The role of cell division in leaf morphogenesis

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*Nicotiana tabaccum* leaves. First picture from the left - deformation of the leaf blade resulting from increased cell proliferation induced at the primordium stage; picture in the middle - control leaf; last picture - deformation of the leaf blade resulting from inhibition of cell division introduced at the primordium stage.
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The aim of this study was to better understand the role of cell division in leaf initiation and morphogenesis. To do this, experiments were designed to modulate cell division and observe the influence on leaf development. Two aspects of cell division were the targets of this manipulation: the frequency and the plane of cell division. The strategy taken was to produce transgenic plants containing either components of the cell cycle machinery (for the manipulation cell division frequency) or elements of the cytokinetic machinery (for the manipulation of the plane of cell division) under transcriptional control of a tetracycline inducible promoter. To locally microinduce gene expression in meristem or young leaf primordia a new technique was developed. The genes selected for this approach were *Nicta;*cycA3;2 (encoding a plant cyclinA), *Sp;*cdc25 (encoding a yeast cdc25 phosphatase) and *Glyma;*phragmoplastin (encoding a plant dynamin-like protein). By local application of tetracycline onto meristems or young leaf primordia we aimed to precisely control the local overexpression of these gene products and, thus, to alter either the proliferation rate or the orientation of cell division.

In the meristem, both increased cell proliferation and disruption of the pattern of cell division (indicated both by histological analysis and marker gene expression patterns) did not lead to altered meristem function or leaf initiation. However, altered cell proliferation rate on the flank of young leaf primordia dramatically affected final leaf shape, with a transient increase in cell division leading to a final decrease in lamina expansion. Conversely, transient inhibition of cell division in this tissue (via local application of a cyclin dependent kinase inhibitor) led to an increase in lamina expansion. These data show that the role of cell division in plant morphogenesis is context dependent. The meristem displays adaptability to altered cell division frequency and orientation whereas there is a certain stage of leaf development when local cell division rate can have a major impact on morphogenesis. Taken in conjunction with other data, the results reported here identify the local cell proliferation rate as potentially a key step in the control of leaf shape. The mechanism by which this might occur is discussed.
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Joanna Wyrzykowska, Stephane Pien, Wen Hui Shen, and Andrew Fleming

**Manipulation of leaf shape by modulation of cell division**

*Development (in press)*
ZUSAMMENFASSUNG


Im Meristem führten weder eine erhöhte Teilungsfrequenz noch die Störung der Zellteilungsmuster (durch histologische und Markergenanalyse nachgewiesen) zu einer veränderten Meristemfunktion oder Blattinitiierung. Hingegen hatte eine veränderte Zellteilungsraten an der Seite junger Blattprimordien einen dramatischen Effekt auf die Blattform, wobei eine vorübergehend erhöhte Zellteilungsraten zu einer Abnahme der Laminaexpansion führte. Diese Daten zeigen, dass die Rolle der Zellteilung in der Blattmorphogenese Kontext abhängig ist. Das Meristem zeigt gegenüber geänderter Zellteilungsfrequenz und -ebene hohe Toleranz, während in einer gewissen Stufe der Blattentwicklung die lokale Zellteilungsraten einen ausgeprägten Effekt auf die Blattmorphogenese haben kann. Zusammen mit anderen
Daten, zeigen die hier präsentierten Resultate, dass die Kontrolle der lokalen Zellteilungsraten ein Schlüsselmechanismus in der Ausbildung der Blattform darstellen könnte.

Ein Teil dieser Arbeit ist veröffentlicht worden oder befindet sich zur Zeit im Druck:

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Joanna Wyrzykowska, Stephane Pien, Wen Hui Shen, and Andrew Fleming Manipulation of leaf shape by modulation of cell division *Development (in press)*
INTRODUCTION

All living organisms are made up of cells. In their development from embryo to adult organism they undergo morphogenesis by cell division and enlargement of pre-existing cells that results in the generation of complete organisms with all the necessary organs of appropriate size and shape. Although it seems obvious that cell production must be a key issue in creating a new organism, there is accumulating evidence supporting the possible separation of cell division from morphogenesis (Doerner et al., 1996; Foard, 1971; Haber, 1962; Steeves and Sussex, 1989). Thus, whether cell division is a driving force in plant morphogenesis or an independent process remains debatable.

Contrary to animals, plants develop new organs in a sequential manner. While animals have the fundamental organisation of the future adult body (including the presence and arrangement of future organs) inscribed in their embryos, plants have a plan for only a primary structure that will become a base to develop the whole plant body. This primary structure contains organs which are the sources of cells, which initiate and organize morphogenetic events and which assure future development into an adult organism. These organs are called the primary meristems and are located (in the case of Angiosperms) at the top of both the shoot and root of the embryo and, later, of the adult plant. The main functions of these meristems are to self-renew and stay in an undifferentiated state and, via the generation of daughter cells, to produce the cells required for the formation of organs such as leaves, stem and root. All these processes involve cell division.

This work investigates aspects of leaf initiation and morphogenesis and their relation to cell division in *Nicotiana tabacum*. In order to understand the background and logic of the experiments presented, an understanding of the structure and function of the shoot apical meristem as well as the cell cycle machinery is required. These are described in the following part of the introduction. All parts describing morphogenesis and meristem function are based on Angiosperm development with the focus on two model plants: *Arabidopsis thaliana* and *Nicotiana tabacum*. 
INTRODUCTION

1. THE SHOOT APICAL MERISTEM AND PLANT MORPHOGENESIS

Postembryonic organogenesis in higher plants is meristem mediated. The shoot apical meristem (SAM) and root apical meristem (RAM) are two driving forces in the development and expansion of the shoot and root system. In the case of shoots, new leaves appear in an orderly sequence (phyllotaxis). This suggests the existence of a program dictating the initiation of new organs at particular times and places. Many scientists have investigated the SAM and have shown that it is highly organized in terms of morphology, histology and genetic regulation (Lyndon, 1998; Steeves and Sussex, 1989). One of them showed amazement at this structure by stating: "The meristem of the shoot apex is a remarkable structure: it is about the size of pinpoint, often only about a tenth of a millimeter in diameter, may consist of fewer than a thousand cells, not apparently very different from each other, yet it gives rise to the whole of the shoot." (Lyndon, 1998)

1.1. Morphology of the shoot apex. The meristem is placed in the apical bud of the shoot and is surrounded by existing leaves and leaf primordia, creating a positional relationship between all these structures. All leaves occur first as primordia in predictable positions on the meristem. The sequential development of leaves can be numerically described, as can the areas of the meristem that will produce leaf initials in the future. Thus, the portion of the meristem that is about to produce a new initial is called I1 (sometimes termed P0) and the next one, still silent and waiting for its turn to initiate is termed I2. Primordia that have already initiated are called P1, P2, P3 and so on, with P1 being the most recently formed primordium (Fig.1). This ordered arrangement of organs around the meristem creating a spiral (by drawing a line linking the sequence of leaf initiation) is known as one of three types of phyllotaxy (Simon, 2001; Steeves and Sussex, 1989).
1.2. The meristem is organised into domains and zones. As a result of histological analyses and the analysis of chimeras the meristem has been subdivided in two different manners. On the one hand the meristem can be viewed as consisting of layers in which the clonal derivatives of a layer are limited in their fate. Thus, there are 3 layers distinguished (L1, L2 and L3) in which L1 derivatives form the epidermis, L2 derivatives generally form mesophyll tissues and L3 derivatives generally form inner tissues and vasculature. At the histological level, the L1 and L2 layers create the tunica and L3 the corpus (Fig.2a). In the tunica there are only anticlinal divisions which align the cells into layers while in the corpus cells divide in all directions and thus do not generate any defined layered organization (Steeves and Sussex, 1989; Vaughan, 1952). Another way of subdividing the meristem distinguishes the presence of three zones: the central zone (CZ), the peripheral zone (PZ) and the rib zone (RZ) (Fig.2b). These zones differ between each other in the frequency of cell division. It is now generally accepted that CZ acts as a population of stem cells with an underlying organizing centre generating the
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Fig. 2 Histology of shoot apical meristem (SAM). (adapted from J.L. Bowman and Y. Eshed, 2000)
a) Meristem consists of 3 layers - L1, L2 that create tunica and L3 - corpus
b) There are 3 zones distinguished in meristem: CZ - central zone, PZ - peripheral zone and RZ - rib zone.
initials for the two other zones and at the same time maintaining itself. Cells in
the CZ divide much slower than in PZ and RZ which are sources of initials for
leaves and shoot tissues, respectively (Barton, 1998; Lenhard and Laux, 1999;
Schoof et al., 2000; Vernoux et al., 2000a) The separation of the meristem into
different zones has also been interpreted as reflecting the presence of
symplastic fields. (groups of cells connected preferentially via secondary
plasmodesmata). According to this model, in this way fields are created within
the meristem, which might facilitate the rapid spread of signals inside the field
but prevent spread of a signal between fields. The symplastic field model
approximately corresponds to the organisation envisaged in the zonation model
and provides an added functional interpretation of this model. All these models
envisage a sophisticated signalling network designed to help cells sense their
neighbourhood and their position in the meristem. Such positional information
is then translated into specific gene expression which finally dictates fate (Irish
and Jenik, 2001; Rinne and Schoot, 1998; Schoot and Rinne, 1999)

1.3. Maintaining the meristem in an undifferentiated state. One of the
meristem’s functions is to remain in an undifferentiated state during a plant’s
life span, thus assuring continuous organ production. Recent research shows
that a variety of genes contribute to the maintenance of a tight genetic
regulation of the undefined state of meristematic cells. In particular, the self-
regulatory system called the wuschel-clavata feedback loop plays an important
role. The homeobox gene WUSCHEL (WUS), expressed in the organizing
centre just proximal to the central zone, is required to promote stem cell fate
within the central zone. Stem cell fate is associated with the expression of
another gene (CLV3) which appears to be an extracellular ligand for a receptor
kinase complex encoded by CLV1. CLV1 is expressed in a group of cells at the
heart of the meristem encompassing those expressing WUS. The model thus
envisages a feedback loop in which excessive WUS expression leads to
increased CLV1 expression in the adjacent stem cells which, via CLV3, acts to
suppress WUS expression. (Fig3) (Bowman and Eshed, 2000; Clark et al., 1993;
Fig. 3 Expression patterns of genes involved in meristem maintenance (adapted from J.L. Bowman and Y. Eshed 2000).

a) *CLAVATA* mRNA is restricted to the tunica layers (L1 and L2) of central zone, *CLAVATA* mRNA is detected in the corpus (L3) of the central zone, *WUSCHEL* mRNA is restricted to a small group of cells called organizing center localized within the L3 of central zone.

b) Signaling interactions between *CLAVATA* and *WUSCHEL* genes, the yellow arrow indicates inductive activity of *WUSCHEL*, the pink bars indicate *CLAVATA* genes restrictive activity.
Clark and Schiefelbein, 1997; Mayer et al., 1998; Schoof et al., 2000). Thus the size of the stem cell population in the active shoot apical meristem is continually checked by the WUS/CLV interactions.

Other gene products also influence the size and cellular organization of the meristem and, in particular, the PZ. Thus mutations in MOGUN (MGO1 and 2) and FASCIATA (FAS 1 and 2) genes lead to enlarged meristems by the accumulation of cells in the PZ (Kaya et al., 2001; Laufs et al., 1998a; Laufs et al., 1998b). How these products are integrated into the WUS/CLV loop model is as yet unclear.

Meristem function is also influenced by expression of members of the Knotted gene family. Initial studies on Kn1 (Knotted-1) from maize, the first homeobox gene identified in plants, showed that ectopic expression led to the switching of cell fate towards more juvenile characteristics (Lincoln et al., 1994; Vollbrecht et al., 1991). Later studies in which the gene (or its homologue from Arabidopsis, KNAT1) was constitutively overexpressed in transgenic plants led to the formation of ectopic meristems along the leaves. Furthermore, mutants in a Kn-related gene, stm, in Arabidopsis led to the loss of meristem formation. Taken together, these data indicate an important role for Kn-like genes in meristem initiation and function, presumably via an effect on cell differentiation and division, although the precise mechanism remains obscure (Barton and Poethig, 1993; Bowman and Eshed, 2000; Long et al., 1996).

2. LEAF MORPHOGENESIS

2.1. Events in the meristem leading towards leaf initiation. Leaf initiation is first visible as a bulging on the meristem flank at the III position. Histologically, the first event associated with leaf initiation is the occurrence of periclinal divisions in the second tunica layer followed by the recruitment of a number of cells from the PZ to form the mass of the leaf primordium. Clonal analysis showed that the new primordium originates from all three cell layers of SAM (Poethig, 1987) and in tobacco comprises about 100 cells (Poethig and Sussex, 1985; Steeves and Sussex, 1989)
At the molecular level, a number of genes have been shown to be involved in leaf initiation. Thus, the event of initiation is correlated with downregulation of expression of *Knotted*-like genes at the II position. This step seems to be necessary to allow a portion of the cells in the PZ to enter the pathway to differentiation. A group of genes encoding Myb transcription factors show a complementary pattern of gene expression to *Knotted*. Transcripts for genes such as *PHANTASTICA* (*PHAN*) from Antirrhinum, *ROUGH SHEATH 2* (*RS2*) from maize and *ASYMETRIC LEAVES* (*AS2*) from Arabidopsis accumulate at the site of leaf initiation and it seems that the expression of these Myb transcription factors leads to the downregulation of *Knotted*-like genes (Schneeberger et al., 1998; Semiarti et al., 2001; Waites et al., 1998). At about the same time genes such *CUC1* and *CUC2* (*CUP-SHAPED COTYLEDONS*) from Arabidopsis or *NAM* (*NO APICAL MERistem*) from petunia are expressed. These gene products, which encode putative transcription factors, are associated with organ separation and mutation of these genes leads to a phenotype involving organ fusion. These transcription factors are also required for *Knotted*-like gene expression suggesting a role in meristem establishment (Aida et al., 1999; Souer et al., 1996; Vernoux et al., 2000b). Genetic analysis of two other genes, *MOGUN* and *FASCIATA* indicates a function in primordia formation, in addition to their role in meristem maintenance already described. *Fas* and *mgo* mutants show similar phenotypes of decreased organ number. Interestingly, double mutants between *fas* and *mgo* develop shoots that are almost incapable of forming leaves. Although both mutations show similar effects it is not known if they act on the same pathway (Kaya et al., 2001; Laufs et al., 1998a; Laufs et al., 1998b).

2.2. Phyllotactic patterning. For each leaf initiation a decision about the position of initiation has to be taken. The mechanism of leaf arrangement is not known although several theories in this subject have emerged during the last 70 years. Thus, in early experiments dating back to 1931 M.Snow and R.Snow successfully applied a surgical approach to remove some parts of the apex of *Lupinus*. The conclusions from their experiments were that the position of primordium initiation is influenced by the pre-existing leaf primordia adjacent to the site of initiation and that the new primordium arises in the first space that
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becomes available (Steeves and Sussex, 1989). Further theories suggested that positioning of primordia is determined by morphogenetic fields in the apex dictated by inhibitory signals emanating from pre-existing primordia (Steeves and Sussex, 1989; Wardlaw, 1949). Finally some recent studies have shed light on potential genetic influences on phyllotaxy. One of the genes shown to be involved in controlling phyllotaxy is ABPHYL (aberrant phyllotaxy) from maize. Mutation of this gene leads to opposite and decussate phyllotaxy as well as to an enlarged meristem (Jackson and Hake, 1999), suggesting a possible correlation between meristem size and phyllotaxy. A disruption of phyllotaxy is also associated with other mutations leading to altered meristem size, such as FAS and MGO (described in the section above). Another gene product which influences leaf positioning is encoded by TERMINAL EAR in maize. Transcripts for this gene accumulate in a horse-shoe like expression pattern within the meristem with a gap at the 11 position, suggesting that the gene product’s function is to prevent non-11 cells in the peripheral zone from entering the leaf initiation process. Mutant analysis of terminal ear confirm this hypothesis in that the phenotype involves the generation of a superfluous number of leaves in a disorganised order (Scanlon, 2000; Veit et al., 1998).

2.3. Axes of leaf development. A leaf acquires three axes of growth during its three-dimensional expansion. The first axis emerges already during initiation and it is a proximodistal axis. Genes influencing primordium initiation, such as the Knotted-like family or Phantastica-like genes are associated with regulation of this growth axis in early stages. At later stages of development the LEAFY PETIOLE (LEP) gene seems to be responsible for patterning along the proximal part of the axis. Thus, mutants of lep show conversion of the Arabidopsis leaf petiole into leaf blade (Graaff et al., 2000).

Growth in the other two axes (lateral expansion of the leaf blade and dorsiventrality) seem to be closely entwined and significant progress has been made in the identification of gene products which regulate this aspect of leaf development. The main concept that has emerged is that adaxial cell fate is dependent on signals from the shoot apex which activate proteins needed to repress expression of genes determining abaxial identity. Furthermore, lateral
expansion of the leaf blade seems to require the interaction of cells expressing adaxial and abaxial identity genes. Thus, in Antirrhinum, a recessive mutation in a gene encoding a Myb transcription factor phantastica leads to loss of dorsiventrality (i.e., radial leaves) and acquisition of abaxial cell identity. leafbladeless 1 in maize seems to perform a similar function. On the other hand, semi-dominant mutants in Arabidopsis in phabulosa-1d leads also to the formation of radial leaves but the acquisition of adaxial cell identity. PHABULOSA encodes an HD-ZIP transcription factor and the protein is predicted to require binding of a lipid ligand for activation. Since all identified mutants in phb show sequence changes in the region of predicted ligand binding, it has been proposed that the mutant proteins might be constitutively active, thus leading to the dominant phenotype. These data suggest that during leaf initiation a lipid signalling molecule activates PHB protein in the presumptive adaxial domain of the leaf leading to the acquisition of adaxial cell identity. Adjacent, non-activated cells would by default acquire abaxial cell identity. The acquisition of abaxial cell identity involves a further family of genes encoding YABBY proteins as well as members of the KANADI gene family. Since adaxial and abaxial cell identity appears to be mutually exclusive, there must be an interaction between PHB-like, YABBY-like and KANADI-like genes, although the details of this have yet to be examined. Similarly, there also appears to be an interaction of PHAN, RSI and RS2, AS1 and AS2 expression and Knotted-like gene expression, suggesting a feedback interaction between cells recently fated to be incorporated into leaves with the meristem from which those cells are derived.

2.4. Cellular parameters in leaf development. Although major advances have been made in our understanding of the genetic regulation of leaf development, virtually all of the genes described in the previous section encode transcription factor-like proteins. This leaves the fundamental question of the target genes and processes involved in the translation of the patterns set out by the transcription factors into actual changes in form and differentiation.

It is clear that plant hormones probably play a significant role in leaf formation, both in determining the position of leaf initiation and the co-ordination of
events during leaf growth. In particular, auxin has been implicated in many aspects of leaf development (Berleth and Sachs, 2001). Thus, disruption of auxin transport within the apex causes leafless shoot production and localised application of auxin on the flank of a meristem of such shoot causes initiation of a leaf (Reinhardt et al., 2000). Studies of the lop1 mutant from Arabidopsis showed that defective auxin transport is linked in leaves with altered development of the midvein which, in turn, results in abnormal rotation of the normal dorsoventral axis shape (Carland and McHale, 1996). Data such as these lead to the prediction of gradients of auxin concentration existing in both leaves and the SAM which are necessary for normal organ development and primordia initiation. However, as yet there is no confirmation for the existence of such gradients. Other plant hormones such as cytokinins and brassinosteroid have also been implicated in leaf development and alteration of levels of these hormones at the whole plant level results in many shape alterations such as stunted shoots, smaller meristem, dwarfism and altered leaf morphology (Sekimata et al., 2001; Werner et al., 2001). However, it must be remembered that hormones are simply signals or triggers. The question still remains, therefore, as for transcription factors, what are the target processes which might be modulated by altered hormone levels?

In this respect, the control of cell division rates and planes and the direction of cell expansion must somehow be involved in the generation of final leaf form. The question is: which of these cellular parameters provides the driving force in the control of leaf morphology?

Recent data have provided evidence supporting the idea that modulation of tissue growth via modulation of cell wall extensibility might play a key role in plant morphogenesis and, in particular, in leaf development. Thus, ectopic application of the cell wall protein expansin (a modulator of cell wall extensibility) was sufficient to induce morphogenesis on the meristem (Fleming et al., 1997). Moreover altered expression of a specific gene encoding an expansin in leaf vascular tissue led to changed leaf size (Cho and Cosgrove, 2000). In addition, the transcripts for specific expansin genes have been shown to accumulate at the site of leaf initiation (Reinhardt et al., 1998). These data
are consistent with expansin (and, thus, modulation of cell wall extensibility) being a target for gene products involved in leaf initiation.

However, it is also clear that co-ordinated patterns of cell division occur during leaf development. This has been most clearly demonstrated in a recent paper from Nancy Dengler’s group. Using a fusion construct of a cyclin B expressed exclusively during mitosis with the GUS reporter gene, the authors could characterise the spatial and temporal patterns of cell cycling in leaves (Donnelly et al., 1999). The conclusions of the work were: 1) that during leaf development, cell proliferation shows a strong proximodistal gradient with cells at the distal tip of the leaf terminating cell division first, 2) cells along the margin of a young leaf primordium undergo a transient burst of division at about the same time as leaf blade formation is initiated. The authors postulated that this burst of cell division represents a key event in leaf blade formation. It is important to distinguish this transient marginal cell division activity from the classically defined marginal meristem. This concept, which proposed that leaf blade size and shape was defined by the extent of clonal derivatives from cells dividing at the leaf margin, has been shown not to be true (Poethig and Sussex, 1985). Rather, in the new work the authors postulate that the marginal cell division somehow dictates the future growth of the blade by some as yet undefined process. It is certainly true that earlier histological work showed that leaf margin cells undergo a specific path of differentiation. However, the functional significance of these observations remains unknown.

3. IS THE CELL CYCLE IMPORTANT FOR MORPHOGENESIS?

The role of the cell cycle in plant development has been debated for a long time, reflecting the general issue of the relationship between the cell and the organism. Simply stated, do plants divide their bodies into cells or do cells divide to create the plant body?

Novel tools to investigate this problem have recently become available as a result of studies on the plant cell cycle describing its elements, their interactions and possible points of integration with developmental cues in plants. However, an
understanding of these experiments first requires an understanding of the plant cell cycle, which is described in the next section. It should be noted that our view of the plant cell cycle is almost entirely based on our understanding of these processes in other model systems. Thus, the majority of experimental evidence comes from research in budding yeast (S. cerevisiae), fission yeast (S. pombe) and animals (mostly mammalian cell cultures and the fruitfly D. melanogaster). Although many elements of the plant cell cycle have been identified via sequence similarity of genes in these model organisms, it must be borne in mind that biochemical and functional data supporting the purported role of these plant proteins is often limited. A further, complication is that the classification of cell cycle elements in yeast and mammals has tended to evolve independently, leading to re-naming of elements. To avoid confusion, both the recent and previous names for cell cycle genes are reported.

3.1. The basic molecular control of the cell cycle. The cell cycle consists of four phases: G1, S, G2, and M. G1 and G2 phases temporally separate the process of DNA synthesis (S phase) and mitosis (M phase). The progression through the cell cycle is driven by a highly conserved group of cyclin dependent kinases (CDK). CDKs are specific serine/threonine kinases the activity of which rises at the transition points (G1>S) and (G2>M) of the cell cycle. CDK activity is regulated by association with cyclin regulatory subunits and the associated specific phosphorylation/dephosphorylation events.

The first identified CDK was cdc2 (CDK1) from Schizosaccharomyces pombe which encodes a 34kDa protein (p34cdc2) and it is the only type of CDK present in yeast (in S. cerevisiae CDK is encoded by cdc28). In the plant family of CDKs five subtypes have been identified: A-E (known before as the cdc2-like family) (Stals and Inze, 2001). Biochemical analyses revealed that CDKA;1 (previously CDKA, CDC2a) is closely related to yeast cdc2/cdc28 and animal CDK1 and CDK2 by sharing the conserved PSTAIRE motif that is located in the cyclin binding region. The PPTALRE or PPTTLRE motif (instead of PSTAIRE) is characteristic for the second subgroup of CDKs comprising CDKB;1 (previously CDKB, CDC2b). CDKA;1 and CDKB;1 differ in their expression pattern during the cell cycle. CDKA;1 is expressed constitutively
while CDKB1 expression is limited to S phase and the G2/M transition, as shown in *Arabidopsis thaliana*, *Medicago sativa*, *Zea maize*, *Glycine max* and *Nicotiana tabacum* (Boer and Murray, 2000; Hemerly et al., 1999; Mironov et al., 1999). CDKs belonging to C, D and E class contain divergent motifs and do not fit into either of the two other classes. Not much is known about those kinases and their function and there are only few identified genes encoding these CDKs. An example are CDKC (previously CDC2c) and CDKD (previously CDC2d) from *Antirrhinum*. They belong to different classes and their expression patterns differ as well. *Antma;CDKC* is preferentially expressed in S phase and G2/M transition, while *Antma;CDKD* is expressed in G2 and M phase. In E class only one member has been identified till now – CDKE (previously CDC2e) in *Medicago sativa* (Hemerly et al., 1999).

CDK protein kinase activity depends on association with cyclins. Cyclins can be generally classified into two groups: mitotic cyclins (including A- and B-type cyclins) and G1 cyclins (including D- and E-type cyclins). Both, A- and B-type cyclins commit the cell to mitosis at the G2–M transition while A-type cyclins have additionally an essential function in S phase. Both groups are characterised by a conserved domain called the cyclin box that allows the interaction with the appropriate kinase subunit. B-type cyclins carry also a domain called the mitotic disruption box that mediates cyclin degradation in late mitosis. D-type cyclins are responsible for G1 phase progression. One of the known functions of cyclin D/CDK complex is phosphorylation of the Rb protein (retinoblastoma protein) which regulates the Rb/E2F transcriptional program. In addition, it seems that cyclin Ds are involved in the integration of hormonal responses and sensing sugar levels (fig.4) (Boer and Murray, 2000; Francis and Sorrell, 2001; Stals and Inze, 2001). Within each type of cyclins there are subgroups described that differ slightly in sequence, expression pattern and activity (Hemerly et al., 1999).

In addition to cyclins there are at least two other levels of CDK regulation. Firstly, inhibitory phosphorylation on threonine 14 and/or tyrosine 15 residues at the N-terminus of the CDK catalytic subunit can block CDK-cyclin complex
Fig. 4: Simplified scheme of the plant cell cycle.
formation. In animals and yeast this phosphorylation is carried out by the wee1 kinase and a similar gene has recently been identified in maize (Boer and Murray, 2000; Sun et al., 1999). Secondly, the activation of complexes requires dephosphorylation by cdc25 phosphatase. Both processes are essential for cell size control. Although no cdc 25 homologue has been reported in plants it has been shown that tobacco CDKA;1 is responsive to fission yeast cdc25 (McKibbin et al., 1998; Zhang et al., 1996). In addition, all CDKs require phosphorylation of a conserved threonine residue (usually T160 or 161) for their activation via opening of the ATP-binding-site. This reaction is performed by a CDK activating kinase (CAK) (Boer and Murray, 2000).

CDK inhibitors (CKI) have been also identified. They can become associated with the activated CDK-cyclin complex and prevent it from phosphorylating substrates. The inhibition state is reversible and does not affect the state of the complexes (Buchanan et al., 2000; Wang et al., 1998; Wang et al., 2000). Summarising, the entire cell cycle consists of cyclic reactions of CDKs with different cyclin classes (according to the phase) and additional phosphorylation/dephosphorylation reactions which leads the cell through cell cycle phases (Fig.4)

3.2. Manipulation of cell cycle. The identification of genes involved in the cell cycle has made it possible for investigators to check the expression patterns of these genes (as described in the previous section) and also to investigate the link between cell cycle manipulation and plant morphology.

Hemerly and colleagues generated a mutated ArathCDKA;1 (previously CDC2aAth) to generate a dominant negative form of the protein which was predicted to inhibit the cell cycle (Hemerly et al., 1995). When this construct was constitutively overexpressed in transgenic tobacco, the plants showed a reduced number of cell divisions resulting in fewer but larger cells producing no overall effect on plant shape (Hemerly et al., 1995). On the other hand manipulation of a particular phase of the cell cycle by overexpression of cyclins can produce a tremendous effect. Thus, constitutive expression of
Arath;cycD3 in Arabidopsis led to leaf curling, a disorganized meristem and delayed senescence (Boer and Murray, 2000; Riou-Khamlichi et al., 1999). Interestingly, D-type cyclins have a very specific expression pattern in the meristem. In snapdragon, cycD1 and D3b are expressed throughout whole meristem while cyc D3a is restricted to the peripheral region of the meristem and organ primordia (Gaudin et al., 2000). Other over-expression experiments with Arath;cycD2 and Arath;cycB1 in Arabidopsis or Sp;cdc25 in tobacco led to increased growth rates but no influence on organ morphology (Cockcroft et al., 2000; Doerner et al., 1996). In the case of Sp;cdc25 overexpression in yeast resulted in premature division and reduced cell size. Similar experiments performed in tobacco roots led to more frequent primordium initiation resulting in smaller primordia comprising smaller cells. However further development of these roots was not disturbed (McKibbin et al., 1998). Some other data coming from experiments on cell cycle inhibitors complement the picture of interactions between cell cycle modulation and growth rates. Wang and co-workers showed the first functional data for a plant cell cycle inhibitor - previously identified in Arabidopsis ICK1. They created transgenic Arabidopsis plants constitutively expressing Arath;ICK1. The observation of this experiment was that the plants were smaller with some modifications in morphology, such as disturbed shape and serration of leaves and petals. Additional histological analyses showed that the cells were bigger, suggesting that the plant organism compensated to an extent the decrease in growth ratio (Wang et al., 2000). These data confirm a tight connection between cell division and expansion in order to accommodate size and growth. They show the possible influence of cell cycle modulation on plant development, however they contradict the data presented by Hemerly et al. where dominant negative overexpression of Arath;CDKA;1 in tobacco also inhibited cell division with no overall influence on plant shape.

The evidence so far accumulated does not allow any absolute conclusion about the connection between the cell cycle and morphogenesis. It seems that increasing or decreasing the rate of cell division at the whole plant level tends not to affect plant morphogenesis but rather growth rate. Additionally, it seems that whenever there is a morphogenetic response to cell cycle variations it
happens at early developmental stages. Exactly at this time decisions about cell cycle progression can have a very dramatic influence. Very recent work of the Bouget group showed response of the Fucus zygote to the inhibition of CDKs. Inhibition of CDKA blocked not only S phase entry but also photopolarisation, suggesting that both processes are controlled by CDKA (Corellou et al., 2001). This evidence for the first time shows the clear connection between morphogenesis of a multicellular organism and the cell cycle.

3.3. Interactions between growth factors and the cell cycle machinery. There are many points of the cell cycle where growth factors play a regulatory role. Thus, auxin and cytokinin are known mitogens and recently it has been shown that they induce mitotic cyclins and cycD gene expression as well as elevated CDKA;1 transcript levels. All these events are associated with the induction of cell division (Francis and Sorrell, 2001; Stals and Inze, 2001; Werner et al., 2001). The plant growth regulator abscisic acid (ABA) has been shown to induce the expression of a CKI with the effect of decreasing CDKA;1 activity. This result provides a possible mechanism by which ABA could induce a general retardation of growth (Doonan, 2000; Wang et al., 1998).

4. THE ROLE OF ORIENTATION OF CELL DIVISION IN LEAF MORPHOGENESIS.

In most cases division consists of two stages - kariokinesis (that is the division of the nucleus) followed by cytokinesis (the phragmoplast formation resulting in the new cell wall). The basics of plant cell cytokinesis have been recognised over 100 years ago. One of the first observations made was that the new cell walls are usually formed in a plane perpendicular to the main axis of expansion (perpendicular to the long axis of the mother cell). Also a rule was formulated stating that the plane of division for most plant cells corresponds to the shortest path that will halve the volume of the parental cell. Experimental evidence supporting the idea of a direct link between cell shape and the plane of division came from experiments showing spherical cells (clumps of callus or isolated single cells suspended in medium) dividing in random directions (Smith et al., 2001). A significant progress has been made in the study of cytokinesis
showing many cytological and molecular aspects of this process. Cytokinesis is
accomplished by progressive deposition of membranes and associated wall
synthesising compounds onto a cell plate, which is first localised centrally in the
equatorial zone between recently formed daughter nuclei. The growing cell plate
then spreads out radially as an expanding interlacing membranous network until it
joins with the parental walls at the periphery. A cytoskeletal structure called a
phragmoplast (PP) not only guides vesicles to the equatorial zone but also directs
the growing cell plate toward a specific site at or near the membrane of the
parental cell wall – the cortical division site (Sylvester, 2000). These processes are
facilitated by several proteins – products of recently identified genes like
Phragmoplastin, KNOLLE, KEULE, GNOM, FASS and TANGLED (reviewed in
(Nacry et al., 2000)) although the exact function of the proteins is not described
for all listed genes. knolle and keule mutants have similar phenotype – defective
cytokinesis resulting in incomplete cell walls and enlarged cells with one or more
large nuclei. KNOLLE is a syntaxin-like protein and is expressed during
cytokinesis with accumulation along the plane of division around the growing
phragmoplast. It has been concluded that, like other syntaxins, it takes part in
vesicle trafficking, docking or fusing at the plane of division. Mutants of tangled,
gnom and fas exhibit another phenotype where the new cell walls are produced
but in an abnormal orientation.

Discovery of the genes involved in cytokinesis allowed scientists to study
the link between division orientation and morphogenesis. Morphological analyses
of mutant plants delivered interesting evidence. Mutants such as tangled and fas
produce plants of normal form despite changes in cell division orientation. This
observation suggests that development and division events are not linked.
However, other mutants such as keule, knolle or gnom affect drastically embryo
development leading to termination of development.

Study on phragmoplast formation delivered more data on cytokinesis.
One of the proteins identified recently in soybean root tips - phragmoplastin (plant
dynamin-like protein/PDL) is localised to the growing phragmoplast. An
overexpression study in BY2 cells showed that cell division is disrupted and that
the new cell walls are often arranged in an oblique manner (Gu and Verma, 1996;
Gu and Verma, 1997). An Arabidopsis homologue of phragmoplastin – ADL1 has been studied for its effects on plant development. Interestingly, homozygous adl1 mutants were seedling defective and development was arrested shortly after germination. However, they were not defective in cytokinesis and showed normal patterns of cell division during embryogenesis and seedling germination (Kang et al., 2001).

The evidence from these mutant analyses suggests that cell division, depending on its time and localisation, may play a crucial role in plant development or might be just a mechanism for tissue partitioning. Thus, the question about the exact role of the cell division plane in morphogenesis still remains open.

5. THE AIM OF THIS WORK.

The aim of this study was to better understand mechanisms of cellular patterning during leaf development. There have been many studies carried out up to now concerning the link between temporal and spatial patterns of cell division and morphogenesis resulting in often contradictory conclusions. On the one hand there is evidence showing that plants can incorporate even drastic changes in the cell cycle and follow a normal developmental program but on the other hand altered growth and perturbation of final organ shape are sometimes observed. However, these observations have been based on cell cycle modulation at the whole plant level which excludes the possibility of observing the response to local changes. To test the hypothesis that local changes in cell division play a role in morphogenesis, we created transgenic tobacco plants in which gene expression can be chemically induced in small portions of tissue. In this system overexpression of a transcripional repressor is used to prevent the constitutive expression of an introduced gene. Addition of a chemical (anhydrotetracycline (Ahtet)) that binds to the repressor derepresses transcription and the expression of the gene occurs only in the cells that have been in contact with Ahtet (Gatz et al., 1992; Pien et al., 2001). To allow for local induction of gene expression we developed a microinduction technique in which small portions of Ahtet soaked lanolin were positioned on the objects of our research – the meristem and leaf.
primordia. For modulation we chose two parameters of the cell cycle: cell division frequency and cell division orientation. The targets for the frequency manipulation were the cell cycle genes, Nicta;cyCA3;2 and Sp;cdc25 (Chaubet-Gigot, 2000; McKibbin et al., 1998) (see also Introduction 3.1 and 3.2). For changing the orientation of cell division we used a gene shown to be involved in phragmoplast formation Glyma;phragmoplastin (see also Introduction 4). Overexpression of this gene in BY2 cells resulted in reorientation of cell division. By introducing these genes into tobacco plants under control of the Ahtet inducible promoter we wanted to: 1) promote cell proliferation or altered cell division plane in developmentally active tissues; 2) observe whether such alterations led to changes in meristem function and leaf formation. The results of this work are presented in three papers focussed on these two aspects as well as on the validation of the system for the inducible regulation of gene expression.

REFERENCES:


INTRODUCTION


Laufs, P., Dockx, J., Kronenberger, J. and Traas, J. (1998a). *MGOUN1* and *MGOUN2*: two genes required for primordium initiation at the


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RESULTS

Results of this work have been presented in following papers:

1. Local expression of expansin induces the entire process of leaf development and modifies leaf shape.
   Stephane Pien, Joanna Wyrzykowska, Simon McQueen-Mason, Cheryl Smart, and Andrew Fleming
   *PNAS September 25, 2001 vol.98 no.20 pages 11812-11817*

2. Manipulation of leaf shape by modulation of cell division
   Joanna Wyrzykowska, Stephane Pien, Wen Hui Shen, and Andrew Fleming
   *Development (in press)*

3. Overexpression of phragmoplastin modulates planes of cell division in the meristem but does not disrupt leaf initiation.
   Joanna Wyrzykowska and Andrew Fleming
   *Manuscript*
Local expression of expansin induces the entire process of leaf development and modifies leaf shape

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Communicated by Hans J. Kendie, Michigan State University, East Lansing, MI, July 23, 2001 (received for review May 30, 2001)

Expansins are a family of extracellular proteins proposed to play a key role in cell wall stress relaxation and, thus, in cell and tissue growth. To test the possible function of expansins in morphogenesis, we have developed a technique that allows transient local microinduction of gene expression in transgenic plants. We have used this system to manipulate expansin gene expression in various tissues. Our results indicate that local expansin expression within the meristem induces a developmental program that recapitulates the entire process of leaf formation. Moreover, local transient induction of expansin expression on the flank of developing primordia leads to the induction of ectopic lamina tissue and thus modulation of leaf shape. These data describe an approach for the local manipulation of gene expression and indicate a role for expansin in the control of both leaf initiation and shape. These results are consistent with the action of cell division-independent mechanisms in plant morphogenesis.

It has been proposed that modulation of cell wall extensibility could play a key role in plant morphogenesis (1). In addition to theoretical considerations supporting this hypothesis, data have accumulated indicating that a family of extracellular proteins (expansins) functions in vivo to modulate cell wall extensibility and thereby regulates organ growth and morphogenesis (2, 3). Expansins were first identified as cell wall-associated proteins that could function in vitro to increase cell wall extensibility (4). It has subsequently become apparent that expansins are widespread in the plant kingdom, and that they tend to be encoded by relatively large gene families whose patterns of transcript accumulation and activity frequently correlate with specific processes of growth, morphogenesis, and differentiation (2). Moreover, data have accumulated supporting a functional role for expansins in these processes. For example, experiments in which expansin expression was suppressed in differentiating vascular tissue in transgenic Arabidopsis plants led to a marked phenotype of dwarfed leaves and altered morphology (5), suggesting that expansin supply might affect only the outermost epidermal cell wall, and that this pattern was very different from the endogenous accumulation of expansin transcripts, which defined a zone encompassing many cell layers of the meristem in the presumptive region of leaf initiation (3, 8). We therefore hypothesized that the formation of incomplete leaf structures after exogenous expansin supply might reflect a technical limitation leading to a failure of the exogenous protein to mimic the endogenous pattern of expansin expression observed during leaf initiation.

To test this hypothesis, we have generated transgenic tobacco plants in which expansin gene expression can be chemically induced. At the same time, we have developed a microinduction system that allows us to induce gene expression in very small tissue parts (fractions of a meristem). Using this system, we demonstrate that local induction of ectopic expansin expression in the meristem induces morphogenesis leading to the formation of leaves that are phenotypically similar to normally generated leaves. Moreover, we show that local manipulation of expansin expression during the earliest stages of leaf development is sufficient to alter local lamina growth leading to modification of leaf shape.

Materials and Methods

Plant Material and Transformation. R7 Nicotiana tabacum seedlings (a gift of C. Gatz, University of Goettingen, Goettingen, Germany) were transformed (3). Regenerants were grown in a greenhouse and F1 seeds collected for analysis. For microinduction experiments, plants were grown in soil in a growth chamber (16 h light at 24°C/8 h dark at 20°C) or on half-strength Murashige and Skoog medium (pH 5.6), 1% (wt/vol) agar (16/8 h light/dark cycle, 24°C, 100 μmol m−2 s−1). For RNA blot and expansin activity analyses, ~100 4-week-old seedlings were grown with gentle shaking (60 rpm) in 2-liter Erlenmeyer flasks containing 200 ml of liquid MS medium (pH 5.6), with or without anhydrotetracycline (Ahtet), at the concentrations and for the times given in Results.

DNA Manipulation. The CsExp1 coding sequence (10) was inserted as a transcriptional fusion into the KpnI/SalI sites of the pBinHyg-Tx vector (a gift of C. Gatz, University of Goettingen, Goettingen, Germany) and thereby transformed (3). Regenerants were grown in a greenhouse and F1 seeds collected for analysis. For microinduction experiments, plants were grown in soil in a growth chamber (16 h light at 24°C/8 h dark at 20°C) or on half-strength Murashige and Skoog medium (pH 5.6), 1% (wt/vol) agar (16/8 h light/dark cycle, 24°C, 100 μmol m−2 s−1). For RNA blot and expansin activity analyses, ~100 4-week-old seedlings were grown with gentle shaking (60 rpm) in 2-liter Erlenmeyer flasks containing 200 ml of liquid MS medium (pH 5.6), with or without anhydrotetracycline (Ahtet), at the concentrations and for the times given in Results.

Microinduction. Ahtet dissolved in DMSO was mixed with melted lanolin/3% paraffin at 60°C and rapidly cooled to generate a paste. Ahtet concentrations used ranged from 0.1 to 100 μg/ml. Portions of paste were applied by using stretched plastic tips to the surfaces of dissected meristems and primordia under a Leica MZ12 (Deerfield, IL) microscope. Controls were performed by using DMSO/lanolin/pauffman paste without Ahtet. At the time of apex dissection, plants had 10–15 leaves, 2–3 of which were left intact on the meristem before microinduction experiments. For lamina experiments, primordia at stage P2 or P3 were used.

Abbreviations: Ahtet, anhydrotetracycline; GUS, ß-glucuronidase; Tet, tetracycline. See commentary on page 10981.

Communicated by Hans J. Kendie, Michigan State University, East Lansing, MI, July 23, 2001 (received for review May 30, 2001).
**Fig. 1.** Tet-inducible expansin expression. (A) RNA blot analysis of total RNA (10 μg/lane) from transgenic tobacco plants containing either the GUS reporter gene (G25) or CsExp1 sequence (lines E1.7 and E1.8) under Tet-inducible transcriptional regulation. Plants were treated with 15 μg/ml Ahtet (+) or buffer (−) for 24 h before RNA extraction. Blots were hybridized with a radiolabeled probe for CsExp1. Methylene blue staining of 25S rRNA is shown as a loading control. (B) Time course of CsExp1 transcript accumulation by RNA gel blot analysis of E1.8 and G25 plants after treatment with 15 μg/ml Ahtet for the times indicated. Hybridization was as in A with 10 μg of RNA/lane. (C) Ahtet concentration dependence of CsExp1 transcript accumulation in line E1.8 by RNA gel blot analysis. Plants were treated for 24 h with the concentration of Ahtet indicated before hybridization as in A with 10 μg of RNA/lane. (D) Expansin activity measurements in plants from lines E1.7, E1.8, or G25 treated with Ahtet (2.5 μg/ml) (shaded columns) or buffer (open columns) for 24 h. Bars represent SE (n = 12).

**Fig. 2.** Strategy to locally induce gene expression in the shoot apex. The meristem (m) is surrounded by primordia (P2, P1). Ahtet-loaded lanolin (t) placed on the meristem acts as a local source of inducer (arrows), leading to the localized induction of transgene transcription.

**Fig. 3.** Microinduction of gene expression. (A) Hand section of a tobacco apex showing localization of GUS expression (blue) to a portion of the apical meristem after manipulation of Ahtet-impregnated lanolin onto the meristem surface. (B) Section of an apex treated as in A showing localized GUS expression in several cell layers on one flank of the meristem. (C) Localized GUS expression on the leaf lamina in two spots corresponding to areas of lanolin-Ahtet treatment (arrows). (D) Localized GUS expression (arrow) in the hypocotyl after treatment, as in C. (E) Whole seedling induction of GUS expression after induction (i) or noninduction (ni) with Ahtet. m, meristem; p, primordium.

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**Table:**

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>G25</th>
<th>E1.7</th>
<th>E1.8</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>48</td>
<td>+</td>
<td>-</td>
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<td>+</td>
</tr>
<tr>
<td>48</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

**Chart:**

- **A:**
  - CsExp1: + + - - +
  - 25S rRNA: - + - + -

- **B:**
  - Time (h): 24 48 0 6 12 24 48
  - CsExp1: G25 E1.8
  - 25S rRNA: + -

- **C:**
  - Ahtet (μg/ml): 0 0.5 5 10 15
  - CsExp1: + + + + +
  - 25S rRNA: - - - - -

- **D:**
  - Extension (μm/min): 0 50 100 150 200 250
  - G25: 0 50 100 150 200 250
  - E1.7: 50 100 150 200 250 250
  - E1.8: 50 100 150 200 250 250
Fig 4. Expansion-induced morphogenesis. (A) Scanning electron micrograph (SEM) of an apex from an E17 plant in which Ahtet-impregnated lanolin was manipulated onto the 12 position of the meristem (m) between primordia P1 and P2. After 72 h, a bulge has formed (arrow) at this position opposite the expected II (B) SEM of an E17 apex treated with buffer at the 12 position. No morphogenesis has occurred. (C) SEM of expansion-induced primordium. (D) SEM of normally formed primordium. (Bar: A and B = 150 μm; C and D = 25 μm.)

light/dark cycle, 24°C. 100 μmol m−2s−1 for 2 weeks before transfer to multiwell plates with water. After growth and root regeneration under the same growth conditions, plantlets were transferred to soil for further growth and analysis.

RNA Analysis. For blots, total RNA was extracted from 4-week-old seedlings by using RNeasy columns (Qiagen, Chatsworth, CA). Gel electrophoresis, blotting, and hybridization with a radiolabeled probe for CsExp1 was by standard methods (11). In situ hybridization was as previously described (12), by using digoxigenin-labeled sense and antisense riboprobes for CsExp1.

Expansin and GUS Activity Assays. Expansin activity assays were performed as previously described (13). Briefly, equivalent amounts of cell wall protein from Ahtet- and control-treated plantlets were added to a cellulose/xylan-rich matrix in the presence of 50 mM sodium acetate (pH 4.5). Expansin activity was calculated as the rate of extension of the material in the first 10 min after protein addition minus the prior rate of tissue extension. GUS activity was visualized in intact apices (14) and either hand sections taken or tissue embedded in Technovit resin (Harka, Bern, Switzerland) for thin-section analysis (according to the manufacturer’s instructions).

Electron Microscopy. Cryoscanning electron microscopy was as previously described (7) of apexes 3 days after manipulation.

Results

Table 1. Induction of expansin expression leads to leaf initiation and reversed phyllotaxis

<table>
<thead>
<tr>
<th>Plant line</th>
<th>Treatment</th>
<th>Apices analyzed</th>
<th>Leaf initiation</th>
<th>Reverse phyllotaxy</th>
</tr>
</thead>
<tbody>
<tr>
<td>E17</td>
<td>+</td>
<td>73</td>
<td>14</td>
<td>14</td>
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<tr>
<td>E18</td>
<td>+</td>
<td>55</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>G25</td>
<td>+</td>
<td>49</td>
<td>0</td>
<td>1</td>
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<tr>
<td></td>
<td>−</td>
<td>28</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

Meristems were induced on 12 position with Ahtet (+) or treated with buffer (−). The number showing novel leaf initiation and reversed phyllotaxis was counted. ND, not determined.

sequence. Ahtet binds to the repressor protein to alleviate this transcriptional repression. Seeds were collected from 11 independent primary transformants and each line tested for the accumulation of CsExp1 transcript with or without prior induction with Ahtet. This experiment led to the identification of six lines showing accumulation of CsExp1 transcripts after induction with undetectable expression under noninducing conditions. The results for two of these lines (E17 and E18) are shown in Fig. 1A. These lines were used for further analysis. A time course of CsExp1 induction by using 15 μg/ml of Ahtet revealed a transient transcript accumulation with a maximum after 12 h (Fig. 1B). Incubation with various concentrations of Ahtet indicated that relatively low levels were sufficient to induce transcript accumulation with a maximum at 5 μg/ml; at higher concentrations, induction was reduced (Fig. 1C). Controls with plants engineered to express the GUS reporter gene under Ahtet-inducible transcription (Tet::GUS) revealed no cross hybridization of the CsExp1 probe with endogenous transcripts under the conditions used (Fig. 1A and B). To test whether the observed changes in CsExp1 transcript level had an influence on
endogenous expansin activity, we performed extensibility tests by using an in vitro system (13). The results (Fig. 1D) indicate that in both lines E1.7 and E1.8, Ahct induction led to an increased level of endogenous expansin activity (5-fold over noninduced levels for line E1.7, 17-fold over noninduced levels for line E1.8).

Microinduction of Gene Expression. To locally induce gene expression, we took the following strategy. Small portions of lanolin were impregnated with Ahct and then manipulated onto the surface of dissected apical meristems, the aim being to generate a local source of Ahct and, thus, a localized area of induction (Fig. 2). To validate this method, we first performed a series of experiments with Tet::GUS plants. Local induction of GUS expression was obtained to a resolution of a fraction of a meristem (Fig. 3A). Analysis of thin sections (Fig. 3B) revealed that GUS expression was induced in several cell layers in a restricted area within the meristem. By using this approach, local GUS induction could be achieved (to varying resolution) in various organs and tissues, including leaves (Fig. 3C), hypocotyls (Fig. 3D), and roots (Fig. 3E). By varying the Ahct concentration, the amount of lanolin, and the time for which the lanolin was left on the tissue, the target tissue area and signal intensity could be controlled (data not shown). That virtually all tissues could respond to the inducer is shown in Fig. 3F by the induction of a seedling after immersion in Ahct.

Local Induction of Expansin Leads to Leaf Initiation and Reversal of Phyllotaxis. Having established a method for the local induction of gene expression, we proceeded to analyze the outcome of
localized expansin induction in various plant organs. In this analysis, we concentrated on the apical meristem, because our previous data indicated that local alteration in expansin activity was likely to lead to altered morphogenesis in this tissue (3). Ahtet-impregnated lanolin was manipulated onto the 12 position of the apical meristem. This is an area that will not normally generate a leaf primordium until after the 11 position (138° distant) has undergone organogenesis. When Ahtet had been locally applied to the 12 position of a Tet::E1.7 meristem, morphogenesis occurred at the 12 position to generate a bulge between the P2 and P1 primordia (Fig. 4A). In control experiments in which either Ahtet was manipulated onto the 12 position of Tet::GUS meristems or buffer was manipulated onto the 12 position of Tet::E1.7 plants, no altered morphogenesis was observed (Fig. 4B). In these cases, a primordium bulge arose at the expected time at the 11 position. Comparison of the expansin-induced bulges (Fig. 4C), and normal leaf primordia at a similar stage of development (Fig. 4D) revealed no overt differences in terms of morphology, epidermal cell size, or structure.

Initiation of leaf structures was observed after localized expansin induction in 19% (Tet::E1.7) and 18% (Tet::E1.8) of cases for the two independent transgenic lines analyzed, with no such altered morphogenesis observed in mock-treated Tet::E1.7 lines (28 plants tested) or Tet::E1.8 lines (26 plants tested), or in an Ahtet-treated Tet::GUS line (49 plants treated) (Table 1). To confirm that the manipulations of the meristem did lead to a local accumulation of CsExp1 transcripts, we performed in situ hybridizations of induced and noninduced meristems. Local application of Ahtet led to accumulation of the CsExp1 transcript principally in the meristem, although occasionally signal was observed in adjacent primordia (Fig. 5A). The CsExp1 probe used did not cross hybridize with endogenous expansin transcripts, shown by hybridizations with buffer-treated control tissue (Fig. 5B).

After initiation, the expansin-induced primordia grew to form leaf structures that were indistinguishable from normally formed leaves. The expansin-induced leaves (Fig. 6D) were ovate and had a system of venation and lamina growth comparable to that observed in normally formed leaves (Fig. 6F). Histological analysis confirmed the presence of all of the major expected cell types in the appropriate position within the induced leaves, both in the region of the vascular tissue (Fig. 6C) and the lamina (Fig. 6D).

After the formation of an Ahtet-induced leaf, the apical meristem continued to generate phenotypically normal leaves but with a reversed phyllotaxis (Table 1). For example, a plant in which the original phyllotaxis was anticlockwise generated leaves 1–3–17 in a clockwise fashion subsequent to the formation of an expansin-induced leaf at the 12 position (Fig. 6E and F). Observation of the order of leaf initiation for the plant shown in Fig. 6E is facilitated by the side view of the plant, where the insertion points of the leaves along the stem can be seen (Fig. 6G).

**Manipulation of Leaf Shape by Local Induction of Expansin.** In addition to local induction of expansin expression within the meristem, inductions were also performed on the flanks of young leaf primordia (P2–P3 stage). Local induction of expansin on the primordium flank led to increased local growth of the lamina and to altered leaf shape in 11 cases of 20 (Fig. 7). Three examples are shown in Fig. 7 A–C. In each case, the outgrowth of the lamina has occurred only on the side of the leaf where the induction was performed, the opposite side of the lamina showing normal morphology. Manipulations in which Tet::E1.7 primordia were mock-treated, or Tet::GUS primordia were induced with Ahtet, did not lead to any change in leaf morphology (Fig. 7D).

**Discussion**

**Microinduction of Gene Expression.** To test the molecular basis of morphogenesis, it is necessary to develop techniques that allow the expression of specific genes in specific cells and to observe the effect of altered gene expression on the process under investigation. Constitutive overexpression of morphogenetically important gene products may be lethal, may tend to highlight early acting pathways in development, or may even induce compensatory mechanisms so that any phenotype is either obscured or variable. One way to circumvent this problem is to use promoter elements that direct gene expression to specific tissues at particular time points (16). However, the number of elements at present characterized is rather limited, and there may be patterns of gene expression that are impossible to reconstruct by using such an approach. The results reported in this paper describe an alternative strategy. This strategy involves the adaptation of the well-established Tet-inducible promoter system (15) by locally applying the inducer to various plant tissues. This approach allowed the local induction of gene expression in all tissues tested. By varying the concentration and time of application, a variety of patterns of gene induction could be achieved. At the extreme, we were able to transiently induce transgene expression down to a resolution of less than 50 μm. This manipulation would not have been possible by using any of the promoter elements presently available. Our microinduction approach thus represents a powerful adjunct to methods already established for the manipulation of gene expression.
Local Expansin Expression Is Sufficient to Initiate the Entire Program of Leaf Development. By using the microinduction technique, we were able to locally induce expansin gene expression within the meristem. This manipulation led to the initiation of leaf development. These results corroborate earlier data indicating that local ectopic application of expansin protein to meristems induced morphogenesis (3, 7). The data also extend these observations by demonstrating that induction of expansin endogenously in several cell layers of the meristem initiates a program of development generating leaves that, at the level of overall morphology and histology, are indistinguishable from normally formed leaves. Expansin-induced leaves also influence the subsequent phyloaxis of the plant, consistent with previous observations and theories on the function of newly generated leaves in generating signals that influence meristem activity (3, 17). Taken together with data showing a specific accumulation of expansin transcripts at the presumptive site of leaf initiation (8, 18), our data fit with a model according to which local increase in growth (via modulation of cell wall extensibility) is a key event in leaf initiation. These data support the concept that alterations in the biophysical context of a tissue can influence development (1, 19).

Local Expansin Expression Modulates Leaf Shape. That local increase in cell wall extensibility can influence morphogenesis was also demonstrated by experiments in which local induction of expansin expression on the flank of leaf primordia led to local altered growth and eventual modification of leaf shape (increased lamina formation). The histology of the ectopic lamina was very similar to that of normally formed leaves (data not shown), indicating the close interaction between cell growth, division, and differentiation. In this context, cell division is not driving morphogenesis; rather, there is a programmed pattern of cell division/differentiation that appears to fill the available space within the organ. This is further evidence for the existence of cell-division-independent mechanisms controlling morphogenesis (20, 21).

Whether local modulation of expansin expression plays a role in the endogenous mechanism controlling leaf morphology remains to be determined. Our analysis shows that expansin genes are expressed in young tobacco leaf tissue (data not shown), and our future research will be focused on identifying expansin genes that might play a role in the mechanism of leaf morphogenesis.

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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 in 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 (Nicota;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 Nicota;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 which 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 which 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, \textit{Nicta}; \textit{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 \textit{Nicotiana tabacum} seedlings (a gift of 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 Whatmann paper submerged in Murashige and Skoog (MS) medium under a 16h light/8h dark regime. For induction, 1 \textmu g/ml Ahtet was added in 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 in 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 of C. Gatz). The resultant clone (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 digoxygenin 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 of C. Gatz, University of Goettingen, Germany). In such a background transcriptional activity from the TetO sequence is repressed until the addition of anhydrotetracycline (Ahtet). Eight independent transgenic lines (Tet::Nicta;CycA3;2) were obtained which showed single locus inheritance (data not shown) and were analyzed 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 both 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 h of induction with a maximum occurring between 4- 8 h, 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 an 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 of 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 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). Due 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 h (Fig. 2B and 2C). 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 h 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 and 2F. Stereological analysis revealed an average cell size in the induced area of 1881 ± 683 µm$^3$ (n = 24) compared with 12'432 ± 2098 µm$^3$ (n = 24) in the non-induced area. This difference in average cell volume indicates a difference in the rate of cellular partitioning between the induced and non-induced flanks. That this local increase in cellular proliferation was due to an increase in Nicta;CycA3;2 activity was supported by in situ hybridisation analysis which showed an increased area of tissue in the induced flank expressing the Nicta;CycA3;2 gene compared with the non-induced flank (Fig. 2G) and with the mock-induced flank of Tet::Nicta;CycA3;2 primordia (Fig. 2H).

The influence of local Nicta;CycA3;2 gene expression on cell proliferation was qualitatively different from that resulting from local expression of the Sp;cdc25 gene, which included an element of disruption of cellular patterning rather than simple increase in cell proliferation. Thus, as shown in Fig. 2I for a Tet::Sp;cdc25 primordium 72 h after microinduction, the induced flanks were characterised by an alteration in the ordered layered structure of the lamina 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 and 2K. 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 resulting from control experiments 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 (Binarova 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 treatment 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 \( (41931 \pm 16125 \ \mu m^3, \ n=16) \) compared with non-treated laminas \( (34620 \pm 1643 \ \mu m^3 \ n=16) \), 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 and 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 either by roscovitine or expansin contrasts with the inhibition of lamina growth produced by local induction of \( \textit{Nicta};\textit{CycA3};2 \) 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. For meristem inductions, the Ahtet-impregnated lanolin was positioned onto the I2 position between primordia P2 and P1, the area at which a leaf will form subsequent to the initiation of an organ at the I1 position 137• distant (Fig. 6A). Local induction of Tet::Nicta;CycA3;2 or Tet::Sp;cdc25 apices at this position with Ahtet did not lead to any overt change in meristem shape or pattern of leaf initiation (0 cases, n = 65 for Tet::Nicta;CycA3;2; 0 cases, n = 9 for Tet::Sp;cdc25) (Fig. 6B). However, histological analysis revealed a disruption to the normal pattern of cell division in the meristem (Fig. 6C and 6D). A local accumulation of smaller cells relative to the rest of the meristem was observed, particularly in the epidermis. Further evidence that the manipulations performed led to altered patterns of cell division came from in situ hybridisation analysis using a histone H4 probe. An increased frequency of cells expressing the S-phase marker gene was observed in Ahtet-treated meristems compared with buffer treated controls (Student t test, p = 0.01, n = 17) (Fig. 6E and 6F).

**DISCUSSION**

**Microinduction of Nicta;CycA3;2 and 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 cyle (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. In this paper we provide functional data that an A-type tobacco cyclin \((Nicta;\text{CycA};3:2)\) and a heterologous \(cdc25\) from \(S.\ pombe\) can modulate plant cell division.

Overexpression of \(Nicta;\text{CycA}3:2\) promoted cell proliferation, as shown by the accumulation of relatively small, cytoplasmically dense cells at the site of induction relative both to surrounding non-induced cells and cells which 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 \(Nicta;\text{CycA}3:2\) 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 \(Nicta;\text{CycA}3:2\) induction. Since \(Nicta;\text{CycA}3:2\) is an endogenous gene of tobacco whereas \(Sp;cdc25\) is of yeast origin, the difference in cellular outcome following \(Nicta;\text{CycA}3:2\) 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 \(Nicta;\text{CycA}3:2\) increased the rate or prolonged the time over which cell division was occurring rather than 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 $Sp;\text{cdc25}$ and $Nicta;\text{CycA3};2$ 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 $Nicta;\text{CycA3};2$ 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, a 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 $Nicta;\text{CycA3};2$ (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 previous studies (Hemerley et al., 1995; Doerner et al., 1996; Cockcroft et al., 2000).

**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 which 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 affect 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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FIGURE LEGENDS

Fig. 1. Analysis of Ahtet-inducible Nicta;CycA3;2 transcript accumulation. (A) Northern blot analysis of Nicta;CycA3;2 gene expression in Tet::Nicta;CycA3;2 (lines S7b and S7c) and Tet::GUS (Con) seedlings treated either with (+) or without (-) Ahtet. Blots were hybridised with a probe for Nicta;CycA3;2 (upper panel) or histone H4 (lower panel) (10 µg RNA/lane). (B) RT-PCR analysis of Nicta;CycA3;2 transcript accumulation in leaf discs from Tet::Nicta;CycA3;2 plants (line S7b) induced for 24h with various concentrations of Ahtet. (C) As in (B) except that leaf discs were incubated with (+) or without (-) Ahtet (0.02 mg/ml) for the times given before analysis.

Fig. 2. Local induction of Nicta;CycA3;2 and Sp;cde25 gene expression leads to cell proliferation on the leaf margin (A) Cross-section through a tobacco shoot apex to show site of Ahtet application (red spot) on the abaxial flank of primordium P2. (B) Cross-section of primordium P2 from a Tet::Sp;cde25 plant 24h after local induction on one flank (red line) (C) Cross-section of primordium P2 from a Tet::Nicta;CycA3;2 plant 24h after local induction on one flank (red line). (D) Tangential section through a primordium from a Tet::Nicta;CycA3;2 plant 72h after Ahtet induction on one flank. An accumulation of small cytoplasmically dense cells is apparent on the induced flank (i) compared with the non-induced flank (ni). (E and F) Magnifications of the induced flank (E) and non-induced flank (F) shown in D. Cells in E are small, dense and meristematic. Cells in F have undergone vacuolation. (G) In situ hybridisation of a section of a primordium P2 from a Tet::Nicta;CycA3;2 plant 72h after Ahtet induction on one flank. The section has been hybridised with a probe for Nicta;CycA3;2. A larger area of signal
(blue/purple) is present in the induced flank (i) compared with the non-induced flank (ni). (H) As in G except that the primordium was mock-induced (i) with buffer on one flank. (I) Cross section of primordium of Tet::Sp:cdc25 plant 72 h after Ahtet induction on one flank. The induced flank (i) is slightly thicker than the non-induced (ni) and the lateral vein (v) differentiating in the non-induced flank is not apparent in the induced flank. (J and K) Examples of Ahtet induced flanks of Tet::Sp:cdc25 primordia. Note the disorganised cellular patterns leading in some areas to apparent cell death and compression and poorly developed vascular tissue (v). (L) Cross section of leaf blade from a non-induced flank showing highly ordered cellular structure and appropriate vascular differentiation (v). Bars: 40 μm in E and F; 60 μm in G and H; 75 μm in B and C; 100 μm in D, J, K, and L; 120 μm in I.

Fig. 3. Altered lamina growth and leaf shape resulting from local induction of Nicta;CycA3;2 and Sp;cdc25 gene expression. (A) Microinductions were performed at various positions along the flanks of a number of Tet::Nicta;CycA3;2 primordia, as shown in the schematic diagrams (red = area of induction, t = Ahtet induction, b = buffer induction). The resultant leaf shape is shown below each schematic. (B) As in A except that the microinductions were performed on primordia of Tet::Sp;cdc25 plants. Bar: 5 mm.

Fig. 4. Histology of asymmetric leaves (A) Cross-section of an asymmetric leaf resulting from local induction of Nicta;CycA3;2 in a primordium at stage P2. The lamina resulting from the induced flank (inset, i) shows altered cellular patterning compared with the non-induced flank (inset, ni) and a lack of epidermal hairs. (B) Cross-section of a symmetrical leaf formed after local induction (i) of a Tet::Nicta;CycA3;2 primordium with buffer only. Bar: 80 μm in A; 250 μm in B.
Fig. 5. Induction of ectopic lamina by local application of roscovitine. (A) Leaf shape resulting from application of roscovitine-loaded lanolin on one flank of a P2 primordium. Ectopic lamina has formed on one side (arrow). (B) Leaf shape resulting from mock-induced control primordium. (C) Cross-section through ectopic lamina resulting from roscovitine treatment. (D) Cross-section through lamina of a normally formed leaf. (E) Leaf shape resulting from local induction of expansin gene expression on one flank of a primordium at the P2 stage. Ectopic lamina has formed on one side (arrow). (F) Leaf shape resulting from local induction of *Nicta;CycA3;2* gene expression on one flank of a primordium at the P2 stage. Retarded lamina growth has occurred at the site of induction (arrow). Bars: 2 mm in A and B; 250 μm in C and D; 5 mm in E and F.

Fig. 6. Altered cell division pattern does not affect meristem function. (A) Cross-section of tobacco apex to show position of Ahtet induction (red spot) on the meristem (m) between primordia P2 and P1. (B) Scanning electron micrograph of shoot apex 72 h after micro-induction of *Nicta;CycA3;2* gene expression. A primordium has arisen at the expected position (I1) with no overt change in morphology at the I2 position. (C) Section through the apical meristem 24 h after local induction of *Nicta;CycA3;2* gene expression. Proliferation has occurred on one flank (arrow) to generate smaller cells than in the surrounding meristem. (D) Schematic to show the cellular boundaries in C. (E) In situ hybridisation of an Ahtet-induced meristem from a Tet::Nicta;CycA3;2 plant hybridised with an antisense probe for histone H 4. Signal (blue/black) is localised to individual cells in the apex. (F) As in E except the meristem was mock-induced with buffer. Bars: 30 μm in C; 50 μm in E and F; 100 μm in B.
Fig. 1
Fig. 3

A  Tet::Nicta;CycA3::2

B  Tet::Sp;Cdc25
Fig. 6
Overexpression of phragmoplastin modulates planes of cell division in the meristem but does not disrupt leaf initiation.

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SUMMARY

The shoot apical meristem consists of an organised cellular structure comprising an outer tunica layer (in which cell division occurs exclusively in an anticlinal orientation) and an inner corpus (in which cell division orientation is more random). The functional significance of this conserved structures is unclear, with various strands of data both supporting and contrary to a causal role for patterns of cell division in leaf initiation. To test the role of cell division orientation in leaf formation, we generated transgenic plants containing a cDNA encoding phragmoplastin under transcriptional regulation of a chemically-inducible promoter. Phragmoplastin is a dynamin-like protein involved in phragmoplast formation whose overexpression has previously been shown to disrupt the orientation of cell division. Using a microinduction technique, we show that local expression of this protein leads to a disruption of the ordered structure of the tunica layer but that meristem function (including leaf initiation) is unaffected. These data show that phragmoplastin overexpression is sufficient to influence cytokinesis in intact tissue and can be used as a tool to manipulate the cell division pattern. Moreover, they support the hypothesis that the pattern of cell division in the apical meristem is not causal for leaf initiation.
INTRODUCTION

Plant cells, being trapped in rigid walls, cannot migrate as animal cells do during development. Thus, once a new cell is formed within a plant its relative spatial position is fixed. A tightly coordinated process of cell division with its plane and frequency and cell elongation might therefore be expected. However, to date accumulating evidence indicates that cell division aberrations do not influence plant shape and general development. Even in the case of complete inhibition of cell division, plants are able to undergo further development for a period of time (Foard, 1971; Foard and Haber, 1961). Furthermore, it has also been shown that minor variations in the pattern of cell division are a normal feature of leaf development (Poethig, 1987) and mutants have now been described in which the cell division plane is severely disrupted throughout the plant and yet the main processes of organogenesis still occur (Cleary and Smith, 1998; Laufs et al., 1998). However, these data show that plants respond to changes occurring in cell division orientation throughout the plant and it has been suggested that local differences in cell division frequency and plane may be important for morphogenesis and that these local gradients are still present in the mutants so far described. Indeed, our own previous data have shown that local induction of cell wall extensibility and cell proliferation can have a major impact on, for example, leaf growth and form (Pien et al., 2001; Wyrzykowska et al., in press). However, our previous work did not address the question of whether cell division plane could also influence morphogenesis. In order to answer this question, we have taken a similar approach to that already described for the local manipulation of cell wall extensibility and cell proliferation, i.e., we have generated transgenic plants containing a
cDNA whose encoded protein, when ectopically expressed, is predicted to disrupt cytokinesis and thus lead to disorganised planes of cell division. The cDNA we chose to use for this strategy encodes the dynamin-like protein phragmoplastin.

Phragmoplastin is a GTP binding protein belonging to the dynamin family. Similar to other dynamins it has been associated with vesicle or membrane transport. The beginning of new cell wall formation in a plant cell starts with production of the phragmoplast – a structure composed of vesicles growing outwards from the center of the cell. Phragmoplastin presumably takes part in vesicle trafficking and is mostly present at the edge of growing phragmoplast (as was shown in dividing cells of soybean root tip cells). Phragmoplastin seems to be an important part of the mechanism of cell wall formation as overexpression of the phragmoplastin gene caused cell wall reorientation (as shown in the tobacco cell suspension culture (BY2)) (Gu and Verma, 1996; Gu and Verma, 1997).

In this paper, we report on the generation of transgenic plants in which phragmoplastin gene expression can be chemically induced by Anhydrotetracycline (Ahtet). We show that microinduction of this gene in the apical meristem leads to a disruption of the normal pattern of cell division, but that this disruption does not lead to any effect on meristem function or leaf initiation. These data are consistent with the hypothesis that plant organogenesis does not depend on particular patterns of cell division.
MATERIALS AND METHODS

Plant material and transformation. R7 Nicotiana tabacum seeds (a gift of C.Gatz, University of Goettingen, Goethingen, Germany) containing constitutively expressed Ahtet repressor were grown on half-strength MS medium (pH 5.7), 1% (w/v) agar (16/8 h light/dark cycle, 24°C, 100 μmol m⁻² x s⁻¹). 3-week old seedlings were transformed using leaf discs and Agrobacterium mediated transformation (Rossi et al., 1993). 10 regenerants were grown in a greenhouse and F1 seeds were collected for analysis. For microinduction experiments, plants were grown in soil in a growth chamber (16h light at 24°C/ 8h dark at 20°C) and for RNA analysis and DNA analysis on half-strength Murashige and Skoog (MS) medium (pH 5.7) 1% (w/v) agar (16/8 h light/dark cycle, 24°C, 100 μmol m⁻² x s⁻¹). For inducibility tests, leaf discs from young leaves of 2 month old plants grown on soil were used. Leaf discs were incubated in liquid MS (pH 5.7) with or without anhydrotetracycline (Ahtet), at concentrations and for times given in the Results section. For phenotype analysis, seeds were grown on half-strength MS medium (pH 5.7) 1% (w/v) agar (16/8 h light/dark cycle, 24°C, 100 μmol m⁻² x s⁻¹) for 4 – 5 weeks and analyzed under a stereoscopic microscope.

Construct preparation. The Glyma;Phrag (or Glyma;PDL) cDNA (Gu and Verma, 1996) (kindly provided by Desh Pal S. Verma, The Ohio State University, Columbus, US) was inserted in Smal/SalI sites of pBinHyg-TX vector (a gift of C.Gatz). The obtained clones, pBinHyg-TX-Phrag and the parallel pBinHyg-TX-β-glucoronidase (GUS) construct (Pien et al., 2001), were introduced into R7 tobacco
plants. All DNA manipulation techniques were standard as described in Sambrook, Fritsch and Maniatis (1992).

RNA analysis. Total RNA was extracted from 5-week old seedlings using RNeasy columns (Qiagen) according to the manufacturer's instructions. Gel electrophoresis, blotting and hybridisation with DIG-labeled probe for Glyma;Phrag were carried according to standard methods (Sambrook et al., 1989). In situ hybridisation was as previously described (Pien et al., 2001), using DIG-labeled sense and antisense riboprobe for Glyma;Phrag or Nicta;NTH15 (NTH 15 cDNA was kindly provided by Makoto Matsuoko, Nagoya University, Japan, (Tamaoki et al., 1997))

DNA analysis. Genomic DNA was extracted from 5-week old seedlings using Nucleon Phyto Pure Plant DNA Extraction Kit (Pharmacia Biotech) according to manufacturer's instruction. Gel electrophoresis, blotting and hybridisation with DIG-labeled probe for Glyma;Phrag were carried according to standard methods (Sambrook et al., 1989). For estimation of number of gene copies inserted during transformation, DNA was digested with HindIII. Positive and negative controls were wild type DNA with or without addition of Glyma;Phra in pBluescript (respectively) treated the same way.

Microinduction method. Localised induction of meristem and leaf primordium was performed using lanolin paste (3% paraffin in lanolin) with 10 mg/ml Ahtet (anhydrotetracycline, dissolved first in DMSO) (Pien et al., 2001). Very small portions of lanolin were applied onto either the meristem or leaf primordium using stretched plastic tips. For all inductions, controls were performed using the same mix without Ahtet and the same application method.
Histological analysis. Samples were taken from appropriate parts of plants and fixed overnight at 4°C in AA fixative (ethanol:glacial acetic acid 3:1 v/v). Tissues were washed in 75% ethanol, dehydrated, infiltrated and embedded in Paraplast. Sections (5-7μm thick) were stained in 0.1% (w/v) toluidine blue, mounted and examined using light or fluorescent microscopy.

Tissue clearing. Tissues were fixed as described above, dehydrated and cleared in a solution of chloral hydrate (trichloroacetylaldehyde monohydrate – 200g; glycerol – 20g; distilled water – 50g) (Candela et al., 1999).

RESULTS

Generation of transgenic plants with inducible phragmoplastin gene expression.

A cDNA encoding soybean phragmoplastin (Gu and Verma, 1996) was cloned into the vector pBinHyg-Tx behind the TetO operator sequence. The construct was then transformed into tobacco plants manipulated to overexpress the tetracycline repressor ((R7 line, a kind gift of C. Gatz, University of Bielefeld). 10 independent lines were regenerated and tested initially by RT-PCR for inducible expression of the phragmoplastin gene. The results of this analysis led to the identification of two lines showing non-detectable background accumulation of the soybean phragmoplastin gene expression but good inducibility of phragmoplastin transcript accumulation. These lines (Nt.phrl, Nt.phr3) were taken for further characterisation by northern blot analysis.
Seedlings were incubated in liquid MS medium with either various concentrations of Ahtet for 8h (Fig 1a) or with and without 0.02mg/ml Ahtet for various times (Fig. 1b). As shown in Fig. 1a, an optimal accumulation of phragmoplastin transcript was achieved with an Ahtet concentration of 0.02 mg/ml. The Ahtet concentration used seemed to be critical since both higher and lower Ahtet concentrations led to the accumulation of barely detectable levels of phragmoplastin mRNA. A time course of phragmoplastin transcript accumulation using 0.02 mg/ml Ahtet revealed a rapid but transient accumulation of the mRNA, with maximal levels being attained within two hours of treatment (Fig. 1b). After 20 hours the level of phragmoplastin transcript accumulation was barely detectable.

Southern blot analysis (Fig. 1c) of the lines Nt.phrl and Nt.phr3 used in the northern blot analysis confirmed that the plants were transgenic. These lines were used in the microinduction experiments described in the next section.

**Induction of phragmoplastin gene expression in the meristem**

As shown in Fig. 2a, the outer tissue of the meristem dome consists of ordered layers of cells which form the classically defined tunica. This cellular partitioning of the outer layers of the meristem is shown schematically in Fig. 2b. Each of the two outermost layers of the meristem have been colour coded to demonstrate that cells of a particular colour share predominantly anticlinal oriented walls. In order to test whether expression of phragmoplastin in this tissue would disrupt this ordered pattern of cell division, we performed a series of microinduction experiments with the Nt.phrl and Nt.phr3 transgenic tobacco lines. As shown in Fig. 2c and d this manipulation led to the
occurrence of many oblique cell walls. This was particularly obvious in the second and third cell layers of the meristem. This alteration in cell division plane was also reflected in a more variable cross-sectional area of cells in the induced meristems.

Despite this disruption of cellular partitioning within the meristem, plant development was unaffected. Thus, all induced meristems of Nt.phrl and Nt.phr3 apices (n = 15) regenerated to form plants in which both the pattern of leaf initiation and leaf form were no different from buffer treated control plants (n = 25) or Ahtet treated Nt.GUS plants (n = 42) (data not shown).

**In situ hybridization analysis of induced gene expression in the meristem**

To corroborate that our microinduction experiments did lead to a local induction of phragmoplastin gene expression, we performed a series of in situ hybridisations using an antisense probe for phragmoplastin. As shown in Fig. 3a, 6 h following microinduction of the Nt.phrl meristems there was a local accumulation of phragmoplastin transcripts on one flank of the meristem. Analysis of buffer-treated control plants or Ahtet-treated Nt.GUS plants revealed no signal above background (Fig. 3b).

The data reported above indicated that although local induction of phragmoplastin gene expression led to an altered pattern of cell division in the meristem, meristem function (as assayed by leaf initiation) was unaffected. To investigate whether the expression patterns of genes involved in meristem function also were unaffected by the disruption of cell division plane, we performed a series on in situ hybridisations using an antisense probe for a *Knotted*-like
homeobox gene, NTH15. As shown in Fig. 3c, transcripts encoding this transcription factor accumulate throughout the meristem but are excluded from one flank, the site of incipient leaf formation (Tamaoki et al., 1997). In the meristems induced to express the phragmoplastin gene, this pattern was altered so that NTH15 mRNA was absent from a large area of the meristem dome, with only a band of cells extending from the inner tissue to the outer cells layers expressing the gene.

In addition to microinduction of phragmoplastin gene expression in the meristem, we also performed microinductions on the flanks of young leaf primordia. Our previous data had shown that local induction of cell proliferation on the flanks of leaf primordia led to a significant change in leaf morphology (Wyrzykowska et al., in press). In contrast, local induction of phragmoplastin did not lead to any consistent change in leaf shape (data not shown).

DISCUSSION

In this study we have analyzed the influence of cell division plane on organogenesis. In order to be able to do this, we constructed transgenic tobacco plants in which phragmoplastin (a dynamin-like protein from soyabean) was under inducible transcriptional regulation by Ahtet. Phragmoplastin is a protein involved in phragmoplast formation (Gu and Verma, 1996; Gu and Verma, 1997) and it has been shown that expression of this protein in tobacco suspension cells (BY2 cells) leads to the generation of cells with misorientation of cell walls. Our data support and extend these results by showing that transient local expression of phragmoplastin in intact plant tissue leads to altered planes of cell division. The exact mechanism by which this occurs is unclear, but presumably the accumulation of high levels of
phragmoplastin during an extended time of the cell cycle leads to disruption or misdirection of vesicle transport or fusion during the process of phragmoplast formation. This leads to cytokinesis becoming disjointed from cell growth and thus misorientation of the new cell wall. The mechanism by which this orienatation is set in the first place remains a mystery, although accumulating evidence indicates that cytoskeletal elements play a role in directing both the position of the new phragmoplast as well as ensuring the correct orientation of mitotic spindle formation (Cleary and Smith, 1998; Gachet et al., 2001).

In our experiments only microinduction of phragmoplastin gene expression in the meristem led to a clear disruption of the normal pattern of cell division. Thus, inductions at the level of the whole seedling, or even on the flanks of developing leaves did not lead to any clear disruption of cell division plane. Cells in the meristem are the most rapidly dividing cells in the plant body, thus it is possible that the cytokinetic apparatus is working towards the limit of its capability and, thus, is sensitive to any disruption of this process. Alternatively, it could be that since the meristem displays a relatively highly ordered cellular structure, any disruption to the plane of cell division is most apparent in this tissue.

Irrespective of the precise mechanism, local expression of the phragmoplastin gene led to altered cell division patterns in the outer tunica layers of the meristem. A number of cells underwent oblique or even periclinal divisions in positions of the meristem where in untreated meristems only anticlinal divisions were normally observed. As a consequence, cell size in the induced meristems was more variable than in control-treated meristems and the tunica layers took
on a more corpus-like cellular partitioning. Whether our manipulations also influenced cell division plane in the corpus itself was difficult to assess due to the endogenous variability of cell division orientation in this region.

Classical analysis of cell division frequency and orientation has led to the observed correlation of the site of leaf initiation with a concentration of periclinal cell divisions or, at least, a randomisation of cell division in this area. Whether this change in cell division pattern is causal to or a consequence of leaf initiation has been long debated (Steeves and Sussex, 1989). Our data indicate that local randomisation of cell division plane alone within the meristem is not sufficient to induce organogenesis. We cannot exclude the possibility that a causal randomisation of cell division in leaf initiation has to be highly localised in time and space and that our manipulations did not precisely replicate this pattern. However, taken in conjunction with our previous data on leaf initiation and development via local manipulation of cell wall extensibility and cell division, the simplest interpretation of our data are that within the meristem changes in cell division frequency and division plane are not sufficient for leaf formation. This interpretation also fits with the analysis of mutants such as fas and tangled in which altered division planes throughout the plant did not prevent the basic processes of organogenesis occurring. Rather, it seems that local changes in cell wall extensibility are the key cellular process involved in leaf initiation.

Our attempts to manipulate cell division orientation along the flanks of young leaf primordia did not lead to any overt change in leaf shape. This is in contrast to previous experiments in which local induction of cell proliferation led to clear changes in leaf morphology. This lack of
effect may, on the one hand, be due to this target tissue being less sensitive to disruption of cytokinesis by simple overexpression of phragmoplastin as cells in the meristem. Alternatively, it could be that the more variable endogenous patterns of cell division in the target tissue led to a masking of any change induced by local expression of the phragmoplastin gene. The role of cell division orientation in leaf morphogenesis therefore remains open, although the published analysis of mutants, such as tangled, argues against an essential role.

Although our manipulation of phragmoplastin gene expression in the meristem did not lead to any change in meristem function in terms of leaf initiation, they did lead to a change in the pattern of accumulation of the endogenous transcripts encoding NTH15. These transcripts normally accumulate throughout the corpus of the meristem except in the region of presumptive leaf initiation. Following local induction of phragmoplastin gene expression, NTH15 transcript accumulation was excluded from a large area of the meristem. The functional significance of this is unclear, but they suggest that simple downregulation of Knotted-like homeobox gene expression in the meristem is not sufficient for leaf initiation. Further experiments to examine the expression patterns of other marker genes for meristem function and leaf formation (e.g., phantastica-like) may shed more light on this phenomenon.

The disruption of the cell division pattern in the meristem led to cells generating daughters which became incorporated into neighboring cell layers. Thus, as shown in Fig. 2, following local induction of phragmoplastin gene expression daughter cells generated in, for example, the outermost tunica became part of the inner cell layer. The organs subsequently generated showed a normal histology with all cell
types being present in the correct position. This is further evidence to support the general observation that plant development is not cell-lineage dependent but depends on cell position.

In conclusion, our data support the theory that the orientation of cell division is not vitally important for the acquisition of plant shape. The appropriate orientation of cell division is more likely to be significant in the generation of appropriate cell types at the right place to allow for correct differentiation.
REFERENCES:


Figure Legends

Fig. 1 Molecular analysis of Nt.phr lines. (a) Total RNA was extracted from 1-month-old seedlings of line Nt.phr3 induced with Ahtet at the concentrations given for 24h. Equal amounts (30 µg) of RNA were loaded in each lane and blots hybridised at 42°C using a DIG-labelled antisense RNA probe. (b) As in (a) except that the seedlings were incubated for various times (as indicated) with 0.02 mg/ml Ahtet. (c) Genomic DNA was extracted from 1-month old seedlings of lines Nt.phr1 and Nt.phr3 and equal amounts (20µg) loaded on each lane. After hybridisation at 42°C using a DIG-labelled DNA probe, the blot was washed to a final stringency of 0.1xSSC at 68°C. WT= non-transgenic DNA either with (+) or without (-) Glyma;Phr cDNA.

Fig. 2 Histological analysis of Ahtet induced Nt.phr3 meristems. (a) mock-induced meristem. (b) line drawing to show cell outlines in (a). The lower drawing has been coloured to highlight the ordered outer two cell layers. (c) Ahtet induced (24h) meristem. (d) line drawing as in (b). The lower drawing has been coloured to highlight the altered patterning in the outer cell layers. T= tunica layer. (bar= 100µm).

Fig. 3 In situ hybridisation of Ahtet induced Nt.phr3 meristems. (a) Section from an apex induced for 7h then hybridised with an antisense probe for Glyma;Phr. (b) as in (a) but hybridised with a sense probe. (c) As in (a) but induced for 24h and hybridised with an antisense probe for NTH15. (d) As in (c) but hybridized with a sense probe. (bar= 100 µm)
The aim of the research reported here was to study the role of cell division in the mechanism of leaf initiation and development. The use of Ahetet-inducible system allowed us to manipulate the cell cycle locally in the meristem and at the edge of leaf primordia. As a result of such manipulations we made observations that led us to constructive conclusions adding more information to the far from complete picture of the mechanism of leaf development. This work demonstrates the influence of cell division on the development of a leaf in two aspects: 1) when the division frequency is changed, 2) when the orientation of division is changed.

Microinduction of cell cycle genes leads to locally increased cell proliferation. Recent research has shown that the modulation of expression of single components of the cell cycle can manipulate cell division. Thus, by constitutive overexpression of different types of cyclins or kinases and phosphatases (cell cycle elements) in transgenic a plants modulation of the cell cycle is possible and often results in increased growth (Doerner et al., 1996; Hemerly et al., 1995; McKibbin et al., 1998). In our study we employed genes of an A type cyclin from tobacco – (Nicta; cyclinA3;2) and a yeast phosphatase – (Sp; cdc25) in a novel transcriptionally regulated system allowing the local over-expression of these genes. In both meristem and primordia the local microinduction of Nicta; cyclinA3;2 gene expression resulted in increased cell proliferation. The increased cell proliferation was confirmed by histological analysis. The cells in the induced area appeared to be relatively smaller with big nuclei, dense cytoplasm and a few small vacuoles indicating a meristematic character. The number of cells was also checked and in the induced area was higher than in corresponding non-induced tissue. Overexpression of Sp; cdc25 resulted in a similar effect with additional modulation of cell size. Cdc25, together with wee1, are responsible for cell size regulation in yeast. However, it is unclear if the size variation in our experiments occurred due to the particular activity of cdc25 or if the observed variation was due to the expression of a heterologous gene in a plant system. Analyses at the molecular level were performed in order to check gene expression after induction. RNA in situ hybridization analyses showed
increased transcript levels at the induced positions. By using cell cycle marker
genes, such as histones, we could visualize the number of cycling cells which was
higher than in the case of non-induced or mock induced tissue. This suggested that
the cell cycle was stimulated resulting in a larger number of cells. However, it is
difficult to distinguish whether the time over which proliferation occurred was
prolonged or whether the rate of the cell cycle was increased in order to obtain the
observed higher number of cells. Concluding, the microinduction technique was
applied successfully resulting in local cell proliferation in both meristem and
primordia. In addition, the manipulation of the cell cycle via the Ahtet-inducible
system could be used as a useful tool to investigate the role of cell division in
plant development.

Localized increase of cell number in the meristem does not induce leaf
initiation. Our data (Pien et al., 2001) showed that local induction of cell wall
loosening by expansin at the I2 position of the meristem is sufficient to initiate a
new leaf. Taking into consideration the close interaction between cell elongation
and cell division, the induced cell growth provoked cell division but in this
context cell division was not the driving force in leaf development. In this study
we present data which help to answer the question whether induced cell division
can be the driving force in leaf initiation. We performed experiments in which the
I2 position of the meristem was induced to undergo higher rates of proliferation.
This resulted in a higher number of smaller cells in this area. However, this did
not produce altered organogenesis (i.e., leaf initiation and reversed phyllotaxy).

These observations can be interpreted according to a mechanistic theory in
which mechanical forces are responsible for meristem function (Green, 1980).
The I2 position is surrounded by two primordia (P1 and P2) which are positioned
very close to each other, thus creating a very narrow space (fig.5). On the other
hand, the I1 position occupies a very broad space which allows the bulging of the
future P1 primordia (fig.5). In such a situation, cells overproduced at the I2
position will be restricted in their expansion and so will reach a smaller cell
volume in order to fit into the narrow space maintained by the pattern of forces
within the meristem. In addition, the induced cells maintain division in an
anticlinal orientation, thus the direction of growth is maintained. Organogenesis
requires a shift in the direction of growth (which involves a change in cell wall extensibility) and this parameter is not affected by simple manipulation of the cell cycle. This maintenance of anticlinal cell division presumably reflects a maintenance of cytoskeletal organization which directs cell wall organization (Lyndon, 1998). Taken together, it seems that following local induction of cell proliferation the direction of growth (and, thus, the pattern of forces) within the meristem is maintained and no morphogenesis occurs. As the meristem cells are indeterminate and possess the capacity to adjust to alterations in division rate, the final shape and growth rate of the meristem is maintained as normal. As a consequence, the excess cells produced at the I2 position become removed from the meristem and become incorporated into new leaves and stem tissue at an appropriate time.

Fig.5 Scanning electron micrograph of shoot apical meristem of *Nicotiana tabaccum*. m – meristem, I1 and I2 primordia initiation places, P1 and P2 – primordium 1 and 2, the arch lines indicate the space on the meristem between the existing primordia.
An alternative interpretation (complementary rather than contrary to that described above) is that cells within the meristem are organised into symplastic fields (as described in the introduction). According to this theory, cells at the I2 position belong to two types of symplastic fields: the tunica field and the peripheral zone field that includes the corpus field. One interpretation is that although cells have been overproduced in this area, there was no change in the link between both fields, i.e., that the plasmodesmal separation between the zones was maintained. According to this theory, induced cell wall loosening would have to lead to some type of new inter-zonal link (presumably via changing the structure of the cell walls) and that this results in leaf initiation. In conclusion, cell division is not the key step in leaf initiation.

Localized changes of cell proliferation at the primordium flank leads to alteration of future leaf shape. Data presented here indicate that local induction of cell proliferation at the edge of a young leaf primordium (P2-P3 stage) leads to drastic changes in the future leaf shape. In the wide range of investigated primordia (P1-P7) only this narrow range of developmental stages showed the response to the induction by increasing the number of cell divisions and altering the shape of the leaf blade as a final result. This observation suggests that at a certain developmental age the primordia contain tissue which is sensitive to this manipulation. We suggest that this sensitivity is linked to a relatively high mitotic activity. This proposal is supported by data presented by the group of Nancy Dengler (Donnelly et al., 1999). Their experiments on Arabidopsis showed that a developing primordium at the stage P2-P3 undergoes a transient burst of divisions along the margin. Additionally they postulated that this event has a crucial role in leaf blade development. Our observation that modulation of cell division at the primordium edge at this time changes leaf development supports this hypothesis. In addition to experiments promoting cell proliferation (which inhibited lamina expansion) we performed experiments where the primordia were treated with roscovitine, a drug preferentially inhibiting CDKs and, as a result, causing retardation of the cell cycle. This manipulation produced a leaf deformation with the contrary morphological effect. In the area of cell division inhibition there was more leaf blade tissue produced than in the corresponding non-induced area.
Obtaining indentation of the leaf blade (suggesting the presence of a lower number of cells in this area, confirmed by statistical analysis – for details see Wyrzykowska et al. in press, Results section) after increasing the number of cells in the primordium is a non-intuitive result. A number of interpretations is possible. Firstly, introducing increased proliferation at a certain point along the primordium edge might cause differences in the time point of entering the differentiation pathway between induced and non-induced groups of cells. The cells that stayed longer in a proliferating state could possibly miss the entry into differentiation. For example, rapid proliferation adjusts cells to decreased expansion rate which is contrary to most differentiation processes in plants. If at the same time the non-induced cells begin differentiation (which is linked with increased growth rate) then an imbalance in growth rate between neighboring tissues would result, thus generating the observed indentation in the leaf blade (fig.6). The same logic can be applied in the case of cells with a retarded cell cycle resulting from roscovitine action. If inhibition of cell division is linked with increased growth rate (premature differentiation) then an increase in lamina expansion would result, as observed. A similar effect of increased leaf lamina growth was observed in our previous study when an increased level of expansin was introduced at the edge of primordia (Pien et al., 2001). Thus, the balance between tissue growth and cell proliferation at an early stage of leaf development seems to be the cause of producing less or more final leaf tissue. This explanation is supported by recent data from overexpression experiments of a G protein in Arabidopsis (Ullah et al., 2001). The group of A. Jones identified a plant homologue of a heterodimeric animal G protein that regulates growth and differentiation. They showed that the plant G protein is a modulator of cell proliferation. By overexpression and antisense approach they could increase or decrease (respectively) cell proliferation. Increased proliferation resulted in smaller leaves while inhibited proliferation resulted in leaves with a larger surface area than wild type. This observation corroborates our data suggesting that increased proliferation is the key point in retarding differentiation and, thus, growth.
Fig. 6 Model to show differences in cell size and organ shape upon response to locally increased proliferation. A) a primordium at P2-3 stage induced with a portion Ahtet soaked lanoline (yellow ball), the violet colour indicates normally (for this stage of primordium) proliferating tissue. B) the primordium after 36-48 h of induction, pink colour indicates the group of cells where proliferation was induced, the light blue colour indicates the cells that start entering the differentiation process (beginning of expansion). C) a young leaf 5-7 days after induction, the dark blue colour indicates the cells that differentiate (intensive expansion), the red colour indicates the cells that missed the switch to differentiation and kept the meristemmatic characteristic.
An alternative interpretation of our results could be derived from a hypothesis that each cell is set to undergo a certain number of cell cycles (Granier and Tardieu, 1998; Tardieu and Granier, 2000). In this light, by increasing cell proliferation rate just at a part of the edge of a primordium the group of cells that underwent such changes would use up the preset number of cycles earlier without reaching the time point of entering final differentiation. This would cause an inhibition of further growth. In the contrary situation, when proliferation is slowed down the cells could divide over a longer period of time with a switch to differentiation occurring that does not terminate division. Our histological analyses of disformed leaves support this in that the parts of the leaves induced to proliferate faster exhibit a more undifferentiated state (round shape, dense cytoplasm, lack of trichome differentiation) while in the case of leaves with decreased proliferation the structure of the leaf is normal.

The explanations presented above complement rather than exclude each other and lead to the same point of underlining the importance of the timing of cellular switch points in developmental programs.

The role of cell division in leaf morphogenesis is context dependent. Local cell proliferation in the meristem and leaf primordia resulted in different responses. The meristem could incorporate the alterations without influence on its overall structure and function while primordia translated the changes into an abnormal leaf shape. This indicates that the influence of cell division on morphogenesis is context dependent. This context dependence is limited even to the time and place within a primordium when a response to local changes in proliferation was observed. It suggests that the pattern of cell division is significant when the main decision about future organ shape is taken. Whether cell cycle elements represent targets for genes (often encoding transcription factors) responsible for leaf shape remains to be investigated.

Although our data do not show the actual mechanism by which leaf shape is altered in response to the modulation of cell division, they indicate a connection
between modulation of the cell cycle and leaf development that has not been obvious from previous investigations.

**Microinduction of phragmoplastin gene causes disruption of tissue organisation.** In recent studies on plant cytokinesis a number of genes have been identified involved in new cell wall formation. It has been shown by an overexpression approach that it was possible to redirect the new cell wall in dividing BY2 cells (Gu and Verma, 1997). In our study we show that overexpression of phragmoplastin is sufficient to change the orientation of new cell walls in tobacco plants. We first generated tobacco plants transformed with a cDNA encoding phragmoplastin from soybean under control of a chemically inducible promoter system. Previous data have shown that overexpression of this protein in a heterologous system led to disruption of cytokinesis (Gu and Verma, 1997). Using the inducible transcription system we avoided possible problems with transformant regeneration due to lethality of transgene expression. Apices of transgenic plants (selected for their inducibility of transgene expression) induced to overproduce phragmoplastin exhibited a disturbed tissue organization. Histological analyses of such meristems showed that the cells of tunica layers were dividing not only anticlinally but also in an oblique manner. This led to disruption of the orderly layers of tunica and resulted at some points in 3 instead of 2 layers. In the case of the corpus of the meristem it was difficult to judge the cell wall orientation as in this part of the meristem divisions in all possible directions occur naturally. To confirm the induction of gene expression we performed in situ hybridization analysis. Elevated levels of transcript were visualized as a localised signal corresponding to the induced I2 position. The signal appeared to be localized not only to the tunica layers but also was present in the meristem corpus.

**The reorientation of cell division plane has influence on tissue structure but does not affect morphogenesis.** One of the first events associated with leaf initiation at the histological level is periclinal division in the second tunica layer (Steeves and Sussex, 1989). This observation led us to the question whether disrupting the anticlinal pattern of divisions in the tunica layers in the I2 position of the meristem would initiate the program of leaf initiation, i.e. is the
orientation of cell division one of the key steps in leaf initiation? Meristems induced to overexpress phragmoplastin at the I2 position showed a disrupted order of the tunica layers (as described above). Although the altered divisions were not really periclinal, a change in the organization of the tunica was introduced. However, this did not influence leaf initiation or the phyllotaxy of the plant.

The induction of phragmoplastin gene expression changed not only the tunica organization but also the expression pattern of the NTH15 Knotted-like gene. In normal conditions NTH15 expression decreases at the I1 position and is thought to be involved in promoting this portion of cells to enter differentiation. In situ hybridization analysis suggested that a decrease of NTH15 mRNA accumulation was correlated with the induction of phragmoplastin over-expression at the I2 position. Thus, even though two symptoms associated with leaf initiation (altered cell division and down-regulation of Knotted-like gene expression) were observed at the I2 position of the induced meristems, the initiation of a new leaf was not promoted. These data indicate that these events are not causal in leaf formation and again corroborate the concept that the changes in cell wall extensibility rather than cell division frequency or orientation appear to be the key to this process.

Primordia were also tested for their response to rearranged direction of divisions along the margin. Such induced primordia exhibited a complete and normal development. Thus both meristem and leaf primordia, indicate by their responses that cell division orientation is not the key issue in organ morphogenesis.

This conclusion is also indicated by study on the tangled-1 mutant from maize showing that although disturbing cell division planes in the whole leaf may influence the leaf size, leaf shape is not changed, i.e., the direction of cell division and the developmental program for leaf formation can be uncoupled. Our data also indicate that induction of phragmoplastin expression at the whole seedling level does not affect plant development and leaf shape. In conclusion, this study substantiates previous theories that the orientation of cell division is not vitally important for the acquisition of plant shape.
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PUBLICATIONS


