes encoding putative effectors of the cell cycle, a tobacco A-type cyclin and a yeast cdc25. The results show that local expression of these genes leads to modulation of cell division patterns. Moreover, whereas altered cell division in the apical meristem had no influence on organogenesis, local induction of cell proliferation on the flanks of young leaf primordia led to a dramatic change in lamina development and, thus, leaf shape. These data indicate that the role of cell division in plant morphogenesis is context dependent and identify cell division in the leaf primordium as a potential target for factors regulating leaf shape.

Key words: Cell division, Morphogenesis, Leaf, Meristem, Tobacco

INTRODUCTION

Significant progress has been made in our understanding of the plant cell cycle (reviewed by Huntley and Murray, 1999; Mironov et al., 1999). One conclusion from this work has been that although the plant cell cycle shows many similarities with that found in other eukaryotes, differences are also apparent. In particular, genome sequencing strategies have revealed a plethora of genes encoding putative components of the cell cycle machinery (e.g., cyclins, cyclin dependent kinases). Whether all of the proteins encoded by these genes function in the cell cycle in an analogous way to that described for similar genes in other eukaryotes is at present unclear. Expression profiles provide some clues as to the potential function of such gene products. For example, analysis of synchronised tobacco cell cultures led to the identification of an A-type cyclin (\textit{Nicta};\textit{CycA3};2) which, according to the pattern of transcript accumulation during the cell cycle and nuclear localisation of the encoded protein, showed the expression pattern expected of a cyclin involved in G1-S phase transition (Reichheld et al., 1996; Chaubet-Gigot, 2000). However, data supporting a functional role of the \textit{Nicta};\textit{CycA3};2 encoded protein in promoting progress through the cell cycle is lacking.

In addition to this lack of understanding of the role of specific gene products in the cell cycle, the actual function or necessity for cell division in plant development, and specifically morphogenesis, has long been debated (Doonan, 2000). Thus, although modulation of the expression of gene products involved in the cell cycle machinery can result in altered plant growth rate (Doerner et al., 1996; Cockcroft et al., 2000), the morphology of such plants is surprisingly normal. In addition, mutants have been identified that show altered planes of cell division throughout the plant, yet organ morphology is unaffected (Smith et al., 1996). Even mutants in which the cell division plane is severely disrupted can still generate basic elements of plant anatomy (Traas et al., 1995). These data support the hypothesis that plant morphogenesis can occur by cell division-independent means (Kaplan, 1992), with modulation of cell wall extensibility being a prime candidate as an alternative mechanism to cell division as the driving or restraining force for morphogenesis (Fleming et al., 1997; Pien et al., 2001a). However, the experiments so far reported do not disprove a role for cell division in morphogenesis and, in particular, it has been argued that local gradients in cell division might still be present in the transgenic and mutant plants described and that these local gradients might be crucial for appropriate morphogenesis (Meyerowitz, 1996). We set out to test this hypothesis by using a system that allowed us to locally manipulate patterns of cell division and observe the outcome on morphogenesis. As a target tissue, we focussed on the early stages of leaf development since data in the literature suggested that a specific pattern of cell division might be important for morphogenesis in this organ.

Leaves arise in a co-ordinated pattern from a specific organ, the apical meristem (Steeves and Sussex, 1989). This organ consists of indeterminate dividing cells. Some daughter cells from the meristem are incorporated into leaf primordia via organogenesis. Cells within leaf primordia generally continue to divide for a period of time but are determinate. There are no
fixed patterns of cell division during leaf development, rather
a stochastic gradient of termination of cell division, with cells
at the distal tip of the leaf exiting the cell cycle before the more
proximal ones (Donnelly et al., 1999). One exception to this
stochastic process involves cells along the periphery of the
young primordium at the presumptive leaf margin. Cells in this
region undergo a burst of cell division (Donnelly et al., 1999)
which is followed by a specific phase of differentiation in
which marginal cells undergo cell wall thickening and
expansion parallel to the margin (Poethig and Sussex, 1985).
At about the same time the process of lamina extension occurs
and this has led to the proposal that the specific pattern of
cellular events at this early stage of leaf development is
causally involved in the process of lamina formation.

To test this hypothesis, we utilised a novel technique to
locally and transiently induce the expression of genes in small
tissue parts (Pien et al., 2001a) This microinduction approach
allowed us to manipulate the expression of genes postulated
to play a role in the cell cycle in intact plant tissue. This
allowed us both to test the functionality of gene products
proposed to play a role in the cell cycle (in particular, Nicta;CycA3;2) and to observe the outcome of such altered
patterns of cell division on morphogenesis. The results
indicate that the influence of cell division on plant
morphogenesis is context dependent.

MATERIALS AND METHODS

Plant transformation, regeneration and microinduction

R7 Nicotiana tabacum seedlings (a gift from C. Gatz, University of
Goettingen) were transformed and regenerated as previously
described (Pien et al., 2001a). For RNA analysis, F1 seeds were
germinated on sterile Whatman paper submerged in Murashige and
Skoog (MS) medium under a 16 hour light/8 hour dark regime. For
induction, 1 μg/ml Ahtet (anhydrotetracycline) was added to the
medium and the culture medium renewed every third day. Two-week-
old plantlets were used for RNA blot analysis. For RT-PCR analysis,
leaf disks from line S7b were incubated on MS medium with or
without Ahtet, at the concentrations and for the times given in
the Results section, before RNA extraction, as previously described (Pien
et al., 2001a). Tet::Sp;cdc25 seeds were obtained from D. Francis
(University of Cardiff, UK) and have been characterised by McKibbin
et al. (McKibbin et al., 1998).

DNA manipulation

The full length Nicta;CycA3;2 cDNA was inserted as a transcriptional
fusion into the pBinHyg-Tx vector (a gift from C. Gatz). The resultant
cloned pBinHyg-Tx-Nicta;CycA3;2 was transformed into R7 tobacco
plants. All DNA manipulations were by standard procedures
(Sambrook et al., 1992).

RNA analysis

RNA blot analysis was as previously described (Pien et al., 2001a)
using a radioactively labelled probe for Nicta;CycA3;2. Quantitative
RT-PCR was performed as previously described (Fleming et al.,
1996). After reverse transcription, cDNA substrate dilutions were
amplified within the linear range with primers for Nicta;CycA3;2. In
situ hybridisation was as previously described using digoxigenin-
labelled antisense riboprobes for Nicta;CycA3;2 and histone H4 (Pien
et al., 2001b).

Histology and electron microscopy

For histological analysis, samples were embedded in Technovit
according to the manufacturer’s instructions for thin section analysis.
Cryo-SEM was as previously described (Fleming et al., 1999).

RESULTS

Generation and characterisation of transgenic plants

The Nicta;CycA3;2 coding region was cloned behind a TetO
promoter in the pBin-Tx vector (Gatz et al., 1992) and the
resulting construct transformed into tobacco plants engineered
to overexpress the Tet repressor protein (a kind gift from C.
Gatz, University of Goettingen, Germany). In such a
background transcriptional activity from the TetO sequence is
repressed until the addition of Ahtet. Eight independent
transgenic lines (Tet::Nicta;CycA3;2) were obtained which showed
single locus inheritance (data not shown) and these were analysed for Ahtet-inducible accumulation of
Nicta;CycA3;2 transcripts. Results for two lines are shown in
Fig. 1. Tobacco plants engineered to contain the GUS reporter
gene under tetracycline-inducible transcriptional regulation
(Pien et al., 2001a) were used as a control (Tet::GUS).

In non-induced Tet::Nicta;CycA3;2 and Tet::GUS lines a
low level of endogenous Nicta;CycA3;2 gene expression was
detectable (Fig. 1A). However, after induction with Ahtet a
large accumulation of Nicta;CycA3;2 transcripts occurred in
lines S7b and S7c. This was not observed in control Tet::GUS
plants treated with Ahtet. RT-PCR analysis indicated that
Nicta;CycA3;2 transcript accumulation was detectable in
Tet::Nicta;CycA3;2 tissue after induction with Ahtet
concentrations as low as 0.002 mg/ml, with maximal
accumulation occurring at concentrations between 0.02 and 0.2
mg/ml (Fig. 1B). Using an Ahtet concentration of 0.2 mg/ml,
a time course of Nicta;CycA3;2 transcript accumulation
showed an increase within 2 hours of induction with a
maximum occurring between 4 and 8 hours, followed by a
decrease in transcript level (Fig. 1C). Lines S7b and S7c were
used for the microinduction experiments described below.

In addition to the Tet::Nicta;CycA3;2 lines, we also
performed microinduction experiments using plants transgenic
for a construct containing the cdc25 coding region from
Schizosaccharomyces pombe under tetracycline inducible
transcriptional control (Tet::Sp;cdc25). Previous work has
shown that Sp;cdc25 is able to dephosphorylate CDK/cyclin
complexes from tobacco (Zhang et al., 1996) and that
constitutive and inducible expression of Sp;cdc25 in plants
leads to increased rate of cell division and a phenotype
including altered lateral root initiation and twisted leaves (Bell
et al., 1993; McKibbin et al., 1998). We obtained seeds from
these plants (a kind gift from D. Francis, University of Cardiff,
UK) and used them in the experiments described below.

Local induction of Nicta;CycA3;2 and Sp;cdc25
alters cell division in leaf primordia

Since both our data and those of other workers had identified
cell division in the primordium flank as a potential site involved
in the control of leaf lamina formation (Donnelly et al., 1999;
Pien et al., 2001a), we first performed a series of experiments
using both Tet::Nicta;CycA3;2 and Tet::Sp;cdc25 plants in
which transgene expression was microinduced on one flank of
a primordium (stage P2-P3). The microinduction technique

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involves the positioning of Ahtet-impregnated lanolin onto the surface of dissected apices, leading to a localised source of inducer for gene expression (Pien et al., 2001a). Owing to the anatomy of the dissected shoot apex, this induction tended to be towards the abaxial side of the initiating lamina (Fig. 2A).

The earliest effects of local induction of either Sp;cdc25 or Nicta;CycA3;2 expression were observed after 24 hours (Fig. 2B,C). Induced Tet::Sp;cdc25 primordia displayed a disruption of cellular patterning at the site of induction leading most overtly to a disruption of vascular differentiation, which proceeded normally in the uninduced flank (Fig. 2B). The initial changes observed in the induced flanks of primordia from Tet::Nicta;CycA3;2 plants were more subtle but included a lack of vascular differentiation in the induced flank and a slight alteration of cellular patterning (Fig. 2C). However, after 40 hours the flanks of induced Tet::Nicta;CycA3;2 primordia were easily distinguished from non-induced or mock-induced flanks by the accumulation of a large number of small cytoplasmically dense cells (Fig. 2D). This difference in cytology between induced and non-induced flanks is shown in more detail in Fig. 2E,F. Stereological analysis revealed an average cell size in the induced area of 1881±683 μm³ (n=24) compared with 12432±2098 μm³ (n=24) in the non-induced area. This difference in average cell volume indicates that the induced flank lacks the ordered layered structure observed in the non-induced flank. Cell size in the induced lamina was more variable than in the non-induced flank and division orientation was disrupted. This disruption of cellular patterning was most obvious in the region of provascular formation leading to the lack or retardation of vascular differentiation. In some cases, local induction of Sp;cdc25 gene expression led to drastic changes in lamina morphology, two examples of which are shown in Fig. 2J,K. There was a general tendency for an increased number of cell layers compared with non-induced flanks (Fig. 2L), a concomitant decrease in lateral extension, a disruption of vascular patterning, a variability in cell size and shape and, in some instances, evidence of local cell death or compression.

**Altered cell division on the primordium flank leads to altered lamina growth and leaf shape**

The alterations in cell division pattern observed immediately following the transient microinduction of Nicta;CycA3;2 and Sp;cdc25 gene expression in primordia flanks led to a major change in lamina growth and leaf shape. Fig. 3A shows the results of a series of experiments in which Ahtet-impregnated lanolin was placed at various positions along the flank of young primordia (P2-P3 stage) of Tet::Nicta;CycA3;2 plants. In each case, the position of Ahtet induction was equivalent to the later formation of a lamina indentation, with induction along the entire primordium flank leading to the reduction of lamina expansion along the entire side of the leaf. In each case, the opposite, non-induced flank underwent morphogenesis to generate a normal ovate leaf structure. A series of similar experiments with Tet::Sp;cdc25 primordia led to similar results (Fig. 3B). Lamina indentation occurred only in the area corresponding to Ahtet induction and the extent of inhibition of lamina expansion was even more drastic than that observed after induction of Nicta;CycA3;2 gene expression.

Alteration in lamina shape was observed at high frequency, both in Tet::Nicta;CycA3;2 apices (46 cases, n=65) and Tet::Sp;cdc25 apices (18 cases, n=22). Control experiments in which Ahtet was manipulated onto the flanks of primordia of Tet::GUS plants very rarely led to alteration in lamina growth (2 cases, n=40), as was also observed with buffer inductions of Tet::Nicta;CycA3;2 apices (1 case, n=31).

A cross section of an asymmetric leaf induced by microinduction of Nicta;CycA3;2 gene expression is shown in Fig. 4A. In addition to the difference in lamina expansion between induced and non-induced flanks, histological differences are also apparent. Thus, as shown in the insets, the induced flank lacks the ordered layered structure observed in the non-induced lamina. This lack of order is reflected in limited vascular differentiation and, most notably, in the lack of hairs originating from the epidermal cells of the induced lamina. In addition, stereological analysis indicated that
average mesophyll cell size in the induced lamina (8598±2647 μm³, n=9) was larger than that in the non-induced lamina (5143±1378 μm³, n=9) and that this difference in size was statistically significant (P=0.02). This increase in mean cell size coupled with the decrease in lamina expansion led to the induced lamina of Tet::Nicta;CycA3;2 leaves consisting of fewer cells than the uninduced lamina. The resulting lamina asymmetry can be compared with the symmetrical leaves of the controls in which tetracycline was locally applied to Tet::GUS primordia or primordia of Tet::Nicta;CycA3;2 apices were treated with buffer (Fig. 4B).

The data described above indicate that a transient promotion in cell division on the primordium flank leads to decreased lamina expansion. To test the hypothesis that premature termination of cell division on the primordium flank might lead to promotion of lamina expansion, we performed experiments in which roscovitine (a specific inhibitor of CDK activity (Binarová et al., 1998)) was placed on the flanks of primordia of wild-type plants. The results, shown in Fig. 5, showed that there was a moderate but distinct increase in lamina expansion in the area corresponding to that treated with roscovitine compared with control Ahtet-treated primordia from Tet::GUS plants (10 cases, n=20) (compare Fig. 5A and 5B). Histological analysis of the roscovitine-induced lamina revealed the presence of all appropriate cell types in the appropriate regions (Fig. 5C) similar to that observed in non-induced lamina (Fig. 5D). Stereological analysis of roscovitine-induced and non-induced laminas revealed a slight increase in average parenchyma cell size in roscovitine-treated tissue (41931±16125 μm³, n=16) compared with non-treated laminas (34620±1643 μm³, n=16), but this difference was not statistically significant. The roscovitine-induced change in lamina shape can be compared with that...
observed following local induction of expression of a gene encoding the cell wall protein expansin on the flank of a leaf primordium (Fig. 5E) (Pien et al., 2001a). Expansin increases cell wall extensibility (Cosgrove, 2000) and our previous data showed that local induction of expansin expression on the flank of leaf primordia led to local promotion of tissue growth (Pien et al., 2001a). The increase in lamina growth promoted by either roscovitine or expansin contrasts with the inhibition of lamina growth produced by local induction of \textit{Nicta;CycA3;2} gene expression (Fig. 5F).

**Ectopic cell division in the meristem does not result in altered meristem form or leaf initiation**

Our previous experiments had indicated that local alteration in cell wall extensibility in the meristem (via altered expression of the cell wall protein expansin) led to morphogenesis and leaf initiation (Fleming et al., 1997; Pien et al., 2001a). To test whether altered cell division pattern could also influence meristem function, we performed a series of experiments with both Tet::Nicta;CycA3;2 and Tet::Sp;cdc25 plants in which transgene expression was microinduced in the apical meristem.

**DISCUSSION**

**Microinduction of \textit{Nicta;CycA3;2} and \textit{Sp;cdc25} gene expression leads to local cell proliferation**

A number of studies have shown that plant cell division can be manipulated by the modulation of expression of individual
components of the cell cycle machinery. Thus, altered expression of D-type cyclins (Riou-Khamlichi et al., 1999; Cockcroft et al., 2000), B-type cyclins (Doerner et al., 1996), and kinases and phosphatases associated with the cell cycle (Hemerley et al., 1995; McKibbin et al., 1998) have been shown to modulate cell division rate in transgenic plants via constitutive overexpression or dominant-negative strategies. We provide functional data that an A-type tobacco cyclin (Nicta;CycA3;2) and a heterologous cdc25 from S. pombe can modulate plant cell division.

Overexpression of Nicta;CycA3;2 promoted cell proliferation, as shown by the accumulation of relatively small, cytoplasmically dense cells at the site of induction relative to both surrounding non-induced cells and cells that had been mock-induced. This direct visualisation of meristematic activity is supported by the analysis of marker genes associated with progress through the cell cycle. Thus, an increased frequency of cells expressing a histone S-phase marker was observed in tissue induced to express Nicta;CycA3;2 compared to non-induced or mock-induced tissue. Although local induction of Sp;cdc25 also led to some alteration in cell proliferation, this was accompanied by a disruption of cytokinesis leading to the production of daughter cells of more variable size than observed following Nicta;CycA3;2 induction. Since Nicta;CycA3;2 is an endogenous gene of tobacco, whereas Sp;cdc25 is of yeast origin, the difference in cellular outcome following Nicta;CycA3;2 or Sp;cdc25 induction might reflect either the different roles of these gene products in the cell cycle or the different origins of the genes introduced into the transgenic plants.

Both tissues in which micro-inductions were successfully performed (meristem and leaf primordium) contain proliferating cells. Thus, it is likely that ectopic expression of Sp;cdc25 and Nicta;CycA3;2 increased the rate or prolonged the time over which cell division was occurring rather than caused re-entry into the cell cycle. Whether modulation of cdc25 or cyclinA activity reflects an endogenous mechanism for modulating cell proliferation in plants is unclear, particularly in the case of cdc25 for which no plant homologue has yet been identified. However, our data show that both Sp;cdc25 and Nicta;CycA3;2 have the potential to influence the cell cycle and can be used as tools to locally manipulate patterns of cell division.

The influence of cell division on plant morphogenesis is context dependent

Local ectopic expression of Nicta;CycA3;2 led to local increase in cell proliferation, both in the meristem and leaf primordia. However, whereas local cell proliferation in the meristem led to no overt effect on morphogenesis, similar manipulation on the flanks of leaf primordia led to a dramatic change in leaf morphology. These data indicate that the influence of cell division on plant morphogenesis is context dependent. Cells in the meristem are indeterminate and possess a great capacity for accommodating to altered rates of cell division to restore appropriate meristem size and function. The recent description of interacting endogenous regulators of cell proliferation in the meristem provides a system by which meristems might respond to and counteract disturbances to the balance of cell division within this organ (Schoof et al., 2000). In addition, individual cell size appears to be highly regulated within the meristem. Our previous work in which local expression of expansin was used to promote growth, led to leaf initiation without any overt change in cell size, suggesting a tight linkage between cell volume and division (Pien et al., 2001a). Promotion of cell division by overexpression of Nicta;CycA3;2 (reported here) led to the local accumulation of smaller cells in the meristem. However, this was transient and appropriate cell volume was later restored with no overt disturbance to meristem function. Thus, there appear to be powerful mechanisms to maintain a balance between cell size and division frequency within the plant. Constitutive modulation of the cell cycle machinery may lead to constitutive activation of such mechanisms, leading to the limited influence of such manipulations on morphogenesis, as observed in
Cell division and leaf morphogenesis

Transient local cell proliferation in primordia (consequent to induction of either Nicta;CycA3;2 or Sp;cdc25 gene expression) led to the later formation of lamina indentation at the site of induction, i.e., decreased final growth of the tissue and fewer cells, whereas transient inhibition of cell division (via local action of roscovitine) led to the formation of local lamina expansion. How should these counter-intuitive observations be interpreted? One possibility is that a transient increase in cell proliferation leads to subsequent cessation of cell division. Thus, while tissue surrounding the area of Nicta;CycA3;2 or Sp;cdc25 gene induction continues to grow by division-associated expansion, the tissue that has been transiently induced into extra divisions can afterwards only grow by expansion. This might lead to an increase in average cell volume in this tissue (as was observed), but the final total number of cells in the induced area is decreased and, thus, final tissue volume (lamina growth) is decreased. Conversely, transient inhibition of cell division by roscovitine might lead to a transient increase in tissue expansion, i.e., cell proliferation and expansion rate are inversely related. As cell proliferation is later resumed following the transient effect of roscovitine, this increased tissue volume (lamina expansion) would become divided into cells of appropriate size as the normal relationship between cell size and division was re-established. This situation would be comparable to that subsequent to a transient local increase in expansin activity (Pien et al., 2001a) in which local increase in lamina growth is accompanied by cell division to generate an appropriate internal histology.

It should be noted that in addition to its interaction with tissue growth, altered cell division is also likely to impinge on cell differentiation. This was observed in our experiments both in the epidermis (decreased hair cell formation) and in internal tissues, most notably in altered vascular differentiation. Interference with vascular formation would disrupt the flux of carbon and water and, thus, have a major impact on local tissue growth.

Although the exact mechanism by which altered cell division impacts on leaf morphogenesis is still to be ascertained, our data show that discontinuities in cell division status within the primordium can drastically alter local growth rates and, thus, leaf shape. This is consistent with the hypothesis that in determinate organs (such as leaves) there are at least some stages of development (e.g., lamina initiation) when specific patterns of cell division are causally involved in morphogenesis (Donnelly et al., 1999). Taken in conjunction with recent advances in the identification of transcriptional networks involved in the regulation of leaf development (Hudson, 2001), our data suggest that elements of the cell cycle machinery might be key downstream targets of these regulatory systems. Finally, our data highlight the importance of the cellular decision to either remain in or to exit the cell cycle as a key step which can influence not only individual cell fate but also, at a higher level of organisation, the morphology of an organism.

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