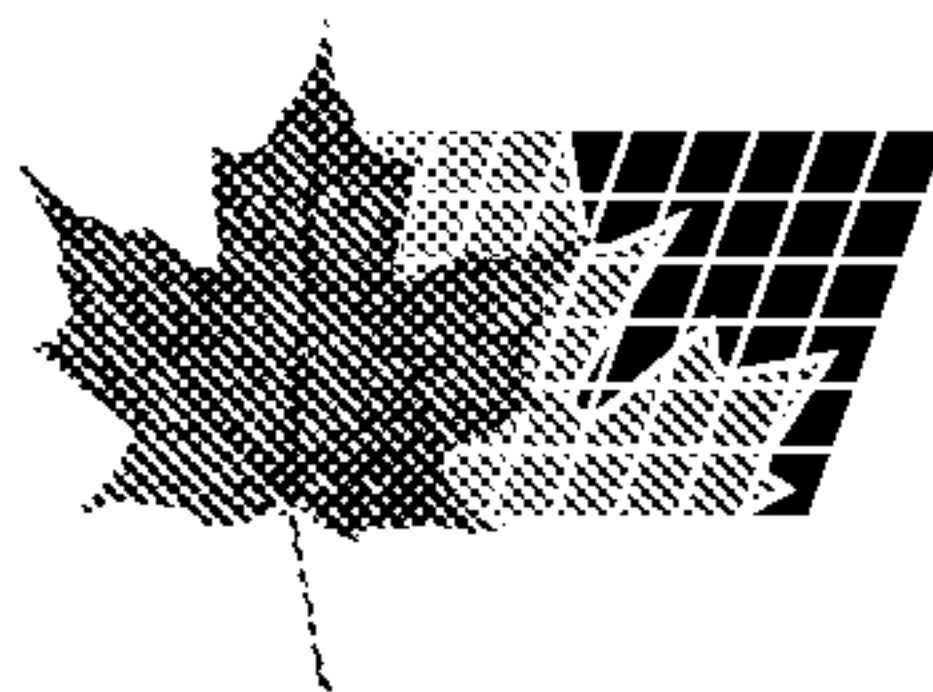




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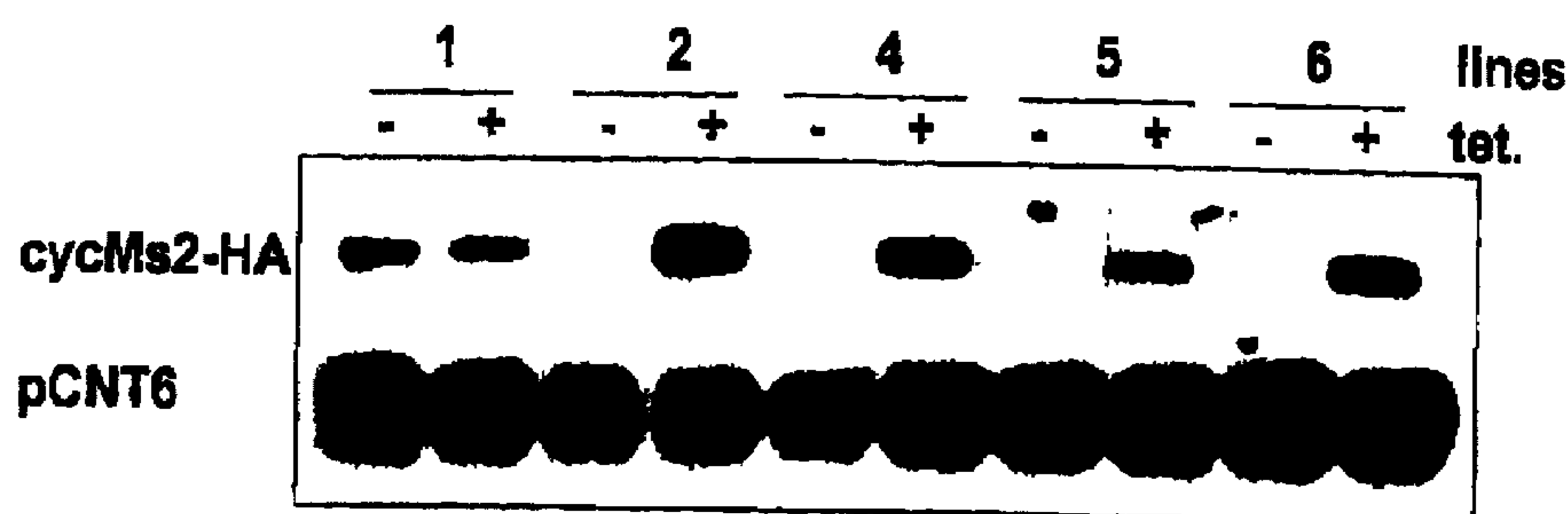
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(54) **METHODE DE MODIFICATION DU METABOLISME ET DU
DEVELOPPEMENT DES PLANTES**

(54) **METHOD OF MODIFYING PLANT METABOLISM AND
DEVELOPMENT**



(57) The present invention provides a method of modifying one or more morphological, biochemical and physiological properties or characteristics in a plant, such as one or more developmental processes and/or sink/source-related processes and/or plant cytokinin-mediated processes and/or gibberellin-mediated including but not limited to the modification of cell fate, the advancement of cell division, the initiation, promotion, stimulation or enhancement of 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 root apical dominance and/or the delay of senescence and/or the modification of bushiness in the plant, said method comprising expressing a cell cycle control protein, in particular cyclin B, 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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ABSTRACT

The present invention provides a method of modifying one or more morphological, biochemical and physiological properties or characteristics in a plant, such as one or more developmental processes and/or sink/source-related processes and/or plant cytokinin-mediated processes and/or gibberellin-mediated including but not limited to the modification of cell fate, the advancement of cell division, the initiation, promotion, stimulation or enhancement of 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 root apical dominance and/or the delay of senescence and/or the modification of bushiness in the plant, said method comprising expressing a cell cycle control protein, in particular cyclin B, 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 METABOLISM AND DEVELOPMENT

FIELD OF THE INVENTION

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 modification of cell fate, the advancement of cell division, the initiation, promotion, stimulation or enhancement of seed development and/or tuber formation and/or shoot initiation 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 root apical dominance and/or the delay of senescence and/or the modification of shoot apical dominance (i.e. bushiness) in the plant, said method comprising expressing a cell cycle control protein, in particular cyclin B, 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. 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 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 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.

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) 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* *RoIC* gene (European Patent No. EP-A-334,383). These approaches also produce undesirable side-effects in the plant and, even in cases where *ipt* or *roIC* is expressed under the control of tissue-specific promoters, these side-effects are observed in other tissues, presumably because the cytokinin is transported readily between cells and tissues of the plant.

30 SUMMARY OF THE INVENTION

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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
5 pleiotropy associated with the prior art.

Surprisingly, the inventors discovered that the targeted ectopic expression of a cell cycle control protein such as cyclin B in particular cells, tissues or organs of the plant produces localised specific modifications to cytokinin-mediated cellular metabolism and
10 cell fate compared to otherwise isogenic non-transformed plants.

More particularly, the inventors have discovered that the G2 phase of the cell cycle in plants can be shortened by the ectopic expression of the alfalfa CycMs2 protein therein. This shortening of the G2 phase results in altered cell fate and sink/source
15 relationships in plants expressing CycMs2, thereby mimicking many cytokinin-mediated and/or gibberellin-mediated developmental and biochemical processes in relation to carbon partitioning, senescence, grain filling, seed size, seed development, shoot development and root development, amongst others.

20 The shortening of the G2 phase by ectopic expression of alfalfa CycMs2 in tobacco is shown in Example 3. Whilst not being bound by any theory or mode of action, it is likely that the ectopic expression of a cyclin B such as alfalfa CycMs2, or ectopic expression of a cyclin B-type protein, in plants advances cell division by advancing entry of cells into mitosis, and that this advancement of cell division alters cellular
25 metabolism via a mechanism that involves modifications to the partitioning of carbon and/or the activities of one or more enzymes involved in carbon partitioning (eg. invertase) and/or the levels of regulatory molecules such as sucrose, ATP, ADP and inorganic orthophosphate. Because cell fate is in some way related to the cell cycle, the altered cellular metabolism which occurs is able to modify cell fate. For example,
30 roots can be regenerated from calli in culture when the cells exit the cell cycle in G2

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phase, while shoot formation is dependent upon an exit from the cell cycle in the G1 phase, such that the formation of roots versus shoots depends upon the phase (G1 or G2) at which cells exit the cell cycle. This proposed mechanism would explain the inhibition of root regeneration observed by the present inventors (see Example 4)
5 when the G2/M transition and/or the duration of the G2 phase is shortened by ectopic expression of CycMs2.

Cyclin B is an intracellular protein, which, unlike exogenously-applied cytokinins or cytokinins produced by ectopic expression of *ipt* or *ro/C* genes, will only exert a
10 localised effect at the site of protein synthesis. This observation has led the present inventors to develop methods for controlled expression of alfalfa CycMs2 in particular cells, tissues and organs of plants, for the purposes of modifying cytokinin-mediated plant morphology and/or biochemistry and/or physiology, and to facilitate the selection of specific cells, tissues and organs which exhibit cytokinin-mediated morphological
15 characteristics and/or biochemical characteristics and/or physiological characteristics.

Accordingly, one aspect of the invention provides a method of modifying cell fate and/or plant development and/or plant morphology and/or biochemistry and/or physiology comprising expressing in particular cells, tissues or organs of a plant, a
20 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 sequences, and organ-specific promoter sequences.

25 In a particularly preferred embodiment of the invention, the cell cycle control protein is the alfalfa CycMs2 protein or a biologically-active homologue, analogue or derivative thereof. The present invention clearly contemplates the use of functional homologues of the alfalfa CycMs2 protein. Accordingly, the present invention is not limited in application to the use of nucleotide sequences encoding the alfalfa CycMs2 protein.

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The present invention clearly extends to the use of substrates of cyclin B that produce the same effects in respect of the present invention as can be produced using cyclin B.

5 Accordingly, in one preferred embodiment of the present invention, the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B is expressed operably under the control of a regulatable promoter that is expressible in a plant cell, to shorten the duration of the G2 phase and/or to shorten the G2/M phase transition of said cell.

10

In another preferred embodiment of the present invention, the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B is expressed operably under the control of a regulatable promoter that is expressible in a plant cell, tissue or organ to modify cell fate and/or
15 plant development.

Other preferred embodiments of the invention relate to the effect(s) of cytokinins and/or gibberellins on plant metabolism. With respect to hormone-mediated effects, the present invention clearly contemplates the broad application of the inventive
20 method to the modification of a range of cellular processes mediated by cytokinins and/or gibberellins, including but not limited to cellular development and/or cell fate; the advancement of cell division; the initiation, promotion, stimulation or enhancement of 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
25 relationships, and/or the modification of root growth and/or the inhibition of root apical dominance and/or the delay of senescence.

Accordingly, in another preferred embodiment of the present invention, the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a modified
30 substrate of cyclin B that mimics the effect of cyclin B is expressed operably under the

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control of a regulatable promoter that is expressible in a plant cell, tissue or organ to modify carbon partitioning between cells.

In another preferred embodiment of the present invention, the alfalfa CycMs2 protein
5 or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B is expressed operably under the control of a regulatable promoter that is expressible in a plant cell, tissue or organ to modify sink/source relationships in the plant.

10 In another preferred embodiment of the present invention, the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B 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
15 operable in the endosperm of the seed or in the storage cotyledon, in which cases the combination of the cell cycle-control protein and endosperm-expressible or cotyledon-expressible promoter provides the additional advantage of increasing the grain size and grain yield of the plant.

20 In still another preferred embodiment of the present invention, the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B 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 and/or leaf senescence.

25

In yet another preferred embodiment of the present invention, the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B is expressed operably under the control of a promoter derived from a meristem-expressible gene or a shoot-expressible gene or
30 root-expressible gene, to reduce bushiness of the plant. In a related embodiment, such

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promoter/cyclin B combinations are used to reduce root apical dominance in plants.

In another preferred embodiment of the present invention, the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that
5 mimics the effect of cyclin B 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.

In another preferred embodiment of the present invention, the alfalfa CycMs2 protein
10 or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B 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.

15 In another embodiment of the present invention, the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B 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 from a gene that is expressed in vascular tissue and/or cambium cells, to increase
20 lignin content therein. Without being bound by any theory or mode of action, the ectopic expression of cyclin B 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 production. Within the vascular tissue, cambial cells contain the highest levels of
25 auxins and are therefore the preferential tissue for cyclin B overproduction.

In a further preferred embodiment of the present invention, the alfalfa CycMs2 protein or a homologue analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B is expressed under the control of a promoter that is
30 operative in meristem tissue of grain crops, to stimulate cell division in the intercalary

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meristem of the youngest stem internode and produce greater elongation of the stem and/or to generate a more extensive photosynthetic canopy.

A second aspect of the invention provides a genetic construct or vector comprising a
5 nucleotide sequence that encodes a cell cycle 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 promoter sequence; and
- 10 (ii) a plant-expressible constitutive promoter sequence, wherein the nucleotide sequence encoding the cell cycle control protein and the plant-expressible constitutive promoter sequence are integrated into a transposable element.

15 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.

20 A third 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
25 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.

30 This aspect of the invention extends to plant propagules and plant parts that contain

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the introduced nucleic acid molecule and have the potential to reproduce one or more of the phenotypes of the primary transformants/transfectants, either by inducing gene expression directly therein or by the application of standard breeding or recombinant technologies.

5

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1-1 is a copy of a photographic representation of a northern blot hybridisation showing the expression of alfalfa *CycMs2-HA* mRNA in the leaves of six transgenic tobacco lines from a tetracyclin-regulatable promoter sequence, either in the absence
10 (-) or presence (+) of 1 mg/dm³ Cl-tetracycline. The positions of *CycMs2-HA* mRNA and a control mRNA (pCNT6) are indicated.

Figure 1-2 is a copy of a photographic representation of a northern blot hybridisation showing the time-dependent accumulation of alfalfa *CycMs2-HA* mRNA in a
15 suspension culture initiated from line 2 (Figure 1-1) on 2,4-D-containing medium and subsequently incubated in medium containing 1 mg/dm³ Cl-tetracycline. The positions of *CycMs2-HA* mRNA and a control mRNA (*CycM*) are indicated. Numbers at the top of the figure indicate elapsed time (hours) following addition of tetracycline.

20 Figure 1-3 is a copy of a photographic representation showing the tetracycline concentration-dependent expression of *CycMs2-HA* fusion protein in cultured tobacco cells. In the top panel, a western blot of total cellular extracts was probed with anti-HA antibody. In the middle panel, a western blot of immunopurified CDK complexes were probed with anti-HA antibodies. In the lower panel, CDK complexes were purified by
25 binding to the p13^{suc1} protein. The position of the *CycMs2-HA* fusion protein is indicated in the upper and middle panels, whilst the position of total p13^{suc1} protein-binding CDK fraction is indicated in the lower panel (p13^{suc1}).

Figure 2 is a copy of a photographic representation of an immunofluorescence
30 micrograph probed with anti-HA antibody, showing the expression of the *CycMs2-HA*

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fusion protein in the nuclei of transgenic tobacco cells following induction by tetracycline. As a control, cells were stained using DAPI to identify the nuclear DNA.

Figure 3 is a copy of a photographic representation showing the histone-kinase activities of CycMs2-HA-associated CDKs (i.e. CycMs2-HA) and p13^{suc1} protein-bound CDKs (p13^{suc1}) in total cellular extracts, and in the nuclear and cytoplasmic fractions of tobacco cells expressing alfalfa CycMs2 as a fusion protein with a haemagglutinin epitope tag (HA).

- 10 **Figure 4-1** is a copy of a graphical representation showing the percentage of aphidicolin-released tobacco cells entering mitosis following tetracycline induction of *CycMs2* gene expression (+) or in the absence of tetracycline induction (-), as a function of time.
- 15 **Figure 4-2** is a copy of a graphical representation showing the percentage of microtubule structures in aphidicolin-released tobacco cells following tetracycline induction of *CycMs2* gene expression (+) or in the absence of tetracycline induction (-), as a function of time.
- 20 **Figure 4-3** is a copy of a graphical representation showing the percentage of aphidicolin-released tobacco cells entering mitosis following tetracycline induction of *CycMs2* gene expression (+) or in the absence of tetracycline induction (-), as determined by flow cytometry and expressed as a function of time.
- 25 **Figure 4-4** is a copy of a graphical representation showing the shortened duration of the G2 phase in aphidicolin-released tobacco cells following tetracycline induction of *CycMs2* gene expression (+) compared to isogenic cells incubated in media lacking tetracycline (-).
- 30 **Figure 5** is a copy of a photographic representation of a northern blot hybridisation

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showing the expression of *CycMs2* mRNA in G2 phase cells ectopically expressing the *CycMs2*-HA fusion protein, compared to the levels of histone *H4* mRNA; the endogenous tobacco *CycM* mRNA; and the control pCNT6. Cells expressing the *CycMs2*-HA fusion protein were incubated in the presence of tetracycline (+ tetracycline), whilst cells not expressing this fusion protein were incubated in the absence of tetracycline (- tetracycline).

Figure 6 is a copy of a photographic representation showing the inhibition of root regeneration from tobacco leaves of transgenic lines that ectopically express alfalfa *CycMs2* mitotic cyclin.

Figure 7 is a copy of a graphical representation of a flow cytometric analysis of the DNA contents in tetracyclin-treated (+tet) and untreated (-tet) tobacco leaves or leaf discs, in the absence of cytokinin and auxin (0/0), or treated with different cytokinin:auxin ratios (i.e. 0.5/0.1; 0.1/0.5) showing the higher percentage of cells with G2 DNA content in leaf cells derived from wild-type plants treated with high auxin:cytokinin [0.5/0.1 (root)], compared to leaf cells derived from wild-type plants treated with low auxin:cytokinin [0.1/0.5 (shoot)] or on equivalent levels of cytokinins and auxins [0.5/0.5(callus)].

Figure 8 is a copy of a schematic representation of non-limiting cell cycle model of shoot/root regeneration.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

One aspect of the present invention provides a method of modifying cell fate and/or plant development and/or plant morphology and/or biochemistry and/or physiology comprising expressing in particular cells, tissues or organs of a plant, comprising expressing in said cells, tissues or organs 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

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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.

5

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

Accordingly, by "cell fate and/or plant development and/or plant morphology and/or biochemistry and/or physiology" is meant that one or more developmental and/or morphological and/or biochemical and/or physiological characteristics of a plant is
15 altered by the performance of one or more steps pertaining to the invention described herein.

"Cell fate" refers to the cell-type or cellular characteristics of a particular cell that are produced during plant development or a cellular process therefor, in particular during
20 the cell cycle or as a consequence of a cell cycle process. Our results presented herein indicate that increased mitotic cyclin expression produces a shortened G2-phase in plant cells. It is likely that mitotic cyclin overexpression has a cytokinin-like effect, evidenced by our finding that mitotic cyclin overproduction results in the suppression of root formation.

25

The shape of a plant is the result of the polarity of auxin and cytokinin. Auxins are produced in the shoot tips where they maintain the shoot meristem. They suppress further shoot formation (shoot apical dominance) and induce root formation. Cytokinins are produced in the root tips where they maintain the root meristem. They
30 suppress further root formation and induce shoot formation. Thus, root suppression

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- by mitotic cyclin overexpression can be seen as a cytokinin effect. A further finding was that, at a critical period in root formation from leaf disks (two weeks after incubating excised leaf disks on an auxin-containing medium), dividing cell populations giving rise to roots had an extended G2 phase. Tetracycline-induced mitotic cyclin
5 overexpression abolished this extended G2 phase and suppressed root formation, suggesting a link between a long G2 phase and root formation. This is the first time that it has been shown that the experimentally controlled length of a phase of the plant cell cycle dramatically alters the developmental fate of these cells.
- 10 The fact that cyclin overexpression affects not only the length of a cell cycle phase but suppresses a specific developmental potency of certain cells such as root cells, indicates that cyclin overexpression mimics a specific effect of a plant hormone and has negative pleiotropic effects, at least in these cells.
- 15 Accordingly, a novel strategy for designing novel crops would therefore be to overexpress or to repress the expression of specific cyclins in specific cell, tissue or organ types via the use of appropriate promoters, in order to modify the pleiotropic effects linked to the specific cyclin. In the case of ectopically-expressed cyclins, the pleiotropic effect of the cyclin in question is enhanced. In the case of repressed
20 expression of cyclins, the pleiotropic effect of the cyclin in question is reversed or suppressed and consequent de-repression of a repressed cyclin, using for example an inducible promoter, induces the formation of organs/cell types/structures/functions that were previously repressed. Persons skilled in the art are aware of means for repressing the expression of genes in plants, for example using antisense, co-
25 suppression, post-transcriptional gene silencing, gene-targeting, ribozyme-mediated approaches, chemical mutagenesis, T-DNA-mediated mutagenesis, and transposon-mediated mutagenesis, amongst others. The present invention clearly extends to all such embodiments.
- 30 Moreover, induced downregulation of cyclins provides for controlled morphogenesis

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in the one or the other or both directions. Non-morphogenic cell cultures, such as those overexpressing a mitotic cyclin and a G1 cyclin, are useful for biomass production, particularly when coupled with autotrophy.

5 "Plant development" or the term "plant developmental characteristic" or similar term shall be taken to mean any cellular process of a plant that is involved in determining the developmental fate of a plant cell, in particular the specific tissue or organ type into which a progenitor cell will develop. Cellular processes relevant to plant development will be known to those skilled in the art. Such processes include, for example,
10 morphogenesis, photomorphogenesis, shoot development, root development, vegetative development, reproductive development, stem elongation, flowering, and regulatory mechanisms involved in determining cell fate, in particular a process or regulatory process involving the cell cycle. For example, in the present context, the inventors have shown that the development of roots from tobacco leaf discs in culture
15 can be inhibited by shortening the G2 phase of the cell cycle.

"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
20 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, storage cotyledon, seed coat, aleurone, fibre, cambium, wood, heartwood, parenchyma, aerenchyma, seive element,
25 phloem or vascular tissue, amongst others.

The suppression of organ formation by cyclin overexpression may be used to specifically design plant architecture, particularly in crops in which apical dominance is weak (e.g. in fruit trees or cereals). Shoot apical dominance has been described as
30 a mechanism by which the dominant organs repress growth elsewhere in the plant by

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acting as an auxin-induced sink for root-derived cytokinins.

"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
5 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,
10 that are produced by plants.

Because most biosynthetic activities of plant cells, such as *in vitro*-cultured cells, are linked to differentiation, such cell cultures may be of interest for fermentation. In a first step, high CDK activity would allow cell mass production, selective downregulation of
15 plant cyclins may derepress morphogenesis and specific biosynthetic activities (e.g. secondary plant metabolite production) may occur.

"Plant physiology" or the term "plant physiological characteristic" or similar term will be understood to refer to the functional processes of a plant, including developmental
20 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 production, secondary growth or wood production, amongst others; responses of a plant to externally-applied factors such as metals, chemicals, hormones, growth
25 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-applied factors,

30 The word "express" or variations such as "expressing" and "expression" as used herein

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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 mRNA molecule to produce a peptide, polypeptide, oligopeptide, protein or enzyme molecule. In the case of expression comprising the production of a sense mRNA
5 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 biological activity, cellular or sub-cellular localization, turnover or steady-state level of the peptide, polypeptide, oligopeptide, protein or enzyme molecule.

10

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 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
15 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 mitosis has not been initiated, and the M phase in which mitosis occurs.

20 Cell cycle 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); Francis *et al.* (1998); and Hirt *et al.* (1994).

25

Preferably, the cell cycle control protein is derived from a yeast or plant cell or animal cell, more preferably, from a plant cell, such as a monocotyledonous or dicotyledonous plant cell. In a particularly preferred embodiment, the cell cycle control protein is derived from an alfalfa plant. Exemplary cell cycle control proteins from alfalfa are
30 described by Hirt *et al* (1992) ; Meskiene *et al.* (1995); Bogre *et al.* (1997), amongst

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others.

- Preferred cell cycle control proteins according to this embodiment of the invention include cyclins A, B, C, D and E including alfalfa CycMs2 and CycMs4 (Evans *et al.* 5 1983; Swenson *et al.*, 1986; Labbe *et al.*, 1989; Murray *et al.*, 1989; Francis *et al.*, 1998; Dahl *et al.*, 1995); 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^{suc1}; (Hayles *et al.*, 1986) and nim-1 (Fantes, 1979; Russell and Nurse, 1986; 1987a; 1987b); homologues of Cdc2 such as Cdc2MsB (Hirt *et al.*, 1993); 10 CdcMs kinase (Bogre *et al.*, 1997) ; cdc2 T14Y15 phosphatases such as Cdc25 protein phosphatase or p80^{Cdc25} (Russell and Nurse, 1986; Kumagai and Dunphy, 1991; Bell *et al.*, 1993; Elledge, 1996) and Pyp3 (Elledge, 1996); cdc2 protein kinase or p34^{cdc2} (Nurse and Bisset, 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 15 kinase (Hemerly *et al.*, 1993); cdc2 T14Y15 kinases such as wee1 or p107^{wee1} (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 Kap1 (Elledge, 1996); cdc28 protein kinase or p34^{cdc28} (Reed *et al.*, 1985; Nasmyth, 1993); p40^{MO15} (Fesquet *et al.*, 1993; Poon *et al.*, 1993); chk1 20 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); MAP kinases described by Wilson *et al.* (1998), Calderini *et al.* (1998), Binarova *et al.* (1998), and Bogre *et al.* (1998).
- 25 Other cell cycle control proteins that are involved in cyclin D-mediated entry of cells 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 30 complex at one or more origins of replication, such as, but not limited to, ORC, CDC6,

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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.

Additional cell cycle control proteins are not excluded. The present invention clearly
5 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.

For the present purpose, the term "cell cycle control protein" shall further be taken to
10 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 involved in the proteolysis of one or more of the above-mentioned cell cycle control
15 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).

20 In a preferred embodiment, the cell cycle control protein of the present invention is involved in controlling or regulating the length of the G2 phase of the cell cycle and/or the transition from the G2 phase to the M phase. As will be apparent from the description contained herein, a cell cycle control protein that is capable of regulating the length of the G2 phase and/or the G2/M transition will be capable of modifying the
25 duration of the cell cycle and the time taken by a cell to exit the cell cycle and commence cell division.

Even more preferably, the cell cycle control protein of the present invention, when expressed or over-expressed in a plant cell, tissue or organ, is capable of shortening
30 the length of the G2 phase of the cell cycle and/or reducing the transition from the G2

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phase to the M phase and, as a consequence, can be used to advance cell division.

Still more preferably, the cell cycle control protein is cyclin B or a homologue, analogue or derivative thereof, including a cyclin B-like protein.

5

In a particularly preferred embodiment of the invention, the cell cycle control protein is a cyclin B comprising the alfalfa CycMs2 protein or a biologically-active homologue, analogue or derivative thereof and, in particular, a plant-derived homologue of the alfalfa CycMs2 protein. The present invention clearly contemplates the use of
10 functional homologues of the alfalfa CycMs2 protein and is not to be limited in application to the use of nucleotide sequences encoding the alfalfa CycMs2 protein.

"Homologues" of a cell cycle control protein such as cyclin B, in particular homologues of CycMs2, are those peptides, oligopeptides, polypeptides, proteins and enzymes
15 which contain amino acid substitutions, deletions and/or additions relative to the alfalfa CycMs2 polypeptide without altering one or more of its cell cycle control properties, in particular without reducing the cyclin B or cyclin B-like activity of the resulting polypeptide with respect to its ability to induce one or more cytokinin-mediated and/or gibberellin-mediated effects in a plant cell, tissue, organ or whole organism.

20

To produce such homologues of a cell cycle control protein such as cyclin B, and in particular CycMs2, amino acids present in alfalfa CycMs2 can be replaced by other amino acids having similar properties, for example hydrophobicity, hydrophilicity, hydrophobic moment, antigenicity, propensity to form or break α -helical structures or
25 β -sheet structures, and so on.

Substitutional variants are those in which at least one residue in the CycMs2 amino acid sequence has been removed and a different residue inserted in its place. Amino acid substitutions are typically of single residues, but may be clustered depending
30 upon functional constraints placed upon the polypeptide; insertions will usually be of

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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*.

- 5 Insertional amino acid sequence variants are those in which one or more amino acid residues are introduced into a predetermined site in the CycMs2 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 sequence will be smaller than amino or carboxyl terminal fusions, of the order of about
- 10 1 to 4 residues.

Deletional variants are characterised by the removal of one or more amino acids from the CycMs2 sequence.

- 15 Amino acid variants of the CycMs2 polypeptide or other cyclin B protein 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
- 20 substitutional, insertional or deletional variants are well known in the art. For example, techniques for making substitution 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.

- "Analogues" of a cell cycle control protein such as cyclin B, in particular analogues of
- 25 CycMs2, are defined as those peptides, oligopeptides, polypeptides, proteins and enzymes which are functionally equivalent to the CycMs2 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 be known to those skilled in the art.

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"Derivatives" of a cell cycle control protein such as cyclin B, in particular derivatives of CycMs2, are those peptides, oligopeptides, polypeptides, proteins and enzymes which comprise at least about five contiguous amino acid residues of a naturally-occurring Cyclin B polypeptide, in particular the alfalfa CycMs2 polypeptide, but which retain
5 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 cyclin B polypeptide. Alternatively or in addition, a derivative may comprise
10 one or more non-amino acid substituents compared to the amino acid sequence of a naturally-occurring cyclin B 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.

15 Other examples of recombinant or synthetic mutants and derivatives of cyclin B, in particular the alfalfa CycMs2 polypeptide, include those molecules 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 cyclin B are also contemplated by the present invention.
20 Additionally, homopolymers or heteropolymers comprising one or more copies of cyclin B, in particular the CycMs2 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.

25

Particularly preferred homologues, analogues and derivatives of the alfalfa CycMs2 polypeptide contemplated for use in performing the present invention are derived from plants or capable of being expressed therein.

30 To effect expression of the cell cycle control protein in a plant cell, tissue or organ,

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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.

- 5 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, dehydration, etc) or a chemical compound such as an antibiotic (tetracycline, 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-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 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.

- 25 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 activating sequences, enhancers and silencers) which alter gene expression in response to developmental and/or external stimuli, or in a tissue-specific manner.

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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 -10 box transcriptional regulatory sequences.

- 5 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.

Preferred promoters may contain additional copies of one or more specific regulatory
10 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, dexamethasone-responsive or tetracycline-responsive regulatory elements may be placed adjacent to a heterologous promoter sequence driving expression of a nucleic acid molecule to
15 confer copper inducible, glucocorticoid-inducible, dexamethasone-inducible, or tetracycline-inducible expression respectively, on said nucleic acid molecule.

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
20 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 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,
25 animal cells and the like.

In the present context, a "regulatable promoter sequence" is a promoter that is capable 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
30 expressed throughout the plant under all conditions. Accordingly, a regulatable

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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, throughout the plant under a specific set of conditions, such as following induction of gene expression by a chemical compound or other elicitor.

5

Preferably, the regulatable promoter used in the performance of the present invention confers expression in a specific location within the plant, either constitutively or following induction, however not in the whole plant under any circumstances. Included within the scope of such promoters are cell-specific promoter sequences, tissue-
10 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 a transposable genetic element (*Ac*, *Ds*, *Spm*, *En*, or other transposon).

15 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.

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

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
25 organ.

As will be apparent from the preceding description, the present invention does not 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
30 gibberellin-mediated effects therein, subject to the proviso that expression is at least

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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 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
5 phenotypes produced in other cell-types or tissue-types or organs.

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

10

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-type or tissue-type or organ of the plant.

15 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.

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

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
25 regulates.

In the construction of heterologous promoter/structural gene combinations it is 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
30 the gene it controls in its natural setting (i.e., the gene from which the promoter is

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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 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.

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

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.*, 1987), and the temperature-inducible heat shock promoter. Such environmentally-inducible promoters are reviewed in detail by Kuhlemeier *et al.* 1987).

20

In an alternative embodiment, the promoter is a chemically-inducible promoter, such as a tetracycline-inducible promoter; 3- β - indoylacrylic acid-inducible *Tip* promoter; IPTG-inducible *lac* promoter; phosphate-inducible promoter; L-arabinose-inducible *araB* promoter; heavy metal-inducible metallothioneine 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 Gatz *et al.* (1996), amongst others.

30 In an alternative embodiment, the promoter is a wound-inducible or pathogen-inducible

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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-inducible promoter (Cleveland *et al.*, 1987), amongst others.

- 5 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 gibberellin-inducible promoter such as the *Amy32b* gene promoter (Lanahan *et al.* 1992), amongst others.

10

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

15

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 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
20 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 expression thereon, as described previously (Ellis *et al.*, 1987). Such modifications can be achieved by routine experimentation by those skilled in the art.

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TABLE 1

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

GENE SOURCE	EXPRESSION PATTERN	REFERENCE
α -amylase (<i>Amy32b</i>)	aleurone	Lanahan <i>et al.</i> (1992); Skriver <i>et al.</i> (1991)
cathepsin β -like gene	aleurone	Cejudo <i>et al.</i> (1992)
<i>Agrobacterium rhizogenes</i> rolB	cambium	Nilsson <i>et al.</i> (1997)
PRP genes	cell wall	http://salus.medium.edu/mmg/tierney/html
barley <i>lir1</i> promoter	endosperm	
synthetic promoter	endosperm	Vicente-Carbajosa <i>et al.</i> (1998)
AtPRP4	flowers	http://salus.medium.edu/mmg/tierney/html
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; http://winetitles.com.au/gwrdc/csh95-1.html
<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	http://salus.medium.edu/mmg/tierney/html
<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 virus</i> gene	phloem	Bhattacharya-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)
β -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	http://salus.medium.edu/mmg/tierney/html

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RD2 gene	root cortex	http://www2.cnsu.edu/ncsu/research
TobRB7 gene	root vasculature	http://www2.cnsu.edu/ncsu/research
AlPRP4	leaves, flowers, lateral root primordia	http://salus.mediam.edu/mmgtierney/html
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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Preferred embodiments of the invention relate to the effect(s) of cytokinins on the determination of cell fate and plant development and cellular processes therefor.

However, the present invention clearly contemplates the broad application of the inventive method to the modification of a range of cellular processes involved in
5 determining cell fate and plant cell development, including but not limited to modifying the length of the cell cycle, and in particular, modifying the length of the G2 phase; modifying the duration of the G2/M phase transition; the advancement of cell division; determination of cell fate and in particular root development and/or seed development; the modification of source/sink relationships, and/or the inhibition of root
10 growth and/or inhibition of root apical dominance and/or the delay of senescence and/or modifying shoot apical dominance.

Preferred embodiments of the invention also relate to specific the effect(s) of hormones such as cytokinins and/or gibberellins on plant metabolism. With respect to such
15 hormone-related effects, the present invention clearly contemplates the broad application of the inventive method to the modification of a range of cellular processes that are mediated by cytokinins and/or gibberellins, including but not limited to cellular development and/or cell fate; the advancement of cell division; the modification of source/sink relationships, and/or the inhibition of root growth and/or the inhibition of
20 root apical dominance and/or the delay of senescence and/or the initiation, promotion, stimulation or enhancement of seed development and/or tuber formation and/or shoot initiation and/or dwarfism and/or pigment synthesis and/or the modification of shoot apical dominance (i.e. bushiness) of the plant.

25 Accordingly, in a particularly preferred embodiment of the present invention, there is provided a method of shortening the duration of the G2 phase of the cell cycle and/or shortening the G2/M phase transition of a cell comprising expressing the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B operably under the control of a
30 regulatable promoter sequence as described *supra*.

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In the present context, the term "substrate of cyclin B" shall be taken to refer to any protein that interacts with cyclin B in regulating the plant cell cycle, including, but not limited to cyclin-dependent kinases (CDKs), the most significant of which is cdc2, which in all cells is the key enzyme driving entry into mitosis.

5

The term "modified substrate of cyclin B" refers to a homologue, analogue or derivative of a substrate of cyclin B that mimics the effect of alfalfa CycMs2 activity described herein. For example, substitution of amino acids in a cyclin-dependent kinase (CDK) can produce one or more cytokinin-like effects in a plant that are similar to those
10 observed following constitutive cyclin B expression in the plant.

Accordingly, similar effects to the cyclin B-induced effects obtained by expressing alfalfa CycMs2 under control of the regulatable promoter, can be obtained by expressing the cyclin B substrate or a modified form thereof operably under control of
15 the same or a functionally-equivalent promoter. The present invention clearly extends to such arrangements.

The present invention extends further to the co-expression of cyclin B, in particular alfalfa CycMs2, and one or more cyclin B substrates and/or one or more modified
20 cyclin B substrates, operably under the control of a regulatable promoter that is selected for a particular application as described herein. The present invention also extends to the co-expression of cyclin B with another synergistic or non-antagonistic cell cycle control protein, such as Cdc25, amongst others.

25 In another particularly preferred embodiment of the present invention, there is provided a method of advancing cell division in a plant cell, tissue or organ comprising expressing the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B operably under the control of a regulatable promoter sequence.

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By "advancing cell division" is meant that the duration between the commencement of the cell cycle and entry of the cell into mitosis or alternatively or in addition, the duration between the commencement of the G2 phase of the cell cycle and entry of the cell into mitosis is shortened. In the present context this is achieved by reducing
5 the duration of the cell in the G2 phase of the cell cycle and/or by reducing the duration of the G2/M phase transition.

In yet another particularly preferred embodiment of the present invention, there is provided a method of altering cell fate or development in a plant cell, tissue, organ or
10 whole plant comprising expressing the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B operably under the control of a regulatable promoter sequence.

Preferably, the cell fate or development that is altered or modified by the performance
15 of the present invention comprises a process that is regulated by the G2 phase of the cell cycle and/or by the duration of G2 and/or by the duration of the G2/M phase transition. In a more preferred embodiment, the cell fate or development comprises root development; and/or seed development, in particular grain production and yield; and/or one or more sink/source relationships of the plant, such as carbon partitioning;
20 and/or tissue senescence. Other processes are not excluded.

In another particularly preferred embodiment of the present invention, there is provided a method of modifying sink/source relationships of a plant tissue, organ or whole plant comprising expressing the alfalfa CycMs2 protein or a homologue, analogue or
25 derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B operably under the control of a regulatable promoter sequence.

As will be known to those skilled in the art, the term "sink/source relationship" refers to the flux of carbon, in particular, in the form of sucrose or triose or triose phosphate,
30 or other carbon-containing compound, from a source organelle, cell, tissue or organ,

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such as but not necessarily limited to photosynthetic tissues (eg. flag leaves), to a particular storage organelle, cell, tissue or organ (the "sink") and to the regulation of carbon flux and/or storage in the sink by such a process. Accordingly, sink/source relationships include such processes as carbon partitioning between sucrose and
5 starch within a particular cell, and the regulation of seed starch deposition by photosynthesis and/or photosynthate transport to the endosperm (i.e. source limitation), and by sink-limiting processes such as endosperm or storage cotyledon ATP/Pi ratio, and the level of starch-metabolising enzymes (eg. ADP-glucose pyrophosphorylase; starch synthase) within the endosperm or storage cotyledon,
10 amongst others. Those skilled in the art will be aware that sink strength is possibly the most important yield component.

Cytokinins are known to promote phloem unloading of metabolites, and in immature seeds cell division activity is correlated with a high endogenous cytokinin level,
15 particularly in maize and legumes). Likewise, hexoses in very young *Vicia faba* seeds stimulate cell division while sucrose stimulates starch formation at a later stage of seed development. Sucrose-to-hexose conversion is controlled by invertase and indeed invertase activity is high in very young seeds. Hexose-to-sucrose conversion is controlled by sucrose phosphate synthase, leading eventually to starch synthesis.
20 Invertase converts sucrose in a sink (meristem, storage organ, seed, etc.) into glucose while glucose stimulates cell division. Cell division produces cell mass in the sink. Cytokinins are part of the signal transduction chain linking the incoming sucrose to the activation of cell divisions. Cytokinins act downstream of the sucrose-to-hexose conversion regulated by invertase, but may have a pleiotropic effect on invertase and
25 the G2/M transition (and possibly also on G1/S transition). At some point in seed development, invertase activity goes down, or glucose is activately converted into starch by activation of sucrose phosphate synthase. Cell division activity is stopped, to be followed by cell expansion (including endoreduplication) and starch biosynthesis. In seeds, grain filling occurs, whilst in leaf initials on meristems, leaf expansion and
30 development occurs. Whilst not being bound by any theory or mode of action, ectopic

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overexpression of a mitotic cyclin, such as CycMs2, has the same effect as cytokinin in that it drives the cell precociously into mitosis, resulting in a shortened G2 phase. A short G2 phase (and/or a short G1 phase) are defining features of a cell that is actively-dividing. Thus, in a mitotic cyclin-overexpressing cell, the G2/M transition is
5 uncoupled from its normal external signal, glucose and cytokinin.

In one application of this phenomenon, such an uncoupling of sink/source relationships is useful in regulating the production of seeds. For example, a transgenically-controlled mitotic cyclin level in young seeds can render these cells more or less responsive to
10 the incoming glucose/cytokinin signal, resulting in more or fewer cells in the endosperm of the seed until glucose-to-sucrose conversion occurs.

In a further application, the production of potato tubers may be regulated. In potato tubers, sucrose induces stolon tips to develop into tubers. However, cytokinin is
15 required to initiate cell divisions in the stolon. A role for glucose in activation of cell division is not known. Overexpression of mitotic cyclins might substitute for the cytokinin effect and stimulate cell division independently from the incoming tuberization signal.

20 Accordingly, it is possible to increase or decrease sink strength of the seed or tuber, and, by extrapolation, of any other sink. Being the work horses of the cell cycle and the endpoints of the signal transduction chain originating from the incoming sucrose. CDKs might be the final and crucial determinants of sink strength.

25 The promoter selected for use according to this embodiment may be any promoter sequence operable in the tissue or tissues in which carbon flux is to be modified.

Accordingly, in a related 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
30 comprising expressing the alfalfa CycMs2 protein or a homologue, analogue or

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derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B, and in particular that mimics the effect of alfalfa CycMs2, operably under the control of a seed-specific promoter sequence.

5 Preferably, the seed-specific promoter is operable in the seeds of monocotyledonous plants, for example the barley *Amy32b* gene promoter, Cathepsin β -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, particularly
10 in those plants having storage cotyledons, for example 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
15 of the amount of starch produced in the endosperm of the seed or in the storage cotyledon. The amount of protein produced in the endosperm or storage cotyledon is also a contributing factor to grain yield.

Accordingly, in a preferred embodiment, the alfalfa *CycMs2* gene or a homologue,
20 analogue or derivative thereof, is placed operably in connection with a promoter that is operable in the endosperm or storage cotyledon of the seed, in which case the combination of the cell cycle-control protein and endosperm-expressible promoter or storage cotyledon-expressible promoter provides the additional advantage of increasing the grain size and grain yield of the plant.

25

For expression of a cyclin B in the storage cotyledon, use of the *lea* gene promoter is particularly preferred.

Endosperm-specific promoters that can be used to drive cyclin B expression have
30 been identified. The components of the promoters responsible for specific expression

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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 be used, including the zein (ZmZ27) gene promoter, the rice glutelin 1 gene (osGT1) promoter, the rice small subunit ADP-glucose pyrophosphorylase
5 (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 expressed in the endosperm during nuclear proliferation are also useful for driving cyclin B expression. Promoters derived from those genes that are
10 expressed in the endosperm at the stage of nuclear proliferation is ending could be ideal for extending this period.

An advantage of the present inventive approach is that the activity of cytokinin metabolising enzymes is circumvented by the direct raising of cyclin B activity in the
15 endosperm, by the ectopic expression of cyclin B, in particular CycMs2 cyclin, 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).

20

In another related 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 alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B, in particular that
25 mimics the effect of alfalfa CycMs2, operably under the 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.

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In another particularly preferred embodiment of the present invention, there is provided a method of inhibiting root development and/or root growth from plant cell, meristem or other tissue, organ or whole plant comprising expressing the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that
5 mimics the effect of cyclin B operably under the control of a regulatable promoter sequence.

By inhibiting "root development" is meant that the formation of a root structure or root-like structure from a meristem is prevented or delayed or repressed, irrespective of
10 whether or not the meristem is developmentally committed to forming a root structure (i.e. irrespective of whether or not the meristem is a root meristem).

By inhibiting "root growth" is meant that the continued growth of a committed root structure or root-like structure from an existing root or root meristem.

15

Accordingly, this embodiment of the present invention relates to the prevention of visible root structures or root-like structures from appearing in plants. As exemplified herein, the present inventors have demonstrated that the ectopic expression of alfalfa CycMs2 in transgenic tobacco plants inhibits the auxin-mediated development of roots
20 from cultured leaf disc tissue. Accordingly, the ectopic expression of cyclin B proteins in plant cells is capable of antagonising auxin-mediated processes in plants, compatible with the concept that to initiate such processes, in particular root development, the cell must remain in the G2 phase of the cell cycle for a longer period than would otherwise be the case. Whilst not being bound by any theory or mode of
25 action, since ectopic expression of CycMs2 shortens G2 and/or the G2/M phase transition, cells do not remain in G2 for a sufficient time to initiate auxin-mediated processes such as root development.

In the performance of this embodiment of the invention, it is preferred that the promoter
30 selected for regulating cyclin B expression is a root-expressible or meristem-

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expressible promoter sequence such as those listed in Table 1 and in particular, the meristem-expressible PCNA promoter sequence. However, since this embodiment of the invention is also applicable to the prevention of root regeneration in cultured cells and tissues, it will also be apparent that any promoter that is operable in the cell or
5 tissue where inhibition of root regeneration is desired will be useful. For example, a leaf-operable promoter is preferred for use in preventing root regeneration from leaf disc tissue. Alternatively, any regulatable constitutive promoter may also be useful in performing this embodiment of the invention.

- 10 In another particularly preferred embodiment of the present invention, there is provided a method of delaying senescence of a plant tissue, organ or whole plant comprising expressing the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B operably under the control of a regulatable promoter sequence. This embodiment of the present invention
15 also relates to the prevention, delay or reduction of leaf chlorosis and/or leaf necrosis in plants.

Preferably, the promoter selected for use in performing this embodiment of the invention is operable in the green tissues of the plant and in particular, in the leaves.
20 Accordingly, the use of a strong promoter such as one of the known *Cab* promoters, the SAM22 promoter, or the *rbcS-1A* and *rbcS-3A* gene promoters listed in Table 1. The SAM22 gene promoter is particularly preferred in light of the developmental regulation of the SAM22 gene and its induction in senescent leaves.

- 25 In another preferred embodiment of the present invention, there is provided a method of modifying shoot apical dominance or bushiness of a plant, comprising expressing the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B, in particular that mimics the effect of alfalfa CycMs2, operably under the control of a meristem-specific
30 promoter sequence or a stem-specific promoter sequence.

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Without being bound by any theory or mode of action, the shortened G2 transition modifies cellular metabolism at the level of carbon partitioning to modify th degree of branch formation in the plant, thereby modifying auxin-induced apical dominance in the plant.

5

In another 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 alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect
10 of cyclin B 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
15 homologue, analogue or derivative thereof.

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 alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a modified
20 substrate of cyclin B that mimics the effect of cyclin B, and in particular that mimics the effect of alfalfa CycMs2, operably under the control of a cambium-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
25 endotransglucosylase (XET) gene promoter sequences, amongst others. The *T. bacilliform* virus gene promoter and the sucrose-binding protein gene promoter are also useful for this application of the invention.

30 Preferred target plant species according to this embodiment are woody plants of

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economic/ agronomic value, in particular hardwood crop plants such as, but not limited 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
5 *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. drepanolobium*, *A. nilotica*, *A. pravissima*, and *A. seyal*; *Acer* spp., in particular *A. pseudoplatanus* and *A. saccharum*. Additional species are not excluded.

10 Without being bound by any theory or mode of action, the ectopic expression of cyclin B 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 production. Within the vascular tissue, cambial cells contain the highest levels of auxins and are therefore the
15 preferential tissue for cyclin B overproduction.

In another preferred embodiment of the present invention, there is provided a method of modifying lateral root production in a plant comprising expressing the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a modified substrate of
20 cyclin B that mimics the effect of cyclin B, in particular that mimics the effect of alfalfa CycMs2, operably under the control of a root-specific promoter sequence.

Preferred promoter sequences according to this embodiment of the present invention include any one of the root-expressible or root-specific promoters listed in Table 1 and
25 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.

In yet another preferred embodiment of the present invention, there is provided a
30 method of modifying the nitrogen-fixing capability of a plant comprising expressing the

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alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B, in particular that mimics the effect of alfalfa CycMs2, operably under the control of a nodule-specific promoter sequence.

5

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.

10

In a further preferred embodiment of the present invention, the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B, in particular that mimics the effect of alfalfa CycMs2, is expressed in one of the specialised minority of plant tissues in which the activation of
15 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.

Accordingly, the present invention preferably provides a method of advancing cell
20 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 alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a substrate or modified substrate of cyclin B that mimics the effect of cyclin B operably under the control of a meristem specific promoter sequence.

25

Without being bound by any theory or mode of action, the shortened G2 phase and/or shortened G2/M phase transition in the intercalary meristem of the youngest stem internode as a consequence of increased cyclin B activity therein results in the production of a more extensive canopy. It is proposed that this leads to an increase
30 in the plant's capacity to support grain production. The stimulatory effect of gibberellic

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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
5 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.
10 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
15 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 a cyclin B gene, in particular the *CycMs2* gene, operably under the
20 control of the regulatable 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 a cyclin B gene, in particular the *CycMs2* gene, operably under the
25 control of the regulatable promoter sequence is stably integrated into the genome of the cell.

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

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- (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
- (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 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 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.

20

Means for introducing recombinant DNA into plant tissue or cells include, but are not limited to, transformation using 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) microparticle bombardment, electroporation (Fromm *et al.*, 1985), microinjection of 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,

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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 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.

10

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

15 A whole plant may be regenerated from the transformed or transfected cell, in 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. 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).

25

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

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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
5 generation (or T1) transformed plant may be selfed to give homozygous second 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
10 forms. For example, they may be chimeras of transformed cells and non-transformed 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).

15 A further aspect of the present invention clearly provides the genetic constructs and 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.

20 In addition to the cell cycle control protein-encoding sequence and regulatable 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
25 which signals termination of transcription. Terminators are 3'-non-translated DNA sequences containing a polyadenylation signal, which facilitates the addition of 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,
30 fungi, viruses, animals and/or plants.

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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
5 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
10 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,
15 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.

20

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
25 a phenotype on a cell in which it is expressed to facilitate the identification and/or 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
30 (*Amp^r*), tetracycline resistance gene (*Tc^r*), bacterial kanamycin resistance gene

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(Kan^r), phosphinothricin resistance gene, neomycin phosphotransferase gene (*nptII*), hygromycin resistance gene, β -glucuronidase (GUS) gene, chloramphenicol acetyltransferase (CAT) gene, green fluorescent protein (*gfp*) gene (Haseloff *et al*, 1997), and luciferase gene, amongst others.

5

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, 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 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.

15

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 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 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.*, *Dorycnium rectum*, *Echinochloa pyramidalis*, *Ehrartia spp.*, *Eleusine coracana*, *Eragrestis spp.*, *Erythrina spp.*, *Eucalyptus spp.*, *Euclea schimperi*, *Eulalia villosa*,

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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*
altissima, *Heteropogon contortus*, *Hordeum vulgare*, *Hyparrhenia rufa*, *Hypericum*
5 *erectum*, *Hyperthelia dissoluta*, *Indigo incarnata*, *Iris* spp., *Leptarrhena pyrolifolia*,
Lespedeza 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*
spp., *Ornithopus* spp., *Peltophorum africanum*, *Pennisetum* spp., *Persea gratissima*,
10 *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*,
Rhopalostylis sapida, *Rhus natalensis*, *Ribes grossularia*, *Ribes* spp., *Robinia*
15 *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*
spp., *Tsuga heterophylla*, *Vaccinium* spp., *Vicia* spp. *Vitis vinifera*, *Watsonia*
20 *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
specifically named above or a tissue, cell or organ culture of any of the above species.

25

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,
Agrobacterium-mediated transformation, protoplast fusion, or electroporation, amongst
30 others.

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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, 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.

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

EXAMPLE 1

Regulatable ectopic expression of alfalfa CycMs2 mitotic cyclin in tobacco

The cycMs2 alfalfa mitotic cyclin was expressed as a fusion protein with haemagglutinin (HA) in tobacco plants under the control of tetracycline-regulatable promoter construct. To facilitate the detection of the transgene, the haemagglutinin epitope tag (HA) was fused to the C-terminus of the CycMs2 coding region, to produce the fusion designated as "CycMs2-HA".

After *Agrobacterium*-mediated transformation, the expression of CycMs2-HA was determined in leaves from 5 independent transgenic lines treated with 1 mg/l Cl-tetracyclin for 24 h (Figure 1-1). In all of these transgenic lines, the mRNA of cycMs2-HA transgene could be detected by RNA-blot hybridisation. In 4 lines, the expression was strictly dependent on the addition of tetracycline.

25

A suspension culture was initiated on 2,4-D-containing medium, using the line 2 plant (Figure 1-1) as starting material. The expression of cycMs2-HA was still strictly dependent on tetracycline in cultured cells and the accumulation of cycMs2-HA mRNA could already be detected after 10 minutes of incubation with 1 mg/l Cl-tetracycline (Figure 1-2).

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To determine the optimal tetracycline concentration for expression of the CycMs2-HA transgene, cultured cells were treated with a range of Cl-tetracycline concentrations for 24 h, and the expression of the CycMs2-HA protein was detected by protein blotting with the HA antibody (Figure 1-3, upper panel). A sharp increase in the production of
5 CycMs2-HA protein was found at 0.01 mg/l Cl-tetracycline concentration.

To determine whether the ectopically-expressed CycMs2-HA protein forms an active complex with cyclin-dependent kinase (CDK), we immunopurified the complex from cell extract treated with different tetracycline concentrations and measured the protein
10 kinase activity of the CDK. The increase in activity of CycMs2-HA complexed with CDK was correlated with the amount of expressed cycMs2-HA cyclin protein in these extracts, indicating that the amount of cyclin is rate-limiting to produce active CDK complexes (Figure 1-3, middle panel). When different CDK complexes were isolated by the binding to the p13^{suc1}-protein, CDK activities were comparable in cells
15 expressing or not expressing the CycMs2-HA protein, indicating that the CycMs2-HA associated CDK activity is only a minor portion of the total CDK activity in these extracts (Figure 1-3, lower panel).

EXAMPLE 2

20 **The CycMs2-mitotic cyclin protein is in the nucleus**

By indirect immunofluorescence microscopy using anti-HA antibody as a probe, the localisation of the ectopically-expressed CycMs2-HA protein was determined (Figure 2). In the presence of tetracycline, the CycMs2-HA protein was detected in the nucleus of around 60% of cells (Figures 2-1 and 2-2). No signal was detectable
25 without incubation of cells in tetracycline (Figures 2-3 and 2-4).

To confirm the nuclear localisation by other means, cells were fractionated into cytoplasm and nucleus and the activity of the CycMs2-HA associated CDK was determined by measuring the protein kinase activity of the complex
30 immunoprecipitated from nuclear and cytoplasmic extracts. The majority of CycMs2-

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HA associated kinase activity was present in the nucleus (Figure 3).

EXAMPLE 3

5 **The length of the G2 phase is shortened in cells that ectopically express
 the CycMs2-HA mitotic cyclin**

Having determined that the amount of cyclin is rate limiting to form an active CDK complex in plant cells, we asked if the ectopic expression of CycMs2 mitotic cyclin in G2 cells is sufficient to enter mitosis.

10 Cell divisions were synchronised by releasing cells from a block by the inhibition of DNA synthesis with aphidicolin in cultured cells. The expression of CycMs2-HA protein was induced by adding 0.1 mg/l Cl-tetracycline, after removing aphidicolin, and the progression of cell cycle was followed by measuring the DNA content of cells with flow cytometry (Pfosser *et al.*, 1995), counting the number of mitotic cells and counting the
15 mitosis-specific microtubule structures (Figure 4).

In tetracycline-treated cells, the number of mitotic divisions started to increase 8 h after aphidicolin release and the maximal number of mitotic divisions were found at 10 h (Figure 5A). In contrast, cells incubated without tetracycline entered mitosis 2 hours
20 later, and the highest number of mitotic divisions was achieved at 12 h. A similar 2 hour advance in the formation of mitotic microtubule structures were observed in cells ectopically expressing the mitotic CycMs2-HA cyclin protein in the presence of tetracyclin.

25 The measurement of DNA content by flow cytometry further confirmed these results (Figure 4-3).

While at 8 h a similar distribution of nuclei with G2 and G1 DNA contents was found in tetracycline-treated and control cells, at 10 h in the presence of tetracycline a high
30 proportion of cells already had nuclei with G1 DNA content, indicating that they passed

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through mitosis.

From these experiments we can conclude that the ectopic expression of cycMs2 mitotic cyclin in G2 cells does not induce mitosis directly but advances cells to enter
5 mitosis 2 hours earlier (Figure 5-4).

As independent molecular proof for the advancement of cell divisions in the cells ectopically expressing the CycMs2-HA protein in G2 phase, mRNA was prepared from these cells and hybridised with an HA fragment to detect the transgene (Figure 5).
10 Additionally, the expression of S phase and mitosis-specific marker genes were also monitored, in particular the histone H4 and an endogenous mitotic tobacco cyclin (cycM) as shown in Figure 5.

Data shown in Figure 5 indicate that histone H4 mRNA was similarly present in
15 samples with aphidicolin and at 3 h both with and without tetracycline, however at 20 h in the presence of tetracycline a higher level of histone H4 expression was observed than that in the control samples, indicating that some cells reached the second S phase at 20 h when the mitotic cyclin is ectopically expressed. The expression of the tobacco cycM mitotic cyclin followed the number of mitotic cells during the
20 synchronous cell division and was similarly advanced by about 2 hours in the presence of tetracycline.

EXAMPLE 4

Ectopic expression of CycMs2 mitotic cyclin inhibits root regeneration 25 in culture from tobacco leaves

Regeneration of shoots and roots from tobacco leaves is dependent on the auxin/cytokinin ratio: high auxin to cytokinin concentration favours root regeneration, while high cytokinin to auxin concentration favours shoot regeneration.

30 In *in vitro* regeneration experiments, with leaf disks of the transgenic line containing the

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tetracycline inducible cycMs2 gene, the regeneration of shoots still required cytokinin even in the presence of ectopic mitotic cyclin expression, but root formation was inhibited by ectopic cyclin expression (Figure 6).

- 5 We measured the DNA content of nuclei from leaves treated for 5 days with different NAA and BAP concentrations in the presence or absence of tetracyclin (Figure 7). A higher percentage of cells with G2 DNA content was found in leaf cells from wild type plants treated with high auxin to cytokinin ratio favouring root formation than in leaves treated with high cytokinin to auxin ratio, favouring shoot regeneration. This
10 observation is compatible with the notion that cells spend more time in G2 when cultured on medium favouring root formation compared to the treatment favouring shoot formation. In leaf disks from transgenic tobacco plants ectopically expressing cycMs2 the number of cells in G2 was reduced indicating a shortened G2 phase.

15

EXAMPLE 5

DISCUSSION

- Plants are multicellular organisms with defined shapes and sizes. A developmentally determined body plan is elaborated by the controlled timing and orientation of cell divisions in restricted zones called meristems. Conserved regulators of cell division
20 in eukaryotes are the cyclin-dependent protein kinases (CDKs). The expression of cyclin genes are tightly regulated in plants, e.g. B-type cyclins were only found in mitotic cells. We used a tetracycline regulatable promoter construct to study if cyclin expression is limiting in the timing of cell divisions. The expression of a mitotic cyclin, the alfalfa cycMs2, results in an elevated CDK activity, and cells with increased cyclin
25 amount entered mitosis earlier. We tested the consequences of a shortened G2 phase on the auxin- and cytokinin-dependent regeneration of shoots and roots from leaf disks. A premature passage through mitosis inhibits root formation, thus having a cytokinin-like effect.

- 30 We propose a model, shown in Figure 8, that in which shoot versus root regeneration

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is regulated by the G1 and G2 exit points from the cell cycle. According to our model, regeneration of roots and root development and/or initiation depends upon whether a cell exits the cell cycle in the G1 or G2 phase. Roots are formed when cells exit the cell cycle in G2 phase, while shoot formation is dependent on an exit from G1 phase or
5 following a shortened G2 phase.

EXAMPLE 5

Expression of CycMs2 under the control of the patatin gene promoter increases tuber size and number in potato plants

- 10 The CycMs2 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
15 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.
- 20 The CycMs2 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 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
25 entire storage parenchyma, cortex and pith, but remains absent from the periderm.

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 CycMs2 expression driven by both Class I and Class
30 II promoters within the same plant. Because the Class I patatin promoter is not

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expressed before the first stolon initiates tuberization, no effects of Class I patatin-CycMs2 transgenes is seen on tuber initiation. However, the Class I patatin promoter drives CycMs2 expression very early after tuber initiation onwards, allowing a maximal impact of CycMs2 activity on organ formation and, as a consequence, on tuber size.

- 5 The fact that the Class I patatin promoter activity subsequently also appears in non-tuberized stolons implies that the Class I patatin – CycMs2 transgene increases both the size and number of tubers.

EXAMPLE 6

- 10 **Expression of CycMs2 under the control of endosperm-specific promoters increases grain size and yield of grain crop plants**

The alfalfa CycMs2 coding sequence is placed operably in connection with the endosperm-specific *ltr1* promoter from barley, or a synthetic promoter containing the
15 endosperm box (GCN motif) of the barley *Hor2* gene (Vicente-Carbajosa *et al.*, 1998). In each case, the CycMs2 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 procedures, in particular microprojectile bombardment or *Agrobacterium*-mediated
20 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.

25

EXAMPLE 7

- Expression of CycMs2 under the control of the *cab-6* or *ubi7* promoters reduces leaf necrosis and chlorosis in lettuce plants**

The alfalfa CycMs2 coding sequence is placed operably in connection with the leaf-
30 specific *cab-6* gene promoter derived from *Pinus* (Yamamoto *et al.*, 1994) or

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senescence-specific *ubi7* gene promoter (Garbarino *et al.*, 1995). In each case, the CycMs2 structural gene is placed upstream of the transcription termination signals of the *Agrobacterium tumefaciens* nopaline synthase (NOS) gene. Lettuce is transformed as described by Bechtold *et al.*, 1993.

5

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-transformed control plants.

10

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WE CLAIM:

1. A method of modifying cell fate and/or plant development and/or plant morphology and/or biochemistry and/or physiology comprising expressing in particular cells, tissues or organs of a plant, comprising expressing in said cells, tissues or organs 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 sequences, and organ-specific promoter sequences.
2. The method according to claim 1, wherein the cell cycle control protein is a cyclin B protein or a homologue, analogue or derivative thereof.
3. The method according to claim 2, wherein the cyclin B protein is derived from a plant.
4. The method according to claim 2 wherein the cyclin B protein is derived from a plant cell, tissue, or organ.
5. The method according to claims 3 or 4, wherein the cyclin B protein is derived from alfalfa or a cell, tissue, or thereof.
6. The method according to claim 1, wherein the cell cycle control protein is a cyclin B substrate or a modified cyclin B substrate.
7. The method according to claim 6, wherein the cyclin B substrate is CDK or a modified form thereof.
8. The method according to claim 1, wherein the regulatable promoter is a tetracycline-inducible promoter sequence.

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9. A method of shortening the duration of the G2 phase of the cell cycle and/or shortening the G2/M phase transition of a cell comprising expressing the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B operably under the control of a regulatable promoter sequence as described *supra*.
10. The method according to claim 9 wherein cyclin B is co-expressed with one or more cyclin B substrates and/or one or more modified cyclin B substrates or with another synergistic or non-antagonistic cell cycle control protein.
11. The method according to claim 9, wherein the substrate of cyclin B comprises CDK.
12. The method according to claim 9 wherein the regulatable promoter sequence is a tetracycline-inducible promoter sequence.
13. A method of advancing cell division in a plant cell, tissue or organ comprising expressing the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B operably under the control of a regulatable promoter sequence.
14. The method according to claim 12 wherein the regulatable promoter sequence is a tetracycline-inducible promoter sequence.
15. A method of altering cell fate or development in a plant cell, tissue, organ or whole plant comprising expressing the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B operably under the control of a regulatable promoter sequence.
16. The method according to claim 15 wherein the regulatable promoter sequence

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is a tetracycline-inducible promoter sequence.

17. The method according to claim 15 wherein the altered cell fate or development is a process that is regulated by the G2 phase of the cell cycle and/or by the duration of G2 and/or by the duration of the G2/M phase transition.

18. The method according to claim 15 wherein the altered cell fate or development is a process that is regulated by the sink/source relationships in the plant or a cell, tissue or organ thereof.

19. The method according to claim 15 wherein the altered cell fate or development comprises reduced or inhibited root development.

20. The method according to claim 15 wherein the altered cell fate or development comprises enhanced or increased seed development.

21. The method according to claim 20 wherein the increased seed development comprises increased grain production and/or yield.

22. The method according to claim 15 wherein the altered cell fate or development comprises enhanced or increased tuberisation.

23. The method according to claim 15 wherein the altered cell fate or development comprises delayed senescence.

24. A method of modifying sink/source relationships of a plant tissue, organ or whole plant comprising expressing the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a modified substrate of cyclin B that mimics the effect of cyclin B operably under the control of a regulatable promoter sequence.

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25. The method according to claim 24 wherein the regulatable promoter sequence is a tetracycline-inducible promoter sequence.
26. A method of increasing tuber formation and/or development in a tuberous crop plant comprising expressing the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a substrate or modified substrate of cyclin B that mimics the effect of cyclin B operably under the control of one or more tuber-specific promoter sequences.
27. The method according to claim 26, wherein the tuberous crop plant is potato.
28. The method according to claim 26 wherein one or more of the tuber-specific promoters is a potato patatin gene promoter sequence.
29. The method according to claim 28, wherein the patatin gene promoter sequence is derived from a class I patatin gene and/or a class II patatin gene.
30. The method according to claim 29, wherein the class I patatin gene promoter is modified to remove sucrose-responsive elements therefrom such as the proximal region of the A repeat.
31. A method of increasing seed set and/or seed production and/or grain yield in a plant comprising expressing the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a substrate or modified substrate of cyclin B that mimics the effect of cyclin B operably under the control of a seed-specific or storage cotyledon-specific promoter sequence.
32. The method according to claim 31, wherein the seed-specific promoter the barley *Amy32b* gene promoter, Cathepsin β -like gene promoter, wheat ADP-glucose pyrophosphorylase gene promoter, maize zein gene promoter, rice glutelin gene

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promoter, legumin gene promoter, *napA* gene promoter, Brazil Nut albumin gene promoter, pea vicilin gene promoter or sunflower oleosin gene promoter.

33. The method according to claims 31 or 32, wherein the seed-specific promoter sequence is operable in the endosperm of the seed.

34. The method according to claim 33 wherein the promoter is the barley *ltr1* promoter sequence.

35. The method according to claim 33 wherein the promoter is a synthetic promoter comprising the endosperm box derived from the barley *Hor2* gene.

36. The method according to claim 31 wherein the storage cotyledon-specific promoter is a *lea* gene promoter sequence.

37. A method of modifying apical dominance or increasing the bushiness of a plant, comprising expressing the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a substrate or modified substrate of cyclin B that mimics the effect of cyclin B operably under the control of a regulatable promoter sequence.

38. The method according to claim 37 wherein the modified apical dominance comprises reduced or inhibited root apical dominance or reduced or inhibited root development.

39. The method according to claim 37 wherein the formation of lateral roots is modified.

40. The method according to claim 37 wherein the promoter is the *LEAFY* promoter sequence.

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41. The method according to claims 37 or 38 wherein the promoter is the *kna1* promoter sequence.
42. The method according to claims 37 or 38 wherein the promoter is the *kn1* promoter sequence.
43. The method according to claims 37 or 38 wherein the promoter is the *CLAVATA1* promoter sequence.
44. The method according to claims 38 or 39, wherein the promoter sequence is the tobacco auxin-inducible gene promoter.
45. A method of modifying the nitrogen-fixing capability of a plant comprising expressing the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a substrate or modified substrate of cyclin B that mimics the effect of cyclin B operably under the control of a nodule-specific promoter sequence.
46. The method according to claim 45, 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.
47. A method of preventing or delaying or otherwise reducing leaf chlorosis and/or leaf necrosis in a plant comprising expressing the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a substrate or modified substrate of cyclin B that mimics the effect of cyclin B operably under the control of a leaf-specific promoter sequence.
48. The method according to claim 47, wherein the promoter is the SAM22 promoter, *rbcS-1A* or the *rbcS-3A* gene promoter.

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49. The method according to claim 47 wherein the promoter is the *cab-6* promoter sequence.
50. The method according to claim 47 wherein the promoter is the *ubi7* promoter sequence.
51. A method of increasing the strength and/or thickness and/or stability and/or wind-resistance of a plant comprising expressing the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a substrate or modified substrate of cyclin B that mimics the effect of cyclin B operably under the control of a stem-expressible promoter sequence.
52. The method according to claim 51, 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.
53. A method of modifying the lignin content of a woody crop plant comprising expressing the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof, or a substrate or modified substrate of cyclin B that mimics the effect of cyclin B operably under the control of a cambium-specific or vascular-tissue-specific promoter sequence.
54. The method according to claim 53, 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.
55. The method according to claim 53 wherein the promoter is the auxin-inducible SAUR promoter sequence.

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56. The method according to claim 53 wherein the promoter is the *rolB* promoter sequence.
57. The method according to claim 53, wherein the woody crop plant is *Eucalyptus* spp., *Populus* spp., *Quercus* spp., *Acer* spp., *Juglans* spp., *Fagus* spp., *Acacia* spp., or teak.
58. A method of modifying one or more plant developmental and/or 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 cell-specific promoter sequence.
59. A method of modifying one or more plant developmental and/or 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.
60. The method according to claim 59, 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.
61. A method of modifying one or more plant developmental and/or 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.
62. The method according to claim 61, 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-

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specific promoter, or stigma-specific promoter.

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

64. The genetic construct according to claim 63, wherein the nucleotide sequence encoding a cell cycle control protein encodes a cyclin B or a cyclin B substrate or a modified cyclin B substrate or a homologue, analogue or derivative thereof.

65. The genetic construct according to claim 63, wherein the regulatable plant-expressible promoter is a tetracycline-inducible promoter.

66. The genetic construct according to claim 63 wherein the regulatable plant-expressible promoter is a patatin promoter sequence.

67. The genetic construct according to claim 63 comprising one or more patatin gene promoter sequences derived from a class I patatin gene and/or a class II patatin gene.

68. The genetic construct according to claim 63 wherein the class I patatin gene promoter is modified to remove sucrose-responsive elements therefrom such as the proximal region of the A repeat.

69. The genetic construct according to claim 63 wherein the regulatable plant-expressible promoter is the auxin-inducible SAUR promoter sequence.

70. The genetic construct according to claim 63 wherein the regulatable plant-expressible promoter is the *ro/B* promoter sequence.

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71. The genetic construct according to claim 63 wherein the regulatable plant-expressible promoter is the barley *ltr1* promoter sequence.
72. The genetic construct according to claim 63 wherein the regulatable plant-expressible promoter is a synthetic promoter comprising one or more endosperm box motifs derived from the barley *Hor2* gene.
73. The genetic construct according to claim 63 wherein the regulatable plant-expressible promoter is the *LEAFY* promoter sequence.
74. The genetic construct according to claim 63 wherein the regulatable plant-expressible promoter is the *kna1* promoter sequence.
75. The genetic construct according to claim 63 wherein the regulatable plant-expressible promoter is the *kn1* promoter sequence.
76. The genetic construct according to claim 63 wherein the regulatable plant-expressible promoter is the *CLAVATA1* promoter sequence.
77. The genetic construct according to claim 63 wherein the regulatable plant-expressible promoter is the *cab-6* promoter sequence.
78. The genetic construct according to claim 63 wherein the regulatable plant-expressible promoter is the *ubi7* promoter sequence.
79. 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 cyclin B protein or a cyclin B substrate or a modified cyclin B substrate or a homologue, analogue or derivative thereof operably in connection with a regulatable promoter sequence selected from the

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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 cyclin B protein or substrate or modified substrate or homologue, analogue or derivative thereof in one or more particular cells, tissues or organs of the plant.

80. The plant according to claim 79 wherein the cyclin B is alfalfa CycMs2.

81. A plant cell, tissue, organ, plant part, propagule or progeny plant of the primary transformed plant according to claims 79 or 80, wherein said plant cell, tissue, organ, plant part, propagule or progeny plant comprises the introduced nucleotide sequence that encodes the cyclin B protein or substrate or modified substrate or homologue, analogue or derivative thereof operably in connection with a regulatable promoter sequence.

82. The plant according to claim 81, exhibiting one or more modified developmental and/or morphological and/or biochemical and/or physiological characteristics compared to otherwise isogenic non-transformed plants.

83. The plant according to claim 82 wherein the modified developmental characteristic comprises altered cell fate and/or reduced G2 duration and/or reduced G2/M transition of one or more cells of said plant.

84. The plant according to claim 82 wherein the modified biochemical characteristic comprises modified sink/source relationships.

85. The plant according to claim 82, wherein the modified morphological and/or biochemical and/or physiological characteristics comprises increased seed set and/or seed production and/or grain yield.

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86. The plant according to claim 85 having increased endosperm size.
87. The plant according to claim 82, wherein the modified morphological and/or physiological characteristics comprises reduced root apical dominance or modified bushiness.
88. The plant according to claim 82, wherein the modified morphological and/or physiological characteristic comprises modified numbers of lateral roots or modified rate of lateral root production.
89. The plant according to claim 82, wherein the modified morphological and/or physiological characteristics comprises increased tuber formation and/or development.
90. The plant according to claim 82, wherein the modified morphological and/or physiological characteristic comprises reduced leaf chlorosis and/or leaf necrosis.
91. The plant according to claim 82, wherein the modified morphological and/or biochemical and/or physiological characteristic comprises modified nitrogen-fixing capability and/or modified nodule size.
92. The plant according to claim 82, 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.
93. The plant according to claim 82, wherein the modified morphological and/or biochemical and/or physiological characteristics comprises increased lignin content.
94. 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 the alfalfa CycMs2 protein or a homologue, analogue or derivative thereof,

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and/or a substrate or modified substrate of cyclin B that mimics the effect of cyclin B, wherein said genetic sequence is placed operably under the control of a meristem-expressible promoter sequence.

95. The method according to claim 82, wherein the promoter sequence is a Proliferating Cell Nuclear Antigen (PCNA) promoter of rice.

96. The method according to claim 94 or 95 wherein the yield of grain is increased.

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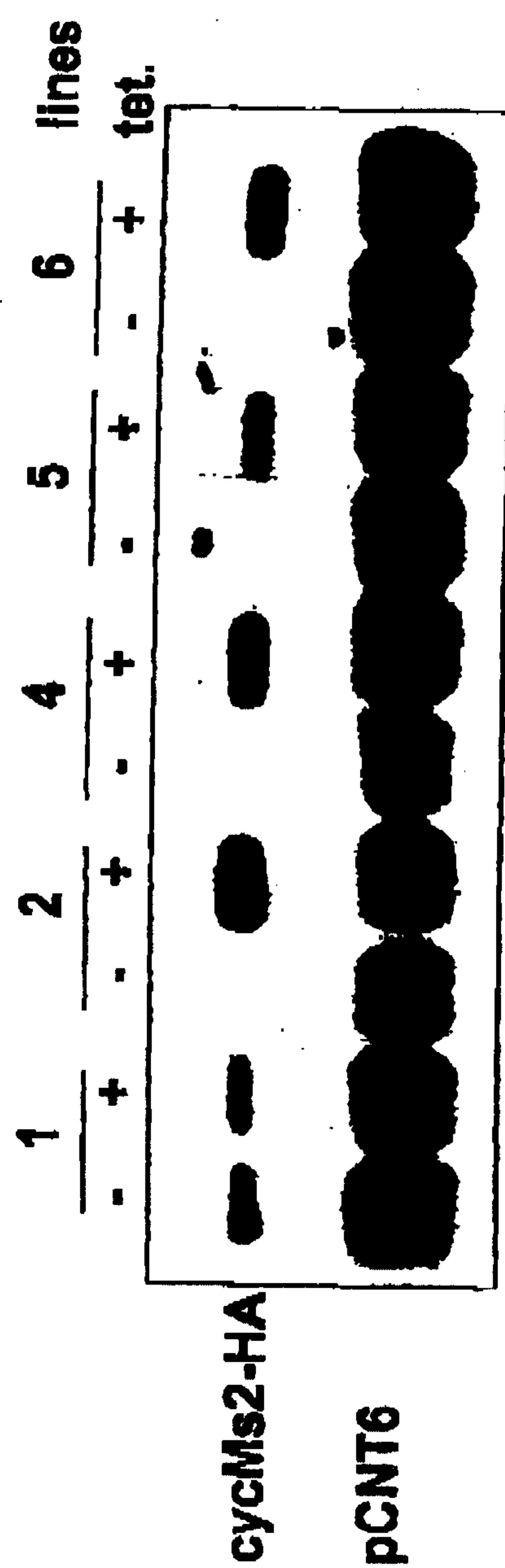
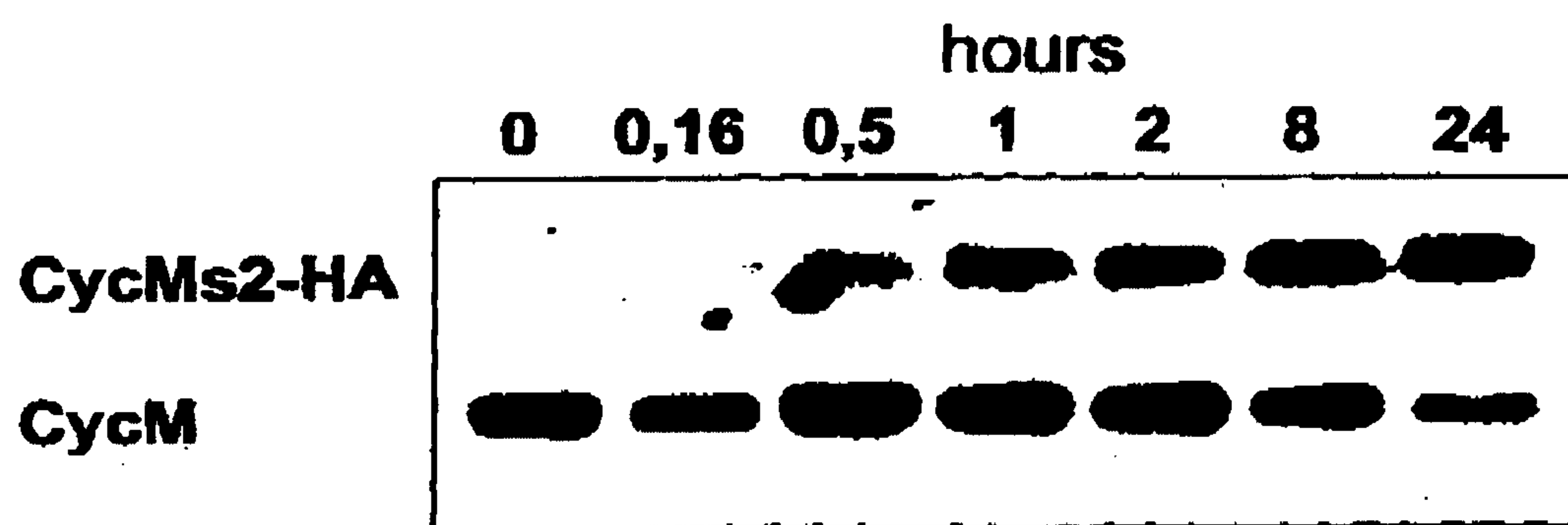


FIGURE 1-1

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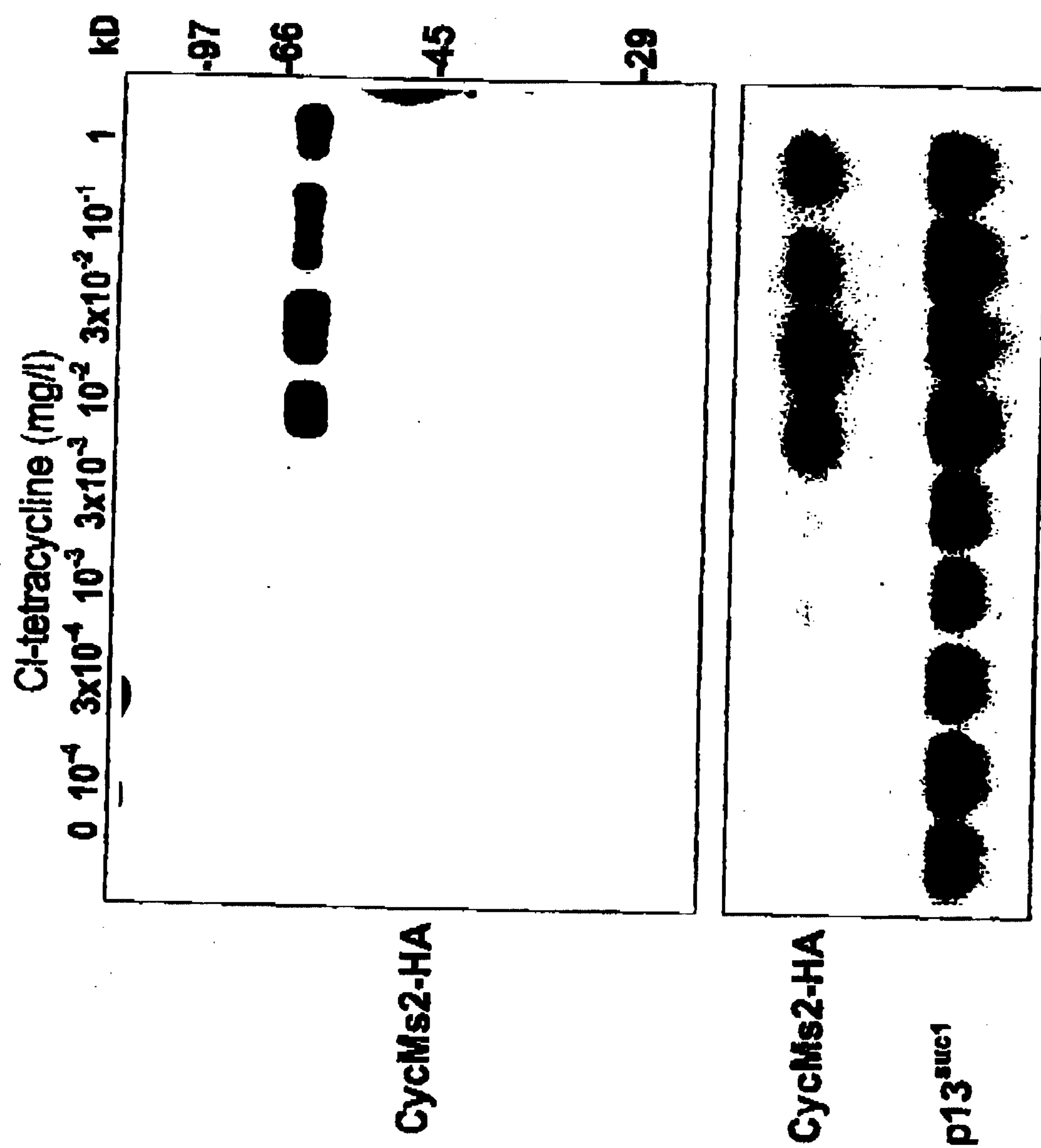


FIGURE 1-3

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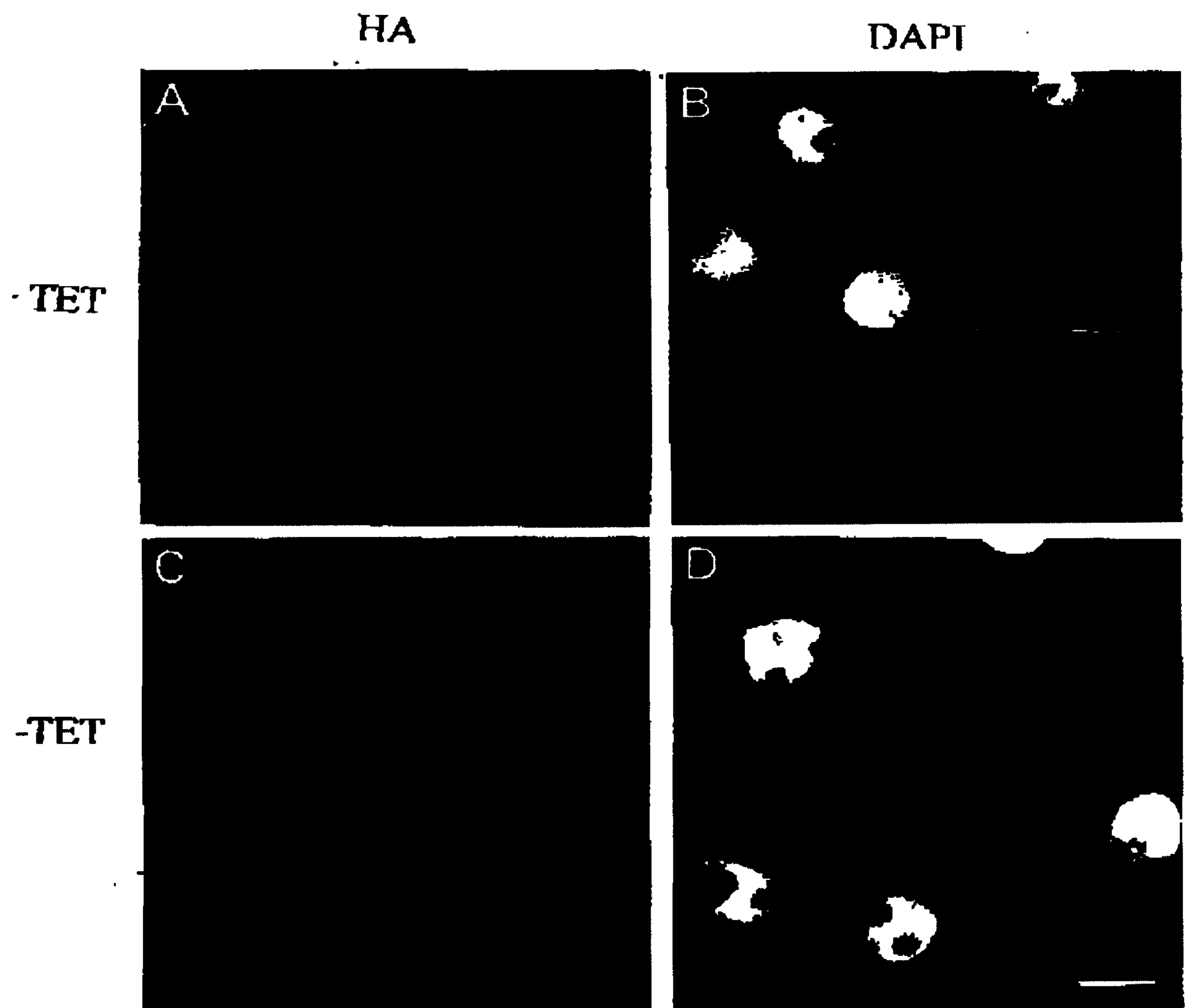


FIGURE 2

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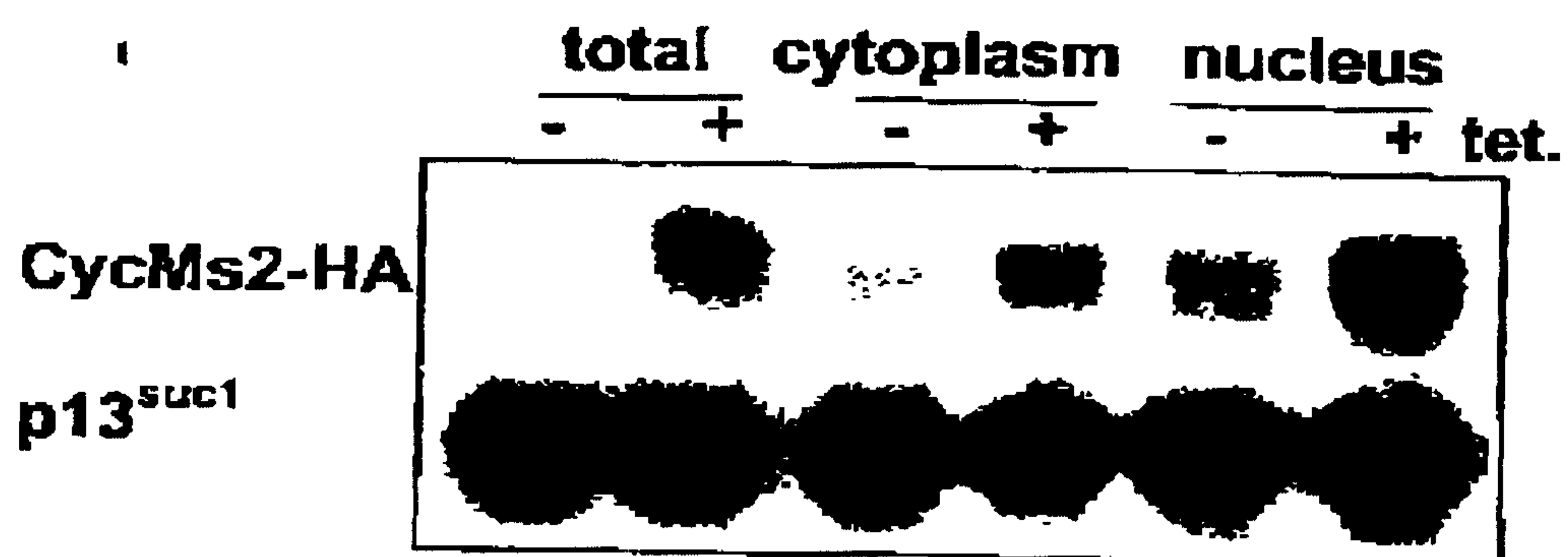


FIGURE 3

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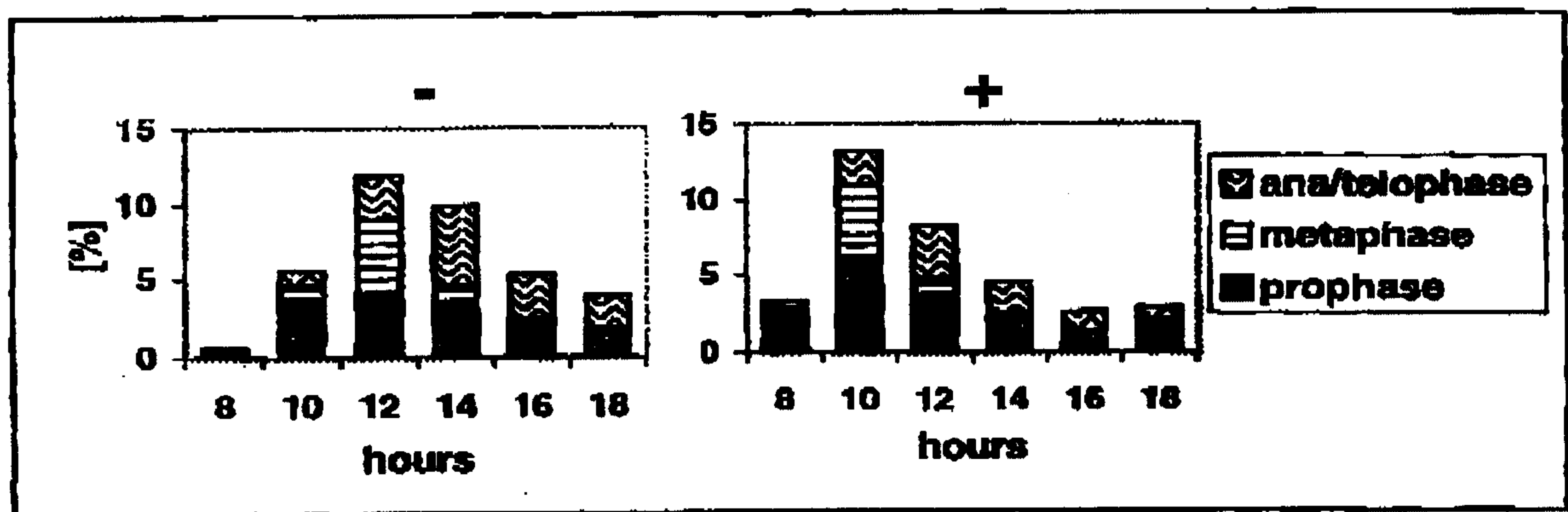


FIGURE 4-1

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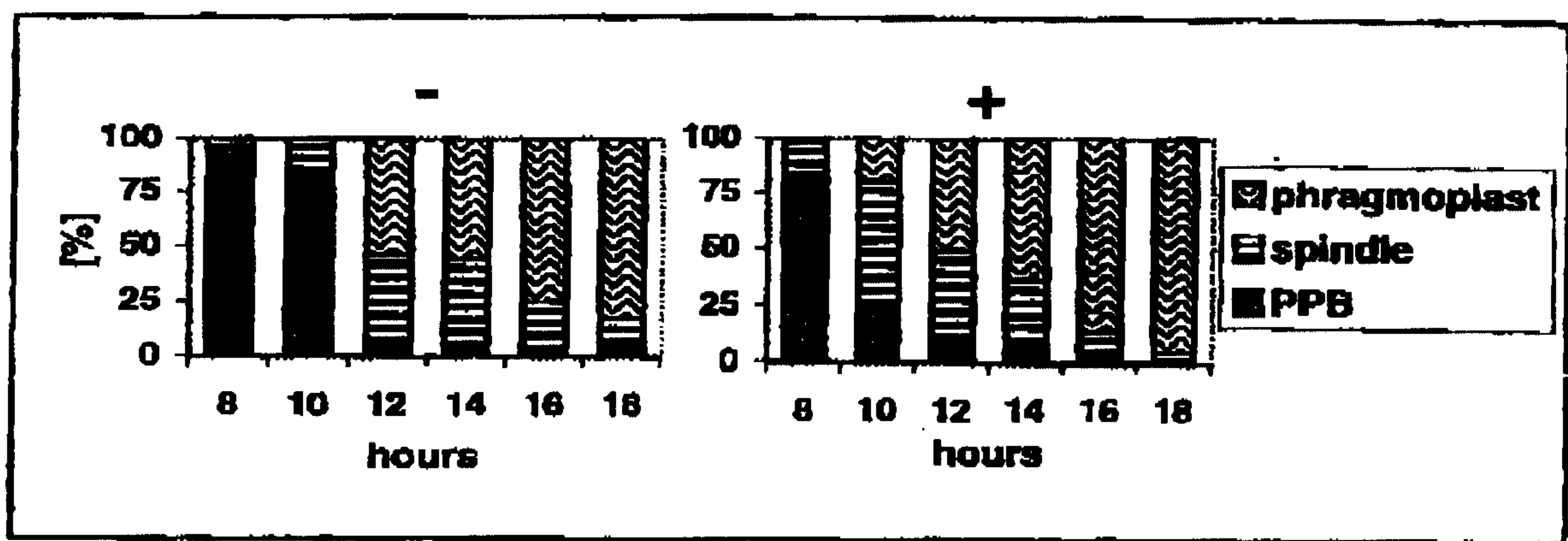
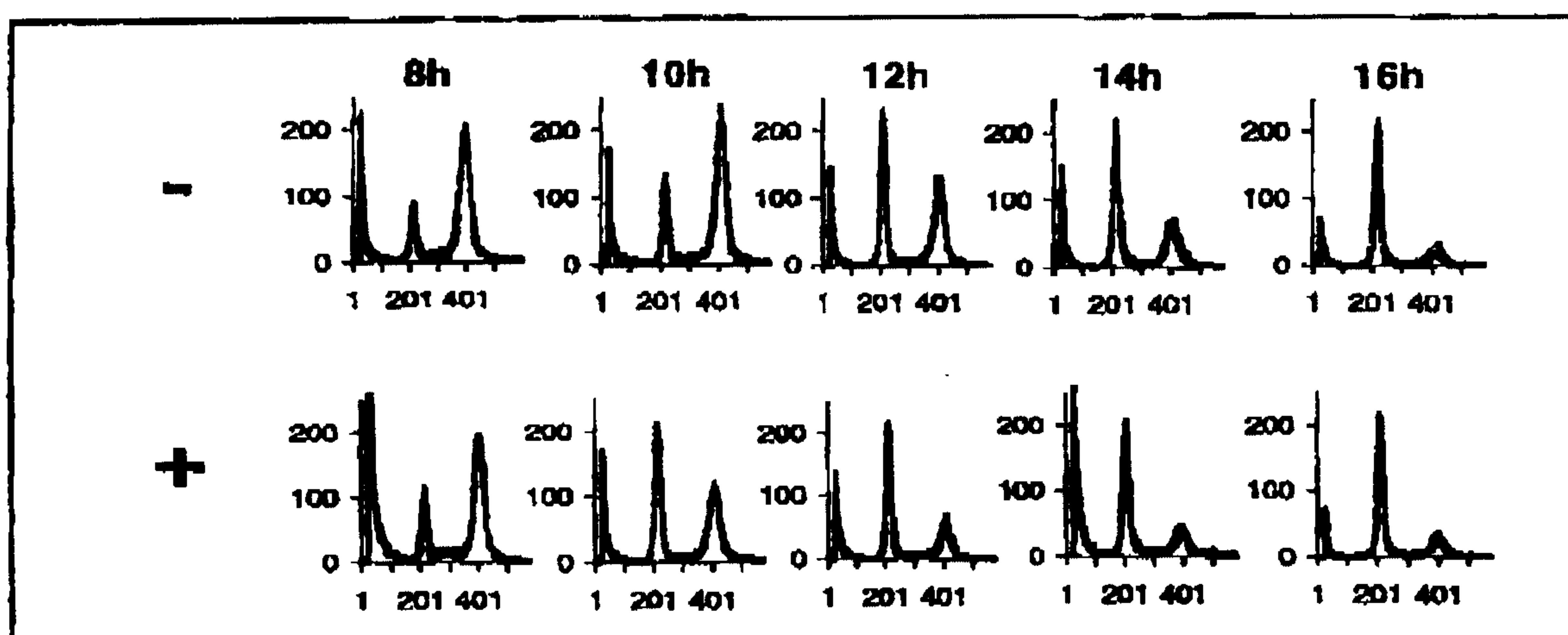
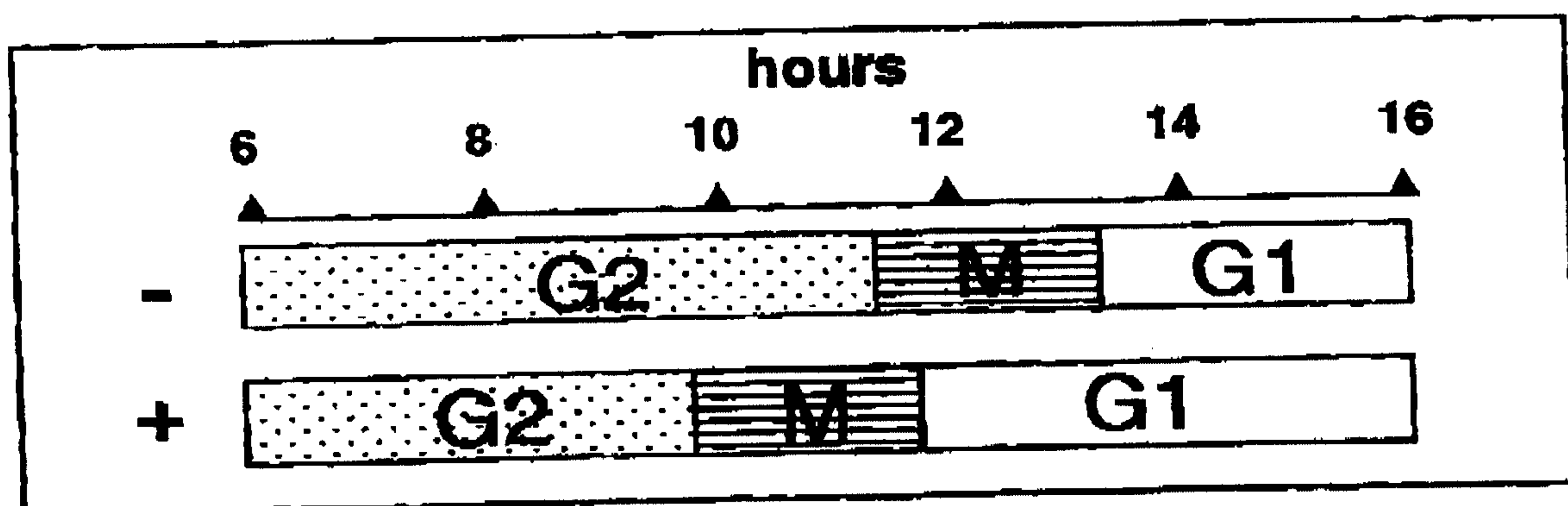
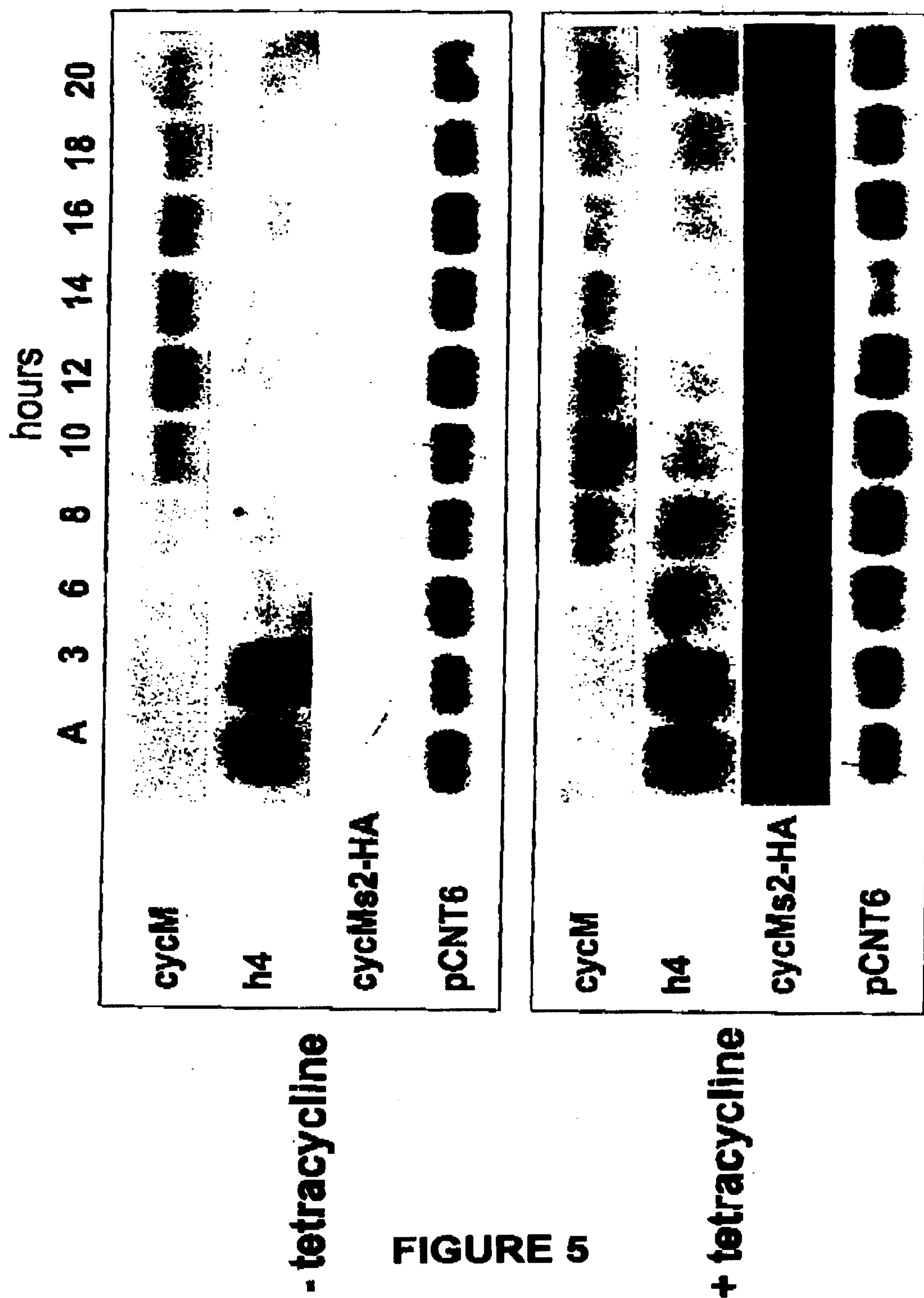


FIGURE 4-2

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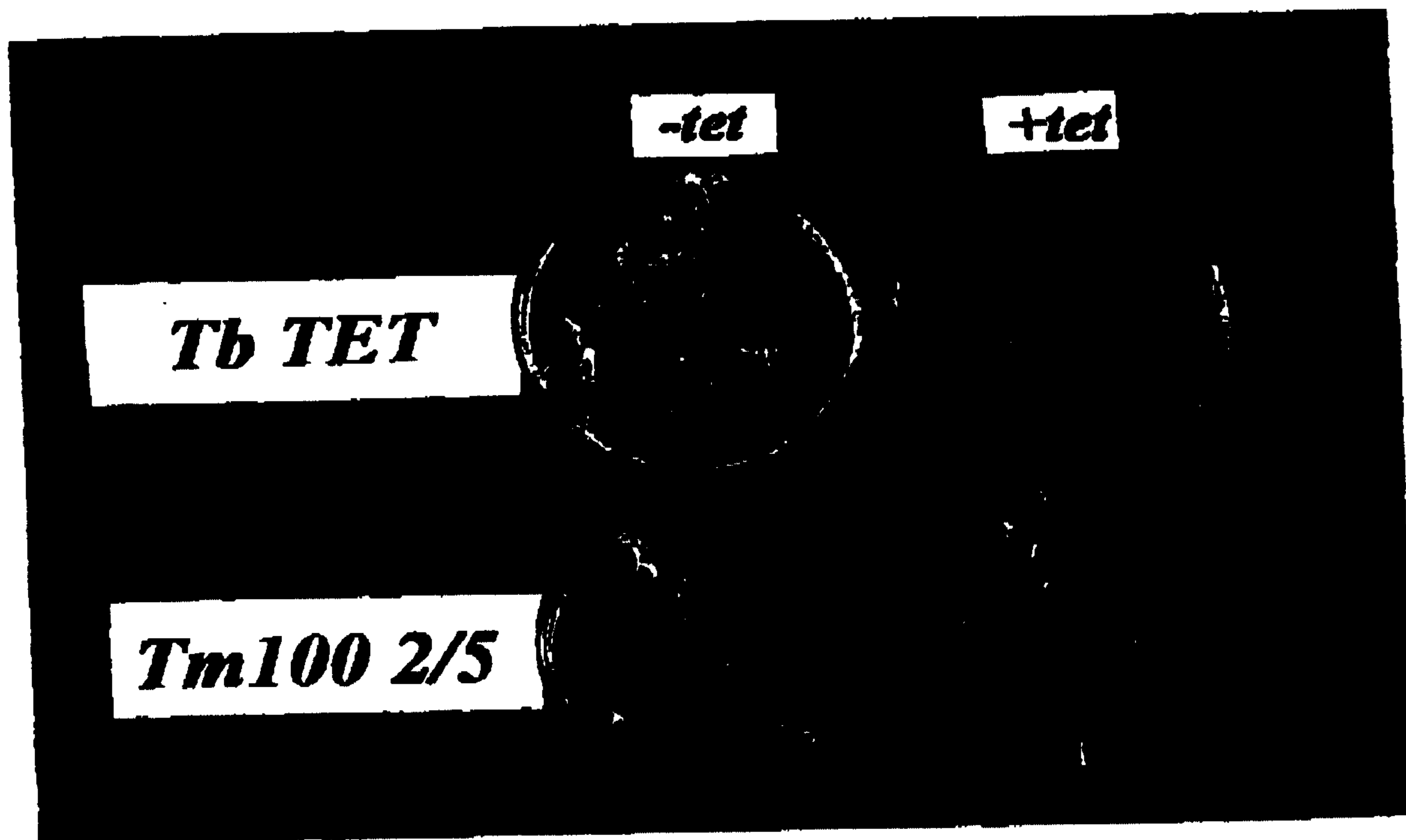


FIGURE 6

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Tm100

NAA/BAP

-tet

+tet

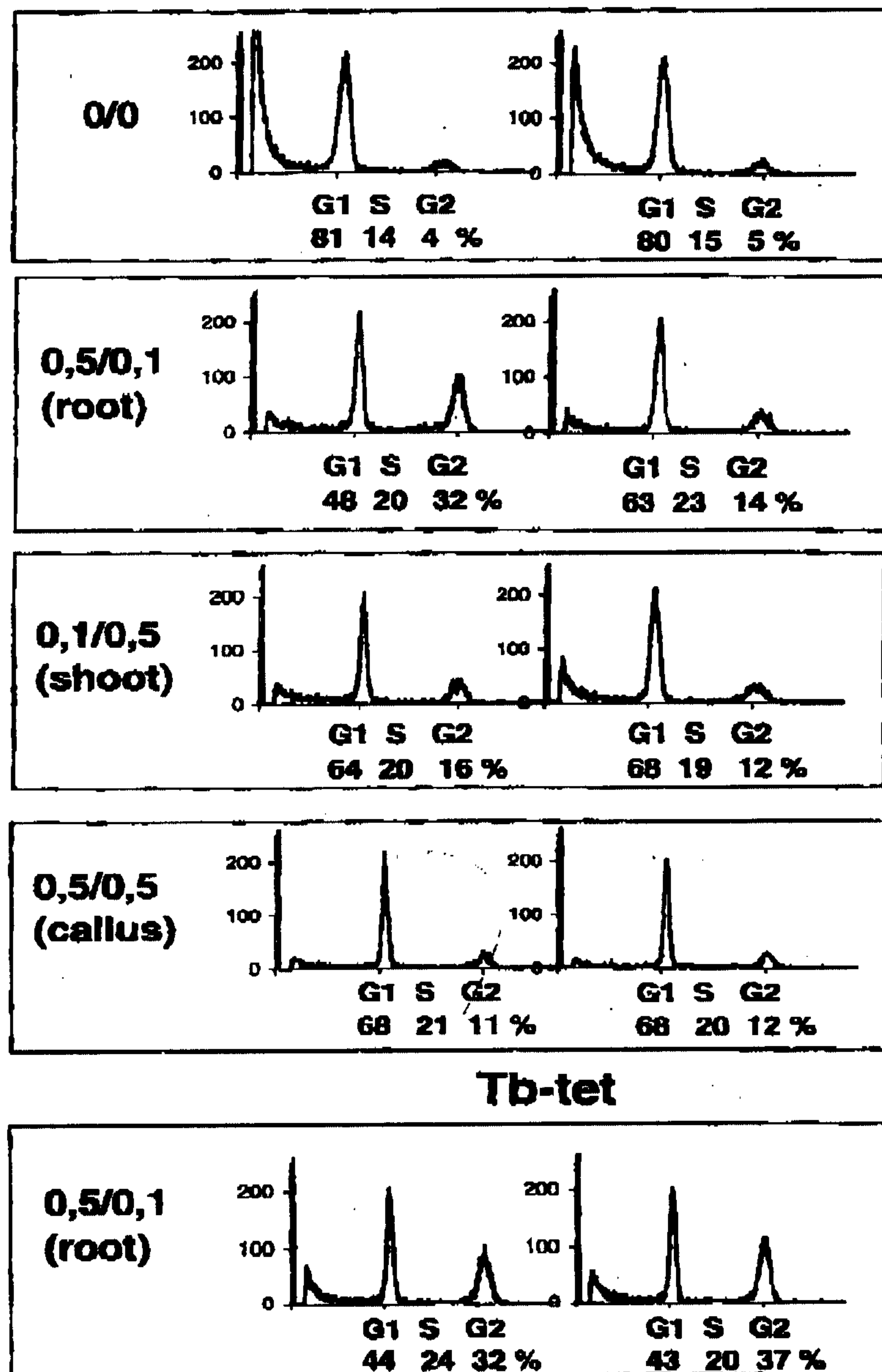


FIGURE 7

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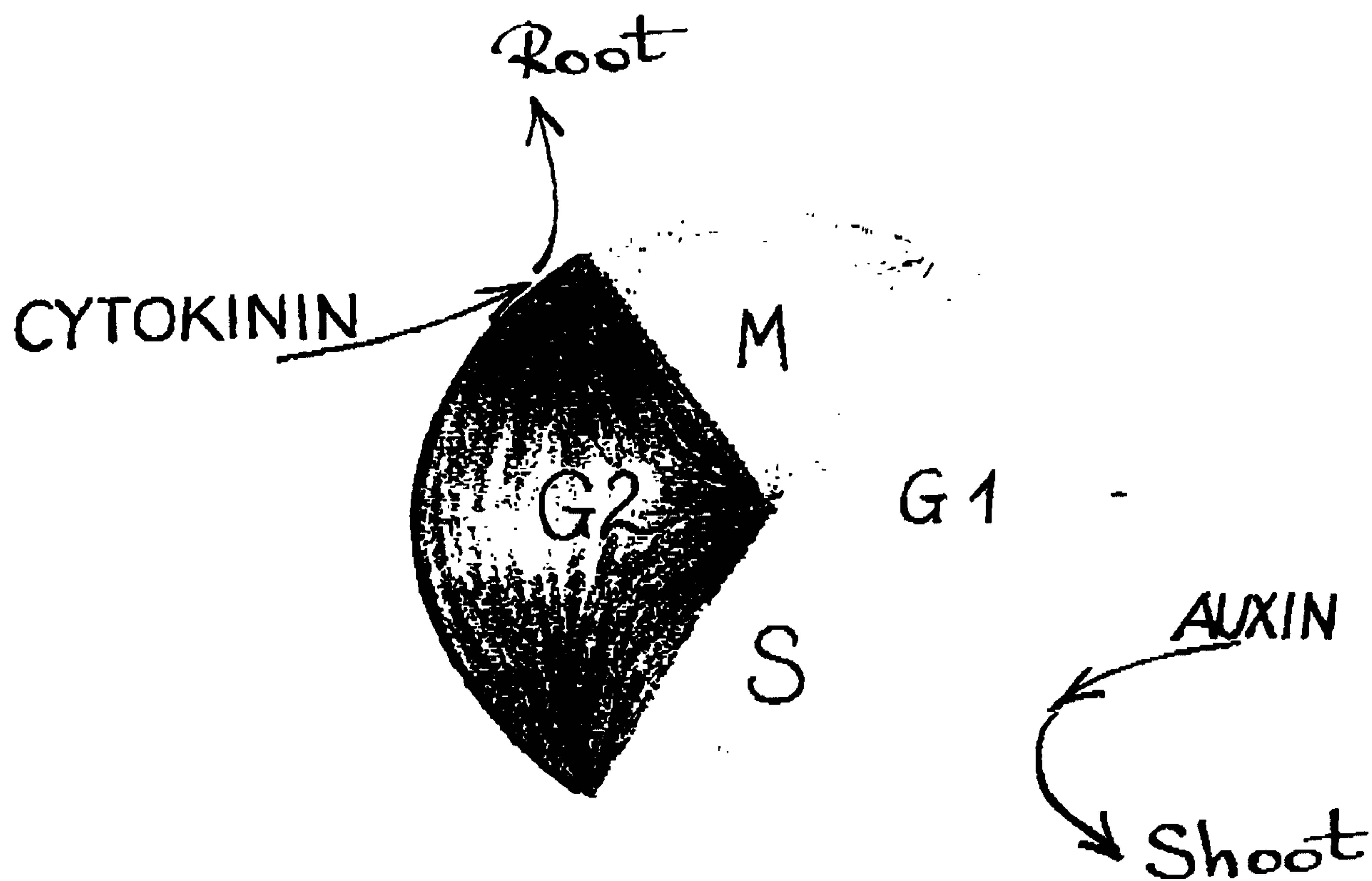


FIGURE 8

