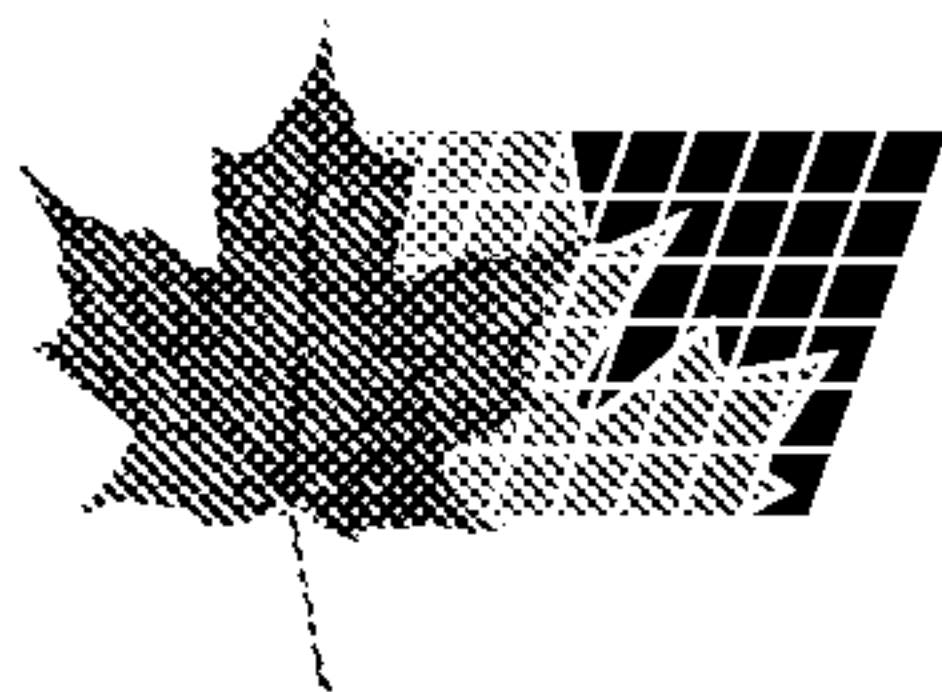




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(54) **METHODE POUR MODIFIER LA MORPHOLOGIE, LA  
BIOCHIMIE ET LA PHYSIOLOGIE DES PLANTES**  
(54) **METHOD OF MODIFYING PLANT MORPHOLOGY,  
BIOCHEMISTRY AND PHYSIOLOGY**

**Dexamethasone ( $\mu\text{M}$ )**

**0    0.01    0.1    1.0    10**



**mRNA cdc25**

(57) The present invention provides a method of modifying one or more plant cytokinin-mediated morphological, biochemical and physiological properties or characteristics, such as one or more environmental adaptive responses and/or developmental processes, including but not limited to the initiation, promotion, stimulation or enhancement of cell division and/or seed development and/or tuber formation and/or shoot initiation and/or bushiness and/or dwarfism and/or pigment synthesis, and/or the modification of source/sink relationships, and/or the inhibition of root growth and/or the inhibition of apical dominance and/or the delay of senescence and/or increased tuberisation and/or increased grain size, said method comprising expressing a cell cycle control protein, in particular the Cdc25 phosphoprotein phosphatase, in the plant, operably under the control of a regulatable promoter sequence such as a cell-specific promoter, tissue-specific promoter, or organ-specific promoter sequence.

**ABSTRACT**

The present invention provides a method of modifying one or more plant cytokinin-mediated morphological, biochemical and physiological properties or characteristics, such as one or more environmental adaptive responses and/or developmental processes, including but not limited to the initiation, promotion, stimulation or enhancement of cell division and/or seed development and/or tuber formation and/or shoot initiation and/or bushiness and/or dwarfism and/or pigment synthesis, and/or the modification of source/sink relationships, and/or the inhibition of root growth and/or the inhibition of apical dominance and/or the delay of senescence and/or increased tuberisation and/or increased grain size, said method comprising expressing a cell cycle control protein, in particular the Cdc25 phosphoprotein phosphatase, in the plant, operably under the control of a regulatable promoter sequence such as a cell-specific promoter, tissue-specific promoter, or organ-specific promoter sequence.

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## **METHOD OF MODIFYING PLANT MORPHOLOGY, BIOCHEMISTRY AND PHYSIOLOGY**

### **FIELD OF THE INVENTION**

5 The present invention relates generally to a method of modifying plant morphological, biochemical and physiological properties or characteristics, such as one or more environmental adaptive responses and/or developmental processes, including but not limited to the initiation, promotion, stimulation or enhancement of cell division and/or seed development and/or tuber formation and/or shoot initiation and/or bushiness  
10 and/or dwarfism and/or pigment synthesis, and/or the modification of source/sink relationships, and/or the modification of root growth and/or the inhibition of apical dominance and/or the delay of senescence, said method comprising expressing a cell cycle control protein, in particular Cdc25 phosphoprotein phosphatase, in the plant, operably under the control of a regulatable promoter sequence such as a cell-specific  
15 promoter, tissue-specific promoter, or organ-specific promoter sequence. Preferably, the characteristics modified by the present invention are cytokinin-mediated and/or gibberellin-mediated characteristics. The present invention extends to genetic constructs which are useful for performing the inventive method and to transgenic plants produced therewith having altered morphological and/or biochemical and/or  
20 physiological properties compared to their otherwise isogenic counterparts.

### **GENERAL**

Those skilled in the art will be aware that the invention described herein is subject to variations and modifications other than those specifically described. It is to be  
25 understood that the invention described herein includes all such variations and modifications. The invention also includes all such steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

30 Throughout this specification, unless the context requires otherwise the word

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"comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

- 5 Bibliographic details of the publications referred to by author in this specification are collected at the end of the description.

As used herein, the term "derived from" shall be taken to indicate that a particular integer or group of integers has originated from the species specified, but has not  
10 necessarily been obtained directly from the specified source.

## BACKGROUND TO THE INVENTION

Development and environmental adaptation are highly regulated processes in plants. These processes are not cell-autonomous but rather involve extensive communication  
15 between different parts of the plant. Amongst the most important mobile signals involved in this long-distance communication are plant hormones such as auxins, cytokinins, abscisic acid, gibberellins, and ethylene. Other signals, so far not defined as plant hormones, include salicylic acid, jasmonic acid and brassinosteroids.

20 There are plethora of data showing that the external application of plant hormones has profound effects on development, metabolism and environmental fitness. For example, the external application of cytokinins produces a variety of morphological, biochemical and physiological effects in plants, including the stimulation of organogenesis, shoot initiation from callus cultures, release of lateral buds from apical  
25 dominance, dwarf growth, alteration of source/sink relationships, stimulation of pigment synthesis, inhibition of root growth, and delay of senescence. Additionally, exogenous cytokinin application following anthesis in cereals enhances grain set and yield and the phase of nuclear and cell division in the developing endosperm of cereal grains is accompanied by a peak of cytokinin concentration, suggesting a role for cytokinins in  
30 grain development in cereals (Herzog, 1980; Morgan *et al.*, 1983). Cytokinins have

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also been implicated in promoting the initiation of tuber formation in potato (International Patent Publication No. WO 93/07272) and in improving the resistance of potato plants to insects (United States Patent No. 5, 496, 732) and in inducing male sterility and partial female sterility in tobacco plants (European Patent No. EP-A-5 334,383).

The effect of cytokinin on plant development and morphology may be attributed, at least in part, to modified biochemistry of the plant, such as a modification to the source/sink relationship in the plant or plant part.

10

Attempts to modify plant cytokinin-mediated and/or gibberellin-mediated growth and developmental responses employ the exogenous application of cytokinins and/or gibberellins respectively. Such approaches are costly and produce undesirable pleiotropic side-effects on the plant tissue.

15

Other approaches to modifying plant cytokinin-mediated growth and developmental responses employ the ectopic expression of an introduced bacterial isopentenyladenosine transferase (IPT) gene (International Patent Publication No. WO 93/07272; United States Patent No. 5, 496, 732; United States Patent No. 5, 689, 042) 20 under the control of a strong constitutive promoter sequence, developmentally-regulated promoter sequence or hormonally-inducible promoter sequence. Alternatively, plant cytokinin-mediated growth and developmental responses have been modified by the ectopic expression of the *Agrobacterium rhizogenes* *RolC* gene (European Patent No. EP-A-334,383).

25

Previously, it had been shown that constitutive expression of yeast Cdc25 in tobacco resulted in precocious flowering, more flowers per flowering head and the presence of "petalless" flowers alongside normal ones. Other changes in development included the positioning of the leaves. When yeast Cdc25 was expressed under control of an 30 inducible promoter, a greater frequency of lateral root formation was observed. Yet,

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the importance of Cdc25 in cytokinin action was so far not recognised (Patent WO 92/09685; Patent WO 93/122239 and, as a consequence, the presented invention and its embodiments could not be envisaged.

## 5 SUMMARY OF THE INVENTION

In work leading to the present invention, the present inventors sought to develop a method of producing specific targeted modifications to plant morphology, biochemistry and physiology, in particular specific target modifications to cytokinin-mediated and gibberellin-mediated plant growth and development, thereby avoiding the problem of  
10 pleiotropy associated with the prior art.

Surprisingly, the inventors discovered that the targeted ectopic expression of a cell cycle control protein in particular cells, tissues or organs of the plant would produce localised specific modifications to plant morphology, biochemistry and physiology,  
15 compared to otherwise isogenic non-transformed plants.

More particularly, the inventors have discovered that the cytokinin-mediated or gibberellin-mediated induction of mitosis in plants can be obtained by the expression of the yeast Cdc25 phosphoprotein phosphatase therein.

20

The cytokinin-mediated induction of mitosis by Cdc25 is shown in Example 2. Whilst not being bound by any theory or mode of action, it is likely that the ectopic expression of yeast Cdc25 phosphatase in plants releases the inhibition of cdc2 activity, which is key enzyme in the control of the cell cycle, and as a consequence, causes isolated  
25 cells to enter mitosis. The Cdc25 phosphatase is an intracellular protein, which, unlike exogenously-applied cytokinins or cytokinins produced by ectopic expression of *ipt* or *rolC* genes, will only exert a localised effect at the site of protein synthesis. This observation has led the present inventors to develop methods for controlled expression of yeast Cdc25 in particular cells, tissues and organs of plants, for the purposes of  
30 modifying cytokinin-mediated plant morphology and/or biochemistry and/or physiology,

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and to facilitate the selection of specific cells, tissues and organs which exhibit cytokinin-mediated morphological characteristics and/or biochemical characteristics and/or physiological characteristics.

5 Accordingly, one aspect of the invention provides a method of modifying plant morphology and/or biochemistry and/or physiology comprising expressing in particular cells, tissues or organs of a plant, a genetic sequence encoding a cell cycle control protein operably under the control of a regulatable promoter sequence selected from the list comprising cell-specific promoter sequences, tissue-specific promoter  
10 sequences, and organ-specific promoter sequences.

In a particularly preferred embodiment of the invention, the cell cycle control protein is the yeast Cdc25 phosphoprotein phosphatase or a biologically-active homologue, analogue or derivative thereof. The present invention clearly contemplates the use of  
15 functional homologues of the fission yeast Cdc25 protein, based upon the evidence provided herein for the presence of Cdc25-like activity and Cdc25-like protein in tobacco (Example 3). Accordingly, the present invention is not limited in application to the use of nucleotide sequences encoding the fission yeast Cdc25 protein.

20 Preferred embodiments of the invention relate to the effect(s) of cytokinins and/or gibberellins on plant morphology and architecture. With respect to cytokinins, the present invention clearly contemplates the broad application of the inventive method to the modification of a range of cellular processes, including but not limited to the initiation, promotion, stimulation or enhancement of cell division and/or seed  
25 development and/or tuber formation and/or shoot initiation and/or bushiness and/or dwarfism and/or pigment synthesis, and/or the modification of source/sink relationships, and/or the modification of root growth and/or the inhibition of apical dominance and/or the delay of senescence. With respect to gibberellins, the present invention clearly contemplates the broad application of the inventive method to the  
30 modification of a range of cellular processes, including but not limited to the initiation,

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promotion, stimulation or enhancement of cell division and/or seed development and/or tuber formation and/or shoot initiation and/or bushiness and/or dwarfism and/or pigment synthesis, and/or the modification of source/sink relationships, and/or the modification of root growth and/or the inhibition of apical dominance and/or the  
5 delay of senescence. In this regard, the identification of substrates of Cdc25 phosphatase other than cdc2 will also reveal the mechanism by which Cdc25 is linked to many cellular processes other than cell division.

In one preferred embodiment of the present invention, the yeast Cdc25 protein or a  
10 homologue, analogue or derivative thereof, or a modified substrate of Cdc25 that mimics the effect of Cdc25 is expressed operably under the control of a promoter derived from a stem-expressible gene, to increase the strength and thickness of a plant stem to confer improved stability and wind-resistance on the plant.

15 In another preferred embodiment of the present invention, the yeast Cdc25 protein or a homologue, analogue or derivative thereof, or a modified substrate of Cdc25 that mimics the effect of Cdc25 is expressed in a tuber-forming plant operably under the control of a promoter derived from a stem-expressible gene or tuber-expressible gene, to increase improve tuber production in the plant.

20

In another embodiment of the present invention, the yeast Cdc25 protein or a homologue, analogue or derivative thereof, or a modified substrate of Cdc25 that mimics the effect of Cdc25 is expressed in a tree crop plant such as, but not limited to, *Eucalyptus spp.* or *Populus spp.*, operably under the control of a promoter derived  
25 from a gene that is expressed in vascular tissue and/or cambium cells, to increase lignin content therein. Without being bound by any theory or mode of action, the ectopic expression of Cdc25 under control of a promoter that is operable in vascular tissue and preferably, in cambial cells, will produce thick-stemmed plants and a higher ratio of vascular tissue-to-pith cells within the stem, thereby resulting in more lignin  
30 production. Within the vascular tissue, cambial cells contain the highest levels of

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auxins and are therefore the preferential tissue for Cdc25 overproduction.

In yet another preferred embodiment of the present invention, the yeast Cdc25 protein or a homologue, analogue or derivative thereof, or a modified substrate of Cdc25 that  
5 mimics the effect of Cdc25 is expressed operably under the control of a promoter derived from a seed-expressible gene, to increase seed production in plants, in particular to increase seed set and seed yield. More preferably, the promoter is operable in the endosperm of the seed, in which case the combination of the cell cycle-control protein and endosperm-expressible promoter provides the additional advantage  
10 of increasing the grain size and grain yield of the plant.

In yet another preferred embodiment of the present invention, the yeast Cdc25 protein or a homologue, analogue or derivative thereof, or a modified substrate of Cdc25 that mimics the effect of Cdc25 is expressed operably under the control of a promoter  
15 derived from a meristem-expressible gene or a shoot-expressible gene or a root-expressible gene, to reduce apical dominance and/or to promote bushiness of the plant and/or to increase or enhance the production of lateral roots.

In still another preferred embodiment of the present invention, the yeast Cdc25 protein  
20 or a homologue, analogue or derivative thereof, or a modified substrate of Cdc25 that mimics the effect of Cdc25 is expressed operably under the control of a promoter derived from a leaf-expressible gene, to prevent or delay or otherwise reduce leaf chlorosis and/or leaf necrosis.

25 In a further preferred embodiment of the present invention, the yeast Cdc25 protein or a homologue analogue or derivative thereof, or a modified substrate of Cdc25 that mimics the effect of Cdc25 is expressed under the control of a promoter that is operative in meristem tissue of grain crops, to stimulate cell division in the intercalary meristem of the youngest stem internode and produce greater elongation of the stem  
30 and/or to generate a more extensive photosynthetic canopy.

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Those skilled in the art will recognise that the present invention may be applied to the selection of *any* cell, tissue, organ or whole organism that expresses exhibit cytokinin-mediated and/or gibberellin-mediated morphological characteristics and/or biochemical characteristics and/or physiological characteristics, from a background of cells, tissues,  
5 organs or whole organisms that do not exhibit such characteristics.

Accordingly, a second aspect of the present invention provides a method of detecting or identifying transformed or transfected plant cells, tissues or organs that are hormone-dependent, comprising expressing the yeast Cdc25 protein or a homologue,  
10 analogue or derivative thereof, or a modified substrate of Cdc25 that mimics the effect of Cdc25 in said plant cell, tissue or organ operably under the control of an plant-expressible inducible promoter, preferably a chemically-inducible promoter for a time and under conditions sufficient for hormone-mediated cell division and/or hormone-mediated tissue differentiation to occur. In an alternative embodiment, the Cdc25  
15 protein is expressed under the operable control of a plant-expressible constitutive promoter sequence, wherein said promoter sequence in operable connection with a nucleotide sequence encoding Cdc25 are integrated into a transposable element to facilitate hormone-mediated cell division and/or hormone-mediated tissue differentiation only in those cells which also contain the transposable element.

20

As used herein, the term "hormone-dependent" means any cell, tissue or organ that requires the exogenous application of a gibberellin or cytokinin to facilitate or produce cell division and/or cell proliferation in primary culture *in vitro*.

25 A third aspect of the invention provides a genetic construct or vector comprising a nucleotide sequence that encodes a cell cycle control protein operably under the control of a regulatable promoter sequence selected from:

(i) a plant-expressible cell-specific promoter sequence, plant-expressible tissue-specific promoter sequence, or a plant-expressible organ-specific  
30 promoter sequence; and

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- (ii) a plant-expressible constitutive promoter sequence, wherein the nucleotide sequence encoding the Cdc25 protein and the plant-expressible constitutive promoter sequence are integrated into a transposable element.
- 5 Preferably, the genetic construct or vector according to this aspect of the invention is suitable for expression in a plant cell, tissue, organ or whole plant and more preferably, the subject genetic construct or vector is suitable for introduction into and maintenance in a plant cell, tissue, organ or whole plant.
- 10 A fourth aspect of the invention provides a plant cell, tissue, organ or whole plant that has been transformed or transfected with an isolated nucleic acid molecule that comprises a nucleotide sequence which encodes a cell cycle control protein, wherein the expression of said nucleotide sequence is placed operably under the control of a plant-expressible cell-specific promoter sequence, plant-expressible tissue-specific
- 15 promoter sequence, a plant-expressible organ-specific promoter sequence, or a plant-expressible constitutive promoter sequence such that said plant-expressible constitutive promoter sequence and said nucleotide sequence encoding a cell cycle control protein are integrated into a transposable genetic element.
- 20 This aspect of the invention extends to cdc2 reproducing said primary transformants/transfectants.

#### BRIEF DESCRIPTION OF THE DRAWINGS

**Figure 1-1** is a copy of a photographic representation of a northern blot hybridisation

25 showing the induction of *Cdc25* mRNA in tobacco cells containing a dexamethasone-inducible *Cdc25* gene, in the absence of exogenous cytokinin. Prior to induction, cells were brought to arrest at the cytokinin control point in late G2 phase by culture without hormone and then with auxin only. Total RNA was extracted from tobacco cells either in the absence of added dexamethasone (lane 0), or after 12 h induction with 0.01  $\mu$ M,

30 or 0.10  $\mu$ M, or 1.00  $\mu$ M, or 10.00  $\mu$ M dexamethasone and then loaded onto agarose

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gels (60  $\mu$ g aliquots RNA per lane), transferred to membrane support and probed with a *Cdc25*-specific probe.

**Figure 1-2** is a copy of a photographic representation of a western blot showing the induction of p67<sup>Cdc25</sup> protein in tobacco cells containing a dexamethasone-inducible *Cdc25* gene, in the absence of exogenous cytokinin. Prior to induction, cells were brought to arrest at the cytokinin control point in late G2 phase by culture without hormone and then with auxin only. Total protein was extracted from tobacco cells either in the absence of added dexamethasone (lane 1), or after 12 h induction with 0.01  $\mu$ M dexamethasone (Lane 2), or 0.10  $\mu$ M dexamethasone (Lane 3), or 1.00  $\mu$ M dexamethasone (Lane 4), or 10.00  $\mu$ M dexamethasone (Lane 5) and then loaded onto SDS/polyacrylamide gels (50  $\mu$ g aliquots total soluble protein per lane), transferred to membrane support and probed with antibody specific for the *Cdc25*-specific probe. p67<sup>Cdc25</sup> was detected by western blot of 50  $\mu$ g aliquots of total soluble p67 protein.

**Figure 1-3** is a copy of a graphical representation showing the induction of cell division in culture, as measured by an increase in cell number, for tobacco cells transformed with a dexamethasone-inducible *Cdc25* gene, in the absence of exogenous cytokinin. Prior to induction, cells were brought to arrest at the cytokinin control point in late G2 phase by culture without hormone and then with auxin only. Cell numbers were determined either in the absence of added dexamethasone, or after 12 h induction with 0.01-10.00  $\mu$ M dexamethasone. Data were also obtained for both transformed cells (O) and for control non-transformed cells ( $\Delta$ ) grown under identical culture conditions.

25

**Figure 2-1** is a copy of a photographic representation showing the activity of *Cdc25* phosphatase (*Cdc25*) and *cdc2* histone kinase (*cdc2*) in transgenic tobacco cells containing a dexamethasone-inducible *Cdc25* gene and progressing from the late G2 phase hormonal control point into division, that have either not been induced with 0.1  $\mu$ M dexamethasone (-D), or alternatively, that have been induced with 0.1  $\mu$ M

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dexamethasone (+D). The activity of Cdc25 was measured by activation of the tyrosine- phosphorylated cdc2 enzyme substrate as determined by assaying for phosphorylation of H1 histone by H1 histone kinase. The Cdc25 enzyme from cells induced for 6 hours with dexamethasone was purified using antibodies against authentic fission yeast Cdc25 protein, or alternatively, using preimmune serum (lane marked p-i) or an antibody that had been pre-competed with repeat-freeze-thaw inactivated GST-Cdc25 fusion protein (lane marked p-c). The cdc2 kinase from cells induced for 12 h with dexamethasone was purified with antibody, or antibody that had been pre-competed with 0.1 mM antigen (lane marked p-c), and assayed by phosphorylation of H1 histone.

**Figure 2-2** is a graphical representation showing the change in activities of Cdc25 phosphatase ( $\Delta$ ) and cdc2 histone kinase (O) in transgenic tobacco cells containing a dexamethasone-inducible *Cdc25* gene progressing from the late G2 phase hormonal control point into division and following induction with 0.1  $\mu$ M dexamethasone. The activities of Cdc25 phosphatase and cdc2 histone kinase were measured as described for Figure 2-1.

**Figure 2-3** is a graphical representation showing the change in cell number (cells/ml  $\times 10^6$ ) of transgenic and non-transgenic tobacco cells containing a dexamethasone-inducible *Cdc25* gene, progressing from the late G2 phase hormonal control point into division and following induction with 0.1  $\mu$ M dexamethasone or cytokinin. Data show cell number for both transgenic cells induced using dexamethasone ( $\square$ ) or cytokinin ( $\Delta$ ), and for non-transgenic cells induced using dexamethasone (O).

25

**Figure 2-4** is a graphical representation showing the change in activities of Cdc25 phosphatase ( $\Delta$ ) and cdc2 histone kinase (O) in transgenic tobacco cells containing a dexamethasone-inducible *Cdc25* gene, progressing from the late G2 phase hormonal control point into division and following induction with cytokinin in the absence of added dexamethasone. The activities of Cdc25 phosphatase and cdc2

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histone kinase were measured as described for Figure 2-1.

**Figure 2-5** is a graphical representation showing the change in activity of cdc2 histone kinase in transgenic tobacco cells containing a dexamethasone-inducible *Cdc25* gene, progressing from the late G2 phase hormonal control point into division and following their stimulation with cytokinin. The cdc2 histone kinase was purified using p13<sup>suc1</sup> beads and treated with GST-Cdc25 fusion protein that had been produced in *Escherichia coli* cells. Data indicate the cdc2 activity before Cdc25 treatment (O), and after treatment (•) with cytokinin.

10

**Figure 2-6** is a copy of a photographic representation showing the activation of cdc2 histone kinase by Cdc25 phosphatase in transgenic tobacco cells containing a dexamethasone-inducible *Cdc25* gene, prior to stimulation with cytokinin (lanes 1-3) or following 3 hours stimulation with cytokinin (lanes 4-6). Detectable cdc2 activity was observed in control samples that had been incubated without added Cdc25 (lanes 1 and 4), or following incubation with (i) immunoprecipitated Cdc25 that had been derived from non-transgenic tobacco cells induced with cytokinin for 6 hours (lanes 2 and 5); or (ii) Cdc25 derived from transgenic tobacco cells containing a dexamethasone-inducible *Cdc25* gene that had been induced with dexamethasone for 6 hours (lanes 3 and 6). The activity of cdc2 histone kinase was measured as described for Figure 2-1. Detection of Cdc25 activity in the immuno-recovered fraction derived from non-transgenic cells indicates the presence of a plant-encoded Cdc25.

**Figure 2-7** is a copy of a photographic representation showing the presence of phosphorylated tyrosine in cdc2a (arrow) following induction of transgenic tobacco cells containing a dexamethasone-inducible *Cdc25* gene with dexamethasone. The cdc2a protein was immuno-precipitated with purified antibody, or with antibody precompeted with repeat-freeze-thaw inactivated GST-Cdc25 (lane marked p-c). The upper band indicated in the Figure represents excess IgG.

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**Figure 3** is a copy of a photographic representation of a western blot showing purified plant-derived Cdc25 protein. The arrow indicates the plant Cdc25 polypeptide. Anti-GST-Cdc25 antibody at a dilution of 1:500 in buffered saline was used to probe affinity-purified plant Cdc25 protein alone (lane 1) or affinity-purified plant Cdc25 protein following incubation for 1 hour with 0.1 mM GST-Cdc25 fusion protein. Molecular weight markers indicating the molecular mass (kDa) of proteins are indicated at the left of the Figure.

**Figure 4** is a copy of a photographic representation showing the cytokinin-dependent proliferation of tobacco cells in culture. Cell proliferation was detected by the incorporation of BrdU into nuclear DNA of excised tobacco pith tissue primary culture on MS medium either without added hormone (panels a,b), or supplemented with 5.4  $\mu$ M NAA (panels c,d) or with 0.56  $\mu$ M BAP (panels e,f) or 5.4  $\mu$ M NAA plus 0.56  $\mu$ M BAP (panels g,h). Cell cultures shown in panels a, c, e, and g have been stained with DAPI, to detect nuclei. Cell cultures shown in panels b, d, f, and h have been incubated with BrdU, and BrdU-containing DNA has been detected by fluorescence of antibody specific for BrdU-containing DNA.

#### DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

One aspect of the invention provides a method of modifying one or more plant morphological and/or biochemical and/or physiological characteristics comprising expressing in one or more particular cells, tissues or organs of a plant, a cell cycle control protein operably under the control of a regulatable promoter sequence selected from the list comprising cell-specific promoter sequences, tissue-specific promoter sequences, and organ-specific promoter sequences.

Preferably, the plant morphological, biochemical or physiological characteristic which is modified is a cytokinin-mediated or a gibberellin-mediated characteristic.

The word "modify" or variations such as "modifying" or "modified" as used herein with

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reference to any specified integer or group of integers shall be taken to indicate that said integer is altered by the performance of one or more steps pertaining to the invention described herein, compared to said integer in the absence of such performance.

5

Accordingly, by "modifying one or more plant morphological and/or biochemical and/or physiological characteristics" is meant that one or more morphological and/or biochemical and/or physiological characteristics of a plant is altered by the performance of one or more steps pertaining to the invention described herein.

10

"Plant morphology" or the term "plant morphological characteristic" or similar term will be understood by those skilled in the art to refer to the external appearance of a plant, including any one or more structural features or combination of structural features thereof. Such structural features include the shape, size, colour, texture, arrangement, and patternation of any cell, tissue or organ or groups of cells, tissues or organs of a plant, including the root, leaf, shoot, petiole, trichome, flower, petal, stigma, style, stamen, pollen, ovule, seed, embryo, endosperm, seed coat, aleurone, fibre, cambium, wood, heartwood, parenchyma, aerenchyma, seive element, phloem or vascular tissue, amongst others.

20

"Plant biochemistry" or the term "plant biochemical characteristic" or similar term will be understood by those skilled in the art to refer to the metabolic and catalytic processes of a plant, including primary and secondary metabolism and the products thereof, including any small molecules, macromolecules or chemical compounds, such as but not limited to starches, sugars, proteins, peptides, enzymes, hormones, growth factors, nucleic acid molecules, celluloses, hemicelluloses, calloses, lectins, fibres, pigments such as anthocyanins, vitamins, minerals, micronutrients, or macronutrients, that are produced by plants.

30 "Plant physiology" or the term "plant physiological characteristic" or similar term will be

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understood to refer to the functional processes of a plant, including developmental processes such as growth, expansion and differentiation, sexual development, sexual reproduction, seed set, seed development, grain filling, asexual reproduction, cell division, dormancy, germination, light adaptation, photosynthesis, leaf expansion, fibre  
5 production, secondary growth or wood production, amongst others; responses of a plant to externally-applied factors such as metals, chemicals, hormones, growth factors, environment and environmental stress factors (eg. anoxia, hypoxia, high temperature, low temperature, dehydration, light, daylength, flooding, salt, heavy metals, amongst others), including adaptive responses of plants to said externally-  
10 applied factors,

The word "express" or variations such as "expressing" and "expression" as used herein shall be taken in their broadest context to refer to the transcription of a particular genetic sequence to produce sense or antisense mRNA or the translation of a sense  
15 mRNA molecule to produce a peptide, polypeptide, oligopeptide, protein or enzyme molecule. In the case of expression comprising the production of a sense mRNA transcript, the word "express" or variations such as "expressing" and "expression" may also be construed to indicate the combination of transcription and translation processes, with or without subsequent post-translational events which modify the  
20 biological activity, cellular or sub-cellular localization, turnover or steady-state level of the peptide, polypeptide, oligopeptide, protein or enzyme molecule.

As used herein, the term "cell cycle control protein" shall be taken to refer to a peptide, polypeptide, oligopeptide, enzyme or other protein that is involved in controlling or  
25 regulating the cell cycle of a cell, tissue, organ or whole organism and/or DNA replication therein. In this regard, those skilled in the art will recognise that the "cell cycle" refers to the growth cycle of an individual cell, including the G1 phase that is entered after the ploidy of the cell has been halved by mitosis, the S phase in which each chromatid is duplicated, the G2 phase in which duplication is complete but  
30 mitosis has not been initiated, and the M phase in which mitosis occurs. Cell cycle

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control proteins and their role in regulating the cell cycle of eukaryotic organisms are reviewed in detail by John (1981) and the contributing papers therein; Nurse (1990); Norbury and Nurse (1992); Ormrod and Francis (1993) and the contributing papers therein; Francis and Halford, (1995); Elledge (1996); Doerner *et al* (1996); and Francis  
 5 *et al.* (1998).

Preferably, the cell cycle control protein is derived from a yeast or plant cell or animal cell, more preferably, from the fission yeast (*Schizosaccharomyces pombe*) or from a plant cell, such as a monocotyledonous or dicotyledonous plant cell.

10

Preferred cell cycle control proteins according to this embodiment of the invention include the cdc2 T14Y15 phosphatases such as Cdc25 protein phosphatase or p80<sup>Cdc25</sup> (Russell and Nurse, 1986; Kumagai and Dunphy, 1991; Bell *et al*, 1993; Elledge, 1996) and Pyp3 (Elledge, 1996); cdc2 protein kinase or p34<sup>cdc2</sup> (Nurse and Bisset,  
 15 1981; Lee and Nurse, 1987; John *et al.*, 1989; Feiler *et al.*, 1990; Colasanti *et al.*, 1991; Hirt *et al.* 1991; John *et al.*, 1993); cdc2a protein kinase (Hemerly *et al*, 1993); cdc2 T14Y15 kinases such as wee1 or p107<sup>wee1</sup> (Russell and Nurse, 1986; 1987a; 1987b; Elledge, 1996), mik1 (Lundgren *et al.*, 1991) and myt1 (Elledge, 1996); cdc2 T161 kinases such as Cak and Civ (Elledge, 1996); cdc2 T161 phosphatases such as  
 20 Kap1 (Elledge, 1996); cdc28 protein kinase or p34<sup>cdc28</sup> (Reed *et al.*, 1985; Nasmyth, 1993); p40<sup>MO15</sup> (Fesquet *et al.*, 1993; Poon *et al.*, 1993); chk1 kinase (Zeng *et al.*, 1998); cds1 kinase (Zeng *et al.*, 1998); growth-associated H1 kinase (GAK; Lake and Salzman, 1972; Langhan, 1978, Labbe *et al.*, 1989; Arion *et al.*, 1988); cyclins A, B, C, D and E (Evans *et al.* 1983; Swenson *et al.*, 1986; Labbe *et al.*, 1989; Murray *et al.*,  
 25 1989; Francis *et al*, 1998); cyclin-dependent kinase inhibitor (CKI) proteins such as Sic1, Far1, Rum1, p21, p27, p57, p16, p15, p18, p19 (Pines, 1995; Elledge, 1996), p14 and p14ARF ; p13<sup>suc1</sup>; (Hayles *et al.*, 1986) and nim-1 (Fantes, 1979; Russell and Nurse, 1986; 1987a; 1987b).

30 Other cell cycle control proteins that are involved in cyclin D-mediated entry of cells

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into G1 from G0 include pRb (Xie *et al.*, 1996; Huntley *et al.*, 1998), E2F, RIP, MCM7C and potentially the pRb-like proteins p107 and p130.

Other cell cycle control proteins that are involved in the formation of a pre-replicative  
5 complex at one or more origins of replication, such as, but not limited to, ORC, CDC6, CDC14, RPA and MCM proteins or in the regulation of formation of this pre-replicative complex, such as, but not limited to, the CDC7, DBF4 and MBF proteins.

10 Additional cell cycle control proteins are not excluded. The present invention clearly encompasses the use of homologues, analogues or derivatives of any of the above-mentioned cell cycle control proteins which also function as cell cycle control proteins, in modifying plant morphology and/or physiology and/or biochemistry.

15 For the present purpose, the term "cell cycle control protein" shall further be taken to include any one or more of those proteins that are involved in the turnover of a cell cycle control protein, or in regulating the half-life of a cell cycle control protein, such as, but not limited to, proteins that are involved in the proteolysis of one or more of the above-mentioned cell cycle control proteins. Particularly preferred proteins which are  
20 involved in the proteolysis of one or more of the above-mentioned cell cycle control proteins include the yeast-derived and animal-derived proteins, Skp1, Skp2, Rub1, Cdc20, cullins, CDC23, CDC27, CDC16, and plant-derived homologues thereof (Cohen-Fix and Koshland, 1997; Hochstrasser, 1998; Krek, 1998; Lisztwan, 1998; Plesse *et al.*, 1998).

25

"Homologues" of a cell cycle control protein such as Cdc25 are those peptides, oligopeptides, polypeptides, proteins and enzymes which contain amino acid substitutions, deletions and/or additions relative to the Cdc25 polypeptide without altering one or more of its cell cycle control properties, in particular without reducing  
30 the ability of the Cdc25 polypeptide to induce one or more cytokinin-mediated and/or

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gibberellin-mediated effects in a plant cell, tissue, organ or whole organism.

To produce such homologues of a cell cycle control protein such as Cdc25, amino acids present in Cdc25 can be replaced by other amino acids having similar properties, 5 for example hydrophobicity, hydrophilicity, hydrophobic moment, antigenicity, propensity to form or break  $\alpha$ -helical structures or  $\beta$ -sheet structures, and so on.

Substitutional variants are those in which at least one residue in the Cdc25 amino acid sequence has been removed and a different residue inserted in its place. Amino acid 10 substitutions are typically of single residues, but may be clustered depending upon functional constraints placed upon the polypeptide; insertions will usually be of the order of about 1-10 amino acid residues. and deletions will range from about 1-20 residues. Preferably, amino acid substitutions will comprise conservative amino acid substitutions, such as those described *supra*.

15

Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in the Cdc25 protein. Insertions can comprise amino- terminal and/or carboxyl terminal fusions as well as intra-sequence insertions of single or multiple amino acids. Generally, insertions within the amino acid 20 sequence will be smaller than amino or carboxyl terminal fusions, of the order of about 1 to 4 residues.

Deletional variants are characterised by the removal of one or more amino acids from the Cdc25 sequence.

25

Amino acid variants of the Cdc25 polypeptide may readily be made using peptide synthetic techniques well known in the art, such as solid phase peptide synthesis and the like, or by recombinant DNA manipulations. The manipulation of DNA sequences to produce variant proteins which manifest as substitutional, insertional or deletional 30 variants are well known in the art. For example, techniques for making substitution

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mutations at predetermined sites in DNA having known sequence are well known to those skilled in the art, such as by M13 mutagenesis or other site-directed mutagenesis protocol.

5 "Analogues" of a cell cycle control protein such as Cdc25 are defined as those peptides, oligopeptides, polypeptides, proteins and enzymes which are functionally equivalent to the Cdc25 polypeptide in inducing one or more cytokinin-mediated and/or gibberellin-mediated effects in plant cells, tissues, organs or whole organisms, but which contain certain non-naturally occurring or modified amino acid residues as will  
10 be known to those skilled in the art.

"Derivatives" of a cell cycle control protein such as Cdc25 are those peptides, oligopeptides, polypeptides, proteins and enzymes which comprise at least about five contiguous amino acid residues of a naturally-occurring Cdc25 polypeptide, in  
15 particular the fission yeast p80<sup>Cdc25</sup> polypeptide, but which retain activity in the induction of one or more cytokinin-mediated and/or gibberellin-mediated effects in a plant cell, tissue, organ or whole organism. A "derivative" may further comprise additional naturally-occurring, altered glycosylated, acylated or non-naturally occurring amino acid residues compared to the amino acid sequence of a naturally-occurring  
20 Cdc25 polypeptide. Alternatively or in addition, a derivative may comprise one or more non-amino acid substituents compared to the amino acid sequence of a naturally-occurring Cdc25 polypeptide, for example a reporter molecule or other ligand, covalently or non-covalently bound to the amino acid sequence such as, for example, a reporter molecule which is bound thereto to facilitate its detection.

25

Other examples of recombinant or synthetic mutants and derivatives of the Cdc25 polypeptide include those incorporating single or multiple substitutions, deletions and/or additions therein, such as carbohydrates, lipids and/or proteins or polypeptides. Naturally-occurring or altered glycosylated or acylated forms of the Cdc25 polypeptide  
30 are also contemplated by the present invention. Additionally, homopolymers or

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heteropolymers comprising one or more copies of the Cdc25 polypeptide are within the scope of the invention, the only requirement being that such molecules possess biological activity in inducing one or more cytokinin-mediated and/or gibberellin-mediated effects in plant cells, tissues, organs or whole organisms.

5

Particularly preferred homologues, analogues and derivatives of the fission yeast Cdc25 polypeptide contemplated by the present invention are derived from plants. As exemplified herein, the present inventors have identified a Cdc25 activity in tobacco cells which is contemplated as being of particular use in performing the various  
10 embodiments described herein.

In a particularly preferred embodiment of the invention, the cell cycle control protein is the yeast Cdc25 phosphoprotein phosphatase or a biologically-active homologue, analogue or derivative thereof and in particular, a plant-derived homologue of the yeast  
15 Cdc25 phosphoprotein phosphatase. The present invention clearly contemplates the use of functional homologues of the fission yeast Cdc25 protein, based upon the evidence provided herein for the presence of Cdc25-like activity and Cdc25-like protein in tobacco (Example 3). Accordingly, the present invention is not limited in application to the use of nucleotide sequences encoding the fission yeast p80<sup>Cdc25</sup> protein.

20

To effect expression of the cell cycle control protein in a plant cell, tissue or organ, either the protein may be introduced directly to said cell, such as by microinjection means or alternatively, an isolated nucleic acid molecule encoding said protein may be introduced into the cell, tissue or organ in an expressible format.

25

By "expressible format" is meant that the isolated nucleic acid molecule is in a form suitable for being transcribed into mRNA and/or translated to produce a protein, either constitutively or following induction by an intracellular or extracellular signal, such as an environmental stimulus or stress (anoxia, hypoxia, temperature, salt, light,  
30 dehydration, etc) or a chemical compound such as an antibiotic (tetracycline,

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ampicillin, rifampicin, kanamycin) hormone (eg. gibberellin, auxin, cytokinin, glucocorticoid, etc), hormone analogue (iodoacetic acid (IAA), 2,4-D, etc) , metal (zinc, copper, iron, etc), or dexamethasone, amongst others. As will be known to those skilled in the art, expression of a functional protein may also require one or more post-  
5 translational modifications, such as glycosylation, phosphorylation, dephosphorylation, or one or more protein-protein interactions, amongst others. All such processes are included within the scope of the term "expressible format".

Preferably, expression of a cell cycle control protein in a specific plant cell, tissue, or  
10 organ is effected by introducing and expressing an isolated nucleic acid molecule encoding said protein, such as a cDNA molecule, genomic gene, synthetic oligonucleotide molecule, mRNA molecule or open reading frame, to said cell, tissue or organ, wherein said nucleic acid molecule is placed operably in connection with a suitable plant-expressible promoter sequence.

15

Reference herein to a "promoter" is to be taken in its broadest context and includes the transcriptional regulatory sequences derived from a classical eukaryotic genomic gene, including the TATA box which is required for accurate transcription initiation, with or without a CCAAT box sequence and additional regulatory elements (i.e. upstream  
20 activating sequences, enhancers and silencers) which alter gene expression in response to developmental and/or external stimuli, or in a tissue-specific manner.

The term "promoter" also includes the transcriptional regulatory sequences of a classical prokaryotic gene, in which case it may include a -35 box sequence and/or a  
25 -10 box transcriptional regulatory sequences.

The term "promoter" is also used to describe a synthetic or fusion molecule, or derivative which confers, activates or enhances expression of a nucleic acid molecule in a cell, tissue or organ.

30

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Preferred promoters may contain additional copies of one or more specific regulatory elements, to further enhance expression and/or to alter the spatial expression and/or temporal expression of a nucleic acid molecule to which it is operably connected. For example, copper-responsive, glucocorticoid-responsive or dexamethasone-responsive  
5 regulatory elements may be placed adjacent to a heterologous promoter sequence driving expression of a nucleic acid molecule to confer copper inducible, glucocorticoid-inducible, or dexamethasone-inducible expression respectively, on said nucleic acid molecule.

10 In the context of the present invention, the promoter is a plant-expressible promoter sequence. By "plant-expressible" is meant that the promoter sequence, including any additional regulatory elements added thereto or contained therein, is at least capable of inducing, conferring, activating or enhancing expression in a plant cell, preferably a monocotyledonous or dicotyledonous plant cell and in particular a dicotyledonous  
15 plant cell, tissue, or organ. Accordingly, it is within the scope of the invention to include any promoter sequences that also function in non-plant cells, such as yeast cells, animal cells and the like.

In the present context, a "regulatable promoter sequence" is a promoter that is capable  
20 of being expressed in a particular cell, tissue, or organ or group of cells, tissues or organs of a plant, optionally under specific conditions, however is generally not expressed throughout the plant under all conditions. Accordingly, a regulatable promoter sequence may be a promoter sequence that confers expression on a gene to which it is operably connected in a particular location within the plant or alternatively,  
25 throughout the plant under a specific set of conditions, such as following induction of gene expression by a chemical compound or other elicitor.

Preferably, the regulatable promoter used in the performance of the present invention confers expression in a specific location within the plant, either constitutively or  
30 following induction, however not in the whole plant under any circumstances. Included

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within the scope of such promoters are cell-specific promoter sequences, tissue-specific promoter sequences, organ-specific promoter sequences and constitutive promoter sequences that have been modified to confer expression in a particular part of the plant at any one time, such as by integration of said constitutive promoter within  
5 a transposable genetic element (*Ac*, *Ds*, *Spm*, *En*, or other transposon).

The term "cell-specific" shall be taken to indicate that expression is predominantly in a particular plant cell or plant cell-type, albeit not necessarily exclusively in that plant cell or plant cell-type.

10

Similarly, the term "tissue-specific" shall be taken to indicate that expression is predominantly in a particular plant tissue or plant tissue-type, albeit not necessarily exclusively in that plant tissue or plant tissue-type.

15 Similarly, the term "organ-specific" shall be taken to indicate that expression is predominantly in a particular plant organ albeit not necessarily exclusively in that plant organ.

As will be apparent from the preceding description, the present invention does not  
20 require the exclusive expression of the cell cycle control protein in a cell, tissue or organ of a plant, in order to induce non-pleiotropic cytokinin-mediated and/or gibberellin-mediated effects therein, subject to the proviso that expression is at least predominantly localised in a particular cell, tissue or organ of the plant. Preferably, the promoter selected for regulating expression of the cell cycle control protein in the plant  
25 cell, tissue or organ, will confer expression in a range of cell-types or tissue-types or organs, sufficient to produce the desired phenotype, whilst avoiding undesirable phenotypes produced in other cell-types or tissue-types or organs.

More preferably, the promoter selected for regulating expression of the cell cycle  
30 control protein in the plant cell, tissue or organ, will confer expression in a limited

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number of cells or cell-types or tissues or tissue-types or organs of the plant.

Even more preferably, the promoter selected for regulating expression of the cell cycle control protein in the plant cell, tissue or organ, will confer expression in a single cell-  
5 type or tissue-type or organ of the plant.

Those skilled in the art will readily be capable of selecting appropriate promoter sequences for use in regulating appropriate expression of the cell cycle control protein from publicly-available or readily-available sources, without undue experimentation.

10

Placing a nucleic acid molecule under the regulatory control of a promoter sequence, or in operable connection with a promoter sequence, means positioning said nucleic acid molecule such that expression is controlled by the promoter sequence.

15 A promoter is usually, but not necessarily, positioned upstream, or at the 5'-end, and within 2 kb of the start site of transcription, of the nucleic acid molecule which it regulates.

In the construction of heterologous promoter/structural gene combinations it is  
20 generally preferred to position the promoter at a distance from the gene transcription start site that is approximately the same as the distance between that promoter and the gene it controls in its natural setting (i.e., the gene from which the promoter is derived). As is known in the art, some variation in this distance can be accommodated without loss of promoter function. Similarly, the preferred positioning of a regulatory  
25 sequence element with respect to a heterologous gene to be placed under its control is defined by the positioning of the element in its natural setting (i.e., the gene from which it is derived). Again, as is known in the art, some variation in this distance can also occur.

30 Examples of promoters suitable for use in genetic constructs of the present invention

- 25 -

include those listed in Table 1, amongst others. The promoters listed in Table 1 are provided for the purposes of exemplification only and the present invention is not to be limited by the list provided therein. Those skilled in the art will readily be in a position to provide additional promoters that are useful in performing the present invention.

5

In an alternative embodiment, the promoter is a tissue-specific inducible promoter sequence, such as but not limited to a light-inducible *rbcs-1A* or *rbcs-3A* promoter, anoxia-inducible maize *Adh1* gene promoter (Howard *et al.*, 1987; Walker *et al.*, 1987), hypoxia-inducible maize *Adh1* gene promoter (Howard *et al.*, 1987; Walker *et al.*,  
10 1987), and the temperature-inducible heat shock promoter. Such environmentally-inducible promoters are reviewed in detail by Kuhlemeier *et al.* 1987).

In an alternative embodiment, the promoter is a chemically-inducible promoter, such as the 3- $\beta$ - indoylacrylic acid-inducible *Tip* promoter; IPTG-inducible *lac* promoter;  
15 phosphate-inducible promoter; L-arabinose-inducible *araB* promoter; heavy metal-inducible metallothionine gene promoter; dexamethasone-inducible promoter; glucocorticoid-inducible promoter; ethanol-inducible promoter (Zeneca); the N,N-diallyl-2,2-dichloroacetamide-inducible glutathione-S-transferase gene promoter (Wiegand *et al.*, 1986); or any one or more of the chemically-inducible promoters described by  
20 Gatz *et al.* (1996), amongst others.

In an alternative embodiment, the promoter is a wound-inducible or pathogen-inducible promoter, such as the phenylalanine ammonia lyase (PAL) gene promoter (Ebel *et al.*, 1984), chalcone synthase gene promoter (Ebel *et al.*, 1984) or the potato wound-  
25 inducible promoter (Cleveland *et al.*, 1987), amongst others.

In a further alternative embodiment, the promoter is a hormone-inducible promoter, such as the abscisic acid-inducible wheat 7S globulin gene promoter and the wheat Em gene promoter (Marcotte *et al.*, 1988); an auxin-responsive gene promoter; or a  
30 gibberellin-inducible promoter such as the *Amy32b* gene promoter (Lanahan *et al.*

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1992), amongst others.

In a further alternative embodiment, the promoter is a constitutive plant-expressible promoter sequence such as the CaMV 35S promoter sequence, CaMV 19S promoter  
5 sequence, the octopine synthase (OCS) promoter sequence, or nopaline synthase (NOS) promoter sequence (Ebert *et al.* 1987), amongst others.

In the case of constitutive promoters or promoters that induce expression throughout the entire plant, it is preferred that such sequences are modified by the addition of  
10 nucleotide sequences derived from one or more of the tissue-specific promoters listed in Table 1, or alternatively, nucleotide sequences derived from one or more of the above-mentioned tissue-specific inducible promoters, to confer tissue-specificity thereon. For example, the CaMV 35S promoter may be modified by the addition of maize *Adh1* promoter sequence, to confer anaerobically-regulated root-specific  
15 expression thereon, as described previously (Ellis *et al.*, 1987). Such modifications can be achieved by routine experimentation by those skilled in the art.

Preferred embodiments of the invention relate to the effect(s) of cytokinins on plant morphology and architecture. The present invention clearly contemplates the broad  
20 application of the inventive method to the modification of a range of cellular processes, including but not limited to the initiation, promotion, stimulation or enhancement of cell division and/or seed development and/or tuber formation and/or shoot initiation and/or bushiness and/or dwarfism and/or pigment synthesis, and/or the modification of source/sink relationships, and/or the inhibition of root growth and/or the inhibition of  
25 apical dominance and/or the delay of senescence. In this regard, the identification of substrates of Cdc25 phosphatase other than cdc2 will also reveal the mechanism by which Cdc25 is linked to many cellular processes other than cell division.

TABLE 1  
EXEMPLARY PLANT-EXPRESSIBLE PROMOTERS FOR USE IN THE PERFORMANCE OF THE PRESENT INVENTION

| GENE SOURCE                          | EXPRESSION PATTERN           | REFERENCE                                                                                                                                                                                |
|--------------------------------------|------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| $\alpha$ -amylase ( <i>Amy32b</i> )  | aleurone                     | Lanahan <i>et al</i> (1992); Skriver <i>et al.</i> (1991)                                                                                                                                |
| cathepsin $\beta$ -like gene         | aleurone                     | Cejudo <i>et al.</i> (1992)                                                                                                                                                              |
| <i>Agrobacterium rhizogenes rolB</i> | cambium                      | Nilsson <i>et al.</i> (1997)                                                                                                                                                             |
| PRP genes                            | cell wall                    | <a href="http://salus.medium.edu/mmg/tierney/html">http://salus.medium.edu/mmg/tierney/html</a>                                                                                          |
| barley <i>ltr1</i> promoter          | endosperm                    |                                                                                                                                                                                          |
| synthetic promoter                   | endosperm                    | Vicente-Carbajosa <i>et al.</i> (1998)                                                                                                                                                   |
| AtPRP4                               | flowers                      | <a href="http://salus.medium.edu/mmg/tierney/html">http://salus.medium.edu/mmg/tierney/html</a>                                                                                          |
| chalcone synthase ( <i>chsA</i> )    | flowers                      | van der Meer <i>et al.</i> (1990)                                                                                                                                                        |
| <i>apetala-3</i>                     | flowers                      |                                                                                                                                                                                          |
| chitinase                            | fruit (berries, grapes, etc) | Thomas <i>et al.</i> CSIRO Plant Industry, Urrbrae, South Australia, Australia;<br><a href="http://winetitles.com.au/gwrdc/csh95-1.html">http://winetitles.com.au/gwrdc/csh95-1.html</a> |
| <i>rbcS-3A</i>                       | green tissue (eg leaf)       | Lam <i>et al.</i> (1990); Tucker <i>et al.</i> (1992)                                                                                                                                    |
| leaf-specific genes                  | leaf                         | Baszczynski <i>et al.</i> (1988)                                                                                                                                                         |
| AtPRP4                               | leaf                         | <a href="http://salus.medium.edu/mmg/tierney/html">http://salus.medium.edu/mmg/tierney/html</a>                                                                                          |
| <i>Pinus cab-6</i>                   | leaf                         | Yamamoto <i>et al.</i> (1994)                                                                                                                                                            |
| SAM22                                | senescent leaf               | Crowell <i>et al.</i> (1992)                                                                                                                                                             |

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|                                      |                         |                                                                                                 |  |
|--------------------------------------|-------------------------|-------------------------------------------------------------------------------------------------|--|
| <i>R. japonicum nif</i> gene         | nodule                  | United States Patent No. 4, 803, 165                                                            |  |
| <i>B. japonicum nifH</i> gene        | nodule                  | United States Patent No. 5, 008, 194                                                            |  |
| GmENOD40                             | nodule                  | Yang <i>et al.</i> (1993)                                                                       |  |
| PEP carboxylase (PEPC)               | nodule                  | Pathirana <i>et al.</i> (1992)                                                                  |  |
| leghaemoglobin (Lb)                  | nodule                  | Gordon <i>et al.</i> (1993)                                                                     |  |
| <i>Tungro bacilliform</i> virus gene | phloem                  | Bhattacharyya-Pakrasi <i>et al.</i> (1992)                                                      |  |
| sucrose-binding protein gene         | plasma membrane         | Grimes <i>et al.</i> (1992)                                                                     |  |
| pollen-specific genes                | pollen; microspore      | Albani <i>et al.</i> (1990); Albani <i>et al.</i> (1991)                                        |  |
| maize pollen-specific gene           | pollen                  | Hamilton <i>et al.</i> (1992)                                                                   |  |
| sunflower pollen-expressed gene      | pollen                  | Baltz <i>et al.</i> (1992)                                                                      |  |
| <i>B. napus</i> pollen-specific gene | pollen; anther; tapetum | Arnoldo <i>et al.</i> (1992)                                                                    |  |
| root-expressible genes               | roots                   | Tingey <i>et al.</i> (1987); An <i>et al.</i> (1988);                                           |  |
| tobacco auxin-inducible gene         | root tip                | Van der Zaal <i>et al.</i> (1991)                                                               |  |
| $\beta$ -tubulin                     | root                    | Oppenheimer <i>et al.</i> (1988)                                                                |  |
| tobacco root-specific genes          | root                    | Conkling <i>et al.</i> (1990)                                                                   |  |
| <i>B. napus</i> G1-3b gene           | root                    | United States Patent No. 5, 401, 836                                                            |  |
| SbPRP1                               | roots                   | Suzuki <i>et al.</i> (1993)                                                                     |  |
| AtPRP1; AtPRP3                       | roots; root hairs       | <a href="http://salus.medium.edu/mmg/tierney/html">http://salus.medium.edu/mmg/tierney/html</a> |  |

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|                                   |                                         |                                                                                                 |
|-----------------------------------|-----------------------------------------|-------------------------------------------------------------------------------------------------|
| RD2 gene                          | root cortex                             | <a href="http://www2.cnsu.edu/ncsu/research">http://www2.cnsu.edu/ncsu/research</a>             |
| TobRB7 gene                       | root vasculature                        | <a href="http://www2.cnsu.edu/ncsu/research">http://www2.cnsu.edu/ncsu/research</a>             |
| AtPRP4                            | leaves; flowers; lateral root primordia | <a href="http://salus.medium.edu/mmg/tierney/html">http://salus.medium.edu/mmg/tierney/html</a> |
| seed-specific genes               | seed                                    | Simon <i>et al.</i> (1985); Scofield <i>et al.</i> (1987); Baszczynski <i>et al.</i> (1990)     |
| Brazil Nut albumin                | seed                                    | Pearson <i>et al.</i> (1992)                                                                    |
| legumin                           | seed                                    | Ellis <i>et al.</i> (1988)                                                                      |
| glutelin (rice)                   | seed                                    | Takaiwa <i>et al.</i> (1986); Takaiwa <i>et al.</i> (1987)                                      |
| zein                              | seed                                    | Matzke <i>et al.</i> (1990)                                                                     |
| napA                              | seed                                    | Stalberg <i>et al.</i> (1996)                                                                   |
| sunflower oleosin                 | seed(embryo and dry seed)               | Cummins <i>et al.</i> (1992)                                                                    |
| LEAFY                             | shoot meristem                          | Weigel <i>et al.</i> (1992)                                                                     |
| <i>Arabidopsis thaliana knat1</i> | shoot meristem                          | Accession number AJ131822                                                                       |
| <i>Malus domestica kn1</i>        | shoot meristem                          | Accession number Z71981                                                                         |
| CLAVATA1                          | shoot meristem                          | Accession number AF049870                                                                       |
| stigma-specific genes             | stigma                                  | Nasrallah <i>et al.</i> (1988); Trick <i>et al.</i> (1990)                                      |
| class I patatin gene              | tuber                                   | Liu <i>et al.</i> (1991)                                                                        |
| PCNA rice                         | meristem                                | Kosugi <i>et al.</i> (1991); Kosugi and Ohashi (1997)                                           |

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In a particularly preferred embodiment of the present invention, there is provided a method of increasing the strength and/or thickness and/or stability and/or wind-resistance of a plant comprising expressing the yeast Cdc25 protein or a homologue, analogue or derivative thereof, or a modified substrate of Cdc25 that mimics the effect  
5 of Cdc25 operably under the control of a stem-expressible promoter sequence.

Preferably, the stem-expressible promoter sequence is derived from the *rbcS-1A* gene, the *rbcS-3A* gene, the *AtPRP4* gene, the *T. bacilliform* virus gene, or the sucrose-binding protein gene set forth in Table 1, or a stem-specific or stem-expressible  
10 homologue, analogue or derivative thereof.

In the present context, the term "substrate of Cdc25" shall be taken to refer to any protein that interacts with Cdc25 in regulating the plant cell cycle, including, but not limited to cyclin-dependent kinases (CDKs), the most significant of which is *cdc2*,  
15 which in all cells is the key enzyme driving entry into mitosis. In all cells, the switch that raises activity of *cdc2* at entry into mitosis is the Cdc25-catalysed removal of phosphate from tyrosine-15 in *cdc2*. In yeasts there is only one CDK (*cdc2*) and the Cdc25-catalysed removal of phosphate from tyrosine-15 in *cdc2* occurs only once in the cell cycle, at the G2/M phase transition. In contrast, higher eukaryotic cells (animal  
20 and plant cells) contain several CDKs. In mammals, the molecular switch of Cdc25-catalysed removal of phosphate from tyrosine-15 in *cdc2* is also used at entry into the S phase, and a separate CDK (CDK2) and a separate Cdc25 (Cdc25A) perform this function. In plants, whilst it is known there are several CDKs, it is not known if there is a single CDK that is controlled at S phase, like CDK2, by tyrosine  
25 phosphate.

Without being bound by any theory or mode of action, the substitution or deletion of the phosphorylation sites of a protein that is a substrate for a cyclin-dependent kinase protein mimics the effect of a constitutive phosphatase activity, such as the effect of  
30 Cdc25 protein phosphatase (p80<sup>Cdc25</sup>) activity, because phosphorylated protein will not

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be produced at high steady-state concentrations in either the absence of phosphorylation or when phosphatases are expressed at high levels. Accordingly, the Cdc25-induced effects described herein can also be obtained by the regulated expression of a modified substrate of Cdc25.

5

The term "modified substrate of Cdc25" refers to a homologue, analogue or derivative of a substrate of Cdc25 that mimics the effect of Cdc25 activity, in particular a non-phosphorylatable Cdc25 substrate that mimics the effect of Cdc25 activity. For example, substitution of threonine and tyrosine at positions 14 and 15 of cyclin-  
10 dependent kinases (CDKs) for alanine and phenylalanine, respectively, can produce one or more cytokinin-like effects in the plant, similar to those observed following constitutive Cdc25 expression in the plant. Notwithstanding that this may be the case, the effects of CDK(A<sub>14</sub>F<sub>15</sub>) expression are inferior to those of naturally-occurring or wild-type Cdc25, possibly because plant cells comprise several Cdc25 substrates.

15

Accordingly, similar effects to the Cdc25-induced effects obtained by expressing Cdc25 under control of the regulatable promoter, can be obtained by expressing the Cdc25 substrate or a modified form thereof operably under control of the same or a functionally-equivalent promoter. The present invention clearly extends to such  
20 arrangements.

The present invention extends further to the co-expression of Cdc25 and one or more Cdc25 substrates and/or one or more modified Cdc25 substrates, operably under the control of a regulatable promoter that is selected for a particular application as  
25 described herein.

In another preferred embodiment of the present invention, there is provided a method of increasing tuber formation and/or development in a tuberous crop plant comprising expressing the yeast Cdc25 protein or a homologue, analogue or derivative thereof,  
30 or a modified substrate of Cdc25 that mimics the effect of Cdc25 operably under the

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control of a tuber-specific promoter sequence.

Preferably, the tuberous crop plant is potato and the tuber-specific promoter is the potato patatin gene promoter. Additional species and promoters are not excluded.

5

In another preferred embodiment of the present invention, there is provided a method of modifying the lignin content of a woody crop plant comprising expressing the yeast Cdc25 protein or a homologue, analogue or derivative thereof, or a modified substrate of Cdc25 that mimics the effect of Cdc25 operably under the control of a cambium-  
10 specific or vascular-tissue-specific promoter sequence.

Preferably, the promoter is a cinnamoyl alcohol dehydrogenase (CAD) gene promoter, laccase gene promoter, cellulose synthase gene promoter and xyloglucan endotransglucosylase (XET) gene promoter sequences, amongst others. The *T.*  
15 *bacilliform* virus gene promoter and the sucrose-binding protein gene promoter are also useful for this application of the invention.

Preferred target plant species according to this embodiment are woody plants of economic/ agronomic value, in particular hardwood crop plants such as, but not limited  
20 to *Eucalyptus spp.*, *Populus spp.*, *Quercus spp.*, *Acer spp.*, *Juglans spp.*, *Fagus spp.*, *Acacia spp.*, or teak, amongst others. More preferably, this embodiment of the invention is applicable to modifying the lignin content of *Eucalyptus spp.*, in particular *E. globulus* and *E. robusta*; or *Quercus spp.*, in particular *Q. dentata*, *Q. ilex*, *Q. incana*, and *Q. robur*; *Acacia spp.*, in particular *A. brevispica*, *A. bussei*, *A.*  
25 *drepanolobium*, *A. nilotica*, *A. pravissima*, and *A. seyal*; *Acer spp.*, in particular *A. pseudoplatanus* and *A. saccharum*. Additional species are not excluded.

Without being bound by any theory or mode of action, the ectopic expression of Cdc25 under control of a promoter that is operable in vascular tissue and preferably, in  
30 cambial cells, will produce thick-stemmed plants and a higher ratio of vascular tissue-

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to-pith cells within the stem, thereby resulting in more lignin production. Within the vascular tissue, cambial cells contain the highest levels of auxins and are therefore the preferential tissue for Cdc25 overproduction.

- 5 In yet another preferred embodiment of the present invention, there is provided a method of increasing seed set and/or seed production and/or grain yield in a plant comprising expressing the yeast Cdc25 protein or a homologue, analogue or derivative thereof, or a modified substrate of Cdc25 that mimics the effect of Cdc25 operably under the control of a seed-specific promoter sequence.

10

- Preferably, the seed-specific promoter is operable in the seeds of monocotyledonous plants, for example the barley *Amy32b* gene promoter, Cathepsin  $\beta$ -like gene promoter, wheat ADP-glucose pyrophosphorylase gene promoter, maize zein gene promoter, or rice glutelin gene promoter. In an alternative embodiment, the seed-specific promoter is operable in the seeds of dicotyledonous plant species, for example
- 15 the legumin gene promoter, *napA* gene promoter, Brazil Nut albumin gene promoter, pea vicilin gene promoter and sunflower oleosin gene promoter, amongst others.

- Those skilled in the art will be aware that grain yield in crop plants is largely a function
- 20 of the amount of starch produced in the endosperm of the seed. The amount of protein produced in the endosperm is also a contributing factor to grain yield. In contrast, the embryo and aleurone layers contribute little in terms of the total weight of the mature grain.

- 25 Accordingly, in a preferred embodiment, the Cdc25 gene is placed operably in connection with a promoter that is operable in the endosperm of the seed, in which case the combination of the cell cycle-control protein and endosperm-expressible promoter provides the additional advantage of increasing the grain size and grain yield of the plant.

30

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Endosperm-specific promoters that can be used to drive Cdc25 expression have been identified. The components of the promoters responsible for specific expression have been identified (Grosset et al (1997) and are interchangeable between agriculturally important cereals (Olsen et al 1992; Russell and Fromm, 1997). Several promoters can  
5 be used, including the zein (ZmZ27) gene promoter, the rice glutelin 1 gene (osGT1) promoter, the rice small subunit ADP-glucose pyrophosphorylase (osAGP) promoter, the maize granule-bound starch synthase (Waxy) gene (zmGBS) promoter surveyed by Russell and Fromm (1997), the Brazil Nut albumin gene promoter, and the pea vicilin gene promoter, amongst others. Promoters derived from those genes that are  
10 expressed in the endosperm during nuclear proliferation are also useful for driving Cdc25 expression. Promoters derived from those genes that are expressed in the endosperm at the stage of nuclear proliferation is ending could be ideal for extending this period.

15 A three way correlation exists between cytokinin level in the endosperm, the number of endosperm cells formed during seed development and grain size, in which cytokinin activates Cdc25 enzyme which in turn activates Cdc2 kinase to drive nuclear division. Accordingly, ectopic expression of the Cdc25 gene in the endosperm enhances Cdc2 activation and nuclear proliferation, resulting in increased grain size, without incurring  
20 the non-specific side effects that application of cytokinin or expression of the *ipt* gene would produce in the plant.

A further advantage of the present inventive approach is that the activity of cytokinin metabolising enzymes is circumvented by the direct raising of Cdc25 activity in the  
25 endosperm, by the ectopic expression of Cdc25 therein. In cases where exogenous cytokinin is used to increase grain size and/or endosperm size, the elevated cytokinin levels and nuclear division in the grain are curtailed by an increase in the activities of cytokinin degrading enzymes, including cytokinin oxidase (Chatfield and Armstrong 1987; reviewed by Morris *et al* 1993).

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In another preferred embodiment of the present invention, there is provided a method of inhibiting or reducing apical dominance or increasing the bushiness of a plant, comprising expressing the yeast Cdc25 protein or a homologue, analogue or derivative thereof, or a modified substrate of Cdc25 that mimics the effect of Cdc25 operably  
5 under the control of a meristem-specific promoter sequence or a stem-specific promoter sequence.

Without being bound by any theory or mode of action, increased cell division in the dormant lateral meristem of plants as a consequence of increased Cdc25 activity  
10 therein results in a higher degree of branch formation in the plant, thereby alleviating auxin-induced apical dominance in the plant.

In another preferred embodiment of the present invention, there is provided a method of increasing lateral root production in a plant comprising expressing the yeast Cdc25  
15 protein or a homologue, analogue or derivative thereof, or a modified substrate of Cdc25 that mimics the effect of Cdc25 operably under the control of a root-specific promoter sequence.

Preferred promoter sequences according to this embodiment of the present invention  
20 include any one of the root-expressible or root-specific promoters listed in Table 1 and in particular, the tobacco auxin-inducible gene promoter described by Van der Zaal *et al* (1991) that confers expression in the root tip of plants, in particular dicotyledonous plants.

25 In yet another preferred embodiment of the present invention, there is provided a method of increasing the nitrogen-fixing capability of a plant comprising expressing the yeast Cdc25 protein or a homologue, analogue or derivative thereof, or a modified substrate of Cdc25 that mimics the effect of Cdc25 operably under the control of a nodule-specific promoter sequence.

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Preferred nodule-specific promoter sequences according to this embodiment of the present invention are listed in Table 1. Additional promoters that are suited for this purpose include the hemoglobin gene promoters derived from *Frankia spp.*, *A. thaliana* or other plants.

5

In still another preferred embodiment of the present invention, there is provided a method of prevent or delay or otherwise reduce leaf chlorosis and/or leaf necrosis in a plant comprising expressing the yeast Cdc25 protein or a homologue, analogue or derivative thereof, or a modified substrate of Cdc25 that mimics the effect of Cdc25  
10 operably under the control of a leaf-specific promoter sequence.

Preferred promoters for use according to this embodiment of the present invention include the SAM22 promoter, *rbcS-1A* and *rbcS-3A* gene promoters listed in Table 1. The SAM22 gene promoter is particularly preferred in light of the developmental  
15 regulation of the SAM22 gene and its induction in senescent leaves.

In a further preferred embodiment of the present invention, the yeast Cdc25 protein or a homologue analogue or derivative thereof, or a modified substrate of Cdc25 that mimics the effect of Cdc25 is expressed in one of the specialised minority of plant  
20 tissues in which the activation of cell cycle progression that is generally contributed by cytokinin is in part performed by other hormones. An example of such a tissue is the youngest stem internode of cereal plants in which gibberellic acid stimulates cell division.

25 Accordingly, the present invention preferably provides a method of stimulating cell division in the intercalary meristem of the youngest stem internode to produce greater elongation of the stem and/or to generate a more extensive photosynthetic canopy of a plant comprising expressing the yeast Cdc25 protein or a homologue, analogue or derivative thereof, or a modified substrate of Cdc25 that mimics the effect of Cdc25  
30 operably under the control of a meristem specific promoter sequence.

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Without being bound by any theory or mode of action, increases in cell division in the intercalary meristem of the youngest stem internode as a consequence of increased Cdc25 activity therein results in greater vigour of the plant due to stem elongation and the production of a more extensive canopy. It is proposed that this leads to an  
5 increase in the plant's capacity to support grain production. The stimulatory effect of gibberellic acid application is thus obtained without side effects on flowering time and seed germination.

Preferred promoters for use according to this embodiment of the invention include  
10 meristem promoters listed in Table 1 and in particular the Proliferating Cell Nuclear Antigen (PCNA) promoter of rice described by Kosugi *et al* (1991).

In each of the preceding embodiments of the present invention, the cell cycle control protein is expressed under the operable control of a regulatable promoter sequence.  
15 As will be known those skilled in the art, this is generally achieved by introducing a genetic construct or vector into plant cells by transformation or transfection means. The nucleic acid molecule or a genetic construct comprising same may be introduced into a cell using any known method for the transfection or transformation of said cell. Wherein a cell is transformed by the genetic construct of the invention, a whole  
20 organism may be regenerated from a single transformed cell, using any method known to those skilled in the art.

By "transfect" is meant that the genetic construct or vector or an active fragment thereof comprising the Cdc25 gene operably under the control of the regulatable  
25 promoter sequence is introduced into said cell without integration into the cell's genome.

By "transform" is meant that the genetic construct or vector or an active fragment thereof comprising the Cdc25 gene operably under the control of the regulatable  
30 promoter sequence is stably integrated into the genome of the cell.

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Accordingly, in a further preferred embodiment, the present invention provides a method of modifying one or more plant morphological and/or biochemical and/or physiological characteristics comprising

- 5 (i) introducing to a plant cell, tissue or organ a genetic construct or vector comprising a nucleotide sequence that encodes a cell cycle control protein operably in connection with a regulatable promoter sequence selected from the list comprising cell-specific promoter sequences, tissue-specific promoter sequences, and organ-specific promoter sequences; and
- 10 (ii) expressing said cell cycle control protein in one or more of said cells, tissues or organs of the plant.

In an alternative embodiment, the present invention provides a method of modifying one or more plant morphological and/or biochemical and/or physiological  
15 characteristics comprising

- (i) introducing into a plant cell a genetic construct or vector comprising a nucleotide sequence that encodes a cell cycle control protein operably in connection with a regulatable promoter sequence selected from the list comprising cell-specific promoter sequences, tissue-specific promoter  
20 sequences, and organ-specific promoter sequences;
- (ii) regenerating a whole plant from said plant cell; and
- (iii) expressing said cell cycle control protein in one or more particular cells, tissues or organs of the plant.

25

Means for introducing recombinant DNA into plant tissue or cells include, but are not limited to, transformation using  $\text{CaCl}_2$  and variations thereof, in particular the method described by Hanahan (1983), direct DNA uptake into protoplasts (Krens *et al*, 1982; Paszkowski *et al*, 1984), PEG-mediated uptake to protoplasts (Armstrong *et al*, 1990)  
30 microparticle bombardment, electroporation (Fromm *et al.*, 1985), microinjection of

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DNA (Crossway *et al.*, 1986), microparticle bombardment of tissue explants or cells (Christou *et al.*, 1988; Sanford, 1988), vacuum-infiltration of tissue with nucleic acid, or in the case of plants, T-DNA-mediated transfer from *Agrobacterium* to the plant tissue as described essentially by An *et al.*(1985), Herrera-Estrella *et al.* (1983a, 1983b, 5 1985).

For microparticle bombardment of cells, a microparticle is propelled into a cell to produce a transformed cell. Any suitable ballistic cell transformation methodology and apparatus can be used in performing the present invention. Exemplary apparatus and 10 procedures are disclosed by Stomp *et al.* (U.S. Patent No. 5,122,466) and Sanford and Wolf (U.S. Patent No. 4,945,050). When using ballistic transformation procedures, the genetic construct may incorporate a plasmid capable of replicating in the cell to be transformed.

15 Examples of microparticles suitable for use in such systems include 1 to 5  $\mu\text{m}$  gold spheres. The DNA construct may be deposited on the microparticle by any suitable technique, such as by precipitation.

A whole plant may be regenerated from the transformed or transfected cell, in 20 accordance with procedures well known in the art. Plant tissue capable of subsequent clonal propagation, whether by organogenesis or embryogenesis, may be transformed with a genetic construct of the present invention and a whole plant regenerated therefrom. The particular tissue chosen will vary depending on the clonal propagation systems available for, and best suited to, the particular species being transformed. 25 Exemplary tissue targets include leaf disks, pollen, embryos, cotyledons, hypocotyls, megagametophytes, callus tissue, existing meristematic tissue (e.g., apical meristem, axillary buds, and root meristems), and induced meristem tissue (e.g., cotyledon meristem and hypocotyl meristem).

30 The term "organogenesis", as used herein, means a process by which shoots and

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roots are developed sequentially from meristematic centres.

The term "embryogenesis", as used herein, means a process by which shoots and roots develop together in a concerted fashion (not sequentially), whether from somatic  
5 cells or gametes.

The regenerated transformed plants may be propagated by a variety of means, such as by clonal propagation or classical breeding techniques. For example, a first generation (or T1) transformed plant may be selfed to give homozygous second  
10 generation (or T2) transformant, and the T2 plants further propagated through classical breeding techniques.

The regenerated transformed organisms contemplated herein may take a variety of forms. For example, they may be chimeras of transformed cells and non-transformed  
15 cells; clonal transformants (e.g., all cells transformed to contain the expression cassette); grafts of transformed and untransformed tissues (e.g., in plants, a transformed root stock grafted to an untransformed scion ).

A further aspect of the present invention clearly provides the genetic constructs and  
20 vectors designed to facilitate the introduction and/or expression and/or maintenance of the cell cycle control protein-encoding sequence and regulatable promoter into a plant cell, tissue or organ.

In addition to the cell cycle control protein-encoding sequence and regulatable  
25 promoter sequence, the genetic construct of the present invention may further comprise one or more terminator sequences.

The term "terminator" refers to a DNA sequence at the end of a transcriptional unit which signals termination of transcription. Terminators are 3'-non-translated DNA  
30 sequences containing a polyadenylation signal, which facilitates the addition of

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polyadenylate sequences to the 3'-end of a primary transcript. Terminators active in cells derived from viruses, yeasts, moulds, bacteria, insects, birds, mammals and plants are known and described in the literature. They may be isolated from bacteria, fungi, viruses, animals and/or plants.

5

Examples of terminators particularly suitable for use in the genetic constructs of the present invention include the *Agrobacterium tumefaciens* nopaline synthase (NOS) gene terminator, the *Agrobacterium tumefaciens* octopine synthase (OCS) gene terminator sequence, the Cauliflower mosaic virus (CaMV) 35S gene terminator  
10 sequence, the *Oryza sativa* ADP-glucose pyrophosphorylase terminator sequence (t3'Bt2), the *Zea mays zein* gene terminator sequence, the *rbcS-1A* gene terminator, and the *rbcS-3A* gene terminator sequences, amongst others.

Those skilled in the art will be aware of additional promoter sequences and terminator  
15 sequences which may be suitable for use in performing the invention. Such sequences may readily be used without any undue experimentation.

The genetic constructs of the invention may further include an origin of replication sequence which is required for maintenance and/or replication in a specific cell type,  
20 for example a bacterial cell, when said genetic construct is required to be maintained as an episomal genetic element (eg. plasmid or cosmid molecule) in said cell.

Preferred origins of replication include, but are not limited to, the *f1*-ori and *colE1* origins of replication.

25

The genetic construct may further comprise a selectable marker gene or genes that are functional in a cell into which said genetic construct is introduced.

As used herein, the term "selectable marker gene" includes any gene which confers  
30 a phenotype on a cell in which it is expressed to facilitate the identification and/or

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selection of cells which are transfected or transformed with a genetic construct of the invention or a derivative thereof.

Suitable selectable marker genes contemplated herein include the ampicillin resistance  
5 (Amp<sup>r</sup>), tetracycline resistance gene (Tc<sup>r</sup>), bacterial kanamycin resistance gene (Kan<sup>r</sup>), phosphinothricin resistance gene, neomycin phosphotransferase gene (*nptII*), hygromycin resistance gene,  $\beta$ -glucuronidase (GUS) gene, chloramphenicol acetyltransferase (CAT) gene, green fluorescent protein (*gfp*) gene (Haseloff *et al*, 1997), and luciferase gene, amongst others.

10

In fact, the cell cycle control protein-encoding sequence, in particular the Cdc25 protein-encoding sequence may also be used as a selectable marker gene as defined herein, by virtue of the altered morphology and/or biochemistry and/or physiology conferred by its regulated expression in plant cells, tissues, organs or whole plants.

15

Those skilled in the art may be aware that plant cells in culture require cytokinin as well as auxin for cell proliferation. In a minority of tissues such as the shoot internode meristem of the Graminae, a hormone other than cytokinin, in particular gibberellin, appears to be required for cell proliferation.

20

The present inventors have confirmed the cytokinin requirement, for tissues other than the shoot internode meristem of the Graminae. In particular, the inventors have observed that whilst auxin alone is able to stimulate the enlargement cells derived from excised tobacco pith tissue, cytokinin is also required for cell division to occur (Figure  
25 4). Only in the presence of both auxin and cytokinin do tobacco cells proliferate and form callus, as measured by the incorporation of bromodeoxyuridine (BrdU) into replicating DNA (Figure 4).

With regard to the dependence of cells upon gibberellin, Sauter *et al* (1995) showed  
30 that within 4 hours of gibberellin application to rice stems, a synchronous decline

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occurs in the number of cells that are in G2 phase, and cells move into mitosis, indicating that the G2-arrested cells had been stimulated. However, the application of gibberellins to plant tissues to promote cell division is also accompanied undesirable pleiotropic side-effects, in particular the induction of flowering, stem elongation, seed  
5 germination, fruit and seed development.

The inability of genetically unmodified plant cells derived directly from the plant to divide without added cytokinin or gibberellin in addition to auxin forms the basis for the positive selection method of the present invention, which depends upon the  
10 unexpected ability of Cdc25 gene expression to replace the requirement for these hormones. In this embodiment of the invention, plant cells that ectopically-express Cdc25 under the control of a regulatable promoter are able to proliferate without added cytokinin or, in the case of certain tissues such as the shoot meristem internode, without added gibberellin, thereby providing a strong positive selection for transformed  
15 or transfected cells on medium lacking cytokinin or gibberellin, as the case may be. The present invention overcomes the disadvantages associated with exogenous gibberellin and/or cytokinin application, by promoting hormone-mediated cell division *via* the ectopic expression of Cdc25 in particular cells, tissues or organs of the plant.

20 This positive selection for transgenic cells is particularly advantageous for plant breeding by gene transfer, wherein the Cdc25 gene operably in connection with a plant-expressible promoter is introduced into the plant cell at the same time as a gene-of-interest. According to this embodiment of the present invention, the Cdc25 gene may be contained in the same plasmid or virus vector as the gene-of-interest, or  
25 alternatively, on separate plasmid or virus vector molecules, in which case these molecules are generally co-introduced to the plant cell.

Accordingly, a further aspect of the present invention provides a method of detecting or identifying transformed or transfected plant cells, tissues or organs that are  
30 cytokinin-dependent, comprising expressing the yeast Cdc25 protein or a homologue,

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analogue or derivative thereof, or a modified substrate of Cdc25 that mimics the effect of Cdc25 in said plant cell, tissue or organ operably under the control of a regulatable plant-expressible promoter, for a time and under conditions sufficient for cytokinin-mediated cell division and/or cytokinin-mediated tissue differentiation to occur in the  
5 absence of cytokinin.

As used herein, the term "cytokinin-dependent" shall be taken to refer to a naturally-occurring plant cell, tissue or organ that at least requires the application of exogenous cytokinin to promote cell division and/or proliferation *in vitro*.

10

A further aspect of the present invention provides a method of detecting or identifying transformed or transfected plant cells, tissues or organs that are gibberellin-dependent, comprising expressing the yeast Cdc25 protein or a homologue, analogue or derivative thereof, or a modified substrate of Cdc25 that mimics the effect of Cdc25 in said plant  
15 cell, tissue or organ operably under the control of a regulatable plant-expressible promoter, for a time and under conditions sufficient for gibberellin-mediated cell division and/or gibberellin-mediated tissue differentiation to occur in the absence of gibberellin.

20 Preferably, the gibberellin-dependent plant tissue is the meristem shoot internode or intercalary meristem of the youngest stem internode derived from a monocotyledonous plant species, in particular the Graminae.

As used herein, the term "gibberellin-dependent" shall be taken to refer to a naturally-  
25 occurring plant cell, tissue or organ that at least requires the application of exogenous gibberellin to promote cell division and/or proliferation *in vitro*.

Preferably, the regulatable promoter used in the transfection/transformation selection systems described herein is a promoter listed in Table 1. Wherein the promoter is  
30 operable in a specific tissue or cell type, selection will only be possible in that cell or

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tissue following transformation, and optionally, following regeneration therefrom, because in other tissues or cells of the transformed plant material the Cdc25 gene will not be expressed.

- 5 More preferably, the plant-expressible promoter is an inducible promoter, such as a tissue-specific inducible promoter sequence, environmentally-inducible promoter, a chemically-inducible promoter, a wound-inducible promoter, a pathogen-inducible promoter, or a hormone-inducible promoter. In this case, dividing cells are selected by switching on the expression of Cdc25 with the appropriate stimulus for the promoter  
10 of choice.

Wherein the promoter is chemically-inducible, the transformed cells can be selected in the presence of the chemical that induces Cdc25 gene expression. In a particularly preferred embodiment exemplified herein, the Cdc25 gene is expressed in tobacco  
15 cells under the control of the dexamethasone-inducible promoter and dividing cells are selected on media containing both the synthetic auxin 2,4-D and dexamethasone, in the absence of added cytokinin.

In an alternative preferred embodiment, the promoter is a constitutive plant-expressible  
20 promoter sequence, wherein the regulatable promoter plus cell cycle control protein-encoding sequence is modified by the insertion of 5'- and/or 3'-transposable genetic element sequences to facilitate movement of genetic construct/vector and selective expression of the cell cycle control protein in a sub-set of cells, tissue or organs of the plant. According to this embodiment, the cell cycle control protein is expressed only in  
25 those cells which also contain the transposable element inserted into their genome.

A further aspect of the invention clearly extends to a plant cell, tissue, organ or whole plant that has been transformed or transfected with an isolated nucleic acid molecule that comprises a nucleotide sequence which encodes a cell cycle control protein,  
30 wherein the expression of said nucleotide sequence is placed operably under the

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control of a plant-expressible cell-specific promoter sequence, plant-expressible tissue-specific promoter sequence, a plant-expressible organ-specific promoter sequence, or a plant-expressible constitutive promoter sequence such that said plant-expressible constitutive promoter sequence and said nucleotide sequence encoding a cell cycle  
 5 control protein are integrated into a transposable genetic element.

The present invention is applicable to any plant, in particular a monocotyledonous plants and dicotyledonous plants including a fodder or forage legume, companion plant, food crop, tree, shrub, or ornamental selected from the list comprising *Acacia*  
 10 *spp.*, *Acer spp.*, *Actinidia spp.*, *Aesculus spp.*, *Agathis australis*, *Albizia amara*, *Alsophila tricolor*, *Andropogon spp.*, *Arachis spp.*, *Areca catechu*, *Astelia fragrans*, *Astragalus cicer*, *Baikiaea plurijuga*, *Betula spp.*, *Bruguiera gymnorhiza*, *Burkea africana*, *Butea frondosa*, *Cadaba farinosa*, *Calliandra spp.*, *Camellia sinensis*, *Canna indica*, *Capsicum spp.*, *Cassia spp.*, *Centroema pubescens*, *Chaenomeles*  
 15 *spp.*, *Cinnamomum cassia*, *Coffea arabica*, *Colophospermum mopane*, *Coronillia varia*, *Cotoneaster serotina*, *Crataegus spp.*, *Cucumis spp.*, *Cupressus spp.*, *Cyathea dealbata*, *Cydonia oblonga*, *Cryptomeria japonica*, *Cymbopogon spp.*, *Cynthea dealbata*, *Cydonia oblonga*, *Dalbergia monetaria*, *Davallia divaricata*, *Desmodium spp.*, *Dicksonia squarosa*, *Diheteropogon amplexans*, *Dioclea spp.*, *Dolichos spp.*,  
 20 *Dorycnium rectum*, *Echinochloa pyramidalis*, *Ehrartia spp.*, *Eleusine coracana*, *Eragrestis spp.*, *Erythrina spp.*, *Eucalyptus spp.*, *Euclea schimperi*, *Eulalia villosa*, *Fagopyrum spp.*, *Feijoa sellowiana*, *Fragaria spp.*, *Flemingia spp.*, *Freycinetia banksii*, *Geranium thunbergii*, *Ginkgo biloba*, *Glycine javanica*, *Gliricidia spp.*, *Gossypium hirsutum*, *Grevillea spp.*, *Guibourtia coleosperma*, *Hedysarum spp.*, *Hemarthia*  
 25 *altissima*, *Heteropogon contortus*, *Hordeum vulgare*, *Hyparrhenia rufa*, *Hypericum erectum*, *Hyperthelia dissoluta*, *Indigo incarnata*, *Iris spp.*, *Leptarrhena pyrolifolia*, *Lespediza spp.*, *Lettuca spp.*, *Leucaena leucocephala*, *Loudetia simplex*, *Lotonus bainesii*, *Lotus spp.*, *Macrotyloma axillare*, *Malus spp.*, *Manihot esculenta*, *Medicago sativa*, *Metasequoia glyptostroboides*, *Musa sapientum*, *Nicotianum spp.*, *Onobrychis*  
 30 *spp.*, *Omithopus spp.*, *Peltophorum africanum*, *Pennisetum spp.*, *Persea gratissima*,

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*Petunia spp.*, *Phaseolus spp.*, *Phoenix canariensis*, *Phormium cookianum*, *Photinia spp.*, *Picea glauca*, *Pinus spp.*, *Pisum sativum*, *Podocarpus totara*, *Pogonarthria fleckii*, *Pogonarthria squarrosa*, *Populus spp.*, *Prosopis cineraria*, *Pseudotsuga menziesii*, *Pterolobium stellatum*, *Pyrus communis*, *Quercus spp.*, *Rhaphiolepis umbellata*,  
 5 *Rhopalostylis sapida*, *Rhus natalensis*, *Ribes grossularia*, *Ribes spp.*, *Robinia pseudoacacia*, *Rosa spp.*, *Rubus spp.*, *Salix spp.*, *Schyzachyrium sanguineum*, *Sciadopitys verticillata*, *Sequoia sempervirens*, *Sequoiadendron giganteum*, *Sorghum bicolor*, *Spinacia spp.*, *Sporobolus fimbriatus*, *Stiburus alopecuroides*, *Stylosanthos humilis*, *Tadehagi spp*, *Taxodium distichum*, *Themeda triandra*, *Trifolium spp.*, *Triticum*  
 10 *spp.*, *Tsuga heterophylla*, *Vaccinium spp.*, *Vicia spp.*, *Vitis vinifera*, *Watsonia pyramidata*, *Zantedeschia aethiopica*, *Zea mays*, straw, amaranth, onion, asparagus, sugar cane, soybean, sugarbeet, sunflower, carrot, celery, cabbage, canola, tomato, potato, lentil, flax, broccoli, oilseed rape, cauliflower, brussel sprout, artichoke, okra, squash, kale, collard greens, and tea, amongst others, or the seeds of any plant  
 15 specifically named above or a tissue, cell or organ culture of any of the above species.

Preferably, the plant is a plant that is capable of being transfected or transformed with a genetic sequence, or which is amenable to the introduction of a protein by any art-recognised means, such as microprojectile bombardment, microinjection,  
 20 *Agrobacterium-mediated* transformation, protoplast fusion, or electroporation, amongst others.

This aspect of the invention further extends to plant cells, tissues, organs and plants parts, propagules and progeny plants of the primary transformed or transfected cells,  
 25 tissues, organs or whole plants that also comprise the introduced isolated nucleic acid molecule operably under control of the cell-specific, tissue-specific or organ-specific promoter sequence and, as a consequence, exhibit similar phenotypes to the primary transformants/transfectants or at least are useful for the purpose of replicating or reproducing said primary transformants/transfectants.

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The present invention is further described with reference to the following non-limiting Examples and to the drawings.

## EXAMPLE 1

### 5 CELL CULTURE, PROTEIN AND ENZYME METHODS

#### **Cell culture**

Suspension cultured cells of *Nicotiana plumbiginifolia* were grown in CS V medium supplemented with 9  $\mu$ M 2,4-dichlorophenoxyacetic acid and 0.23  $\mu$ M kinetin, and were brought to arrest at the cytokinin control point by the omission of kinetin from the  
10 culture medium. Arrest of cell cultures was confirmed by cell counting.

#### **Antibodies**

Polyclonal antibodies were raised in rabbits using the carboxy terminal amino acid sequence of the tobacco cdc2 protein, designated as cdc2a, as an immunogen. This  
15 peptide has the amino acid sequence KRITARNALEHEYFKDIGYVP and has been demonstrated by complementation analyses in yeast to be a functional homologue of cdc2. The cdc2a peptide was synthesised chemically, purified by HPLC and conjugated to keyhole limpet haemocyanin. Antibodies were also prepared against a recombinant GST-Cdc25 catalytic core fusion protein, that had been synthesised in  
20 *Escherichia coli*.

#### **Assay of cdc2 and Cdc25 activities**

Both cdc2 and Cdc25 enzyme activities were extracted from tobacco cells, by grinding the cells in liquid nitrogen. For cdc2 extraction, NDE buffer containing 25 mM HEPES  
25 (pH 7.2) with protease and phosphatase inhibitors was used. For Cdc25 extraction, PDE buffer, containing 25 mM MOPS (pH 7.2), 100 mM NaCl, 10 mM DTT, 5mM EDTA, 1mM EGTA, 1% NP-40, 50 mM NaF, 0.5 mM PMSF, 3  $\mu$ g ml<sup>-1</sup> leupeptin, and 20  $\mu$ g ml<sup>-1</sup> aprotinin, was used.

30 Immunoprecipitates of cdc2 and Cdc25 were obtained by reaction with 25  $\mu$ l protein

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A-purified antibodies against cdc2 and Cdc25 respectively, for 3 h at 4°C, followed by sedimentation of the antigen-antibody complexes using 35 µl protein A beads per sample. The immunoprecipitates were then washed three times, for 10 min per wash, using HDW buffer, followed by similar washing using HBK buffer. In the case of Cdc25  
5 immunoprecipitates, the HBK buffer was supplemented with 2 µM spermidine.

To measure cdc2 activity, the phosphorylation of H1 histone was followed.

To measure Cdc25 activity, assays were conducted in two stages. First, Cdc25  
10 immunoprecipitates from 500 µg total soluble plant protein were incubated for 30 min at 30°C in Cdc25 assay buffer with 0.25 µg tyrosine phosphorylated cdc2 substrate that had been purified with p13<sup>suc1</sup>-beads from 500 µg protein of arrested Cdc25-22 mutant fission yeast. The phosphatase reaction was stopped by removing the complexed Cdc25/cdc2 by sedimentation. In the second stage of the Cdc25 assay, the  
15 supernatant was assayed for yeast cdc2 kinase that had been activated. Assays to be compared directly were run and exposed together in a Phosphorimager.

#### ***cdc2a phosphotyrosine assay***

To assay phosphotyrosine in cdc2a, the cdc2a enzyme fraction was recovered  
20 essentially as described *supra* for the cdc2 activity assay, except that 5 mg of extracted plant protein was used as starting material, and the NDE buffer was modified to include 2.5 mM sodium vanadate and 1 mM phosphotyrosine, and the immune complexes were washed with HDW buffer supplemented with 1 mM with sodium vanadate.

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Western blots of cell-derived protein were probed with anti-phosphotyrosine mouse monoclonal (PY99, Santa Cruz Biotechnology, S.C., USA), followed by [<sup>125</sup>I]-labelled second antibody, and the signal obtained was detected by Phosphorimage analysis.

#### **30 Northern blots**

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RNA was extracted from cells ground in liquid nitrogen, into 2 volumes of 10 mM Tris/HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 1% (w/v) SDS and 2 volumes of phenol:chloroform:iso-amylalcohol 25:24:1 at 4°C and fractionated.

- 5 RNA was electrophoresed on agarose gels, transferred to membrane and probed with the 65- bp *Bgl*II-*Xba*I fragment of the *Cdc25* gene, using standard procedures.

## EXAMPLE 2

### Expression of yeast *Cdc25* makes cell division in plant cells independent 10 of cell division

The effect of ectopic expression of yeast *Cdc25* in plant s was investigated because cells arrested by lack of cytokinin, whether derived from suspension culture or excised freshly from the plant, have abundant *cdc2* protein that is enzymically inactive because  
15 phosphorylated at tyrosine.

Latent *cdc2* protein kinase activity can be released *in vitro* by incubation with the phosphoprotein phosphatase *Cdc25* that is specific for *cdc2*. When cytokinin stimulates entry into mitosis, dephosphorylation of *cdc2* is one of the events that occur,  
20 but it was uncertain whether the hormone might have several effects in the cell cycle. To test this possibility we therefore arranged the inducible expression of the fission yeast *Cdc25* gene in tobacco under the control of a dexamethasone-inducible promoter. Only if the sole essential action of cytokinin is to cause dephosphorylation and activation of *cdc2* kinase can the ectopic expression of the *Cdc25* gene substitute  
25 for presence cytokinin at mitosis.

We now report that the sole essential action of cytokinin in sustaining cell division is activation of *Cdc25* since the hormone can be substituted by expression of this gene.

30 Levels of the fission yeast enzyme *Cdc25* that removes inhibitory phosphate from

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tyrosine in cdc2 kinase were brought under genetic control in the plant by joining the yeast *Cdc25* gene to a modified plant promoter that contained rat glucocorticoid response elements (GREs), which are responsive to rat glucocorticoid receptor protein (GR) in the presence of dexamethasone and therefore allowed induction without  
5 interference from plant hormones. The GRE-Cdc25, together with the constitutively-expressed NOS promoter-GR construct, were inserted into the vector pBin19, which contains pnos:nptII for kanamycin resistance, and introduced into cells of *N. plumbaginifolia* by electroporation into protoplasts. Clones resistant to kanamycin were tested for ability to form a colony on solid medium containing dexamethasone  
10 and auxin but no cytokinin.

At the high concentration of 10  $\mu$ M dexamethasone, cells commonly arrested at prophase in mitotic catastrophe but lower inducer concentrations allowed colony formation and generated cell lines in which inducible expression of Cdc25 was  
15 detected by Western blot analysis using antibody against glutathione-S-transferase (GST)-Cdc25 fusion protein.

Inducible cell lines contained yeast *Cdc25* DNA (detected in Southern blots, not shown) and in 0.01-10  $\mu$ M dexamethasone they accumulated Cdc25 mRNA and  
20 protein (Figure 1-1; Figure 1-2). Effects on division were tested in cells that had been arrested at the G2 phase hormonal control point by depletion of auxin and cytokinin followed by provision of auxin only. Dexamethasone at 0.01-10  $\mu$ M induced division (Figure 1-3) and a sharp optimum concentration of 0.1  $\mu$ M dexamethasone was observed in independent clones, consistent with requirement for a critical optimum  
25 Cdc25 activity. No cell division was observed without inducer, or in untransformed cells treated with dexamethasone (Figure 1-3). Three independent lines were analysed biochemically and had similar properties. Results from one line are shown.

The experimental system used for subsequent experiments involved the prior arrest  
30 of suspension culture cells, at the cytokinin control point in late G2 phase. Arrest at

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this point was obtained by incubation without hormone and then with auxin (2,4-D) without cytokinin. Mitosis could then be induced by addition of cytokinin, or alternative potentially mitogenic treatments could be tested. Progress through prophase is a little slower after this arrest than in cells not emerging from hormonal block and is very  
5 suitable for study of the succession of biochemical events in plant mitosis.

Induced synthesis of Cdc25 in cells at the cytokinin control point in late G2 resulted in appearance of Cdc25 activity, which was detected by its activation of yeast cdc2 H1 histone kinase that was provided a substrate in low activity form, phosphorylated on  
10 tyrosine 15 and amenable to activation by Cdc25 (Figure 2-1 to Figure 2-7). The induced Cdc25 phosphatase activity peaked at 6 h and provides an explanation for the increase in cdc2 kinase activity, which increased while Cdc25 was active (Figure 2-2). Specific recovery of cdc2a and Cdc25 was indicated by precompetition with cdc2a peptide antigen and by preimmune anti-Cdc25 serum or anti-Cdc25 antibody  
15 precompeted with inactive GST-Cdc25 (Figure 2-1).

To test whether the effectiveness of ectopically expressed Cdc25 derived from the operation of mechanisms present in normal mitosis, transgenic cells induced with dexamethasone were monitored for Cdc25 phosphatase and cdc2 kinase activity  
20 (Figure 2-2; Figure 2-3) in parallel with cells induced with cytokinin (Figure 2-3; Figure 2-4). Both showed increase in Cdc25 activity and then cdc2a kinase activity leading to division. A control over Cdc25 activity at post-translational level is indicated by the absence of a higher Cdc25 catalytic activity when yeast enzyme was expressed in addition to the endogenous Cdc25 (Figure 2-2; Figure 2-4). This suggests that the  
25 additional yeast enzyme comes under homeostatic controls that are conserved between yeasts and plants. Post-translational control of Cdc25 activity is known to be complex, tolerant to different levels of the protein, and to involve activating phosphorylations and ubiquitin-directed proteolysis.

30 The temporal correlation of induced Cdc25 phosphatase activity with increase in cdc2

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kinase activity (Figure 2-2; Figure 2-4) suggested that the phosphatase is responsible. We tested this by investigating whether Cdc25 enzyme could activate cdc2 from cells in prophase and whether the extent of activation by Cdc25 declined when activation had already occurred *in vivo*. Data presented in Figure 2-5 show that excess  
5 bacterially-synthesised GST-Cdc25 could activate plant cdc2 enzyme that was extracted in prophase between 3 hours and 12 hours, and that the extent of activation declined in proportion with activation that had previously occurred. These data are consistent with the increase in Cdc25 phosphatase driving prophase progression by dephosphorylating cdc2. After 12 hours, cdc2 activity declined during anaphase and  
10 the enzyme then became unresponsive to GST-Cdc25, consistent with the anaphase decline in activity being due to proteolysis of cyclin, as observed for cyclin 1b in maize mitosis.

The low level of Cdc25 activity in cells that are arrested by limiting cytokinin, as at time  
15 zero (i.e. 0 hours) in Figure 2, indicates that down-regulation of Cdc25 activity is part of the cytokinin control mechanism and that induced Cdc25 therefore provides a biologically relevant signal. The resulting daughter cells were viable; indicating that mitosis driven by induced Cdc25 is functionally normal. These daughter cells could proliferate indefinitely with dexamethasone replacing cytokinin and required nine-fold  
20 dilution every 7 days precisely as in control cultures provided with auxin and cytokinin. They are routinely maintained in dexamethasone without cytokinin. Thus, unexpectedly the data provided herein reveal that the sole essential action of cytokinin in sustaining cell division is activation of Cdc25 and the hormone can be substituted by expression of the *Cdc25* gene.

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**EXAMPLE 3****Evidence for the presence of Cdc25 protein in plant cells**

The ability of yeast Cdc25 to influence cytokinin-mediated cell division in plants suggested to the present inventors that the yeast protein replaces the activity of an endogenous plant Cdc25 enzyme that is activated by cytokinin. To demonstrate that this is the case, the effectiveness of induced yeast Cdc25 produced in transformed plant cells to activate cdc2, was compared to the effectiveness of a putative plant-derived Cdc25 from genetically unmodified cells to activate cdc2.

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The yeast and putative plant Cdc25 enzymes recovered by immunoprecipitation using anti-Cdc25 antibody were compared in reaction with tyrosine phosphorylated plant cdc2 enzyme taken from cells arrested at the G2 control point (Figure 2-6, lanes 1-3). Substrate cdc2 was also taken from cells after 3 hours stimulation with cytokinin (Figure 2-6, lanes 4-6), when partial cdc2 activation had occurred (Figure 2-5; Figure 2-6, lanes 1 and 4).

The activation of plant cdc2 by yeast Cdc25 expressed in plant cells (Figure 2-6, lane 6) demonstrates a mechanism by which Cdc25 can substitute for cytokinin. Furthermore this activation mechanism is a normal part of plant mitosis, because non-transgenic plant cells also contain a Cdc25 activity, unambiguously of plant origin, that is both present following cytokinin stimulation and capable of activating plant cdc2 (Figure 2-6, compare lanes 2 and 5). Moreover, the plant Cdc25 activity is slightly more effective than the heterologous yeast Cdc25 in activating plant cdc2 in the tobacco cells tested (Figure 2-6, compare lanes 5 and 6).

We also assayed phosphotyrosine in the cdc2a kinase that increased in activity when the hormonal block was released. As shown in Figure 2-7, levels of tyrosine phosphate in cdc2a declined after induction of Cdc25, as the catalytic activity of cdc2a increased (Figure 2-2), indicating that a decline in phosphotyrosine caused by

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induction of Cdc25 in transgenic cells stimulates entry of cells into mitosis.

To further test the evidence for Cdc25 presence in genetically unmodified plant cells, we tested for immunological cross-reactivity between plant Cdc25 and authentic fission yeast Cdc25. In western blot analyses, antibodies against fission yeast Cdc25 detected a protein of 67 kDa in a tobacco cell fraction obtained using the mitotic protein p13<sup>suc1</sup> as an affinity ligand to purify cell cycle proteins (Figure 3, lane 1). Moreover, the binding of antibody to this 67 kDa tobacco protein was eliminated by pre-competition with authentic yeast Cdc25 protein (Figure 3, lane 2), suggesting that the yeast and plant Cdc25 protein share protein epitopes, such as primary amino acid sequences, secondary, or tertiary structures. The size of the 67 kDa tobacco protein correlates with the known size of other Cdc25 molecules.

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#### EXAMPLE 4

##### **Expression of Cdc25 under the control of the patatin gene promoter increases tuber size and number in potato plants**

The fission yeast Cdc25 coding sequence is cloned between the promoter of a class I patatin gene (Liu *et al.*, 1991) and the transcription termination signals of the nopaline synthase (NOS) gene of *Agrobacterium tumefaciens*. Preferentially, the B repeat region and the distal region of the A repeat of the patatin promoter is used, without the proximal region of the A repeat. The proximal region of the A repeat of the patatin promoter confers sucrose-responsiveness in various tissues, which is not a desirable characteristic for our purposes (Grierson *et al.*, 1994). This construct is placed in a binary vector, mobilized to *Agrobacterium tumefaciens*, and the introduced into potato plants.

The Cdc25 protein is expressed under the control of the Class I patatin promoter when the first stolon starts to tuberize, consistent with the expression pattern for the patatin

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gene (Liu *et al.*, 1991). At this stage, expression is associated with both internal and external phloem. After tuber induction has occurred, promoter activity is found both in tuberized stolons and in non-tuberized stolons. Expression then expands to the entire storage parenchyma, cortex and pith, but remains absent from the periderm.

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Because the Class II patatin promoters are expressed in the periderm and as such are complementary to the Class I promoters (Köster-Töpfer *et al.*; Liu *et al.*, 1991; Nap *et al.*, 1992), it is beneficial to have Cdc25 expression driven by both Class I and Class II promoters within the same plant. Because the Class I patatin promoter is not  
 10 expressed before the first stolon initiates tuberization, no effects of Class I patatin-Cdc25 transgenes is seen on tuber initiation. However, the Class I patatin promoter drives Cdc25 expression very early after tuber initiation onwards, allowing a maximal impact of Cdc25 activity on organ formation and, as a consequence, on tuber size. The fact that the Class I patatin promoter activity subsequently also appears in non-  
 15 tuberized stolons implies that the Class I patatin – Cdc25 transgene increases both the size and number of tubers.

## EXAMPLE 5

20 **Expression of Cdc25 under the control of the SAUR gene promoter or the *A. rhizogenes rolB* promoter increases lignin in poplar plants**

The fission yeast Cdc25 coding sequence is cloned between the promoter of the soybean SAUR gene (Li *et al.*, 1992 ) and the transcription termination signals of the  
 25 nopaline synthase (NOS) gene of *Agrobacterium tumefaciens*. The SAUR promoter is inducible by auxins. This chimeric genetic construct is introduced between the T-DNA borders of the binary vector pBI121 or similar vector and mobilised into *Agrobacterium tumefaciens*. Poplar is transformed by *Agrobacterium*-mediated transformation using standard procedures.

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Transgenic poplar trees containing this construct show increased lignin content, correlated with an increased stem diameter and the higher ratio of vascular tissue to pith and cortex cells.

- 5 A similar phenotype in poplar is produced when the Cdc25 expression is driven by the *rolB* promoter of *Agrobacterium rhizogenes* (Nilsson *et al.*, 1997), that is expressed in cambial cells (i.e. the dividing cells of the vascular tissue).

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## EXAMPLE 6

### **Expression of Cdc25 under the control of endosperm-specific promoters increases grain size and yield of grain crop plants**

The fission yeast Cdc25 coding sequence is placed operably in connection with the  
15 endosperm-specific *ltr1* promoter from barley, or a synthetic promoter containing the endosperm box (GCN motif) of the barley *Hor2* gene (Vicente-Carbajosa *et al.*, 1998). In each case, the Cdc25 structural gene is placed upstream of the transcription termination signals of the *Agrobacterium tumefaciens* nopaline synthase (NOS) gene. Cereals, in particular rice, maize, wheat and barley, are transformed using standard  
20 procedures, in particular microprojectile bombardment or *Agrobacterium*-mediated transformation systems, with the genetic constructs.

The grain size and starch storage capacity of the endosperm of the seeds of transformed plants is increased relative to otherwise isogenic non-transformed plants.

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### EXAMPLE 7

#### **Expression of Cdc25 under the control of meristem-specific promoters reduces apical dominance in *A. thaliana* and *B. napus* plants**

5 The fission yeast Cdc25 coding sequence is placed operably in connection with the shoot meristem-specific *LEAFY* promoter (Weigel *et al.*, 1992), or the KNOTTED-like *Arabidopsis thaliana knat1* promoter (Accession number AJ131822), or the KNOTTED-like *Malus domestica kn1* promoter ( Accession No. Z71981), or the *Arabidopsis thaliana CLAVATA1* promoter (Accession number AF049870). In each  
10 case, the Cdc25 structural gene is placed upstream of the transcription termination signals of the *Agrobacterium tumefaciens* nopaline synthase (NOS) gene. *A. thaliana* and *Brassica napus* plants are transformed as described by Bechtold *et al.*, 1993.

Transformed plants exhibit cytokinin-like effects at the level of the shoot (and flower)  
15 meristem, resulting in reduced apical dominance.

### EXAMPLE 8

#### **Expression of Cdc25 under the control of the *cab-6* or *ubi7* promoters reduces leaf necrosis and chlorosis in lettuce plants**

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The fission yeast Cdc25 coding sequence is placed operably in connection with the leaf-specific *cab-6* gene promoter derived from *Pinus* (Yamamoto *et al.*, 1994) or senescence-specific *ubi7* gene promoter (Garbarino *et al.*, 1995). In each case, the Cdc25 structural gene is placed upstream of the transcription termination signals of the  
25 *Agrobacterium tumefaciens* nopaline synthase (NOS) gene. Lettuce is transformed as described by Bechtold *et al.*, 1993.

Leaf deterioration (chlorosis and necrosis) in lettuce, for example as a consequence of post-harvest storage, is delayed in transformed lettuce plants compared to non-  
30 transformed control plants.

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**EXAMPLE 9****Cdc25 as a selectable marker gene in transformed plants**

In the present example of the application of the present invention, pith tissue excised  
5 from tobacco plants according to the method described by Zhang *et al* (1996) is  
treated with *Agrobacterium tumefaciens* containing the binary vector pBIN19  
comprising within the T-DNA borders the following genetic constructs:

- (1) the *gfp* gene placed operably under control of the CaMV 35S promoter  
and upstream of the NOS terminator;
- 10 (2) the fission yeast *Cdc25* gene placed operably under the control of a  
modified plant promoter that contains rat glucocorticoid response elements  
(GREs), which are responsive to rat glucocorticoid receptor protein (GR) in the  
presence of dexamethasone (Schena *et al.*, 1991); and
- 15 (3) a NOS promoter-GR gene construct that expresses GR constitutively in  
plant cells.

The green fluorescent protein is targeted to the endoplasmic reticulum by virtue of the  
inclusion of an appropriate signal peptide sequence being encoded in the genetic  
construct.

20 The binary vector is introduced into plant cells, essentially according to Bechtold *et al.*  
(1993).

When the transformed plant tissue is incubated on agar medium that contains auxin  
and 1  $\mu$ M dexamethasone, but lacking cytokinin, only cells containing the binary vector  
25 in a format such that the expression of *Cdc25* occurs therein are capable of  
proliferating and form transgenic callus. Untransformed cells fail to proliferate and  
eventually are overgrown and die. None of the surviving cells fail to express green  
fluorescent protein, indicating that the frequency of escapes using the inventive  
method is very low to negligible.

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Transformed calli are initially on agar containing medium with dexamethasone and auxin but no cytokinin, to obtain selection for transgenic cells, which alone are able to proliferate. The calli resulting from sustained rapid proliferation of these cells are then transferred to medium without dexamethasone, but with both auxin and cytokinin to  
5 allow shoot formation. Finally, to allow root formation, shooting calli are transferred to medium without auxin, or alternatively or in addition, with the root-promoting hormone indole butyric acid.

In broadly applying the inventive method, the *gfp* reporter gene construct is substituted  
10 with a gene-of-interest that encodes a desired characteristic, such as a desired agronomic trait, for which positive selection for successful transfer is sought.

An advantage of the inventive method is that, whilst cytokinin expressed from the *ipt* gene or exogenously supplied from the medium allows the proliferation of neighbouring  
15 non-transgenic cells, the ectopic expression of Cdc25 in transformed cells is highly localised and, as a consequence, highly-specific. Accordingly, the present invention overcomes the need to carry out extensive genetic crossing to eliminate progeny that do not contain the desired gene, in order to establish lines able to transmit the new character to future generations according to standard Mendelian inheritance.

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Moreover, in the present inventive method, the plantlet relies entirely on normal endogenous hormone production during the later regeneration stages and develops into a normal plant. The present method generates plants no longer expressing Cdc25, completely normal in growth, and having gained the new beneficial trait conferred by  
25 gene transfer. In contrast, when cytokinin is expressed from the *ipt* gene or exogenously supplied from the medium, the continuing raised cytokinin synthesis alters development and growth of plantlets.

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**WE CLAIM:**

1. A method of modifying one or more plant morphological and/or biochemical and/or physiological characteristics comprising expressing in one or more particular cells, tissues or organs of a plant, a genetic sequence encoding a cell cycle control protein operably under the control of a regulatable promoter sequence.
2. The method according to claim 1, wherein the cell cycle control protein is a Cdc25 protein or a homologue, analogue or derivative thereof.
3. The method according to claim 2, wherein the Cdc25 protein is derived from fission yeast.
4. The method according to claim 2 wherein the Cdc25 protein is derived from a plant cell, tissue, organ or whole organism.
5. The method according to claim 4, wherein the Cdc25 protein is derived from a tobacco cell, tissue, organ or whole organism.
6. The method according to claim 1, wherein the cell cycle control protein is a Cdc25 substrate or a modified Cdc25 substrate.
7. The method according to claim 6, wherein the modified Cdc25 substrate is CDK(A<sub>14</sub>F<sub>15</sub>).
8. The method according to claim 1, wherein the regulatable promoter is a dexamethasone-inducible promoter sequence.
9. The method according to claim 8, wherein the dexamethasone-inducible promoter sequence comprises one or more modified glucocorticoid response elements (GREs).

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10. A method of increasing the strength and/or thickness and/or stability and/or wind-resistance of a plant comprising expressing the yeast Cdc25 protein or a homologue, analogue or derivative thereof, or a modified substrate of Cdc25 that mimics the effect of Cdc25 operably under the control of a stem-expressible promoter sequence.
11. The method according to claim 10, wherein the stem-expressible promoter sequence is derived from the *rbcs-1A* gene, the *rbcs-3A* gene, the *AtPRP4* gene, the *T. bacilliform* virus gene, or the sucrose-binding protein gene or comprises a stem-specific or stem-expressible homologue, analogue or derivative thereof.
12. A method of increasing tuber formation and/or development in a tuberous crop plant comprising expressing the yeast Cdc25 protein or a homologue, analogue or derivative thereof, or a modified substrate of Cdc25 that mimics the effect of Cdc25 operably under the control of one or more tuber-specific promoter sequences.
13. The method according to claim 12, wherein the tuberous crop plant is potato.
14. The method according to claim 11 wherein one or more of the tuber-specific promoters is a potato patatin gene promoter sequence.
15. The method according to claim 14, wherein the patatin gene promoter sequence is derived from a class I patatin gene and/or a class II patatin gene.
16. The method according to claim 15, wherein the class I patatin gene promoter is modified to remove sucrose-responsive elements therefrom such as the proximal region of the A repeat.
17. A method of modifying the lignin content of a woody crop plant comprising expressing the yeast Cdc25 protein or a homologue, analogue or derivative thereof,

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or a modified substrate of Cdc25 that mimics the effect of Cdc25 operably under the control of a cambium-specific or vascular-tissue-specific promoter sequence.

18. The method according to claim 17, wherein the promoter sequence is a cinnamoyl alcohol dehydrogenase (CAD) gene promoter, laccase gene promoter, cellulose synthase gene promoter or xyloglucan endotransglucosylase (XET) gene promoter.

19. The method according to claim 17 wherein the promoter is the auxin-inducible SAUR promoter sequence.

20. The method according to claim 17 wherein the promoter is the *rolB* promoter sequence.

21. The method according to claim 17, wherein the woody crop plant is *Eucalyptus* spp., *Populus* spp., *Quercus* spp., *Acer* spp., *Juglans* spp., *Fagus* spp., *Acacia* spp., or teak.

22. A method of increasing seed set and/or seed production and/or grain yield in a plant comprising expressing the yeast Cdc25 protein or a homologue, analogue or derivative thereof, or a modified substrate of Cdc25 that mimics the effect of Cdc25 operably under the control of a seed-specific promoter sequence.

23. The method according to claim 22, wherein the seed-specific promoter the barley *Amy32b* gene promoter, Cathepsin  $\beta$ -like gene promoter, wheat ADP-glucose pyrophosphorylase gene promoter, maize zein gene promoter, rice glutelin gene promoter, legumin gene promoter, *napA* gene promoter, Brazil Nut albumin gene promoter, pea vicilin gene promoter or sunflower oleosin gene promoter.

24. The method according to claims 22 or 23, wherein the seed-specific promoter

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sequence is operable in the endosperm of the seed.

25. The method according to claim 24 wherein the promoter is the barley *ltr1* promoter sequence.

26. The method according to claim 24 wherein the promoter is a synthetic promoter comprising the endosperm box derived from the barley *Hor2* gene.

27. A method of inhibiting or reducing apical dominance or increasing the bushiness of a plant, comprising expressing the yeast Cdc25 protein or a homologue, analogue or derivative thereof, or a modified substrate of Cdc25 that mimics the effect of Cdc25 operably under the control of a meristem-specific promoter sequence or a stem-specific promoter sequence.

28. The method according to claim 27 wherein the promoter is the *LEAFY* promoter sequence.

29. The method according to claim 27 wherein the promoter is the *knat1* promoter sequence.

30. The method according to claim 27 wherein the promoter is the *kn1* promoter sequence.

31. The method according to claim 27 wherein the promoter is the *CLAVATA1* promoter sequence.

32. A method of increasing lateral root production in a plant comprising expressing the yeast Cdc25 protein or a homologue, analogue or derivative thereof, or a modified substrate of Cdc25 that mimics the effect of Cdc25 operably under the control of a root-specific promoter sequence.

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33. The method according to claim 32, wherein the root-specific promoter sequence is the tobacco auxin-inducible gene promoter.
34. A method of increasing the nitrogen-fixing capability of a plant comprising expressing the yeast Cdc25 protein or a homologue, analogue or derivative thereof, or a modified substrate of Cdc25 that mimics the effect of Cdc25 operably under the control of a nodule-specific promoter sequence.
35. The method according to claim 34, wherein the nodule-specific promoter sequence is selected from the list comprising *nif* gene promoter, *nifH* gene promoter, ENOD promoter, PEPC promoter, leghaemoglobin gene promoter and hemoglobin gene promoter.
36. A method of preventing or delaying or otherwise reducing leaf chlorosis and/or leaf necrosis in a plant comprising expressing the yeast Cdc25 protein or a homologue, analogue or derivative thereof, or a modified substrate of Cdc25 that mimics the effect of Cdc25 operably under the control of a leaf-specific promoter sequence.
37. The method according to claim 36, wherein the promoter is the SAM22 promoter, *rbcS-1A* or the *rbcS-3A* gene promoter.
38. The method according to claim 36 wherein the promoter is the *cab-6* promoter sequence.
39. The method according to claim 36 wherein the promoter is the *ubi7* promoter sequence.
40. A method of modifying one or more plant morphological and/or biochemical and/or physiological characteristics comprising expressing in one or more particular cells, tissues or organs of a plant, a cell cycle control protein operably under the control

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of a regulatable cell-specific promoter sequence.

41. A method of modifying one or more plant morphological and/or biochemical and/or physiological characteristics comprising expressing in one or more particular cells, tissues or organs of a plant, a cell cycle control protein operably under the control of a regulatable tissue-specific promoter sequence.

42. The method according to claim 41, wherein the promoter is a phloem-specific promoter, cell-wall-specific promoter, root cortex-specific promoter, root vasculature-specific promoter, tapetum-specific promoter, or meristem-specific promoter.

43. A method of modifying one or more plant morphological and/or biochemical and/or physiological characteristics comprising expressing in one or more particular cells, tissues or organs of a plant, a cell cycle control protein operably under the control of a regulatable organ-specific promoter sequence.

44. The method according to claim 43, wherein the promoter is an aleurone-specific promoter, flower-specific promoter, fruit-specific promoter, leaf-specific promoter, nodule-specific promoter, pollen-specific promoter, anther-specific promoter, root-specific promoter, seed-specific promoter, endosperm-specific promoter, embryo-specific promoter, or stigma-specific promoter.

45. A genetic construct comprising a nucleotide sequence encoding a cell cycle control protein placed operably in connection with a regulatable plant-expressible promoter sequence.

46. The genetic construct according to claim 45, wherein the nucleotide sequence encoding a cell cycle control protein encodes Cdc25 or a modified Cdc25 substrate or a homologue, analogue or derivative thereof.

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47. The genetic construct according to claim 46, wherein the regulatable plant-expressible promoter is a dexamethasone-inducible promoter.
48. The genetic construct according to claim 46 wherein the regulatable plant-expressible promoter is a patatin promoter sequence.
49. The genetic construct according to claim 48 comprising one or more patatin gene promoter sequences derived from a class I patatin gene and/or a class II patatin gene.
50. The genetic construct according to claim 49 wherein the class I patatin gene promoter is modified to remove sucrose-responsive elements therefrom such as the proximal region of the A repeat.
51. The genetic construct according to claim 46 wherein the regulatable plant-expressible promoter is the auxin-inducible SAUR promoter sequence.
52. The genetic construct according to claim 46 wherein the regulatable plant-expressible promoter is the *ro/B* promoter sequence.
53. The genetic construct according to claim 46 wherein the regulatable plant-expressible promoter is the barley *ltr1* promoter sequence.
54. The genetic construct according to claim 46 wherein the regulatable plant-expressible promoter is a synthetic promoter comprising one or more endosperm box motifs derived from the barley *Hor2* gene.
55. The genetic construct according to claim 46 wherein the regulatable plant-expressible promoter is the *LEAFY* promoter sequence.

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56. The genetic construct according to claim 46 wherein the regulatable plant-expressible promoter is the *knat1* promoter sequence.

57. The genetic construct according to claim 46 wherein the regulatable plant-expressible promoter is the *kn1* promoter sequence.

58. The genetic construct according to claim 46 wherein the regulatable plant-expressible promoter is the *CLAVATA1* promoter sequence.

59. The genetic construct according to claim 46 wherein the regulatable plant-expressible promoter is the *cab-6* promoter sequence.

60. The genetic construct according to claim 46 wherein the regulatable plant-expressible promoter is the *ubi7* promoter sequence.

61. A transformed plant produced by the method comprising:

- (i) introducing into a plant cell a genetic construct or vector comprising a nucleotide sequence that encodes a cell cycle control protein operably in connection with a regulatable promoter sequence selected from the list comprising cell-specific promoter sequences, tissue-specific promoter sequences, and organ-specific promoter sequences;
- (ii) regenerating a whole plant from said plant cell; and
- (iii) expressing said cell cycle control protein in one or more particular cells, tissues or organs of the plant.

62. The plant according to claim 61, wherein the cell cycle control protein is Cdc25 or a modified Cdc25 substrate or a homologue, analogue or derivative thereof.

63. A plant cell, tissue, organ, plant part, propagule or progeny plant of the primary transformed plant according to claim 61, wherein said plant cell, tissue, organ, plant

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part, propagule or progeny plant comprises the introduced nucleotide sequence that encodes a cell cycle control protein operably in connection with a regulatable promoter sequence.

64. The plant according to claim 61, exhibiting one or more modified morphological and/or biochemical and/or physiological characteristics compared to otherwise isogenic non-transformed plants.

65. The plant according to claim 64, wherein the modified morphological and/or physiological characteristics comprises increased strength and/or thickness and/or stability and/or wind-resistance of a plant stem.

66. The plant according to claim 64, wherein the modified morphological and/or physiological characteristics comprises increased tuber formation and/or development.

67. The plant according to claim 64, wherein the modified morphological and/or biochemical and/or physiological characteristics comprises increased lignin content.

68. The plant according to claim 64, wherein the modified morphological and/or biochemical and/or physiological characteristics comprises increased seed set and/or seed production and/or grain yield.

69. The plant according to claim 64, wherein the modified morphological characteristic comprises increased endosperm size.

70. The plant according to claim 64, wherein the modified morphological and/or physiological characteristics comprises reduced apical dominance or increased bushiness.

71. The plant according to claim 64, wherein the modified morphological and/or

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physiological characteristic comprises increased numbers of lateral roots or increased rate of lateral root production.

72. The plant according to claim 64, wherein the modified morphological and/or biochemical and/or physiological characteristic comprises increased nitrogen-fixing capability and/or increased nodule size.

73. The plant according to claim 64, wherein the modified morphological and/or physiological characteristic comprises reduced leaf chlorosis and/or leaf necrosis.

74. A method of detecting or identifying transformed or transfected plant cells, tissues or organs that are hormone-dependent, comprising expressing a nucleotide sequence encoding the yeast Cdc25 protein or a homologue, analogue or derivative thereof, or a modified substrate of Cdc25 that mimics the effect of Cdc25 in said plant cell, tissue or organ operably under the control of a regulatable plant-expressible promoter, for a time and under conditions sufficient for hormone-mediated cell division and/or hormone-mediated tissue differentiation to occur in the absence of added hormone.

75. The method according to claim 74 wherein the hormone is cytokinin, and the hormone-mediated cell division is cytokinin-mediated cell division, and the hormone-mediated tissue differentiation is cytokinin-mediated tissue differentiation.

76. The method according to claim 74 wherein the hormone is gibberellin, and the hormone-mediated cell division is gibberellin-mediated cell division, and the hormone-mediated tissue differentiation is gibberellin-mediated tissue differentiation.

77. The method according to claim 74, wherein the plant-expressible promoter is a tissue-specific inducible promoter sequence, environmentally-inducible promoter, a chemically-inducible promoter, a wound-inducible promoter, a pathogen-inducible

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promoter, or a hormone-inducible promoter.

78. The method according to claim 74, wherein cells are cultured in the presence of one or more auxins.

79. The method according to claim 74, wherein the chemically-inducible promoter is a dexamethasone-inducible promoter sequence.

80. The method according to claim 74, wherein the regulatable promoter is a constitutive plant-expressible promoter sequence and wherein a genetic sequence comprising:

- (i) the regulatable promoter; and
- (ii) the nucleotide sequence encoding yeast Cdc25 protein or a homologue, analogue or derivative thereof, or a modified substrate of Cdc25 that mimics the effect of Cdc25 operably connected to the regulatable promoter,

is flanked 5'- and 3'- by a transposable genetic element sequence.

81. A method of detecting or identifying transformed or transfected plant cells, tissues or organs that are cytokinin-dependent, comprising expressing a nucleotide sequence encoding the yeast Cdc25 protein or a homologue, analogue or derivative thereof, or a modified substrate of Cdc25 that mimics the effect of Cdc25 in said plant cell, tissue or organ operably under the control of a dexamethasone-inducible promoter in the presence of one or more auxins for a time and under conditions sufficient for cytokinin-mediated cell division and/or cytokinin-mediated tissue differentiation to occur in the absence of cytokinin.

82. A method of detecting or identifying transformed or transfected plant cells, tissues or organs that are gibberellin-dependent, comprising expressing a nucleotide sequence encoding the yeast Cdc25 protein or a homologue, analogue or derivative thereof, or a modified substrate of Cdc25 that mimics the effect of Cdc25 in said plant

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cell, tissue or organ operably under the control of a dexamethasone-inducible promoter in the presence of one or more auxins for a time and under conditions sufficient for gibberellin-mediated cell division and/or gibberellin-mediated tissue differentiation to occur in the absence of added gibberellin.

83. The method according to claim 82, wherein the plant is a monocotyledonous plant.

84. The method according to claim 82, wherein the plant belongs to the Graminae.

85. The method according to claim 84, wherein the plant tissue is shoot internode meristem tissue or intercalary meristem tissue or the cell is a cell derived from said tissue.

86. A method of increasing the extension of photosynthetic canopy in a cereal crop plant by expressing in an internode meristem of stem tissue a genetic sequence encoding a yeast Cdc25 protein or a homologue or derivative thereof or encoding a modified substrate of Cdc25 which mimics the effect of Cdc25 said genetic sequence operably under the control of a meristem-expressible promoter sequence.

87. The method according to claim 86, wherein the promoter sequence is a Proliferating Cell Nuclear Antigen (PCNA) promoter of rice.

88. The method according to claim 86 or 87 wherein the yield of grain is increased.

**Fetherstonhaugh & Co.**  
Ottawa, Canada  
Patent Agents

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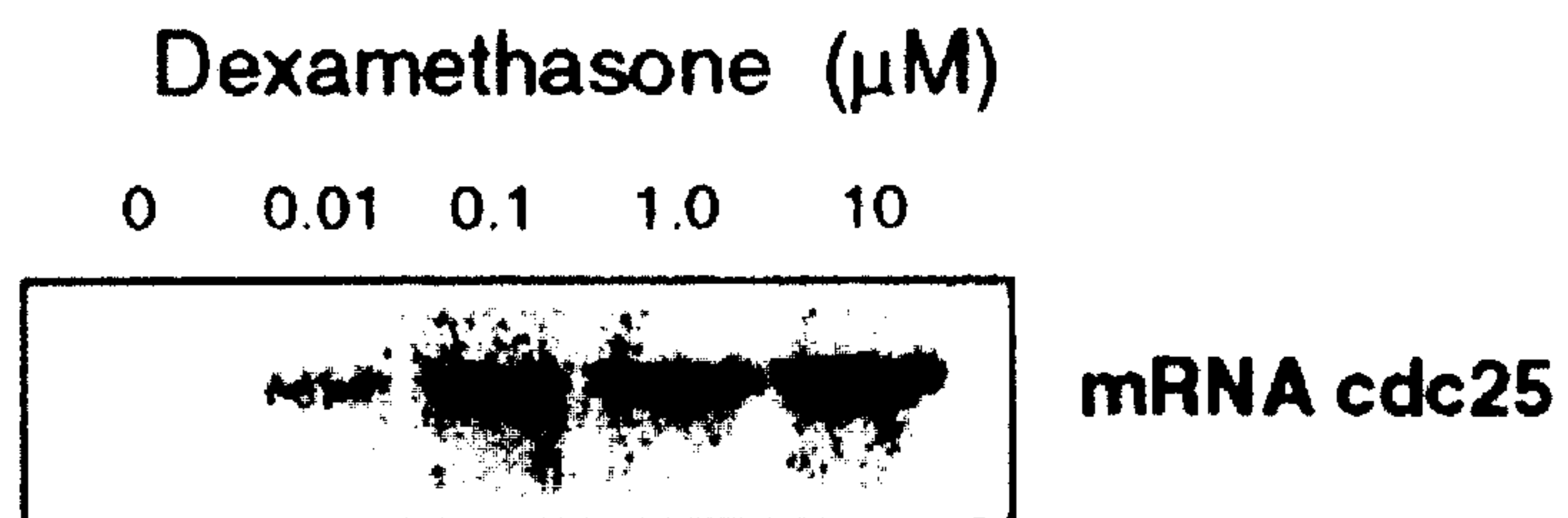


FIGURE 1-1

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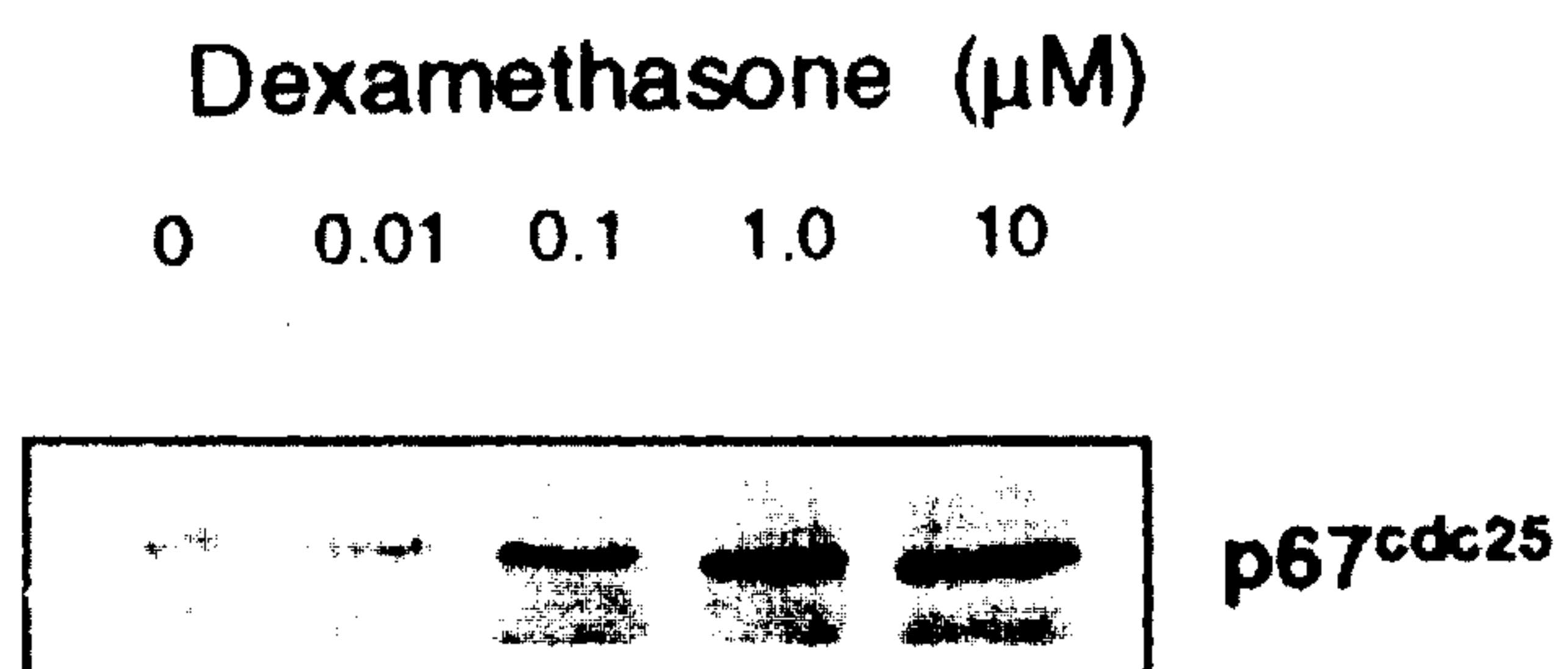


FIGURE 1-2

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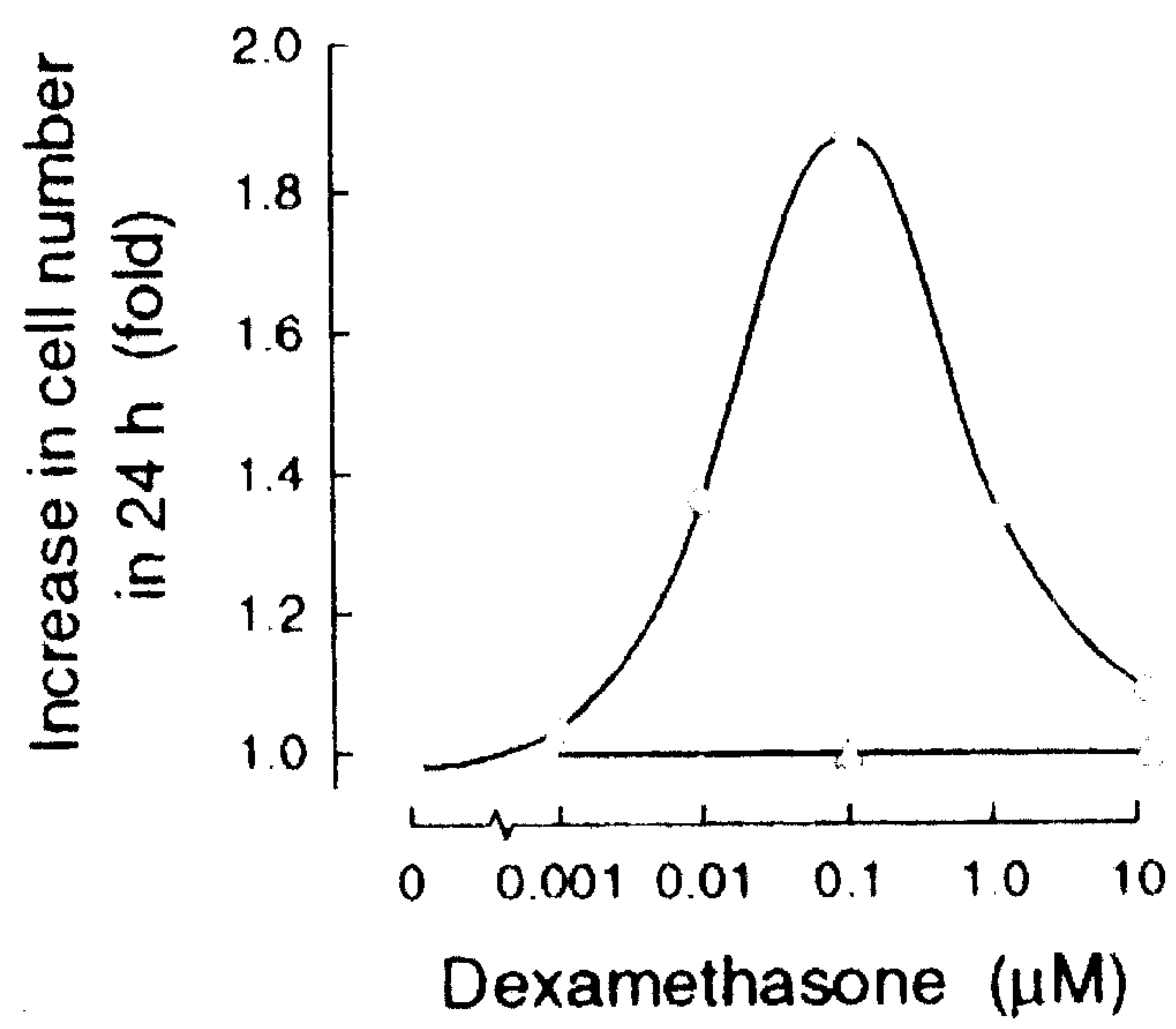


FIGURE 1-3

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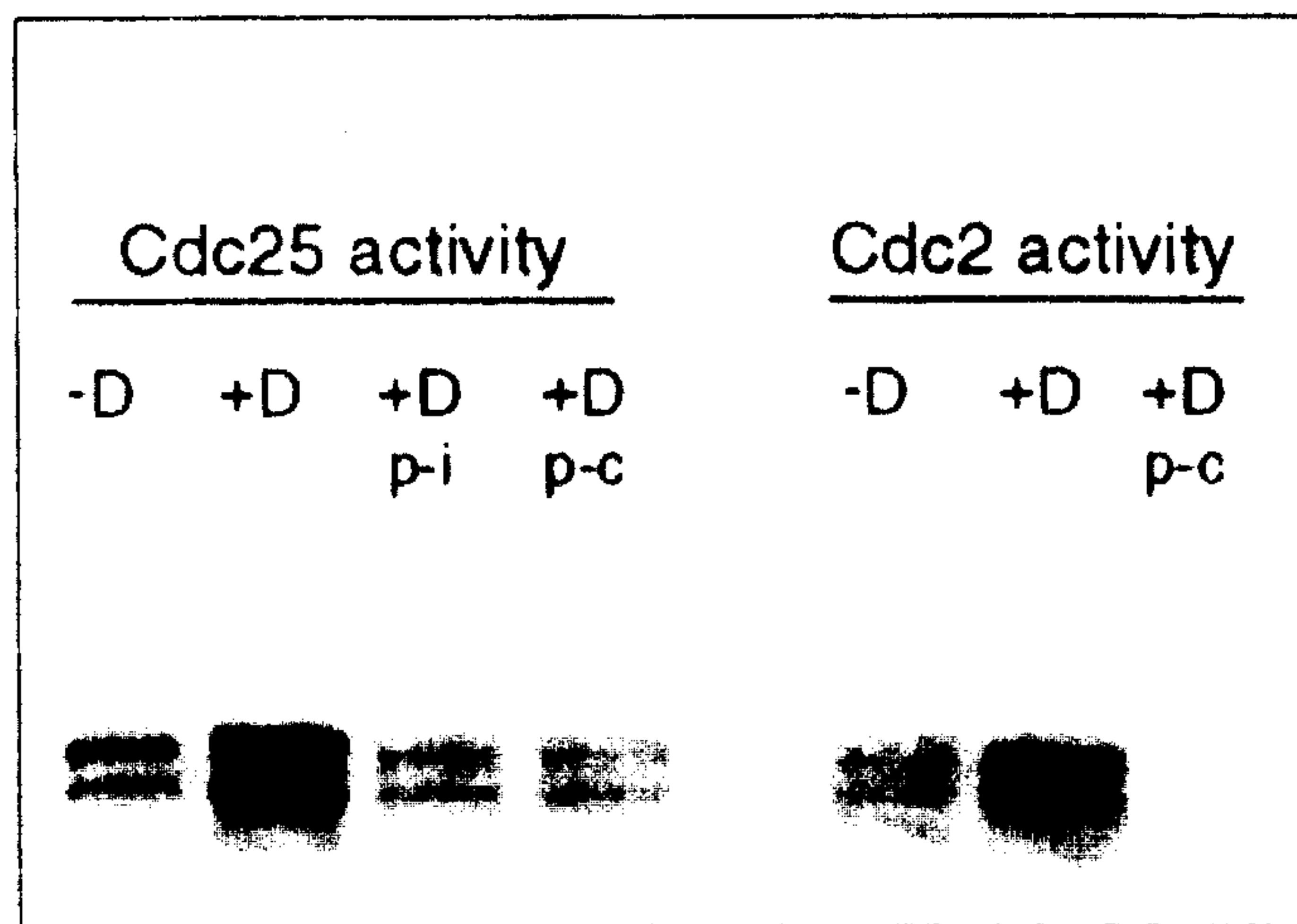


FIGURE 2-1

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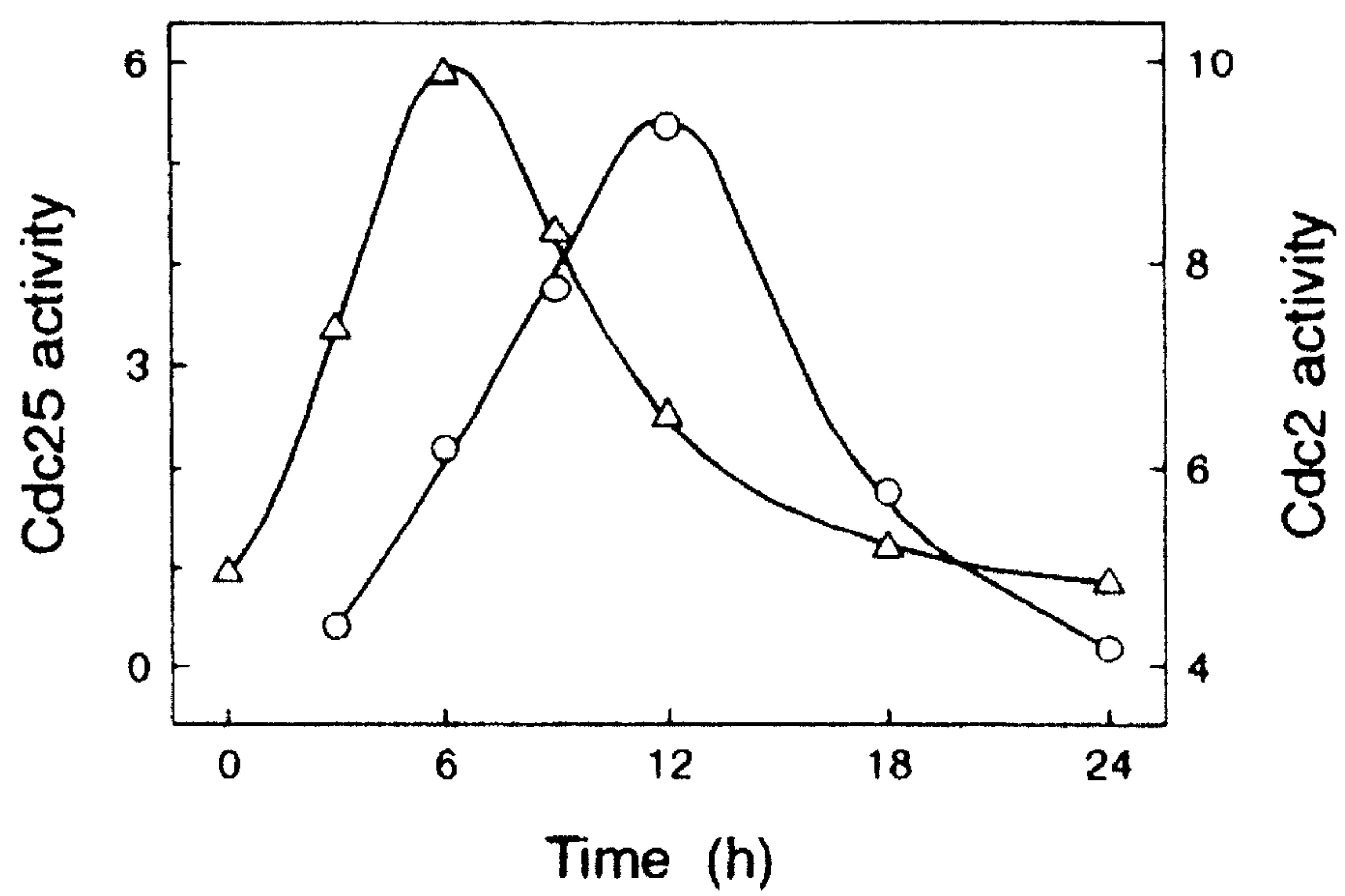


FIGURE 2-2

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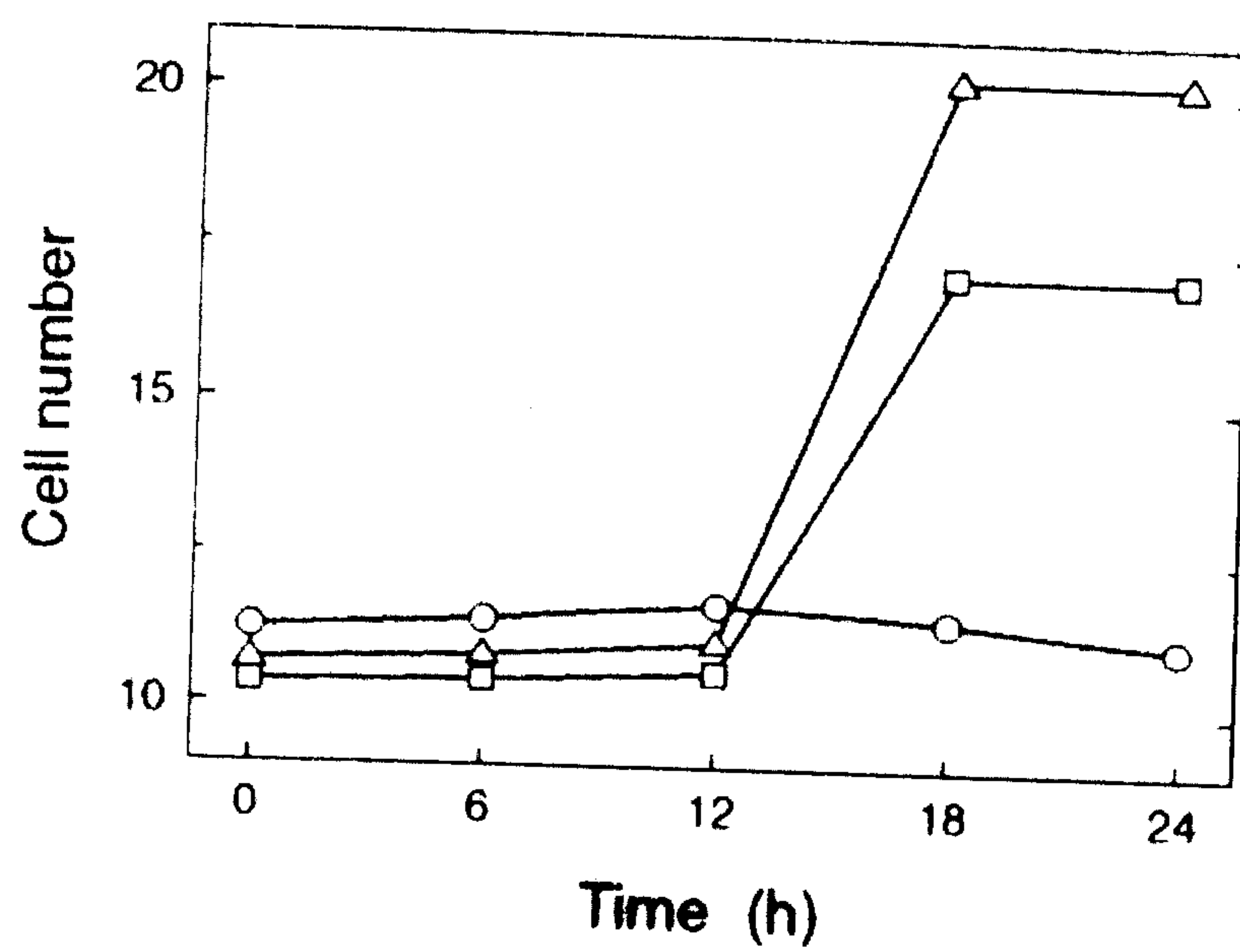


FIGURE 2-3

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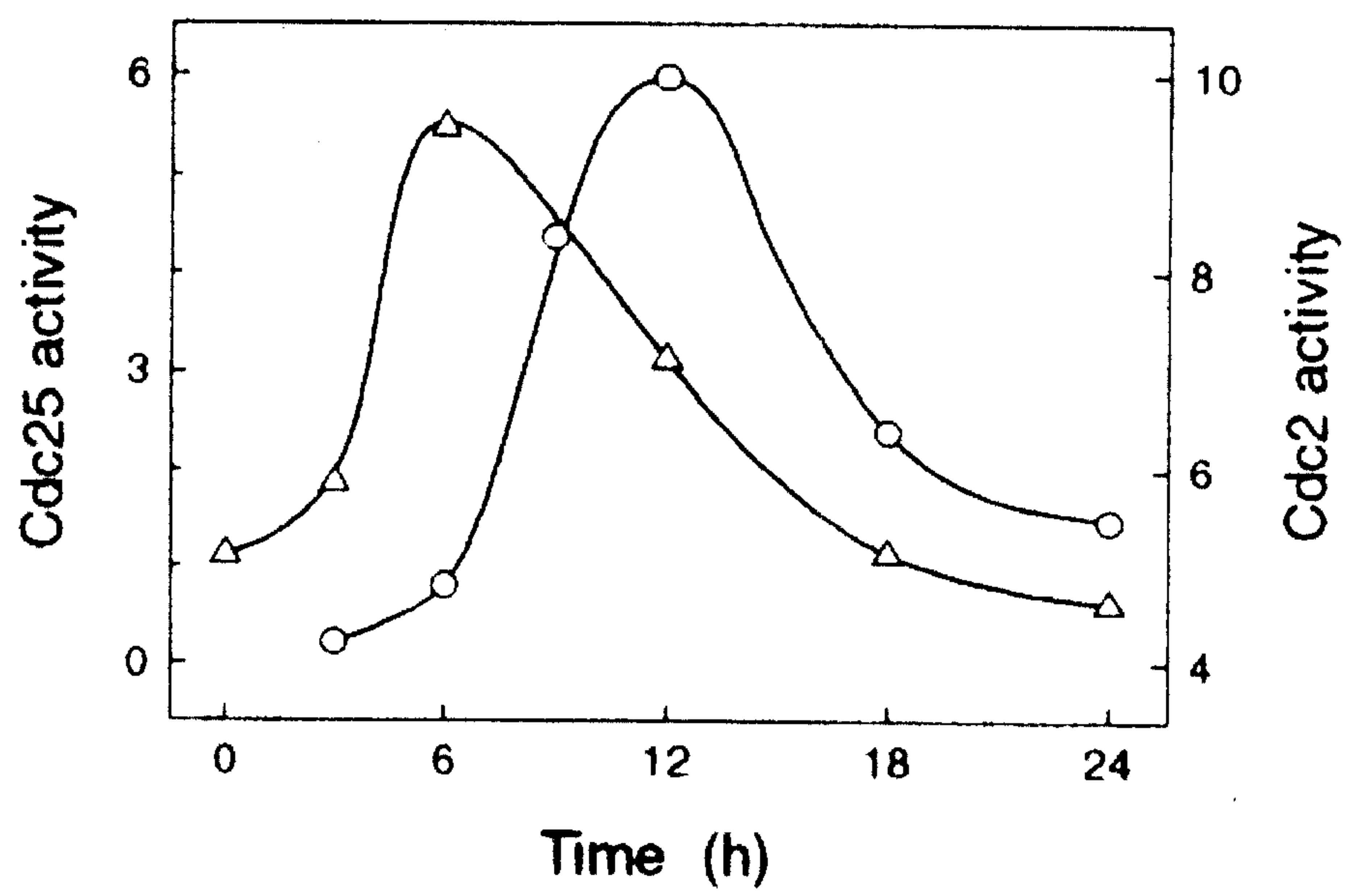


FIGURE 2-4

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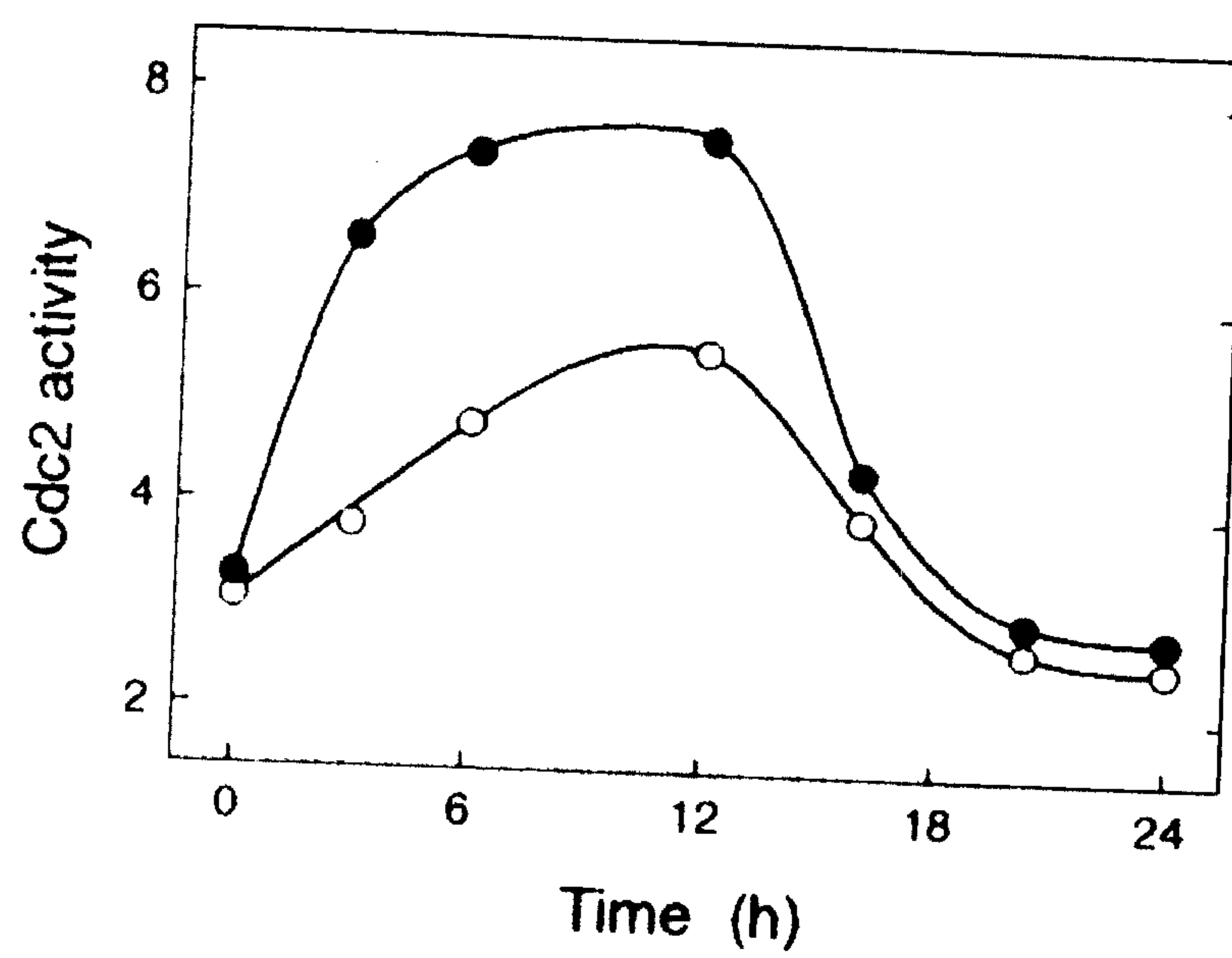


FIGURE 2-5

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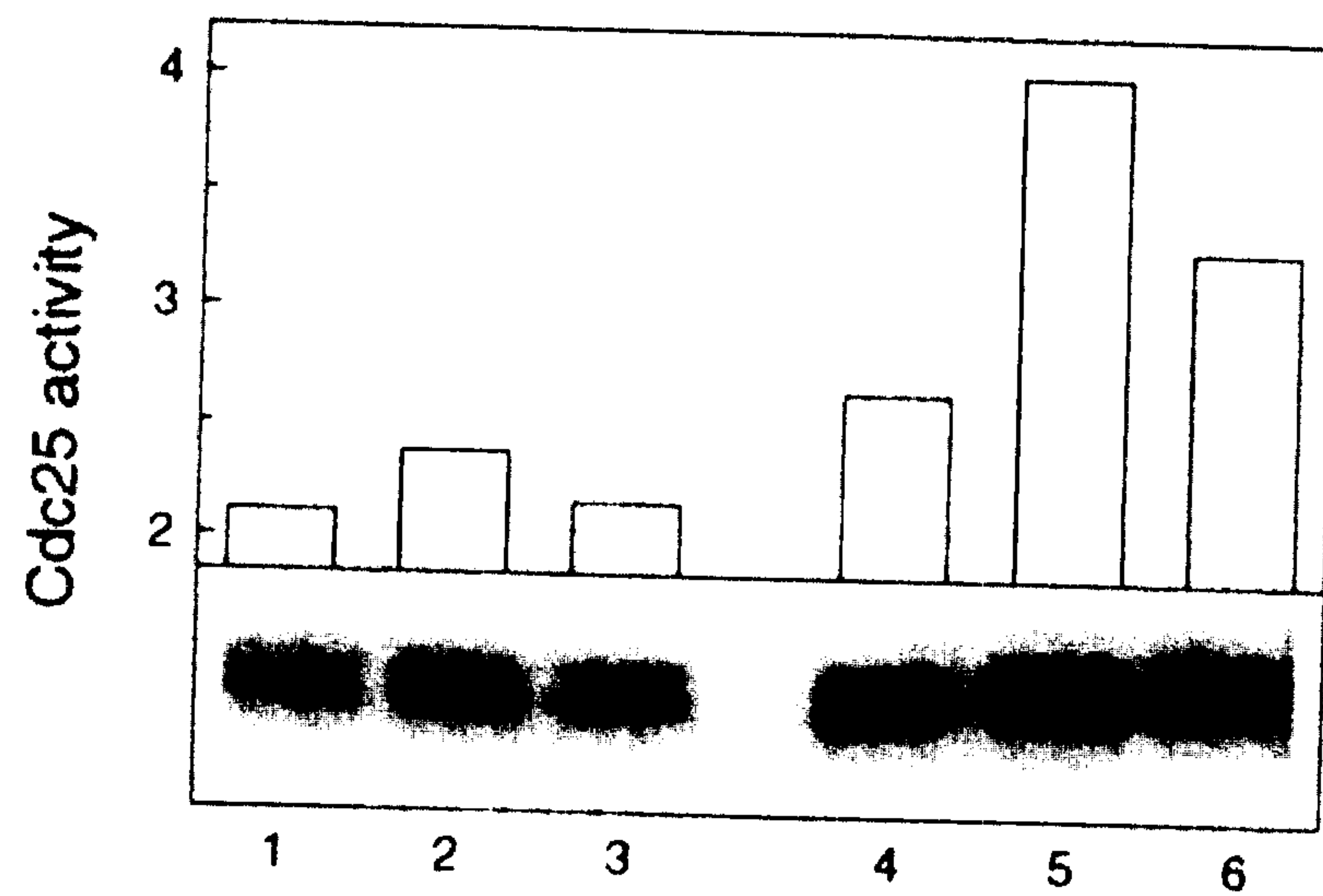


FIGURE 2-6

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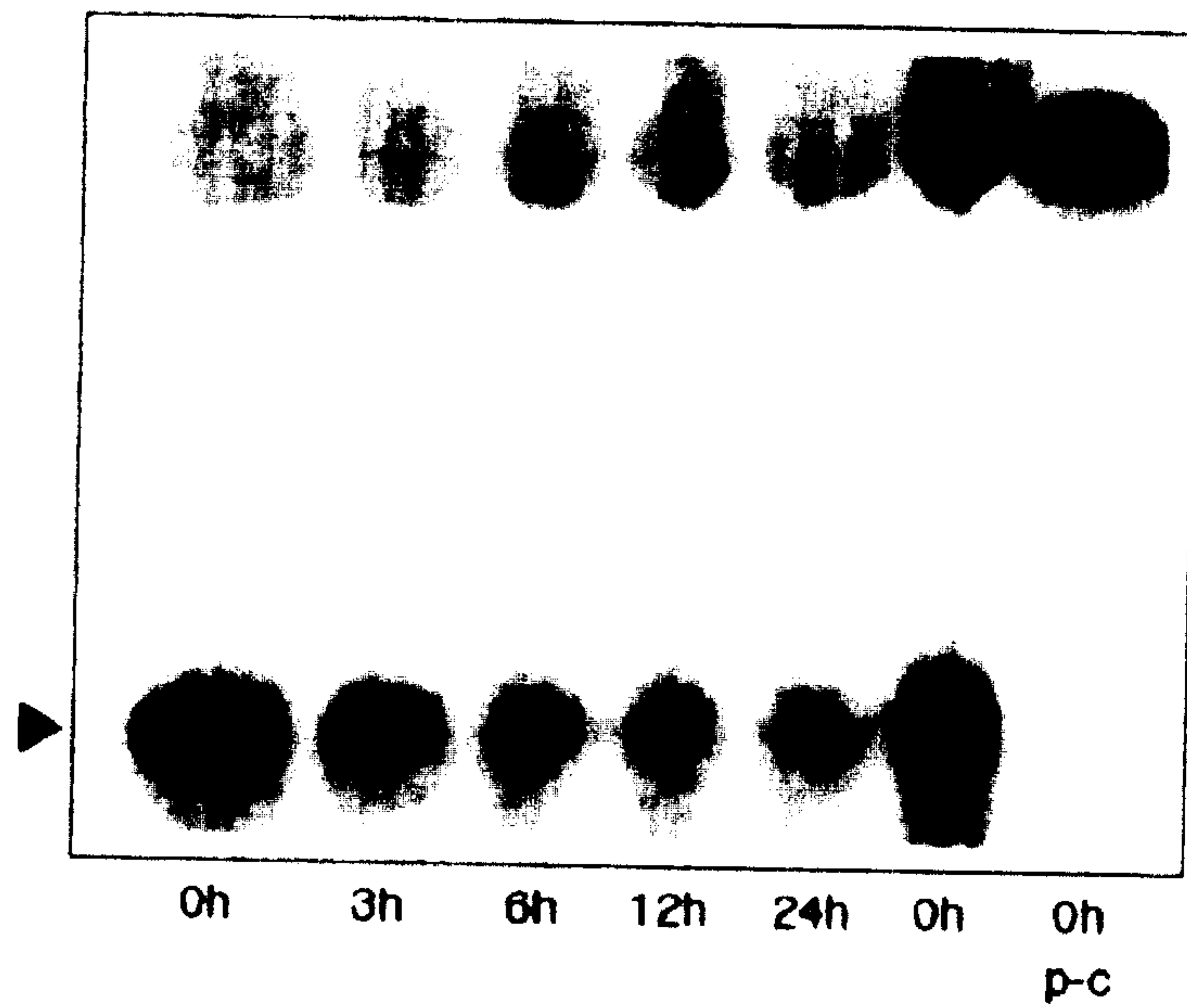


FIGURE 2-7

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42 -

31 -

18 -

1 2

FIGURE 3

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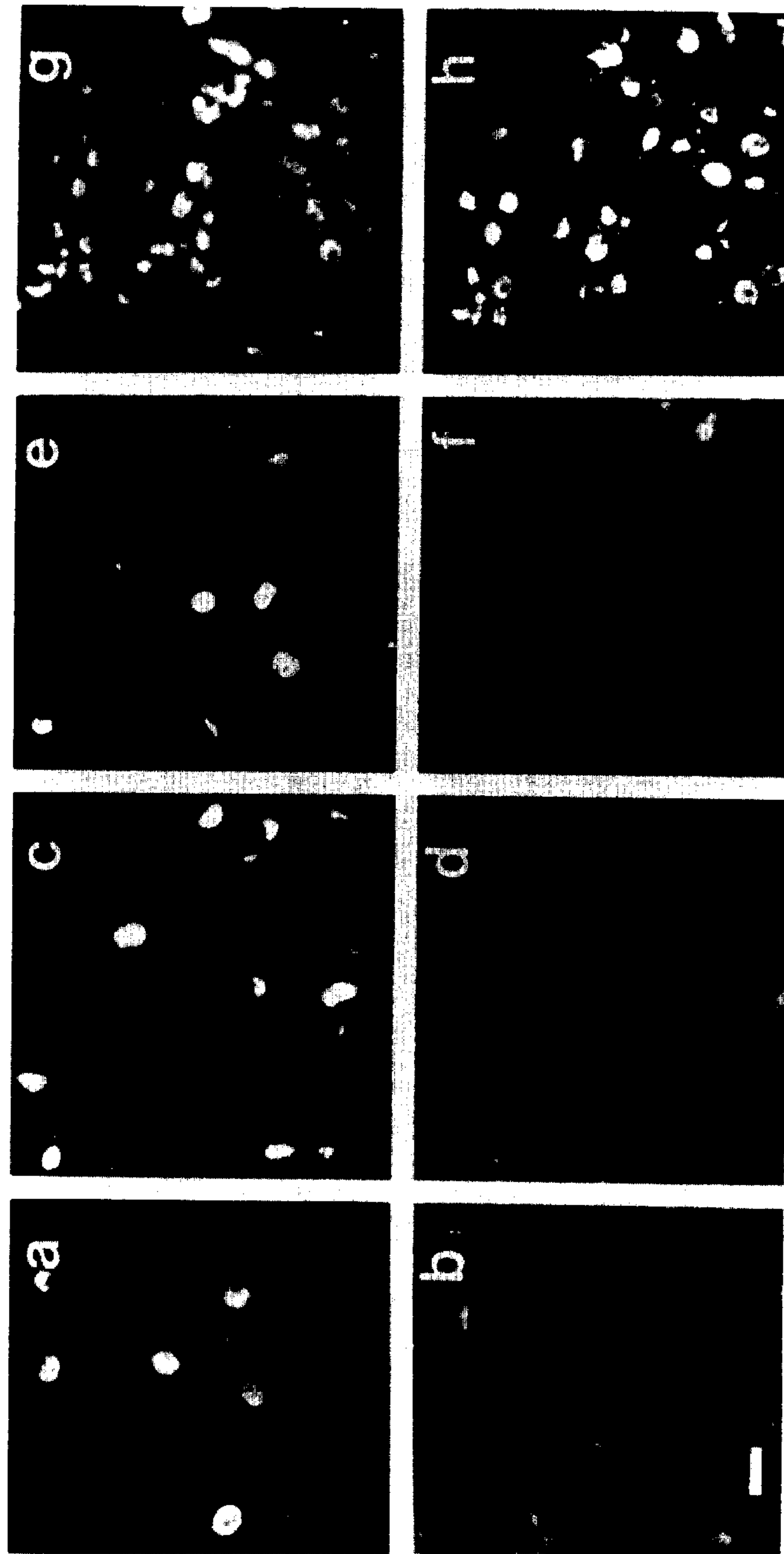


FIGURE 4

Dexamethasone ( $\mu\text{M}$ )

0      0.01    0.1      1.0      10



**mRNA cdc25**