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Chapter 14 Hormonal regulation of cell cycle progression and its role in development

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14.1 Introduction

It is becoming clear that auxin and cytokinin drive progression through the cell division cycle and also define the regions of cell division within apical meristems through the creation of regions of locally amplified hormone concentration. Mutations and transgenes that affect hormone concentration confirm the classic hypothesis that cytokinin is important in promoting shoot meristem identity and slowing root development, while conversely auxin preferentially promotes root development (Skoog and Miller, 1957). A necessity for cytokinin and auxin to initiate DNA replication and mitosis, represents control at the same transitions that are regulated in other eukaryotes but, although DNA replication has marked similarities with metazoa, the initiation of plant mitosis has unique features. At DNA replication, hormonally induced accumulation of D-cyclins directs phosphorylation to RB-related proteins, which then release S-phase transcription, as described in detail in accompanying chapters. More attention is paid here to control of mitosis, which requires increase in CDK enzyme activity, through removal of inhibitory phosphate from CDK as in other eukaryotes, but in plants it is dependent on cytokinin accumulation in the late G2 phase. Cytokinin abundance is essential for mitotic initiation but can be substituted by expression of yeast CDC25 tyrosine phosphatase that is specific for CDK.

Hormones that stimulate cell cycle progression also act at long distances in balancing growth of root and shoot and, additionally, within apical meristems they delimit cell division zones. In the shoot apex, abundance of cytokinin is a key regulatory element. Its induction of the cytokinin biosynthesizing enzyme IPT7 reinforces local cytokinin concentration and sustains cell cycle activity. Whereas in the root apex, auxin is the key hormone that specifies stem cell and meristem areas through local concentration of the hormone, which results from its recirculation under the direction of auxin efflux regulators of the PIN protein family. Hormones in multicellular plants therefore function to organize division zones as well as driving progression through the division cycle.

This review considers first the impacts of hormones on the division of an individual cell, and then presents recent molecular evidence that hormones arriving from long distance at the meristem regions of root and shoot are used to create zones of locally raised hormone concentration within the meristem. Finally, recent evidence is presented that long-distance transport of hormonal signals, and resulting

changes in the ratio of hormones, determines the location of lateral shoot and root development and hence plant architecture.

14.2 Auxin and cytokinin have paramount roles in cell proliferation control

Auxin and cytokinin were initially pinpointed as the most potent regulators of plant cell division by classical studies of plant tissue culture. These revealed stimulation of division and capacity to induce development of root or shoot identity through change in hormone ratio. Appreciation of the key role of auxins and cytokinins is reinforced by the ability of *Agrobacterium tumefaciens* to induce tumours through genes for auxin and cytokinin synthesis, and is further underlined by the absence of any known mutants that are viable while unable to synthesize these hormones. This review narrows its focus to auxin and cytokinin, which are both absolutely required for division and meristem function.

14.3 Growth and cell cycle gene expression induced by auxin and cytokinin

Auxin and cytokinin are classically referred to as growth factors, and some of their effects on cell division derive from growth stimulation. Growth is certainly required for the maintenance of average cell size in proliferating cell populations and in the individual cell there is a widely observed and probably universal need to attain a critical minimum cell size for division (reviewed by Fantes *et al.*, 1975; Fantes and Nurse, 1981; Mitchison, 2003). This is clear in yeasts, where conditional division mutants are available and reveal that S phase is initiated at constant cell size even when growth rate changes (e.g. Carter and Jagardish, 1975). A cell size requirement is also detected in mammalian cells (e.g. Zetterberg and Killander, 1965; Dolznig *et al.*, 2004) and is implied in plant cells from the increasing probability of division as cells enlarge (Canovas *et al.*, 1990) which conforms to the eukaryote-wide observation that cell size is stabilized in proportion with nuclear DNA content by initiation of division at the appropriate cell size (Gregory, 2001). A further, probably universal, coupling to growth comes from requirement for a significant growth rate, seen in animal cells (Zetterberg and Larsson, 1985) and yeast cells, where faster growth allows more efficient translation of essential cell division proteins, in particular the G1 cyclin (CLN3) of budding yeast (Polymenis and Schmidt, 1997) and the mitotic phosphatase CDC25 and B-cyclin (CDC13) of fission yeast (Daga and Jiminez, 1999). These are essential for the key transitions G1/S and G2/M phase (see later) and accumulate only when cells are growing well, since a potential hairpin configuration in their mRNA is prevented from forming by a high density of active ribosomes on the messenger.

Growth is therefore a prerequisite for sustained proliferation of all cells, and one action of plant growth factors that drive the cell cycle is indeed to stimulate growth. Auxin in particular often stimulates biosynthesis and cell enlargement (Skoog and

Miller, 1957; Theologis *et al.*, 1985; Lincoln *et al.*, 1990). This is evident in culture, where cells without auxin cease growth and therefore do not divide, whereas cells with auxin but lacking cytokinin continue in growth but are blocked in division (e.g. Skoog and Miller, 1957). However, an additional direct influence of auxin and cytokinin on division is indicated by their induction of some cell cycle genes.

Hormone-induced expression of cell cycle genes can proceed rapidly in cells primed to respond to hormone, as in the elongation zone of pea roots, where auxin induces a rise in the transcript of *CDKA* within 10 min and a stable, threefold higher level by 3 h (John *et al.*, 1993). Cells in this zone have been recently formed and they have *CDKA* mRNA above the basal level found in fully differentiated cells. In normal development they respond to the tip having grown more distant by initiating local cell division and growth in new lateral root primordia. Such spacing of lateral roots, by initiation at a distance from the root tip, aids the efficient mining of soil for nutrients, and the signal for lateral root induction may be attainment of a sufficiently high ratio of auxin to cytokinin (as will be discussed further); therefore, exposure to auxin can be interpreted as the premature arrival of a signal to which the tissue is primed to respond. Induction of *CDKA* expression in response to auxin has also been detected in whole roots within 2 or 3 days (Martinez *et al.*, 1992; Hemerly *et al.*, 1993) and the CDK protein itself is detectable after 2 days in auxin-treated cotyledon explants (Gorst *et al.*, 1991). These slower induction responses, in cells not programmed to proliferate unless wounded, suggest that auxin often first stimulates cell growth before inducing cell cycle catalysts. A significant basal level of *CDKA* protein and transcript is found in all viable plant cells, even if not actively dividing (Gorst *et al.*, 1991; Martinez *et al.*, 1992), probably reflecting the unique general capacity of plant cells to return to division.

Auxin raises the capacity for division by inducing *CDKA* but additional proteins are required for division and auxin can induce some of these after a lag during which growth occurs or prior cycle events are completed. Two examples are, expression from the mitotic cyclin *CYCA2;1* promoter in *Arabidopsis* root within 12 h of exposure to auxin [synthetic NAA] (Bursens *et al.*, 2000) and from the promoter of mitosis-specific *CDKB* (*CDKB2;1*) of *Medicago*, in a synchronous culture stimulated with auxin (synthetic 2,4-D). The *CDKB* gene was expressed after a 9 h lag during which cells entered late G2 phase and came to require *CDKB* for mitosis (Zhiponova *et al.*, 2006). The particular transcription factors that are most significant in the expression of cell cycle genes are not yet known.

Cytokinins are particularly strong inducers of the D-cyclins, which have well-characterized effects on the induction of S phase in animals and plants through activation of S phase transcription (described later). Induction of D-cyclin is therefore a major mechanism by which cytokinin stimulates division. The question is, can cytokinin also induce accompanying growth? Although development of most animal cell types is remarkably unaffected by the absence of D-Cyclins (Kozar *et al.*, 2004), these cyclins are essential for growth and proliferation of the major lymphoid and myeloid blood cell lines, and furthermore, these become malignant if *D-cyclin* expression is upregulated (Bergsagel *et al.*, 2005). This has been interpreted as a possible indication that D-cyclins stimulate growth as well as division (e.g. Sherr,

1994), perhaps because the substrates of CDK/CYCLIN D could include transcription factors for genes that drive growth, or because CDK/CYCLIN D in animal cells could phosphorylate signal transduction proteins that are normally phosphorylated in response to growth factors. If such mechanisms operated in plants, then cytokinins that induce D-type cyclins and cell division may also be responsible for meristem growth.

Evidence for D-CYCLIN enhancement of growth is not conclusive. Expression of CYCLIN D in cultured mammalian cells accelerates entry into S phase and therefore reduces the number of cells in the G1 phase, but cells were smaller through having accelerated division without accompanying increase in growth (Ohtsubo and Roberts, 1993; Quelle *et al.*, 1993; Resnitzky *et al.*, 1994). Similar reduction in cell size from transgenic stimulation of division occurs in the *Drosophila* wing (Neufeld *et al.*, 1998). But growth stimulation by raised D-CYCLIN sometimes can occur, as in the *Drosophila* eye when CYCLIN D complexes with CDK4, although this CDK is not essential for growth and has no plant analogue (Frei and Edgar, 2004). Cell growth is therefore not an obligatory accompaniment of cell cycle progression or consequence of raised CYCLIN D. In plants also, it is not clear that D-cyclins regulate growth, since in cultured Arabidopsis cells CDK/CYCLIN D2 activity rose only after growth had been resumed for 2 h and CDK/CYCLIN D3 increased even later at 4 h (Riou-Khamlichi *et al.*, 2000). Induction of growth cannot confidently be attributed to CDK/CYCLIN D2 since the *CYCD2;1* gene of Arabidopsis induces faster growth only when heterologously expressed in tobacco seedlings and without the change in abundance of G1 phase cells, which would be expected if it affected the cell cycle (Cockroft *et al.*, 2000). Similarly, overexpression of *CYCD3;1* in Arabidopsis prevents termination of cell division in the developing leaf but it does not increase growth, and therefore reduces cell size (Dewitte *et al.*, 2003). It is therefore difficult to conclude that cytokinin induction of CYCLIN D induces plant growth as well as cell division.

14.4 Does cell cycle progression affect growth?

The foregoing discussion opens the broader topic of whether the formation of new cells results in growth. Hormones that influence division will be of broader importance if control of division also regulates growth. Growth regulation by division might occur according to the hypothesis that growth is cell based, that is growth occurs because progress through the cell cycle itself stimulates growth or daughter cells are programmed to grow to a certain final size. Consistent with cell-based growth, the size of rodents increases when CDK/CYCLIN activity is increased by knock-out of the CKI class inhibitor p27KIP1. The modified mice have more cells and, although cells are unable to quite reach full size, the animals do increase somewhat in size (Nakayama *et al.*, 1996).

A quite different interpretation of growth is that growth itself is regulated and forms an organ or organism of regulated final size, while cells divide as necessary to accommodate the increasing mass. In this case the general stability of cell size

must result from an additional mechanism coupling division to growth. Such adjustment of division to growth is already in operation in the unicellular organisms from which multicells evolved. In unicellular organisms, a general requirement of adequate cell size for commitment to division has been noted in unicellular fungi (Fantes and Nurse, 1981; Mitchison, 2003) and plants (Donnan and John, 1983) and has been conserved through the transition to multicellular animals (e.g. Dolznig *et al.*, 2004) and, available evidence suggests, also to higher plants (Canovas *et al.*, 1990).

Current evidence is against cell-based growth in lateral organs, such as the leaf, where the determinate final size of the organ conflicts with any requirement that cells should reach a postulated programmed size. Cell size was not maintained when cell number in the Arabidopsis leaf was increased by overexpression of *CYCLIN D3* and markedly smaller cells resulted (Dewitte *et al.*, 2003); however, local induction of cell division was to some extent able to attract growth within the developing leaf to the affected region (Wyrzykowska *et al.*, 2002). Cell-based growth might therefore be thought more likely to occur in indeterminately growing terminal apices, where growth might extend to accommodate a programmed final cell size in spite of larger cell number. But our current analysis of ectopic cyclin expression in the root tip (Ruhu Qi and Peter CL John, unpublished observations) indicates that growth there also is not cell based.

Cell division may not therefore be the engine of plant growth, but it remains at least a vital process for the continuation of growth by maintaining cells at a physiologically effective size; small enough to allow adequate levels of gene transcripts and other nucleus-derived elements to permeate the cytoplasm. At the tissue level, cell division is also important in generating daughter cells in the correct number, location and shape to allow formation of tissues and organs of specialized function. For example in the leaf, division in the epidermis generates stomata by subdivision without requiring growth, while internally the apportionment of growth into daughter cells that form palisade and spongy mesophyll layers generates the photosynthetic tissue.

14.5 Division sustains continuation of growth

The dependence of continued growth on cell formation can be seen from the cessation of growth in lateral roots and shoots when cell division is prevented by irradiation or cytoskeleton inhibition. Lateral root formation begins with localized growth in a few pericycle cells (in Arabidopsis as few as 2–3 longitudinal neighbours) which grow radially and divide periclinally maintaining an isodiametric shape (Sussex *et al.*, 1995). This initial primordial growth can occur if cell division is blocked by colchicine, but growth ceases when cells have become unusually large, having nonetheless formed the shape of an incipient primordium. The blocked primordium can resume growth and development if colchicine is removed (Foard *et al.*, 1965). A similar dependence of lateral root outgrowth upon cell division is seen in the inhibitory effect of upregulated expression of the CDK inhibitor CKI/KRP2 on

lateral root formation, which incidentally indicates that CKI protein transcription has a developmental role in addition to a cell cycle regulatory role in plants (Himanen *et al.*, 2002).

In the shoot apex also, leaf primordia can emerge by localized growth even when cell division is blocked by irradiation, as in *Triticum* (Foard, 1971). Furthermore, if division in the developing leaf halts at a smaller cell number, due to suboptimal cytokinin that has been engineered by ectopic expression of cytokinin oxidase, the cells become larger than usual but not sufficiently to compensate for reduced number and a small leaf results (Werner *et al.*, 2003). The eventual cessation of growth for lack of cell division may be caused by physiological inefficiency in abnormally large cells and also, in complex cell division zones such as apical meristems, because further development requires signalling and hormone movement (as will be discussed) between cell layers that have not formed.

14.6 Localized growth

The mechanisms by which growth factors may induce local growth and hence foci of cell division remain mysterious. In the case of lateral root primordia, their initiation occurs opposite protoxylem poles and is negatively regulated by localized expression of the cyclin-dependent kinase inhibitor gene *ICK/KRP2*. This gene reduces lateral root number if ectopically expressed and it is transcribed in interpolar regions of the pericycle where lateral root initiation would disrupt normal radial distribution of laterals (Himanen *et al.*, 2002), but the molecular basis of localized radial expression of *ICK/KRP2* and of the spacing of laterals along the longitudinal axis is uncertain. Longitudinal spacing of laterals is set by the proximity to the apical meristem at which a lateral root can be initiated. This distance and, therefore, the spacing between laterals is reduced at higher auxin/cytokinin ratio (Wightman *et al.*, 1980; Hemerly *et al.*, 1993; discussed below).

Also mysterious is the mechanism by which total organ size is perceived and maintained. In the developing leaf it has been noted that cell division is less extensive if cytokinin level is reduced by cytokinin oxidase, which correlates with cytokinin importance in division, but there is also a correlation with reduced overall size of the leaf and therefore it is not impossible that a gradient of cytokinin extending into the enlarging leaf determines the limits of its growth (Werner *et al.*, 2003). Many aspects of hormonal control over growth are still not understood; however, the mechanisms of hormonal control over cell division are perhaps becoming clearer.

14.7 Hormonal impacts at the G1/S phase progression

A major control event of the cell division cycle is the termination of the G1 phase by commitment to enter DNA replication. In microorganisms this commitment, once taken, is sustained even if growth is subsequently slowed or prevented, for example, by low nutrient (e.g. Carter and Jagadish, 1975) or by absence of photosynthesis

in plant cells (Donnan and John, 1983). At the molecular level, key events in commitment to the S phase are the attainment of a concentration of G1-CYCLINS (D-CYCLINS) that raises CDK/CYCLIN activity and increases phosphorylation of the CDK inhibitor class of proteins (CKI) that then have increased affinity for the ubiquitination machinery and are consequently proteolyzed. This proteolysis of CKI is a key event since microbial cells where gene knockout is easier reveal that all G1-CYCLINS can be deleted and yet cell viability can be maintained if there is also removal of the CKI protein (Sanchez-Diaz *et al.*, 1998). Consistent with this, elevated expression of plant CKI homologues slows cell division (described in accompanying chapters). Further progression to DNA replication involves CDK/CYCLIN D-catalyzed phosphorylation of pocket proteins (RBR) that unless phosphorylated prevent transcription by E2F class transcription factors that transcribe the genes of DNA precursor synthesis and DNA replication as well as the genes that encode mitotic cyclins (reviewed by Gutierrez *et al.*, 2002, and detailed in accompanying chapters). Establishment of the S phase is therefore accompanied by the appearance of a new class of cyclins – the mitotic cyclins, which are distinctive in supporting higher CDK/CYCLIN activity that is necessary for DNA replication and later for mitosis.

Events at the G1/S phase transition therefore require CDKs and CYCLINS, of which CYCLIN D synthesis can be most clearly linked to stimulation of cell division at this time. In cultured plant cells, cytokinin strongly induces CYCLIN D3 whereas CYCLIN D2 is more responsive to sucrose (Murray *et al.*, 1998; Riou-Khamlichi *et al.*, 2000). Sucrose may be considered in this respect a *de facto* hormone and its effects on shoot/root ratio and flowering (Tran Thanh Van, 1981; Galtier *et al.*, 1993) could derive in part from its effects on cell division through induction of CYCLIN D2. Although both auxin and cytokinin have been found to affect the expression of CDKs and CYCLINS, there has been little study of possible hormonal interactions in particular tissues. Competitive effects on gene expression can occur since the rise in CYCLIN D3 transcript that occurs within 4 h of treatment of Arabidopsis callus with cytokinin (kinetin) was prevented if auxin (2,4-D) was present in addition (Murray *et al.*, 1998). Furthermore, post-translational events can affect levels of division catalysts, since in pea root CDK induced by auxin (IAA) is proteolyzed if cytokinin (zeatin riboside) is applied (John *et al.*, 1993). These interactions could have developmental implications, for example as a mechanism of inhibition by root apical meristems of lateral root initiation, as will be discussed.

Induction of CYCLIN D expression is a major component of cytokinin stimulation of the cell cycle since its expression can replace the presence of cytokinin. When leaf tissue of transgenic Arabidopsis expressing 35S::CYCD3 was tested for its ability to form callus on medium with auxin only, or with auxin plus cytokinin, the number of calli able to form without external cytokinin increased from 18% in wild type to between 41% and 50% owing to the elevated CYCLIN D3 transcript (Riou-Khamlichi *et al.*, 1999). This independence from external cytokinin in some lines that expressed CYCLIN D3 indicates that a major effect of cytokinin is to induce expression of this cyclin, but the observation that half of the calli still required cytokinin indicates that it has other essential functions.

14.8 Hormonal impacts at the G2/M phase progression

Cytokinin is also required at the initiation of mitosis, which is a control point that operates in all eukaryotes (Norbury and Nurse, 1992; MacNeill and Nurse, 1997) through inhibition of enzyme activity in CDK/mitotic cyclin complexes by phosphorylation of a tyr15 in the CDK supplemented by a weaker additional inhibition caused by phosphorylation of the adjacent thr14. The inhibited state is established in the G2 phase by the tyrosine kinase WEE1, of which plant homologues have been identified (Sun *et al.*, 1999; Sorrell *et al.*, 2002). Mitosis is then initiated by the progressive removal of the inhibitory phosphates by regulated activity of the protein tyrosine phosphatase CDC25 (Millar *et al.*, 1991; Norbury and Nurse, 1992; Kovelman and Russell, 1996), which can be referred to as a dual-specificity phosphatase in higher organisms in recognition of its dephosphorylation of tyr and thr. While the same dephosphorylation occurs in plant CDK (Zhang *et al.*, 2005) the identity of the enzyme that carries out CDC25 phosphatase function in plants is unresolved, as discussed below. This is a major question as the G2/M control point universally provides a means to block mitosis if DNA is damaged or incompletely replicated (Rhind and Russell, 2001; Preuss and Britt, 2003), but it also operates more frequently in plants than in other kingdoms as a point of arrest under physiological or developmental influence (Van't Hof, 1974; Zetterberg and Larsson, 1985). Progression at G2/M can be delayed by water or oxidative stress (Schuppler *et al.*, 1998; Reicheld *et al.*, 1999) or accelerated by hormone stimulus in excised rice stems (Sauter and Kende, 1992) and is also under developmental control since pericycle cells arrested in the G2 phase are the site of the localized growth that initiates lateral root primordia (Beeckman *et al.*, 2001).

A role of cytokinin in stimulating the G2/M phase progression is indicated by G2 arrest when cytokinin is removed from tissue culture (e.g. Fosket, 1977; Tao and Verbelen, 1996; Valente *et al.*, 1998) and by converging evidence from two independent cultured cell lines. A line of *Nicotiana plumbaginifolia* cells repeatedly cultured with cytokinin (kinetin) was found to have lost the capacity to synthesize sufficient cytokinin to be autonomous and in the absence of external cytokinin could only progress through the G1 and S phases to an arrest point in the late G2 phase, which indicated that cytokinin was necessary for entry into prophase (Zhang *et al.*, 1996). Consistent with this, freshly isolated tobacco cells without cytokinin also execute DNA replication but cannot enter mitosis (Valente *et al.*, 1998). The *Nicotiana tabacum* BY2 cell line is autonomous for cytokinin and increases its level through several orders of magnitude at the late G2 phase (Redig *et al.*, 1996) coincident with the time when the *plumbaginifolia* line arrests if cytokinin is not available. Furthermore, the *tabacum* line, like *plumbaginifolia*, is unable to enter mitosis if cytokinin synthesis is inhibited, but mitosis is released by added cytokinin (Laureys *et al.*, 1998).

The block to mitosis without cytokinin in *plumbaginifolia* was found to involve accumulation of CDK in inactive form phosphorylated at Y15, which could be activated by the catalytic portion of CDC25 (Zhang *et al.*, 1996). Furthermore, cells of alfalfa in the late G2 phase accumulate CDK containing tyr-phosphate,

which can be activated *in vitro* by CDC25 from *Drosophila* (Mészáros *et al.*, 2000). From this evidence it was uncertain whether the primary cytokinin-responsive event may have been at some early point in the G2 phase leading to CDK activation later as a part of mitosis. To test this, the CDK activator CDC25 was investigated for possible ability to substitute for cytokinin in stimulating mitosis. The fission yeast *CDC25* gene, which is of well-established specificity for CDK (Millar *et al.*, 1991) was brought under the control of the steroid inducible promoter, and its expression was induced in cells arrested in the G2 phase by lack of cytokinin. Remarkably, in view of the multiple actions of cytokinin (e.g. Hoth *et al.*, 2003), *CDC25* expression was able to fulfil the cell cycle functions of cytokinin, allowing mitosis and proliferation (Zhang *et al.*, 2005). Both normal and induced mitosis involved an increase in CDC25 tyrosine phosphatase, coincident with a maximum rate of activation of CDK, accompanied by a decline in tyrosine phosphate content in the CDK and a decline in capacity of the plant CDK to be activated *in vitro* by CDC25. In tobacco cells also, a cytokinin block to mitosis (Laureys *et al.*, 1998) was circumvented if *CDC25* was expressed (Orchard *et al.*, 2005).

In cell division, therefore, a key outcome of cytokinin presence was the tyrosine dephosphorylation of CDK in the late G2 phase. During evolution, the tyrosine and adjacent threonine that are negatively regulated by WEE1 phosphorylation have been retained in plant cell cycle CDKs (e.g. Joubès *et al.*, 2000; and accompanying chapters), and furthermore, in the plant, developmental consequences of regulatory CDK phosphorylation are consistent with this being a biochemical signal of cytokinin presence, as discussed later.

The identity of the CDC25-like tyrosine phosphatase(s) in plants remains a fascinating question. The primitive unicellular green plant *Ostreococcus tauri* has a gene with clear homology to the highly conserved CDC25 genes of yeasts, insects, and animals and it complements conditional mutation in *cdc25ts* of fission yeast (Khadaroo *et al.*, 2004). However, the green lineage diverged very early in evolution from *O. tauri* (Bhattacharya and Medlin, 1998) and there has apparently been an accompanying divergence of structure in the mitotic phosphatase(s), or else the adoption of mitotic function in other phosphatase(s), since no known higher plant phosphatase has the highly conserved regions characteristic of CDC25 in other kingdoms. Identification of higher plant mitotic phosphatase may therefore come to depend on tests of their function, which can be ambiguous since complementation of the *cdc25(ts)* mutation in fission yeast is rather easily satisfied, for example, by a human protein tyrosine phosphatase that is unrelated to the cell cycle (Gould *et al.*, 1990). Furthermore, it is not certain which of the several mitotic phosphatases in yeast may be the most closely related to the plant enzyme since in fission yeast CDC25 works in tandem with PYP3 phosphatase. Mitosis is delayed if PYP3 is absent and can be sustained entirely by PYP3 alone if *PYP3* expression is raised (Millar *et al.*, 1992), and furthermore, STP1 can also substitute for CDC25 although it has no mitotic phenotype (Mondesert *et al.*, 1994). Higher plants may therefore have evolved a distinct different phosphatase enzyme with mitotic function, or may use a variant of PYP3 or even STP1. In animal cells there are up to three structurally

related CDC25 enzymes (e.g. Mailand *et al.*, 2000) and we cannot eliminate the possibility that plants could contain multiple, perhaps structurally unrelated, tyr-thr-phosphatases.

A possible CDC25 homologue has been named in *Arabidopsis* and indeed both activates CDK *in vitro* (Landrieu *et al.*, 2004) and advances mitosis when overexpressed in yeast (Sorrell *et al.*, 2005), but it cannot complement absence of CDC25 activity (Landrieu *et al.*, 2004) and is not preferentially expressed in tissues containing dividing cells (Sorrell *et al.*, 2005) although the opposing tyrosine kinase *WEE1* is so expressed (Sorrell *et al.*, 2002). The putative CDC25 has no clear sequence similarity to the CDC25 of other kingdoms, although it is predicted to fold similarly, and it is also unusually small, corresponding to only the catalytic region of other CDC25 molecules. Its failure to complement CDC25(ts) may derive from the absence of regulatory regions, although this absence has not prevented other phosphatases from complementing. The enzyme that has CDC25 function at plant mitosis is therefore far more difficult to identify than in other kingdoms and it may be necessary to gain information from the activity of putative CDC25 enzymes through the cell cycle, under normal progression and with checkpoint arrest, to test for functional relationship to division. It should be noted also that function of the same or related tyr-phosphatase may be required at S phase initiation, as in mammalian cells where the S phase-specific CDK2 is under control of a discrete phosphatase (e.g. Mailand *et al.*, 2000). The multiple CDKs found in plants such as alfalfa (Mészáros *et al.*, 2000) could reflect specific S phase function in some.

It is also uncertain how cytokinin, which is clearly necessary at mitosis, can at that time increase mitotic phosphatase relative to WEE1 kinase activity. It is encouraging that alternative phosphorylation patterns have been found to regulate activity in CDC25 phosphatases (e.g. Kovelman and Russell, 1996; Bulavin *et al.*, 2003) and in the opposing WEE1 kinases (Aligue *et al.*, 1997; Rhind and Russell, 2001) and that the activity changes include upregulation of tyr-phosphatase and downregulation of tyr-kinase, either of which could induce mitotic initiation. It may be significant that cytokinin signal transduction proceeds by transfer of protein phosphate groups (Haberer and Kieber, 2002; Kakimoto, 2003).

Operation of hormonal control over mitotic initiation is consistent with the additional importance of mitosis in plants as leading to the formation of structural elements such as cross walls and as committing continued proliferation rather than switching to endoreduplication that frequently provides a means for cell enlargement in the plant kingdom (Larkins *et al.*, 2001; Boudolf *et al.*, 2004). Control of mitosis through regulation of CDK is consistent with the central importance of CDK in plant cell division, indicated by slower division when CDK is mutated (Hemerly *et al.*, 1995) or is inhibited (e.g. Cleary *et al.*, 2002) and faster mitotic progression when active CDK is microinjected (Hush *et al.*, 1996). Significance of the mitotic control point in the intact plant is seen when CDK is activated by local expression of yeast CDC25, which results in local zones of division (Wyrzykowska *et al.*, 2002), and similarly constitutive expression increases lateral root and shoot initiation (McKibbin *et al.*, 1998; Suchomelova *et al.*, 2004). Lateral root primordia

Figure 14.1 Cytokinin control of cell cycle progression. In the G1 phase, cytokinin stimulates CYCLIN D transcription and the increased CDK/CYCLIN D activity commits the cell to subsequent DNA synthesis, by proteolysis of CDK inhibitor proteins, phosphorylation of RB-related protein and activation of transcription by E2F factors. Resulting expression of deoxyribonucleotide-synthesizing enzymes, DNA-replicating proteins and mitotic cyclins drives DNA polymerization. In the G2 phase, cytokinin stimulates protein phosphatase activity, which has the CDC25-like function of progressively removing inhibitory phosphate placed on CDK by the tyrosine kinase WEE1. Rising CDK/CYCLIN B activity is integral with prophase, driving disassembly of the preprophase band, condensation of chromosomes, assembly of the mitotic spindle and nuclear envelope breakdown, which together establish the metaphase of mitosis.

are also indicated to come under post-translational control since the initials begin division in the pericycle while not initially expressing more cell cycle proteins than their neighbours (Hemerly *et al.*, 1993). Cytokinin therefore makes a dual contribution to the cell cycle (John and Zhang, 2001) in which progression at G1/S is advanced by induction of CYCLIN D and mitosis is initiated by activation of CDK tyr-phosphatase activity (Figure 14.1).

The arrest of cells at either the G1 or G2 phase when cytokinin is limiting may depend on slight differences in endogenous levels of CYCLIN D, or of activated phosphatase and CDK/CYCLIN B that determine which transition first becomes limiting as cytokinin concentration falls. Certainly, in plant tissues cells can arrest at either point (e.g. Van't Hof, 1974) and cells in different experimental systems have different arrest tendencies.

14.9 Roots and shoots provide each other with hormones essential for division

The cell division hormones auxin and cytokinin are more abundant at opposite poles of the plant. Higher extractable levels of cytokinin are found in roots (Chen *et al.*, 1985) but auxins are more abundant in shoot tips and young leaves (Srivastava, 2002). These locations are likely sites of synthesis and this is supported for cytokinin by evidence that in vegetative tissue the biosynthetic gene *ISOPENENTYLTRANSFERASE4* (*IPT4*) is preferentially expressed at the root tip (Miyawaki *et al.*, 2004).

Several aspects of meristem activity in the plant can now be understood as derived from the requirement for hormones from these widely separate sources. The higher concentrations of auxin in the root, and cytokinin in the shoot, imply gradients of declining concentration extending from the major sites of synthesis. A more recently appreciated refinement is that both shoot and root apical meristems amplify the concentration of the limiting hormone that arrives from the opposite pole of the plant and it then locates cell division activity within the apex. Furthermore, the initiation of lateral meristems may be located in response to the gradients of auxin and cytokinin extending through the plant from shoot and root, as will be discussed.

The broad trends in hormone concentration through the plant are shown in Plate 14.2, which also indicates the locally raised concentrations within meristems. Cytokinin in the shoot apex is amplified by local synthesis, while auxin in the root is concentrated through local recirculation. These concentration mechanisms for individual hormones that operate at the limit of their long-distance signalling prevent suboptimal concentrations for division where intensive division is required and they also provide demarcation of the regions of rapid cell division within apical meristems.

14.10 Cytokinin contributions to stem cell and meristem identity at the shoot apex

In the shoot apex, a stable core of indeterminate stem cells in the outer three cell layers is created by the interaction of several genes. The *KNOTTED1*-like homeobox (*KNOX1*) family of shoot homeobox genes is upregulated, perhaps indirectly, by cytokinin (Rupp *et al.*, 1999) and in *Arabidopsis* a key member of this family has been named *SHOOT MERISTEMLESS* (*STM*) for its phenotype when mutated. One function of *STM* is to specify stem cell proliferation in the central region of the shoot apex in concert with the *WUSCHEL* gene (*WUS*), a homeodomain gene of another family. *WUS* maintains a stable number of stem cells through being sensitive to inhibition by the *CLV3* peptide that diffuses from cells that have been induced to stem cell identity, thus creating a negative feedback loop that is stronger if the stem cell area is overlarge (Lenhard and Laux, 2003). In addition, *WUS* enhances the effects of cytokinins, and may thereby stimulate stem cell proliferation, by repressing factors that decrease response to cytokinin (Leibfried *et al.*, 2005). The

type-A response regulator proteins ARR5, ARR6, ARR7 and ARR15 are regulated by their phosphorylation and act to moderate overresponse to cytokinin. Expression of *WUS* leads to tenfold lower expression of these regulators, therefore enhancing response to cytokinin and enhanced stem cell proliferation. Furthermore, the key role of cytokinin in stabilizing stem cell proliferation is indicated by the blocking of stem apical meristem growth by over-expression of ARR7 in a constitutively active form (resembling the phosphorylated form that is generated by signal transduction from high cytokinin) which prevents response to cytokinin (Leibfried *et al.*, 2005).

STM has another major function, which is to induce expression of the cytokinin-synthesizing enzyme IPT7 (Yanai *et al.*, 2005). Overexpression of KNOX1 transcription factors (including STM) in Arabidopsis consistently raised expression of *IPT7* and increased the extractable cytokinin levels 30-fold and the intensity of expression from the cytokinin-inducible promoter *ARR5*. This induction of cytokinin synthesis through *IPT7* is a major function of *KNOX1* since *stm* mutation, which normally renders the plant nonviable, could be rescued in 60% of transgenics if they received *AtIPT7* attached to the *STM* promoter, implying that cytokinin can largely replace *STM* (Yanai *et al.*, 2005). The zone of raised cytokinin expression that can be induced by *KNOX1* expression is normally more extensive than the central zone of *KNOX1* expression. This is consistent with the requirement for cytokinin to support cell proliferation in the leaf (e.g. Werner *et al.*, 2003) and this cytokinin function is also indicated by mitotic activity (reported by the induction of *CYCLINB:GUS* expression) when *STM* is ectopically expressed in the leaf (Lenhard *et al.*, 2002). The absence of *KNOX1* gene expression from the meristem flanks reflects the requirement that it be strictly downregulated in leaf primordial initials to allow their switch out of proliferation to a determinate cell identity (Long *et al.*, 1996).

In spite of these key functions of cytokinin, auxin remains essential to the shoot apical meristem as can be seen from the block to apex development that follows when auxin transport to the apex is limited by mutation in PIN auxin efflux regulator proteins (e.g. Gälweiler *et al.*, 1998; Blilou *et al.*, 2005), consistent with essential contributions being made by both hormones to cell cycle progression.

14.11 Auxin contributions to stem cell and meristem activity at the root apex

In the root apex, stem cells occupy a region designated the quiescent centre and have the complex function of producing root cap initials in the direction of root growth as well as producing initials for all other tissues behind the extending root tip. A point of similarity with the shoot apex may be that the quiescent centre is under control of a homeobox gene *QUIESCENT CENTRE HOMEOBOX (QHB)* that resembles *WUS* and, when overexpressed in rice, causes expansion of the quiescent centre in the manner of *WUS*, inducing stem cell identity in the shoot (Kamiya

et al., 2003). However, *QHB* is not alone in having the capacity to specify stem cell identity since *PLETHORA* (*PLT*) genes, which encode AP2-domain transcription factors, are auxin inducible and can specify root identity when ectopically expressed (Aida *et al.*, 2004). In the root tip, auxin (the *PLT* inducer) is tightly focused on the stem cell region by its recirculation through auxin efflux regulators of the PIN-FORMED (*PIN*) protein family that are asymmetrically located at the periphery of the cell (Gälweiler *et al.*, 1998). Auxin can be tracked arriving at the root tip down the central vascular tissue and, instead of being lost through the central root cap, is recirculated up the epidermis until routed centripetally back to the central stele (Blilou *et al.*, 2005) to reinforce the local high auxin signal in the root tip meristem. Again, the dominant hormone-regulating cell division contributes to meristem organization, since auxin induces *PLT* expression and, therefore, stem cell identity in cells of the quiescent centre, and *PLT* reinforces this pattern through involvement in *PIN* expression, especially of *PIN3* and *PIN7*, which return auxin from the tip through the epidermis and then direct it centripetally to reinforce the reflux loop. The consequent focusing of auxin concentration at the core of the meristem induces *PLT* expression there and specifies the size of the quiescent centre stem cell region (Blilou *et al.*, 2005). In spite of the importance of auxin, cytokinin is also essential for cell proliferation in the root apical meristem, as illustrated by inadequate division that results from the *woodenleg* mutation in the cytokinin receptor gene *AHK4*, which causes insufficient division in vascular precursors and a deficiency of phloem (Mähönen *et al.*, 2000; Nishimura *et al.*, 2004).

14.12 Hormones and the balance of cell proliferation between root and shoot

The dependence of meristems on auxin from the shoot and cytokinin from the root to sustain division provides a mechanism for balancing shoot and root growth. In general terms, a large shoot will tend to dissipate the cytokinin that it receives in a larger volume of shoot tissue, which will tend to slow the further proliferation of cells in the shoot, while the larger shoot will also have a more extensive capacity to produce auxin, which will tend to stimulate root growth and also act to restore plant proportions. Conversely, a more vigorous root can encourage increased shoot growth through cytokinin supply, and furthermore, this cytokinin signal is enhanced under favourable growth conditions since nitrogen nutrient elevates expression of *IPT3* and presumably cytokinin synthesis in the *Arabidopsis* root (Miyawaki *et al.*, 2004). The cytokinin signal to shoot growth is sufficiently substantial to be exploited in agriculture since rice cultivars found by QTL to have genes for reduced expression of cytokinin oxidase are better able to provide a strong cytokinin signal to shoot growth and increased grain yield (Ashikari *et al.*, 2005). The most extensive increase in growth in response to hormonal signals occurs through initiation of new points of growth through outgrowth of laterals (Plate 14.2), examined in the next section.

14.13 Auxin/cytokinin ratio and initiation of cell proliferation in lateral meristems

There is evidence that lateral meristem initiation responds to the ratio of hormone concentration in the root/shoot axis (Plate 14.2) in a way that prevents excessive proximity to the tip and spaces laterals in a manner that provides effective interception of water and nutrient in the soil and access to sunlight for photosynthesis.

Hormonal influence over lateral branching has been indicated by classical demonstrations that the inhibition of lateral shoot outgrowth by an active shoot apical meristem (termed apical dominance) could be continued if the apex was removed but replaced by auxin (IAA) diffusing from the lanoline paste applied to the cut tip (Thimann and Skoog, 1933); an effect opposed by additional presence of cytokinin (Wickson and Thimann, 1958) and confirmed in many systems (reviewed by Cline, 1991). Similarly in the root, inhibition of lateral root formation close to the apex could be mimicked by cytokinin applied to excised pea root segments, and this could be reversed by the additional presence of auxin (Torrey, 1962). A long-held expectation, based on such experiments, has been that the ratio of concentration of auxin and cytokinin is as significant as their absolute concentration (Skoog and Miller, 1957; Cary *et al.*, 2002).

Early experimental systems that involved surgery or excision of tissue might not extrapolate to the intact plant, but this concern is removed by use of transgenes and mutants that alter hormone levels (or the perception of hormones) within the intact plant. An early demonstration of the importance of the ratio of cytokinin to auxin concentrations (Romano *et al.*, 1993; Klee and Romano, 1994) was the detection that reduced auxin level in tobacco was equivalent to increased cytokinin level in stimulating lateral shoot outgrowth. The *iaaL* gene, which leads to the formation of inactive auxin-lysine conjugates, lowered effective auxin concentration. The outcome was a raised cytokinin phenotype, of increased lateral shoot outgrowth, presumably caused by raised cytokinin/auxin ratio although cytokinin concentration was not increased.

More recently, a battery of mutations and transgenes has become available to alter internal levels of auxin and cytokinin, and the resulting phenotypes bear out the classical findings that raised cytokinin is stimulatory to shoot development at the expense of the root, whereas raised auxin is stimulatory to root development but can retard cell division in the shoot. Taking only a few illustrative examples, effects of high auxin/cytokinin ratio have been studied using mutations in the AHK cytokinin receptor family that make the cytokinin signal less effective (Mähönen *et al.*, 2000; Nishimura *et al.*, 2004). Stronger effects come from actual reduction of cytokinin levels by expression of the cytokinin-degrading enzyme cytokinin oxidase (CKX), which has been thoroughly studied in tobacco and Arabidopsis (Werner *et al.*, 2001, 2003). Strong effects are also obtained if signal transduction from cytokinin is extensively inhibited by expression of the signal transducer ARR7 in a form constitutively activated to attenuate the cytokinin signal (with glutamate substituted for asp 85), which blocks shoot apical development (Leibfried *et al.*, 2005). Furthermore, a high auxin/cytokinin ration can be obtained by increasing

auxin synthesis through the regulatory mutation *superroot* (*sur*) in *Arabidopsis* (Boerjan *et al.*, 1995). Phenotypic effects of a high auxin/cytokinin ratio in the root included increased root growth and extent of lateral roots and, with auxin at above normal concentration, a greater density of lateral roots and lateral initiation very close to the apical meristem. While conversely, in the shoot, strong negative effects were observed, including stunting, 20-fold reduction in leaf cell production and, at 4-fold above normal auxin levels, a shoot so reduced that no flowers were produced.

The converse situation of a high cytokinin/auxin ratio has similarly been studied by several means. Mutations that decrease perception of auxin, by reducing the capacity of AUX/IAA negative transcriptional regulator to respond to auxin by becoming ubiquitinated and proteolyzed, have been isolated as a series of alleles with decreasing auxin responsiveness (Rouse *et al.*, 1998). These show increasing stunting of the root system down to less than 20% of wild type and increasing predominance of growth in the shoot, including outgrowth of lateral shoots. These effects obtained through a modified transcription regulator underline the contribution of gene expression in hormonal effects. A further increase in the cytokinin/auxin ratio has been obtained by increasing cytokinin concentration through the *amp1* mutation in *Arabidopsis* (Chaudhury *et al.*, 1993) or by introduction of the bacterial *IPT* cytokinin synthesis gene into many plant species (e.g. Medford *et al.*, 1998). And more recently, the endogenous plant *IPT* genes have been used and the induction of cytokinin synthesis has been shown to be a major mechanism by which homeobox genes direct shoot meristems (Yanai *et al.*, 2005). High cytokinin/auxin ratios cause reduced growth in the root and in the shoot induce extensive initiation and outgrowth of lateral shoots, and can also initiate ectopic foci of division within the meristem causing supernumerary cotyledons and leaves.

14.14 Possible mechanisms for cell cycle response to hormone concentration and ratio

It is appropriate to consider mechanisms that might allow cell cycle gene control to be influenced not just by single hormones but by hormone ratios, and in addition how a hormone that might be essential for cell proliferation can also have the negative effect of providing an inhibitory signal in apical dominance, such as the effect of auxin imposing dormancy in lateral buds and cytokinin opposing lateral root initiation. Possible mechanisms include gene control by complexes of transcription factors that have a different affinity for DNA than do single factors and can contain proteins that have positive or negative effects on transcription. Investigation of these effects is inevitably painstaking and slow because regions as small as 7 base pairs located within 10 kb either upstream or downstream of the open reading frame can act as enhancers (cis regulatory elements) through binding transcription factors (e.g. the reviews by Blackwood and Kadonaga, 1998; Davidson *et al.*, 2002). Regulatory regions can therefore be too small to be recognized by sequence (Kreiman, 2004). Because each gene requires detailed empirical study, almost everything about the