Functional and developmental analysis of cell cycle control genes in alfalfa (*Medicago sativa*) and snapdragon (*Antirrhinum majus*)

A Ph. D. Thesis
presented by

Damla Dedeoglu Bilgin

Supervised by
Prof. Dénes DUDITS

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Sevgili Annem'e

ABSTRACT

Cell division is a complex process that is fundamental to survival and propagation of all organisms, including higher plants. Plants have unique developmental features that distinguish them from other eukaryotes. As they are immobile, morphogenesis is determined only by cell division and expansion. Complex organs are formed by special region called meristems. Most differentiated cells have the potential to dedifferentiate and to reenter the cell cycle that's why the plant cells must maintain a strict control of the temporal, developmental and spatial aspects of cell division and cytokinesis.

In this thesis, by using alfalfa (*Medicago sativa*) and snapdragon (*Antirhinnum majus*) plants as model systems, the cell cycle machinery is studied extensively. The homologues of one of the main components of the machinery, cyclin genes are isolated. By RT-PCR analysis, the transcriptional activity of cdks and cyclins are studied in hormone-stimulated alfalfa mesophyll cells. The effects of different hormones and especially synthetic auxins on the alteration of the gene expression pattern via auxin receptor and formation of somatic embryos are analysed. Effects of other hormones (NAA, kinetin, 24-epibrassinolid and abscicis acid) on cdks and cyclin expressions are studied. Effects of several concentrations of these hormones and their kinetics are determined. For further characterisation of isolated alfalfa cell cycle control genes, they are transiently over-expressed in alfalfa, tobacco and maize protoplasts and histone-GUS chimeric expression cassettes are used as an indicator of S-phase activation and deactivation.

In *A. majus* meristems, expression patterns of two different D-cyclins are analysed by in situ mRNA hybridisation. A MYB transcription factor gene mutant line-*Phantastica* was used to analyse the expression relation between myb gene and D-type cyclins.

This research addressed the fundamental questions on regulation of gene expression during cell division and plant development. Plants are ideal organisms that allow the use of both basic genetic approaches and molecular-cellular tools to investigate such complex mechanisms. As most of the regulatory mechanisms are conserved among eukaryotes, anticipated results would also be used in studies on the "control of growth and cell cycle" in other organisms including mammals. With possible findings, I also expect to contribute to a rather simple but important question "why plants do not get cancer".

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NOMENCLATURE

ABA	Abscisic acid
AldR	Aldose Reductase
BAP	Benzylamino purine
BY-2	Bright yellow 2
Cdc	Cell division cycle
Cdk	Cyclin-dependent kinase
Cyc	Cyclin
2,4-D	2,4-Dichlorophenoxyacetic acid
DMSO	Dimethyl sulfoxide
DTT	$_{DL}$-Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethyl ether)
FCM	Flow cytometry
IAA	Indole acetic acid
MAPK	Mitogen Activated Protein Kinase
MPF	Maturation Promoting Factor
MS	Murashige-Skoog
Msc	Medicago sativa clone
NAA	Napthylacetic acid
PBS	Phosphate Buffered Saline
Phan	*Phantastica*
RT-PCR	Reverse Transcription and Polymerase Chain Reaction
SSC	Saline sodium citrate
SDS	Sodium Dodecyl Sulfate

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CHAPTER I
INTRODUCTION

1.1 *The Cell Cycle Control in Plants*

Cell division is a complex process that is fundamental to the survival and propagation of all organisms, including higher plants. It has been shown that the basic mechanism of cell cycle control is conserved among eukaryotes. Compared to most animals, plants have a somewhat unusual life style, to which many aspects of their morphogenesis and cell division are adapted. Presence of a rigid cell wall prevents the cell migration and consequently organogenesis is dependent on cell division and cell expansion at the site of formation of new organs. Most cell proliferation occurs in specialized regions called meristems. As the cells are displaced from the meristem, cells differentiate and expand. Expansion is sometimes associated with endoreduplication, a process by which the DNA in re-replicated without intervening mitoses. There is many debates as to whether cell proliferation is a driving force in plant morphogenesis or merely a response to growth and sub-divides space. The cells that go to form organ primordia behave differently from those that remain in the meristem. Those that remain in the meristem are "stem" cells, that is they divide relatively slowly but indefinitely and do not differentiate. Their progeny, which are displaced from the meristem, can have one of two fates. One fate involves the formation of stem tissue and cells following this path tend to expand extensively and rarely divide. The other fate involves the formation of leaves. Cells following this path initially divide very rapidly and then differentiate. The meristem identity itself can change from a vegetative to a reproductive phase, resulting in the formation of flowers. A key problem in plant biology is understanding why these different populations of cells behave differently. The plant development is mainly post-embryonic. During embryogenesis, the main developmental event is the establishment of the root-shoot axis. Most plant growth occurs after germination, by iterative development at the meristems. Structurally, the plant cell cycle differs from that of animals, particularly in the early stages of mitosis; before spindle formation, a microtubule array called the pre-prophase band defines the future division plane of the cell. At the end of mitosis, another microtubule array, the phragmoplast, is involved in cytokinesis. Mutants defective in cytokinesis have dramatic effects on plant development (Jurgens, 1997).

1.2 *Conserved and novel cyclin-dependent kinases (cdk) in the plant cell division cycle*

Plants contain an extended cdk gene family. Plant cdk genes were identified in *Pisum sativa* (pea), *Medicago sativa* (alfalfa), *Oryza sativa* (rice), *Petunia hybridia, Glycine max* (soybean),

Antirrhinum majus (snapdragon) and *Arabidopsis thaliana.* In addition to cognate homologs of cdc2, which encode proteins that contain a perfectly conserved PSTAIRE motif, T loop and catalytic domain, plants express unusual cdc2-related genes which may be unique to this group. Structurally, these unusual cdks align with cdc2 (i.e. they belong to the cdk1 group), but they differ from the cdc2 kinases, most notably in that they contain variant PSTAIRE-motifs and these genes will be referred as cdc2-variants.

At least two classes of cdc2-variants are recognized: One contains a PPTALRE motif (Am-cdc2c, At-cdc2b, and Ms-cdc2d) the other contains a PPTTLRE motif (Am-cdc2d, Ms-cdc2f) (Fobert *et al.*, 1996, Magyar *et al.*, 1997, Sauter, 1997) This extended cdc2-like gene family may have arisen by gene duplication in plants soon after the animal and plant lineage diverged; although there are no reports of similar cdc2 variants from animals, the moss *Physcomitrella patens* (a primitive land plant) also expresses a related cdc2-variant, PATTLRE.

Expression of both cognate and variant cdc2 genes is associated with cell proliferation but there are significant differences in the timing (Fobert *et al.*, 1996, Magyar *et al.*, 1997). For cognate cdc2 genes, levels of both transcript and protein tend to be constant throughout the cell cycle although actively proliferating tissues tend to contain significantly higher levels than differentiated tissues. The variant cdc2 genes are expressed at specific phases of the cell cycle, PPTALRE cdc2-variant is expressed in the S-phase through to M-phase where the PPTTLRE variant is expressed for a much narrower window in G2 and early M-phase.

Available data suggest that these closely related cdc2 proteins are recruited into different kinase complexes with distinct periods of activity. Activation of kinase complexes containing cognate cdc2 genes is periodic with peaks at G1/S and G2/M but, in G2 alfalfa cells, the cognate cdc2 complexes are activated as a H1 kinase prior to PPTTLRE-cdc2 complexes. The reason for having an extra G2-activated cdc2 kinase could be because of PPB-is a transient microtubule structure which predicts the division plane, is formed in G2 and disappears by metaphase-.The mid-G2 peak of kinase activity may reflect activation of a preprophase band (PPB) associated cdc2 or that other, as yet undefined, mitotic events are induced by the cdc2-variant kinases. Kinases, probably cdc2-related, are implicated in PPB assembly; both assembly and disassembly are sensitive to kinase inhibitors (Katsuka and Shibaota, 1995) and microinjection of active cdc2 kinase (Hush *et al.*,1996) or algal MPF (Zhang *et al.*, 1996) results in PPB disassembly. A cdc2-related kinase, one of the two cyclin B1 proteins and a cyclin A1 transiently associate with the PPB in maize (Mews *et al.*, 1997). The other cyclin B1 protein is present in the nucleus until the nuclear envelope disassembles at mitosis and remains associated with the spindle. There is therefore a spread of circumstantial evidence to link the pre-mitotic cdc2 activity with PPB formation.

Taken together the data suggest that the cognate and variant cdc2 have distinct roles in the plant cell cycle. The plant cdc2 cognate genes appear functionally very similar to cdc2, because when expressed in yeast, they complement or partly complement selected cdc2/28 mutants (Fobert *et al.*, 1996, Hirt *et al.*, 1993). In planta functions have yet to be attributed to the cdc2 variants but when expressed in yeast, they fail to complement and can even interfere with cell division. This suggests that their ability to interact with the yeast cell cycle machinery is conserved but their function has significantly diverged. Mutants in these genes would be extremely informative.

A variety of other cdk-related kinases have been isolated but their role in cell cycle progression is unknown. The R2 gene from rice encodes a protein that is structurally similar to mammalian cdk7 and R2 transcripts accumulate to higher levels in G1 than during the rest of the cycle (Sauter, 1997). The alfalfa cdc2MsC gene encodes a 57kD protein similar to human CHED kinase and a human PITALRE kinase, which can phosphorylate Rb in vitro (Magyar *et al.*, 1997). A partial cDNA, cdc2MsE, encodes yet another distantly related kinase with a novel PSTAIR motif.

1.3 *A and B-type of Cyclins in Plants*

Cell cycle studies have been triggered in higher plants by the importance of the proper control of the cell division during plant development with respect to meristem activity, secondary organ formation, growth factor response, nodulation and *in vitro* regeneration (Shaul *et al.*, 1996, Jacobs, 1992). The conservation of the basic tools of the cell cycle machinery amongst eukaryotes has enabled the cloning of plant homologues of cyclins. Plants tend to have rather large numbers of cyclins. However, based on detailed comparative analysis of large numbers of cyclins from different plant species, plant cyclins have been classified into three groups of A-type cyclins, two groups of B-type and at least three D-types (Renaudin *et al.*, 1996). From an evolutionary point of view, it is arguable as to whether the major classes of cyclins had evolved and developed specialised functions prior to plant-animal divergence but considerable radiation has occurred since then, giving plants their own distinctive cyclin sub-families.

"A" type cyclins display phase-specific gene expression but their period of expression is quite broad and could not be so dramatically defined as the B-cyclins. Cyclin A transcripts increase and may even peak in S-phase but continue into G2 and M phase (Ito *et al.*, 1997, Reichheld *et al.*, 1996, Shaul *et al.*, 1996). An alfalfa cyclin A gene, cycMs3, is induced very early (within an hour) after quiescent cells are induced by wounding and incubated in hormone containing medium (Meskiene *et al.*, 1995).

The B1 cyclins, the best-characterized group, are expressed at, and may be rate-limiting, for transition across the G2/M boundary. Cyclin B1 transcripts accumulate specifically in dividing cells in G2 and M phases (Doerner *et al.*, 1996, Fobert *et al.*, 1994, Ito *et al.*, 1997, Kouchi *et al.*, 1995) and specific

cyclin B antibodies identify active cdk (the identity of the cdk is unknown) complexes during G2 in synchronized alfalfa cells (Magyar *et al.*, 1997). Ectopic expression of the Arabidopsis B1 cyclin gene, cyc1At, under the control of the cdc2a-At promoter accelerated root growth without inducing any developmental abnormality (Doerner *et al.*, 1996). The extra growth was due to the formation of extra cells, suggesting that progression through the cell cycle had been significantly enhanced. Although the phase of the cycle affected was not determined experimentally, the cyclin B protein is probably only stable during G2 and so the simplest explanation is that G2 would be shortened in these plants. Given that at least some plant cells contain a cytokinin-dependent G2 checkpoint, this seems a reasonable expectation.

Cyclin B promotes G2/M progression and its availability limits growth. Several groups have shown 5' regions of the cyclin B gene can direct G2 specific expression (Ito *et al.*, 1997, Shaul *et al.*, 1996). Four repeats of an 9bp element, similar to MYB protein binding sites and termed MSA (for "M-specific activators") elements were found in the 5' regions of other M-phase specific genes, including B-cyclins and kinesin-like genes, from other plant species. (Ito *et al.*, 1997). The cdc2a-At gene contains multiple binding sites for MYB proteins in a region of its promoter that enhances gene expression (Chung *et al.*, 1995)

1.4 *D-type of Cyclins and the Rb/cyclin D pathway for regulating G1/S specific gene expression.*

The various lines of evidence point to D-type of cyclins and Rb acting as integrators of both positive and negative signals for cell proliferation. D-type cyclins are an important mechanism, which the extracellular growth factors, induce their expression by way of a MAP-kinase signal transduction pathway. These signals may be proliferative and promote the commitment to division. On the other hand they may stimulate differentiation, probably by by the dual mechanism of slowing G1 progression, accompanied by by the concomitant activation of specific sets of genes that cause alternative pathways to be followed.

A cyclin D/CDK4 kinase phosphorylates the Retinoblastoma protein (pRb); E2F complex, an inhibitor of S-phase gene expression. E2F is then released to promote S-phase gene transcription. An analogous pathway has been proposed whereby plant growth substances affect cell proliferation by controlling the production of G1 cyclins (Murray 1997). Three different D-type cyclins were isolated by complementation of yeast G1 deficient mutants with plant cDNA libraries (Dahl *et al.*, 1995, Soni *et al.*, 1995). Cyclin D3-At gene expression in quiescent cells is strongly stimulated by the plant growth factor, cytokinin, while cyclin D4-Ms is induced prior to S-phase by wounding. The expression of other D-cyclins response to other types of extracellular factors important for plant growth; for instance, cyclin D2-At expression requires a carbon source but not cytokinin and some of them (cyclin D3At and D1At)

show a tissue specificity of expression that is not directly related to the proliferative state of the tissue involved.

Plant D-cyclins contain an Rb binding motif LxCxE in the N-terminus that enhances their binding to both plant and animal pRb (Huntley *et al.*, 1997). Rb related proteins are also developmentally regulated during leaf growth, being expressed more highly in non-dividing regions. Maize pRb undergoes changes in level and phosphorylation state concomitant with endoreduplication, and it is phosphorylated in vitro by an S-phase kinase from endoreduplicating endosperm cells (Grafi *et al.*, 1996). The maize Rb protein has the capacity to prevent DNA replication as judged by its effect on virus replication (Xie *et al.*, 1996) and, when expressed in mammalian cells, can prevent E2F mediated gene transcription pRb (Huntley *et al.*, 1997). Together, these results suggest that the maize Rb is a representative of the pocket protein family and may play a role in cell cycle progression.(Horvath *et al,* 1998)

1.5 *Cyclin-dependent kinase inhibitor (cdk inhibitor) genes in plants.*

Despite the isolation of several cyclin-dependent kinase inhibitor genes in mammalian system, there are very few examples in plants. The first cdk inhibitor like gene was isolated from *Arabidopsis thaliana* (Wang *et al,* 1997). It was named ICK1 and structurally it shares limited similarity, with the mammalian p27Kip1 cdk inhibitor. The region of similarity (77%) is restricted to a 31 amino acid stretch, corresponding to the C-terminal portion of the p27Kip1 cdk inhibitory domain (Polyak *et al,* 1994). Otherwise, ICK1 is structurally distinct from p27Kip1. The region of similarity is located in the C-terminal of ICK1, in contrast to the N-terminal in p27Kip1, and ICK1 contains a strong coil-coiled domain absent in the mammalian protein. Outside the 31 amino acid conserved region, the remainder of ICK1 shows no homology to p27Kip1 or to any other known proteins. Recombinant ICK1 specifically inhibits the histone H1 kinase activity of plant cdc2-like kinases, but has no effect on human $p34^{cdc2}$ activity. When the ICK1 was cloned under CaMV 35S promoter and transformed into tobacco leaf protoplasts, flow cytometry analysis revealed a 50% decrease in the number of cells entering into S-phase (Dedeoglu *et al,* submitted). Another plant G1 cyclin-dependent kinase inhibitor gene from a photoautotrophic cell suspension culture of *Chenopodium rubrum* (red goosefoot) was isolated (Fountain *et al,* submitted).

1.6 *MYB Transcription Factors in Plants*

The cloning of the first transcription factor from plants, the C1 from maize, indicated that plants use transcription factors that are structurally related to those of animals and are implicated in regulating cell proliferation with a large number of gene regulatory roles (reviewed by Martin and Paz-Ares, 1997). In plants there is good evidence for distinct functions for different MYB proteins, some controlling

secondary metabolism, some regulating cellular morphogenesis and some serving in the signal transduction pathways responding to plant growth regulators. Within these groups there are sub groups of MYB proteins with overlapping functions. One plant MYB gene is closely related to the c-MYB sub-group, a factor which enhances G1/S progression by activating transcription of the mammalian cdc2 kinase gene. A MYB protein has been shown induce ectopic cell divisions, albeit within a restricted developmental context: expression of a myb gene, MIXTA, from Antirrhinum in tobacco results in the formation of multicellular trichromes from epidermal cells which would otherwise have exited from the cell division cycle. Taken together, these data strongly implicate MYB proteins as playing a role in regulating cell cycle progression in plants.

The *Phantastica* mutant of *Antirrhinium majus* used in this study is a *MYB* transcription factor mutant (personal communication). The mutation is reflected to the phenotype when the plants are grown under 25°C. This mutation have several effects on the morphogenesis of the *A. majus* plant. When the plants are grown under 25 °C, they form needle like and heart shaped leaves. The flowers are wrinkled and an asymmetry is observed in the leaf formation. When the *Phantastica* mutant were grown at 15 °C, they cease growth and the plant cannot develop at all. It is proposed that the effects of mutations on leaf morphogenesis is equally applicable to development of petal lobes (Waites and Hudson 1995).

1.7 *Cell cycle mutants in higher plants*

To understand the spatial and temporal regulation of mitosis during plant development, it is not enough to search the plant homologues of yeast and animal genes. An essential tool in the elucidation of the cell-cycle phosphorylation cascade has been the use of mutational genetic dissection. However it is important to select the set of characteristics that will allow us to identify relevant mutations affecting directly the spatial regulation and mechanisms of higher plant cell cycle control.

In plants, most of the mitotic activity is restricted to the meristems and the young growing tissue surrounding them. The primary meristems have to initiate the formation of organs and determine their basic structure whereas another type of meristematic cell, the cambial cell, is responsible for secondary growth and increasing the width of stems and branches. The functional complexity is reflected to meristem structure, the layers in the apical meristem L1, L2, L3 corresponds to epidermis, mesophyll, and vascular tissues, respectively. In the shoot apex there are two different zones, central zone, peripheral zones which are thought to be involved in meristem maintenance and organ initiation, respectively. Between zones there are differences in the mitotic activity. Cell division does not only occur within the meristem, meristemoids -the cells that will form the stomatal complexes- and the cells in the young growing organs retain mitotic activity.

The search for mutants perturbed in the spatial control of cell division is concentrated on *Arabidopsis* because of shorter life cycle and the smaller genome size compared to other higher plants. Mutants from other plants also exist.

There are partially characterized sets of genes regulating the cell proliferation in the meristems. The first gene of interest is KNOTTED (KN1) in maize which is a member of a family of a homeobox genes necessary for installing and maintaining meristematic activity. KN1 was characterized by extra cell divisions in and around the vascular tissues (Jackson *et al.*, 1994, Vollbrecht *et al.*, 1991). mRNA *in situ* hybridizations showed that this gene was only expressed in vegetative meristems in wild-type plants. It has been introduced into tobacco under the control of 35S promoter and again extra cell divisions were observed in and around the vascular tissues (Sinha *et al.*, 1993). The exact role of KN1 is not known, as mutations reducing its activity have not been isolated yet. Another member of the KN1 family was identified by mutation; the SHOOTMERISTEMLESS (STM) gene (Barton *et al.*, 1993, Long *et al.*, 1996). Inactivation of this gene resulted in complete absence of meristematic and mitotic activity at the shoot apex. Later on, two loci were identified that, when mutated block outgrowth of the root meristem, ROOTMERISTEMLESS (RML) in *Arabidopsis* (Cheng *et al.*, 1995). The mutants were able to form embryonic root meristems however development is blocked only postembryonically, as root cells are unable to divide further.

The second group of genes controlling meristem activity functions in a different way. Mutations in these genes cause meristems to increase in size dramatically, which can be accompanied by either an increased or a reduced production of organs. They seem to restrict the size of the meristem and, therefore, the number of mitotically active cells in the growing plant *clavata* (*clv* 1-3) (Clark *et al.*, 1993, 1995), *fasciata* (fas1-2) (Leyser *et al.*, 1992) and *mgoun* (*mgo*)in *Arabidopsis*. Similar phenotypes are found in many species (reviewed by Gorter, 1965).

Cell proliferation in the meristems is also dependent on environmental factors and light is the best characterized factor which is an absolute prerequisite for activation of the shoot and (to a lesser extent) the root apical meristems after germination, in the dark cell proliferation is inhibited by a set of genes called *DEETIOLATED* (*DET*) and *CONSTITUTIVE PHOTOMORPHENIC* (*COP*), respectively (Chory *et al.*, 1989, 1994, Deng *et al.*, 1991). When these genes are inactivated the shoot and root meristems are active in the dark, forming leaves and lateral roots. *DET* and *COP* genes must be necessary to keep the meristematic cells in a 'non-competent' state in the dark.

There are also genes that are involved in cell division control outside the meristems. When the group of cells has left the shoot apical meristem, they go through a limited number of divisions before mitotic activity ceases and the cells are arrested in the certain stage of the cell cycle. There are sets of genes necessary to inhibit the cell divisions outside the meristem. Mutants that are examples of this

group are; *mgoun* -showed ectopic cell division and the formation of meristemsalong the stem-, *SUPERROOT* (*SUR*) gene -inhibits extra cell divisions and root meristem formation in the root and the hypocotyl (Boerjan *et al.*, 1995). Some set of genes has the opposite function and they are necessary to keep populations mitotically active, until sufficient cells are generated otherwise the number of cells in the differentiated organs is reduced. Such as; *pale cress* (*pac*) and *cristal* -mutants have reduced numbers of mesophyll cells (Reiter *et al.*, 1994, Delarve *et al.*, 1997), *pin* and *pinoid* -mutants show reduced capacity to generate mature flowers, flower organs and cauline leaves (Okada *et al.*, 1991, Bennett *et al.*, 1995).

Division plane determination is another aspect, which characterizes the dividing cells in the developing plant. Microscopical analysis revealed that there is a structure responsible of predicting and fixing the plane of division, it is called preprophase band (PPB), appears at G2 and disappears at late prophase. It is assumed that presence of PPB is essential for normal morphogenesis however it is not known to what extension the division plane alignment is essential for plant morphogenesis. Several mutants were identified from different plant species which showed perturbed division plane alignment, such as; *gib-1* in tomato (Barlow *et al.*, 1993), *hyp-2* in *Nicotiana plumbaginifolia* (Traas *et al.*, 1995a), *TAN* in maize (Smith *et al.*, 1996), *GNOM, FASS, TONNEAU,* in *Arabidopsis thaliana* (Mayer *et al.*, 1993, Tores-Ruiz and Jurgens 1994, Traas *et al.*, 1995b). *gib-1, hyp-2* mutants showed abnormal cell alignment in the root meristems by forming extra cell layers, *TAN* mutant has an altered shape and arrangement of cells in all tissues and has perturbed longitudinal division planes, *GNOM* showed abnormal first division plane, *FASS, TONNEAU* mutants had no PPBs.

It is unlikely to catch a basic cell cycle machinery mutant because the gamets carrying such mutations would not be viable. It is also possible to find leaky mutations where the protein retains some of its activity. Moreover, most of the cell cycle control genes are shown to be multigene families (cyclins and cdks) so the related genes may substitute for the inactivated members of the same family.

1.8 *Phytohormones and Somatic embryogenesis in Plants.*

Plants need light (sugar), water, oxygen, carbon dioxide, minerals, good temperature and freedom from external disturbances, including diseases, to survive and thrive. The major plant hormones are made by plant cells in order to transmit information, and carry out actions which are necessary in the case of inadequacy or abundance of stimulators. There are two general classes of hormones found in animal system and probably also occurs in plant systems; steroids and peptides. The steroid class forms a hormone/receptor complex in the cytoplasm which is then transported into the nucleus where mRNA is synthesized, resulting in a given response. The peptide hormones which bind to a receptor at the plasmamembrane, altering the enzyme adenylate cyclase and activating cyclic AMP

from ATP, which acts as a second messenger for a given response. In plants phytohormones are grouped as follows; Auxins, cytokinins, giberellins, abcisic acid, brassinosteroids, ethylene, salicylates and jasmonates.

Auxin is a generic term representing a class of compounds which are characterized by their capacity to induce elongation in shoot cells. They are made by any meristematic cell faced with an abundance of shoot derived nutrients (sugar, carbon dioxide and oxygen). It causes the initiation of new roots in order to balance out the excess of shoot nutrients. Exogenous application of auxins can also promote root initiation and early development of the root. However it has no effect on root elongation or is inhibitory at higher concentrations (Blakesley *et al.*, 1991). It stimulates the storage of shoot derived nutrients, including gases. Besides of root initiation and cellular elongation there are other known responses of auxins; phototropism, geotropism, apical dominance, ethylene production, fruit development, parthenocarpy, abscission.

Cytokinin is made by any meristematic cell faced with an abundance of root derived nutrients (water and minerals). It stimulates the initiation of new shoots in order to balance out its root nutrients. It also stimulates the storage of root derived materials for future scarcity. Endogenous cytokinins have an inhibitory effect on root formation and growth (Okoro and Grace, 1978), moreover exogenous applications of cytokinins are not beneficial to adventitious root formation (van Staden and Harty, 1989)

Brassinosteroid is made by meristematic cells under good growth conditions. It is released when there is no significant external treat from extreme temperature, disease, physical trauma, such as wind or insects or toxins of various biological or man made sources. The principal function of brassinostereoids is the regulation of photomorphogenesis and cell elongation. It promotes a generalized storing of all nutrients, balancing this with growth.

Giberellins are synthesized via the mevalonic acid pathway in young actively growing shoots and developing seeds. When any non-meristematic cell is faced with a scarcity of shoot derived nutrients and causes the release of stores of shoot derived nutrients, as well as the shrinking of unneeded root cells. It also attempts to increase shoot derived materials by bolting which brings the plant into a better atmosphere and light. Giberellins have been shown to be involved in many other physiological processes; stem growth, bolting/flowering, seed germination, dormancy, sex expression, senescence, parthenocarpy, fruit set and growth.

ABA is made by any non-meristematic cell faced with a lack of root derived nutrients. The main known function of ABA is the regulation of adaptation to dessication and osmotic stress. ABA also adjusts root and shoot size in order to increase water and mineral consumption. It releases the stores of root derived materials and may stimulate a form of root bolting. However there are conflicting reports on the effects of exogenously applied ABA on adventitious rooting (Davis 1989, Hartmann *et al.*, 1990).

Ethylene is a simple unsaturated hydrocarbon, produced in all higher plants. It is also involved in many physiological processes in plants from seed germination through senescence and death of the plant including fruit ripening, dormancy, abscission, flowering, senescence, shoot and root growth and apical dominance.

Salicylates are a class of compounds having similar activity to salicylic acid (SA) which is a plant phenolic. SA has been identified in leaves and reproductive structures of plants, with the highest levels reported in the inflorescence of thermogenic plants and plants infected by necrotizing pathogens (Raskin 1992a, 1992b). SA has major effect on a variety of plant processes; Flowering, heat production in thermogenic plants and promotion of disease resistance.

Jasmonates have been shown to be biosynthesized in stem apex, young leaves, immature fruits and root tips (Sembdner and Parthier 1993). When it is exogenously applied, it promotes senescence, petiole abscission, root formation, tendril cooling, ethylene synthesis, and β-carotene synthesis (Stanswick 1992). Moreover it has been shown to inhibit seed germination, callus growth, root growth, chlorophyll production and pollen germination (Anderson 1989, Parthier 1990, Vick and Zimmermann 1986). Endogenous jasmonic acid levels have been shown to increase in response to external stimuli such as wounding, mechanical forces, elicitors from pathogen effect and osmotic stress (Sembdner and Parthier 1993). No synthetic forms of jasmonates are commonly used.

The cell division is more discretely localized in plants compared to dispersed cell division and cell death in animals. The cells of the plant zygote are unable to migrate so the plant morphogenesis is dependent on the precise spatial and temporal control of the cell division. Wide adaptation capability of plants that can be explained as a quantitative estimation of the range of environmental conditions to which an individual of a particular genotype can adapt. The high capability of adaptation of an individual depends on the phenotypic plasticity of its every character. An individual genotype assumes particular characteristics in a given environment. In a second environment it may remain the same or it may be different. The amount by which the expressions of individual characteristics of a genotype are changed by different environments, is a measure of the plasticity of these characters (Bradshaw, 1965). There are three principal features of phenotypic plasticity (Smith, 1990).

1) Plasticity is manifested as the flexible expression of a constant genotype.

2) Plasticity is intimately connected with the environmental variations.

3) Plasticity confers adaptive value in respect of the capacity of the individual organism to adapt or acclimate to environmental conditions.

Therefore, plasticity is shown by a genotype when its expression is able to be altered by environmental influences. In most of the plants the flexibility of the differentiation program enables the generation of the embryogenic cell stage in a fully differentiated cell under certain conditions. This can

include instability in morphogenic and developmental programs and responses to damaging external conditions in order to ensure survival and reproduction. The embryogenic response in cultured plant cells can be positively correlated with the increasing potential of single character for developmental plasticity. A totipotent state can be generated in somatic cells by reprogramming of the gene expression pattern. In somatic embryogenesis, the induction of the totipotent state is under the control of several external and internal factors. Especially exogenously applied synthetic auxins i.e. 2,4-D (2,4-Dicholorophenoxy Acetic Acid) can reprogram differentiated somatic cells to become totipotent and to reach the developmental potential similar to that of the egg cells after fertilization (Dudits *et al*, 1991a, Brummel *et al*, 1987, Sung *et al*, 1983). It triggers the embryogenic pathway of somatic cells both in dicotyledons (Ammirato *et al*, 1977, Sung *et al*, 1984) and monocotyledons (Vasil *et al*, 1987, Wernicke *et al*, 1987).

Other than the involvement of auxin transport in the initiation of the somatic embryogenesis The possible roles of calmodulin (Overvoorde and Grimes, 1994) and lea proteins (Kiyosue *et al.*, 1993, Vivekananda *et al.*, 1992, Wurtele *et al.*, 1993) are also considered. Extracellular lipid carrying proteins (EP) are also known to be involved in the regulation of cell expansion and the maintenance of biophysical features required for morphogenesis (Sterk *et al.*, 1991, van Engelen *et al.*, 1992).

When the comparison of events of fertilization in sea urchin embryos and auxin treatment in somatic cells has been accomplished, it is seen that most of the molecular changes occur in both cases (Dudits *et al*, 1995).

	FERTILIZATION	AUXIN TREATMENT In Somatic Cells
Membrane depolarization	+	+
PIP_2 hydrolysis	+	+
Ca^{2+} release	+	+
-cortical granule exocytosis		
-NAD kinase		
Na^+-H^+ exchange	+	+
-pH_i	increase	decrease
Oxygen consumption increase	+	+
-H_2O_2 production	+	?
Actin polymerization	+	?
K^+ conductance	+	+
Transport changes	+	+
Endocytosis	+	?
Protein synthesis	+	+
Pronuclear movements	+	?
Cyclin accumulation	+	?
DNA synthesis	+	+
MPF activation	+	?
Nuclear envelope breakdown	+	+
Cell division	+	+

Even with these findings, the complete pathway of somatic embryogenesis is yet unclear. Alfalfa (*Medicago sp.*), which is used in this study, is an open pollinated plant and only some of its genotypes are able to produce callus or cell suspension with the ability to regenerate plants. This regeneration ability is an inherited character (Kao, 1980, Dijak, 1987, Song, 1990). Differences in auxin sensitivity of the cells can be suggested as a limiting factor in the complex interaction between cells and synthetic hormones. Application of stresses to plant systems can induce the embryo formation, therefore, it is important to observe the changes in the gene expression of the cell cycle control genes. Not only 2,4-D but other stress factor such as; giberellic acid (GA), salicylic acid (SA), abscisic acid (ABA) heat

treatment, cold treatment, osmotic changes can provide in formation as well as the analysis of embryo defective mutants in maize and *Arabidopsis* (Meinke, 1995).

1.9 *Use of Histone H3::GUS::H3 terminator construct as an indicator of S-phase.*

The replication-dependent class of histone genes from higher plants including alfalfa, maize and wheat is under tight transcriptional control during the cell cycle (Chaubet and Gigot, 1998 and Iwabuchi *et al.*, 1998). A histone H3 promoter ALH3-1.1 cloned and characterized from alfalfa (Wu *et al.,* 1988) specifies expression in S-phase as studied in synchronized cell suspension cells (Kapros *et. al.,* 1993). In transgenic tobacco plants, alfalfa histone H3.1 promoter was shown to be active in meristem tissues and its activity was linked to DNA synthesis after reinitiation of cell divisions in fully differentiated leaf mesophyll cells (Kapros *et al.* 1993). Similarly, we found that the wheat histone H4 promoter region isolated by Tabata *et al.* (1983) is active in meristematic tissues of transgenic maize plants (Omirulleh *et al.,* 1993). Further characterization of this regulatory element, similar to the well characterized wheat histone H3 (Nakayama. *et al.* 1992, Mikami. *et al.*, 1993, Ohtsubo *et al.* 1997) and maize histone H3/H4 promoters (Brignon *et al.* 1993, Chaubet *et al.* 1991), revealed its preferential activity in maize cells at the S-phase of the cell cycle (Bilgin *et al.*, unpublished). S-phase specific expression of histone genes is also regulated at posttranscriptional level. Many of the well characterized plant histone genes share several conserved sequence motifs at their 3' ends and these sequences were shown to be involved in modulating S-phase associated activity of replication-dependent histone genes (Mikami K, *et. al.*, 1993, Ohtsubo *et. al.*, 1994).

Protoplast transformation is a widely used procedure for the introduction of DNA into plant cells. As a powerful analytical tool, transient gene expression assays allow comparisons of different treatments with significant sensitivity, specificity and rapidity. Regulatory properties of promoters, introns as well as transactivation or suppression processes among complex mixture of cloned genes can be evaluated in protoplast preparations of appropriate sources (reviewed by Fehér and Dudits, 1994).

We established a sensitive and reliable transient expression assay to identify the functional roles of cloned cell cycle regulatory genes in cultured plant cells. We have utilized the replication-dependent, S-phase-specific activity of histone gene promoters and 3' untranslated regions (3'UTR) from alfalfa and wheat. The chimeric histone promoter-β-glucuronidase (GUS) reporter gene fusions possessing all conserved motifs that is known to drive S-phase-specific activity were introduced into monocot (maize) and dicot (alfalfa, tobacco) protoplasts along with putative cell cycle genes of alfalfa under the control of viral CaMV 35S promoter. Reporter gene function in the form of GUS enzyme activity (Jefferson *et al.* 1987) was used to monitor the influence of transient expression of putative alfalfa cell cycle control genes on G1-S phase transition in protoplast derived cells.

1.10 *Aim of this study*

In this thesis, several approaches have been used to characterize the cell cycle control genes. Mainly it has been tried to understand how the cell cycle is altered when some of the cell cycle control genes were over expressed (transient over-expression experiments). Hormone experiments were done as they play a central role in cell cycle induction, suppression and somatic embryogenesis with unclear mechanisms. A mutant was partially characterized to understand the better mechanism that led to dorsoventality in the lateral organs of plants and the role of a cell cycle control gene in this phenomena (cycD3A Antma;*CycD3*;1 gene and *phantastica* mutant of *Antirrhinum majus*). New techniques are optimized and used to over come the limited sample source and complications of already existing methods (expressional analysis of cell cycle related genes with RT-PCR in leaf protoplast and use of CyQuant cell proliferation assay). All this study was done to get a better picture of plant cell cycle control and development.

CHAPTER II

MATERIALS and METHODS

2.1 *Materials*

2.1.1 *Plant Materials*

Two alfalfa suspension cell cultures were used;

A2 (*Medicago sativa*) cell line was maintained in Muroshige-Skoog medium with 1mg/l 2,4-Dichlorophenoxyacetic acid and 0.2 mg/l kinetin (Bogre *et al*).

KS10 (*Medicago sativa*) was maintained in SHF medium with 10 μM NAA and 1μM kinetin. *In vitro* plants of these lines were maintained on hormone free UM medium.

In vitro plants of Streptomycin resistant tobacco line (SR1) were used in mesophyll leaf protoplast isolation experiments. The plants were maintained on hormone free UM medium.

For mRNA *in situ* hybridization experiments wild type *Antirrhinum majus* plants and a mutant line *Phantastica* were used. They were grown in temperature and humidity controlled growth chambers, the soil mix was JI #1 (John Innes)

2.1.2 *Molecular Materials*

The cDNA library which was used for cloning of CycB2;ms;2, was prepared from auxin treated alfalfa micro-callus suspension (Györgyey, 1991).

The pRT101 and pRT104 plant expression vectors were kindly provided by Dr. Reinhart Töpfer (Töpfer, *et al.*, 1993). pTHG2 construct containing wheat histone H4 promoter and β-glucronidase marker gene was prepared as in Bilgin *et al.*, 1999. The cyclin-dependent kinase inhibitor like gene from *Arabidopsis thaliana* (Wang *et al.*, 1997) was kindly provided by Dr. John Doonan.

2.2 *Methods*

Plant Tissue Culture and Cell Biology Techniques

2.2.1 *Polyethylene Glycol-Mediated Direct Gene Transfer to Alfalfa (A2 Cell Suspension)*

1) Alfalfa A2 cell suspension was transferred weekly to the cell density of $5X10^5$ cells/ml.

2) 24 hours after the transfer, the culture was mixed with E1 enzyme to a ratio of 1:1 and put on shaker (50 rpm) overnight in the dark.

3) Next day the mixture was filtered through 100 μm (60 μm if necessary) steel mesh.

4) The filtrate was centrifuged at a speed of 1000 rpm (swingout rotor) for 3-4 min.

5) The supernatant was removed and the pellet was washed with UM washing solution as gently as possible to remove enzyme.

6) The protoplasts were suspended in 10 ml of MaMg solution and they were counted with a heamocytometer.

7) They were diluted to a density of 5X 10^6 protoplasts/ ml.

8) They were distributed into separate tubes and DNA was added up to 5 µg DNA/ 1X10^6 protoplasts.

9) The protoplasts were mixed gently but throughly with DNA and incubated for 10 minutes.

10) Equal volume of 40 % PEG 4000 was added very slowly and gently and were incubated for 15 minutes.

11) PEG was washed away by gently adding of 10 volumes of UM washing solution.

12) The protoplasts were centrifuged at a speed of 1000 rpm for 3-4 min.

13) They have been resuspend in K75 medium containing 2,4-D (0.2 mg/l, NAA (0.5 mg/l), zeatin (1 mg/l) to a density of 500,000 protoplasts/ ml and kept in dark at 25 °C.

14) Samples for transient GUS essay were collected on the 2nd and 3rd days.

2.2.2 *Polyethylene Glycol-Mediated Direct Gene Transfer to Tobacco Leaf Mesophyll Protoplasts*

1) 3-4 fully expanded leaves were taken and wetted in the enzyme solution and the midribs were removed.

2) The leaf halves were cut into small pieces and embedded in 12 ml enzyme solution over night at 25 °C in the dark, without shaking.

3) The digest was gently agitated by slowly shaking for 10 minutes, the protoplast suspension was passed through a 100-µm steel mesh.

4) The protoplast suspension was distributed into 10 ml tubes and equal amount of W5 solution was added onto it and centrifuged down at 100 *g* for 3-5 minutes.

5) After removing the supernatant, the cells that was collected at the pellet was resuspended in 5 ml of 0.6 M sucrose and on the top of it 1 ml of W5 solution was slowly added.

6) After centrifugation the protoplasts that were floating at the interphase was collected. They were washed once with W5 solution and collected in 10 ml of W5 and counted in a hemocytometer.

7) For each treatment 5X10^5 protoplasts were resuspended in 300 µl of transformation buffer, reporter and effector genes were added as a 20 µg DNA mix at a 1:3 ratio and incubated for 5 minutes.

8) 300 µl of PEG solution was added slowly and was mixed carefully. The transformation mix was incubated for 10 minutes at room temperature with occasional shaking.

9) Gradually 10 ml of W5 solution was added and the protoplasts were centrifuged down.

10) The protoplasts were resuspended in hormone containing K3 medium (5X10^4pp/ml) and kept at 25°C at dim light, the samples were collected on the 3rd day.

2.2.3 *GUS enzyme assays, cell proliferation assay and flow cytometry*

The activity of the reporter GUS gene product was assayed according to Gallagher (1992), with slight modifications. Briefly, at 72 hours after direct gene transfer, protoplasts were lysed in 400 µl of extraction buffer and subjected to 3 rounds of freeze-thaw cycles. The lysates were cleared by centrifugation and the supernatant containing 2-10 µg protein was used for fluorometric GUS assays. The fluorescence was measured in a mini fluorometer TKO 100 (Hoefer Scientific, USA). The results were corrected for endogenous activity, auto-fluorescence and equal protein concentrations as determined by the Bradford method (Bradford, 1976). The data were calculated as nmoles methylumbellyferon (MU) produced per mg protein per hour.

DNA synthesis was monitored with CyQUANT® Cell Proliferation Assay Kit (Molecular Probes, Germany) according to the protocol provided by the manufacturer. Daily samples were collected from transformed protoplast cultures and frozen in lysis buffer. Prior to the addition of the CyQuant-GR dye and fluorometric measurements (excitation: 480 nm, emission: 520 nm), the samples were treated with RNase A (20 units/ml) for a hour at ambient temperature.

Nuclei isolation and flow cytometry were performed as described in Savouré *et al,* (1995), using propidium iodide (10 µg/ml) as nuclear stain. For each sample, 10-20 thousands of nuclei were counted from defined fluorescence gates in a Becton Dickens FacsCalibur™ flow cytometer. The proportions of cell cycle phases and S-phase percentages were determined with the Modfit computer program.

2.2.4 *mRNA in situ Hybridization*

1) 4% paraformaldehyde was used as fixative. Tissues were cut with clean blades and dipped into fixative. 2-3 times of 1 minute of vacum was applied (tissues should settle down and should not float)

Fixation and Embedding

Day 1

4% paraformaldehyde	overnight	4 °C

Day 2

0.85% saline	30 min.	on ice.
50% EtOH/0.85 saline	1.5 hours	on ice.
70% EtOH/0.85 saline	1.5 hours	on ice.
85% EtOH/0.85 saline	1.5 hours	4 °C.
95% EtOH	1.5 hours	4 °C.
100% EtOH	1.5 hours	4 °C.
100% EtOH	overnight	4 °C.

Day 3

100% EtOH/0.3% safranin	30 min.	room temp.
100% EtOH	1.5 hours	room temp.
50% EtOH:50% histoclear	1 hour	room temp.
100% histoclear	1 hour	room temp.
100% histoclear	1 hour	room temp.
100% histoclear	1 hour	room temp.
50% histoclear/50% wax chippings	overnight	40-50 °C

Days 4-6

The tissues were kept in wax at 60 °C and the wax was changed each morning and evening.

Day 7

Some wax was poured to a suitable sized mould and the tissue was palced before it solidifies. The rest of the mould was filled with wax and the wax was solidified by floating on cool water and stored in the fridge.

Sectioning

1) The wax blocks were cut into trapezoid shape, leaving about 2mm of wax around the tissue.

2) The blocks were mounted such that the longer of the two parelle faces was at the bottom.

3) Ribbons of sections were cut at 7-10 μm thickness and floated on sterile water on coated slides, placed on 42 °C hotplate until the ribbon flattens out.

4) The excess water was drained off and the left on hotplate overnight to dry.

5) Tissue pre-treatment was done as follows;

Histoclear	10 minutes
Histoclear	10 minutes
100% EtOH	1 minute
100% EtOH	30 seconds
95% EtOH/0.85% saline	30 seconds
85% EtOH/0.85% saline	30 seconds
50% EtOH/0.85% saline	30 seconds
30% EtOH/0.85% saline	30 seconds
0.85% saline	2 minutes
PBS	2 minutes

Pronase (0.125 mg/ml in pronase buffer	10 minutes
Glycine (0.2% in PBS)	2 minutes
PBS	2 minutes
Formaldehyde(4% in PBS)	10 minutes
PBS	2 minutes
PBS	2 minutes
Acetic anhydride	
(3 ml in 600ml 0.1M triethanolamine pH8)	10 minutes
PBS	2 minutes
0.85% saline	2 minutes

6) Dehydration was done through EtOH series up to the first 100% then washed in fresh 100% EtOH.

Preparation of RNA probes

1) Template is cloned into a T7, SP6 promoter containing vector and linearized.

2) The reaction was set up at room temperature because the buffer contains spermidine, which precipitates the DNA on ice.

3) 1 μl template (0.5-1 μg)
2.5 μl 5mM (ribo) ATP
2.5 μl 5mM (ribo) GTP
2.5 μl 5mM (ribo) CTP
2.5 μl 1mM DIG-(ribo) UTP
5 μl 5X reaction buffer
1 μl RNasin
7 μl distilled water
1 μl T7 RNA polymerase

Mix well and spin briefly

4) Incubate at 37 °C for 30 min-2 hours.

5) Stop reaction with: 75 μl 1XMS buffer
2 μl tRNA (100 μg/μl)
1 μl DNase (RNase-free)

Mix well and spin briefly.

6) Incubate at 37 °C for 10 minutes

7) Precipitated with 100 μl ammonium acetate (3.8 M)

600 μl EtOH (ice cold)

Leave overnight at -20°C.

8) Centrifuged down for 10-15 minutes at 4 °C, washed with ice cold 70% EtOH:0.5M NaCl and dried.

9) The pellet was resuspended in 50 μl of RNase free water and 50 μl of 200 mM carbonate buffer was added (pH 10.2) and was mixed thoroughly

10) Incubation was done at 60 °C depending on the probe length.

11) 10 μl acetic acid (10%)

12 μl sodium acetate (3M)

312 μl ethanol (ice cold) was added over the reaction.

12) It was incubated overnight at -20 °C, spinned and washed with 70% EtOH and dried.

13) Resuspended in 50 μl TE

Hybridization

1) Previously prepared riboprobes were heated upto 80 °C for 2 minutes and kept on ice. The final hybridization mix contained 1 part probe and 4 parts hybridization buffer (see appendix A)

2) 25-40 μl hybridization mix was put on each slide and covered with coverslip.

3) Hybridization was done at 50 °C, overnight in sealed boxes.

4) Nextday the slides were washed with wash buffer (see appendix A) at 50 °C for 30 minutes.

5)2XNTE (see appendix A)		5 minutes
Wash buffer	50 °C	1 hour
1XSSC	room temp	2 minutes

6) Slides were air-dried

Detection of DIG

1) Slides were put on a tray and incubated as follows;

Buffer 1	5 minutes
Buffer 2	1 hour
Buffer 3	1 hour
Buffer 4	1 hour

(see appendix A for buffers)

2) Washed 4 times 20 minutes with buffer 3

3) Equilibrated 5 minutes in buffer 1 and 5 minutes in buffer 5

4) Buffer 5 was removed and 15 ml of buffer 6 were put on each tray and left at dark for 36 hours or

longer.

5) To stop the enzyme reaction slides were washed as follows;

H_2O	5 minutes
70% EtOH	5 minutes
95% EtOH	5 minutes
100% EtOH	5 minutes
100% EtOH	5 minutes
Histoclear	3 minutes
Histoclear	3 minutes

6) The slides were air-dried and 2-3 drops of Euparal were on slides and covered with coverslip and dried for overnight in the fumehood.

7) Results were viewed under light microscope and photographed.

Molecular Biology and Recombinant DNA Techniques

2.2.5 *cDNA Library screening*

1) cDNA library which was prepared from 2,4-D (100µM) induced alfalfa cell line RA3 was screened.

2) *E. coli* host strain LE392 was grown to logaritmic phase in 5 ml of LB medium supplemented with 50µl of 1M $MgSO_4$ and 50 µl of 20 %(w/v) maltose.

3) The bacteria were centrifuged at 4000 rpm for 10 minutes.

4) The supernatant was removed and the bacteria were resuspended in 0.5 volume of sterile 10 mM MgSO4, it was kept at +4 °C and used within a week.

5) To check the titer of the library several dilutions of the stock library was made with SM buffer and 500µl of bacteria was mixed with these dilutions and incubated at 37°C for 20 minutes.

6)The inoculate was mixed with 3 ml of melted top agar (0.7% agar in NYZ medium) at 50°C and pour onto a plate containing fresh, hardened, prewarmed NYZ bottom agar containing 13 cm plates.

7) The plates were inverted and incubated for 6-10 hours at 37°C.

8) After determining the appropriate dilution, 50000 pfu/plate (20 plates were poured) was plated for the first screening of the library.

9) After the plaques became confluent the plates were placed to +4°C for several hours.

10) Hybond nylon filter was placed on the plate for 30 seconds and was stabbed from 2 places with Indian ink containing syringe in order to determine the orientation.

11) After the first filter removed second filter was placed on the plate for 5 minutes and was stabbed from the same places.

12) The filters were placed on denaturation solution saturated Whatman papers for 5 minutes and then 2 times 5 minutes each on to neutralization solution saturated papers, finally were soaked into 5XSSC and air dried. This process was done to all filters and phage containing side was always kept up.

13) The filters were exposed to UV crosslink for 5 minutes.

2.2.6 *Nucleotide Hybridization*

1) The filters have been prehybridized in a 50% formamide containing solution (15 ml 20XSSC, 2.5 ml 10% milk powder, 5 ml 1M Tris pH 7.4, 5 ml 10% SDS, 1 ml of 0.5M EDTA, 10 mg Torulla yeast total RNA, 50 ml formamide, completed to 100 ml) at 42 °C for 24 hours.

2) The cycMs1 clone was used as hybridization probe and was labeled with dCTP P^{32} and hybridization was done at the same temperature for 48 hours.

3) The filters were washed with 2XSSC, 0.1% SDS, for 20 minutes, times with 0.5XSSC, 0.1% SDS for 20 minutes and exposed to X-ray for 3-5 days.

4) The positive clones were picked with the end of a sterile Pasteur pipette and kept in 500 µl SMC buffer at 4 °C o/n.

5) For the second screening the picked plaques were used and the lysis was done in 9 cm petri plates with an appropriate dilution to pick a single plaque.

6) From the single plaque which was kept in SMC buffer 5 µl was taken and PCR was performed with SP6 verses T7 primers in 100 µl reaction mixture.

7) The amplified fragment was isolated by using Sephaglas isolation kit (Pharmacia) and subcloned into pUC 19 Sma I site according (*et al*) and cloning was confirmed with sequencing.

2.2.7 *Cloning of Cell Cycle Control Genes under a Constitutive Promoter*.

1) For this purpose the plasmids pRT101 and pRT104 carrying the 35S RNA promoter of the cauliflower mosaic virus (CaMV), and the corresponding poly(A) signal sequence inserted into pUC18/19 were used (Töpher *et al* 1993).

2) cDNA clones of alfalfa cdc2MsA, Medsa;*CycB*;3 and cyclin-dependent kinase inhibitor ICK1 (Wang *et al.*, 1997) were cloned into multicloning sites of pRT101. cdc2*Ms*A was cloned into Xba I/EcoR I sites, Medsa;*CycB*;3 into KpnI/EcoR I sites and ICK1 cDNA was cloned into SstI / BamH I sites. These construct were named as; pDA1, pDA2, pDA4, respectively.

3) For Medsa;?*CycB*;3, (120 bp missing from 5' of the full clone) translational fusion vector pRT104, which carries an ATG codon, was used. Medsa;?*CycB*;3 was cloned into XbaI/ Xho I sites and called pDA3.

4) By using the symmetrical arrangement of restriction site Pst I, bordering both regulatory elements in pRT101 and pRT104 vectors, the chimeric constructs of 35S::cdc2*Ms*A, 35S::Medsa;*CycB*;3, and 35S::Medsa;*CycB*;3 were cloned into Pst I site of pTHG2 plasmid (Omirulleh *et al.,* 1993), these constructs were named as; pDM1, pDM2, pDM3, respectively.

2.2.8 *Single strand cDNA Synthesis*

1) To treat the RNA with DNase, 5-20 µg total RNA was disolved in 44 µl of water and 5 µl of 10X DNase buffer (400mM Tris, pH8, 100mM NaCl, 60 mM $MgCl_2$, 100 mM $CaCl_2$) and 1µl of RQ1 RNase-free DNase (Promega 1U/ul) were added.

2) The samples were incubated at 37 °C for 30 minutes. DNase was inactivated by incubating at 65 °C for 10 minutes.

3) After phenol: chloroform extraction the RNA was precipitated with 0.1 volume of Na-acetate, pH5.2 in 2.5 volume of ethanol.

4) The amount of RNA was measured and checked on agarose gel. 5-20 ug RNA was diluted to 11.5 µl water and 1 µl of dT primer was added (1µg/µl) and denatured at 70 °C for 10 minutes.

5) Reverse transcription was done by adding 5 µl of 5X reverse transcription buffer (GibcoBRL), 10mM DTT, 0.5 mM dNTP, 1 µl (200 U) Superscript II reverse transcriptase (Gibco BRL, lacking RNase H activity) and 0.5 µl (Promega, 0.4 U) RNase inhibitor. The mixture was incubated at 42 °C for one hour and reverse transcriptase was inactivated by incubating in boiling water for 5 minutes.

6) 5 units of RNase H (Gibco BRL 1U/µl) was added and the mixture was incubated at 37 °C for 30 minutes. and inactivation of enzyme was done by incubating in boiling water for 5 minutes.

7) The synthesized cDNAs were spinned through microspin column-400 (Pharmacia) in order to clean them. The amounth of cDNAs were determined spectrophotometrically and stocks were diluted to 50 ng/µl.

2.2.9 *RT-PCR Analysis*

1) 200-400 ng of cDNA was used for PCR analysis. 5 µl of PCR buffer (10X concentrated), 5µl of dNTP mixture (2 mM) primers (0.25 µM each) and 1µl of Taq DNA polymerase (Pharmacia 5U/µl) were added, the reactions were done in 50µl of total volume.

2) Annealing temperature of the PCR reactions were determined according to the T_m value of primers (see Appendix B). The elongation temperature was 72°C.

3) The number of cycles was optimized for each gene separately.

CHAPTER III
RESULTS

For cloning of CycB2;ms;2, a cDNA library which was prepared from auxin treated alfalfa micro-callus suspension (Gyorgyey, 1991) was used.

To test the changes in the expression level of cell cycle genes due to hormonal response of abscisic acid (ABA), 2,4-dichlorophenoxy acetic acid (2,4-D), naphthaleneacetic acid (NAA), cytokinin (kinetin) and brassinosteroid (24-epibrassinolide), KS10 and A2 (*Medicago sp*) cell suspensions were used. The expression levels of the some of the cell cycle related genes isolated from *Medicago sativa* (cdc2*Ms*A, Medsa;*CycB2*;1, Medsa;*CycB2*;2, Medsa;*CycA2*;1, Medsa;*CycD3*;1, histone H3.1, MAP kinase MMK1, *Medicago* Aldose Reductase (MAldR)) were detected by northern analysis. For alfalfa and maize transformation experiments, the plant expression vectors pRT101 and pRT104 were used (Töpfer, 1993). These vectors enabled the expression of cell cycle genes under the control of 35S RNA promoter of Cauliflower mosaic virus (CaMV) and its corresponding polyadenylation signal.

To study the cell cycle events, alfalfa mesophyll protoplast system was used. The expression of the cell cycle genes (cdc2*Ms*A, cdc2*Ms*B, cdc2*Ms*D, Medsa;*CycB2*;1, Medsa;*CycB2*;2, Medsa;*CycA2*;1, *Medicago* MAP kinase MMK1) were detected by RT-PCR.

The results are given in detail in the following sections.

3.1. *Cloning of Medsa;CycB2;2* .

A partial cDNA clone of cyclin Medsa;*CycB2*;2 was cloned previously (Hirt *et al.*, 1992). The cDNA library which was prepared from synthetic auxin induced (100 μM 2,4-D treatment for 1 hour) microcallus suspension culture was screened with partial Medsa;*CycB2*;2 clone in order to pick the full length. At the end of the second screening of the library 2 positives clones were picked and one of them was containing the 200 bp 5' missing end of the gene. This clone contained three internal EcoR I sites instead of one as the library was constructed into bacteriophage LambdaGEM-2 vector. To subclone the cDNA from bacteriophage vector into a plasmid, 5' end of the cDNA clone in lambdaGEM-2 was sequenced shortly and primers were designed for the 5'end and 3'end of the cDNA clone (see Appendix B; cyc2 forward1.primer and cyc2 reverse1.primer) for PCR amplification. *Pfu* DNA polymerase (Stratagene) enzyme was used for PCR amplification. The PCR fragment was subcloned into pBluescript II SK(-) vector Sma I site and sequenced throughly (see Appendix B for amino acid sequence).

3.2. *Hormonal Responses in alfalfa (M. sativa) and snapdragon (A. majus).*

A) *In vitro* alfalfa KS10 line plants were used for this purpose. The petioles of these plants were cut (1cm long) and placed on SH media (see Appendix A) containing different hormone combinations (15 µM NAA/ 10 µM kinetin, 15 µM NAA/ 1 µM kinetin, 15 µM NAA, 10 µM NAA/ 10 µM kinetin , 10 µM NAA/ 1 µM kinetin, 10 µM NAA and 2.25 µM 2,4-D). The best callus formation was obtained with 10 µM/ 1µM kinetin combination and were maintained in liquid medium (figure 3.2.1 A) Figure 3.2.1 B and C are two examples showing poor calli formation, these were maintained in liquid media too. All of them are tested for somatic embryo formation by inducing with 100 µM 2,4-D for 1 hour and washed and placed on SH regeneration medium (see Appendix A). As control alfalfa RA3 line was used. Alfalfa KS10 cell suspension maintained in 10 µM NAA/ 1 µM kinetin containing SH liquid medium was found to be 3 times more embryogenic than the control. Later on, this cell suspension was used in induction experiments.

B) Surface sterilized mature green house *Antirrhinum majus* leaves were cut into 1mm^2 pieces and placed on different hormone combinations containing MS medium (0.5 µM NAA/ 1µM BPA, 5 µM NAA/ 1µM BPA, 50 µM NAA/ 1µM BPA 0. 5 µM NAA/ 10µM BPA, 5 µM NAA/ 10µM BPA, 50 µM NAA/ 10µM BPA and 4.5µM 2,4-D/0.6 BPA, 9µM 2,4-D, 0.2 g/l casamino acids). The best calli formation was obtained with 0.5 µM NAA/ 1µM BPA, 0.5 µM NAA/ 10µM BPA and these were maintained in liquid medium (figure 3.2.2).

Figure 3.2.1

Alfalfa KS10 line plants petioles were placed on SH media containing different hormone combinations. The most calli were obtained with the following combinations.

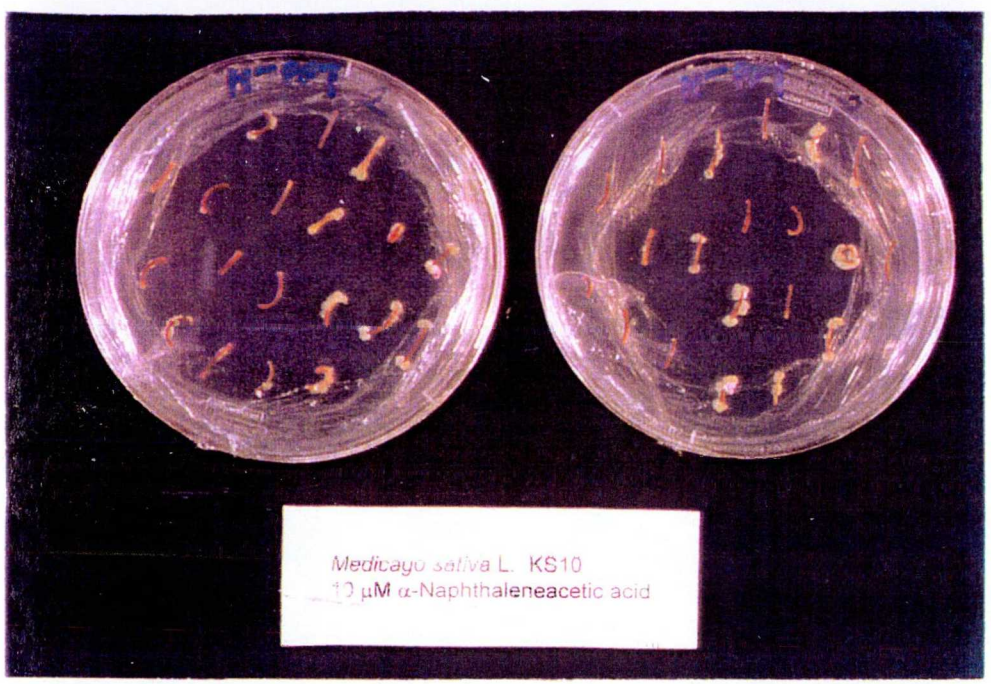
A) 2.25 µM 2,4-D

B) 10 µM NAA

C) 10 µM NAA/ 1 µM kinetin


Medicago sativa L. KS10
2.25 µM 2,4- Dichlorophenoxyacetic acid

A


Medicago sativa L. KS10
10 µM α-Naphthaleneacetic acid

B

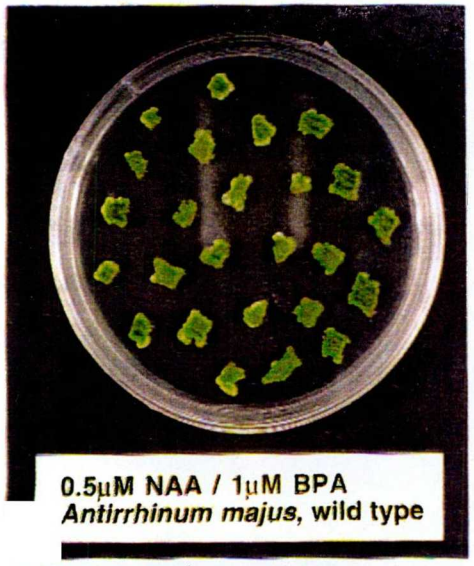

Medicago sativa L. KS10
10 µM α-Naphthaleneacetic acid / 1 µM Kinetin

C

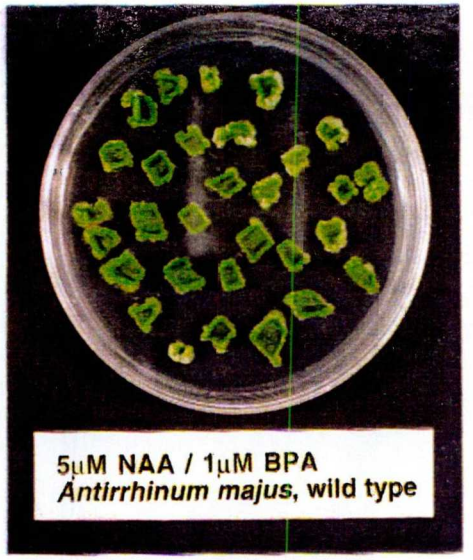
Figure 3.2.2

Antirrhinum majus leaf pieces were placed on MS media containing different hormone combinations

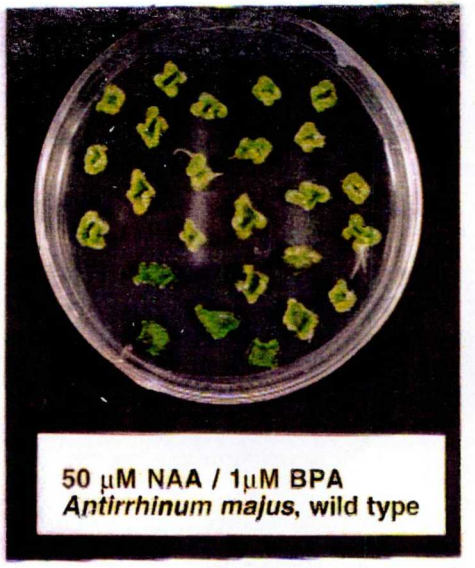
A) 0.5 µM NAA/ 1µM BAP
B) 5 µM NAA/ 1µM BAP
C) 50 µM NAA/ 1µM BAP
D) 4.5µM 2,4-D/0.6 BAP
E) 0. 5 µM NAA/ 10µM BAP
F) 5 µM NAA/ 10µM BAP
G) 50 µM NAA/ 10µM BAP
H) 9µM 2,4-D, 0.2 g/l casamino acids.



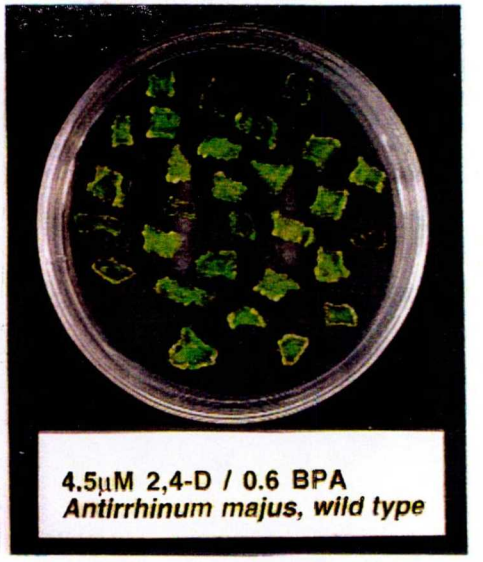
0.5µM NAA / 1µM BPA
Antirrhinum majus, wild type



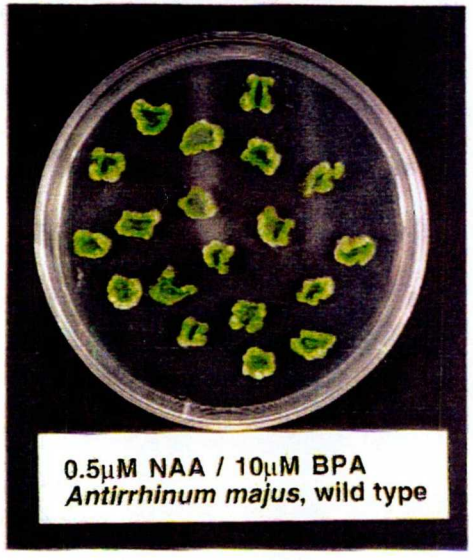
5µM NAA / 1µM BPA
Antirrhinum majus, wild type



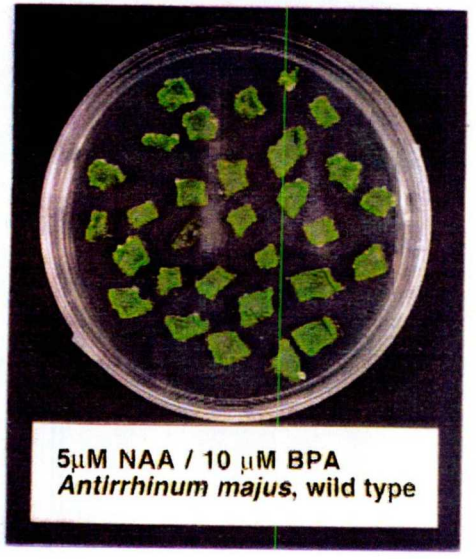
50 µM NAA / 1µM BPA
Antirrhinum majus, wild type



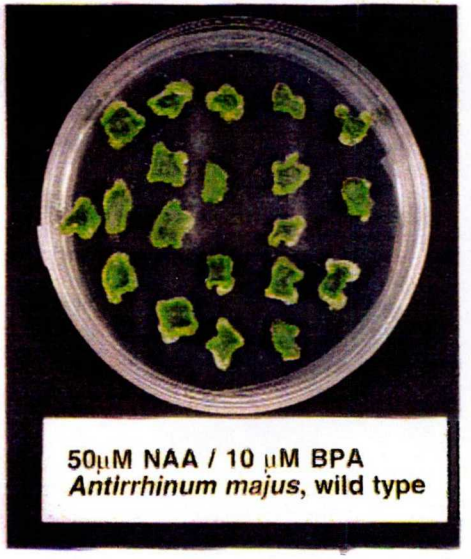
4.5µM 2,4-D / 0.6 BPA
Antirrhinum majus, wild type



0.5µM NAA / 10µM BPA
Antirrhinum majus, wild type



5µM NAA / 10 µM BPA
Antirrhinum majus, wild type



50µM NAA / 10 µM BPA
Antirrhinum majus, wild type

9µM 2,4-D, 0.2 g/L casamino acids
Antirrhinum majus, wild type

Activation of cell cycle genes due to hormonal treatment in alfalfa

3.3 *Effects of different hormones and concentrations on the expression of cell cycle related genes*

Hormone starved alfalfa cell suspension culture (A2 line) was treated with different hormones (NAA, kinetin, 24-epibrassinolide) and concentrations (0.01, 0.1, 1 mg/l) for various duration (8, 24 hours). The mRNA expression patterns of cdc2*Ms*A, cdc2*Ms*B, Medsa;*CycA2*;1, histone ALH3-1.1 and MAP kinase MMK1 were detected by northern analysis.

cdc2*Ms*A and cdc2*Ms*B were slightly induced with hormone treatments compared to control sample. cdc2*Ms*A was significantly induced with 1μM 24-epibrassinolide and 0.01 mg/l NAA+1 μM 24-epibrassinolide treatment for 24 hours (figure 3.3.1 A). With Medsa;*CycA2*;1 the strongest signal was observed at 0.01 mg/l NAA+0.1 mg/l kinetin treatment for 24 hours (figure 3.3.1 C). MAP kinase MMK1 has shown a constitutive expression (figure 3.3.1 D).

ABA treatments

The alfalfa cell suspension culture which was subcultured in 1 mg/l 2,4-D and 0.2 mg/l kinetin containing MS medium, was treated with 0.1, 1, 10 and 100μM ABA and the viability of cells were checked by FDA (florecein diacetate) and no difference was observed between the control and ABA treated cells (85% viability). By using 75μM ABA, time course experiments were performed and samples were collected at 10, 30 minutes, 1, 2, 4, 8, 24 hours. The mRNA expression analysis of cdc2*Ms*A, Medsa;CycB2;2, Medsa;*CycA2*;1, Medsa;*CycD3*;1, histone ALH3-1.1, MAPK MMK1 and MaldR were done. Northern analysis of Medsa;CycB2;2, Medsa;*CycA2*;1, histone ALH3-1.1, MAPK MMK1 did not show any difference in response to different concentrations of ABA (data not shown) and time course (figure 3.3.2 B, C, E, F, respectively). cdc2*Ms*A mRNA expression was mainly constitutive, slightly peaking at 8th hour (figure 3.3.2 A). Medsa;CycD3;1 mRNA expression was induced by time, starting from 1 hour and peaking at 8th hours (figure 3.3.2 D). MaldR mRNA expression was induced by the increasing ABA concentration. In the time course experiment, an increase in the expression was observed starting from 1 hour till 8 hours sampla, peaking at 2 hours (figure 3.3.2 G)

Figure 3.3.1

Hormone starved alfalfa cell suspension culture (A2 line was treated with different hormones and concentrations for various duration.

C) Control - Hormone suspension cells

1) 0.01 mg/l NAA

2) 1 mg/l NAA — 8 hours of treatment

3) 0.1 mg/l Kinetin

4) 0.01 mg/l NAA + 0.1 mg/l Kinetin

5) 0.01 mg/l NAA

6) 1 mg/l NAA — 24 hours of treatment

7) 0.1 mg/l Kinetin

8) 0.01 mg/l NAA + 0.1 mg/l Kinetin

9) 0.1 µM 24-epibrassinolide

10) 1 µM 24-epibrassinolide — 24 hours of treatment

11) 0.01mg/l NAA + 0.1 µM 24-epibrassinolide

12) 0.01mg/l NAA + 1 µM 24-epibrassinolide

Northern analysis of several cell cycle related genes were done.

A) cdc2*Ms*A (lower band) and cdc2*Ms*B (upper band)

B) Medsa;*CycA2*;1

C) Histone ALH3-1.1

D) MAPK MMK1

Figure 3.3.1

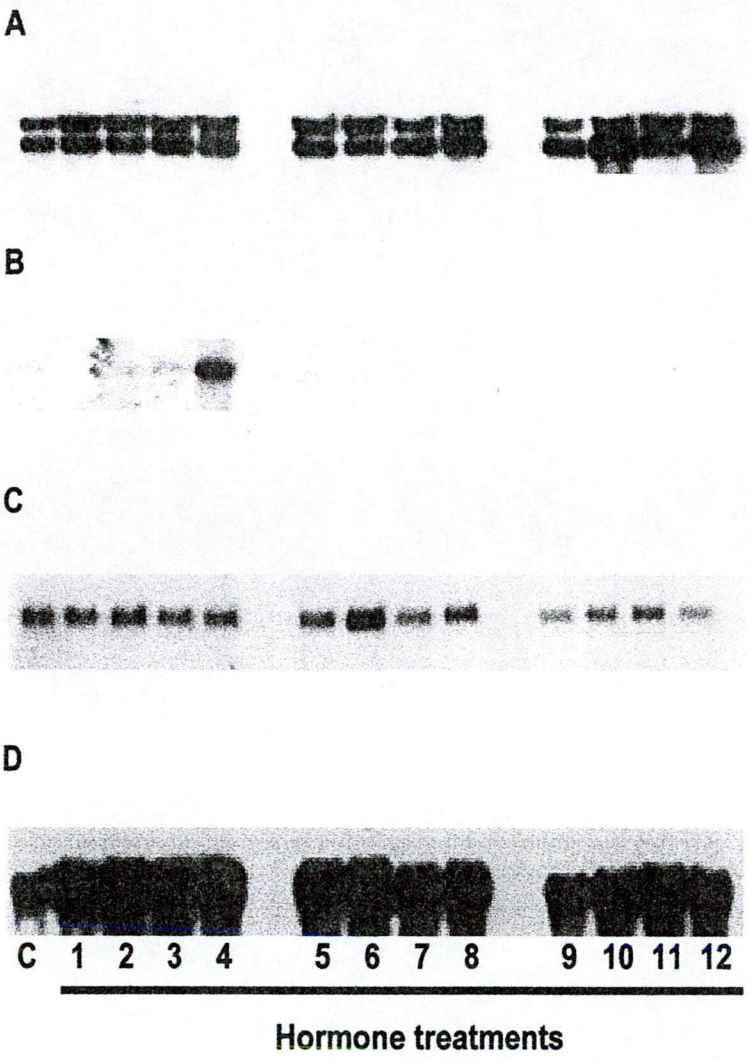


Figure 3.3.2

The alfalfa cell suspension was treated with 0.1, 1, 10, 100μM ABA. time course experiments were performed by using 75μM ABA and samples were collected at 10, 30, 60 minutes, 2, 4, 6, 8, 24 hours. C is the control sample from non-ABA treated cell suspension. The mRNA expression analyses of following genes were done.

A) cdc2MsA

B) Medsa;CycB2;2

C) Medsa;CycA2;1

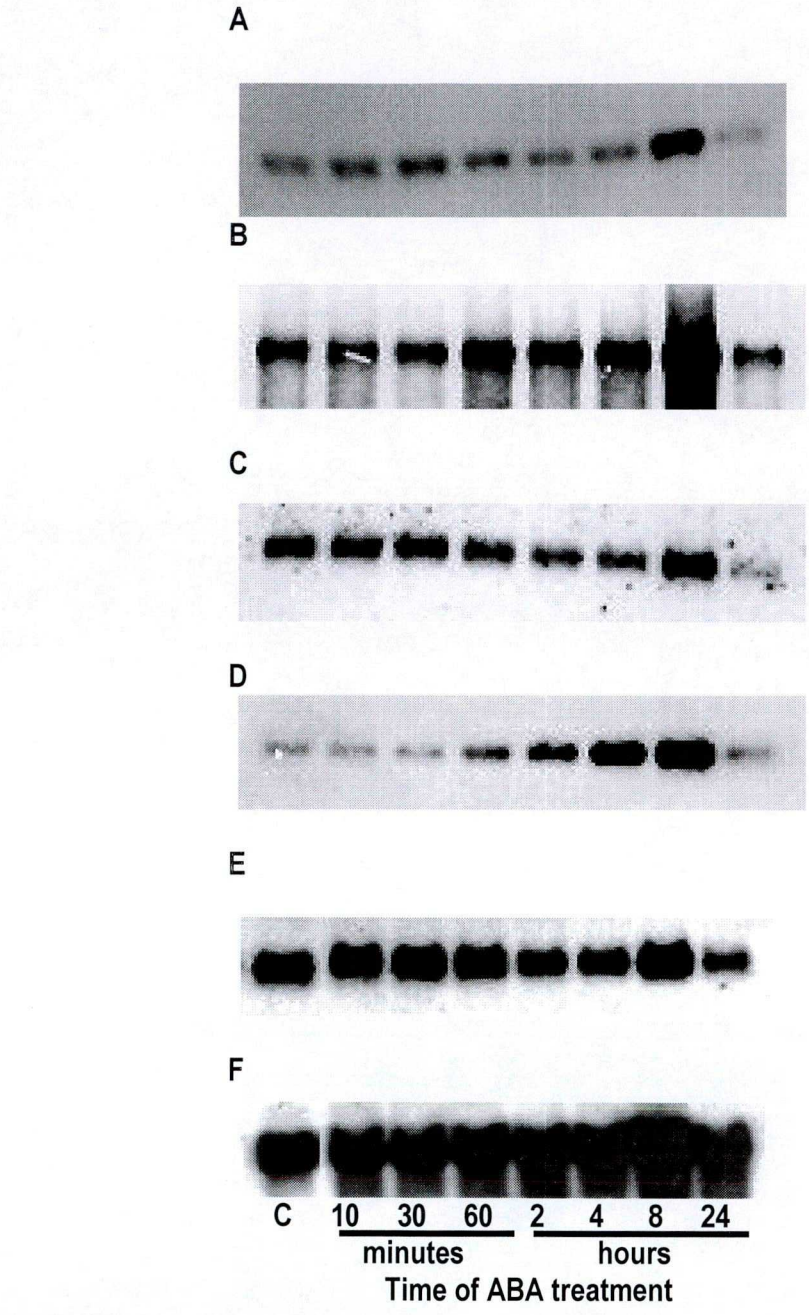
D) Medsa;CycD3;1

E) Histone ALH3-1.1

F) MAPK MMK1

G) MAldR

Figure 3.3.2


A
B
C
D
E
F
C 10 30 60 2 4 8 24
minutes hours
Time of ABA treatment
G
C 0.1 1 10 100
10 30 60 2 4 8 24
µM ABA treatment
minutes hours
Time of ABA treatment

3.4. *Induction of somatic embryogenesis in alfalfa cell suspension by 2,4-D*

Alfalfa cell suspension was induced with 100 μM 2,4-D for one hour and put on regeneration medium (see Appendix A) and samples were collected (0, 1, 2, 3, 8, 24, 48, 96hrs, 1, 2, 3, 4, 6, 10 weeks). The northern analysis revealed that Medsa;*CycB2*;1 (figure3.4.1 D) and Medsa;*CycB2*;2 (data not shown) expression was induced on the 24th hour and it was continuing till the 7th day and disappeared by time. Medsa;*CycD3*;1 expression was rather different from the other two mitotic cyclins, there was a strong induction on the 8th hour and the signal was present on 1st and 2nd days –being the most strong on these days and the signal never vanished (figure 3.4.1 A). cdc2*Ms*A expression was constitutive till the 6th week after that a decline was observed (figure 3.4.1 E). MAPK MMK1 showed a fluctuating signal pattern, being strongest at 1hour, 24, 48 hours and 1 week (figure 3.4.1 B).

Figure 3.4.1

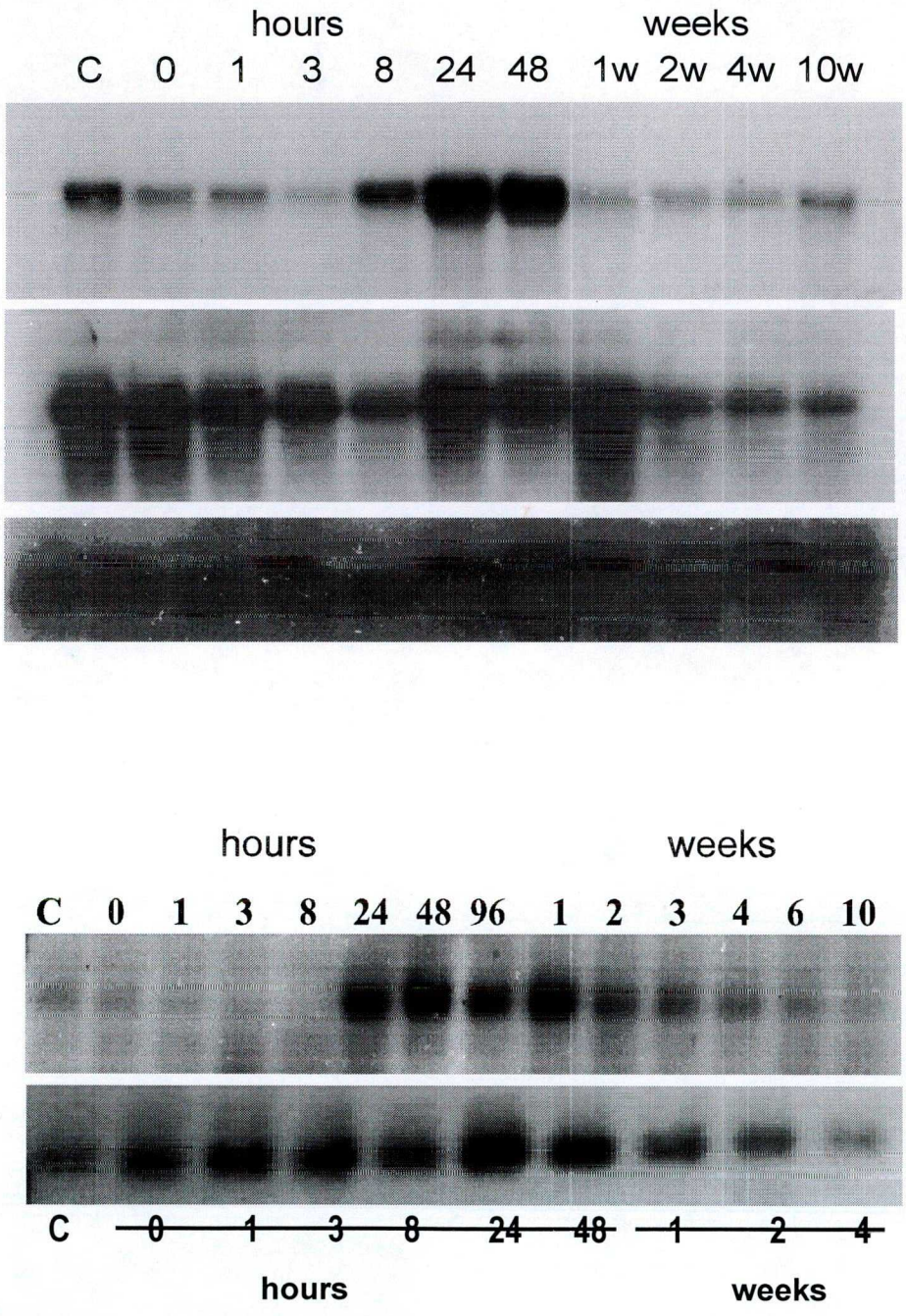


Figure3.4.1

Alfalfa suspension cells were induced with 100µM 2,4-D. Samples for northern analysis were collected at different time points (0, 1, 2, 3, 8, 24, 48, 96 hours, 1, 2, 3, 4, 6, 10 weeks)

A) Medsa;*CycD3*;1

B) MAPK MMK1

C) Histone ALH3-1.1

D) Medsa;*CycB2*;3

D) cdc2*Ms*A

3.5. *Analysis of an Antirrhinum majus mutant "phantastica"*.

Two different D-cyclins are expressed throughout the cell cycle but only in proliferating cells; Antma;*CycD3*;1 and Antma;*CycD3*;2 (figure 3.5.3 and 3.5.4). To understand the better mechanism that led to dorsoventality in the lateral organs of plants, mutants at the *phantastica* (*phan*) locus of *Antirrhinum majus* have been used. The leaves and bracts and petal lobes of *phan* mutants show varying degrees of reduction in dorsal tissues at a restricted temperature of 15 °C (figure 3.5.1 B, C). The seeds of *phan* mutant and the wild type *Antirrhinum majus* were planted at 20 °C and after germination were shifted to 15 °C for a month and shoot apex samples were collected at 4 weeks. RT-PCR analysis and mRNA *in situ* hybridization were performed for the characterization of Antma;*CycD3*;1 gene (figure 3.5.3 and 3.5.5). The RT-PCR analysis and mRNA *in situ* hybridization revealed that Antma;*CycD3*;1 mRNA expression was affected from temperature shift experiments (figures 3.5.2).

Figure 3.5.1

Temperature shift experiments of wild type and mutant (*Phantastica) Antirrhinum majus* plants, 4 weeks after germination.

A) Wild type *Antirrhinum majus* kept at 20 °C. No phenotypic difference was observed between 20 °C and 15 °C wild type plants.

B) *Phantastica* mutant kept at 20°C.

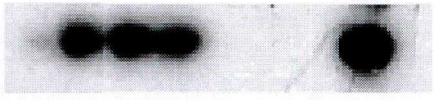
C) *Phantastica* mutant kept at 15°C.

A

B

C

Figure 3.5.2



Temperature ***15 20 20 15*** ***C***

(°C)

Phantastica **Wild type**

RT-PCR analysis of *Phantastica* mutant and wild type *Antirrhinum majus* shoot apices by using Antma;*CycD3*;1 gene specific primers. Samples were collected both for RT-PCR and mRNA *in situ* analysis. C is the control PCR amplification from Antma;*CycD3*;1 plasmid.

Figure 3.5.3

mRNA *in situ* hybridization of longitudinal sections of *Antirrhinum majus* apices probed with Antma;*CycD3*;1. A, B, and C are vegetative, inflorescence and floral apices, respectively. Sections were probed with digoxigenin labelled antisense RNA and viewed under bright field.

Figure 3.5.3

A

B

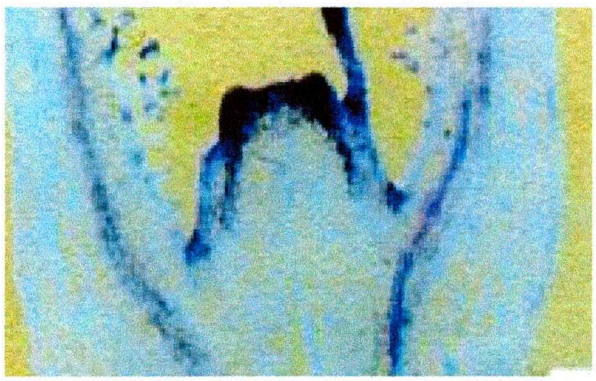
C

Figure 3.5.4

mRNA *in situ* hybridization of longitudinal sections of *Antirrhinum majus* apices probed with Antma;*CycD3*;2. A, B, and C are vegetative, inflorescence and floral apices, respectively. Sections were probed with digoxigenin labelled antisense RNA and viewed under bright field.

Figure 3.5.4

A



B

C

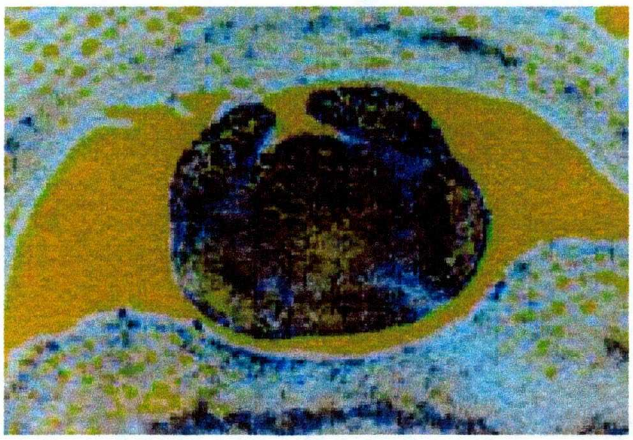


Figure 3.5.5

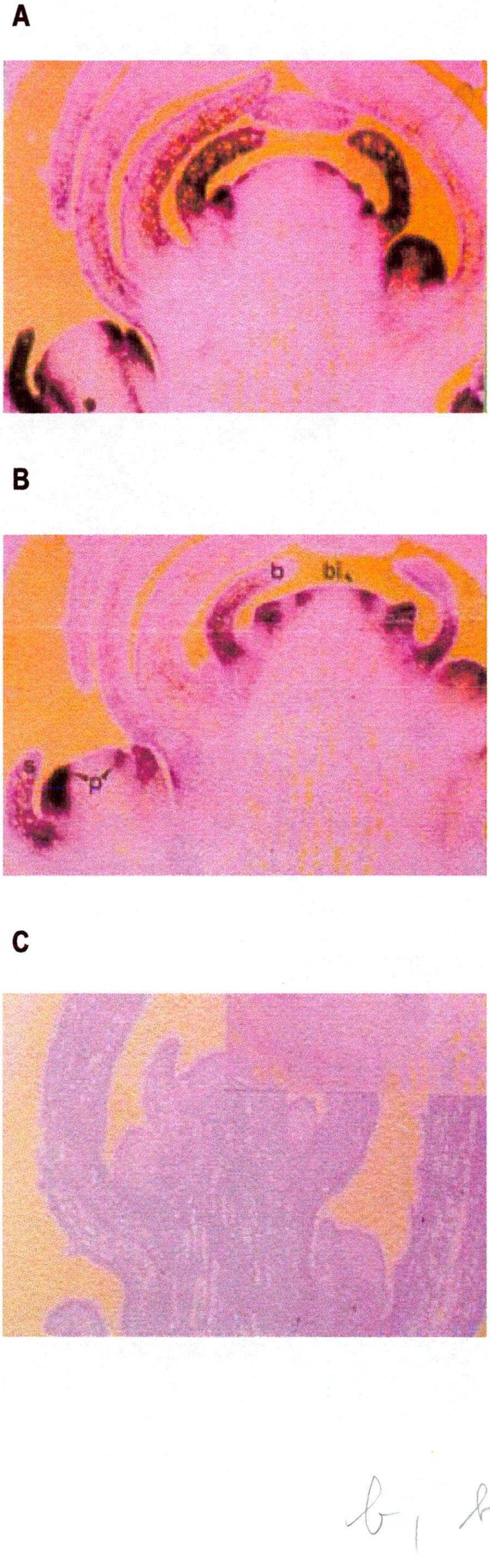
mRNA *in situ* hybridization of longitudinal sections of *Antirrhinum majus* wild type and mutant *Phantastica* inflorescence apices. Apices hybridized with digoxigenin labelled antisense (**A** and **B**) and sense RNA (**C**) of Antma;*CycD3*;1. They were viewed under bright field.

A is the inflorescence shoot apex of wild type grown at 15 ^{o}C.

B is the inflorescence shoot apex *of Phantastica* mutant grown at restrictive temperature of 15 ^{o}C.

C is the inflorescence shoot apex *of Phantastica* mutant grown at restrictive temperature of 15 ^{o}C.

Figure 3.5.5



b, bl - mian?

3.6 *Transient over expression of cell cycle genes*

For this purpose pRT101 and pRT104 plant expression vectors were used (Töpfer *et al.*, 1993) These vectors contained a 35S RNA promoter of CaMV and its corresponding poly A signal with a polylinker sequences between them. PRT101 was suitable for transcriptional fusions and pRT104 was suitable for translational fusion which carries an ATG codon. These vectors have symmetrical arrangement of restriction sites bordering both regulatory elements and these restriction sites provide the possibility for simple transfer of a given chimeric construct to other vectors (table 3.6.1).

cdc2*Ms*A (XbaI/EcoRI), Medsa;*CycB2;3* (KpnI/EcoRI) cDNAs were cloned into pRT101 and , ΔMedsa;*CycB2;3* (XbaI/XhoI) without destruction box was cloned into pRT104 on indicated restriction sites, ICK1 was cloned into BamHI/SstI sites of the pRT101. They were named as pDA1, pDA2, pDA3 and pDA4, respectively. These constructs were used for alfalfa and tobacco protoplast co-transformation together with pH3.1::GUS construct (table 3.6.1).

By using the symmetrical arrangement of the PstI restriction endonuclease sites bordering both regulatory elements in pRT101 and pRT104 vectors, the chimeric over-expression cassettes of cdc2*Ms*A and Medsa;*CycB2*;3 cDNAs were fused to pH4GUS (pTHG2) plasmid containing wheat histone H4 promoter-GUS translational fusion and wheat H3 polyadenylation signal (Omirulleh et al. 1994). The resulting vectors were designated as pDM1, pDM2 and pDM3 for cdc2MsA, Medsa;*CycB2*;3, and truncated *CycB2*;3 Δ cDNAs, respectively (table 3.6.1).

Histone H3-1.1 gene promoter from alfalfa acts as an S-phase marker in tobacco protoplast-derived cells.

We first determined the timing of the re-initiation of DNA synthesis in protoplast-derived tobacco mesophyll cells and how transiently expressed alfalfa histone H3-1.1 promoter responds during the early stages of the culture under experimental conditions applied. At the time of protoplast isolation, 90% of the tobacco cells were at the G0-G1-phase, as judged by flow cytometry (figure 3.6.3 A). We introduced the reporter plasmid (alfalfa histone H3::GUS::H3 3'UTR) or the reference control vector (CaMV 35S::GUS::NOS poly A) into tobacco leaf

protoplasts and then monitored kinetics of both the GUS enzyme activity and DNA synthesis (figure 3.6.1). S-phase was determined by flow cytometry and also a cell proliferation assay kit (CyQuant®-GR, Molecular Probes) which is based on fluorometric quantitation of the total DNA amount. Both data indicated that DNA synthesis activity of freshly isolated protoplast predominantly take place during the 2nd to 4th days of culture (figure 3.6.1). The GUS activities of the reference plasmid exhibited a sustained level after a peak at 24 hours whereas the H3::GUS::H3 3'UTR reporter plasmid fluctuated with a highest level at 3rd day. In the second part of the experiments the transition of G1 cells to S-phase after transformation with the reporter plasmid was followed by flow cytometry in parallel with daily measurements of GUS enzyme activity (figure 3.6.1). The highest GUS activity was measured in 3-days-old protoplast cultures at the same time as the flow cytometry data indicated a major increase in the number of S-phase cells in agreement with the cell proliferation assay. Based on these results, the alfalfa H3::GUS::H3 3'UTR reporter construct was used further as a marker of G1-S transition and GUS activities were measured at 72 hours post-transformation.

To investigate the effect of the two putative alfalfa cell cycle control genes on G1/S-phase progression characterized with histone promoter activity, the reporter and effector plasmids (cyclin Medsa;*CycB2;3* or cdc2*Ms*A, see table 3.6.1) were co-introduced into tobacco mesophyll protoplasts. Both treatments resulted in significantly higher GUS activity than the protoplast population treated with the reporter gene construct only. (figure 3.6.2). Also the transient expression of cyclin Medsa;*CycB2;3* increased GUS expression by a factor of 2.5 fold. A greater stimulation (3.5 fold) was observed in cdc2*Ms*A over-expression. In an attempt to check whether the observed changes in histone promoter function indeed reflect the cell cycle events, we have analysed the actual distribution of cells in different phases by flow cytometry (figure 3.6.3). In comparison to the control, the frequency of S-phase cells was ~10-20% higher in cultures co-transformed with either the kinase or cyclin over-expression plasmid. However, transient expression of a 5' truncated cyclin Medsa;*CycB2;3* cDNA, lacking the 120 bp 5' coding sequence,

resulted in a reduced response and lower S-phase frequency with respect to the full length cyclin cDNA. The cyclin-dependent protein kinase inhibitor; ICK1 over expression caused a decrease in the GUS activity as well as in the percentage of S-phase cells (figure 3.6.2).

Transient expression of the full length cdc2MsA and* Medsa;*CycB2;3* *cDNAs increases the response of S-phase specific histone promoters in alfalfa protoplast-derived cells

In alfalfa cell suspension protoplasts similar histone promoter activation was observed. Here, the expression of either the kinase or the cyclin gene resulted in increased GUS activity relative to control cells. The cyclin Medsa;*CycB2;*3 was found to be slightly more effective inducer than the other treatments (figure 3.6.4). For cdc2*Ms*A the activation was dose dependent; up to 8-fold in comparison to control. GUS activity elevated with increased amounts of effector plasmid used in transformation. Simultaneous introduction of the two effectors (kinase and B-type cyclin) resulted in high GUS activity almost equivalent to the reporter activation obtained with 30 µg of cdc2*Ms*A plasmid alone (figure 3.6.4). Similar to the data obtained in tobacco cells, the 5' truncated cyclin Medsa;*CycB2*;3*;* cDNA could enhance GUS activity with respect to the control treatment, but approximately two times less effective than the full length cyclin cDNA.

Transient over-expression of alfalfa cell cycle genes in maize cells

In maize cells, we constructed vectors carrying both the reporter cassette (wheat histone H4 promoter::GUS::wheat histone H3 3'UTR) and the effector cell cycle gene component (table 3.6.1). Here, the kinetics of the GUS enzyme activity and DNA replication were measured daily for 4 days after DNA uptake. Our data indicated that the DNA synthesis and maximum reporter gene function controlled by wheat histone H4 promoter coincide at 72 hours post-transformation (data not shown). In the maize protoplasts, the expression of the alfalfa *cdc2MsA* gene resulted in the highest histone promoter response (figure 3.6.5) whereas cyclin Medsa;*CycB2*;3 also increased GUS activity but not as much as in the dicot cells.

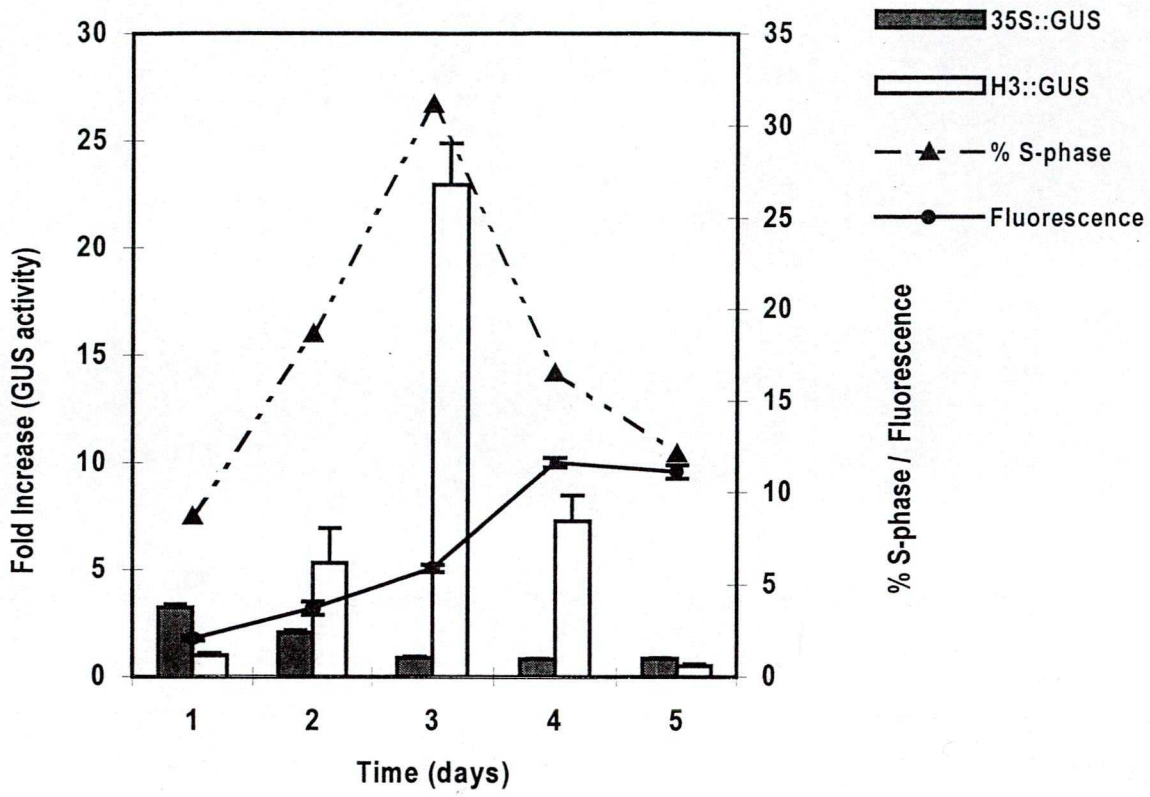
Table 3.6.1

A plasmid	promoter	gene	poly A	Origin
pH3GUS	histone ALH3-1.1	GUS	Histone H3 3'UTR	Kapros *et al.*; 1993
pDA1	CaMV 35S	cdcMs2A	CaMV 35S	this study
pDA2	CaMV 35S	CycB2;3	CaMV 35S	this study
pDA3	CaMV 35S	CycB2;3Δ	CaMV 35S	this study
pDA4	CaMV 35S	ICK1	CaMV 35S	this study
P35SGUS	CaMV 35S	GUS	CaMV 35S	Töpfer *et al.*; 1993
B				
pH4GUS	wheat histone H4	GUS	wheat histone H3	Omirulleh *et al.*; 1994
pDM1*	CaMV 35S	Cdc2MsA	CaMV 35S	this study
pDM2*	CaMV 35S	CycB2;3	CaMV 35S	this study
pDM3*	CaMV 35S	CycB2;3Δ	CaMV 35S	this study

*All pDM constructs contain a copy of pH4GUS reporter cassette on the same vector.

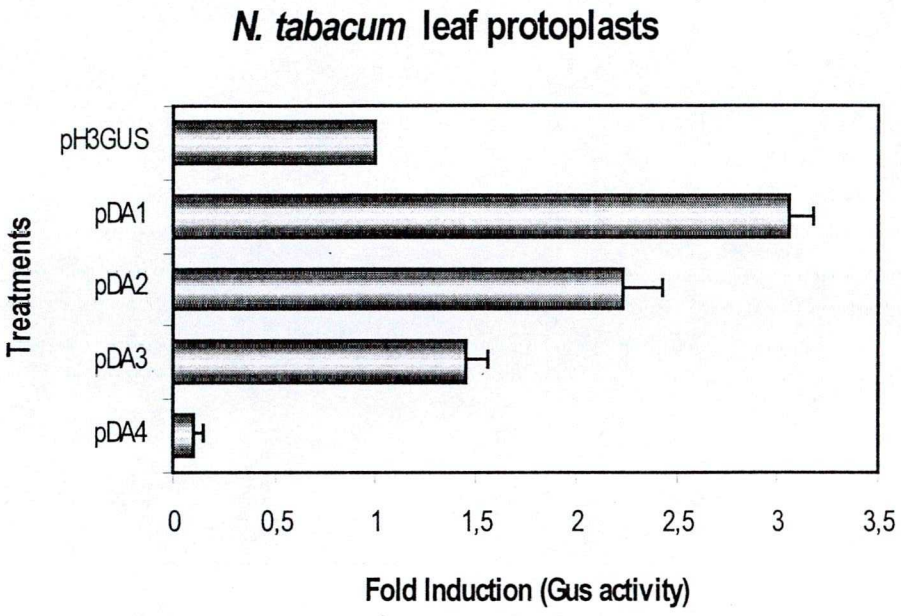
Composition of plant expression vectors used in this study. In panel A, reporter and effector plasmids used in co-transformation of dicot species, alfalfa and tobacco are shown. Panel B shows the characteristics of effector and reporter cassettes used for transient assays in maize cell suspension protoplasts.

Figure 3.6.1



Transient assays showing daily monitored activity of two different reporter plasmids and corresponding cell cycle parameters measured by flow cytometry and relative DNA amount by CyQuant cell proliferation assay in *N.tabacum* leaf protoplasts. The bar graph corresponds to GUS activity driven by histone H3 and CaMV 35S promoters given as fold change in reporter enzyme activity with respect to 0 time of protoplast transformation. All assays repeated for at least three times, each assay containing replicate readings.

Figure 3.6.2



Transient assays showing increased reporter activity upon transient over-expression of cdc2 and cyclin genes in *N.tabacum* leaf protoplasts. Data shown are average fold-inductions over four repetitions. The raw data was also statistically tested for "independence" from the control by *t*-test and both *cdc2MsA* and cyclin over-expressions was found to be significantly different from the control.

Figure 3.6.3

Flow cytometric analysis of *N.tabacum* leaf mesophyll protoplasts.

Freshly isolated leaf protoplasts

Control; 3 days after DNA uptake; transformed with H3::GUS::H3 3' UTR

3 days after DNA uptake; transformed with 35S::cdc2MsA+H3::GUS::H3

3 days after DNA uptake; transformed with 35S::*CycB2*;3+H3::GUS::H3

3 days after DNA uptake; transformed with 35S::Δ *CycB2*;3+H3::GUS::H3

3 days after DNA uptake; transformed with 35S::ICK1+H3::GUS::H3

These results showed increases in S-phase frequencies in cdc2*Ms*A and cyclin *CycB2*;3 overexpression and decrease with ICK1 overexpression, consistent with the reporter activity. Shown values represent percentage of cells at different stages of the cell cycle in two different assays and similar patterns were obtained from independent DNA treatments

Figure 3.6.3

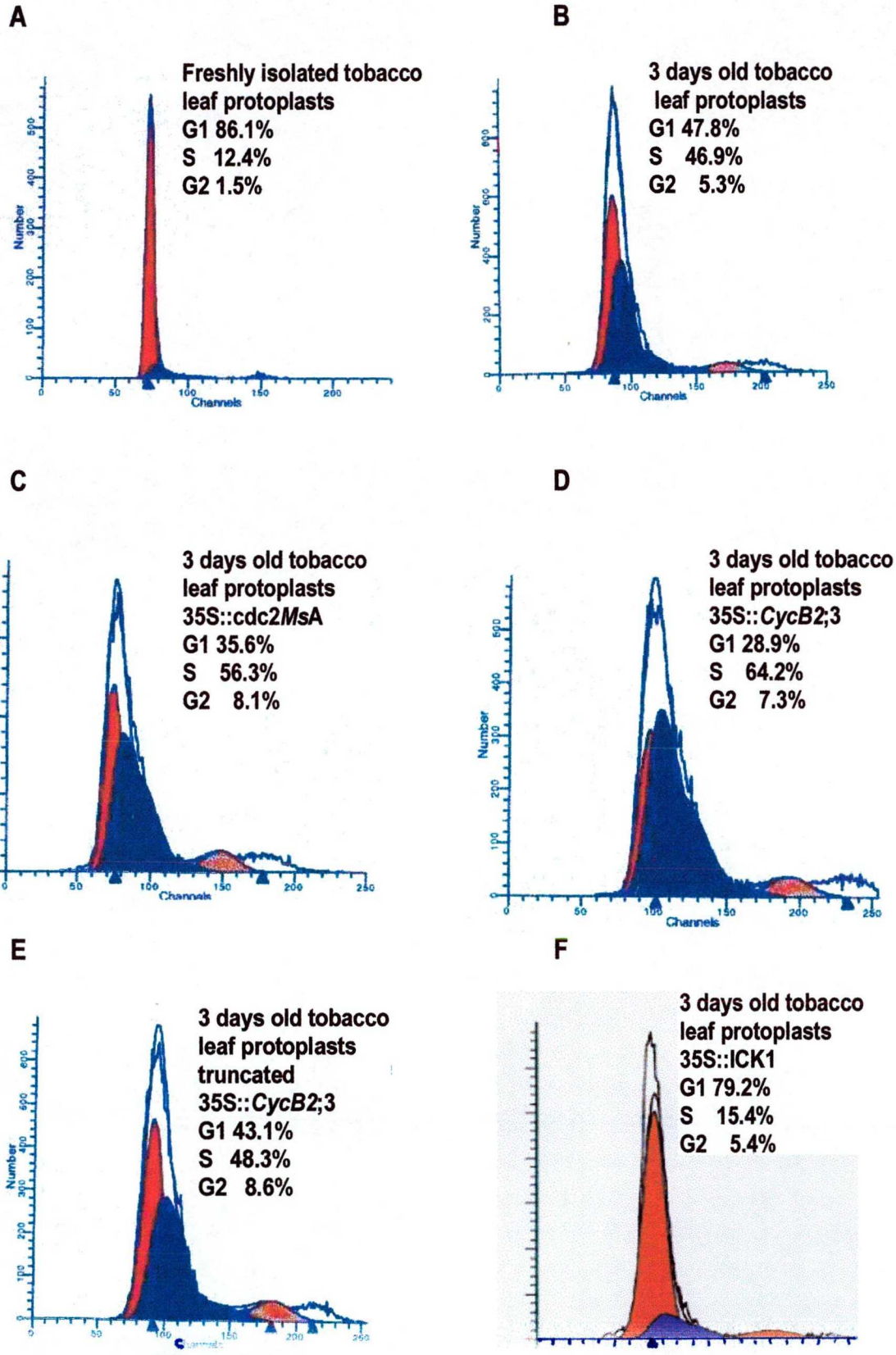
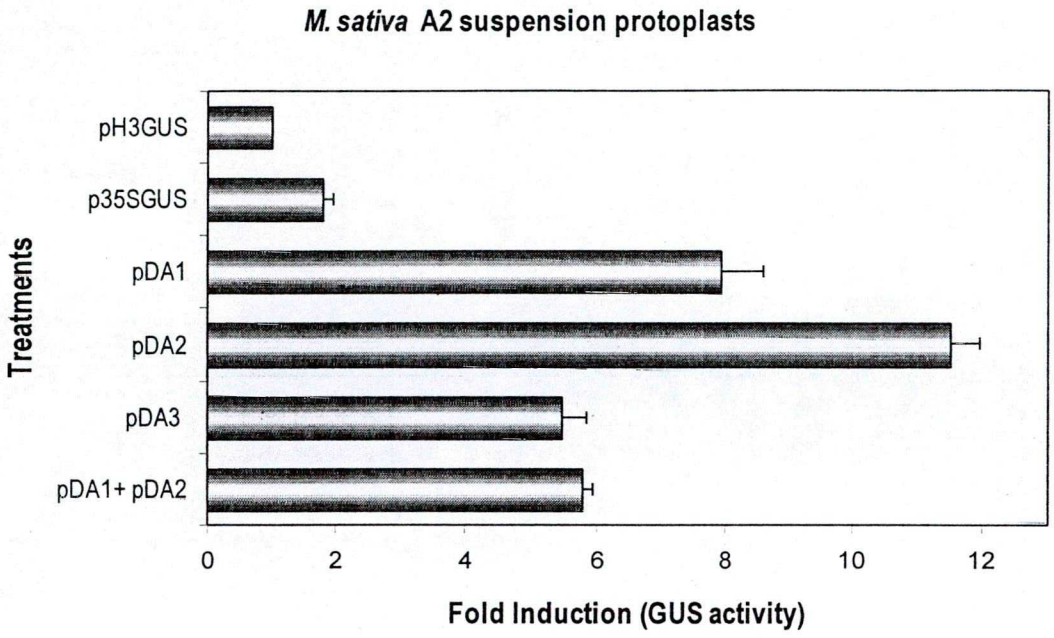

A
Freshly isolated tobacco leaf protoplasts
G1 86.1%
S 12.4%
G2 1.5%
B
3 days old tobacco leaf protoplasts
G1 47.8%
S 46.9%
G2 5.3%
C
3 days old tobacco leaf protoplasts
35S::cdc2MsA
G1 35.6%
S 56.3%
G2 8.1%
D
3 days old tobacco leaf protoplasts
35S::CycB2;3
G1 28.9%
S 64.2%
G2 7.3%
E
3 days old tobacco leaf protoplasts truncated
35S::CycB2;3
G1 43.1%
S 48.3%
G2 8.6%
F
3 days old tobacco leaf protoplasts
35S::ICK1
G1 79.2%
S 15.4%
G2 5.4%
Number
Channels

Figure 3.6.4



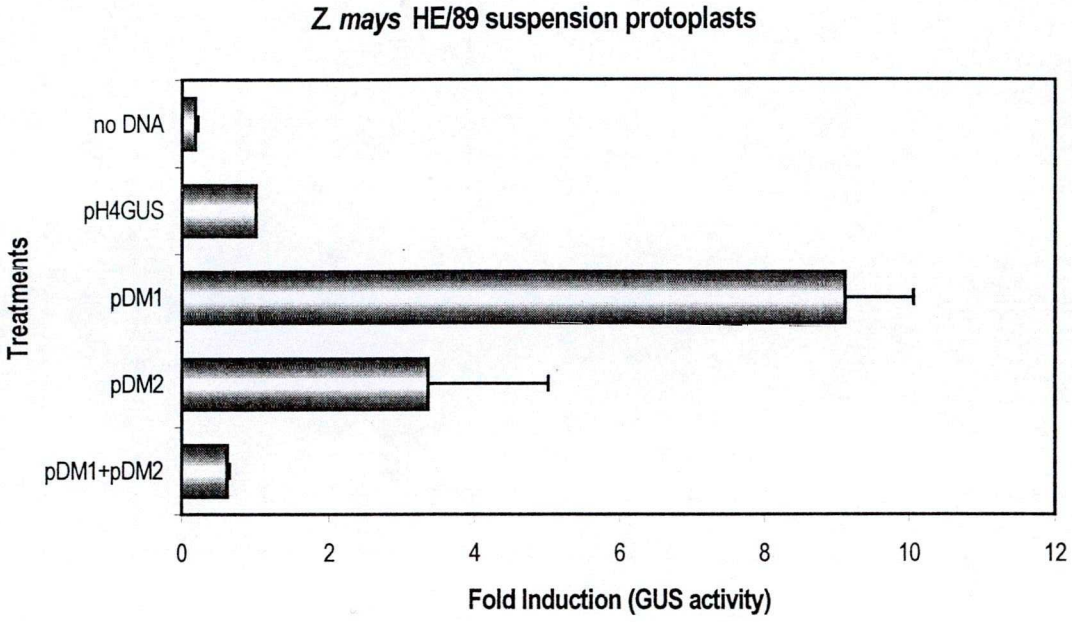
Transient assays using alfalfa A2 suspension cell protoplasts. All assays repeated for at least six times, each assay containing replicate readings. The raw data was also statistically tested for "independence" from the control by *t*-test. Both cdc2*Ms*A and cyclin over-expressions was found to be significantly different from the control. Standard errors are shown as error bars.

pDA1 35S::cdc2*Ms*A

pDA2 35S::*CycB2*;3

pDA3 35S::Δ *CycB2*;3

Figure 3.6.5



Transient assays using maize HE/89 suspension cell protoplasts. cdc2*Ms*A over-expression was found to be significantly different from control in *t*-tests. All assays were repeated for at least six times, each assay containing replicate readings. Standard errors are shown as error bars.

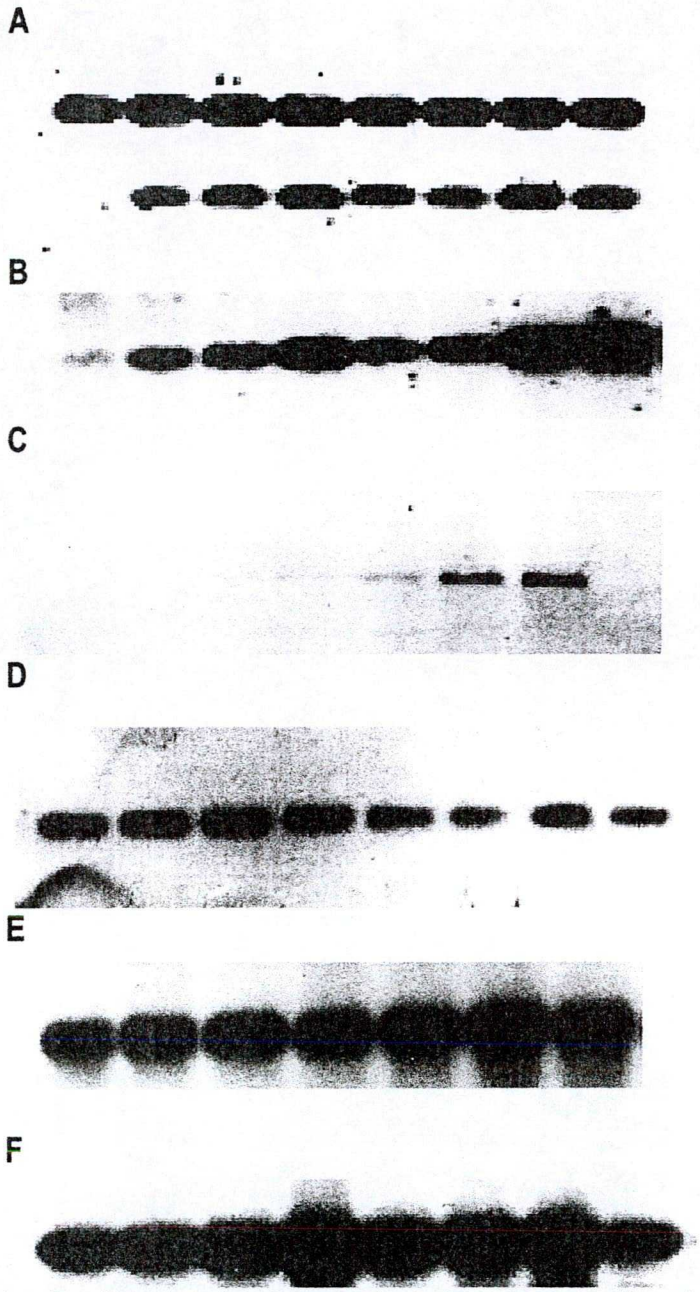
pDM1 35S:: cdc2*Ms*A+wheat H4::GUS::H3 terminator

pDM2 35S::*CycB2;*3+wheat H4::GUS::H3 terminator

3.7 *Expression analysis of cell cycle related genes in alfalfa mesophyll protoplasts during induction of cell cycle by wounding and hormone treatment*

Leaf mesophyll protoplasts were isolated from embryogenic alfalfa A2 line The expression patterns of cdc2*Ms*A, cdc2*Ms*B, cdc2*Ms*D, Medsa;*CycB2*;3, Medsa;*CycA2*;1, MAPK MMK1 and *Msc*27 were checked by RT-PCR. Cdc2*Ms*A and cdc2*Ms*B were detected by using one common and two gene specific primers in a single tube reaction. Their expressions were constitutive except cdc2*Ms*A expression could not be detected at 0 time (figure 3.7.1 A, lower band). Cdc2*Ms*D expression was detected starting from1 day sample and showed an increase on 6^{th}, 7^{th}, 10^{th} day samples (figure 3.7.1 B). *CycB2*;3 expression was detected only at 5^{th} and 6^{th} days which corresponds to G2-M phases (figure 3.7.1 C). MAPK MMK1 and *Msc*27 showed a constitutive expression (figure 3.7.1 E, F).

Figure 3.7.1



RT-PCR analysis of cell cycle related genes by using gene specific primers. Leaf mesophyll protoplast samples were collected daily up to 7 days.

A) cdc2*Ms*A (lower) and cdc2*Ms*B (upper band)

B) cdc2*Ms*D

C) Medsa;*CycB2*;3

D) Medsa;*CycA2*;1

E) *Msc27*

F) MAPK MMK1

CHAPTER IV

DISCUSSION

Activation of cell cycle genes due to hormonal treatments in alfalfa.

4.1 *Effects of different hormones and concentrations on the expression of cell cycle related genes*

The presence of auxins in the culture medium with or without cytokinins can initiate either formation of callus tissues or development of organs, or alternatively somatic embryos from *in vitro* cultured cells (reviewed by Dudits *et al.*, 1991). We have used two approaches for the analysis of response of cell cycle related genes to hormone treatments. First approach was to treat hormone starved suspension cells with low concentrations of NAA, kinetin and 24-epibrassinolide for different duration and second was to induce the suspension cells with high concentration of 2,4-D.

After application of NAA, kinetin and 24-epibrassinolide, when we examined the response of cdc2*Ms*A and cdc2*Ms*B. Their expression was slightly induced in response to NAA and kinetin treatments. A difference was observed in 24-epibrassinolide treatment, cdc2*Ms*A expression was induced in the presence of 1μM 24-epibrassinolide with or without NAA in the medium. Brassinosteroids are essential for normal plant growth and development (Clouse, 1996) and enhance the cell division rates significantly as shown in Chinese cabbage mesophyll protoplasts (Nakajima *et al.*, 1996), *Petunia hybridia* leaf mesophyll protoplasts (Oh *et al.*, 1998) and in cultured explants of *Helianthus tuberosus* (Clouse and Zureck *et al.*, 1991). They appear to be in close relationship with auxins (IAA, NAA) typically acting synergistically (Arteca, 1996) so induction of in the expression of cdks can be expected. However cdc2*Ms*A and cdc2*Ms*B are two cdks showing high sequence similarity (91% amino acid identity) and since now no difference was found in their expressional analysis (Hirt *et al.*, 1991, Magyar *et al.*, 1997). Medsa;*CycA2*;3 expression was only detected when the cells are treated with NAA and kinetin together. Medsa;*CycB2*;2 expression showed the same expression pattern (data not shown). Histone ALH3-1.1 was expressed all through the treatments though it was stronger in the cells treated with 1 mg/l NAA. The effect of hormones on histone promoters can be indirect. For example; in transgenic maize plants wheat histone 4 promoter was activated by 1 μM 2,4-D. The GUS staining pattern indicated a strong expression in root meristem and the root vascular tissues. On the other hand ABA diminished the signal in the root tip and in lateral roots. Zeatin application to those transgenics showed a patchy pattern just above the proliferating zone of the root tip indicating its possible role in activation of partially differentiated cell types (Bilgin *et al.*, 1999).

MAPK MMK1 expression was stronger than the control in all of the treatments and did not show any difference. There are reports on the hormone effect on MAP kinase activity (Hutley and Phillips,

1995, Knetsch *et al.* 1996, Mizoguchi *et al.* 1994) however very limited data is present on the effects of hormones on the transcriptional activation or inhibition of MAPKs.

ABA treatments

In plants, ABA, like environmental stresses or wounding effect can induce expression of a number of highly conserved genes. The accumulation of these gene products could protect plants from stress-induced damage. Many of these genes are expressed during embryogenesis (Sheen, 1996). In water-stressed plants, ABA facilitates stomata closure. ABA causes K^+ to leak out of the guard cells while H^+ and organic acids enter in causing the guard cells to close. Once closed ABA prevents opening in response to light. The signal transduction mechanism by which ABA produces these changes has not been fully established. However, there is evidence suggesting that $Ca^{2+}$and GTP-binding proteins have a role in signal transduction chain (Davies and Jones 1991, Li and Assmann 1993). ABA levels also increase in response to salt, cold and high-temperature stress, each of which are known to cause a deficiency of water. It has been suggested that ABA synthesis is regulated at the transcriptional level and following an increase in ABA there is a modification of gene expression in stressed plants (Davies and Jones 1991). Although Meszaros *et al.*, have analyzed the $p34^{cdc2}$ kinase level in alfalfa suspension culture treated with ABA and observed an ABA concentration-dependent inhibition above 50 µM in the phosphorylation of histone H1 in $p13^{suc1}$-Sepharose bound fraction. No transcriptional inhibition was observed in cdc2*Ms*A expression (figure 3.3.2). Wang *et al.* (1998) showed that ABA induced the expression of cdk inhibitor ICK1 but did not induce or inhibit the expression of *cdc2a* from *Arabidopsis thaliana* or ICK3.

We also could not detect a significant change in the Medsa;*CycB2*;2, Medsa;*CycA2*;1, Histone ALH3-1.1 and MAPK MMK1 expressions in ABA treated alfalfa suspension cells. To exclude the possibility of ABA was not taken up by the suspension cells we analyzed the induction of ABA inducible alfalfa aldose reductase (AldR). It was reported that NADPH-dependent aldose reductase shows ABA induced transcriptional activation and is one of the responsible genes of ABA-induced freezing tolerance in bromegrass cell cultures (Lee *et al.*, 1993). The aldose reductase gene from barley is also shown to be expressed constitutively during embryo maturation and is modulated by the plant hormones abscisic acid (ABA) and gibberellic acid (GA) (Bartels *et al.*, 1991). When we analyzed the expression pattern of *Medicago sativa* AldR (Obershall *et al.*, unpublished) in ABA treated cells we obtained an induction with the increasing concentration of ABA and through the time course experiment a strong induction was observed after 2 hours. This indicated the effective entrance of ABA to the suspension cells.

ABA was thought to have an inhibitory effect on cell cycle progression. For example; reduction of cell division and/or DNA synthesis in root tips (Newton, 1977, Barlow and Pilet 1984), in apical, axillary shoot meristem (Fraser and Mathews, 1983, Jacqmard *et al.*, 1995) and in embryo (Phillips,

1971, Myers *et al.*, 1990) were reported. However, when the correlation between cell cycle progression and endogenous levels of plant hormones was studied in synchronized tobacco BY-2 cell suspension cultures. No significant correlation could be identified for IAA and ABA. In contrast, there were sharp peaks in the levels of specific cytokinins (zeatin- and dihydrozeatin-type) at the end of the S phase and during mitosis (Redig *et al.,* 1996). In intact roots, ABA is found in root cap and thought to be the cap inhibitor then placing root caps on growing cleoptiles would be expected to inhibit elongation. However, an auxin like stimulation (instead of ABA-like inhibition) of growth was observed in response to this treatment (Moore *et al.*, 1984).

When we analyzed the expression of D-type cyclin Medsa;*CycD3*;1 we observed an induction 2-8 hours of ABA treatment. The induction can be caused by the increase in the Ca^{2+} concentration in the cell due to ABA treatment. The induction by ABA is a short-term one as the induction of the Medsa;*CycD3*;1expression decreases at 24 hours. During this short-term only Medsa;*CycD3*;1can be induced but not the others because it is one of the first genes to be activated in the cell cycle cascade. ABA can be involved indirectly in the induction of D-type cylins by the changes it caused in the cell state. For example; wounding induces several cyclin expression (Bögre *et al.* 1995) and it is known that ABA is involved in mediating the wound response and can induce several wound inducible genes in the absence of a wound signal (Bowles 1993).

Since the discovery of ABA, researchers have been trying to provide evidence that it is the dormancy hormone but they have been unsuccessful. Early work by Eagles and Wareing (1963) showed that under short days ABA levels were increased in leaves and buds resulting in dormancy and that exogenous application of ABA to non-dormant buds promoted dormancy. It has also been shown that short-day treatments, which induce dormancy, in some species, do not promote increases in ABA levels correlating with the induction of dormancy. Previously ABA was thought to be responsible for causing abcission of leaves, fruits and flowers, it is now generally accepted that it is not. ABA probably acts indirectly by causing premature senescence which causes a rise in ethylene production, which turns on numerous genes involved in abscission (Osbourne 1989)

4.2 *Induction of somatic embryogenesis in alfalfa cell suspension by 2,4-D*

The development of the plant embryo comprises two processes; first the establishment of the precise spatial organization of the component cells derived from a single-fertilized egg or in somatic embryogenesis case from stimulated totipotent cells, second, the generation of cellular diversity within the developing embryo-cytodifferentiation. With our experimental system we focused on the expressions of the cell division related genes after application of an external stimuli- 100µM 2,4-D. Several auxin-regulated genes have been isolated (rewieved by Sakai, 1992) however regulatory sequences involved

in hormonal activation of transcription of these genes are not clear (Meinke, 1995). The stimulation of totipotent cells to form somatic embryos involves the reactivation of cell cycle. The artificially induced series of cell divisions requires coordinated expression of set of responsible genes and the post-translational modifications of the regulatory proteins. The cell cycle control genes play a crucial role in this event (Dudits *et al.* 1995). The key regulatory elements of the cell cycle control, cdc2 and cyclin expression were analysed as well as MAPK MM1 and histone H3.1 after 2,4-D induction.

Induction of cell division in hormone treated cells is a consequence of the activation of a multicomponent cascade system that includes binding of the hormone to the receptor molecules, triggering of the signalling pathway, reprogramming of gene expression and structural reorganization of the cyto-architecture (reviewed by Dudits *et al.* 1993). Various stimuli including wounding, elicitors and drought have been shown to activate rapidly protein kinases (reviewed by Machida *et al.* 1997). It is believed that in plants MAPKs play an important role in cellular transmission of signals generated by various stimuli. In our experimental system, 2,4-D triggers the cascade of events that is why we analysed the expression pattern of MAPK MM1. The transcript of MAPK MM1 never wanished and appeared in fluctuations. At the first hours and especially at 24, 48 hours and 1 week samples it was strongly transcribed (see figure 3.4.1 B). It is suggested that MAPK MM1 play a role in cell proliferation as it is detected at higher level at the S and G2 phases than the other phases. 24 hours must be a crucial time point after the induction because all of the tested genes showed an increase-slight or strong-at this time point. The mitotic cyclin Medsa;*CycB2*,3 expression was first observed at 24 hours. The D-type of cyclin Medsa,*CycD3*,1 was also expressed strongly at 24 hours however its transcript appeared earlier than the mitotic cyclin. Cdc2*Ms*A expression was mainly constitutive, started to vanish when embryos are formed. Histone H3.1 expression was stronger after 24 hours.

Under stress conditions (cold, drought), the accumulation of steady state transcripts of a MAP kinase was observed (Jonak *et al.* 1996). Several hormones have been reported to positively or negatively affect the enzymatic activity of MAP kinases. ABA induces the activation of a MAP kinase in barley (Knetsch *et al.* 1996) Giberellic acid affect the MAP kinase pathway in a negative way (Hutley and Phillips, 1995). Refeeding auxin to auxin starved cells leads to MAP kinase activation (Mizoguchi *et al.* 1994). So it has been suggested that MAP kinases might be involved in auxin signalling. 2,4-D induction triggers the MAPK MM1 transcription. As a consequence of these first the D-type cyclin and then the mitotic cyclin transcriptions are induced. Cdc2*Ms*A has shown a very slight increase in its mRNA expresion at 24-48 hours. Its regulation is mainly post-transcriptional and compared to cyclins the cdc2MsA transcript is always present except its transcript decreased as cells differentiate and form an embryo.

During the hormone response analysis of the cell cycle related genes, several cdks and cyclin genes were used. It has been always tried to involve cdc2*Ms*A and cdc2*Ms*B, and one of the mitotic cyclins in the analysis. Medsa;*CycB2*;2 was cloned and shown that it exerts a sequence similarity to Medsa;*CycB2*;3 around 95%. Due to high similarity it was preferred to used Medsa;*CycB2*;3 as it was a full clone. When both of mitotic cyclin were analysed no difference in mRNA expression was observed.

4.3 *Analysis of an Antirrhinum majus mutant „phantastica".*

It has been shown that certain cyclins two B type and one A-type, Fobert *et. al* 1994) and two cdks (Fobert *et al* 1996) are expressed during specific phases of the cell cycle in *Antirrhinum majus* (snapdragon) meristems. Two different D-cyclins are cloned and characterized by *in situ* hybridization that they are expressed throughout the cell cycle only in proliferating cells Antma;*CycD3*;1 and Antma;*CycD3*;2 (figure 3.5.3 and 3.5.4, respectively). One of these, the Antma;*CycD3*;1 is up-regulated just as cells leave the meristem and participate in leaf primordia formation. D-cyclins are implicated in regulating entry into the cell cycle and are excellent candidates for mediating developmental influence over cell proliferation by controlling entry into the cycle. The expression of Antma;*CycD3*;1 therefore defines a distinct population of proliferating cells within the phytomer which are committed to a particular developmental fate (Gaudin *et al;* submitted). This population is characterized by two other molecular markers: The homeobox protein, *knotted KN1* (Jackson *et. al* 1994), and the *MYB* gene *phantastica* (*PHAN)* (Waites and Hudson 1995). Knotted protein is down regulated at approximately the same stage as Antma;*CycD3*;1 is switched on and may be a negative regulator (Jackson & Doonan unpublished); while the *MYB* gene, *phantastica*, is expressed in approximately the same domain as Antma;*CycD3*;1 and may be a positive regulator (Hudson & Doonan unpublished).

Four mutations were described at the locus of *PHAN* of *Antirrhinum majus*. The mutant phenotypes suggest that *PHAN* expression is involved in all aspects of dorsoventality in leaves, bracts and petals: from specifying the position of laminal initiation early in organ development, to determination of dorsal cell types at a later stage (Waites *et al.*, 1995). All four mutants are more similar to wild-type when grown at higher temperatures. However when they are grown at 20 °C, they show a phenotypic difference (figure 3.5.1 B). They produce two types of leaves; needle like young leaves showing no evidence of dorsoventrality, they lack laminae and all cell types associated with the dorsal region of the wild type leaf and heart shaped wrinkled leaves containing more cells in transverse section (Waites *et al.*, 1995). They also produce wrinkled flowers. It is also known that the mutation in *PHAN* locus causes the meristem to cease growth at low temperature (17 °C and lower). We have performed the temperature shift experiments and examined the expression of Antma;*CycD3*;1 which is thought to be positively regulated by *PHAN* gene. Our results both by RT-PCR and *in situ* mRNA hybridization,

showed that Antma;*CycD3*;1 transcript is dramatically reduced in *PHAN* mutant grown in 15 °C and no transcript reduction was observed in the control plants grown at the same temperature (figure 3.5.2, 3.5.5). Understanding how the transcription of the Antma;*CycD3*;1 is regulated will provide insights into the spatial and temporal control of cell proliferation during development of the phytomer. We have also observed a colour change in the flowers of the *PHAN* mutant when they are shifted to 15 °C , they became dark pink.

4.4 *Transient over expression of cell cycle genes.*

Alfalfa and wheat histone H3 gene promoters are well-characterised regulatory sequences conferring S-phase-specific expression (Kapros *et al.,* 1993, Ohtsubo *et al.,* 1993). In transient expression assays, Lepetit *et al.,* (1992) could not detect replication-dependent GUS activity when *Arabidopsis* histone H4 promoter GUS reporter gene fusions were tested in tobacco mesophyll protoplasts. This observation was linked to the fact that tobacco leaf protoplasts did not possess any DNA replication activity at 24 hours after DNA uptake. It was also postulated that regulated activity of replication-dependent histone promoters is exclusively dependent on integration into the chromatin for transcription. In our experiments we were able to detect substantial GUS activity, particularly at 72 hours, in parallel to the increased frequency of S-phase cells. In a similar assay, a constitutive CaMV 35S promoter exhibited a constant level of induction for five days after a major peak at 24 hours, indicating its replication-independent expression (figure 3.6.1). As the *in vitro* protoplast culture, PEG mediated DNA uptake and mode of detection were similar, the observed difference between the *Arabidopsis* H4 and alfalfa histone H3 promoters may arise from the presence of the histone H3 termination sequence in the reporter plasmid used in our transient assays. The 3' UTR sequences of histone genes were shown to be involved in S-phase specific post-transcriptional 3' processing and prolongation of mRNA half-life of histone genes resulting 5-10 fold more transcripts (Chaubet and Gigot., 1998). Many of the well characterized plant histone genes share several conserved sequence motifs at their 3' ends and these sequences were shown to be involved in modulating S-phase associated activity of replication-dependent histone genes (Mikami et. al., 1993, Ohtsubo et. al., 1994).

In plants, homologues of several cdc2 and cyclin genes were isolated and characterized (Ferreira *et al.,* 1991; Hirayama *et al.,* 1991; Hirt *et al.,* 1991, Renaudin *et al.,* 1994, Fobert *et al.,* 1997). However, there are only few reports on their involvement in plant cell cycle regulation *in vivo.* Currently available information on plant cell cycle originate from expression analysis of mRNA/protein species in synchronized cell cultures (Magyar, *et al.,* 1997; Fuerst, *et al.,* 1996), *in situ* mRNA analysis in shoot meristems (Fobert *et al.,* 1994); complementation of yeast cell cycle mutants or unfertilized Xenopus eggs via the expression of plant cDNAs (Hirt *et al.,* 1993, Renaudin *et al.,* 1994), interaction of putative

partner proteins in yeast two hybrid screens (De Veylder *et al.,* 1997a and 1997b) and analogies based on structural characteristics among proteins derived from different organisms (Renaudin *et al.,* 1996, Dudits *et al.,* 1998).

The modularly organized promoter regulatory elements of eukaryotic histone H3/H4 gene promoters and the cognate transcription factors have been characterized within the context of cell cycle-dependent regulatory parameters indicates presence of phosphorylation–dependent modifications in transcription factor interactions (Heintz, 1991). In humans, the S-phase transcription factor complexes assembling at the histone promoters include cdc2, cyclin A, and a Rb related protein (van Wijnen *et al.,* 1994). Thus, reflecting an integration of phosphorylation-mediated control of histone gene expression at the G1-S phase transition point. In transgenic tobacco leaf protoplasts, the activation of *Arabidopsis cdc2 At* promoter driven transcription was shown to occur at the G1/S transition (Hemerly *et al,* 1993). Similarly, in fission yeast, the temporal order of S-phase was shown to be determined by $p34^{cdc2}$ kinase-B cyclin complexes (Hayles *et al..,* 1994). In *Xenopus,* over-expression of Cdks (cdc2 and cdk2) were enough to induce S-phase in Cdk depleted egg extracts (Chevalier *et al.,* 1995). Stimulation of histone promoter activity and S-phase upon over-expression in plant protoplasts, suggests similar structural and functional roles for the alfalfa *cdc2MsA,* in the assembly of cell cycle regulatory complexes of histone gene promoters. In higher plants, the S-phase specific binding activity of a wheat histone binding protein HBP-1 (bearing a putative cdc2 like kinase phosphorylation site) was also shown to be modulated by phosphorylation/dephosphorylation treatments of nuclear extracts in wheat (Iwabuchi, *et al.,* 1998).

Our experiments revealed that transient over-expression of the *Cdc2* kinase induced high level of histone promoter mediated GUS activity both in monocot and dicot species. Compared to high GUS activity obtained with *Cdc2* kinase over-expression, the alfalfa B-type cyclin showed significant differences in GUS activity between dicot and monocot systems. The C*dc2* kinases are highly conserved among plant species as compared to cyclins and this conservation may be functional among plant species (Renaudin *et al.,* 1996; Dudits *et al.,* 1998). The conserved nature of the plant Cdk's can probably overcome the potential incompatibilities together with the other putative partners of activation complexes. The ectopic expression of relevant Cdk's may complement the function of host counterparts in heterologous systems. The dose-dependent stimulation of histone promoter by alfalfa *cdc2MsA* reflects a „threshold" concentration of DNA introduced; effective at 30µg of the plasmid. The use of both homologous and heterologous systems not only showed the reliability and broad usage of this method but also indicated differential activity in monocot and dicot species.

To observe whether the increases in the reporter gene function are linked with cell cycle progression, the actual distribution of cells in different cell cycle phases were analyzed by flow cytometry in transformed tobacco leaf protoplasts. We have also tried to analyze the state of cells in alfalfa and

maize transient expression experiments however we failed to get a clear analysis of the cells and the reason for this could be that they are not dividing as synchronous as tobacco leaf protoplasts do. The second method; cell proliferation assay based on fluorescence enhancement of a dye upon binding to DNA not only provided information on daily progress in DNA synthesis but also confirmed our results obtained from flow cytometry in tobacco leaf protoplasts. In *cdc2MsA* and cyclin *CycB2*;3 over-expressions, the increased percentage of S-phase cells in the cultures at 72 hours could either result from the stimulation of more cells to progress from G1 to S phase or by inhibition of exit from the S-phase. As the frequency of mitotic divisions among treatments was similar, we excluded the second case (data not shown).

Based on the previous characterization of Medsa;*CycB2*;3 it is expected to be involved in G2/M transition. However, its mRNA was also detected at the G1 phase of the synchronized alfalfa cells (Hirt *et al.,* 1992, Savouré *et al.,* 1995). Interestingly, in alfalfa and tobacco cells, its over-expression resulted in elevated histone promoter activity and increased number of S-phase cells as shown in our gene expression assay. After over-expression of a 5' truncated Medsa;*CycB2*;3 lacking the destruction box, the observed GUS activity was less than the levels induced by full length Medsa;*CycB2;*3. The B-type cyclins are destroyed towards the end of mitosis and at the time of sampling, the tobacco protoplasts progress through G1-S phases. We propose that G1-S interference by the B-type cyclin was through protein-protein interaction and thus this interaction was less effective with its truncated form. Studies on other eukaryotes have also indicated the ability of B-type cyclins driving G1-S phase progression; in fission yeast a mitotic cyclin $p56^{cdc13}$ could promote progression into DNA replication and mitosis (Fischer, 1996). In *Arabidopsis* the expression of a B-type cyclin, *CycB2;at;1* under the control of the *cdc2At* promoter significantly accelerated root growth in *Arabidopsis* plants (Doerner *et al.,* 1996) which may also reflect its positive effect on cell cycle re-activation. Most recently, two D-type cyclins from tobacco was shown to accumulate prior mitosis rather than its predicted G1 phase presence (Sorrell *et al.*, 1999). This finding also suggests that plant cyclins may govern roles other than predicted from their protein sequences.

The use of a cyclin dependent protein kinase inhibitor; ICK1, isolated from *Arabidopsis thaliana*; (Wang *et al.*, 1998) in tobacco experiments revealed a low GUS activity and low percentage of S-phase cells determined by flow cytometry analysis (figure 3.6.2.B). It has been shown by *in vitro* binding assays that ICK1 protein could interact independently with both Cdc2a and CycD3 from *Arabidopsis thaliana*; (Wang *et al.*, 1998). It acts as an inhibitor of Cdc2 kinases *in vitro.* Our results support the inhibitory effect of ICK1.

The production of transgenic plants have several drawbacks, such as the time required to produce transgenic plants, the unusual behaviour of the transgene due to the site of integration (copy

numbers, position effects), post-integration modifications (gene silencing, formation of anti-sense transcripts due to downstream host promoter). Perhaps the most serious problems occur when plants are re-generated; since this process requires all divisions. All cells carrying these transgenes must be able to divide and thus, one cannot use this approach if the gene interferes significantly with the cell division and plant development. Considering these factors, plant protoplasts and transient expression assays provide valuable research tools for studying fundamental cellular processes in plants and can be utilised for functional analysis of cloned cell cycle regulatory genes.

The transient over-expression of cell cycle regulatory genes in protoplast-derived cells cultured *in vitro* and lack of signals generated by spatial organization of tissues created an artificial state concerning the actual presence of mRNAs/proteins during the cell cycle. For that reason, it is of great importance to emphasize that these results may not directly reflect absolute functions of the genes tested. One should also consider that in „PEG mediated DNA uptake" it is possible to introduce DNA into a certain number of the target cells and these may in turn be studied in the reporter gene assays. However, flow cytometry and cell proliferation assay reflect the overall profile of the population including cells that were not transformed or incompetent for reporter gene function. Nevertheless, this transient assay offer the possibility of testing functional roles of genes particularly during the activation of S-phase machinery and/or histone gene expression

4.5 *Expression Analysis of cell cycle related genes in alfalfa mesophyll protoplasts during induction of cell cycle by wounding and hormone treatment.*

To study the cell cycle events one needs a synchronously dividing cell population. There are several ways of obtaining a synchronous cell population. The suspension cultures are synchronized by chemical (hydroxyurea, aphidicolin, treatments) or physical (phosphate starvation) means (Dedeoglu 1992). However these chemical or physical synchronization methods are either start at S-phase or do not obtain high synchrony to study all the phases of the cell cycle. Isolation of leaf mesophyll protoplasts is an alternative way of obtaining a synchronous cell population (85-90% at G1-phase at 0 time). As the cells of a mature leaf are mostly in G0-phase, the isolated protoplasts enter to the cycle to the cycle from G1-phase by the effects of several external stimuli (wounding and presence of hormones). The only handicap with this method is the amount of material to do the northern analysis of several genes especially the low copy ones like cyclins. Here I used the RT-PCR method to overcome this handicap and showed the expressional analysis of several cell cycle related genes with a very limited amount of protoplasts. RT-PCR analysis is a more sensitive method compared to conventional northern analysis. For this reason, for each gene of interest the PCR reaction was optimized and stopped at the logarithmic phase. When the reaction mixtures were loaded on the denaturing gel, practically nothing

was visible, results were obtained after non radioactive nucleic acid hybridization. Design of gene specific primers enabled me to do the expression analysis of genes showing high similarity such as; cdc2*Ms*A and cdc2*Ms*B –91% amino acid identity. Such a protoplast population of alfalfa A2 line are known to enter to S-phase on the 3rd day and M phase on 5th day and after (Bögre *et al.,* 1990). Cdc2*Ms*A could not be detected on the 0 time which ~90% of the cells are in G1-phase. Cdc2*Ms*D signal showed an increase on 3rd day and after that 6th and 7th days that corresponds to S and M-phases, Medsa;*CycB2;*3, a mitotic cyclin, expression was only detected at 5th and 6th days. The same expression pattern was observed with another mitotic cyclin Medsa;*CycB2;*2 (data not shown). Medsa;*CycA2;*1 expression was constitutive as this is termed as a replacement cyclin. It is known that in the cultured cells MAPK MMK1 expression was detected at higher levels at S and G2 phases of the cell cycle than at the G1 phase (Jonak *et al.,* 1993 and Bögre *et al.,* 1997). I observed the same pattern in which there was an increase in MAPK MMK1 expression on the 3rd day and after. Msc27 did not show any fluctuation in their expression as it has a cell cycle independent expression and used as a control. One should always keep in mind that after 5-6 days the synchrony of cells starts to decrease.

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APPENDIX A

SOLUTIONS

Moroshige-Skoog Medium (1000 ml)

NH_4NO_3	1.65 g
KNO_3	1.9 g
$MgSO_4.7H_2O$	0.37 g
KH_2PO_4	0.17 g
$FeSO_4$+EDTA (5.5 g/L + 7.45 g/L)	5 ml
$CaCl_2.2H_2O$ (15 %)	2.9 ml
MS microsalt stock solution	1 ml
B5 vitamin stock solution	1ml
Casein	0.25 g
Sucrose	20 g

pH=5.7 (with KOH)

Hormone concentrations: 1 mg/L 2,4D and 0.2 mg/L Kinetin.

0.2% gerlit or 0.8 % agar.

Sterilized by autoclaving.

MS Microsalt Stock Solution (100ml)

$MnSO_4.4H_2O$		2.23 g
H_3BO_3		0.63 g
$ZnSO_4.7H_2O$		0.82 g
$Na_2MoO_4.2H_2O$	(dissolved separately)	25 mg
$CuSO_4$		2.5 mg
$CoCl_2.6H_2O$		2.5 mg
KI		0.083 g

B5 Vitamin Stock Solution (100 ml)

Nicotinic acid	0.1 g
Thiamin.HCl (vit 1)	1 g
Pyridoxin.HCl (vit B6)	0.1 g
Myo-inositol	10 g

SHF Medium (1000 ml)

KNO_3	2.5 g
$MgSO_4.7H_2O$	0.37 g
$NH_4H_2PO_4$	0.3 g
$CaCl_2.2H_2O$(15% stock)	1.3 ml
$FeSO_4$+EDTA (5.5g/l +7.45 g/l)	2.7 ml
Saccharose	30 g
SH microsalt stock solution	1 ml
SH vitamin stock solution	20 ml

pH=5.6 (with KOH)

0.8% agar

Hormone Concentrations (for KS10 line) 10 μM NAA + 1 μM Kinetin .

SH Microsalt Stock Solution (100 ml)

$MnSO_4$		1 g
H_3BO_3		0.5 g
$Na_2MoO_4.2H_2O$	(dissolved separately)	0.01 g
$ZnSO_4.7H_2O$		0.1 g
$CuSO_4$		20 mg
$CoCl_2$		10 mg
KI		100 mg

SH Vitamin Stock Solution (1000 ml)

Myo-inositol	50 g
Thiamin-HCl	250 mg
Nikotinic acid	250 mg
Pyridoxin-HCl	25 mg

SH Regeration Medium

$NH_4H_2PO_4$ (7.5%)	4 ml
Proline (3M)	10 ml
$(NH_4)_2SO_4$ (13.5%)	10 ml

First these three components are mixed and filter sterilized. Then the volume is completed to 1000 ml with sterile SH medium (hormone free, pH=5.6-5.7 containing 0.8% agar and autoclaved).

E1 Enzyme

The following enzymes were disolved in 100 ml of A/2 solution.

Cellulase (R10)	2 g
(Yakult Honsha co,Ltd for pp. isolation)	
Driselase	0.5 g
(Fluka 26.5 u/mg)	
Macerozyme	0.5 g
(R10 for living cells, Yakult)	
Pectinase	1 g
(Serva 0.2 u/mg)	
Pectolyase	0.1 g
(Sigma 3.2 u/mg)	

A/2 solution (100 ml)

MES	58.5 mg
NaH_2PO_4	10.0 mg
$CaCl_2.2H_20$	100 mg
Mannitol	6.37 g (0.35 M)
Sorbitol	6.37g (0.35 M)
pH =6.5	

The enzyme mixture was centrifuged and the clear phase was taken, its pH was adjusted to 5.6 (with KOH) and filter sterilized

UM Washing Solution (1000 ml)

D Glucose	68.4 g
$CaCl_2.2H_2O$	200 mg
pH = 6.0 (with 0.1 N NaOH)	
Sterilized by autoclaving.	

MaMg (Alfalfa transformation buffer, 1000 ml)

0.4 M Mannitol	72.84 g
15 mM $MgCl_2$	3.05 g
0.1% MES	1 g

pH = 5.6

Sterilized by autoclaving.

PEG 4000

40% PEG 4000

0.4 M Mannitol

0.1M $Ca(NO_3)_2.4H_2O$

pH=7-9

Filter sterilized, stored at - 20 °C

K75 protoplast medium (1000 ml)

NH_4NO_3	0.6 g
KNO_3	1.9 g
$MgSO_4.7H_2O$	0.3 g
KH_2PO_4	0.17 g
KCl	0.3 g
$FeSO_4$+EDTA (5.5 g/l+7.45 g/l)	5 ml
KI (75 mg/100 ml)	1 ml
Sucrose	250 mg
Casein	250 mg
Glucose	70 g
B5 micro	1 ml
K75 vitamin	1 ml
Organic acids	2 ml
Sugar stock soln.	10 ml
Nuc. acid stock soln.	1 ml
Aminoacid stock soln.	10 ml
Coconut milk	5 ml
$CaCl_2$ (15%)	4 ml
Mannitol (0.2 M)	36 g

$\Rightarrow$ pH=5.6 (NaOH)

Filter sterilized

B5 Microsalt Stock Mixture (100 ml)

$MnSO_4$		1 g
H_3BO_3		0.3 g
$ZnSO_4.7H_2O$		0.2 g
$Na_2MoO_4.2H_2O$	(dissolved separetely)	25 mg
$CuSO_4$		2.5 mg
$CoCl_2.6H_2O$		2.5 mg

K75 Vitamin Mixture (100 ml)

Nicotinamide	10 mg
Pyridoxin. HCl	10 mg
Thiamin.HCl	10 mg
D-Calcium-Pantot-henat	10 mg
Folic acid	4 mg
p-Aminobenzoicacid	0.2 mg
Biotin	0.1 mg
Cholinchloroid	10 mg
Riboflavin (B2)	2 mg
Ascorbicacid	20 mg
Vitamin A	0.1 mg
Vitamin D3	0.1 mg
Vitamin B12	0.2 mg
Inozit (myo-inositol)	10 mg

$\Rightarrow$ pH=6.5

Nucleic Acid Stock Solution (100 ml)

Adenine	10 mg
Guanine	3 mg
Thymine	3 mg
Uracil	3 mg
Hypoxantine	3 mg
Cytocine	3 mg

$\Rightarrow$ pH=6.5

Organic Acid Stock Solution (100 ml)

Sodium-pyruvat	200 mg
Citrate	400 mg
Malate	400 mg
Fumarate	400 mg

$\Rightarrow$pH=6.5 (NH_4OH)

Sugar Stock Solution (100 ml)

Fructose
Xylose
Mannose
Rhamnose
Cellobiose
Sorbitol
Mannitol

All sugars 2500 mg

pH=6.5

Aminoacid Stock Solution (100 ml)

Glutamine	56 mg
Alanine	6 mg
Glutamicacid	6 mg
Cystein	2 mg

Asparaginic acid, arginine, cystin, glycine, histidine, isoleucine, leucine, lysin, Methayinine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, aspargine $\Rightarrow$ 1 mg

$\Rightarrow$pH=6.5

K3 Medium (Tobacco protoplast culturing Medium)

Macroelements (mg/l)

KNO_3	2500
NH_4NO_3	250
$CaCl_2X2H_2O$	900
$MgSO_4X7H_2O$	250
$(NH_4)_2SO_4$	250
$NaH_2PO_4XH_2O$	150
$CaHPO_4$	50

Microelements (mg/l)	
Na_2EDTA	37.3
$FeSO_4X7H_20$	27.8
H_3BO_3	3.0
KI	0.75
$MnSO_4\ XH_20$	10.0
$ZnSO_4X7H_2O$	2.0
$CuSO_4X5H_2O$	0.025
$Na_2MoO_4X2H_2O$	0.25
$CoCl_2X6H_2O$	0.025
Carbohydrates (g/l)	
D(+) Sucrose	102.69
D(+) Xylose	0.25
myo-Inositol	0.1
Vitamins (mg/l)	
Pyridoxine HCl	1.0
Thiamine Hcl	10.0
Nicotinic acid	1.0
Hormones (mg/l)	
2,4-dichlorophenoxyacetic acid	0.1
1-naphthylacetic acid	1.0
6-benzylaminopurine	0.2

mRNA In situ Hybridization Solutions

Fixative (4% Paraformaldehyde)

For 300m1 of fixaUve, 150 ml dH2O was heated to 60 Oc and 2 drops of 2M NaOH was added. 12 g of paraformaldehyde was dissolved in this in fumehood. 150 ml of PBS (phosphate buffered saline, pH 6.5-7) was added on and the pH was adjusted with 30% H_2S0_4 to 7.

Preparation of slides and coversups

1)The slides were soaked in concentrated nitric acid for 30 minutes.

2)Washed with distilled water for 1 hours, in several changes of water and drained

3)Washed in acetone for 15 minutes.

4)After draining they were baked at 180 °C for 2 hours.

5)When the slides were cooled down 8~l of poly-L-lysine (1 mg/mi in water) was added and drew into a film over the slide using a coversup.

6)The slides were left on 42 °C hotplate overnight to dry, then stored in a box with dessicant in fridge, they are stable for up to 1 week.

7)The coversups were also washed with acetone and baked.

Stock Solutions

10X saline (1l)

NaCl	89 g/l

10X PBS (1l)

1.3M NaCl	74g
0.07M Na_2HPO4	9.94g
0.03M NaH_2PO4	4.14g

20X Pronase buffer

1M Tris-HCl pH 7.5

0.1M EDTA

Diluted to 1X and 1ml pronase was added.

Pronase (Sigma type XIV)

Dissolved 40mglml in H_20.

Predigested by incubating for 4 hours at 37 ^{0}C and stored at -20 ^{0}C in 1 ml aliquotes.

Triethanolamine 2M

As triethanolamine cannot be autociaved, 149g of was poured into a autoclaved bottle and made up to 500ml with autoclaved water and the pH was adjusted to 8 with HCl.

tRNA

Sigma type XXl was used and made up in water 100mg/ml.

Washing solutions (after hybridization)

Wash bufter

2XSSC

50% formamide

De-gas before use.

10XNTE

5M NaCl

l00mM Tris-HCl pH 7.5

l0mM EDTA

RNase A (Sigma type I-A)

Stock solution of 10mg/ml was made in water and stored in 1.2ml aliquots in water.

Solutions to detect the signal

All buffers should be fresh.

Buffer 1

100mM Tris-HCl

150 mM NaCl, adjust pH to 7.5, keep at 20 ^{o}C

Buffer 2

Buffer 1 containing 0.5% (w / v) blocking reagent.

Buffer 3

Buffer 1 containing 1% (w / v) BSA and 0.3 (v/v) Triton-X-100

Buffer 4

Buffer 3 containing Anti-digoxigenin-AP in a concentration of 1:3000

Buffer 5

100 mM Tris-HCl

100 mM NaCl

50 mM $MgCl_2$ pH 9.5

Buffer 6

Buffer 5 containing 2µl NBT and 1.5 µl X-P Per ml buffer 5

NBT: 75 mg/ml nitroblue tetrazolium salt in 70% (v/v) dimethytformamide

X-P: 50 mg/ml 5-bromo-chloro-3-indolyl phosphate in dimethylformamide

APPENDIX B

Primer Sequences

A) cdc2AB common forward primer

5' GGA GTA ACT TCA TTG CCT 3'

Tm=52.3 °C

B) cdc2A reverse primer

5' AGC AAA ATA ACA CAC CCA 3'

Tm=54.7 °C

C) cdc2B reverse primer

5' ATG CCA GTA GGA GTA ACA 3'

Tm=50.9 °C

D) cdc2DF common reverse primer

5' ATC CCC TGG AAA CAA AGC 3'

Tm=59.9 °C

E) cdc2D forward primer

5'ACG CTG GTT CAG AGT TTT 5'

Tm=55.7 °C

F) cdc2F forward primer

5' GAC CGG AAA ACA ATG ATG 3'

Tm=57.1 °C

G) CYC1 Forward primer (for Medsa;CycB2;3)

5' GAG ATC TAT GAA GTT TTC TGA GGA GAA G 3'

Tm=54.2 °C

H) CYC1 Reverse primer (for Medsa;CycB2;3)

5' CGG AAT TCA ATA AGC AGT CAA CCA GTA T 3'

Tm-=59.2 °C

I) CYC2Forward primer (for Medsa;CycB2;2)

5' CGG ATC CAT GGA TGA ACA ACT TGA CCT C 3'

Tm=65.3 °C.

J) CYC2Reverse primer (for Medsa;CycB2;2)

5' CGA GCT CTT ATG GCT GGT TCT TGT TCT CC 3'

Tm=-65.1 °C.

K) CYC3 Forward primer (for Medsa;CycB2;2)

5' CGG ATC CAT GGC CAT TGA TGA TGA ACT CAA G 3'

Tm=70.4 °C

L) CYC 3 Reverse primer (for Medsa;*CycA2;* 1)

5' GCT CTA GAT GCT TGC AAT TTG TAT CCC TC 3'

Tm=-61.5 °C

M) MAP1 Forward primer (for MAPK MM1)

5' ACA ATG GAA GGA GGA GGA 3'

Tm=57.9 °C

N) MAP1 Reverse primer (for MAPK MM1)

5' ACA CTA CTG CTG GTA CTC AGG 3'

Tm=56.9 °C

O) CycD3 common primer (*A. majus*)

5' GAG GAA TCA AAG TAT GTG TTT GAG 3'

Tm=66 °C

P) CycD3a reverse primer

5' ATG GTT CTA GTT GAG TAG TTG TAA 3'

Tm=62 °C

Q) CycD3b reverse primer

5' GAT TGC TAA GCA CCA GTC GTC ACA 3'

Tm=62 °C

Amino acid sequence of cloned Medsa;CycB2;2

M.varia mRNA for mitotic cyclin 2 (Medsa;CycB2;2)

ORGANISM: Medicago sativa subsp. X varia
Eukaryota; Viridiplantae; Streptophyta; Embryophyta; Tracheophyta;
euphyllophytes; Spermatophyta; Magnoliophyta; eudicotyledons;
Rosidae; Fabales; Fabaceae; Papilionoideae; Medicago.
sub_species="A2"

QN**RRALGGIN**QNFVHGRPYPCVVHKRVLSEKHEICEKKQADLGHRPITRRFAAKIAGSQQ
SYAEKTKNSNPLNLNEFGNSIAIDDELKSPEDQPEPMTLEHTEPMHSDPLEMEEVEMEDIE
GEMILDIDSCDANNSLAVVEYIEDLHAYYRKIEYLGCVSPTYMDEQLDLNERMRAILVDWLI
EVHDKFDLMQETLFLTVNLIDRFLAKQNVVRKKLQLVGLVAMLLACKYEEVSVPVVSDLIHI
ADRAYTRKDILEMEKLMLNTLQYNMSLPTAYVFMRRFLKAAQADKKLELVAFFLVDLSLVE
YEMLKFPPSLVAAAAVYTAQCTVSGFKHWNKTCEWHTNYSEDQLLECSMLMVGFHQKA
GAGKLTGVHRKYGSAKFSFTAKCEPACFLLENKNQP"

Bold characters shows the destruction box motif
The cyclin box is shown by underlined characters
Pest rich sequences are found between123-180.
Accession number is X82040

This thesis is based on following publications

1) Dudits, D., Bögre, L., Bako, L., **Dedeoglu, D.**, Magyar, Z., Kapros, T., Felföldi, F., Györgyey, J., (1993) "Key components of cell cycle control during auxin-induced cell division" In :J.C. Ormond, D. Francis (editors), *Molecular and cell biology of the plant cell cycle*, Dordrecht, Kluwer Academic Publishers, pp 111-132.

2) Magyar, Z., Bako, L., Bogre, L., **Dedeoglu D.**, Kapros, T. and Dudits, D. (1993) "Active cdc2 genes and cell cycle phase specific cdc2 related kinase complexes in hormone-stimulated alfalfa cells" *Plant Journal*, 4(1), 151-161.

3) Bilgin, M., **Dedeoglu, D.**, Peres, A., Engler, G., Inze, D., Dudits, D., and Feher, A. (1999) "Meristem and cell division associated expression of wheat histone H4 promoter is modified by 2-4,D and ABA in transgenic maize plants" (*Plant Science*, in press).

4) Doonan, J.H., **Dedeoglu, D.**, Fobert, P., Gaudin, V. and Lunness, P. (1997) "Spatial and temporal regulation of cell division during plant growth" *Cell Biol. Int.*, 21:861-863.

5) **Dedeoglu, D.**, Bilgin, M., Setenci, F., Kapros, T., Feher A., and Dudits D., (1999) "Transient overexpression of cyclin or cyclin dependent kinase genes activates S-phase in protoplast-derived plant cells" (submitted to *Plant Cell Reports*).

Unrelated Publication

A. PERES , K. NIKOVICS, J. DE ALMEIDA-ENGLER, **D. DEDEOGLU**, G. ENGLER, D. INZÉ , D. DUDITS , A. FEHÉR

Functional analysis of the *Arabidopsis thaliana* cycB1;1*At* promoter region in transgenic maize (submitted to *Cereal Research Communications*)