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Utilization of transgenic maize plants for functional analysis of a histone promoter and host-geminivirus interactions

Ph.D. Theses

by

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ABSTRACT

The ability to create transgenic maize with ease and speed is a significant advantage for improving agronomic characters of maize and for using transformation as a tool to explore basic biological questions on maize genetics and development.

In this study, I have used the embryogenic maize suspension cell line HE/89 as a target for genetic transformation. After polyethylene glycol mediated direct DNA uptake into the protoplasts of HE/89, these cells retained their capacity to form embryos and then fully differentiated fertile plants. Thus, this efficient transformation and regeneration system allowed me to investigate the activities of foreign genes and regulatory elements during plant development and pathogenesis.

Functional analysis of wheat histone H4 gene promoter in transgenic maize plants have demonstrated the conserved nature of its meristem and S/phase- dependent activity and its potential to be used as a versatile indicator of cell cycle progression under different phytohormone conditions. Moreover, mRNA profiles of maize cell cycle genes (histone H4, *PCNA*, histone *H2B*, Zeam;*CycB2*;1) were demonstrated in partially synchronized cells; fluctuating throughout the cell division cycle in correlation with their predicted function.

Viruses lacking the proper organelles and machinery for proliferation totally depend on host factors while interacting with host proteins responsible for the maintenance of a permissive environment for their replication. *Maize streak virus* (MSV) genes responsible for its perpetuation was investigated to elaborate the interaction with the host replication machinery. Here, I used wheat histone H4::GUS carrying transgenic maize plants to monitor the kinetics of cell cycle during virus infection. I showed that MSV infection and transient expression of *MSV* Rep gene activates the replication/S phase-specific activity of the wheat histone H4 promoter. Transcriptional activation of other host cell cycle genes taking part in G1-S phase progression upon MSV infection was also demonstrated.

In an attempt to produce virus resistance maize plants, transgenic maize plants expressing the MSV capsid protein were also tested within the work-frame of this thesis. The plants accumulating significant amounts of MSV capsid mRNA and protein did not show resistance to virus infection. I have utilized these plants to determine the sub-cellular localization of capsid protein –carrying a nuclear localization signal- and found that the coat protein is transported and maintained in the nucleus supporting a proposed function in viral DNA transport.

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ABBREVIATIONS

ABA	Abscisic acid
BSA	Bovine Serum Albumin
CaMV	Cauliflower Mosaic Virus
2,4-D	2,4-Dichlorophenoxyacetic acid
DNA	Deoxyribonucleic acid
dsDNA	double stranded DNA
GUS	β-glucuronidase
gusA	β-glucuronidase gene from *E. coli*
MU	4-methylumbelliferone
mRNA	messenger ribonucleic acid
MSV	Maize Streak Virus
4-MUG	4-methylumbelliferyl β- D-glucuronide
NOS	Nopaline synthase gene of *Agrobacterium*
NPT II	Neomycin phosphotransferase II gene: Tn 5 of *E.coli*
PAT	Phoshinotricin acetyltransferase
PEG	Polyethylene glycol
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl Fluoride
RT-PCR	Reverse transcription-coupled polymerase chain reaction
WDV	Wheat dwarf virus
ssDNA	Single stranded DNA
T-DNA	Transfer DNA, of *Agrobacterium*
X-Gluc	5-bromo-4-chloro-3-indolyl β-D-glucuronide

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"Whatever you can do or dream you can, begin it. Boldness has genius, power, and magic in it. Begin it now"

Johann Wolfgang von Goethe, 1749-1832

CHAPTER I

INTRODUCTION

Plants, like all other higher eukaryotes go through a life cycle with a vegetative and a reproductive phase of growth. Plant organs develop throughout the vegetative growth period of a plant. Unlike other higher eukaryotes, most fully differentiated cells have the potential to dedifferentiate and to reenter the cell cycle. Due to the lack of cell migration, plant embryogenesis and development solely depend on cellular functions that regulate spatial and temporal control of cell division and expansion (Dudits *et al.* 1997; Jacobs *et al.*, 1997; Miranov *et al.* 1997). To be able to build complex organs, plants must maintain strict control of cell division. Thus, in order to understand how plants regulate cell division and identify the nature of plant gene function during plant development a number of tools have been developed and utilized.

Cereals were among the first domesticated plants when agriculture started to replace hunting and gathering more than 10000 years ago. Maize, rice, wheat and millets account for almost half of the worlds food and nutrition and for this reason they have been the major focus of plant breeding efforts over the last 50 years. Rapidly emerging technologies of plant cell and molecular biology have provided powerful and novel methods that enhanced our understanding of plant development, as well as complemented conventional breeding methods. As the need for high grain quality and agronomic performance of cereals increased by the consumption, cereals became prime targets of genetic manipulations particularly for tolerance to diseases and stresses (Jähne, 1995).

Modern plant biotechnology is based on the delivery, integration and expression of defined foreign genes into plant cells, which have the capacity to regenerate *in vitro*. The extreme recalcitrance of cerals to genetic manipulation is mainly due to the difficulties in normal and fertile plant regeneration. That is why, most of the efforts turned towards finding target tissues with high

capacity of differentiation under defined conditions (Potrykus, 1990; 1991). Embryogenic callus tissues derived from shoot meristems, mature and immature zygotic embryos became the primary tissues suitable for genetic transformation. Over the past five years, transgenic strains of various major cereals have been produced, with transformation of rice and maize being most common. A majority of the cereal transformants obtained to date have been generated by protoplast transformation, particle bombardment technique and *Agrobacterium*-mediated transformation which is rapidly becoming the method of choice. Rice and maize, the plants in which transformation-related technology is most advanced, appear to be models for basic and applied studies (Vasil, 1996; Komari *et al.*, 1998).

PART 1

GENETIC TRANSFORMATION IN PLANTS

1.1 Principles of genetic transformation

Major prerequisites of genetic transformation were previously outlined by Potrykus (1991). The requirements for the proof of integrative transformation can be summarized as follows:

- Negative controls for treatments and analysis;
- Strong correlation between treatment and predicted results
- A tight correlation between physical data that consists of complete Southern analysis containing the predicted signals in high-molecular-weight DNA, including hybrid fragments between host DNA and foreign gene, and the presence of the complete gene, and evidence for the absence of contaminating DNA fragments or identification of such fragments;
- Phenotypic data supported by enzyme or metabolite assays;
- Previously available information that allows the discrimination between false positives and correct transformants in the evaluation of the phenotypic evidence;
- Correlation of the physical and phenotypic evidence with transmission to sexual offspring supported by complete molecular and genetic analysis of offspring populations.

1.1.1 Genetic transformation methods

Since the first reports on transgenic plant production, the number of plant species that were modified by recombinant DNA technology has reached to more than 120. Most of these are dicot plants with high capacity and genotype independent competence for genetic manipulation and their capacity for efficient *in vitro* regeneration. Only after 1990, plants from all of the important cereal crops could be regenerated from cultured tissues or single cells. During the last 9 years, genetic transformation and fertile plant regeneration of cereals from highly embryogenic genotypes were successfully demonstrated (reviewed by Vasil, 1994; Birch, 1997).

The three most versatile methods of plant genetic transformation are PEG-mediated direct DNA uptake, *Agrobacterium* mediated transformation and biolistic delivery. In the early stages of genetic transformation, the "direct DNA uptake into protoplasts" has been a popular experimental choice. Genetic transformation through direct DNA uptake into protoplasts rely on embryogenic suspension cultures and need well established plant regeneration protocols from protoplasts. This has now been achieved in a considerable number of plant genotypes. Protoplasts can take up DNA

when treated with PEG and/or electric pulses causing reversible, non destructive permeabilization of the plasma membrane (Lazzeri *et al.* 1995). The protoplast isolation procedure can promote the shift of potentially competent cells into the competent state through induction of a wound response by cell wall digestion. The exogenous DNA introduced into protoplasts is easily integrated into genomic DNA through non-homologous recombination. (Lyznik *et al.* 1991). The transformation of protoplasts allows obtaining hundreds of independent transformation events in a single experiment. In addition, the direct gene transfer to protoplasts is a convenient method for fast assessment of promoter activity by analyzing transient gene expression (reviewed by Fehér and Dudits, 1993)

Some other methods have been developed to supplement direct DNA uptake into plant protoplasts, utilizing cationic liposomes like lipofectin (Zhu *et al.* 1993) or ultrasonication (Zhang *et al.* 1991) which may allow introduction of DNA -restriction enzyme complexes, large DNA molecules otherwise which could be difficult by standard PEG mediated delivery method.

The biolistics or particle gun bombardment allows the transport of genes into many plant cells without too much manual effort. The enormous investment into this technique has paid off, and transgenic plants have been recovered (Klein *et al.* 1992; 1993; Christou *et al.* 1997; Vasil *et al.* 1999) otherwise that would have been difficult to produce by other methods. In recalcitrant plants like cereals with limited amount of cells competent for transformation-regeneration events, the particle has to reach those rare cells by a random hit, and the DNA has to integrate into the genome of these cells.

Another potential problem and limitation of biolistics is connected with the use of multicellullar structures for transformation experiments that results in recovery after selection of chimeric tissues composed of a mixture of transformed and nontransformed cells. Most of the published works in biolistics report obtaining chimeras in their experiments (Barcelo and Lazzeri 1995).

Cereals were among the plant species where successful T-DNA integration was not shown to until recently, due to host responses lacking tumor formation upon interaction with virulent *Agrobacterium* strains. For the first time Grimsley *et al.* (1987), have proven by agroinfection, the ability of *agrobacterium* to transform maize cells. This method was based on injection of a dense Agrobacterium solution into the lower shoot meristems of 5-10 days old maize seedlings (Grimsley, 1990; Boulton, 1995). It was later on demonstrated that the efficiency of such T-DNA insertions was comparable to other dicot counterparts and *Vir A* gene of the pathogen is a key regulator of the interaction between the bacterium and the plant (Raineri *et al.* 1993). This method was mostly used to study geminivirus replication in monocot plants where maize streak virus DNA was transferred into

maize cells. After agroinfection, the transformed plants were then recognized by the symptoms of viral infection caused by partially disarmed viruses. First Hiei *et al.* (1997) using rice calli induced from scutella as a target for *Agrobacterium* mediated gene transfer showed that genetic transformation is feasible in a cereal species. A successful delivery was shown to depend on the use of tissues containing 'competent' cells and the induction of virulence genes with acetosyringone. Recently, over 2,600 transgenic rice plants were regenerated from >500 independently selected hygromycin-resistant calli after *Agrobacterium*-mediated transformation in rice. These plants were transformed with modified synthetic *crylA(b)* and *crylA(c)* coding sequences from *Bacillus thuringiensis* as well as the hygromycin phosphotransferase and GUS marker genes (Cheng *et al.* 1998). The studies of transformation of rice suggested that numerous factors, including the genotype of plants, types and ages of inoculated tissues, vectors, strains of *Agrobacterium*, selection marker genes and selective agents, and various conditions of tissue culture are of critical importance.

1.1.2 Factors affecting genetic transformation and transgenics: competence

Plant cells differ in their capability to respond to exogenous stimuli, a phenomenon, and called competence. Cells capable of regenerating transgenic plants must be competent both for integrative transformation and regeneration. Although, theoretically, all plant cells are totipotent, the conditions for plant regeneration are not fully understood for every plant tissue culture system. Plant tissues are composed from cells competent for many responses. However, since the competence for transformation and regeneration are the most important ones, it should be taken in consideration that few cells are competent for both integrative transformation and regeneration. Most of the plant cells are competent for transformation or regeneration. A rather large portion of the cells are potentially competent, thus they can be triggered to shift towards re-initiation of the ontogenic program if they are exposed to a proper treatment; a variable portion of cells is in a non-competent stage (Dudits, 1991). The relative composition of cell population is determined by the genotype, the tissue origin of the organ, the developmental state of the organ, the physiological state of cells.

As shown by a large number of experimental observations, wounding and hormone shock are potent triggers for shifting potentially competent cells towards a competent state. Plants and tissues differ in their wound responses. Only plants with a pronounced wound response develop larger populations of wound-adjacent competent cells for regeneration and transformation. Monocots with wound response (e.g. Asparagus) are as easy to transform as dicots with wound response; and dicots without proper wound response (e.g. grain legumes) are probably as difficult to transform as cereals (Potrykus, 1991).

Another factor worth mentioning is the size of DNA being used for genetic transformation. Fleming *et al.,* (1995) showed that the size of the DNA fragment containing the selectable marker used to monitor transformation can directly affect the efficiency of stable transformation both in tobacco and maize protoplasts, where the smallest DNA fragments gave the highest stable transformation frequencies.

Torres *et al.,* (1993) demonstrated that transient expression of plasmid DNA in plant protoplasts can be strongly influenced by the bacterial strain used for plasmid propagation. Results suggest that DNA methylation may be partly responsible for deregulating promoter activity in the transient expression.

1.1.3 Factors affecting gene expression: post transformation

There are numerous conditions that can interfere with transgene expression in plants. These include poor recognition of promoter elements, differences in transcription efficiency between the source of the promoter and the transformed material, inefficient mRNA termination, polyadenylation, processing and/or stability, inefficient translation initiation and/or inappropriate codon usage and poor design of expression vectors with potential regions for gene inactivation by recombination. Some of these potential problems can be eliminated by appropriate design of the plant transformation vectors. However, there are several endogenous mechanisms that can interfere with gene expression and lead to non-mendelian inheritance of introduced traits.

It is generally accepted that the amounts of transcripts are mainly regulated by cis-acting sequences, mainly located on the 5' end of the gene. Impact of *cis*-acting enhancers, which have been found in the vicinity of some genes, cannot account for the huge differences in the expression of the same chimeric gene in individually regenerated transgenic plants. Several factors might play a considerable role:

1. Position effect: The expression of foreign genes is influenced by their chromosomal position. This position effect might be related to chromatin structure at the integration site and/or the location of endogenous *cis*-acting regulatory sequences nearby. Studies on scaffold attachment regions (*SAR*) demonstrated that constructs carrying marker genes flanked by yeast *ARS-1* element reduced the position effect variation most probably by mediating *in vivo* binding to the nuclear scaffold. In addition to this, *SAR*s not only insulate the introduced DNA from the influence of factors in the chromatin surrounding the site of integration but also bring *cis* acting elements close to the scaffold and physically resist to heterochromation, allowing continued access by polymerases and transcription factors (Allen *et al.* 1993; 1996).

2. Gene inactivation by DNA methylation, co-suppression and copy number : The impact of copy number on gene expression varies from one system to another. While some authors have found that the copy number of the independently integrated genes can be in positive correlation with the level of gene expression (Deak *et al.* 1988). However, Klein *et al.* were not able to find such a correlation (1989). Mittelsten-Scheid *et al.* (1996) have found that single copy results in higher expression when compared with multiple insertions of the gene. The last phenomenon has been linked to the methylation of genes.

There is a tendency towards the integration of multiple copies of the introduced genes in cereal transformation. It has been suggested that when multiple copies of introduced gene are co-suppressed, again methylation of sequences accounts for the phenomenon (Matzke and Matzke 1998). Alterations in phenotype are commonly observed in regenerated plants in which both stable and reversible genetic alterations have been linked to changes in patterns of DNA methylation (Kumpatla *et al.* 1997). The most common DNA modification in plant cells is cytosine methylation at CG dinucleotides and CNG trinucleotides where methycytosine can interfere with protein-DNA interactions. Recognition and inactivation of introduced genes might be regarded as a defense response preventing the expression of potentially deleterious foreign DNA molecules. Renckens *et al.*, (1992), showed that petunia plants escape from negative selection against a transgene by silencing the foreign DNA via methylation. Their observations demonstrated that reversible DNA methylation was the main cause of silencing of the transgene.

In *N. benthamiana*, systemic, post-transcriptional silencing of transgenes was initiated in localized regions of the plant by introduction of transgene-homologous DNA fragments, including those without a promoter. The nature of silencing mechanism, the mode of its initiation, and the ability of the signal to move long distances indicated the existence of a sequence-specific signaling mechanism in plants that may have roles in developmental control as well as in protection against transposons and viruses (Voinnet *et al.* 1998).

A recent analysis of transgenic plants expressing *Fab* antibody fragments suggested that epigenetic effects result in different transgene expression profiles, thus giving a fine example of the mentioned phenomena (De Neve, 1999). When the stability of antibody and *Fab* expression was assessed in transgenic *Arabidopsis* lines, each of these lines showed silencing of the transgenes that encode the antibody polypeptides, leading to instability of antibody production. The characteristic variation in the level of antibody accumulation in each line as a function of developmental stage, indicated that the T-DNA integration pattern played a role in triggering silencing, and also that the history and the integration position of simple transgene loci can influence

the susceptibility to epigenetic silencing. In different lines with low antibody accumulation levels, methylation was found either in the promoter alone, in both the promoter and the transcribed region, in the transcribed region only, or in the transcribed region and downstream sequences.

3. *Trans*-acting factors. Promoter-binding proteins and other regulatory factors involved in signal transduction can differ from species to species, and this might cause altered expression of a transgene.

5. Physiological state of the plant, *in vitro* culture system, plant to plant variability and epigenetic factors may result in disturbances of the gene expression. Transient expression and stable transformation experiments in two *Nicotiana* species have indicated that despite the constant and large differences in stable transformation efficiency in favor of *Nicotiana tobacco. N. plumbaginifolia* exhibited significantly higher levels of gene activity in transient expression assays. The results suggest that the major differences in transfection ability were not in DNA uptake, but rather in the specific fate of the transforming DNA in the two species (Negrutiu, 1990).

6. Anatomical and physiological characters and differences in the secondary metabolism may play a considerable role and contribute to the production of controversial results when different systems are used (Blaich *et al.* 1992).

1.1.4 Plant Protoplasts and gene expression analysis by genetic transformation

Protoplasts as "naked cells" represent a unique experimental system in plant biology. Viable plant protoplast are easily isolated from a wide variety of species using cell wall digesting enzymes and when cultured, they form new cell walls as a first step to regenerate into plants. Protoplasts can be used to study *de novo* cell wall formation and in investigations on membrane structure and transport. Furthermore, ultrastructural changes like microtubule rearrangement can be monitored within the cell. In cell biology, for the isolation of plant cell organelles again protoplast serve as donors. On the other hand, plant protoplast can also be utilized for genetic manipulation techniques, like somatic hybridization and direct gene transfer (Negrutiu *et al.* 1992).

Much of the attention and interest in modern molecular biology is focused on the regulation of gene expression. Since many regulatory components are responsible for spatial and temporal restriction of gene activity, the use of gene fusions can be a tool of functional studies. Factors influencing or mediating such regulation are often best studied using gene fusions (or chimeric genes). Gene fusions can be defined as DNA constructions composed of the coding sequences from one gene (reporter) being transcribed and/or translated under the direction of the controlling sequences of another gene (controller) in an assay performed *in vitro* or *in vivo*. Gene fusions can be

of two general types. Transcriptional fusions are defined as fusions in which all protein coding sequences are derived only from the reporter, while in translational fusions the polypeptide produced is the result of coding information provided by both controller and reporter. Most frequently, the reporter encodes an enzyme. Use of reporter enzymes can greatly enhance the sensitivity of gene fusion detection, and can also facilitate many types of analyses (Jefferson *et al.* 1987). Precisely constructed gene fusions simplify the way of the monitoring of gene expression with enhanced sensitivity. They facilitate the comparison of different or altered regulatory sequences and allow study of individual members of gene families without the interfering effect of another genes. Moreover, the analysis of large number of samples that is a basic requirement for the use of statistical methods might be an overwhelming task, unless routine, high resolution techniques are available similar to the sensitive assays developed for detection of expression of the GUS gene fusions (see below). The use of *in vitro* generated gene fusions followed by their introduction into the plant genome has a tremendous impact on modern plant molecular biology.

Other reporter gene systems include genes derived from firefly luciferase *Luc,* maize anthocyanin biosynthetic pathway genes (*C1, B* and *R* genes) and green fluorescent protein of jellyfish *Aquaria victora*. Although bacterial and firefly luciferases are sensitive reporter genes, they were particularly engineered for short-term stability. The need for sophisticated and expensive chemiluminesence readers is a major drawback. Differing from GUS and luciferase reporter genes, anthocyanin gene offers an *in vivo* detection and particularly useful in cereal transformation as an indicator of transformation efficiency.

Recently a new non-destructive and sensitive marker gene system based on the green fluorescence protein (GFP) of *Aqurina victora*, has been developed (Sheen *et al.* 1995; Leffel *et al.* 1997). This reporter system, allows vital monitoring of the gene activity under UV illumination and can tolerate both N-terminal and carboxy-terminal fusions. Several laboratories already demonstrated its use, particularly in virus research, in precise tracking of plasmodesmatal trafficking where viral movement proteins genes or promoters were tagged with GFP (Baulcombe *et al.*, 1995, Cruz *et al.* 1996).

1.1.5 Maize and Genetic Transformation

Maize *(Zea mays L.* or 'Indian corn') a member of the grass family the *Gramineae* is a large kerneled, highly domesticated, vigorous annual plant of tropical origin, which is well suited for biological research, including molecular studies. Teosinte (*Zea mays* ssp. *parviglumis*) is considered to be its wild ancestor though maize exhibits an extreme morphological divergence from its apparent

wild progenitor. As a crop plant maize is remarkably dissimilar to its recent wild ancestor, teosinte, making it an extremely interesting model also for the study of evolution. Investigations into the evolution of maize are currently being performed at the molecular and morphological levels (White, 1998). Despite the fact that the domestication of maize from teosinte occurred only about 7000 years ago, maize and teosinte are completely interfertile, and show no greater divergence in their chromosomes, gene structures, or nucleotide sequences than one is apt to observe between two varieties of maize. Genetic analysis suggested that only four or five loci are responsible for the key morphological differences in these two species (Doebley 1992; Nelson *et al.* 1995).

Most of the plant body of maize is leaf tissue. Its main stem, or culm, is a slender, segmented shaft similar to the stalk of a bamboo or sugarcane. The enlarged joints along the stem, the nodes, mark the points of leaf attachment; the stem segment between nodes is called the internode. Each node bears a single leaf in a position opposite that of the neighboring leaf, giving the plant two vertical rows of leaves (so-called distichous phyllotaxy). Maize has unisexual flowers. The male (staminate) flowers are located at the apical tip of the main stem in the tassel, a branched inflorescence. The female (pistillate) flowers are found in one to several compact ears, located on the ends of short branches near the middle of the stem. This partitioning of male and female flowers in separate structures distinguishes maize from other cereals and is one of the principal reasons that have resulted in the convenient exploration of its genetics. Thus making controlled pollinations in maize requires only placing a bag over the tassel and ear shoot, while in other cereals it is necessary to emasculate each flower used as a female parent. Another biological feature that aids enormously in performing genetic studies is the size of an ear. A normal, vigorous maize plant will usually produce an ear bearing 400 to 600 kernels with embryos consisting of many thousands of cells and often having a fresh weight of 50 mg or more. The kernels can be cross- or self-pollinated in a single act, so that very large numbers of progeny can be obtained with a minimal amount of effort (Walbot, 1994).

The first report on genetic transformation of maize was by De Wet *et al.,* in 1985. In this work, pollen was used as a vector. The pollen tube, at germination, lacks a cell wall near its tip where DNA fragments might be absorbed through this pore into the pollen tube and possibly into the sperm cell enabling incorporation in the DNA of the fertilized egg cell. Though this method produce phenotypically transformed kernels but lacked molecular evidences of stable-inheritable integration into maize genome.

Numerous publications on transient gene expression analysis as well as stable transformation experiments have been reported using maize protoplasts (Rhodes *et al.* 1988; Lyznik

et al. 1989; Sinibaldi *et al.* 1992, Marrs *et al.* 1995, Gallie *et al.* 1994). Although, protoplast based direct gene transfer methods have provided detailed information on conditions of transformation and co-transformation of maize cells. Dudits and his co-workers also demonstrated that protoplasts of an embryogenic maize cell line HE/89 can be used efficiently for PEG mediated direct DNA uptake transformation and fertile plant regeneration. Despite the difficulties of protoplast cultures this system has produced hundreds of independently transformed lines and fertile plants without any chimeras (Morocz *et al.* 1990, Omirulleh *et al.* 1993).

Genetic transformation methods using target cells/tissues other than protoplast have been also developed:

Gould *et al.,* (1991), after *Agrobacteium* inoculation of isolated shoot apices, obtained the seed progeny of two chimeric maize transformants. Until recently it was thought that *Agrobacterium*-mediated transformation of cereals has very low potential due to the availability and accessibility of cells competent for integrative transformation and regeneration (Potrykus, 1991). Ishida *et al.* in 1996 have demonstrated the stable integration, expression and transmission of transgenes to the progeny after *Agrobacterium* mediated transformation of maize.

The biolistics transformation or in other words accelerated particle delivery was also proved to be efficient in both transient gene expression studies (Klein *et al.* 1989; Daniell *et al.* 1997) and for the recovery of fertile transgenic maize (Fromm *et al.* 1990; Gordon-Kamm *et al.* 1990; Walters *et al.* 1992; Koziel *et al.* 1993). The major source of target was either embryogenic calli or zygotic embryos dissected aseptically and used for transformation. A major drawback of the system lies in the low efficiency of transformation and high number of chimeric lines appearing as a result of treatment and selection of multicellular structures (Barcelo *et al.* 1995). Recently, construction of sophisticated versions of biolistic bombardment devices, careful establishment of tissue culture conditions, using tissues retaining their dedifferentiation capacity after genetic transformation and refining the pre and post-bombardment conditions improved the outcomes of this method (reviewed by Christou *et al.* 1997). For instance, Zhong *et al.,* (1996) have developed a transformation system using maize shoot meristems for integrative transformation by microprojectile bombardment. Here, maize shoot meristems, more precisely the shoot apices were carefully dissected from germinating seedlings and young plantlets and grown in the presence of plant growth hormones, 2,4-D and benzyladenine. The callus and partially differentiated shoot meristems were successfully used as targets for genetic transformation and further regeneration of plants. This achievement further broadened the possibility of successful genetic manipulation of maize as it offers a genotype-independent *in vitro* culture and plant regeneration system.

Another effective transformation method of maize is electroporation. D'Halluin *et al.*, in 1992, have described the transformation of regenerable maize tissues by electroporation where wounded immature embryos were electroporated with a chimeric gene encoding neomycin phosphotransferase (NPTII). Although 25 independent transgenic lines were obtained and fertile plants were regenerated after kanamycin selection, some of the putative transformant lines exhibited chimeric nature.

The most recently developed technique for maize transformation involves the vortexing of silicon carbide whiskers with maize cells in the presence of plasmid DNA. Fertile transgenic plants have been regenerated using embryogenic callus cultures (Frame *et al.* 1994)

So far fertile transgenic maize plants have been obtained using selectable markers for resistance to chlorsulfuron (Fromm *et al.* 1990), phosphinotricin (Gordon-Kamm *et al.* 1990, Omirulleh *et al.* 1994), hygromycin (Walters *et al.* 1992), kanamycin (D'Halluin *et al.* 1992) and to methothrexate (Golovkin *et al.* 1993).

Based on the embryogenic cell suspension HE/89 and PEG mediated protoplast transformation, Omirulleh *et al.*, (1993) demonstrated modification of a tissue specific promoter activity by fusing CaMV enhancer sequences upstream of the α-amylase promoter from wheat.

Later studies based on the production and utilisation of transgenic maize plants concentrated on answering problems in relation to regulation of gene expression and improvement of grain quality in maize as a result of genetic transformation. The tissue-specific, developmental, and genetic control of four endosperm-active genes (maize granule-bound starch synthase "Waxy" gene, a maize 27 kDa zein gene, a rice small subunit ADP-glucose pyrophosphorylase gene, Russell *et al.* 1997 and maize caffeic acid O-methyltransferase, Capellades *et al.* 1996 was studied via expression of GUS reporter genes in transgenic maize plants under the control of a promoter from soybean (Lyznik *et al.* 1995).

PART 2

FUNCTIONAL ANALYSIS OF THE WHEAT HISTONE H4 PROMOTER IN TRANSGENIC MAIZE PLANTS AND ITS UTILISATION FOR CELL CYCLE STUDIES

Cell division is the most important basic biological event in the embryonic and post-germinative development of higher plants. Although a conserved biochemical regulatory network controls cell cycle progress in plants, animals, and yeast, the fate of individual plant cells appear to be determined mainly by their spatial organization within the proliferating tissue, the meristem. This is a striking difference from other higher eukaryotes where the fate of the cell is determined in the early stages of embryogenesis. Therefore, in order to understand how plants develop and respond to their environment, it is necessary to understand the mechanism that controls the progression of the cell cycle (Doonan, 1998; Jacobs; 1998).

In eukaryotes, progression through the cell cycle is maintained by the expression of cdc2 and cyclin genes, encoding the catalytic and regulatory subunits of the cell division related protein kinase complexes. As a results of their activity, plant cells progress through the cell division cycle. Regulation of cell cycle in plants requires synthesis of certain mRNAs and proteins that are enzymatically or structurally needed to proceed through phase transitions. The transition from G1-S phase requires the synthesis of proteins that function directly on DNA metabolism; such as ribonucleotide kinases, proliferating cell nuclear antigen and histone proteins whose synthesis is closely related to DNA replication. Therefore, investigating the regulation of histone genes expression would reveal information on signal transduction mechanisms that link G1-S progression to S-phase specific histone gene expression in plant cells (Jacobs, 1995; Mikami and Iwabuchi, 1993, Chaubet *et al.* 1998, Iwabuchi *et al.* 1998).

2.1 Activity of Histone genes in plants

Histones, major protein components of the eukaryotic chromatin have a primary role in DNA assembly into nucleosomes in eukaryotes. They are encoded by a multi-gene families constituting five major classes, *H2A, H2B, H3, H4 and, H1*. Expression of these structural genes is coupled to DNA synthesis and coordinately regulated at transcriptional and post-transcriptional levels through the cell cycle (Heintz, 1989).

Like their animal counterparts histone genes from higher plants can be grouped in two major types (reviewed by Chaubet and Gigot 1998).

- The predominant, replication-dependent variants whose expression is tightly linked to the S(-ynthesis) phase of the cell cycle and the meristematic tissues
- Replication-independent and/or tissue-specific replacement variants that accumulate in terminally differentiated non-dividing tissues.

Structural properties and regulation of histone gene expression have been extensively studied in distantly related eukaryotes like yeast and animals, and despite some differences, major roles and modes of expression were shown to be very similar. In contrast, little is known about the histone gene activity in higher plants. In the last ten years, several plant histone genes have been cloned from various higher plants of most of which belong to the class of replication dependent variants (Waterborg *et al.* 1996; Chabouté *et al.* 1993). As in vertebrates, plant histone genes lack introns except a few genes encoding for replication independent/tissue specific variants. In contrast to the animal species, plant histone genes are polyadenylated like in lower eukaryotes. (Fabry *et al.* 1995)

Most plant histone genes are dispersed in the genome. As an exception, rice histone H2A, H2B and H4 genes are clustered within 6.5 kb chromosome fragment remains. In *Arabidopsis*, only few copies of H3 and H4 genes exits while their number can reach to 10-100 in maize, wheat and alfalfa. The coding regions of the same subtype of histone genes are highly conserved among different plant species (Waterborg, 1992).

The fluctuations in synthesis of replication dependent histones in proliferating cells are paralleled by similar changes in their corresponding mRNAs. The mRNA levels of the two major classes of histones are low during the G1, G2 and M phases whereas highly accumulate during the S phase of the cell cycle (Osley, 1991). This regulation involves both transcriptional and post-transcriptional factors, initiation of transcription, the 3' processing of pre-mRNA and the degradation of mRNA. Since the transcription rate in S-phase is only 2-5 times higher than that during G1 or G2. As this variation alone can not fully account for the much greater fluctuations in the mRNA levels. In other words, during the S-phase of the cell cycle, replication dependent histone genes are transcribed at high levels while mRNAs have longer half lives. Moreover, although histone genes are expressed with different efficiencies, the relative ratios of the transcripts from individual histone genes seem to remain constant indicating that they are controlled by a common mechanism. Thus, regulation of histone genes during cell cycle, account for the fact that all histones are controlled coordinately by transcriptional and post-transcriptional processes. Moreover, regulation of histone

gene expression provides a link between the cell division cycle and plant development, particularly during embryogenic growth.

For the regulation of histone gene expression in *Saccharomyces cerevisiae* and animals both 3' and 5' flanking regions are required. The primary structure and function of plant histone genes has also indicated several conserved nucleotide sequences as *cis* acting control elements at both the 3' and 5' flanking regions. In the 5' flanking regions of plat histone H3 and H4 genes several conserved sequences, hexamer (**ACGTCA**), octamer (**CGCGGATC**), and nonamer (**CATCCAACG**) motifs were found (reviewed by Mikami K and Iwabuchi M, 1993; Iwabuchi *et al.* 1998). To further elucidate the regulatory mechanisms responsible for cell cycle dependent expression of plant histone genes, chimeric gene constructs comprised of proximal promoter regions of histone genes regulating the expression of GUS reporter gene have been introduced into plant cells (Lepetit *et al.* 1992; Kapros *et al.* 1993; Atanassova *et al.* 1992; 1998; Tareda *et al.* 1993). Expression analyses of transgenic cell lines indicated the significant roles of various conserved sequence motifs (*ACGTCA, CGCGGATC, and CATCCAACG*) that serve as 5' *cis*-acting control elements. 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.* 1992). In transgenic tobacco plants, alfalfa histone H3.1 promoter was shown to be active in meristem tissues and this activity was linked to DNA synthesis after re-initiation of cell divisions in fully differentiated leaf mesophyll cells (Kapros *et al.* 1993). The cell cycle phase-specific expression of the wheat histone H3 promoter was shown to be dependent on 5' regulatory elements, particularly the *type I element* composed of octamer and nanomer motifs (Nakayama *et al.* 1992; Ohtsubo *et al.* 1993; Iwabuchi *et al.* 1993). Mutations disrupting the sequence of *type I element* of wheat histone H3 promoter diminished the meristem and S-phase specific activity in transgenic rice cells indicating that this *cis*-acting element is responsible for the S-phase specific expression of plant histone genes (Nakayama *et al.* 1993; Ohtsubo *et al.* 1997). The hexameric sequence *ACGTCA* also functions in transcriptional regulation of many plant genes including the wheat histone genes. The cauliflower mosaic virus (CaMV) 35S RNA promoter has the same hexameric sequence and mutation analyses confirmed that the hexamer contributed greatly to transcription from the 35S promoter in sunflower cells (Kawata *et al.*, 1989).

Transient expression experiments revealed that the promoter region of *Arabidopsis* H4 gene (H4A748) could drive a basal replication-independent expression of the GUS reporter gene. Transgenic tobacco plants showed that this promoter could confer both basal expression in non dividing cells and a strong meristem specific expression. These results reported by Lepetit *et al.*

1992, for the first time demonstrated that a histone H4 promoter is able to direct both replication dependent and independent gene expression. When the activity of the maize histone H4 *H4C7* was examined in transgenic tobacco plants the replication independent basal activity of this promoter was found to be linked to the conserved nanomeric sequence present in the 5' flanking regions of the histone genes as a cis- acting element.

Tareda and his co-workers in 1993, investigated the developmental regulation of wheat histone H3 gene expression, H3 promoter was fused to GUS reporter gene and introduced into rice protoplasts. Detailed histochemical analysis revealed two distinct types of GUS expression in transgenic plants. One was cell cycle dependent activity found in the apical meristem of shoots, roots and young leaves and the other a cell division-independent expression occurring in flower tissues, the anther wall and the pistil. It was also confirmed by deletion analysis that the proximal promoter region containing the conserved hexamer, octamer and nonamer motifs were sufficient to direct both cell division-dependent and -independent expression. Further studies on the well conserved regulatory elements (*Type I*: element :CC**ACGTCA**CC**GATCCGCG**) found in the proximal promoter region of a certain class of plant histone genes suggested that the hexamer and the octamer motifs may play important roles in the regulation of replication-dependent but not replication- independent expression of the wheat histone H3 gene.

When a 1023 bp fragment and truncated derivatives of the maize histone H3C4 gene promoter were fused to the GUS gene and introduced via *Agrobacterium tumefactions* into the genome of *Arabidopsis thaliana*, GUS activity was found in various meristems of transgenic plants like in the case of other plant histone promoters. Deletion studies indicated that both the proximal and distal regions of the promoter were necessary to direct S phase-dependent activity (Attanosova, 1998)

In 1983, the wheat histone H4 gene (TH011) was isolated from a genomic library by Tabata *et al.* Studies on the genome structure of the gene revealed that it exits as 100-125 copy per hexaploid wheat genome. A variant of the same gene were found to be similar to TH011 but with one amino acid substitution in the protein coding region. However, the variant gene did not contain the nonamer motif at the regulatory sequence of the gene.

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, 1998; Iwabuchi, 1998). However, the functional activity of the wheat H4 histone gene promoter, which contains all the conserved sequences presumably directing S-phase dependent histone gene expression, has not been studied so far in homologous cereal system. After *Agrobacterium*-mediated introduction of

wheat histone genes into sunflower cells it has been shown that the amount of H4 histone transcripts is seven times less then that of H3 histone, the values comparable to the transcription rate in wheat seedlings (Tabata, 1987). The authors concluded that histone H3 and H4 gene expression is controlled by a similar regulatory mechanism during transcription in these two species.

In further studies on the central roles of sequence motifs, a wheat histone H3 promoter region was used in a heterologous assay system utilizing cultured rice and tobacco cells. These experiments showed that some of the cis regulatory sequences in the far upstream of the H3 promoter function differently in monocot and dicot cells. In tobacco, the transcriptional system was sensitive to changes in the orientation of *cis*-acting motifs, whereas in rice were functioning independently in gain of expression.

Two recently isolated histone *H2B* genes (*TH123 and TH153*) showed motifs similar or identical to positive cis-acting elements of histone H3 gene. Analysis of the promoter activity showed that H2B promoters were preferentially active in meristematic tissues and suggesting that *H2B* genes are regulated in part by the same mechanism which exist in H3 and H4 gene transcription (Yang *et al.* 1995).

Although a considerable amount of biochemical and genetic information has accumulated on the structure and functions of plant histones upon cDNA and genomic cloning, their interaction with DNA binding proteins and the molecular mechanism underlying regulation of histone gene expression remains to be further elucidated. Several histone binding proteins were identified as putative transcription factors regulating the activity of histone gene promoters with basic leucine-zipper (HBP-1a, HBP-1b Nakayama *et al.* 1997; Mikami *et al.* 1994; 1995), single-strand DNA binding, (ssDBP-1, ssDBP-2 Takase *et al.* 1991) and zinc finger (WZF1 Sakamoto *et al.* 1996) characteristics. These genes are regulated in tissue specific and developmental manner indicating their links to the similarly regulated histone genes (Iwabuchi *et al.* 1998). It is also shown that the nuclear proteins HBP-1a and HBP-1b specifically bind to hexamer motif *in vitro* in wheat. These DNA binding proteins are shown to have leucine-zipper and basic motifs, which are characteristics of *bZIP* type transcription factors of animal and yeast cells. A zinc-finger-type transcription factor *WZF-1* was found to bind to a novel *cis*-acting element of histone gene promoters and repress its own promoter, (Sakamoto *et al.* 1996). Moreover, when the Gal4-recognition sequences (Gal4 DNA-binding domain 1-94) of *Saccaromyces cerevisiae* were put upstream of the core histone promoter, it was able to trans-activate the wheat histone H3 promoter *in vivo*. This result demonstrates the link between the conserved transcriptional activation mechanism of two eukaryotes with respect to histone gene promoter function (Nakayama *et al.* 1995).

The positive effect of histone terminators have been reported for other plant species to increase the reporter gene activity and disruption of certain motifs resulted in the loss of this substantial increase but not tissue or cell cycle phase specific expression. The hairpin structure in the 3' untranslated region (3' UTR) of the histone pre-mRNAs, increased efficiency of 3'-processing, and prolonged mRNA half-life results in 5-10 fold increase in the amounts of histone mRNAs during the S-phase (Ohtsubo *et al.* 1994).

Molecular characterization of putative cell cycle genes and their regulatory sequences require efficient synchronization of cell populations reversibly blocked at a certain stage of the cell division cycle (Reicheld *et al.* 1996; Magyar *et al.* 1997). Many of the studies on characterization of histone genes and regulatory sequences mentioned above utilized chemical synchronization of transgenic suspension cells (Iwabuchi *et al.* 1993; Ohtsubo *et al.* 1994) or re-initiated the cell division cycle in fully differentiated leaf mesophyll protoplast to monitor the kinetics of transcriptional activity (Kapros *et al.* 1993).

Histone genes have recently gained further importance due to their strict S-phase specific expression and potential to be used as S phase marker of the cell division cycle or as highly active promoters for regulated expression of seed proteins in transgenic rice (Huang N, pending patent application, unpublished).

2.2 Phytohormones, cell cycle and histone gene expression

Auxins and cytokinins play a major role in plant growth and development. To understand the effects of these phytohormones on the cell division cycle, it is important to find out basic regulatory switches controlling the transition from G_1-S, G_2-M phases of the cell cycle and how hormones affect them. Cell cycle genes are thought to be candidates to play a role in mediating the effect of various growth regulators (Dudits, 1998; Jacobs, 1997).

Among the plant hormones that regulate plant cell division, differentiation and development, auxins were identified as potent inducers of proliferation as well as phototropism, gravitropism, vascular differentiation and lateral branching of roots and shoots (Arteca, 1996). They are also involved in re-initiation of the dedifferentiation in mature plant tissues as positive regulators of cell division and initiation of several associated developmental programs in higher plants (Dudits, 1992). One of the most potent form of synthetic auxins is 2,4-D which was previously shown to trigger cell cycle dependent kinase activity and cell divisions in alfalfa (Magyar *et al.* 1993). Studies on the effect of auxins on cell cycle components in alfalfa have revealed a stimulatory role of the synthetic

analogue 2,4-D reflected by accumulation of Cdk-cyclin mRNAs/proteins and histone H1 kinase activity via activation of Cdk complexes.

Cytokinins are plant hormones that regulate plant cell division. They are also involved in developmental processes including photomorphogenesis, chloroplast biogenesis and senesence (Kakimoto *et al.* 1998). When the correlation between cell cycle progression and endogenous levels of cytokinins were studied, sharp peaks in the levels of were observed at the end of the S-phase and during mitosis (Redig *et al.* 1996). Exogenously applied cytokinins also effect cell division by inducing the expression of cell cycle control genes and/or through interacting with endogenous growth factors in specific tissues (Hemerly *et al.* 1993). Zhang *et al.*, (1996) have demonstrated that auxins and cytokinins together induce a 40 fold higher activity of cdc2-like protein in excised parenchyma cells of *N. tabacum*. In wheat leaves, $p34^{cdc2}$ related proteins, the control of cell cycle progression, the switch between division and differentiation in tissue development was shown to be linked to stimulation by auxin and cytokinin (John *et al.* 1990; 1993). Correlation between cell cycle progression and endogenous levels of plant hormones was studied in synchronized tobacco BY-2 cell suspension cultures using solid-phase anion exchange chromatography in combination with immunoaffinity purification and mass spectrometry. Sharp peaks in the levels of specific cytokinins (zeatin- and dihydrozeatin-type) at the end of the S phase and during mitosis suggested that zeatin might play a specific regulatory role in the progression of the plant cell cycle (Redig *et al.* 1996). Another key regulator of G1-S phase transition, the D-type cyclin *CycD3* was found to be elevated in a mutant of *Arabidopsis* with a high level of cytokinin and to be rapidly induced by cytokinin application in both cell cultures and whole plants (Fuerst *et al.* 1996). Constitutive expression of *CycD3* in transgenic plants allowed induction and maintenance of cell division in the absence of exogenous cytokinin. These results suggest that cytokinin activates *Arabidopsis* cell division through induction of *CycD3* at the G1-S cell cycle phase transition (Riou-Khamlichi *et al.* 1999).

Abscisic acid (ABA) mediated growth control is a fundamental response of plants to adverse environmental cues. The linkage between ABA perception and growth control is currently being unraveled by using different experimental approaches such as mutant analysis and microinjection experiments (Himmelbach *et al.* 1998). There are few data concerning the direct involvement of ABA on cell division cycle. It was reported that ABA interferes with replication forks and suppresses the effect of cytokinin. It was also suggested that the balance between ABA and cytokinin levels is a major factor controlling the rate of DNA replication and cell division in shoot meristems (Jackmard *et al.* 1995)

2.3 Histone promoters as markers for S-phase progression

We have recently demonstrated (Dedeoglu, unpublished) the utilization of histone H4 promoter GUS reporter fusions as a marker in transient over-expression of cloned cell cycle genes cyclin *cycMs1* and *cdc2BMs* in maize protoplasts. Plant expression vectors carrying two independent transcriptional units wheat histone H4 promoter GUS fusion and full length cell cycle genes mentioned above under the control of CaMV 35S promoter and termination sequences were introduced into maize protoplasts and changes in the GUS enzyme activity were monitored after 48 hours.

Furthermore, transgenic maize lines of H4-GUS can also be used to monitor changes in the host replication mechanism upon geminivirus infection. Nagar *et al.*, 1995, demonstrated the subcellular co-localization and induction of viral particles with another DNA replication-associated protein, proliferating cell nuclear antigen (*PCNA)*. The reliability of these findings were shattered by the fact that *PCNA* is also known to be induced by DNA damage possibly caused by virus invasion. The reports on involvement of viral genes in host cell cycle machinery will be discussed in lthe following part, devoted to maize streak virus biology.

PART 3

STUDIES ON MAIZE STREAK VIRUS: VIRUS RESISTANCE AND HOST-VIRUS INTERACTIONS

3.1 Plant viruses and their biological importance

Viruses provide useful tools and model systems, which can contribute to our understanding of cellular mechanisms, providing insights into processes that would otherwise be difficult to study (Fraser *et al.* 1990; Hillman *et al.* 1998).

Although the origin of plant viruses is uncertain, they are possibly co-evolved with animal viruses from the same ancestor, an insect virus. Plant viruses utilize several mechanisms to generate inter-species and intra- species: genetic diversity. This is mostly due their replication mechanisms that are prone to errors resulting in numerous mutations. One striking example of this is the difference of two isolates of the same plant DNA virus in nucleotide level is often more than the difference between two plant or animal species of the same genus. Also, another source of this variation is recombination and re-assortment and occasionally other common mechanisms of genetic variation, such as gene duplication and overprinting. In these aspects, plant DNA viruses are not exception and thus provided an excellent model system for studying general mechanism of gene expression, host pathogen interactions and molecular evolution (Roossnick *et al.* 1997).

3.1.1 Geminiviruses and MSV

Geminiviruses constitute a family of plant DNA viruses with a single-stranded circular genome that is encapsidated in unique icosahedral twinned (or geminate) particles (Lazarowitz, 1992; Timmermans *et al.* 1994). Geminiviruses are transmitted by whiteflies, leafhoppers, or treehoppers. The whitefly species *Bemisia tabaci* is the most efficient vector of dicot-infecting geminiviruses whereas *Cicadulina mbila* is a common vector for monocots. Their genomes are composed of either one or two DNA segments. Monopartite geminiviruses absolutely require a functional coat protein (CP) for infectivity, whereas bipartite geminivirus CP null mutants can infect plants systemically. These viruses cause significant damage to crop plants, especially in tropical and sub-tropical regions. They have also proved to be particularly useful as experimental systems for plant molecular biology studies, for example in the study of DNA replication and plant gene expression (Wright *et al.* 1997).

The geminiviruses are classified into three subgroups, dependent on their genome organization, host range and vector species. Maize streak virus, MSV, is a member of subgroup I of the *Geminiviridae*, along with wheat dwarf virus (WDV) and digitaria streak virus (DSV). Members of this subgroup have a monopartite genome and are transmitted by leafhoppers, mainly to cereals and other grasses. Transcription of the viral genome is bi-directional, resulting in the production of V1 (movement protein, MP) and V2 (coat protein, CP) from the virion-sense. The same intergenic region produces C1 (*RepA*) and C1:C2 (*Rep*) from unspliced or spliced complementary-sense transcripts (Palmerand Rybicky, 1998).

A recent study revealed the presence of a satellite DNA in a geminivirus, a structure mostly associated with several plant RNA viruses. It was reported that a 682-nt circular DNA satellite is associated with tomato leaf curl geminivirus (TLCV) infection. This is the first demonstration that satellite molecules are not limited to RNA viral systems. The DNA satellite (TLCV sat-DNA) is strictly dependent for replication on the helper virus replication-associated protein and is encapsidated by TLCV coat protein. (Dry, 1997).

All geminiviruses, replicate in the nucleus of susceptible cells by rolling circle replication. Minichromosome like double-stranded replicative form DNA generated during rolling circle replication, serves as templates for transcription of viral genes by RNA polymerase II and associated cellular transcription machinery (Stenger *et al.* 1991).

The *Rep* protein has been identified as the replication initiator protein. It is the sole protein required for geminiviral replication. Both the dicot and monocot-infecting viruses possess this protein (Laufs, 1995). The *RepA* protein, encoded by subgroup I viruses, is required for transcriptional activation of the virion-sense genes. When transformed with plasmid constructs containing greater than unit length DNA of tomato leaf curl geminivirus, soil bacterium *Agrobacterium tumefaciens* accumulates viral replicative form DNAs indistinguishable from those produced in infected plants (Rigden *et al.* 1996). The observation that "geminivirus DNA replication functions are supported by the bacterial cellular machinery", provides evidence for the theory that these circular single-stranded DNA viruses have evolved from prokaryotic episomal replicons.

MSV is a typical member of single stranded DNA plant viruses. The nucleotide sequence of MSV has been determined in 1984 by Mullineaux PM *et al.* Unlike previously characterized geminiviruses to possess two circles of DNA, the data were accommodated into one DNA circle of 2687 nucleotides. Analysis of open reading frames revealed seven potential coding regions for proteins, three in the viral (+) sense and four in the complementary (-) sense. The position of likely transcription signals on the MSV DNA sequence would suggest a bi-directional strategy of

transcription. To complement this initial finding, a population of 'nested' DNA molecules isolated, from MSV preparations. These molecules had ribonucleotides covalently linked to the DNA species' discrete 5' deoxyribonucleotide terminus suggesting that these molecules have been used to prime the synthesis in vitro of a complementary strand to virion DNA, initiating this reaction (Donson *et al.* 1984). The intergenic region containing promoter elements required for rightward transcription includes an upstream activating sequence (UAS) which endows the promoter with full activity in a maize transient expression system (Fenoll *et al.* 1990). The UAS contains two GC-rich repeats (GC boxes) and a long inverted repeat or hairpin with a loop harboring a TAATATTAC sequence common to all geminiviruses. This region includes the two GC-rich boxes, which are similar in sequence to *Sp1* binding sites binds maize nuclear factors *in vitro*.

Further studies revealed the presence of three RNA transcripts encoded by MSV DNA in virus-infected maize leaves. Two of the transcripts, a major 0.9 kb and a minor 1.05 kb RNA, were mapped on the virion (+) sense DNA and the other minor transcript of 1.2 kb was mapped on the complementary (-) sense DNA, demonstrating that transcription of MSV DNA was bi-directional (Morris-Krsinich, 1985). Similar to its mammalian counterparts, the single-stranded DNA geminiviruses produce transcripts from both strands (virion- and complementary-sense) of a nuclear double-stranded DNA molecule. In MSV-infected maize plants, approximately 80% of the complementary-sense transcripts produce the C1 protein, whilst the remaining 20% are spliced to remove a 92 nucleotide long intron and produce a C1:C2 fusion protein (Rep) (Wright *et al.* 1997). Disruption of the complementary-sense 3' splice site abolished virus replication. Spliced and unspliced forms of each virion-sense transcript were also produced, but differed in splicing efficiency. These multiple transcript initiation sites and different splicing efficiencies suggest that splicing is an important feature in the regulation of both early and late gene expressions in MSV.

Cloning of two distinct symptomatic variants of the geminivirus streak virus from maize plants infected with the Nigerian strain (MSV-N) (Boulton, *et al.* 1991) facilitated to reveal mechanisms of symptom development. Although these two isolates are highly homologous and have identical restriction enzyme maps and differs by only three nucleotides. Following "agroinoculation" to maize plants, MSV-Nm produced narrow, mildly chlorotic discontinuous streaks, whereas MSV-Ns-infected tissue had wide, severely chlorotic streaks. Symptom appearance was delayed in MSV-Nm inoculation with respect to MSV-Ns and the former had a narrow host range within the *Gramineae* comprising a fraction of that of the wide host range isolate MSV-Ns. The determinants of host range, severity of chlorosis, streak length, and timing of symptom appearance was mapped to a fragment which includes the large intergenic region and the 5' terminus of the complementary sense

C1 gene. On the other hand "streak width" was determined by the virion-sense portion of the genome, which is consistent with the observation that the virion-sense gene products (V1 and V2) are required for spread of the virus (Boulton *et al.* 1989). Insertion and deletion mutagenesis of the two virion-sense genes, V1 and V2, of MSV prevented symptomatic infections following 'agroinoculation' of maize seedlings. Mutants containing insertions or deletions in the coat protein gene, V2, were able to replicate to low levels, producing dsDNA although virion ssDNA was not detected and symptoms were not observed. Hence, unlike the bipartite geminiviruses, MSV requires coat protein to produce symptomatic systemic infection.

Further studies on MSV coat protein (CP) showed that it required for virus movement within the plant. Deletion or mutation of MSV CP does not prevent virus replication in single cells or protoplasts but leads to a loss of infectivity in the inoculated plant. The mechanism by which MSV CP mediates the transfer of MSV DNA from cell to cell and through the vascular bundle is still unknown. Towards understanding the role of MSV CP in virus movement Liu *et al.*, 1997, investigated the interaction of the CP with viral DNA was investigated using the 'south-western' assay. Wild-type and truncated MSV CPs were expressed in *E. coli* and used to investigate interactions with single-stranded (ss) and double-stranded (ds) DNA. The results showed that MSV CP bound ss and ds viral and plasmid DNA in a sequence non-specific manner. The binding domain was mapped to within the 104 N-terminal amino acids of the MSV CP. The presence of a nuclear localization signal in MSV CP suggests that it is presumebly involved in nuclear transport of other viral proteins. These facts indicate that the binding of CP to MSV DNA is involved in viral DNA nuclear transport as well as encapsidation and thus may have a role in intra- and inter-cellular movement as well as systemic infection.

3.1.2 MSV as an episomal gene vector

Geminiviruses are also used as convenient autonomously replicating vectors for foreign gene amplification in plants. Using tissue culture techniques, these plasmid-like extrachoromosal replicons have been adapted for the analysis of the regulation of gene expression in a wide range of hosts, including both mono- and dicotyledonous species. In monocotyledonous plants that are particularly recalcitrant to transformation, geminivirus symptom-induction has been used as a sensitive marker for DNA uptake and transient gene expression. (Stanley 1993)

A study by Shen *et al.*, 1995, demonstrated the usefulness of MSV as an efficient replicating vector in cells of maize plants, where a reporter gene (bar) coding for phosphinothricin acetyltransferase was inserted into the small non-coding region of the MSV genome. When the

recombinant bar-containing MVS vectors were introduced into maize seedlings via agroinfection, the chimeric viral DNA was found to replicate to high copy numbers in maize leaves. These leaves were resistant to the application of the herbicide Basta®.

MSV and related geminiviruses were also used to analyze the activity of plant transposases, such as the Ac/Ds elements of maize. MSV was used as a vector to introduce the maize transposable element Dissociation (Ds) and to study its excision in maize plants. MSV carrying *Ds1* in its genome was introduced into maize plants by agroinfection. Excision of the *Ds1* element from the MSV genome was detected only when functions from the transposable element Activator (Ac) were supplied in trans, either endogenously by the recipient maize plant or by co-transformation with *Agrobacterium* carrying a genomic *Ac* clone. The excision of *Ds1* could easily be visualized by the appearance of viral symptoms induced by the revertant virus. These results showed that the use of the MSV replicon provides a rapid and simple tool for the investigation of the excision of transposable elements in maize plants.

3.2 Non conventional virus resistance: engineered resistance in transgenic plants

Transgenic plants carrying nucleotide sequences derived from plant viruses can exhibit increased resistance to viral disease. Many viral sequences confer some level of either resistance to infection or suppression of disease symptoms (tolerance). These include segments of viral genomes encoding capsid or coat proteins, sequences encoding proteins that are or may be subunits of the viral replicase, sequences incapable of encoding proteins, entire genomes of defective interfering viruses and satellite viruses, and complete genomes of mild strains of virus. The transgene may act on initiation of infection, replication of virus, spread of the infection throughout the plant, and symptom development. More than one of these processes can be impaired by a single transgene derived from a single viral gene. The level of protection ranges from very low to high, while the breadth of protection ranges from very narrow, where protection is only observed against closely related strains of the virus from which the transgene was derived, to moderately broad, extending to other viruses. Data are insufficient to establish a molecular mechanism of resistance for most of the described examples. In addition, although the use of a particular segment of the viral genome confers resistance in one virus-host system, analogous sequences from a different virus in another host may be ineffective (Hull and Davies 1992; Lomonossof, 1995).

Development of virus-resistant varieties using classical breeding has also been limited for several crops due to the lack of known resistance genes and/or genetic barriers or complexity of the target crops. Engineered protection offers a new approach to manage virus diseases. Engineered

protection is referred to as resistance or protection conferred in plants by viral-derived nucleic acid sequences that are introduced into the plant genome via genetic engineering. Transgenic plants developed by this approach express viral sequences and are likely to be protected against infections by the virus from which the resistance gene is derived, and closely related viruses.

Engineered protection, a form of parasite-derived resistance (Hull and Davies, 1992), is now commonly used as a strategy for controlling virus diseases. Numerous reports have shown that this approach is generally applicable to many plant viruses without the drawbacks of classical protection (Beachy *et al.* 1990; Lomonossoff, 1995).

3.2.1 Coat protein mediated protection against plant viruses

Replication, long-distance spread, and vector transmission depends on the presence of coat protein. Expression of genes encoding nonfunctional CPs in transgenic plants can be tried in order to interfere with normal CP function. CP-mediated resistance describes the resistance that results from the expression of a virus CP gene in transgenic plants. There are several proposed mechanisms of CP-mediated protection and cross-protection in plants. Although both have common features, cross-protection is thought to be a complex response caused by the replication and expression of the entire viral genome. Here the resistance conferred by the expression of a virus coat protein gene is more limited. The term genetically engineered cross-protection is frequently used because in many cases the phenotype of resistance mimics that of cross-protection. However, in CP-mediated resistance, more accurately describes the resistance that results from the expression of a virus CP gene in transgenic plants. (Hackland *et al.* 1994)

One of the first reports in virus resistance by non-conventional means was by Tümer *et. al* (1987) where alfalfa mosaic virus (AlMV) coat protein was introduced into tobacco and tomato plants using Ti plasmid-derived plant transformation vectors. In the transgenic progeny, the plants were challenged by the same virus. These plants were producing high levels of the chimeric viral coat protein and had significantly reduced numbers of lesions and accumulated lower amounts of coat protein due to virus replication than the control plants. Later on, numerous reports on using the same strategy against plant RNA viruses were published as summarized below.

The expression of the viral coat protein gene in transgenic plants has been shown to induce tolerance against number of different but closely related virus infection particularly in dicot plants (Beachy, 1990). Transgenic tobacco plants expressing the coat protein gene of tobacco mosaic virus were tested for resistance against infection by five other tobamoviruses sharing 45-82 % homology in CP amino acid sequence with the CP of tobacco mosaic virus. The transgenic plants (CP+)

showed significant delays in systemic disease development after inoculation with tomato mosaic virus or tobacco mild green mosaic virus compared to the control (CP-) plants, but showed no resistance against infection by ribgrass mosaic virus.

In the first field trial ever of transgenic plants engineered for virus resistance, Nelson *et al,* (1988) evaluated two tomato lines expressing the CP gene of the TMV U1 strain. Transgenic plants displayed nearly complete resistance to mechanical infections by TMV and only 5% were symptomatic by at the end of the trial compared with 99% of the control plants.

When transgenic tobacco plants expressing the coat protein of tobacco mosaic virus (TMV) under the control of two different promoters; the promoter from a ribulose bisphosphate carboxylase small subunit (rbcS) gene and cauliflower mosaic virus 35S promoters differences in resistance was observed. The 35S::CP constructs gave higher resistance than the rbcS:CP constructs. Leaf mesophyll protoplasts isolated from both plant lines were equally resistant to infection by TMV indicating that the difference in resistance between the lines was due to the difference in tissue-specific expression (Clark *et al.* 1990). In agreement with this report, two different tissue specific promoters, *Phaseolus vulgaris* pal2 promoter (expression in the upper leaf epidermis-xylem) and the rolC promoter from *Agrobacterium rhizogenes* leading phloem and leafhair tip cells specific expression were tested along with CaMV 35S promoter driving TMV coat protein gene expression. Transgenic tobacco plants exhibited substantial differences in their responses to virus inoculation. The results indicated that highest level of protection obtained from constitutive 35S promoter rather that vascular tissue specific Pal2 and rol-C promoter was due to inhibition of infection rather than to effects on long-distance spread through the phloem (Reimann-Philipp *et al.* 1993)

Developments in genetic transformation and the agronomic importance of controlling virus diseases in crop plants lead to further evaluation of the trait in field conditions. Transgenic tomato plants expressing the coat protein gene of cucumber mosaic virus (CMV) strain WL, a member of CMV subgroup II, were evaluated for resistance to CMV infections under field conditions for 2 years. The four transgenic tomato lines exhibited a high level of resistance to CMV infections, since all 747 transgenic plants remained symptomless throughout the crop cycle. (Fuchs *et al.* 1996)

Studies on cp-mediated virus resistance in cereals demonstrated that CP-mediated resistance to virus infection can be extended to cereals and to the viruses transmitted by an insect vector (plant hopper). Hayakawa T *et. al.,* 1992, demonstrated that the coat protein gene of rice stripe virus exhibited a significant level of resistance to virus infection. Moreover, the virally encoded strip disease-specific protein was not detected in transgenic plants expressing CP 8 weeks after inoculation, indicating protection occurred before viral multiplication.

In maize, the maize dwarf mosaic virus strain B (MDMV-B) coat protein gene was introduced into sweet corn cell suspension cultures via particle bombardment or electroporation. When plants regenerated from a highly expressive line were challenged with a virus inoculum and the presence of the MDMV-cp provided resistance to inoculations with MDMV-A or MDMV-B and to mixed inoculations of MDMV and maize chlorotic mottle virus (Murry *et al.* 1993).

3.2.2 Other non-conventional strategies against plant viruses

Other than CP-mediated protection, using viral DNA sequences or genes from other organisms several other strategies were develop and virus resistant plants were obtained via genetic transformation.

Genetically engineered plants expressing either intact or mutant forms of the virus-encoded replicase subunit were resistant to infection by the virus from which the transgene was obtained. In many instances, the resistance is very effective and will be useful in the field (Baulcombe *et al.* 1994). A truncated form of the C1 gene of tomato yellow leaf curl geminivirus (TYLCV) expressing the N-terminal 210 amino acids (act) of the *Rep* protein, was cloned under the control of the CaMV 35S promoter and introduced into Nicotiana benthamiana. The same sequence was also cloned in anti-sense orientation. Transient and stable gene expression systems showed that the expression of the N-terminal 210 aa of the TYLCV *Rep* protein efficiently interferes with virus infection. Primary transformants were tested for resistance to TYLCV by agroinoculation, some plants proved to be resistant, particularly in the sense lines Infection of leaf discs derived from transgenic plants showed that expression of the transgene correlated with a substantial reduction of viral DNA replication. In another study by MacFarlane *et al.,* (1992), *Nicotiana benthamiana* plants transformed with the open reading frame (ORF) encoding the 54-kDa protein, were resistant to infection by purified PEBV.

Application of antisense RNAs to interfere with the disease caused by TYLCV resulted in resistance to the virus. The target of the anti-sense RNA is the rare messenger RNA of the Rep protein, encoded by the C1 gene. Transgenic *Nicotiana benthamiana* plants expressing C1 anti-sense RNA were shown to resist infection by TYLCV (Bendahmane *et al.* 1997)

Hong *et al.,* 1996 have recently described a novel strategy for engineering resistance to African cassava mosaic virus (ACMV) in transgenic *Nicotiana benthamiana* plants using a virus-inducible promoter to control the expression of a plant ribosome-inactivating protein (RIP) transgene. Later, they have used a potato virus X (PVX) vector to express the ACMV transactivator protein, AC2, *in planta* and confirm that amplification of RIP activity in transgenic plants is mediated by AC2 (1997).

Transgenic tobacco plants which express untranslatable sense or anti-sense forms of the tobacco etch virus potyvirus (TEV) coat protein gene sequence has been generated. One of seven transgenic plant lines expressing a CP gene anti-sense transcript showed an attenuation of symptoms when inoculated with TEV. Three of ten transgenic plant lines expressing untranslatable sense transcripts did not develop symptoms when inoculated with TEV. These lines were resistant to either aphid or mechanically transmitted TEV. In contrast to CP-mediated resistance reported for other viruses, resistance was (1) mediated by an RNA molecule; (2) TEV-specific (i.e., "broad-spectrum resistance" was not observed); (3) independent of inoculum levels; (4) not dependent on plant size and; (5) due to decreased levels of virus replication. Protoplast experiments were used to demonstrate that resistant plant lines did not support the production of virus protein and progeny virus at wild-type levels (Lindbo *et al.* 1992).

One interesting approach involves the use of vertebrate enzymes mediating the catalysis of RNA decay by the "interferon-regulated 2-5A system" upon virus infection. Ectopic expression of two human cDNAs in transgenic plants resulted in substantial reduction of tobacco etch virus yield as measured by ELISA assay. These results indicated that expression of a mammalian 2-5A system in plants provides resistance to virus infections (Mitra *et al.* 1996).

Another novel strategy was based on exogenous application of pokeweed antiviral protein (PAP), a ribosome-inhibiting protein found in the cell walls of *Phytolacca americana* (pokeweed), that protects heterologous plants from viral infection. Transgenic plants that expressed either PAP or a double mutant derivative of PAP showed resistance to infection by different viruses introduced by mechanical or aphid transmission. Analysis of resistance in transgenic plants suggests that PAP confer viral resistance by inhibiting an early event in infection. Expression of PAP in transgenic plants offers the possibility of developing resistance to a broad spectrum of plant viruses by expression of a single gene.(Lodge *et al.* 1993, Tümer *et al.* 1997). Similarly, to develop an antiviral agent and virus-resistant plants, a cDNA clone encoding *Phytolacca insularis* antiviral protein (PIP) was isolated. Transgenic potato plants expressing the PIP cDNA under the control of the cauliflower mosaic virus 35S promoter were resistant to viruses, such as potato virus X, potato virus Y, and potato leafroll virus. These results suggested also that the PIP cDNA could be used for the development of an antiviral agent and transgenic plants resistant against a broad spectrum of plant viruses infecting through both mechanical and aphid transmission (Moon *et al.* 1998).

3.3 Virus Resistance in Geminiviruses

There are few reports concerning the resistance to geminiviruses. Transgenic tomato plants expressing the tomato yellow leaf curl virus capsid protein were resistant to the virus. Kunik T, *et al.,* (1994), used the tomato yellow leaf curl virus (TYLCV) gene that encodes the capsid protein (V1) under the transcriptional control of the cauliflower mosaic virus 35S promoter and cloned into an *Agrobacterium* Ti-derived plasmid to transform plants from an interspecific tomato hybrid, *Lycopersicon esculentum* X L. pennellii (F1), sensitive to the TYLCV disease. Transgenic progeny, expressing the V1 gene, were inoculated with TYLCV using white flies fed on TYLCV-infected plants, they responded either as untransformed tomato or showed expression of delayed disease symptoms and recovery from the disease with increasingly more resistance upon repeated inoculation. Transformed plants that were as sensitive to inoculation as untransformed controls expressed the V1 gene at the RNA level only. All the transformed plants that recovered from disease expressed the TYLCV capsid protein. In other words, CP-mediated protection was dependent on presence of protein.

The C1 gene of tomato yellow leaf curl geminivirus (TYLCV) encodes a multifunctional protein (Rep) involved in replication. A truncated form of this gene, capable of expressing the N-terminal 210 amino acids (act) of the *Rep* protein, was cloned under the control of the CaMV 35S promoter and introduced into Nicotiana benthamiana using *Agrobacterium tumefaciens*. The same sequence was also cloned in antisense orientation. When self-pollinated progeny of 19 primary transformants were tested for resistance to TYLCV by agroinoculation, some plants proved to be resistant, particularly in the sense lines. Two such lines were further studied. The presence of the transgene was verified and its expression was followed at intervals. All plants that were resistant to TYLCV at 4 weeks postinoculation (wpi) contained detectable amounts of transgenic mRNA and protein at the time of infection. Resistance was overcome within 9-15 weeks post inoculation. Infection of leaf discs derived from transgenic plants showed that expression of the transgene correlated with a substantial reduction of viral DNA replication.

Bendahmane *et. al.,* (1997) recently reported application of anti-sense RNA to interfere with the disease caused by the same geminivirus TYLCV targeting the mRNA of the Rep protein encoded by C1 gene. Transgenic *Nicotiana benthamiana* plants expressing C1 anti-sense RNA were shown to resist infection by TYLCV. Some of the resistant lines were symptomless, and the replication of challenged TYLCV was almost completely suppressed also in the next two generations of the progeny.

Diseases caused by geminiviruses are widespread in the tropical-subtropical tomato production areas. Tomato yellow mosaic geminivirus (ToYMV) is a member of the whitefly-transmitted subgroup (III), which is vectored by the whitefly *Bemisia tabaci.* Several accessions of *Lycopersicon chilense*, *L. hirsutum* and *L. peruvianum* var. *glandulosum* were identified that exhibit tolerance or a high degree of resistance to a Venezuelan isolate of ToYMV in field and greenhouse studies using mechanical inoculation and viruliferous vectors. Polymerase chain reaction analysis showed that viral DNA is present in infected plants and absent in resistant ones. Mechanical and insect transmission gave different results in some species or cultivars suggesting a distinct mechanism for resistance.

Resistance to maize streak virus (MSV) is an essential trait of improved maize varieties in sub-Saharan Africa. Weltz *et al.,* 1998 mapped quantitative trait loci (QTL) for resistance to MSV in a population of derived from a cross between the maize inbred lines CML202 (resistant) from CIMMYT-Zimbabwe and Lo951 (susceptible) from Italy. Field tests were in Zimbabwe, inoculated with viruliferous leafhoppers (*Cicadulina mbila*) and the QTL's partially dominant gene action was consistent with the nearly intermediate resistance of the F1 generation. This gene seems to be allelic or identical to Msv1, a major resistance gene that was previously identified. Moreover, in efforts to develop maize hybrids resistant to maize streak virus, it was found that the growth and yield of maize was significantly influenced by plant stage at time of challenge (Barrow *et al.* 1992).

PART 4

4.1 MSV and Maize Cell Cycle

4.1.1 Structure and Function of Geminivirus Replication proteins

Geminiviruses constitute a genus of plant single stranded DNA viruses that replicate through double stranded DNA intermediates in plant nuclei. As has been shown with the well-characterized mammalian DNA viruses, their replication depends on host replication proteins and transcription factors as well as virus encoded products. Therefore, geminiviruses can be useful for exploring some aspects of the mechanism of plant DNA replication and cell division through a study of viral-host protein interactions (Lazarowitz, 1992; Palmer and Rybicky, 1998).

Replication Strategy of ssDNA Viruses

1. Conversion into dsDNA (=host repair process?)
2. Early transcription (by host enzymes)
3. Translation of (regulatory) protein and
   "rolling circle" ssDNA replication
4. Late transcription (usually mediated by viral proteins)
5. Synthesis of late (=structural) proteins
6. "Sequestering" of viral genomic ssDNA
7. Assembly into virions

Studies on the replication of geminiviruses during infection indicate the role of a viral protein (*Rep*) in creating a permissive environment for viral DNA replication (Oroczo *et al.* 1998). This protein first recognizes the replication origin, then acts as a site-specific endonuclease to initiate a nick on the DNA before displacing a single stranded copy during rolling circle replication (Laufs *et al.* 1995).

Geminivirus *Rep* proteins have additional roles in specific DNA recognition associated with viral origin recognition (Fontes *et al.* 1994), they possess a ATP/GTPase activity (Desbiez *et al.* 1995) and the AC2 product of the bipartite viruses acts as a transactivator of other viral genes. The

carboxy terminal domain of the *Rep* protein of all geminiviruses shares some homology to the DNA binding domain of the *myb*-like class of plant transcription factors (Hofer *et al.* 1992). The transactivation of the CP promoter by C-sense gene products of subgroup I viruses has been demonstrated for WDV (Hofer *et al.* 1992), Chloris striate mosaic virus (Zhan *et al.* 1993), and MSV (Mazithulela *et al.* 1997), but it is not known if the transactivation of host genes is also possible.

4.1.2 Host-virus interaction during the cell division cycle

The first demonstration that geminivirus infection induces the host cell cycle was using digitaria streak virus (DSV). DSV replicative forms were found to predominate in S-phase nuclei of infected plants. Thus, DSV replication appears to be synchronized with host DNA replication primarily under the control of host factors available only at G1/S boundary of cell division cycle (Accotto *et al.* 1993). Recently, it has been shown that the bipartite geminivirus tomato golden mosaic virus (TGMV) and its Rep protein induce the accumulation of proliferating cell nuclear antigen (PCNA), a protein associated with DNA replication, in differentiated leaf cells of *Nicotiana benthamiana*. However, this report does not provide direct proof of the correlation of geminivirus replication and cell division activity because PCNA may also be induced by DNA damage (Nagar *et al.* 1997). Other links between the requirement for cell cycle activation and geminivirus replication have been made recently. The discovery of a "retinoblastoma" (Rb) binding motif in WDV and other monocot-infecting geminiviruses has led to the isolation of a Rb homologue from maize (Xie *et al.* 1995; 1996). Rb proteins are negative regulators of the cell division cycle by binding transcription factors and cyclin-cyclin dependent kinase complexes that act as maturation-promoting factors at the G1/S phase boundary (Wiman *et al.* 1993). Mammalian DNA viruses, like SV40, adenovirus E1A (Moran *et al.* 1993) and human papilloma virus type 16 (Vousden *et al.* 1993) have been shown to modify the cellular environment to render it permissive for viral replication. This is mediated through binding of viral proteins to Rb and other associated protein complexes. Two recently cloned cDNAs from Zea mays, *RRB1* and *RRB2*, encode RB-related proteins (Ach *et al.* 1997). RRB1 was a 96-kDa nuclear protein that can physically interact with two mammalian DNA tumor virus oncoproteins, *simian virus* 40 large-T antigen and *adenovirus* E1A, and with a plant D-type cyclin. RRB1 can also bind to the AL1 protein from tomato golden mosaic virus (TGMV), a protein which is essential for TGMV DNA replication. Further understanding of the mechanism by which geminiviruses manipulate DNA replication and cell cycle control was obtained using the yeast two-hybrid system. It was shown for WDV, that both RepA and Rep can bind Rb. Since an intact Rb binding motif (LxCxE) is required for efficient WDV replication in cultured wheat cells, it is highly likely that the C-sense viral gene

product(s) are responsible for linking of the viral and cellular DNA replication cycles (Xie *et al.* 1996). Collin *et al.*, (1996) suggested that the WDV CP gene expression activated by *RepA* might be dependent upon an interaction between *RepA* and the Rb-like protein. Using a yeast two-hybrid assay, Liu *et al*, 1999 have investigated whether the complementary-sense gene products of bean yellow dwarf virus, a mastrevirus that is adapted to dicotyledonous plants, also bind maize Rb protein. They demonstrated RepA binds to Rb protein, Rep does not, suggesting that RepA alone regulates host gene expression and progression of cells to S-phase.

We have recently used the yeast two hybrid system to demonstrate the binding of the MSV RepA protein to the human Rb and its maize homologue and also to identify several functionally important domains of *Rep* and *RepA* (Horvath *et al.* 1998). The MSV RepA protein activates transcription of both *HIS3* and *lacZ* reporter genes in *Saccaromyces cerevisae*. The activation domain resides between residues 219-231 of the *RepA* protein. The full-length *Rep* did not transactivate gene expression, but an additional activity was identified between residues 252-270 when a truncated version (terminating at residue 271) was tested. Therefore the *Rep* and *RepA* proteins contain two independent putative activators in yeast cells. The first activator domain (residues 219-231) is not present in *RepA* which is produced from a spliced transcript (sequences encoding these residues are present within the intronic region). It is now important to determine whether either (or both) of these activation domains are required for V-sense gene expression and whether they affect host gene expression in maize cells. Additionally, although association of the MSV Rep protein with Rb-like proteins was not seen in yeast cells, it could be that both Rep and RepA might interact *in vivo*.

The yeast two-hybrid data also showed that the MSV Rep and RepA products are capable of forming homo- and hetero-dimers and oligomerisation domains have been identified. These interactions likely are important for regulation of viral replication and transcription as well as host gene expression as most transcriptional activators and transcription factors act as such dimers. Furthermore, there is evidence that the origin recognition and processing activities of the geminivirus Rep proteins of WDV and tomato yellow leaf curl virus (TYLCV) are mediated by the amino-terminal region (Heyraud-Nitschke *et al.* 1995). In TGMV, the Rep protein (AL1 or AC1), has a ssDNA binding site within its oligomerisation domain and binds specifically to the viral origin of replication. The oligomerisation of this protein is a pre-requisite for DNA binding and thus viral replication (Thommes *et al.* 1993, Fontes *et al.* 1994). Moreover, the protein oligomerises with protein AL3 (AC3), an enhancer of viral DNA accumulation, indicating the existence of functional hetero-protein complexes (Oroczo *et al.* 1997). The MSV oligomerisation domain is sited in the amino-terminal region of the

RepA protein and it may therefore be equivalent to a region from TYLCV that determines the specificity of viral replication origin recognition. Expression of a peptide containing the 210 amino acid N-terminal of C1 gene from TYLCV interferes with viral replication in transgenic tobacco plants (Noris *et al.* 1996).

These results suggest that G1 regulation in plant cells is possibly affected by viral proteins in interaction with host factors, for maintaining a permissive environment for virus replication. It is therefore logical that geminiviruses, which also depend upon host DNA replication, would use a similar strategy to effect replication in non-dividing, differentiated tissues.

In another study, Hur *et al,* investigated the mechanism of BCTV-induced symptom development, emphasis on the callus like structures that form on 'hyper-susceptible' ecotypes. Quantitative analysis of GUS reporter activity in virus infected transgenic plants containing constructs with the cdc2, cyclin cyc1 and SAUR promoters indicated that auxin regulated promoter activity is induced concomitantly with cell cycle gene promoter activities in symptomatic tissues. Interestingly, auxin resistant mutants and auxin transport mutants of *Arabidopsis* showed reduced infectivity and symptom development. These data strongly suggest that altered auxin levels be involved in the induction of cell division in BCTV-infected plants association with symptom development and systemic infections. (Hur JG, Davis KR, unpublished). Same laboratory reported on ORF L4 of BCTV, playing a major role in symptom development in *Arabidopsis;* a two-hybrid screen of a cDNA library from a susceptible *Arabidopsis* ecotype was done in order to identify plant factors that interact with BCTV ORF L4. Three clones were isolated that show specific interaction with ORF L4. Two clones correspond to known genes. One of the known genes encodes a ubiquitin conjugating enzyme, which plays a role in cell cycle progression. The other encodes an *Arabidopsis* shaggy-like kinase. The shaggy-like kinases are related to mammalian glycogen synthase kinase, a protein involved in several signaling pathways, some of which are associated with regulating cell division. (Buckley KJ,. Ware DH,. Davis KR, unpublished). Most recently, the C4 product of beet curly top geminivirus was shown to induce cell division and hyperplasia in transgenic *Nicotiana benthamiana* plants (Latham *et al.* 1997).

There only few reports on the host-MSV interactions. Using mRNA *in situ* analysis and immunohistological assays, it was not possible to co-localise the transcripts of *Rep* genes and S-phase-specific histone H2B and PCNA transcripts or proteins in MSV-infected maize plants (Lucy, 1996). Moreover, MSV was not found in the shoot apical meristem. It was previously reported that *Agrobacterium* preferentially transfers T-DNA into cells of leaf primordia, which are formed at the time of inoculation but not into the meristematic region (Shen *et al.* 1994).

All these increasing body of evidence supports a tight link between the cellular and geminiviral replication cycles and suggests that the cell cycle may be reset in a manner analogous to that used by tumor inducing animal DNA viruses. To better understand the host- cell division cycle and MSV replication, I have used transgenic maize lines carrying a replication-dependent- wheat histone H4 promoter-β glucuronidase marker gene fusions for further identifying the role of viral genes in S-phase activation. Both actively dividing cell suspensions and non-proliferating fully differentiated leaf tissues have been used for virus infection studies. The simian virus 40, a model for the mammalian replicon, is a uniquely powerful system for the study of drugs and treatments that target enzymes of the mammalian replication apparatus (Snapka *et al.* 1993). Most of the studies on mammalian DNA viruses were conducted on simian virus 40 and its gene product the large T-antigen. In these studies aphidicolin was shown to induce topological and recombinational events in simian virus 40. During aphidicolin exposure, highly compacted SV40 DNA replication intermediates were immediately broken down (Snapka *et al.* 1991). It was also shown that the mechanism by which T antigen stimulates homologous recombination in human fibroblasts involves DNA replicative synthesis and can be suppressed by inhibitors of DNA replication such as aphidicolin and hydroxyurea (Shammas *et al.* 1997). Purification and characterization of *Pseudorabies* virus (PRV) DNA polymerase revealed that it is sensitive to aphidicolin. Aphidicolin inhibited *in vitro* PRV DNA polymerase activity and completely blocked viral growth *in vivo* at 4.4 μM (Verri *et al.* 1994). Based on the similarities between mammalian DNA viruses and *geminiviridae*, role of DNA inhibitor aphidicolin was assessed to support the hypothesis that MSV is dependent on host DNA replication.

CHAPTER II

MATERIALS AND METHODS

2.1 Bacterial material and techniques

2.1.1 General techniques:

All bacterial manipulations were done according to standard aseptic techniques under a laminar flow hood cabinet.

For plasmid cloning *E.coli* strains DH5α or HB101 were used. Competent cells for bacterial transformation was prepared with a modified $CaCl_2$ procedure and transformations were done with standard heat shock procedure as described in Sambrook *et al,* 1989.

For basic DNA cloning purposes, pUC19 or pBluescript SK^+ plasmids were used.

Bacterial colonies grown and maintained on Luria-Bertani medium supplemented with appropriate antibiotics; carbenicillin at 100-200 µg per ml, kanamycin at 25-50 µg per ml, on 0.8% bacterial agar plates. These plates were incubated at 37° C overnight and for further storage kept at 4° C for several months. Liquid cultures were grown in Luria-bertani broth or occasionally a rich medium 2XTY, after inoculation flasks at least 4 times bigger than nutrient volume shaken at 250-300 rpm at 37° C. A liquid aliquot of each bacterial clone carrying a specific plasmid was also kept at -80° C in 7% DMSO for longer storage.

Plasmid DNA isolation was performed by a commercial plasmid DNA isolation kit (Qiagen GmBH, Germany) according to the manufacturer's recommendations. This system was based on alkaline lysis followed by column chromatography. Purity and integrity of the DNA was confirmed by spectrophotometry and agarose gel electrophoresis, respectively. For precise quantitation of DNA fluorimetric methods utilizing a DNA binding dye bisbenzimide (Hoechst 33258) was used in a TKO 100 mini fluorimeter (Hoefer Scientific Instruments, U.S.A). Plasmid DNA was precipitated in the presence of sodium acetate and ethanol. After washing the DNA pellet with 70 % ethanol, DNA was dissolved sterile conditions in water or TE buffer (pH=8) and kept at -20° C until usage.

2.1.2 Construction of plant expression vectors and transformation plasmids

The wheat histone H4 gene and histone H3 genomic clones (pTH012 and pTH011) in pBR322 vector were provided by Dr. M. Iwabuchi, Japan.

The promoter region of 720 bp was cloned as a Hind III/Xho I fragment and after polishing the ends by Klenow DNA polymerase, this fragment was ligated to the coding region of GUS reporter

gene from pIDS011. Termination sequences were different for two transformation vectors. In pH4G, polyadenylation signals from nopaline synthase gene (NOS) of *Agrobacterium tumefaciens* was used whereas in pTHG2 vector a 850 bp Sau IIIA/Hind III fragment of wheat histone H3 gene was cloned as the termination signal.

The p35Ac gene construct carrying a synthetic phosphinotricin acetyltransferase (PAT) gene under the control of CaMV 35S promoter and NOS termination signal was kindly provided by P.Ecker of Hoechst, Germany.

The plasmid pKM794 was kindly provided by Drs. E. Fejes and contains double enhancer sequence of the CaMV 35S promoter (-208 to -46) inserted upstream of the minimal promoter region (-90 to +8) linked to GUS reporter gene and NOS terminator sequence.

The plant transformation vector pAHC25 (Christiensen, 1996) carries both PAT gene and GUS genes under the control of maize polyubiquitin gene promoter, followed by its first intron and a small portion of first exon. Transcription termination was maintained by the NOS termination sequence.

For the studies on the maize streak virus resistance, a series of plant transformation vectors were constructed. The 814 bp long EcoRI/Hind III fragment of Maize Streak Virus V2 gene was sub-cloned into a plant expression vector pJIT60 carrying a doubled CaMV 35S promoter and termination signal from the same virus sequence both on sense and anti-sense orientations. In addition to this vector, same V2 gene was first blunt ended and then replaced the GUS reporter gene in pAHC25. The resulting plasmid vectors were suitable for direct transformation as the selectable marker gene expression cassette also existed on the same alignment.

2.2 Plant cell, tissue and protoplast culture techniques

2.2.1 Maintenance of maize cell suspension culture HE/89

An embryogenic maize cell suspension line HE/89 and its derivatives Ke2/2, Ke2/22 were used in these studies (Morocz, 1989). It was maintained in NM6M medium, a modified version of Chu's N6 medium. It consists of standard MS salts supplemented with 500 mg/l bacto-tryptone, 3% sucrose and 0.5 mg/l 2-4, dichlorophenoxyacetic acid at pH=5.6. Sterilization was done by autoclaving at 120 p.s.i for 25 minutes.

Fifty milliliters of cultures started as 0.4% (fresh weight/volume) cell suspension in 100 ml Erlenmeyer flasks grown for 3 weeks with continuos orbital shaking at 100-150 rpm under light. At the end of this period, cultures reached to 8% (w/v) were further grown for 2 days with the addition of fresh medium.

For the establishment of transgenic cell suspensions from callus tissues different sub-culturing and hormone regime was used. Transgenic calli were first kept in 1 mg/l 2,4-D at 1% (w/v) cell density for 2 weeks and than the small pieces of this suspension sieved through 500 µm meshes to form a fine starter culture to be maintained as control suspensions.

2.2.2 Protoplast isolation and culture from HE/89

For the enzymatic removal of the cell walls of maize suspension cells, a coctail consist of 2% Cellulase RS and 0.2% Pectolyase Y23 was used. The digestion solution was supplemented with 0.3 M mannitol as an osmoticum and 0.1 % methlyethane sulfonate (MES) as a pH stabilizer. This preparation was first filtered to remove insoluble contaminants and than heat treated at 55° C for 15 minutes to inactivate potential proteases. Finally, filtered through 0.2 µm filters for sterilization.

Two grams of the fresh log phase HE/89 culture was first washed with a washing solution "MaCa" that consists of 0.2 M mannitol, 80 mM $CaCl_2$ and 0.1% MES at pH=6. While the cells were kept in 10 ml of MaCa solution in a glass petri dish 5 ml of the enzyme cocktail and incubated in the dark with constant mild shaking for 2-4 hours. At the end of this period, petri dishes were examined under the inverted microscope for rough determination of protoplast release efficiency. Occasionally, enzyme solution is made in protoplast culture medium ppNM6 and incubation times of 10-16 hours used without mechanical agitation. After the enzyme treatment, the protoplast suspensions were passed sequentially through 200, 100 and 50 µm-mesh size stainless steel sieves with continuous addition of MaCa washing solution. Enzyme solution was removed by 3-minute centrifugation at 100g. The pellet was washed 2 more times with MaCa with standard centrifugation and finally the number of protoplasts was determined by counting in a haemocytometer chamber. In most of the cases, sucrose gradient centrifugation for further purification of the protoplasts from cell debris omitted as extensive washings provide relatively pure protoplast for further manipulations.

With or without transformation, HE/89 protoplasts were cultured in protoplast culture medium ppNM6 that consists of micro and macro elements of the N6M medium with modifications where Mg and Ca ion concentrations raised and as sugar source along with sucrose other monosaccharides were used. NAA and zeatin mixed isomer was included for better growth. *L*-glutamine and serine was also added before filter sterilization of this medium.

HE/89 protoplasts were cultured at a density of 0.5-1 million /ml in liquid cultures at 23°C under dim light for several weeks to facilitate micro colony formation. After this period cultures can be diluted with NM6M medium and spread onto solidified medium for further growth and callus formation.

2.2.3 Protoplast transformation, selection and plant regeneration

Freshly isolated protoplasts were gently re-suspended in MaCa solution as 1 million protoplasts per 0.1 ml. Typically 1-3 million protoplasts were used per each DNA transformation treatment and distributed to sterile 15 ml Wassermann tubes. Plasmid DNA that was dissolved in sterile water as 1µg/µl was added onto the protoplast as 10µg per million protoplasts. During a period of 10 minutes DNA was allowed to settle evenly on protoplast by several gentle swirling of the tube. At the end of this period equal volume of PEG (1500) dissolved in 0.4 M mannitol, 100 mM $MgCl_2$ was added onto the protoplast/DNA suspension drop by drop. In order to facilitate the precipitation of DNA on the plasma membrane and intake of plasmids tubes were left in the hood for 10 minutes with occasional gentle mixing. Protoplasts were released from the osmotic action by drop-wise addition of 15 ml of MaCa solution over a period of 20 minutes. Excess DNA and transformation solution with PEG removed my standard centrifugation.

Protoplast treated with PEG and DNA (along with control treatments without PEG and/or DNA) was observed under the microscope for possible damage exerted by these two components just after re-suspending them in protoplast culture medium ppNM6 at 1 million/ml cell density.

Protoplast cultures were incubated in dim light at 23° C for 3-4 weeks before the selection process was initiated. In case of serious evaporation of the liquid media small amounts of pNM6 medium was added to the cultures.

For the selection of putative transformant protoplast derived micro colonies, protoplast cultures were diluted to 10 ml in NM6 medium and gently spread onto petri dishes with NM6 medium supplemented with appropriate selective agents and 0.2% Gelrite®; a clear agar substitute, as solidifying agent. In case of selection by phosphinotricin, a preparation consists of 10 mg/l *L*-PPT was used. When the selection was based on kanamycin resistance, 150 µg/ml kanamycin concentration was used. After a period of 3-6 weeks, resistant micro callus colonies were distinguishable from the non-transformant on the petri dishes. Individual colonies were transferred onto fresh petri plates for further perpetuation of the callus material. After 3-4 weeks each putative transformants were transferred on media lacking hormone for initiation of somatic embryo formation and subsequently shoot-root regeneration. At this point, a small amount of calli was sampled for PCR analysis, as well as another piece was kept on hormone plus high sucrose (12% sucrose) containing media for securing the fate of the clone.

When proper embryo formation, shoot and root regeneration was obtained in jars, platelets of 8-15 cm. were transferred into soil for further growth and seed production. These plantlets were

first transferred to climate chambers with 20-23° C with high humidity (70-85%) and then kept in green house conditions for the development of sexual organs. Plants with normal immature ears were cross-pollinated with control lines and set for seed production for an additional period.

2.2.4 Biolistic transformation by electric discharge apparatus: Accell®

For transient assays and virus transmission, biolistics was used as a tool to introduce and express foreign genes in maize cells. A sophisticated particle bombardment gun Accell® utilized was leased from Agrocetus Inc, USA. The plasmid DNA was loaded onto 1.5µm gold beads (Alpha chemicals Inc., USA) at a rate 40µg/mg gold using standard $CaCl_2$-spermidine protocol of Klein et al, 1997. The coated beads were then pipetted onto metalized myler carrier sheets (18x18mm). After a brief period of settling the ethanol drained from the carrier sheets. The gun was loaded by placing 10µl of water between the spark points. The target tissues were pre-treated with a osmoticum (0.35 M sorbitol) in a petri dish for 4 hours to minimize physical damage. The target was then positioned in a way that the desired area to be exposed as it was inverted above retaining screen. The assembly was evacuated to 600 millibars before the discharge activated by charging the capacitor at 14kV. After bombardment, the tissue was transferred onto N6M culture media and left until harvest for reporter gene assays.

2.3 Molecular and Biochemical methods

2.3.1 Plant genomic DNA isolation, PCR and Southern analysis

Genomic high molecular weight DNA from maize tissues was basically isolated according to a modified protocol of Dellaporta *et al*, 1983. Briefly, plant tissues of 0.1-2 g were homogenized into a fine powder in liquid nitrogen. At least 4 volumes of extraction buffer (50 mM Tris pH 8, 100 mM NaCl, 1% SDS, 10 mM EDTA pH 8 and 10 mM β-mercaptoethanol) and 10µg/ml Proteinase K (Serva) was added to the homogenate and tubes incubated at 65° for 20 minutes. After the addition of ice cold KOAc (5 M) samples were left on ice for 20 minutes. Debris was removed by centrifugation at 10000g and nucleic acids were precipitated with the addition of equal volume of isopropanol. After a brief wash with 75% ethanol, the pellets were dissolved in water. The genomic DNA was precipitated once more in the presence of 2.5 M ammonium acetate and 2 volumes of absolute ethanol. Final pellet was washed once with 75% ethanol and dried under mild vacuum.

For Southern analysis of the isolated genomic DNA, restriction enzyme digestions were performed. Typically twenty µg of DNA was digested in a volume of 200 µl containing One -Phor-All + reaction buffer (Pharmacia), 120 units of restriction enzyme, 2 mM spermidine for 16 hours at

appropriate temperatures optimum for the used enzyme. Occasionally, 10 µg/ml DNase free RNase was added to remove residual RNA. At the end of the reaction, complete digestion of the DNA was checked by agarose gel electrophoresis and restricted DNA was brought to a smaller volume by NaOAC/ethanol precipitation.

Agarose gel electrophoresis was performed with 0.8-1% gels in TAE buffer, ran at 3-4 Volts/cm for 10-16 hours. The gels were blotted onto nylon membranes (Hybond N, Amersham, U.K) by mild alkaline/ downward capillary blotting as described by Chomszinky (1994). DNA on the filters were than immobilized in a Stratalinker (Stratagene, U.S.A) UV cross-linker and kept at -20° C until use.

For PCR screening of putative transgenic maize lines, genomic DNA isolated from these lines under special care to avoid contamination with foreign DNA using standard methods. For each reaction 200-500 ng of DNA was mixed with a master mix solution containing 20 pmols of each gene specific primer, 0.2 mM dNTP mix, Taq DNA polymerase reaction buffer and 2 units of Taq DNA polymerase in a total volume of 30-50µl. Reaction tubes were mixed gently but immediately and then PCR was initiated just after the addition of paraffin wax beads as barriers against evaporation. Cycling parameters were consist of a initial denaturation of 5 minutes at 85° C and 2 minutes of 96° C followed by 25-30 cycles of 20 seconds of denaturation at 94°C, 30 seconds at the Tm temperatures of the specific oligonucleotide pairs and 30 seconds at 72°C for extension. Amplified sequences often consisted of 200-500 bp long sequences and these cycling parameters were kept similar for all different genes.

An aliquot of amplification products were separated on 2% agarose gels in TAE buffer. Gels were visualized under UV transilluminators and photogarphed.

2.3.2 Plant Total and mRNA isolation, RT-PCR and northern analysis

Plant total RNA isolation was performed according to acid phenol-guanidine thicyanide method of Chomczynski and Saachi, (1987). Typically, 500mg of plant tissues were homogenized into a fine powder and "solution D" was added to this homogenate. After the additions of 1/10 volume 2M NaOAc (pH=4), one volume of water equilibrated phenol and 1/5 volumes of chloroform; the homogenate was mixed well and kept on ice for 30 minutes. Debris was removed by centrifugation at 10000g for 30 minutes at 4°C. The aqueous phase was mixed with 1/3 volume of cold absolute ethanol to precipitate the RNA. Incubation on ice for 20 minutes followed by a centrifugation at 10000g for 20 minutes. The precipitate was once washed with 80% ethanol and then briefly air-dried. Further purification was performed by dissolving the pellet in TE buffer supplemented with 0.5%

SDS. After the addition of 150µg/ml Proteinase K, the tubes were kept at 52°C for 20 minutes. Degraded proteins and the enzyme was removed by a round of acidic phenol:chloroform (1v:1v) extraction. The RNA was finally precipitated at -20°C for 2 hours in the presence of 200 mM NaCl and 3 volumes of absolute ethanol. To remove residual salts, the pellet obtained from previous step washed 2 times with 80% ethanol and air-dried. Final pellet was dissolved in sterile distilled water and kept at -20°C until use. Spectrophotometric measurements and agarose gel electrophoresis, respectively, was used to determine the quantity and integrity of the RNA.

Reverse-Transcription mediated PCR was performed using 5 µg of total RNA. Prior to reverse transcription RNA was treated with a RNase free DNAse (Promega, Germany) for 20 minutes. Heat denatured samples (70°C for 15 minutes) were reverse transcribed with Superscript II reverse transcriptase (GIBCO-BRL, U.K) in the presence of 1µg of oligo dT primer, 1mM of dNTP mix, reaction buffer, 1 mM DTT and 200 U of the enzyme. The reaction was performed at 42°C for 50 minutes. The reaction was stopped and heating the tube to 96° C for 5 minutes inactivated the enzyme. PCR analysis was done with 400 ng of cDNA in a total volume of 50 µls where 0.2mM dNTPs, 20 pmols of each gene specific primers and 1 unit of Taq DNA polymerase (Pharmaicia) in PCR buffer provided. Cycling parameters were 85°C for 5 minutes 94°C for 2 minutes for one cycle and then 15-25 cycles (depending on the genes of interest) at 94°C for 20 seconds, 40 seconds at annealing temperature (50-65°C) and an extension at 72° C for 40 seconds. One third of the reactions were run in a agarose gel comprising 2% in TEB electrophoresis buffer. Denatured, neutralized and blotted by downward capillary action onto neutral nylon membranes (Hybond N, Amersham, England) o/n with either with 20xSSC or 1 M ammonium acetate.

All hybridizations were done under stringent conditions. Briefly, the filters were pre-hybridized with a buffer containing 250 mM Na-phosphate pH 7.2, 7% SDS, 10 mM EDTA pH 8 and 1% BSA. Radioactively labeled DNA probes prepared by random priming method using a commercial kit (multiprime, Amersham, U.K) were added into hybridization tubes after 2-4 hours for a further 16 hours at 65°C. The background and unincorporated radioactivity was carefully removed by successive washes with descending concentrations of salined sodium citrate solution (from 2XSSC-0.1 %SDS to 0.1XSSC-0.1%SDS) at 65°C. Then the filters were exposed to X-ray films (KODAK, safe film) in safe-cassettes at -70°C. Exposure times were different according to the strength of signals initially detected by a hand hold monitor but often varied between 24-96 hours.

2.3.3 Plant total protein isolation and western analysis

Plant tissues (100-500 mg) were homogenized in 4 volumes of a buffer consisting of 50 mM Tris pH=7.6, 0.5 % SDS, 0.1% NP-40 14mM ß-mercaptoethanol and 10mM PMSF. The

lysates were cleared by high speed centrifugation; typically 15 minutes in a microfuge and then protein concentrations were determined by Bradford (1976) dye-binding assays. Before loading protein samples onto 12% SDS-denaturing polyacrylamide gels, samples were immersed in boiling water for 5 minutes.

The SDS-denaturing polyacrylamide gel electrophoresis for size fractionation of the total proteins was performed according to standard protocols. The gels were prepared in 12.5% TRIS-Glycine buffer (25mM Tris, 192mM glycine, 0.1% SDS pH=8.3) and each sample (10-30µg protein) in sample buffer (supplemented with 10% glycerol, 0.01% bromophenol blue) was loaded. A low molecular weight marker (SIGMA, USA) and semi-purified whole MSV (400 ng) was also run along with the samples. For a 9x12 cm gel, 20mA of constant current applied until the dye reaches to the edges, typically in 4 hours. Before transfer of proteins onto nitrocellulose filters, the stacking gel was removed. Then the transfer of proteins onto nitrocellulose filters was done using an electroblotting apparatus (Bio-Rad, USA) in transfer buffer /10mM imidozole, 30 mM glycin-glycin, 20% methanol, 0.5% SDS) at a current of 2.5mA/cm^2 for 15-2 hours. After the transfer, the molecular weight marker lane was cut out and stained with 0.05% amidoblack in 20% methanol-5% acetic acid solution.

The filters were then blocked with a solution containing 5% milk powder, 0.02% sodium azide and 0.2% Tween 20 in standard phosphate buffered saline. Polyclonal antibody raised against whole MSV particles were diluted in (1:500) blocking solution and the filters were incubated at room temperature for 2 hours. Excess antibody was removed with brief washes in PBS prior to incubation with a secondary anti-rabbit alkaline phosphatase conjugated *IgG* (SIGMA) similarly diluted in blocking buffer at 1:8000. After an incubation for 2 hours, the filters were washed in PBS, equilibrated in alkaline phosphatase buffer (100mM NaCl, 5 mM $MgCl_2$, 100mM Tris, pH=9.5). Choromogenic reaction was initiated by the addition of nitro blue tetrazolium (66 µl from 5% stock) and 5-bromo-4-chloro-3-indolyl phosphate (33µl from 5% stock) in 10 ml of reaction buffer. The signals often appeared within 10-20 minutes and the reaction was terminated by washing the filter in 10mM EDTA solution.

2.3.4 Virus replication assay

Protoplast isolation and PEG mediated transformation of control and transgenic maize cell suspensions were done according to the protocol given above with slight modifications. Amount of DNA per treatment was increased to 15-20 µg per million protoplasts. At different

sampling times, protoplast cultures were collected by low speed centrifugation, the culture medium was removed and then the samples were briefly homogenized with a glass rod to a fine suspension. DNA isolation was performed with another method that ensures the recovery of single stranded DNA species as well. Briefly, the homogenate was mixed with a extraction buffer consists of 100mM Tris, 10mM EDTA and 200 mM NaCl, 0.5% SDS and this suspension was once extracted with phenol chloroform. The aqueous phase was carefully removed after centrifugation and the nucleic acids precipitated with 1/20 volumes of NaOAc and 2.5 volumes of absolute ethanol. The pellet was washed twice with 75% ethanol, blotted dry and re-suspended in TE buffer.

2.3.5 Fluorimetric and histochemical GUS enzyme assays

All protocols related to the enzyme activity of GUS was determined by protocols based on the work of Jefferson *et al.,*1987 and Jefferson, 1990 with modifications as explained by Gallagher ,1992.

For fluorimetric GUS assays, total proteins were extracted with a neutral phosphate buffer (50 mM sodium phosphate, 10 mM EDTA pH=7), supplemented with strong detergent and protease inhibitors (0.1%SDS, 14mM ß-mercaptoethanol). The protein concentrations were determined by Bradford dye-binding method and all samples were diluted to 0.10 mg protein per ml. Five to twenty micrograms of total protein was mixed with a fluorigenic substrate 4-Methyl-umberiferly-glucurunide (BioSynth AG, Switzerland) at 1 mM final concentration in homogenization buffer. Reactions were incubated at 37° C for 0, 1 and 4 hours where 0.2 M Na_2C0_3 was used to stop the reaction. Mini DNA fluorimeter TKO 100 with fixed excitation and emission wavelengths was used to quantitate the enzyme activity. The fluorimeter was calibrated with a 1nM solution of methyl umberiferron in water (Sigma).

During the studies with MSV-host interaction a luminometric GUS measurement kit (Tropix, U.S.A) was utilized due to its wide dynamic range. After cell homogenization, the extract is transferred to a microcentrifuge tube and briefly clarified by centrifugation. A small aliquot of extract (25 µl containing 20-50µgof protein) was transferred to a luminometer tube. Reaction buffer containing Glucuron® substrate was added and the reaction was incubated for 30 to 60 minutes at room temperature. After placing the sample in a luminometer, the light emission accelerator (100µl) was added with automatic injection devices and light emission was measured after a 10 second pause for 5 seconds. Data was stored as relative light units per µg protein used in each sample.

Histochemical GUS assays were done after brief fixation with 96% cold acetone in -20° C for one hour. The fixed tissues were then washed with 100mM Na-phosphate buffer pH=7.2 to remove residual acetone. The samples were than incubated after a brief vacuum infiltration in a phosphate buffer containing 1 mM chromogenic substrate; X-GluC-Na salt (BioSynt AG, Switzerland) at 37° C for 16 hours. The samples were than cleared and fixed in a solution consisted of chloral hydrate (2): lactic acid (1): phenol (1). For the staining of somatic embryos and callus pieces both the fixation and tissue clearing steps were omitted.

For evaluation and documentation samples were examined under the light microscope using dark-field and Nomarski phase contrast condenser options. Photos were taken using highly sensitive color reversal films (Kodak T1000) under artificial light.

2.3.6 Cell synchronization and flow cytometry

In order to synchronize the transgenic maize suspension cells at the border of G1/S phases, log phase cell suspension cultures were treated with hydroxyurea (10mM) for 36 hours at 8% cell density (fresh weight/volume). After extensive washes with fresh media (1 liter for each 200ml of suspension cells), the suspension was further grown in 20% conditioned media. Samples for RNA, flow cytometry and mitotic index were taken at 2 hours intervals for 21 hours. Alternatively, aphidicolin dissolved in DMSO was used at a final concentration of 8µg/ml under similar culture conditions. The mitotic phase inhibitor colchicine was dissolved in distilled water and used at 0.05% (w/v) final concentration for the time periods indicated.

Nuclei isolation and flow cytometry was performed according to Savouré *et al.*, 1993. Briefly, cells were burst in Galbraith buffer with the mechanical help of automatic pipettes, and then sieved through 25µm stell meshes to remove large pieces of cellular debris. After staining with propidium iodide (10µg/ml) 10-20 thousands of nuclei per time point was monitored with a Becton Dickinson *FacsCalibur* flow cytometer. Data were evaluated with the computer program ModFit™ and represented as the percentage of the cells in G1, S or G2 phases. Mitotic indices were determined by counting ~1000 cells fixed in Farmer's fixative (3:1 ethanol:acetic acid) at 4 C for 24 hours and then stained with 4',6-diamidino-2-phenylindol HCl, (DAPI). The mitotic cells versus interphase nuclei was determined under a fluorescent inverted microscope with appropriate UV band filters.

2.3.7 Immunocytochemistry and microscopy

For the determination of sub-cellular localization of MSV capsid protein in transgenic cells, first protoplasts were isolated as explained above. A shorter (2 hour) enzymatic removal of cell walls

was coupled with mild fixation of the protoplast on microscope slides with a buffer containing 0.3% formaldehyde in standard PBS buffer. The brief fixation was for one hour; then the slides were washed through PBS buffer (3 times 5 minute each) in joplin jars to remove excess formaldehyde and re-hydrate the fixed protoplasts. The slides were first dipped into blocking solution containing 3%BSA in PBS at room temperature for 30 minutes and then covered with the same solution but containing pre-absorbed (acetone washed crude control protein incubated with the MSV-CP antibody for 16 hours at 4-8ºC) polyclonal MSV antibody MSV3 at 1:200 dilution. The immuno-reaction was stopped by washes with PBS (3 times 10 minute each) after a hour. Secondary TRITC conjugated anti-rabbit rat IgG antibody (Southern Biotechnologies, USA) was used as a secondary antibody in blocking buffer at 1:2000 dilution for one hour. After the washing out excess unbound antibody, the slides were mounted with 50 glycerol in 20mM TRIS buffer pH=6.8 and kept in dark until microscopic observation.

The slides were examined in epi-fluoresence microscope, Olympus, BH-2, (Japan) and microphotographs were taken using the attached camera at appropriate magnifications onto negative color films optimized for artificial light.

"De lo que veas, cree muy poco, De lo que te cuenten, nada"

Of what you see, believe very little, Of what you are told, nothing.

Spanish proverb

CHAPTER III

RESULTS

3.1 Functional analysis of wheat histone H4 promoter in transgenic maize plants

In this study, I have analyzed the expression of wheat histone H4 promoter in transgenic plants carrying GUS reporter gene under the 720 bp long 5' regulatory sequence from the genomic DNA clone TH011 (Tabata, 1983). Detailed sequence analysis of this genomic DNA clone, as well as its closely related, well studied counterparts from other higher plants revealed the presence of conserved motifs responsible from tissue specificity, responses to external stimuli, and replication-dependent activity. In order to confirm the putative roles of these DNA sequence motifs and compare transcriptional control mechanisms of cereals we have produced several independent transformants. Construction of transformation plasmids, genetic transformation and recovery of these putative clones is a subject of another Ph.D. thesis (Omirulleh, S 1993, JATE) and will not be included in this manuscript. Thus, this study concentrates on functional analysis of wheat histone regulatory sequences, elucidation of cell cycle associated expression of histones and other cell cycle related genes in maize, and utilization of these transgenic plants as indicators of G1-S phase progression upon external stimuli.

3.1.1 Molecular characterisation of putative transformants

Protoplast transformation was performed using plasmids pH4G, pTH2G and p35SAc (Figure 1) as described in Omirulleh, 1993.

Putative transgenic maize clones carrying wheat histoneH4::GUS fusions were selected and further grown for regeneration into full fertile plants. After selection on 0.5mM L-PPT, somatic

embryogenesis was induced by the removal of 2,4-D from the culture medium and plantlets were subsequently obtained in glass tissue culture jars (Figure 2).

The putative transformants were first screened by genomic DNA PCR with oligonucleotide primers specific for the GUS coding sequence (PgusF and PgusR, appendix A). As shown in figure 3A, the presence of the transgene was confirmed in several independent callus lines using oligonucleotide primers specific for *E.coli* β-glucuronidase gene amplifying a 1200bp internal fragment.

To demonstrate the relative levels of transgene mRNAs in different clones northern blot hybridization was performed. Northern blot analysis of these clones also indicated the presence and expression of the transgene in correct order as judged by transcript size (Figure 3B).

Several independent transgenic clones were chosen and further characterized. In the selected clones, integration of the GUS gene into high molecular weight plant DNA was confirmed by Southern hybridization (Figure 4).

The reporter gene activity was further evaluated by quantitative and qualitative tests for ß-glucuronidase. Fluorimetric assay and histochemical GUS staining was used to confirm the reporter gene activity in tested clones. Quantitative assessment of GUS enzyme activities of the different calli derived from independent transformation events are given in Figure 5. Here, the activity of wheat histone H4 promoter was briefly compared with a transgenic maize line KM10 carrying minimal CaMV 35S promoter (-46 t0 +8) and two *cis*-acting enhancer elements (-208 to -56) from the same viral regulatory sequence. The histone H4 promoter exhibited higher GUS activity than the CaMV 35S promoter in the callus tissues of different transformants. The transgenic clone TH2, carrying the GUS gene under the control of wheat histone H4 promoter and wheat histone H3 terminator exhibited 6-10 fold higher activity as compared to the other clones with the same promoter but NOS termination signal. The relative amount of GUS mRNA from the clone TH2 however, was only 2-4 fold higher than in the case of other transformants.

The observed pattern of signal intensity in northern analysis was compared with the quantitative GUS enzyme measurements and found to be in good correlation.

3.1.2 Wheat histone H4 promoter is preferentially expressed in proliferating cells and meristematic tissues

In order to reveal tissue specific regulation of the histone H4 promoter, reporter enzyme and transgene mRNA expression patterns of different tissues, both proliferating and differentiated from selected transgenic clones, were performed. The histone promoter activity in cell suspensions,

embryogenic calli and differentiated tissues such as roots, leaves and shoot apices composed of both quiescent and dividing cells, was analyzed by northern hybridization and GUS enzyme assays. Combined results of fluorimetric, and histochemical analysis clearly demonstrated the meristem specific activity of the wheat histone H4 promoter in proliferating tissues composed of actively dividing cells (Figure 6B, 6C, Figure 7). After histochemical GUS activity staining of embryo forming calli exhibited a range of reporter activity (Figure 6A). The activity of the promoter was observed in relatively young tissues, basal parts of the leaves and shoot apices consisting of differentiating meristematic cells. Distal parts of the leaves were found to have low levels of the GUS reporter protein. In agreement with fluorimetric quantitation of GUS activity, RT-PCR analysis of GUS expression at the distal and basal parts of young leaves showed significant differences in favour of the basal regions with higher activity (Figure 7A and B). These results were coherent with previous reports on proliferation and differentiation of maize tissues and mitotic, as well as cyclin dependent kinase activities exhibited in the used tissues (see discussion).

Histochemical GUS assays were also performed on different tissues of the transgenic lines, particularly on the roots that consist of both fully differentiated and differentiating tissues. Similar to the results of fluorimetric assays, GUS activity was clustered in meristematic tissues, root tips, emerging lateral root buds and vascular tissues (Figure 12, left column) . I was not able detect any GUS staining in mature leaves or pollen that might exhibit basal replication independent activity.

3.1.3 Expression of wheat histone H4 promoter driven GUS and other cell cycle associated genes in synchronized maize cell suspensions

First, I have examined the GUS reporter enzyme activity in freshly isolated protoplast from transgenic maize suspension cells and monitored the kinetics for 7 days. The fluorimetric assay revealed a sustained moderate level of reporter enzyme activity in the first 3 days of *in vitro* culture. On the 4th day, the activity dropped to a low level and then increased for the next 3 days reaching the highest at the end of 7 days (Figure 8).

Alternatively, I have treated transgenic suspension cells with cell cycle blockers and monitored the mRNA accumulation for an almost full division cycle after release from the block. After treatment of transgenic cell suspensions either with hydroxyurea, aphidicolin or colchicine, I have examined mRNA accumulation by northern analysis in two independent transgenic clones (Figure 9). The GUS mRNA accumulated when grown in the presence of two G1-S phase blockers, (20 µg/ml aphidicolin or 10 mM hydroxyurea) while the addition of 0.05% colchicine in the medium diminished its presence; thus indicating cell cycle and S-phase-dependent activity of the promoter.

In further experiments I released the cells from the S-phase block and followed the cell cycle progression taking samples at 2-3 hour intervals for 21 hours. I monitored the cell cycle progression by flow cytometry along with the expression of the GUS and various cell cycle marker genes by northern analysis. Mitotic indices were also determined at each time point. Figure 10, shows the results of flow cytometry, mitotic index and northern blot hybridization. After the release of the hydroxyurea block, within four hours, the expression of the wheat histone H4 promoter driven GUS reporter gene reached to a peak within the first 3 hours and than gradually decreased after 8 - 10 hours with a parallel increase in the accumulation of the mitotic cyclin *CycB1;zm;1* mRNA. The histone *H2B* and histone *H4* genes were highly expressed right after the removal of inhibitors (hydroxyurea or aphidicolin) blocking near the G1/S phase border, and their expression gradually declined as the cells progressed into mitosis. The mitotic index have reached a peak after 15 hours when ~14 % of the cells were in mitosis while the expression of GUS and histone H4 gene decreased to lowest levels during the synchrony.

The *PCNA* gene had a more sharp increase in expression probably towards the end of the S-phase in comparison with the histone and GUS mRNAs already present at a relatively high concentration right after the release from the block. Expression of the maize B type cyclin *CycB1;zm;1* gene, as a putative mitotic cyclin was also monitored. As predicted, its mRNA level was elevated at time points where high percentage of cells reached mitosis. The level of maize glutathione reductase (G. Creissen, unpublished) mRNA was monitored as a control, with cell cycle-independent expression during synchronous growth of cell suspensions. The mRNA of maize glutathione reductase exhibited a sustained level of transcript accumulation during the cell synchronization.

Aphidicolin as an inhibitor of DNA polymerase has exhibited similar pattern for both GUS and maize histone H2B but their transcript accumulated earlier than in hydroxyurea suggesting a difference in timing and/or mode of action (Figure 11).

3.1.4 Hormones modify the activity of histone promoter

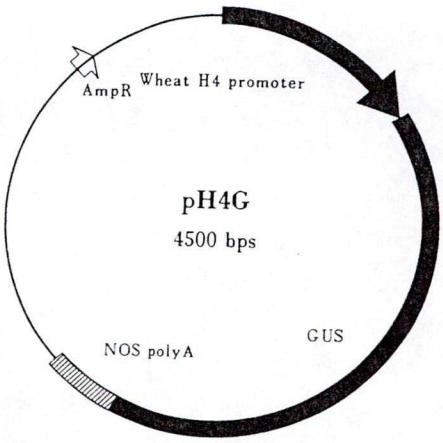
The activity of wheat histone H4 promoter was further studied in hormone treated transgenic maize (Figure 12). For this study, root segments from primary root tip and secondary lateral roots of transgenic clones were used. For RT-PCR analysis, in order to avoid interference of signals clustered in close alignments root tips were not processed. Mostly the upper regions of roots which are prone to produce lateral roots were treated with different plant growth regulators; such as 2,4-D and zeatin, at concentrations known to drive callus growth of maize callus/suspension.

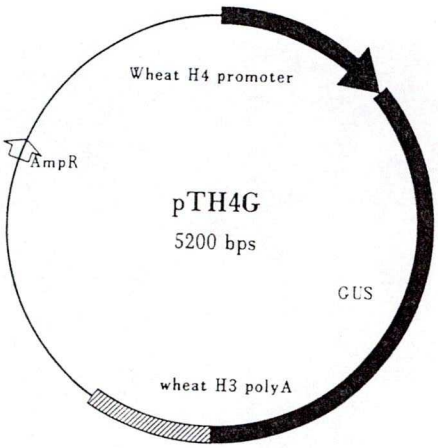
Figure 1

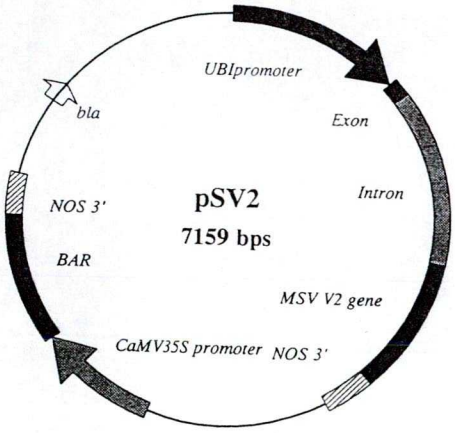
Schematic drawings of plasmids used in production of stable and transient transformation of maize cells.

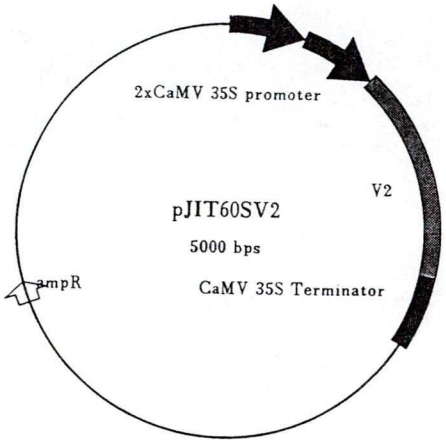
The construction details are given in chapter III, materials and methods.

pH4G	wheat histone H4 promoter::GUS::NOS terminator
pTHG2	wheat histone H4 promoter::GUS::wheat histone H3' terminator
pJIT60SV2	CaMV 35S promoters (2X)::MSV-V2::NOS terminator
pSV2	maize ubiquitin promoter::MSV-V2::NOS terminator
pREP	maize ubiquitin promoter::MSV-C1:C2::NOS terminator


AmpR
Wheat H4 promoter
pH4G
4500 bps
NOS polyA
GUS


Wheat H4 promoter
AmpR
pTH4G
5200 bps
GUS
wheat H3 polyA


UBIpromoter
bla
Exon
NOS 3'
pSV2
7159 bps
Intron
BAR
MSV V2 gene
CaMV35S promoter
NOS 3'


2xCaMV 35S promoter
V2
pJIT60SV2
5000 bps
ampR
CaMV 35S Terminator

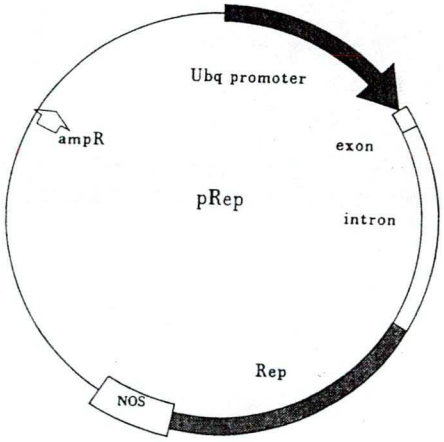

Ubq promoter
ampR
exon
pRep
intron
Rep
NOS

Figure 2

Selection and *in vitro* regeneration of transgenic maize plants

A) Selection of putative transformants on *L*-phosphinotricine (0.5 mM).

Control and herbicide resistant colonies appeared 8-10 weeks after PEG-mediated protoplast transformation.

B) Shoot formation in embryogenic calli recovered from protoplast-derived PPT resistant colonies

C) *in vitro* culture forming full organ differentiated plantlets

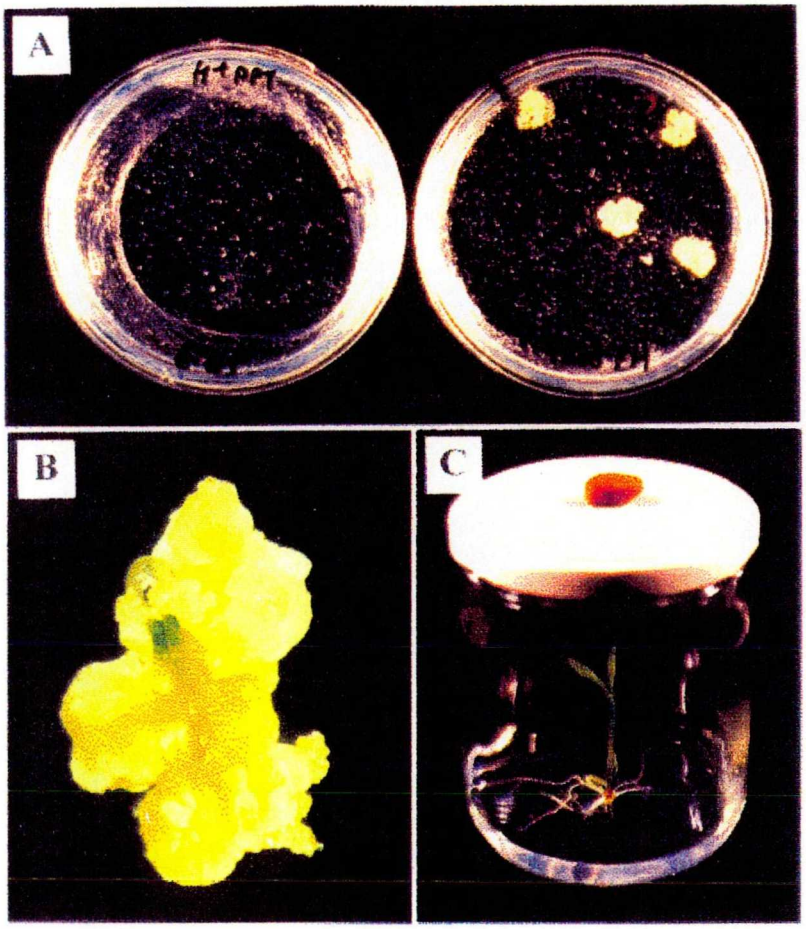

A
B
C

Figure 3

Molecular characterisation of putative transgenic maize lines for plasmid DNA integration, mRNA expression and reporter enzyme activity.

A) Southern blot analysis of maize genomic DNA after PCR amplification of GUS sequences. Number of PCR cycles was restricted to 15 to reflect the transgene copy number differences between different clones. 1, untransformed control, 2-8 different clones. Clone TH2 derived from a similar plasmid vector but carries wheat histone H3 3'UTR instead of NOS termination signal. KM10 carries CaMV 35S promoter elements.

B) Northern blot analysis of selected clones. Each lane consist of 10µg of total RNA hybridized against the coding region of GUS gene from the plant expression vector plasmid pH4G,. The signal appeared at expected mRNA size, ~1890 bp.

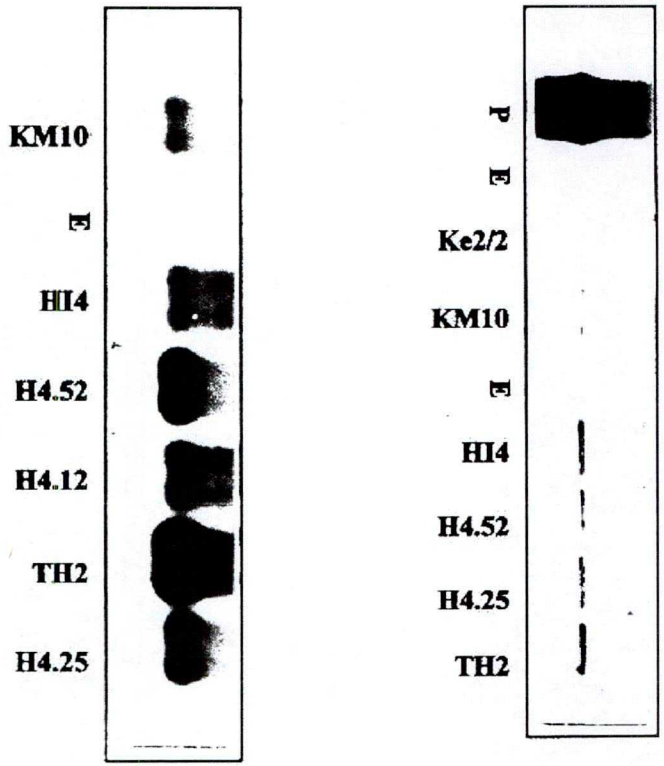
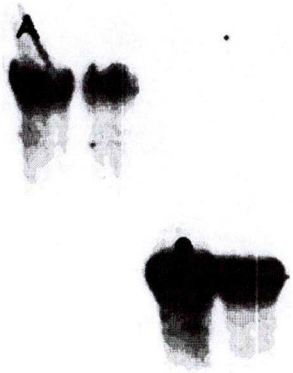

KM10
E
HI4
H4.52
H4.12
TH2
H4.25
P
E
Ke2/2
KM10
E
HI4
H4.52
H4.25
TH2

Figure 4

Southern blot analysis of transgenic maize clones H4.25 (lanes 1, 3) and H4.52 (lanes 2, 4).

First two lanes are uncut-high molecular weight DNA from transgenic maize plants. Lanes 3-4 are 6µg genomic DNA digested with restriction endonucleases *EcoRI* and *HindIII* releasing coding region of GUS gene from the plant expresion vector plasmid pH4G, ~1890 bp.



1 2 3 4

Figure 5

Fluorimetric GUS assay of different putative transgenic maize callus lines one week after subculturing.

For each clone replicate readings of 3 independent measurements were averaged. The clone TH2 carries a wheat histone H3 3'UTR sequence instead of NOS termination signal. KM10 is a transgenic line where GUS gene is driven by a minimal CaMV 35S promoter and 2 enhancers from the same viral regulatory region.

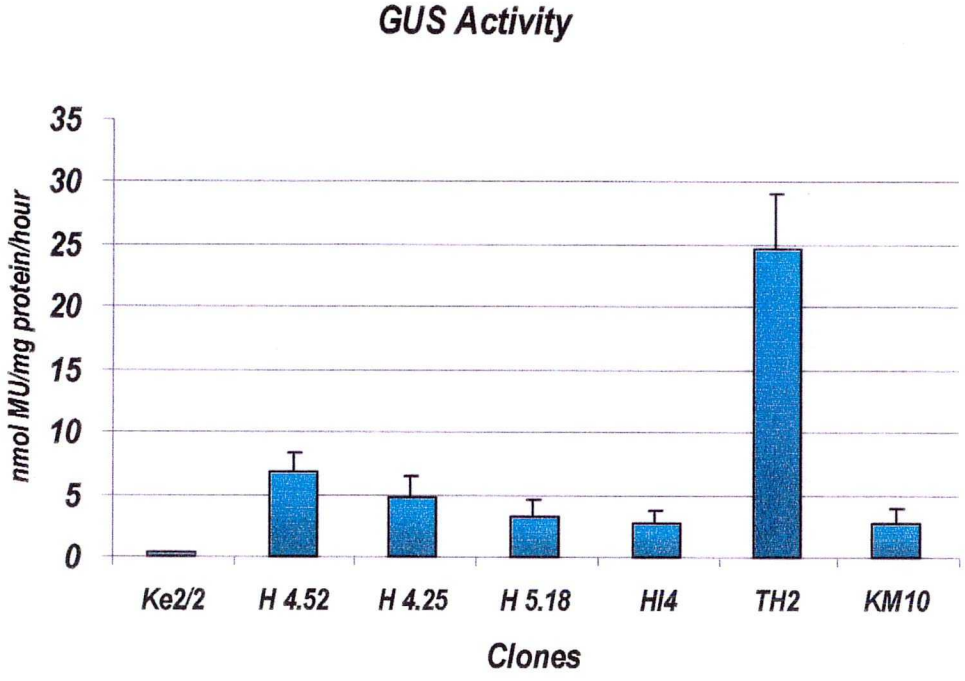

GUS Activity
nmol MU/mg protein/hour
35
30
25
20
15
10
5
0
Ke2/2
H 4.52
H 4.25
H 5.18
HI4
TH2
KM10
Clones

Figure 6

Histochemical GUS analysis of putative transgenic maize lines

A) Histochemical GUS staining of callus tissues from selected clones

Upper lane from left; HE/89, H5.18, HI4, KM10

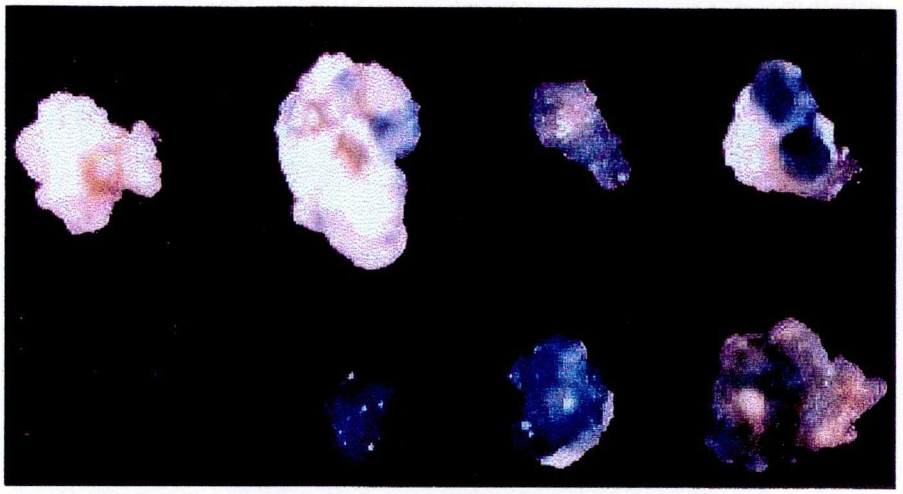
Lower lane from left; TH2G, H4.52, H4.25, H4.12

Histochemical GUS staining of somatic embryos during shoot formation

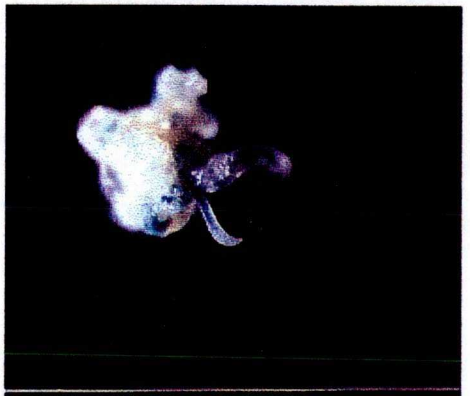
B) H4.25

C) H4.52

A



B



C

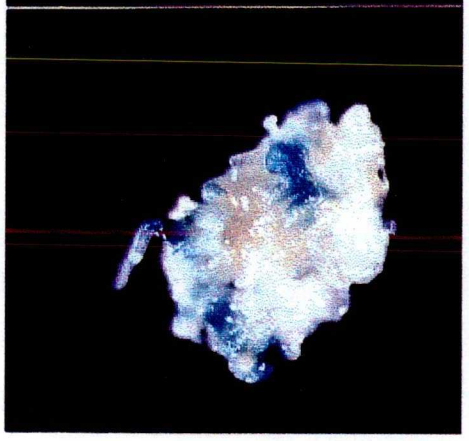


Figure 7

Expression of GUS reporter gene in different tissues of transgenic maize plants

A) Fluorimetric GUS assay of different tissue parts. Leaf tissues and shoot apical meristems (SAM) of two independent transgenic clones (1:clone H4.25; 2: clone H4.52) were tested. Leaf base was harvested from the first one cm part of the leaf whereas leaf tips upper one cm of the transgenic leaves. Each bar represents mean values of four independent measurements of GUS activity at the indicated regions of tissues (five leaf stage). Error bars represent standard variation from the mean values.

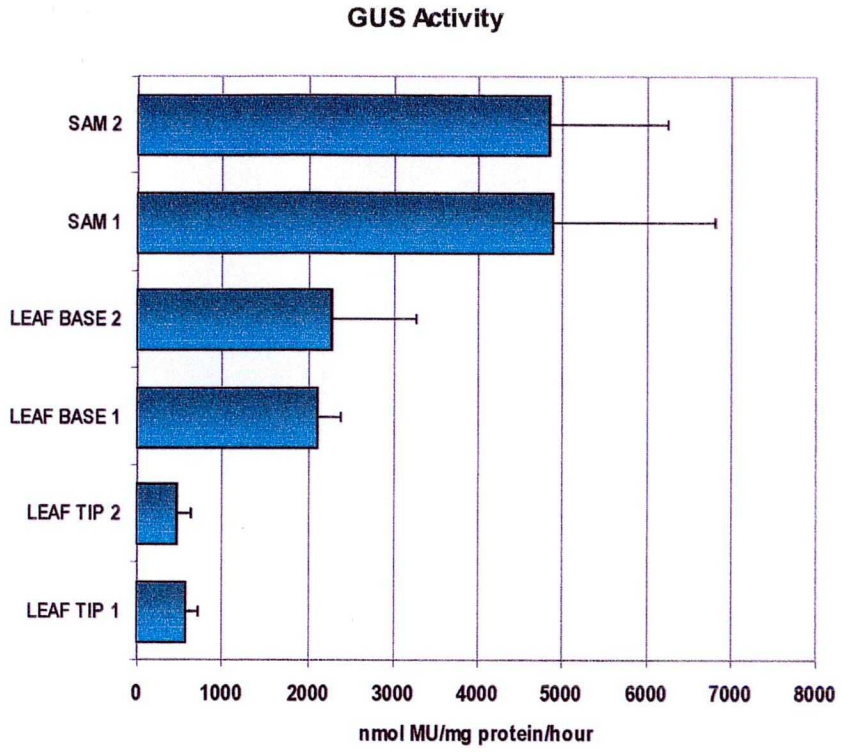
B) RT-PCR analysis of GUS reporter mRNA expression in different tissue parts.

Lane 1: Shoot apical meristem, (SAM)

Lane 2: Leaf base

Lane 3: Leaf tip

A


GUS Activity
SAM 2
SAM 1
LEAF BASE 2
LEAF BASE 1
LEAF TIP 2
LEAF TIP 1
0
1000
2000
3000
4000
5000
6000
7000
8000
nmol MU/mg protein/hour

B

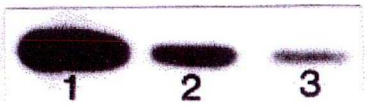

1
2
3

Figure 8

GUS activity in protoplast derived cells.

Daily monitored GUS reporter enzyme activity in transgenic maize protoplast of clone H4.52. Fluorimetric measurements of protoplast cultures sampled at daily intervals for seven days and measured. Means of replicate readings of four independent experiments are given.

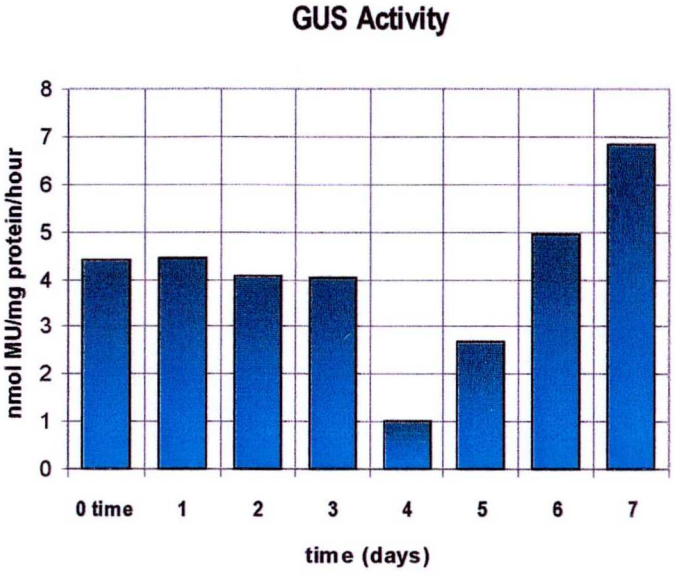

GUS Activity
nmol MU/mg protein/hour
8
7
6
5
4
3
2
1
0
0 time
1
2
3
4
5
6
7
time (days)

Figure 9

Transgene mRNA accumulation in cultured suspension cells with cell cycle inhibitors.

Northern blot analysis was performed with clones H4.52 (A) and H4.25 (B) treated for 24 hours with inhibitors. Twenty µg of total RNA was hybridized against GUS probe.

Lane 1: untreated control

Lane 2: 0.05% (w/v) colchicine

Lane3: 10mM hydroxyurea

Lane 4: 20µg/ml aphidicolin .

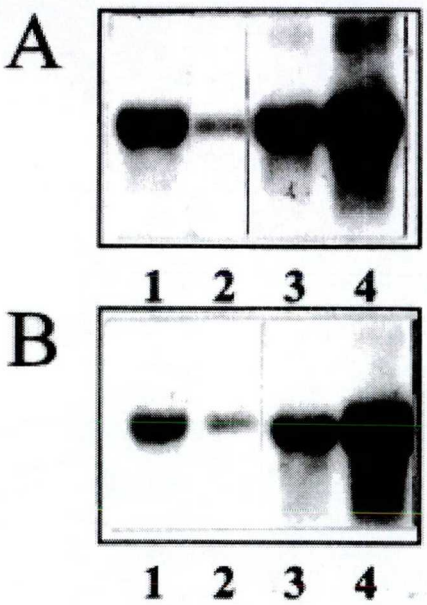

A
1 2 3 4
B
1 2 3 4

Figure 10

Flow cytometry, mitotic index and northern analysis of in partially synchronized maize cells of clone 4.52

A) Kinetics of DNA synthesis monitored by flow cytometry. Nuclei isolated at different time points were analyzed for DNA content. Mitotic index is also indicated on the upper x-axis of the bar graph.

B) The mRNA expression patterns of GUS reporter gene driven by the wheat histone H4 promoter and other cell cycle associated genes.

a) Proliferating cell nuclear antigen (PCNA)
b) GUS
c) histone H4
d) Zeama;*CycB2*;1
e) Glutathione reductase (control)

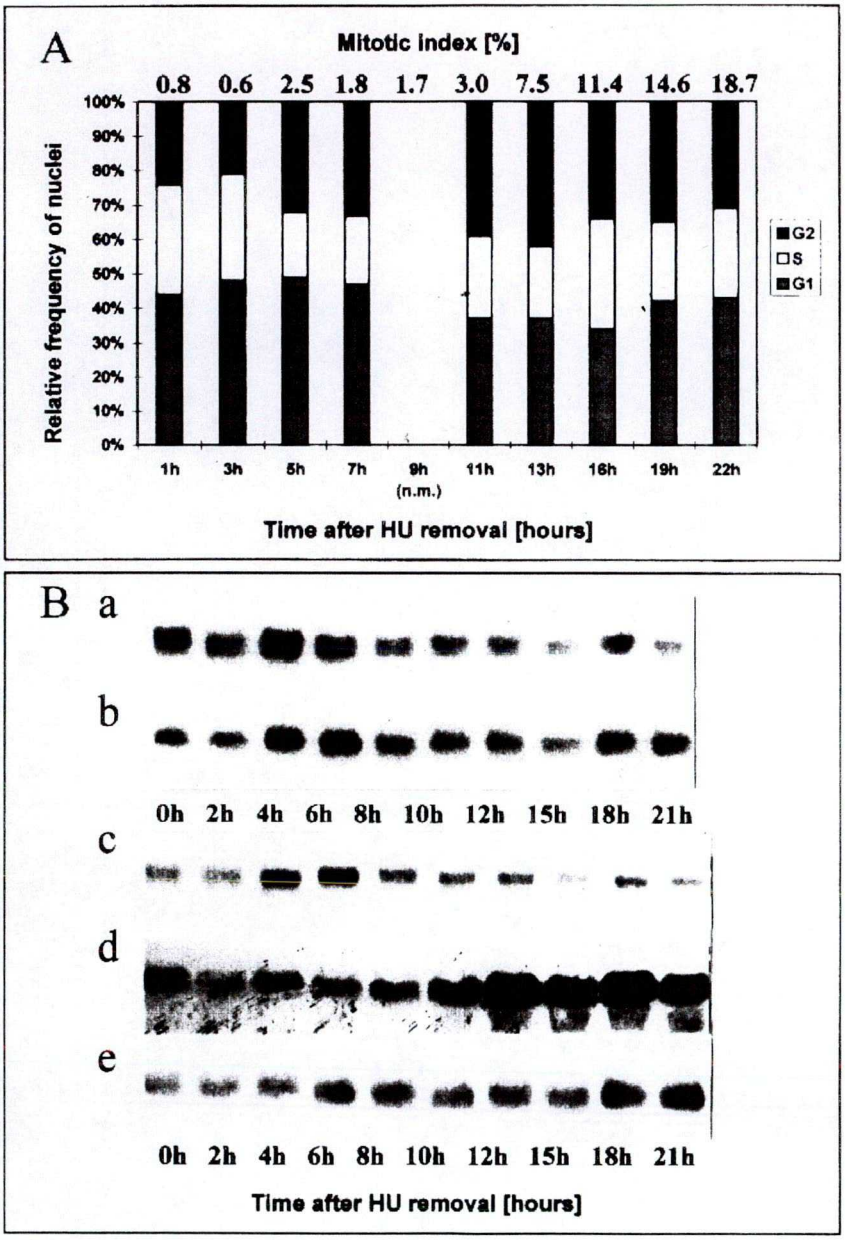

A
Mitotic index [%]
0.8 0.6 2.5 1.8 1.7 3.0 7.5 11.4 14.6 18.7
Relative frequency of nuclei
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
G2
S
G1
1h 3h 5h 7h 9h (n.m.) 11h 13h 16h 19h 22h
Time after HU removal [hours]
B
a
b
0h 2h 4h 6h 8h 10h 12h 15h 18h 21h
c
d
e
0h 2h 4h 6h 8h 10h 12h 15h 18h 21h
Time after HU removal [hours]

Figure 11

Northern analysis of GUS reporter and histone *H2B* mRNA of the clone H4.25 in partially synchronised cells with 10 µg aphidicolin for 30 hours. Samples were taken at indicated time points for 18 hours after washing out the aphidicolin in the culture medium (0 hr). Twenty µg of total RNA was loaded onto each lane and blots were hybridized by indicated gene specific probes.

A) GUS

B) Maize histone H2B

C) Maize glutathione reductase gene (control for cell cycle independent expression)

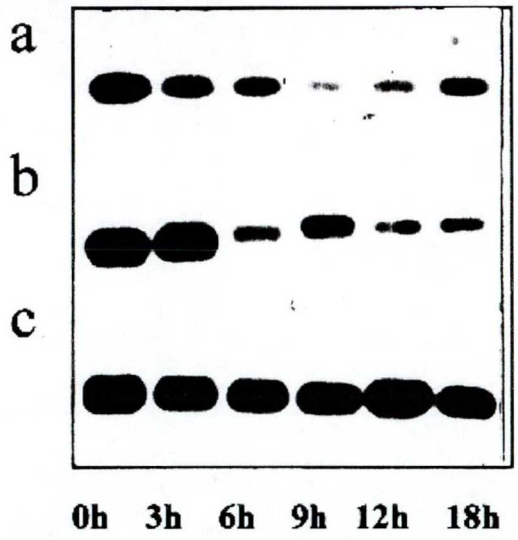

a
b
c
0h
3h
6h
9h
12h
18h

Figure 12

Analysis of phytohormones on wheat histone H4 promoter activity in transgenic maize plants by histochemical GUS assays and RT-PCR.

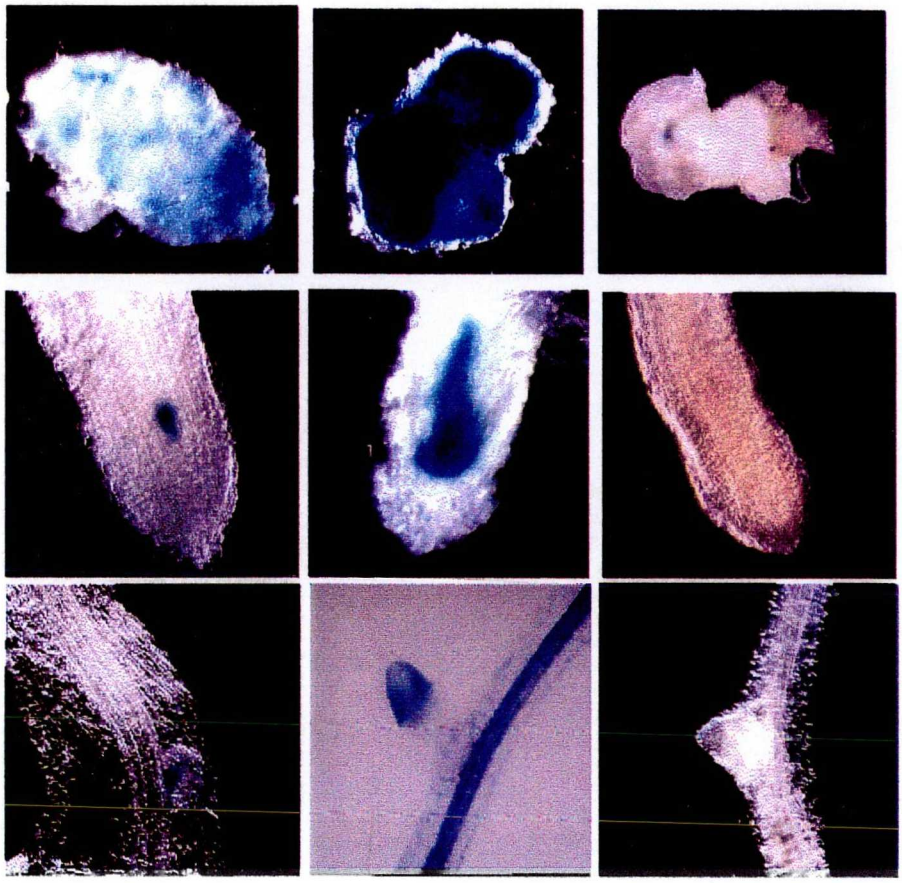
The tissues were treated with phytohormones 2,4-D at 1μM, ABA at 10μM and zeatin at 50 μM for 48 hours prior to histochemical GUS assays.

A) Histochemical GUS assays with callus, primary root tip and secondary (lateral) root tissues of clone H4.52 with and without (control) hormone treatment.

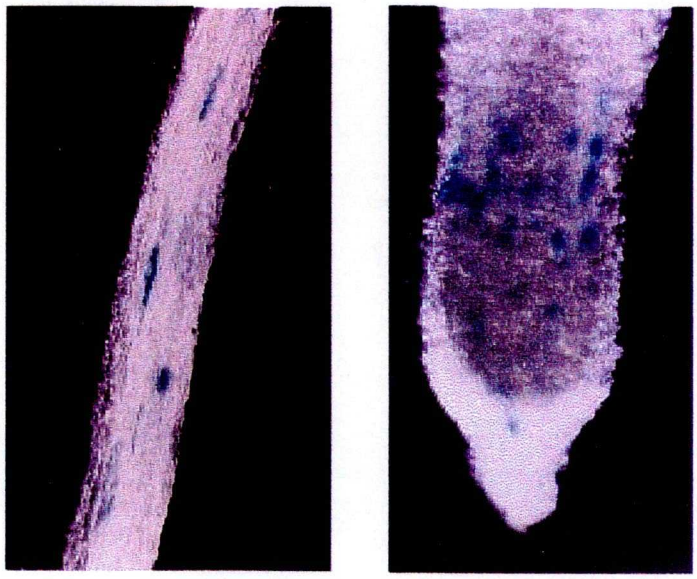
B) Histochemical GUS assays on root tip and upper root segments treated with 50μM zeatin (mixed isomer)

C) Histochemical GUS assays on whole plants where individual roots of the same plantlet immersed in two different hormones as indicated above. Upper panel demonstrates the transcript levels of GUS reporter mRNA upon hormone treatment obtained by RT-PCR analysis for from the same experiment. Lower panel shows roots from the same planlet immersed into different hormones from the same RT-PCR experiment.

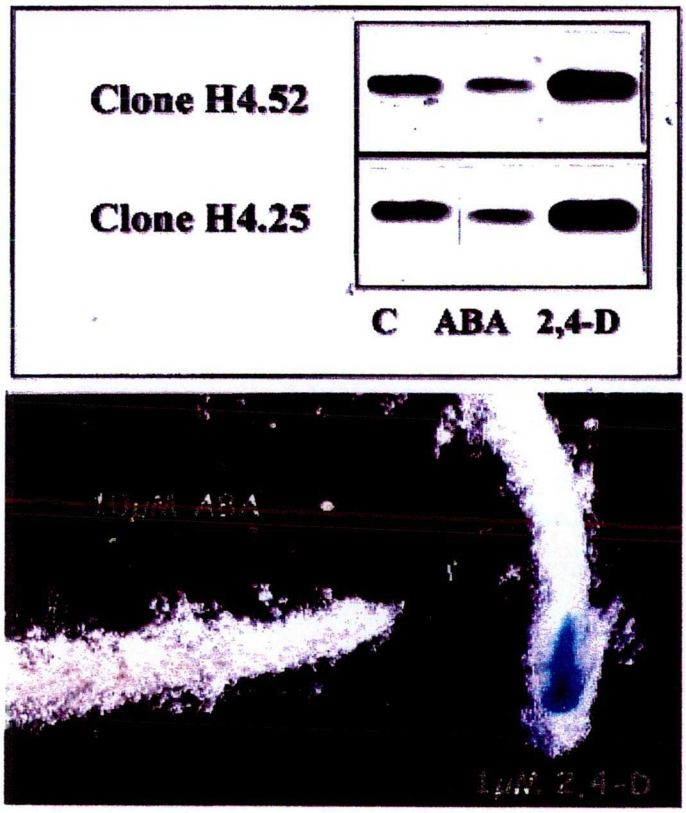
A



B



C



Transgenic plant tissues, root segments or embryogenic calli were fully dipped into medium semi-solidified with 0.1% gelrite and incubated in the presence of plant growth regulators for 48 hours. These explants and calli were previously maintained on hormone free medium for at least two weeks. In the culture medium, 2,4-D at 1 µM or zeatin was used at 50µM final concentration. In addition to these, abscisic acid also used as a stress related factor at 10µM final concentration. The response of histone promoter was determined at conditions that permit cell division within the normal physiological conditions for *in vitro* cultures. The histochemical assays were performed after 48 hours of treatment in different hormones (Figure 12 A). For 2,4-D and ABA, the activity of the histone H4 promoter was also evaluated by RT-PCR. Figure 12 C, shows the results of RT-PCR analysis of different hormone treatments; where 2,4-D significantly increased promoter activity. The reporter transcript diminished from the ABA treated root tissues at 10µM concentration. Here, histochemical staning was done one two root tips of the same plantlet that immersed into two different hormone containing medium.

Histochemical assays also revealed a similar pattern, confirming the result of RT-PCR analysis. Embryogenic calli showed intense staining pattern in auxin treated samples. In ABA treated cells there was no staining (Figure 12 A, right panel). The ABA treatment totally diminished the GUS reporter enzyme activity in root tips, lateral roots and embryogenic calli. The reduction in GUS expression showed that the lateral root buds responded to the ABA signal earlier than the vascular tissues at 24 hours. Later on, vascular tissues were also lacking GUS activity as detected at 48 hours.

In intact, differentiated root segments, 2-4 D (1µM) treatment increased the zone of activity in root meristems on and around the vascular tissues. Embryogenic calli gave similar responses to hormone treatments.

Zeatin induced a patchy staining pattern at upper meristems of primary roots, above the most active zone indicating that the promoter was active in tiny clusters over the root segments (Figure 12 B). The number of active spots were even less on upper fully differentiated root tissues.

3.2 Expression of viral sequences in transgenic plants for protection against MSV

The objective of this project was to produce transgenic maize plants expressing viral sequences from MSV and test these plants for resistance against the virus.

3.2.1 Production and molecular characterization of transgenic lines expressing MSV-CP

A protoplast transformation scheme based on embryogenic HE/89 maize cell suspension was utilized to stably transform and express MSV- CP sequences in maize plants.

In the first set of experiments, the transformation work was first carried out using the plant expression vector pJIT60, which carries a doubled CaMV 35S promoter sequence. The 814-bp long V2 gene coding for the MSV capsid protein was inserted into the EcoRI site of pJIT60 to make pJIT60SV2 (Figure 1). The gene transfer technology was based either on PEG mediated co-transformation of the above plasmid and another vector pNA2G, carrying kanamycin (NPT II) selection marker genes under the control of CaMV 35S promoter (Omirulleh, 1993). The transformation vectors constructed in JII, Norwich were used for PEG-mediated direct DNA uptake into maize embryogenic protoplasts. The putative transformants were identified on the basis of kanamycin (150µg/ml) resistance and further regenerated for analysis.

Putative transformants carrying the virus capsid gene sequence were first identified by PCR amplification of the coat protein gene region in callus lines. Genomic DNA (100-500 ng) was used for PCR experiments in the presence of MSV-specific primers V816 and V817. As a positive control either a full length of MSV (plasmid BH-10, 10ng/PCR reaction) or crude extracts of MSV infected leaf tissues were used. A 325 bp long coat protein fragment was amplified after 30 cycles of PCR.(20 sec at 94 °C, 30 sec at 45 °C and 30 sec at 72 °C). Figure 13A, shows the result of PCR analyses. Several of the selected plants were further characterized for DNA integration, mRNA expression and protein accumulation. Two of these transformants were further investigated, as other lines were either only carrying the selectable marker gene or show poor *in vitro* regeneration (Figure 13B, C,D). The analysis indicated the integration and expression of the transgene in two lines. Southern, northern and western blotting analysis of cp6 and cp8 confirmed the integration and expression of the transgene in these clones. The genomic DNA was first restricted by EcoRI to release the V2 coding sequence, blotted onto membrane and hybridized against a PCR amplified 320 bp long DNA fragment. The correct size of a signal at 813 was detected in the blot. Same clones were tested for mRNA accumulation in northern blots hybridized against full length of V2 gene. The result of northern was confirmed with 3'RACE analysis (Figure 13D). Using polyclonal antibodies raised against the MSV-CP, the transgene product was detected in callus tissues of cp8 and cp6. (Figure 13 E, F). The level of CP was found to accumulate more in callus tissues than in leaves.

Selected plantlets were first transferred onto pith-discs to induce root formation and than potted into soil mixtures for further growth and seed set at the greenhouse.

The transgenic clone CP8 was then tested for MSV replication in a transient protoplast replication assay (Figure 17A). Protoplasts from suspension cells of cp8, were transfected with BH10, possessing a copy of MSV genome, by PEG mediated-direct DNA uptake. Accumulation of viral DNA replicative forms were detected 14 days post-transfection, indicating that the transgene did not interfere with the viral replication and ssDNA formation. Several plants were also tested in virus transmission test utilizing insects as vectors. Transgenic plants developed typical diseases symptoms, as wide streaks over the leaves with a 4-7 day delay with respect to the control MSV-susceptible maize seedlings of *Golden Bantham.*

As the primary goal was to produce plants with significant capsid protein mRNA and accumulation the experiments using the pJIT60SV2 vector and kanamycin selection system was terminated. For the second part of transformation experiments MSV-V2 gene coding the capsid protein was put under the control of maize ubiquitine promoter in plant expression vector pAHC25. The selection of transformants was facilitated by the presence of a synthetic phosphinotricine acetyl transferase gene on the same plasmid vector, again driven by the same promoter. The putative transformants, hereafter will be named as SV_2, were selected on the bases of their growth on 0.5mM *L*-PPT; active ingredient of the herbicide BASTA®. Figure 14 illustrates steps taken during selection and regeneration of SV_2 plants.

Selected clones were screened by genomic DNA-PCR (Figure 15A, B). The DNA isolated from these clones was both tested for presence of the capsid protein gene in PCR with primers (V2F-V2R) and the selectable marker (patF-patR). Both genes were found to co-exist in selected clones.

The mRNA accumulations of these transgenic lines were performed using standard protocols for northern blotting as well as RT-PCR and 3'RACE (Figure 15C, D and E). Most of the selected clones exhibited significant accumulation of transgene (MSV-CP) mRNA.

After this primary screen, Southern analysis of selected clones were performed using on uncut genomic DNA (Figure 16A), with a restriction endonuclease SacI or EcoRI that cuts at 3' or 5' of the transcriptional units (Figure 16C) and with Hind III that releases the transgene (Figure 16B). The analysis showed the integration of the transformation vector into high molecular weight genomic DNA as well as its position dispersed at several sites in the target genome with an average of 1-5 copies.

These results were also confirmed with genomic DNA -PCR where the number of cycles was restricted to remain at the logarithmic phase of the amplification (12 cycles). The signals were

Figure 13

Molecular analysis of transgenic maize lines produced by co-transformation of selectable marker gene and CaMV 35S promoter driven MSV-V2 gene into maize protoplasts. Selected calli were tested with genomic PCR, Southern, northern and western blottings:

A) Genomic DNA PCR for the CaMV 35S promoter region. A PCR fragment of 310 bp was amplified from kanamycin-resistant protoplast derived cells. First lane is a positive control containing a mixture of non-transformed maize DNA and 10 ng of the plasmid p35SAc. Lanes 2-6 are different putative MSV-CP transgenic maize lines. Lane 7 contains DNA from non-transformed maize calli.

B) Southern blot analysis of genomic DNA digested with EcoRI that releases the V2 gene from the plant expression vector, pJIT60SV2. Hybridization was performed with radioactively labelled full length V2 gene excised with the same enzyme from the plant transformation vector. Lane 1-4 putative transformants; Lane 5 plasmid (100 pg) DNA.

D) Expression of the transgene in two clones CP6 and CP8 as detected by 3'RACE PCR. Lane one contains cDNA from MSV infected plants. Other lanes: Lane 1: MSV infected leaf, lane 2: clone CP, lane 3: clone CP8

E) Northern analysis of the same two clones, CP6 and CP8. Twenty µg of total RNA was loaded onto denaturing gels and filter was hybridized with full-length MSV-V2 gene.

F) Western analysis of transgene product, the MSV capsid protein in two transgenic maize lines. Immunoblots were prepared as described in Chapter II, Materials and Methods.

Lane 1: control (non-transformed) maize Lane 2: clone CP6

Lane 3: clone CP4 Lane 4: clone CP8

G) Western analysis of transgene product, the MSV capsid protein in two different tissues of the clone CP8. Lane 1: Control (non-transformed) Lane 2: Callus tissue of CP8 Lane 3: Leaf tissue of CP8

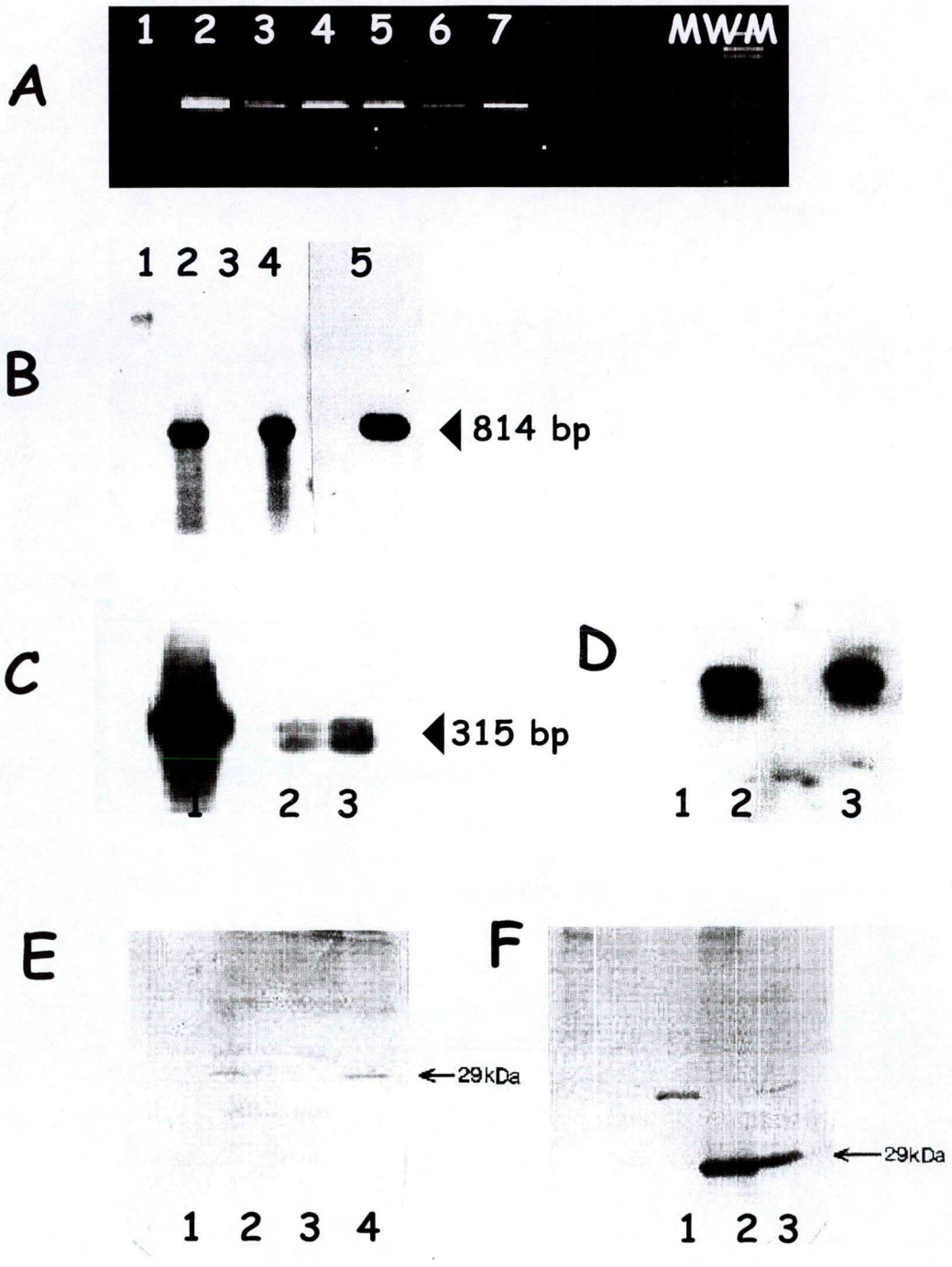

A
1 2 3 4 5 6 7 MWM
B
1 2 3 4 5
814 bp
C
1 2 3
315 bp
D
1 2 3
E
29kDa
1 2 3 4
F
29kDa
1 2 3

Figure 14

Selection and *in vitro* plant regeneration of SV_2 lines

A) Selection of putative transgenic lines on 0.5mM L-PPT

1: control (non-transgenic for PAT gene)

2: putative transformants with plasmid pSV2 (Ubi::V2::NOS—p35SAc)

3: control; transformed with plasmid p35SAc (CaMV 35S::PAT::NOS)

B) Formation of embryogenic calli on hormone free, L-PPT containing medium

C) *In vitro* plant regeneration

D) Fertile plant regeneration in the glass house

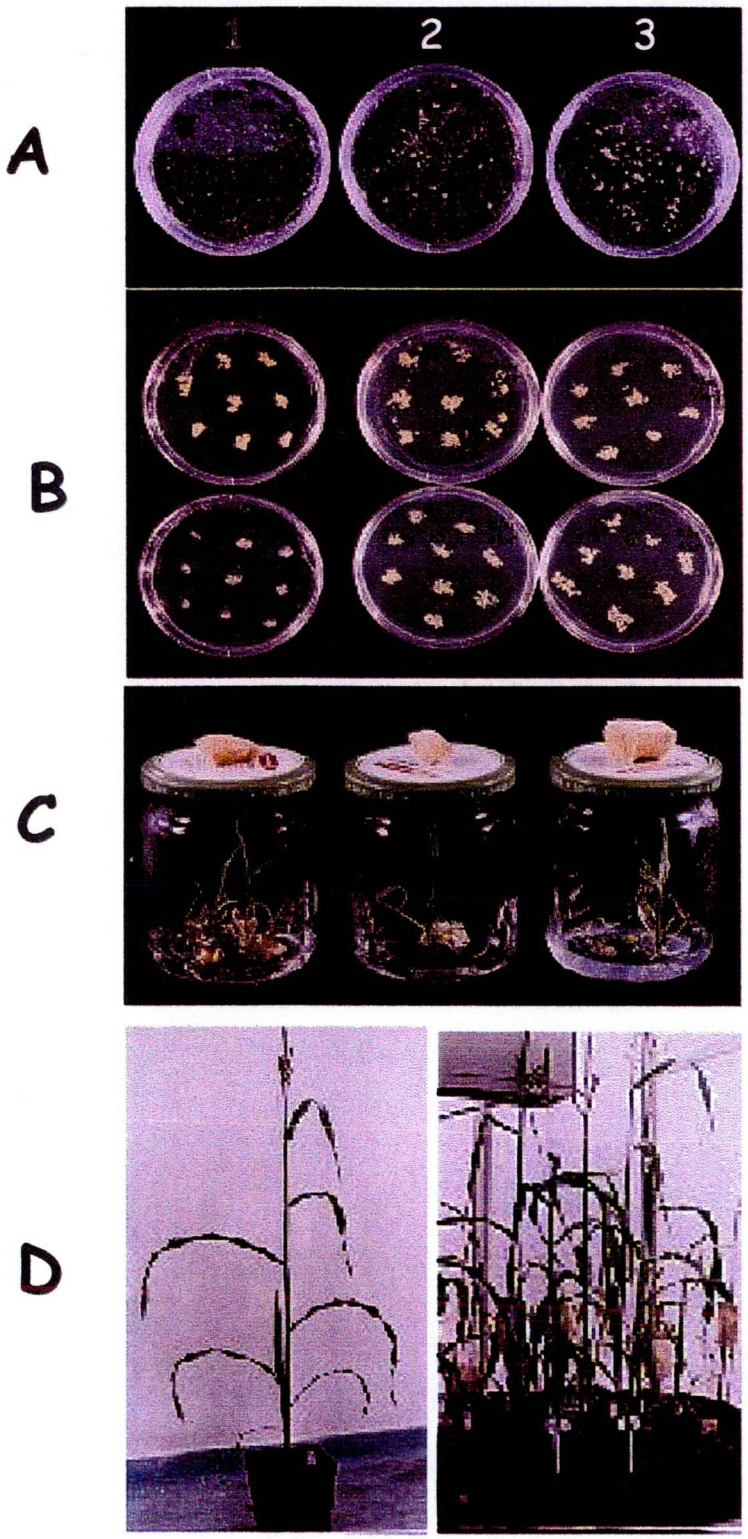

A
1
2
3
B
C
D

Figure 15

Molecular characterisation transgenic maize plants

A) Genomic DNA PCR for the MSV-V2 gene. A 318 bp PCR fragment was amplified. Ethidium bromide stained gel (2% agarose) contained 1/5 of the reaction mixture. Lane 1 contains 10 ng of SV_2 plasmid DNA. Lanes 2-8 are different putative SV_2 transgenic maize lines. Lane 9 is DNA from non-transformed calli.

B) Genomic DNA PCR for the selectable marker gene PAT. A PCR fragment of 235 bp was amplified from PPT-resistant protoplast derived cells. PCR was restricted to 15 cycles and the signal was amplified by Southern blotting and autoradigraphy. First lane is a positive control containing a mixture of non-transformed maize DNA and 10 ng of the plasmid p35SAc. Lanes 2-9 are different putative SV_2 transgenic maize lines selected on L-PPT.

C) Quantitative PCR for copy number estimations of SV_2 lines. Lanes 1-9 contains different clones. PCR reaction was restricted to 12 cycles and the signals were amplified after Southern hybridization. Lanes a, b, c, d contains 2, 5, 10, 20 of transgene copy equivalents were mixed in transgenic maize DNA, respectively.

D) Transgene (MSV-V2) mRNA accumulation in selected SV_2 transgenic lines. 3'RACE was performed using ss cDNA from the same selected clones. Ethidium bromide stained gel (2% agarose) contained $1/4^{th}$ of the reaction mixture. Lanes 1-8 are different putative SV_2 transgenic maize lines. Lane 9 contains plasmid DNA, pSV2 and lane 10 is a cDNA from MSV infected tissue. First lane is non-transformed control maize cDNA.

E) Northern blot analysis of selected SV_2 transgenic lines, in lanes 2-8 . Lane 1 contains RNA from clone CP8.

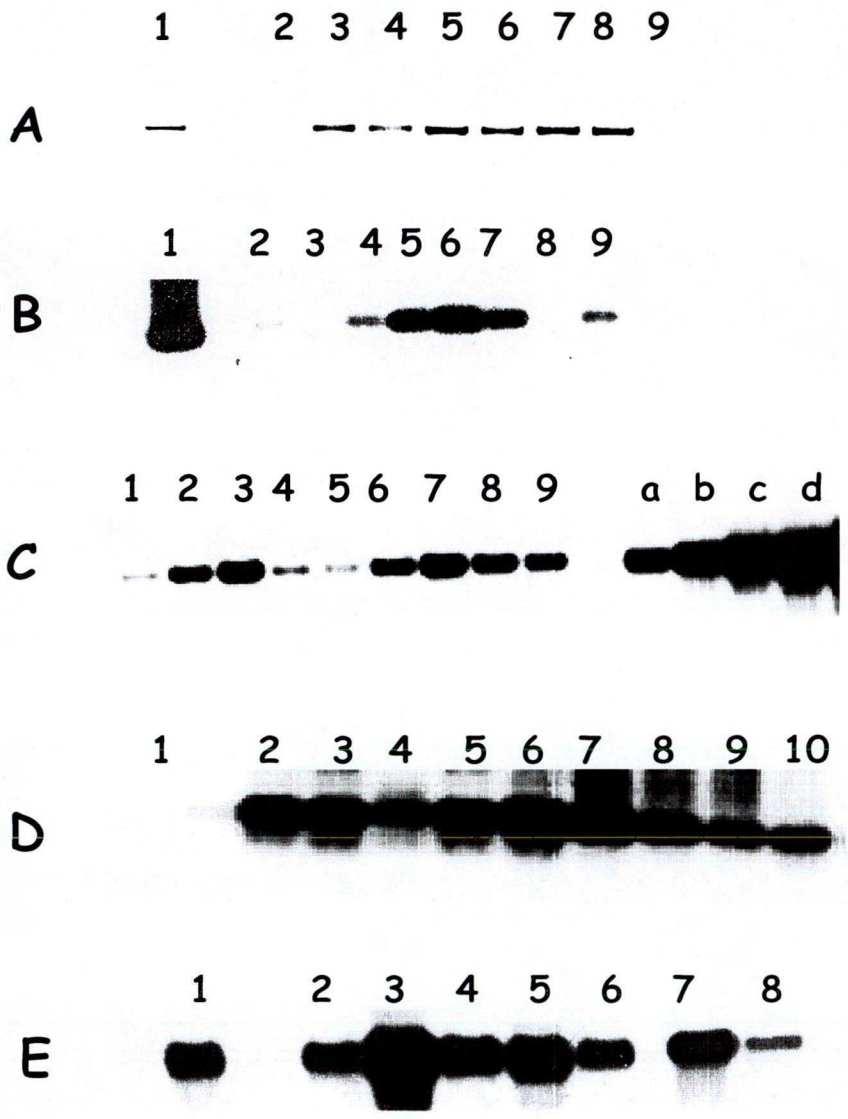

1 2 3 4 5 6 7 8 9
A
1 2 3 4 5 6 7 8 9
B
1 2 3 4 5 6 7 8 9 a b c d
C
1 2 3 4 5 6 7 8 9 10
D
1 2 3 4 5 6 7 8
E

Figure 16

Southern blot hybridization and confirmation of the transgene integration into maize genomic DNA.

A) High molecular weight (uncut) genomic DNA (6µg) hybridised against a PCR amplified MSV-V2 gene. Lane 1-9 contains SV_2 transgenic lines.

B) Genomic DNA from SV_2 transgenic lines digested with restriction endonuclease EcoRI and HindIII that releases the expression unit (1840 bp). Lane 1 is a negative control, lanes 2-5 contains SV_2 transgenic lines. A series of pSV_2 plasmid DNA was mixed with control maize DNA to give a series of copy numbers/haploid maize genome: lane 6 one copy, lane 7 two copies, lane 8 five copies and lane 9 ten copies equivalents of the transgene. Lane 10 contains plasmid DNA fragment (100 pg).

C) Genomic DNA from SV_2transgenic lines digested with restriction endonuclease EcoRI or SacI that cuts either from the 5' or 3' of the expression unit and hybridised by MSV-V2 gene. Lane 1 is a negative control, lanes 2-6 contains SV_2transgenic lines cut with EcoRI. Lanes 7-11 are DNA digested with SacI prior to blotting and Southern analysis.

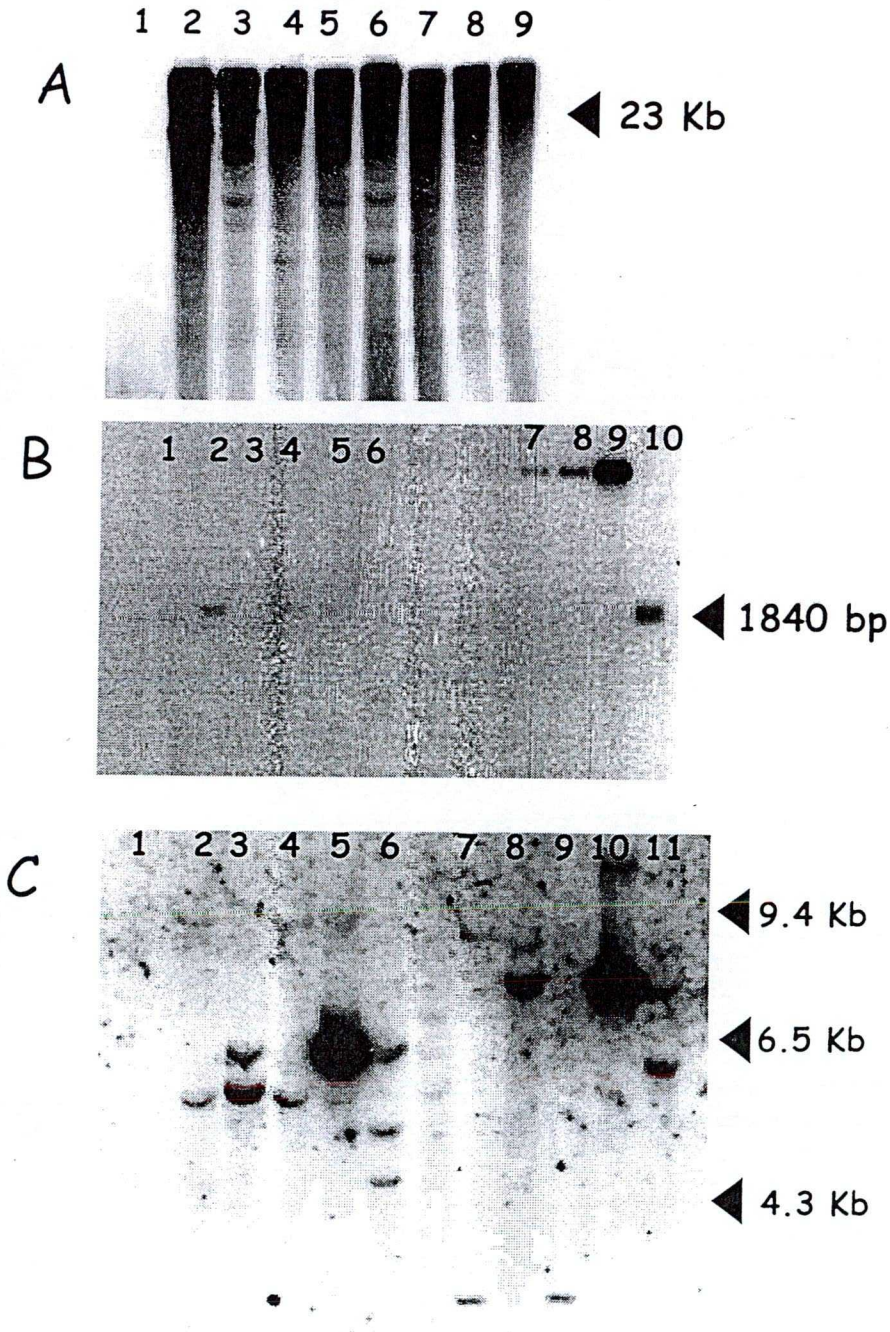

A
1 2 3 4 5 6 7 8 9
23 Kb
B
1 2 3 4 5 6 7 8 9 10
1840 bp
C
1 2 3 4 5 6 7 8 9 10 11
9.4 Kb
6.5 Kb
4.3 Kb

Figure 17

Virus replication assays using protoplasts from transgenic maize lines expressing MSV-CP. The protoplasts were transfected with BH10 possessing a single copy of MSV genome; pBSK+. Protoplast derived colonies were harvested for DNA isolation and subsequent Southern analysis, 12-14 days after PEG mediated plasmid DNA delivery. Filters were hybridized with a radiolabelled V2 gene fragment

A) Protoplast replication assay for clone CP8; lane one contains non transfected control.

Lane 1: Control, HE/89 not infected

Lane 2: Control, HE/89 at 12 days after transfection

Lane 3: CP8 at 5 days after transfection

Lane 4: CP8 at 8 days after transfection

Lane 5: CP8 at 12 days after transfection

B) Protoplast replication assays for SV_2 clones

Lane 1: Control, HE/89 just after transfection

Lane 2: Control, HE/89 at 14 days after transfection

Lane 3: Sv_23 just after transfection

Lane 4: Sv_23 at 14 days after transfection

Lane 5: SV_25 just after transfection

Lane 6: SV_25 at 14 days after transfection

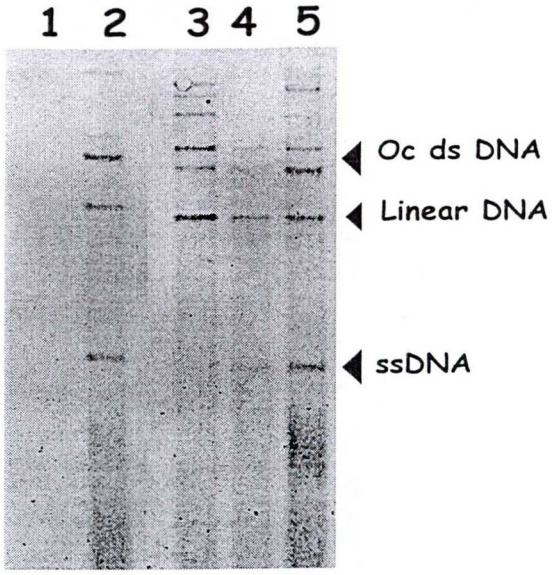
Lane 7: SV_217 just after transfection

Lane 8: SV_217 at 14 days after transfection

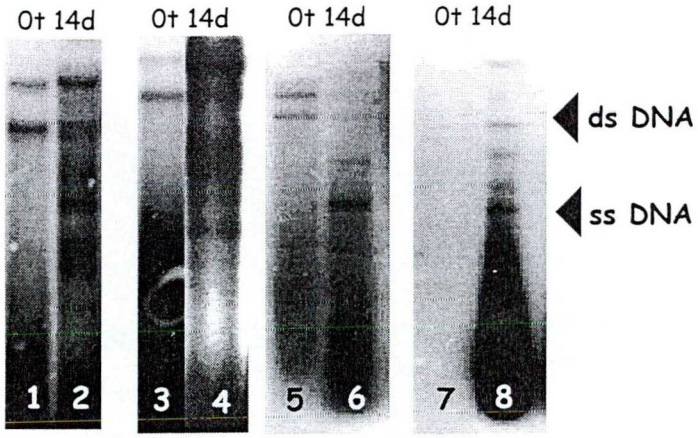
C) Replication assays using transgenic maize callus after biolistic delivery of the MSV genome; plasmid BH10. Transgenic were harvested at 8 days post transfection. As controls several other transgenic lines were used: PT2 carries a CaMV::PAT; KM10 carries CaMV::GUS.

Lane 1: Control DNA	Lane 2: SV_23	Lane 3: SV_28
Lane 4: SV_25	Lane 5: SV_211	Lane 6: KM10
Lane 7: SV_212	Lane 8: SV_217	Lane 9: SV_26
Lane 10:PT2	Lane 11: CP8	

A


1 2 3 4 5
Oc ds DNA
Linear DNA
ssDNA

B


0t 14d
0t 14d
0t 14d
0t 14d
1 2
3 4
5 6
7 8
ds DNA
ss DNA

C

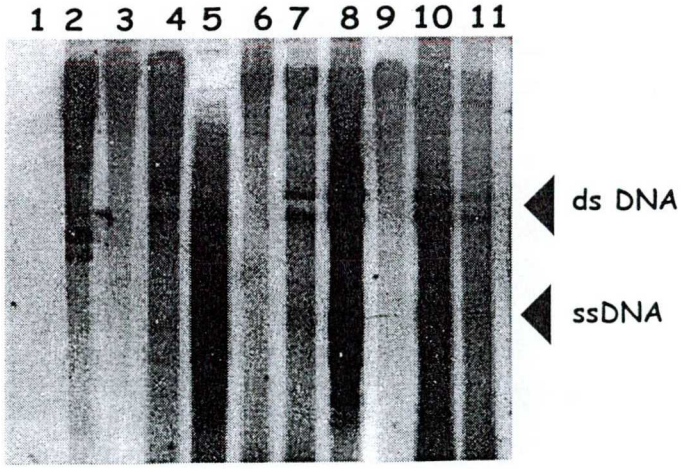

1 2 3 4 5 6 7 8 9 10 11
ds DNA
ssDNA

Figure 18

Virus replication in transgenic maize plants after insect transmission.

Photographs were taken 14 days post-insect transmission. For each individual plant 6-10 leafhopper previously fed on MSV (mild Nm and severe Ns strains) infected control plants (golden bantham) were used. At the end of the transmission period, insects were taken back to healthy control plants and their ability to transmit the disease was evaluated.

A) Clone $Sv_2$17

Lane 1: Leaf sample of $SV_2$5 infected with MSV-Ns

Lane 2: Leaf sample of $SV_2$5 infected with MSV-Nm

Lane 3: control, (golden bantham) infected with MSV-Ns

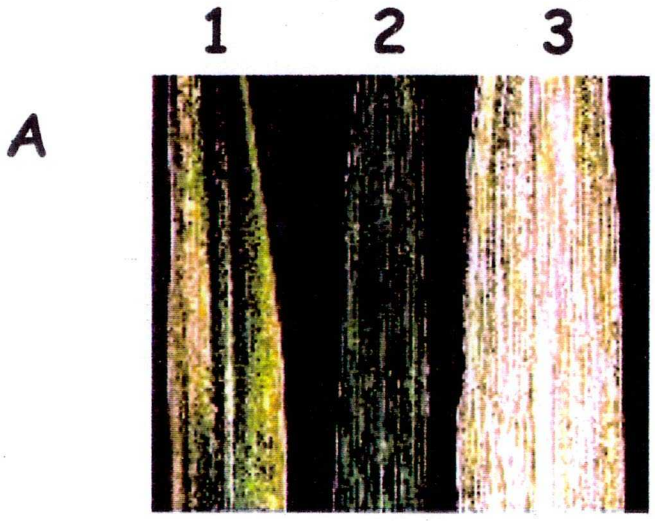
B) Clone $SV_2$3

Lane 1: Leaf sample of $SV_2$17 infected with MSV-Ns

Lane 2: Leaf sample of $SV_2$17 infected with MSV-Nm

Lane 3: control, (golden bantham) infected with MSV-Nm

Lane 4: control, (golden bantham) infected with MSV-Ns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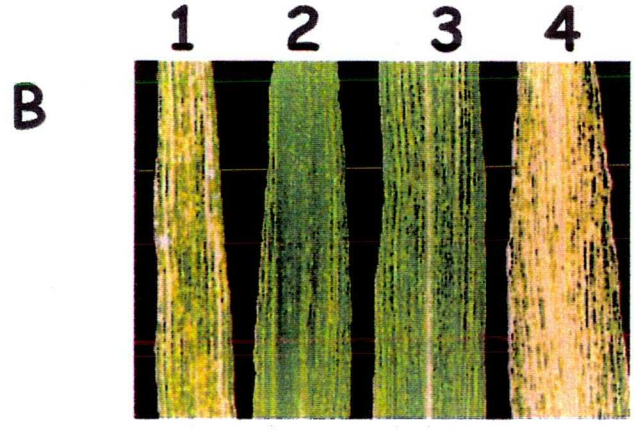
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4
B

Figure 19

Impaired growth of transgenic maize lines expressing MSV-capsid protein. These plants are shown are grown for 4 weeks in phytotron chambers and greenhouse.

A) Transgenic maize line carrying a CaMV 35S promoter GUS fusion; KM10

B) Transgenic maize line carrying CaMV 35S promoter MSV-CP fusion; CP8

C) Transgenic maize line carrying CaMV 35S promoter-PAT fusion; PT2

A B C

visible only after Southern blotting where signal intensity appeared as a function of copy numbers integrated in the genome (Figure 16D).

These plants were further grown as callus, cell suspension cultures and as mature plants to be utilized in further experiments for the assessment of virus resistance.

Protoplast replication assays were performed from selected clones using cell suspensions of transgenic lines. 12-14 days post-transfection with BH10 plasmid, DNA samples from MSV transfected protoplasts were blotted and analyzed by Southern analysis (Figure 17). Results indicated that the viral transgene, in sense orientations did interfere with viral replication as judged by the accumulation of replicative viral DNA forms in protoplast replication assays and insect transmission assays performed on whole plants (Figure 18). From two different clones the mature plants were subjected to MSV by transmission facilitated by insects previously fed on infected plants. All transgenic maize plants including controls carrying another plant expression vector; pH4G have developed symptoms after 14 days of growth in glass house conditions. The non-transgenic maize, *Golden Bantham* plants however accumulated these symptoms earlier than the transgenic lines within 10-12 days.

In an attempt to see whether the capsid protein can block the *C.mbili*'s receptors for the virus, leaf hoppers (5 leafhopper/ plant) were first fed on transgenic $SV_2$3, $SV_2$5 plants and than on MSV infected plants. Finally these insects were transferred onto non-transgenic maize, *Golden Bantham* plants. After 12 days plants were scored for symptom development. All plants after the period developed wide typical chlorotic streaks indicating that the transgene product did not block the insects' receptors prior to feeding on MSV infected plants.

3.2.2 Subcellular localization of MSV capsid protein in transgenic maize cells

The transgenic plants carrying the MVS capsid protein gene were also tested for the subcellular localisation of the viral gene in host cells. Based on the assumption that the capsid protein might be imported to the nucleus, immunohistochemical detection of the capsid protein was done on protoplasts of transgenic suspension cells. Polyclonal antibodies raised against the capsid protein (sap from infected tissue) were first depleted by absorption to crude protein (non-transformed maize). The anti-MSV CP antibodies were coupled with an anti-rabbit rat TRITC conjugated secondary antibody for detection under epi-fluoresence option of a Zeiss Laser Scanning Microscope. In three different clones, arising from two different experiments and where expression is driven by different promoters, CP was found to accumulate in the nuclei of the host cell (Figure 20). The DAPI stained nuclei also confirmed the cell structure and signal localisation. There was no

signal in samples without the secondary antibody or in transgenic clone KM10 carrying a CaMV35S::GUS reporter fusion.

3.3 Interaction of MSV-host genes during viral infection

The primary goal of these experiments was to see whether the MSV replication is dependent on host cell cycle machinery and also find out the host factors and viral genes interacting during the formation of new viral DNA.

3.3.1 Wheat histone H4 promoter is trans-activated by MSV infection

First, transgenic maize plants carrying the chimeric gene construct, wheat histone H4::GUS fusions were infected with two infectious strains of MSV either by agroinoculation or insect transmission. These two strains *Ns* and *Nm* were differing in symptom development and severity of disease progress, latter being "milder". The primary goal of this approach is first to determine whether the activity of GUS is induced (via the wheat histone H4 promoter) following MSV infection and also to link the magnitude of the promoter activity to the "severity" of infectious MSV strains.

To determine whether GUS expression is linked to the stimulation of host DNA replication machinery by the presence of virus proteins, GUS enzyme assays, northern blotting analysis was performed with infected and non infected transgenic and also with non-transgenic control (golden bantham) maize plants. Another transgenic maize clone expressing GUS gene driven by CaMV 35 promoter and two enhancers from the same regulatory unit was tested along with the histone H4::GUS plants. The luminometric GUS assays performed at 12 days post-infection (via insect transmission) and enzyme activities at expanded leaf pieces were measured. (Figure 21) Wheat histone promoter activity was significantly induced upon MSV infection. After fluorimetric assessment of GUS enzyme activity, it was found that, MSV infection trans-activated the replication-dependent histone H4 promoter 12 days post inoculation, in transgenic maize plants. The GUS activity was increased by several folds with respect to the control plants. These results also indicate that the S phase specific expression of the histone promoter was activated both in lower stem and expanded leaves in MSV infected plants.

The presence MSV infection was also tested both by observations on phenotypic changes (streak abundance, streak width) and also by Southern blotting against MSV DNA. The degree of activation was correlating with the streak size; thus the strain used for transmission though the single stranded DNA and other DNA-replication intermediate forms equally existed in the tested plants (not shown).

3.3.2 Transient expression of MSV Rep induces histone promoter activity

Changes in "viral and host gene expression" mediated by the Rep/RepA was further examined using a transient expression system. Responses to wild type MSV in binary vector MB105 and pREP carrying Rep overexpression cassette viral sequences were examined after their transient expression in maize cells. Full length, *Rep/RepA* genes were inserted into the plant expression vector pAHC25 (Christensen, 1994); between ubiquitin promoter-first intron and exons and the NOS termination signal and delivered using particle bombardment using electric discharge bombardment device developed by Agrocetus. The target was transgenic maize suspension cells carrying wheat histone H4::GUS fusions. After 3 days, reporter gene function was assessed by chemiluminescence GUS enzyme assays which offer a wide dynamic response range and insensitive to endogenous GUS-like activities. The relative changes in enzyme activity as measured on the 3rd day after the transfection shows a significant activation of the histone promoter by both plasmid treatments with respect to the control treatment that also coincides with the start of viral replication (Figure 22). The degree of this trans-activation was several folds higher in pREP than in wild type dimeric copy of MSV. The negative control, p35SAC (Ecker, unpublished), carrying a synthetic phosphinotricine acetyl transferase gene under the control of CaMV 35S promoter and terminator sequences, did not show any significant increase in reporter gene activity.

3.3.3 Transcription of other cell cycle genes during MSV infection

The host cell cycle-MSV interaction was further evaluated in MSV infected maize tissues by northern analysis. The northern blot hybridization analysis of the MSV infected maize plants indicated that a significant amount of histone *H4*, histone *H2B* and *PCNA* mRNAs accumulated in differentiated leaf and stem tissues where proliferation activity are clustered in tight zones along the vascular bundles (Figure 23). The most dramatic increase in transcript level was in cdc2ZmA; a cyclin-dependent kinase assumed to govern a role in G1-S phase progression. Interestingly, the expression of the B-type putative mitotic cyclin *CycB1;zm;1* also increased in infected plant leaves. In transgenic maize plants carrying another developmentally regulated but cell cycle independent promoter GUS fusion (CaMV::GUS::NOS), the transcription of the endogenous histone H4 was increased indicating a common upstream factor that controls initiation of transcription during MSV infection.

In other transgenic plants where non-infectious leafhoppers were used, there was no change in mRNA accumulation of histone or other cell cycle gene in response to insects.

Both severe (Ns) and mild (Nm) strains of the MSV induced host mRNA transcription, in correlation with their capacity to produce active *Rep* proteins. Figure, shows that both in golden bantam plants highly susceptible to MSV infection and transgenic lines possessing wheat histone H4:GUS or CaMV 35S:GUS chimeric expression units, the expression of S-phase associated genes increased. When two different strains of the virus, causing severe (Nigerian isolate; Ns) or mild (Nigerian isolate, Nm) symptom development were compared, the severe isolate induced higher expression of the *cdc2ZmA*, histone H2B/H4 and PCNA.

3.3.4 A DNA polymerase inhibitor interferes with virus replication

In attempt to see whether a DNA polymerase inhibitor can block host and subsequent virus DNA replication, a transient expression assay was performed. Maize cell suspensions were bombarded with an infectious dimeric copy of the MSV genome in binary vector MB105 and grown in the presence of 8µg/ml aphidicolin for 3 days. At the end of the period DNA from callus was tested for single stranded DNA accumulation by PCR. To eliminate the inoculum (double stranded plasmid DNA targets), the DNA was digested with restriction endonuclease EcoR I to completion before the reaction. The PCR reaction was performed with primers specific for V2 gene and the amplification was stopped at 20 cycles before target saturation. The MSV replication was significantly impaired in the presence of aphidicolin; the DNA polymerase -α inhibitor (Figure 24).

Figure 20

Subcellular localisation of MSV capsid protein in transgenic maize lines.

Selected clones were tested with immunocytochemistry on protoplasts from several clones carrying and expressing the MSV-V2 gene in maize. Signals were obtained after incubation with a fluorescent dye (TRITC) conjugated secondary antibody after appropriate excitation of the fluorochrome. Microphotographs were stored and processed digitally.

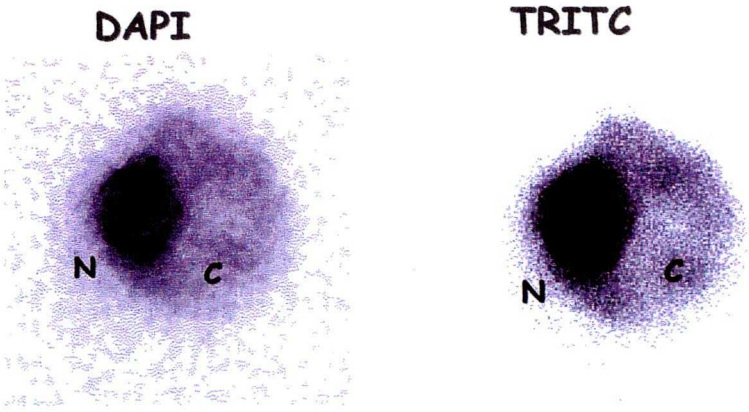
The nucleus (N) and cytoplasm (c) are designated in panel A.

A) Analysis of the clone CP8 Localization of the nucleus (DAPI) and CP (TRITC)

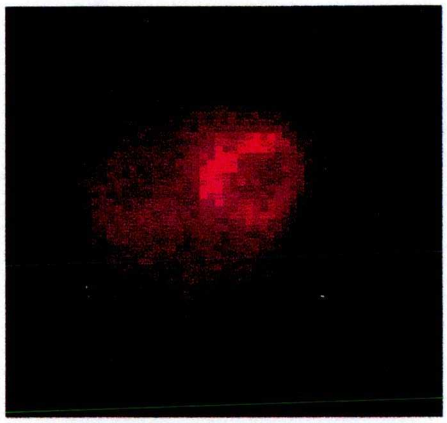
B) Analysis of the clone $SV_2$3 (artificial colour)

C) Analysis of the clone $SV_2$17

A



B



C

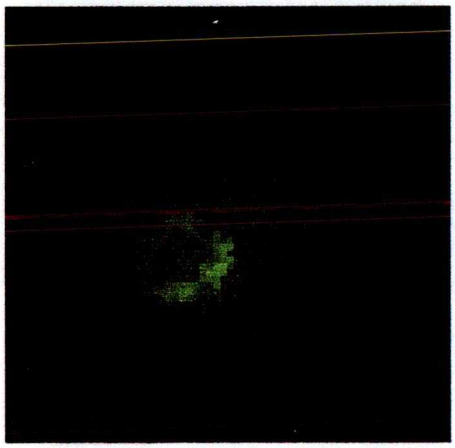


Figure 21

Wheat histone H4 promoter activity in transgenic maize plants.

Maize plantlets were agro-infected by vectors (MB105) bearing dimeric copies of MSV-Ns. The plants were harvested 10 days post-inoculation and relative luminesence units (RLU) were measured using GUS-Light Chemiluminesence Kit. The leaf (mid portion of the leaf) or shoot apical meristems (first 3 cm of the lower stem) from two selected clones were tested. The first set of bars represent results obtained from H4.52 and the other from clone H4.25. Purple bars represent healthy (control) plants whereas green bars are MSV infected plants. Error bars represent standard variation among samples.

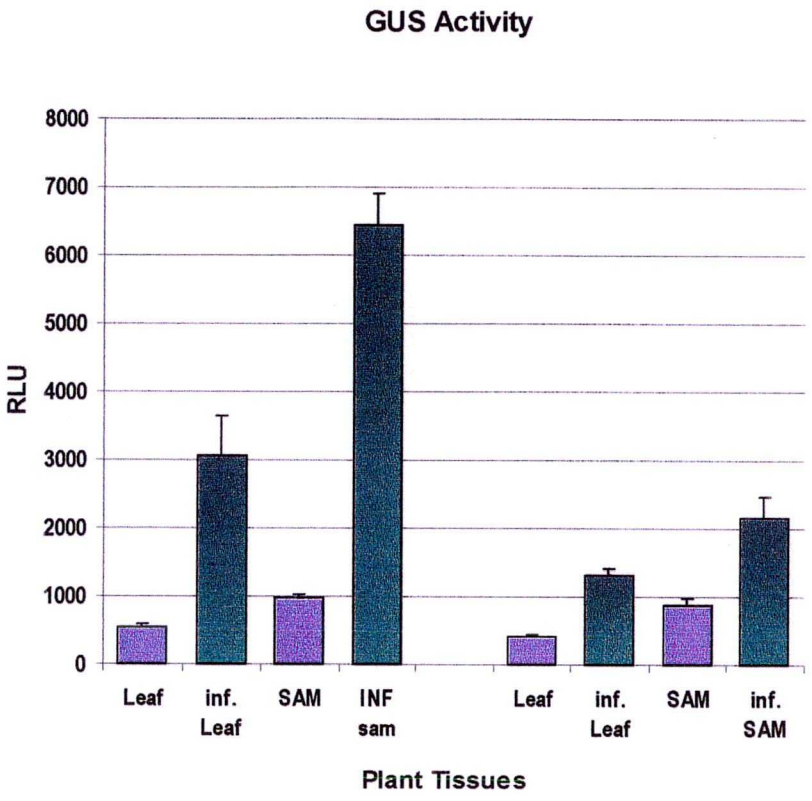

GUS Activity
8000
7000
6000
5000
4000
3000
2000
1000
0
RLU
Leaf
inf. Leaf
SAM
INF sam
Leaf
inf. Leaf
SAM
inf. SAM
Plant Tissues

Figure 22

Transient activation of histone H4 promoter in maize cells.

GUS enzyme activity was measured after delivery of MSV DNA into transgenic maize cells by biolistic transfection of the plasmid pBH10 and pRep. On the third day post-transformation, samples were processed for GUS activity. Data was derived from GUS assay values (RLU) of replicate readings of six independent experiments for each plasmid. Control treatment is a plant expression vector p35SAc (CaMV 35S::PAT::NOS). Error bars indicate variation among different set of experiments for each plasmid.

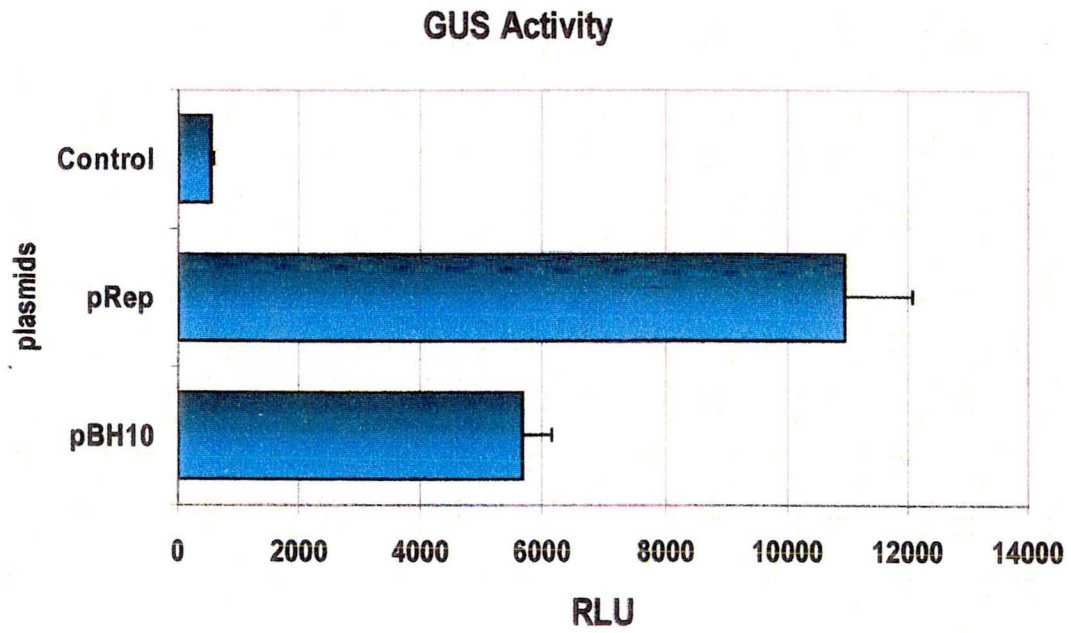

GUS Activity
Control
pRep
pBH10
plasmids
0
2000
4000
6000
8000
10000
12000
14000
RLU

Figure 23

mRNA expression of maize cell cycle genes in MSV infected plants

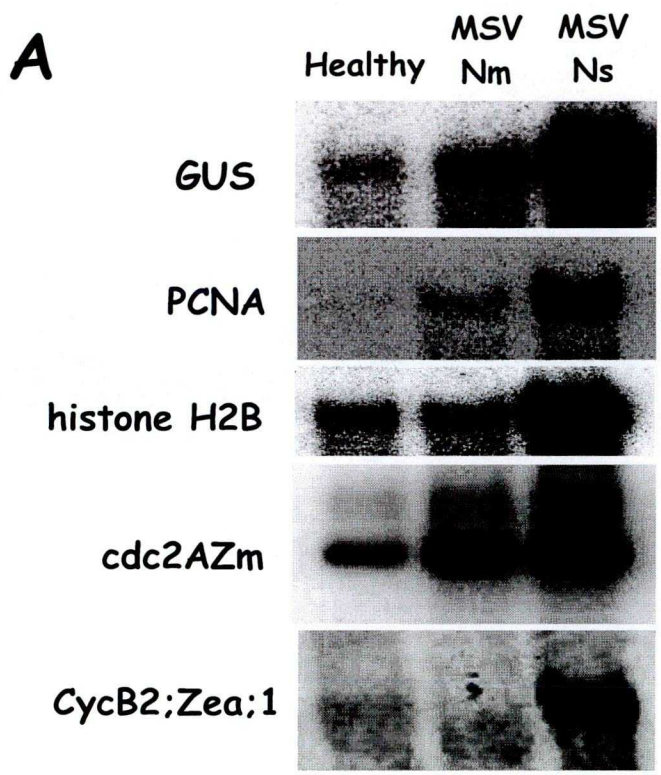
Northern blot analysis of S-phase associated maize cell cycle genes in different tissues of MSV infected non-transgenic (Golden bantham) and H4::GUS transgenic lines with two virus strains, Nigerian mild (Nm) and Nigerian severe (Ns).

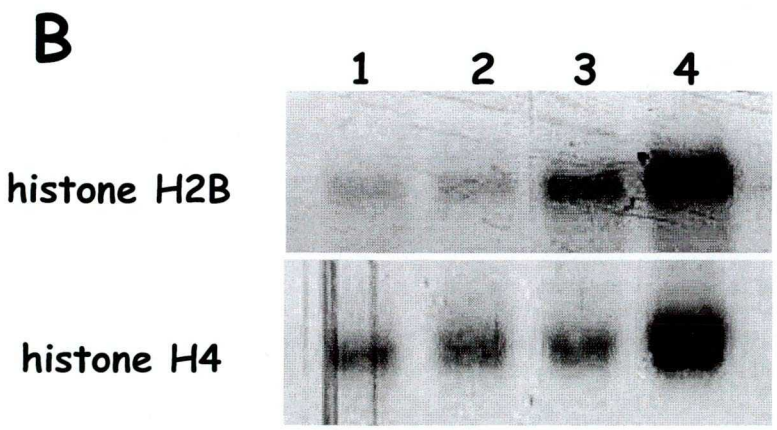
A) Northern analysis of maize histone H4 gene in MSV infected leaf tissues of non transgenic (golden bantham) plants 10 days post infection. Twenty µg of total RNA was loaded on each lane.

Lane 1: Healthy non-MSV infected leaf
Lane 2: MSV-Nm infected leaf
Lane 3 MSV-Ns infected leaf

B) Northern analysis of maize histone H2B (upper) and H4 (lower) genes in transgenic plants carrying wheat histone H4::GUS (H4.25) and CaMV35S promoter::GUS (KM10) chimeric genes. During MSV infection RNA samples were taken 10 days post infection by virus transmission. Twenty µg of total RNA was loaded onto each lane.

Lane 1: Leaf (clone H4.25) as control, non MSV infected
Lane 2: Leaf (clone KM10)
Lane 3: Stem (clone H4.25)
Lane 4: Leaf (clone H4.25)


A
Healthy
MSV Nm
MSV Ns
GUS
PCNA
histone H2B
cdc2AZm
CycB2;Zea;1


B
1
2
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4
histone H2B
histone H4

Figure 24

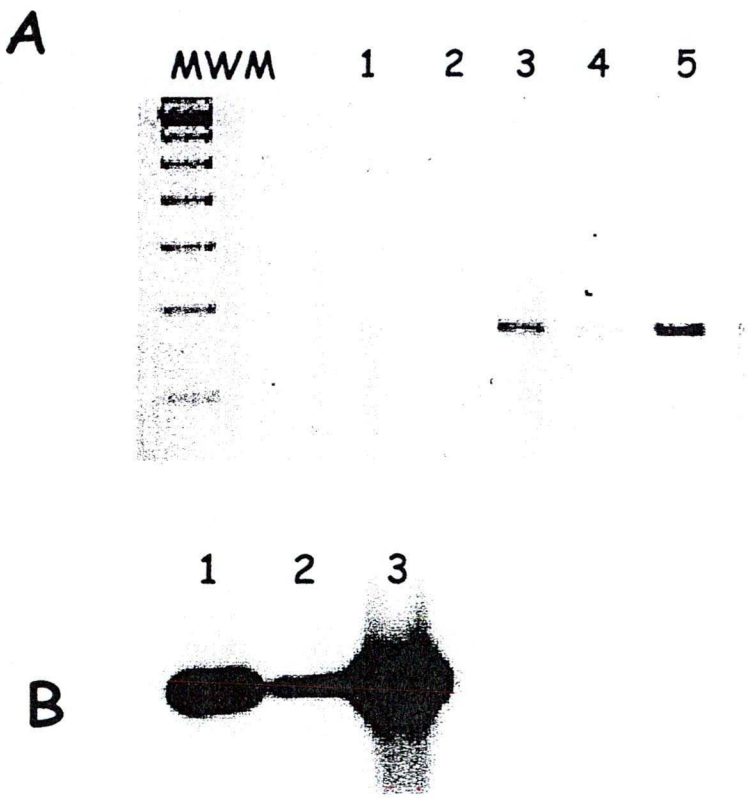
Aphidicolin blocks the replication of MSV

A) Accumulation of ssDNA in aphidicolin treated HE/89 maize suspension cells, two days after biolistic delivery of MSV DNA; BH10 (monomer of MSV in pBSK+). Prior to PCR, samples were incubated with the restriction endonuclease EcoRI and HindIII (5units/µg DNA for 6 hours) to eliminate ds DNA inoculum. These two endonucleases cut inside the MSV-V2 gene within the amplified region.

Lane 1: control DNA (healthy suspension cells; HE/89)
Lane 2: Eco RI/HindIII digested control DNA + 1µg of plasmid MB105
Lane 3: Eco RI/HindIII digested, no aphidicolin, transfected with MB105
Lane 4: Eco RI/HindIII digested, aphidicolin treated (8µg/ml) DNA
Lane 5: Transfected with MB105, without EcoRI/HindIII digestion

B) Expression of MSV-V2 gene (capsid protein) in aphidicolin treated cell suspensions bombarded with MSV DNA (BH10). The samples were taken two days after biolistic delivery and total RNA was treated with DNaseI to eliminate inoculum.

Lane 1: No aphidicolin
Lane 2: Aphidicolin (8µg/ml)
Lane 3: Control; MSV-infected leaf tissue


A
MWM
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3
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B

"What you want are facts, not opinions"

from Notes on Nursing

Florence Nightingale

CHAPTER IV

DISCUSSION

4.1 GENETIC TRANSFORMATION AND MAIZE

Cereals represent the most important food source of the mankind. Although the transformation of cereals has been a difficult task, all of the major species were transformed by direct DNA delivery into regenerable protoplasts by osmotic or electric shock treatments or by the biolistic method and recently by *Agrobacterium* mediated transformation.

The term "genetic transformation" used in this thesis work, refers to revealing mechanisms of gene function in plants, complementing and supplementing traditional methods of crop improvement and have a profound impact of food production. This thesis also aims to demonstrate the basic science and applied aspects of genetic transformation through production and utilization of transgenic maize plants.

Few plants share maize's importance to both agronomy and basic biology. Thus, the ability to create transgenic maize plants would be a tremendous advantage for those who are trying both to improve the commercial traits of maize and those using transgenics to explore more on the basic questions of "plant gene expression" in higher plants.

PEG mediated DNA uptake into protoplasts is an efficient and reliable way of gene transfer and subsequent analysis of the gene introduced (Negritiu *et al.* 1987; 1990). The use of protoplasts in transient expression experiments provides an opportunity to make a rapid assessment of the functional activity of different promoters, but there are certain limitations of the system. First, the protoplast is not a 'normal' plant cell. The lack of cell wall and the physiological stress caused by the combined action of cell wall-digesting enzymes, the change of surrounding milieu, the signals released by the wounded and dying cells can affect

the metabolism of the cell and produce some misleading results (Harkins, 1990). The successful uptake of plasmid DNA and the subsequent expression of the introduced genes are influenced by many parameters. Some of them are of technical characteristic: purity and viability of protoplasts, plasmid DNA and reagents, the physiological state of the plant source.

A successful protoplast transformation and plant regeneration is also based on the choice of proper genotypes and tissues as the source of starting material, to the efforts to minimize the stress exerted during the whole procedure and improvement of culture and regeneration systems. In these aspects, maize suspension HE/89 provided a perfect target for both transient and stable transformation experiments particularly with its competence for PEG mediated direct DNA uptake method, high frequency of transformation of and retained capacity of regeneration into whole mature plants after transformation. However, major problem encountered during these studies was the fertility of the regenerated maize plants. Most of the cases, male sterility was observed in transgenic maize lines. This was than reflected to seed settings and resulted in very few numbers of seeds from the primary transformants. Particularly, during the study on "Coat protein mediated protection against MSV", this has been major limitation, as all the modes of assessment of resistance to MSV infection is by agro-inoculation or by insects feeding on young seedlings. Thus, primary transgenic population was in turn used for insect transmission assays. For this reason, segregation and subsequent expression analysis of the transgene in the progeny was not monitored. The lack of seed production can be due to technical or biological reasons. The growing conditions could have been inappropriate at that time in our greenhouse or possibly the viral DNA influences growth and fertility, since in parallel with this virus resistance project I successfully produced large number of fertile plants transformed with other constructs.

In this study, I have used three different gene delivery and genetic transformation protocols utilizing the chemical (PEG mediated), physical (biolistic) and biological (agro-inoculation) means to drive DNA into plant cells and tissues. These methods have different characteristics and advantages over each other however, PEG mediated direct DNA uptake into protoplast stands to be the most versatile system for stable transformation into maize plants in my hands. Biolistics on the other hand, offered quick assessment of gene function. It has allowed me to test kinetics of reporter gene function when transiently expressed viral DNA in suspension cells and embryogenic calli. Moreover, it was possible to process statistically significant numbers of samples at a time.

Though developed for viral DNA transmission in maize, agro-inoculation can be used for other genes tested though the limitation is the stability of the DNA introduced and interference by the vector organism at the site of inoculation. Thus this method enables to express genes as extrachromosomal units, regardless of the size of the plant expression cassette.

In agreement with other reports on protoplast derived transgenic cereal plants, the transformation efficiency, number of regenerable colonies of these experiment showed that PEG-mediated protoplast transformation is a feasible tools for genetic manipulations. Used selection schemes based on kanamycin and

L-PPT resistance were stringent enough to minimize the number of "escapers"; however, NPT II gene in some cases caused poor root regeneration. Thus, L-PPT was utilized in further experiments.

The analysis of plasmid DNA integration and copy number estimations indicated that the used method has delivered 1-4 copies of the transgene DNA into the target, maize genome. The transgene DNA as single transcriptional units, was mostly dispersed to several integration sites, though in some cases several copies were aligned in close vicinity. As a technical tool, quantitative PCR offered an easy tool to estimate copy numbers without tedious Southern analysis. The comparisons of transgene copy numbers, mRNA transcript levels and protein accumulation did not suggest significant post-integrational influences, all correlating with the DNA integrated and the nature of the regulator sequences in plasmid vector introduced.

Several promoter regulatory sequences derived from virus (CaMV 35S) and plant genomic DNA (maize polyubiquitine, wheat histone H4) were used to drive the expression of the transgene. Both CaMV and ubiquitin promoters exhibited constitutive but developmentally regulated expression. In transient assays, expression vector pAHC25 driven expression provided significant transgene product to reach a "sound" level. Wheat histone H4 promoter on the other hand, has the capacity to be used as a promoter for gene modifications and crop improvement purposes when an agronomic trait is linked to early development.

The production of transgenic plants have several drawbacks, such as the time required to produce transgenic plants, the unusual behavior 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 regenerated, since this process requires 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. These studies on production and utilization of transgenic maize plants also underlined the tight link between the presence of the right source of target tissue, the method of genetic transformation, the nature of the plant expression vector introduced and established plant regeneration schemes. Development of genotype independent transformation schemes leading to transgenic progeny thus should be considered as a major factor among all listed criteria. Use of plant protoplasts and transient expression assays can provide valuable research tools for studying fundamental cellular processes in plants and can be utilized for functional analysis of cloned cell cycle regulatory genes.

4.2 FUNCTIONAL ANALYSIS OF WHEAT HISTONE H4 PROMOTER

The histone gene is a paradigm for transcriptional control at the G1/S phase transition point in the cell cycle. Our current understanding of the basic structure and function of chromatin in plants illuminates the fact that it plays a critical role in the regulation of gene expression and its components like histone proteins have additional roles other than storing DNA. Chromatin structure, nucleosome organization and gene-nuclear matrix interactions facilitate crosstalk between regulatory sequences and targeting of transcription factors to cognate binding domains. Thus, understanding of structure and function of histones has important

implications for the design of new strategies for the developmental growth control, gene expression, disease states and development of transgenic plants.

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). 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 (Dedeoglu, unpublished). 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 and Mikami 1998), thus, reflecting an integration of phosphorylation-mediated control of histone gene expression at the G1-S phase transition point. Here, the histone gene promoter provides a "blueprint" for integration of regulatory signals, which mediate responsiveness to factors controlling competency for cell cycle progression at the initiation of DNA replication. During unfavorable conditions for the growth of the plant, the down-regulation of proliferation at the onset of differentiation is functionally linked to modifications in protein-DNA and protein-protein interactions at histone gene promoter regulatory elements.

Histones like most genes and regulatory sequences whose activity has been studied in transgenic plants are expressed in functionally relevant patterns in respect to cell and organ specificity, developmental timing and response to environmental cues. Analysis of the activity of the wheat histone H4 promoter in maize also exhibited expected expression patterns indicating its link to cell division dependent expression clustered in meristem tissues of the regenerated plants. Similar expression patterns were obtained in roots of other transgenic plants carrying histone H3 or H4 promoters linked to marker genes both from monocot and dicot species.

Molecular characterization of cell cycle regulated genes and their regulatory sequences requires transcriptional, translational or in situ analysis of their activity in synchronized cell population. Until now, for maize there are no report regarding successful synchronization using chemical inhibitors of cell division cycle. Some studies on maize cell cycle dynamics and division-related microtubule reorientation were based on root tissues of maize (Baluska *et al.* 1993; Lee *et al.* 1996), however the progress of cell cycle stages and activity of cell cycle genes was not monitored in details. Thus, the function of maize cell cycle genes as predicted from their nucleic acid and/or protein sequences and their resemblance to other fully characterized counterparts, still remained to be explored. We attempted to apply this method to maize cell suspensions *in vitro.* In rice, partial synchronization of transgenic suspension cells carrying wheat histone H3 promoter-GUS translational fusions with a reversible cell cycle inhibitor, aphidicolin, demonstrated that an enhanced histone promoter activity coincides with the S-phase (Ohtsubo *et al.* 1993; 1997). The careful dissection of the

patterns of mRNA accumulation originating from histone promoter-reporter gene cassettes also indicated that the replication-dependent activity started just before DNA replication at late G1 phase and is controlled independently of the DNA synthesis (Atanassova *et al.*, 1998). Our results are in agreement with these observations. Several reports suggest discrepancies between the expression of the reporter gene driven by the histone promoter and the endogenous counterpart in different plant systems and with different chemical inhibitors of the cell cycle progression (Chaubet *et al.*, 1998). However, in our case, both the GUS and endogenous histone H4 mRNA accumulation after aphidicolin treatment of roots indicated that the re-activation of gene expression coincides with the G1/S phase transition. The presence of the mitotic spindle stabilizer colchicine in the culture medium, however, arrested the majority of the cells at mitosis and subsequently the histone promoter regulated GUS mRNA levels decreased. These were further confirmed by northern analysis after cell synchronization using hydroxyurea.

Use of two different S-phase blockers also indicated that reporter gene activation takes place between the restriction points of hydroxyurea and aphdicolin. These inhibitors have different modes of action and assumed to act at different but overlapping periods where initiation of histone gene expression most likely occurs. In the presence of colchicine on the other hand, which acts as an inhibitor of microtubule dynamics totally diminished GUS mRNA supporting the S-phase specific role of the wheat histone H4 promoter.

The transcriptional activities of cell cycle related genes in maize such as histone *H4* (Chaubet *et al.* 1991), *cdc2* (Colisanti *et al.* 1991), histone *H2B* (Joanin *et al.* 1992), *PCNA* (Lopez *et al.* 1995) and B-type cyclins (Renaudin *et al.* 1994) were shown to be proliferation-dependent but the differential gene expression in certain phases of the cell division cycle have not been demonstrated yet. We have used partially synchronized cell populations transiently arrested at the G1/S phase border of the cell cycle and monitored the differences in mRNA accumulation with gene specific cDNA probes during progression from G1/S-phases to mitosis. We were able to reach a level of enrichment enough to recognize phase-dependent oscillations in mRNA accumulation as 35-45% of the cells progressed synchronously after the release of the hydroxyurea block. The activities of histone *H4*, histone *H2B*, *PCNA* genes exhibited late G1-S phase dependence, while the mRNA accumulation of B-type cyclin *CycB1;zm;1* suggested a role towards mitosis. Thus, in the present experiments, the expression of the analyzed maize genes showed characteristic cell cycle phase-specific patterns common to other members of the same gene families identified from other plant species.

Preliminary experiments on cell cycle phase specific activity of wheat histone H4 promoter was conducted on protoplasts of transgenic lines. Re-initiation of cell division cycle after protoplasting resulted in sustained GUS enzyme activity for the first 3 days of culture and only after the 4th day exhibited an increase. These results suggested that the GUS reporter enzyme activity cannot be utilized due to its stability. Although GUS is widely used as a reporter gene to study transcriptional regulation in transgenic plants, there are several reports on its inconvenience in cell cycle studies. Shaul *et al,* 1999, examined tobacco BY-2 cultures transformed with *Arabidopsis* cyclin promoters fused to GUS and detected discrepancies in levels of the GUS mRNA and the GUS protein activity. These observations highlight the need for special precautions while using

the GUS reporter gene in conditions that represent a rapid change in a developmental or a metabolic state; particularly the cell cycle. These precautions dictated further synchronization experiments to be limited to the reporter transcript levels. Moreover, suspension protoplasts after protoplasting does not constitute a synchronous population and the protoplast assay can be utilized only as a indicator of proliferation activity not for the determination of phase specific activity of the histone H4 promoter in maize.

Partial synchronization in protoplast cultures and treatment of cell suspensions with reversible cell cycle inhibitors clearly demonstrated that an enhanced gene expression takes place during S-phase. These results also demonstrate that the transcriptional mechanisms within the monocotyledonous plants do not differ in replication-dependent histone gene activity. Moreover, the expression driven by the replication dependent wheat histone H4 promoter maintained its specificity without its own termination signal which was also shown to be involved in post-transcriptional regulation of histone gene expression. In other words, 5' cis-acting sequences were enough to drive replication dependent, S-phase specific activity in transgenic maize plants. 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). Although we have also produced a transgenic line under the control of same promoter but carrying wheat histone H3 terminator sequence for polyadenylation signal, it was possible to generalize the observation of higher reporter gene activity in comparison to other lines with NOS terminator. However, considering the fact that, these transgenic lines with different terminator sequences possessing similar copy numbers, the differences in mRNA accumulation can be linked to the regulatory effect of 3' UTR sequences. Moreover the fold difference in mRNA levels and corresponding GUS enzyme activities suggested a mechanism based on mRNA stability rather than cis and trans factors at the site of transgene integration.

Phytohormones play critical roles in the coordination of plant growth and development. There are very few reports on activity of histone genes and regulatory sequences when intact cells or tissues are exposed to phytohormones. Transcriptional analysis or *in situ* detection of their activity thus would provide valuable information on progression of cell cycle upon administration of different hormones, growth factors, metabolic/cell cycle inhibitors and under stress conditions. Wheat histone H4 promoter activity in transgenic maize is developmentally regulated in a replication dependent manner predominantly expressed in pre-meristems and meristem tissues and can be modified by exogenous application of phytohormones. Due to the strict meristem specific expression pattern of the marker gene in these transgenic cell lines I utilized these plants for monitoring responses to different phytohormones. Here transgene expression serves as an indicator of the direction and multitude of the cell cycle activation as histone gene expression is linked to the S-phase. As shown, wheat histone H4 activity could be modulated by phytohormones like 2-4 D, ABA and zeatin. These results support the potency of auxins triggering the G1/S phase progression while proving the negative

regulatory effect of ABA as a stress related signal. As a potent cytokinin, zeatin did not significantly effect the histone promoter activity, probably due to its suggested role in late S-phase and M phases (Redig *et al.* 1996).

In an attempt to determine the effects of auxins on root tissues of transgenic maize lines, I have treated the intact root segments to different growth regulators and monitored changes in GUS activity by RT-PCR and histochemical analysis.

The embryogenic calli and differentiated root segments of maize H4::GUS transformants showed enhanced promoter function in the presence of 2,4-D as detected by northern blotting and histochemical GUS assays. When differentiated roots were subjected to 2,4-D for 48 hours, they exhibited an increased zone of expression in vascular tissues towards pericycle but at the same time GUS expression was not altered in the surrounding fully differentiated layers like the cortex and epidermis. In *Arabidopsis*, the *cdc2aAt* promoter driven GUS expression was increased by two folds in transgenic plants treated with auxins. Moreover, the GUS expression exhibited a well defined three zone pattern from root apical meristems towards distal vascular tissues when stimulated with 2,4-D (Hemerly *et al.* 1993). Upon 2-4D treatment, the appearance of a "three zone pattern" in lateral roots agrees with this report that links *Arabidopsis* cdc2A promoter activity to competence for cell division. However, in our case, in spite of the similar expression pattern driven by the wheat histone H4 promoter in response to 2,4-D in root tissues, it cannot be uncoupled from the cell division. In root tissues the earliest response of wheat histone H4 promoter to 2,4-D was recorded after 24 hours by histochemical assay. Induction of cell division by auxins belongs to the late responses that require reprogramming of the transcription pattern and alteration of cell structure (Dudits, 1993). Therefore, here the changes in histone promoter activity may reflect activation of the transcriptional regulatory pathways leading to cell division.

Abscisic acid (ABA) is mostly associated with various stress-related responses such as stomatal closure, the maintenance of seed dormancy and the inhibition of plant growth (Giraudat *et al.* 1995). Reduction of cell division and/or DNA synthesis by exogenously applied ABA has been described in different tissues of plant cells (Barlow *et al.* 1984; Jacmard *et al.* 1995). In the roots of transgenic maize plants, ABA (10 μM) have diminished the wheat histone H4 promoter activity in meristematic regions indicating its upstream regulatory role in cell cycle inactivation. A recent report on the molecular characterisation of a putative cell cycle inhibitor gene from *Arabidopsis*, ICK1 was induced by ABA; thus suggesting a possible mode of ABA action on cell cycle inhibition (Wang *et al.* 1998).

Cytokinins, on the other hand, were found to be directly involved in cyclin mRNA accumulation, a rate limiting regulatory protein of the replication complex. However, their effect on fully differentiated tissues, modes of re-initiation of cell divisions has not been revealed in details. The patchy pattern obtained after exogenous application of zeatin indicated that only few cells were responsive. When the phytohormonal induction of S phase was monitored by transcript levels of the gene for mitogen activated protein kinase (*PMEK1*) and cdc2 in petunia protoplasts, only 2,4-D, and not BA, was able to stimulate *PMEK1* gene

transcription indicating a S-phase engagement (Trehin *et al.* 1998). The increases in endogenous levels of zeatin in synchronized *BY2*-cell suspension cells at the end of the S phase and during mitosis also suggests a role coupled to cdc2 kinases (Redig *et al.* 1996). Despite the presence of differences in transcriptional regulation of dicot and monocot plants, a similar mechanism may be responsible for the effect of zeatin on transgenic maize plants indicating a requirement of both hormones to commit for S-phase. Differences in transcriptional activation by histone promoter may also be linked to their competence for hormone perception in different cells or cell types. In other words, organelle composition, spatial organization, permeability, signal transduction capacity and differential responsiveness of certain cell types may accomplish these differences to external stimuli generated by phytohormones.

Depending on the spatial and temporal organization the stability of GUS reporter mRNA and subsequent enzyme activity is modified (Inze D, personal communication). It was previously proposed that the GUS enzyme has higher turnover rates in actively proliferating tissues than in fully mature differentiated tissues. This observation and statement was probably also true for this study.

This result encouraged me to use cell cycle dependent histone H4/H3 promoter-reporter gene carrying transgenic plants as an indicator of S-phase activation/deactivation during geminivirus-infection and is discussed in the following parts.

4.3 MSV: Coat protein mediated resistance and virus-host interactions

The fact that "plant viral genomes are relatively small", this facilitated their characterization at the molecular level. Eventually, this has prompted research into the development of virus resistance based on interference with the viral multiplication cycle by the introduction of viral sequences into the plant genome. Among the tested strategies, the most successful one so far involves the constitutive expression of the coat protein gene of the virus against which resistance is desired. A decade of research has proven that plants can be genetically engineered to resist virus infection through expression of viral CP genes, as well as other viral genes and sequences. Additional opportunities for development of resistant plants will require research focused on mechanisms of protection, improvements in expression vector design, and transformation of new crop species (Miller *et al.* 1998).

4.3.1 CP-mediated virus resistance

Plant viruses cause considerable losses to crops and none of the current approaches of classical breeding are likely to give the long-term answer to disease control. The new concept of non-conventional protection however holds promises in this area (Hull *et al.* 1993). The practical difference from conventional breeding for resistance is that the resistance is derived from the pathogen rather than the host itself. In non-conventional protection, the expression of a viral or virus-related sequence in the plant genome interferes with the virus infection cycle, holds considerable promise for designing new resistance or protection 'genes'. A series of targets in the viral genome is identified and a range of mechanisms for attacking those targets has been determined in this field. The problems of field deployment of the transgenic plants, especially those

associated with the risk to the environment. Various questions which molecular biologists and plant breeders should have to consider include what are desirable characters to have in protection 'genes' and how these new 'genes' should be deployed.

During the production of transgenic maize plants carrying MSV-CP, the first set of transformation experiments suggested that the vector system used in the original co-transformation vector should be modified for higher expression in all tissues regardless of developmental stage. The coat protein did not interfere with viral replication, moreover the plants derived from two CP expressing lines demonstrated growth abnormalities. The cp8 plants died after a month in greenhouse conditions, after developing dwarf bush like thick leaves. I could not obtain fully grown plants from this line. The seed production from these transformants was also very limited. Moreover, to eliminate the need for screening among a mixed population of transformants carrying only the selectable marker and the ones with both characters, a single vector delivery was chosen to proceed with.

Expression of viral CP genes in transgenic plants can lead to virus resistance by interference of either the transcript or the protein with virus infection. Even in cases in which CP accumulation is required, the degree of resistance does not always correlate with CP levels in transgenic plants. In cases in which CP-mediated resistance can be overcome by inoculation with viral RNA instead of virions, interference with virion assembly is the likely cause of resistance. Measurement of virus accumulation in protoplasts prepared from transgenic plants was used to show interference with early events of virus infection. In our case, protoplast accumulated significant amounts of viral DNA. Moreover there was no evidence for inhibition of local virus spread in selected transgenic plants. A reduced rate of virus accumulation in inoculated leaves can usually also be explained with reduced rate of replication. In addition to virion formation, CP can function in different ways in plant virus infections.

Based on the results from the transgenic plants expressing MSV CP and data available on other virus resistance strategies via genetic modification clearly suggests the use of viral sequences other than the CP against MSV. The interference of *Rep* protein is a potential approach, which would impair virus replication at early stages of virus infection. Thus over-expression of full of truncated copies of MSV replication related genes in transgenic plants might block virus replication without significantly affecting plant development as observed in CP-expressing lines. Moreover, recent publications support the view that in replicase-mediated resistance the degree of resistance was well correlated with the transgene mRNA and/or protein levels (Lomonosoff, 1995).

Knowledge of CP function(s) in a particular plant-virus interaction will be useful to design gene constructs. Since CP accumulation levels in transgenic plants do not always correlate with resistance, newly generated transgenic plant lines are now frequently tested for virus resistance before further characterization. However, as the objective of my transformation experiments was also to study the role of CP in virus infection cycle it was necessary to determine transcript and protein levels in the transgenic plants. Gene constructs used in my experiments, encoding sense and anti-sense CP transcripts was included in the experiment.

However the interference of the CP in host development indicated the need for a mutant-CP vector which might interfere with the virus replication whiteout suppressing the growth of maize plant.

4.3.2 The MSV capsid protein is transported to plant nuclei

Most plant viruses exist in the cytoplasm of their host cells. Geminiviruses, replicating via double stranded DNA templates are one exception. As they replicate in nucleus, this feature makes them an attractive model for studying the role of nuclear import in virus-plant interactions. In all monopartite geminiviruses tested so far, CP is required for viral induction of symptomatic systemic viral infection (Boulton *et al.* 1989; Briddon *et al.* 1989; Woolston *et al.* 1993) and thus indispensable for symptom production and spread regardless of the insect vector.

The mechanism how the geminiviruses reach to the nucleus is not well understood. However, comparisons of amino acid sequences at the amino terminus of geminiviruses, including MSV revealed a potential nuclear localization sequence. I have also tested the hypothesis that NLS sequence in transgenic maize lines carrying MSV coat protein gene under the control of maize ubiquitin promoter directs the transgene product into the nucleus of the plant cell. Protoplasts from several independent transgenic lines, known to accumulate capsid protein in substantial amounts (as determined by western blotting) were tested for subcellular localization using polyclonal antibodies against the MSV capsid protein. In all tested 3 clones, the coat protein was localized in the nucleus but not in the cytoplasm.

Although geminiviruses have been found in the nuclei of phloem-associated cells, the mechanism of viral invasion is poorly understood. The possible role of the capsid protein (CP), the only known component of the viral coat, in virus transport into the host cell nucleus can be investigated by monitoring its specific nuclear accumulation in plant cells. The mechanism by which MSV CP mediates the transfer of MSV DNA from cell to cell and through the vascular bundle is also unknown. However, Liu *et al.* (1997) have demonstrated that MSV CP bounds to ss and ds viral and plasmid DNA in a sequence non-specific manner restricted via the first 104 N-terminal amino acids, *in vitro*. This data suggest that the binding of CP to MSV DNA is involved in viral DNA nuclear transport as well as encapsidation and thus may have a role in intra- and inter-cellular movement as well as systemic infection. Deletion or mutation of MSV CP does not prevent virus replication in single cells or protoplasts but leads to a loss of infectivity in the inoculated plant. In another, dicot infecting geminivirus TYLCV, NLS of the viral CP was capable of translocating the capsid protein across plant and insect nuclear membranes (Kunik *et al.* 1998) suggesting that CP may also play an active role in invasion of insect cells where the virus is subsequently stored.

Nuclear accumulation of the CP has several other biological implications and particular effects on the development of transgenic plants producing MSV-CP in substantial amounts used in this study. First of all, CP may play a essential role in the nuclear import of MSV single-stranded DNA. MSV-CP may also mediate the invasion of phloem-associated cells and the cells that are not actively dividing via actively transporting viral genome significantly exceeding size exclusion limits of nuclear pores, thus the viral nuclear entry independent

of nuclear envelope breakdown during mitosis. Both events would be leading the establishment of viral infection at the early phases of the geminivirus infection. Similar protein-mediated nuclear transport of viral genomes has been suggested for SV40 (Clever *et al.* 1991) and the influenza virus (O'Neill *et al.* 1995). Transgenic plants that were analyzed in this study, have shown several morphological abnormalities in the late stages of their development that were not common in other protoplast-derived transgenic maize plant produced and propagated under similar conditions. Namely, in many cases, the CP-transgenic plants stopped growing before reaching full fertile plants. Their stem thickened, developed bush like semi-curled leaves and subsequently the plants died. This phenomenon was directly correlating with the amount of CP-protein produced and thus suggesting an interference of MSV-CP in impaired growth probably through the blockage of nuclear pores and disrupting nuclear trafficking of the plant cell. Therefore, in production and utilisation of transgenic plants, the foreign DNA introduced into the host should not be interfering with the plant metabolism and DNA sequences should be kept as "inert" as possible that would evoke optimum response (resistance to stresses, production of a metabolite etc.) but not impair plant growth and development..

4.4 Host cell division cycle during MSV infection

Studies on dicot infecting geminivirus Rep proteins and their well characterized animal counterparts were shown to be involved in trans-activation of host gene expression as well as altering cell division control mechanism to favor viral replication. Limited genetic and biochemical analysis of a monocot infecting geminivirus, maize streak virus (MSV) suggests that also MSV codes for proteins (*Rep* and *RepA*) that might interact with key regulators of the plant cell division cycle to facilitate its own perpetuation and systemic infection.

I have utilized the transgenic plants and cell lines carrying a β-glucuronidase marker gene driven by wheat replication-dependent histone H4 gene promoter as an indicator of host DNA replication activation upon viral infection. The reporter gene activity was monitored by fluorimetric measurements after virus transmission either by insect transmission, agroinoculation and where appropriate after biolistic transformation. These results indicate that the late G1/S phase specific expression of the histone promoter was activated both in lower stem and expanded leaves in MSV infected plants. In transgenic plants where non-infections leafhoppers were fed, there was 2-3 times less histone mRNA accumulation and lower GUS activity with respect to the infected clones. In transgenic maize plants carrying another developmentally regulated but cell cycle -independent promoter GUS fusion (CaMV 35S::GUS::NOS), the transcription of the reporter gene was increased indicating an common upstream factor that controls initiation of transcription during MSV infection.

In fully differentiated maize leaf tissues, proliferation is often stopped except in certain cell types in the shoot apical meristem. However, mature leaves posses a limited DNA replication activity but only at vascular tissues where MSV movement through the plant occurs. In the case of transgenic *Nicotiana benthamiana* plants, the C4 product of beet curly top geminivirus; a dicot infecting geminivirus shown to induce cell division and hyperplasia (Lantham *et al.* 1997). As these plants did not develop morphological changes in infected

tissues (neither in shoot nor in leaves) the induction of DNA replication machinery seems to be limited to the activation of G1/S phases of the host cell cycle without host DNA replication. The MSV infection could trans-activate a rather "silent" DNA replication mechanism in mature leaves but not mitosis. Thus, the interaction of MSV Rep protein and G1/S phase control proteins (cdks, Rb-related protein(s), G1 cyclins, cdk inhibitors) are most likely determinants of this partial activation of the cell cycle in terminally differentiated plant cells.

The transcript accumulation of S-phase specific histone genes H4 and H2B; a cyclin dependent kinase *cdc2ZmA*; a mitotic cyclin *Zeam;CycB2;1* and *PCNA* during MSV infection also supports the results obtained from reporter gene in transgenic maize plants. The northern hybridization indicated that a significant amount of histone *H4*, histone *H2B* and *PCNA* mRNAs accumulated in differentiated leaf and stem tissues where proliferation activity are clustered in tight zones along the vascular bundles. Interestingly, the mRNA of the B-type putative mitotic cyclin *CycB2;zm;1* also increased in infected plant leaves. These results indicate that MSV is able to trans-activate the transcription of S-phase related genes as well as a mitotic cyclin. Though the transcriptional activation of division kinases, core histones and the PCNA was expected, the change in cyclin mRNA remains to be explained. One possible explanation links MSV to its mammalian counterpart, SV40 where the expression of large T-antigen induced the transcription and activity of cell division kinases Cdk2, Cdc2 and their regulatory partner cyclins (Ohkubo *et al.* 1994). Moreover, cyclin B promoters were shown to be activated also by cdk, cyclin E and cyclin D proteins depend upon binding of transcription factors to the *CCAAT* elements and is correlated with activation by cyclin-dependent kinases (Katula *et al.* 1997). Further, the interaction of cyclin A-cdk2 with SV40 T antigen is mediated via cyclin A. suggesting that cyclin A and cdk2 are components of the SV40 replication initiation complex (Cannella *et al.* 1997).

Similar to its oncogenic mammalian counterparts, MSV uses a genuine strategy to utilize the host DNA replication machinery for its own perpetuation. The MSV Rep proteins most likely interact with key regulators of G1-S phase progression but probably not with cellular factors controlling progression through mitosis. The lack of neoplastic growth in MSV infection differs from the mammalian DNA viruses and a dicot infecting geminivirus TYCV. Some of these results may be regarded as contradictory to those reported by Lucy *et al.*; 1996 where S-phase associated proteins were shown to be present at same zones but different cells with MSV proteins. However, the modes of detection (mRNA *in situ* and immunolocalisation versus northern blot-RT-PCR analysis), proliferation status (high division rates versus resting cells) and the target tissue used (primary shoot apex versus differentiated, expanded leaf) in these two studies are different.

Recent reports on characterization of viral replication proteins including the complementary-sense gene products of wheat dwarf virus (WDV), maize streak virus, of the genus *Mastrevirus* that infects monocotyledonous plants, bind to human and maize retinoblastoma (Rb) protein. Rb proteins control cell-cycle progression by sequestering transcription factors required for entry into S-phase, suggesting that the virus modify the cellular environment to produce conditions suitable for viral DNA replication. In order to determine the role of the MSV Rep (coded by complementary genes C1:C2) in activation of the histone

promoter in "undifferentiated" proliferating cell populations, transient expression assays were performed. After transfection with infectious copies of the MSV genome or maize ubiquitin promoter::Rep gene fusions a significant activation of the histone promoter by both plasmid treatments with respect to the control treatment was observed. The relative changes in enzyme activity as measured on the 3rd day after the transfection showed that both the whole virus and the *Rep* can induce histone promoter activity, later to a higher extend. These results suggest that the viral proteins, Rep and RepA in particular are able to induce histone promoter activity in maize cells. Both plasmids delivered into "late log phase" cell suspensions of the transgenic maize line H4.52 resulted in significant increases in GUS activity. The activation by MB105, dimeric copy of MSV in pBIN19 was slightly higher than the over-expression of MSV Rep genes. A serious limitation of this transient transfection assay however may lie in the differences between the full MSV replicon and the Rep-RepA overexpression plasmid treatments. The wild-type Rep/RepA plasmid has already been shown to activate expression of LIR - GUS replacement (MP and CP replacements) expression constructs in multiple shoot clump cultures (Mazithulela *et al.,* unpublished data). The fact is, that introduction of wild type plasmid may represent a situation close to actual infection cycle. Concerning the state of the host cell and amount of viral proteins produced, the ubiquitin promoter driven MSV *Rep* construct probably yielded different amounts and/or different ratios of non spliced and spliced *Rep* proteins than the actual virus infection. It was previously shown that most effective transient expression of plant expression vectors takes place with in the first 48 hours due to the destruction of plasmid DNA by host nucleases in later stages. On the other hand, MSV and other related geminiviruses were shown to accumulate only after 48 hours after biolistic delivery in the form of a plasmid. The replication of the MSV vector is linked to excisions of the dimeric copy by recombination and expression of early genes; C1 and C2. Thus, the single stranded virus forms are detected after this period. For these reasons, I have harvested bombarded cells at 72 hours and further processed for GUS activity. In addition to these factors, transfer of target cells on fresh media would elevate promoter activity and mask differences that might be induced by MSV proteins.

The presented data suggest transactivation of the maize histone H4 promoter and endogenous cell cycle genes involved in G1-S phase progression upon MSV infection or transient expression of replication related proteins Rep and/or RepA. These results also indicate that the transgenic maize plants carrying replication-dependent wheat histone H4 promoter: GUS reporter gene can efficiently be utilized for monitoring changes in the host replication machinery. In addition to these, sequence motifs known to drive S-phase specific expression of histone genes "type I element" are also present in the regulatory region of MSV (LIR) and some other plant DNA viruses; probably facilitating a synchronous activation of viral proteins with respect to host (Morozov, 1994). From another point of view, these sequences may play a role in a cell cycle dependent regulation of plant virus DNA replication and transcription. This fact may also suggest a co-evolution of the DNA replication schemes of the host and virus.

Further molecular and biochemical dissection of the induction pathways, identification of the interacting proteins of the MSV and host replication machinery would reveal information on the plant cell cycle and

geminiviruses. Particular emphasis should be given to identifying and characterizing replication initiation complexes that might comprise of cdks, cyclins, transcription factors and viral proteins.

CONCLUSION

Taking into account that plants differ from other higher eukaryotes with their capacity to reinitiate their differentiation program after fully developed, this alternative reproduction pattern should be well known and utilized to solve basic biological problems in plants.

Maize is a versatile model plant for biological studies conducted on "plant cell division and development" and also forms the counterpiece of world agriculture, providing most valuable sources of food and nutrition for human civilization along with rice, wheat and millets. For that reason molecular improvement of cereals has gained more importance with the growing nutrition problems and maize became one of the major focus of genetic improvement through transformation. Current protocols of genetic transformation and molecular analysis of gene function, allows conducting basic research to investigate the fundamental bases of plant development and plant-microbe interactions. Similarly, studies leading to increased food production, renewal of the genetic diversity of the world's crops and reduce inputs of pesticides and herbicides consistent with the view that sustainable food production is necessary for reduced population growth and a healthy environment.

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APPENDIX A

Oligo-nucleotide primer sequences:

gusF: 5' GGTGGGAAAGCGCGTTACAAG 3'
gusR: 5' GTTTACGCGTTGCTTCCGCCA 3'

KE185A28: 5' GATACAGTCTCAGAAGACCAAAGG 3'
KE184A28: 5' TAGAGGAAGGGTCTTGCGAAGGA 3'

V816: 5' ATCGCCATCGACTACCA 3'
V817: 5' AACCCACTCGTGAACTT 3'

V818: 5' GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT 3'
V819: 5' GACTCGAGTCGACATCG 3'

patF: 5' TGAGGTTGAGGGTGTTGTGGCTGG 3'
patR: 5' CCCCTGCCTGTGTATCCCAAAGCC 3'

cdc2ZmAF: 5' CTTCCTCGCCACTTAGTTCG 3'
cdc2ZmAR: 5' TATATGCGCTTCTCGCTGTG 3'

CycB2;1,Zea F: 5' ATTGCTCAGCATGACAGACG 3'
CycB2;1 Zea R: 5' TCCCTGCTGTATGCACTGTC 3'

H4C7F: 5' GCGGCAAGGGCGGCAAGGGTCT 3'
H4CYR: 5' CGCCGTCACCTACACCGAGCAC 3'

APPENDIX B

Maize streak monogeminivirus

Index

- Nomenclature
- Host range, transmission and symptoms
- Physical and biochemical properties
- Taxonomy and relationships
- Comments and References

Data collated by M.B. von Wechmar, 1985. Revised by P.G. Markham and M.I. Boulton, 1989.

Nomenclature

Synonyms

bajra streak virus (Seth et al., 1972a & 1972b), cereal African streak virus, maize streak A virus.

Acronym

MSV

Strains

maize strain, Panicum maximum strain, sugarcane strain, Digitaria setigera strain.

ICTV decimal code

29.0.1.0.006

Host range and symptoms

First reported in Zea mays; from South Africa; by Fuller (1901); Bock et al. (1974); Storey (1925); Rose (1978); Gorter (1953).

Natural host range and symptoms

Symptoms persist

Avena sativa, Hordeum vulgare, Secale cereale, Sorghum bicolor, Triticum aestivum, Zea mays, Saccharum spp., Panicum maximum, Setaria verticillata, Digitaria abyssinica, Digitaria velutina, Cenchrus echinatus, Brachiaria reptens, Coix lacryma-jobi, Chloris gayana, Pennisetum purpureum, Sorghum sudanense, Eleusine africana, Eleusine indica, Rottboellia exaltata, Setaria homonyma, Setaria pallidifusca, Urochloa panicoides, Urochloa trichopus - chlorotic or white streaking or lesions.

Transmission

Transmitted by a vector; an insect; *Cicadulina mbila, C. arachidis, C. bipunctella, C. triangula, C. bimaculata, C. similis, C. latens, C. ghaurii, C. parazeae; Cicadellidae*. Transmitted in a persistent manner. Virus retained when the vector moults; does not multiply in the vector; not transmitted congenitally to the progeny of the vector; not transmitted by mechanical inoculation; not transmitted by contact between plants; not transmitted by seed; not transmitted by pollen.

Ecology and control

Studies reported by Rose (1973a; b; 1974; 1978).

Geographical distribution

Spreads in the African region; India, Madagascar, Reunion, and Yemen.

Experimental host range

Many (>9) families susceptible. Experimentally infected plants mostly show streaks and spots.

Diagnostically susceptible host species and symptoms

Zea mays - systemic chlorotic streaking.

Diagnostically insusceptible host species

Zea perennis, Zea diploperennis, Pennisetum americanum.

Maintenance and propagation hosts

Hordeum vulgare, Triticum aestivum, Zea mays.

Assay hosts (Local lesions or Whole plants)

Hordeum vulgare (W), Triticum aestivum (W), Zea mays cv. Golden Cross Bantam (W).

Susceptible host species

Aegilops cylindrica
Aegilops triaristata
Aegilops triuncialis
Aegilops umbellulata
Agropyron sibiricum
Agrostis gigantea
Alopecurus pratensis
Andropogon gerardi
Avena fatua
Avena sativa
Bothriochloa alta
Brachiaria eruciformis
Brachiaria reptens
Bromus uniloides
Calamagrostis canadensis
Cenchrus argentina
Cenchrus cucullata
Cenchrus echinatus
Cenchrus gayana
Cenchrus radiata
Cenchrus submutica
Cenchrus virgata
Chloris gayana
Coix lacryma-jobi
Cymbopogon distans
Cymbopogon schoenanthus
Cynodon dactylon
Digitaria abyssinica
Digitaria enantha
Digitaria horizontalis
Digitaria longiflora
Digitaria marginata
Digitaria milanjiana
Digitaria sanguinalis
Digitaria ternata
Digitaria velutina
Echinochloa polystachya
Eleusine africana
Eleusine coracana
Eleusine indica
Elytrigia repens
Eragrostis abyssinica
Eragrostis aspera
Eragrostis ciliaris
Eragrostis curvula
Eragrostis valida
Euchlaena mexicana
Festuca arundinacea
Glyceria fluitans
Heteropogon contortus
Holcus lanatus
Hordeum vulgare
Hyparrhenia rufa
Imperata arundinacea
Leptochloa virgata
Lolium multiflorum
Lolium perenne

Lolium subulatum
Panicum bergii
Panicum coloratum
Panicum hallii
Panicum maximum
Panicum miliaceum
Panicum texanum
Panicum virgatum
Paspalum almum
Paspalum notatum
Paspalum scrobiculatum
Paspalum urvillei
Pennisetum clandestinum
Pennisetum purpureum
Rottboellia exaltata
Saccharum
Schedonnardus paniculatus
Schizachrium scoparium
Secale cereale
Setaria chevalieri
Setaria faberi
Setaria homonyma
Setaria italica
Setaria pallidifusca
Setaria verticillata
Setaria viridis
Sorghastrum nutans
Sorghum bicolor
Sorghum sudanense
Trichachne californica
Trichloris crinita
Trichloris pluriflora
Triticum aestivum
Urochloa helopus
Urochloa panicoides
Urochloa trichopus
Vaseyochloa multinervosa
Zea may

Insusceptible host species

Agropyron cristatum
Agrostis canina
Agrostis palustris
Agrostis stolonifera
Agrostis tenuis
Alopecurus aequalis
Andropogon hallii
Arrhenatherum elatius
Bothriochloa barbinodis
Bouteloua gracilis
Bouteloua hirsuta
Bromus carinatus
Bromus erectus
Bromus inermis
Cenchrus canterai
Cenchrus ciliaris
Cenchrus distichophylla
Chloris gayana
Cynodon dactylon
Dactylis glomerata
Dactyloctenium gigantea
Digitaria decumbens
Digitaria valida
Echinochloa colona
Echinochloa crus-galli
Elymus canadensis
Eremochloa ophiuroides
Euchlaena perennis
Festuca ovina
Festuca rubra
Hordeum jubatum
Leersia hexandra
Panicum coloratum var. M
Panicum maximum
Paspalum dilatatum
Pennisetum americanum
Pennisetum clandestinum
Pennisetum macrorum
Pennisetum typhoides
Phalaris arundinacea
Phalaris tuberosa
Phleum pratense
Poa compressa
Poa pratensis
Poa trivialis
Setaria italica
Setaria nigrirostris
Setaria sphacelata
Sorghum bicolor
Sorghum halepense
Sorghum miliaceum
Sorghum propinguum
Sorghum vulgare
Stenotaphrum secundatum
Tripsacum dactyloides
Zea diploperennis
Zea perennis

Families containing susceptible hosts

Gramineae (95/146)

Families containing insusceptible hosts

Gramineae (57/146)

Comments on host-range

All isolates tested infect Zea mays cv. Golden Cross Bantam.

Sources of host-range data

Bock et al. (1974); Rose (1978); Damsteegt (1983); Storey (1925); Storey and McClean (1930).

Physical and biochemical properties

Properties of particles in sap

LV: more than 7 days. Leaf sap contains many virions. Electron microscopy: UA is best. ISEM is most reliable.

Purification method

Von Wechmar and Milne (1982).

Particle morphology

Virions geminate; not enveloped; 18 nm in diameter; 30 nm in length; angular in profile; without a conspicuous capsomere arrangement.

Physical properties

Two sedimenting components in purified preparations (in sucrose (Howell, 1984). However, one could be of damaged virion as most geminiviruses sediment as a single component); sedimentation coefficient of the fastest 76 S; of the other(s) 54 S.

Biochemical properties

Genome consists of DNA; single-stranded; circular. Total genome size 2.69 kb. Genome unipartite; largest (or only) genome part 2.689 kb (Nigerian isolate, Mullineaux, 1984; 2.690, South African isolate, Lazarowitz, 1988; 2.681, Kenyan isolate, Howell, 1984). Base composition 25.7 % G; 25.4 % A; 23.6 % C; 25.3 % T. Infectivity retained when deproteinised with phenol or detergent (via agroinfection). Poly A region absent (but does have polyadenylated transcripts). Additional factor required for infectivity (coat protein is essential for transmission by leafhoppers, but cloned DNA can infect by agroinfection).
Nucleotide sequence references: Mullineaux et al. (1984); Howell (1984); Lazarowitz (1988).

NCBI sequence data (Entrez search)
Sequence database accession code(s)
K02026 Gb(84)_vi:MZS Maize streak virus, complete genome. 6/88 2,687bp.

ID GEMSVCPI standard; DNA; VRL; 992 BP.
AC X71961;
SV X71961.1
NI g575639
DT 15-NOV-1994 (Rel. 41, Created)
DT 15-NOV-1994 (Rel. 41, Last updated, Version 27)
DE Maize streak virus V2 gene for coat protein (clone Y946)
KW coat protein.
OS Maize streak virus
OC Viruses; ssDNA viruses; Geminiviridae; Mastrevirus.
RN [2]
RP 1-992
RA Briddon R.W.;
RL Submitted (18-MAY-1993) to the EMBL/GenBank/DDBJ databases.
RL R.W. Briddon, John Innes Institute, Dept of Virus Research, John Innes
RL Centre for Plant Science Res., Colney Lane, Norwich NR4 7UH, UK
RN [3]
RP 1-992
RX MEDLINE; 95176579.
RA Briddon R.W., Lunness P., Chamberlin L.C.L., Markham P.G.;
RT "Analysis of the genetic variability of maize streak virus";
RL Virus Genes 9:93-100(1994).
DR SPTREMBL; Q67598; Q67598.
FH Key Location/Qualifiers
FH
FT source 1..992

FT /organism="Maize streak virus"
FT /db_xref="taxon:10821"
FT /specific_host="Infected Zea mays "
FT /clone="Y946"
FT /isolate="originating from Nigeria"
FT CDS 55..789
FT /db_xref="PID:g575640"
FT /db_xref="SPTREMBL:Q67598"
FT /gene="V2"
FT /product="coat protein"
FT /protein_id="CAA50759.1"
FT
/translation="MSTSKRKRGDDSNWSKRVTKKKPSSAGLKRAGSKADRPSLQIQTLQHAGTTMITVPSGGVCDL
INTYARGSDEGNRHTSETLTYKIAIDYHFVADAAACRYSNTGTGVMWLVYDTTPGGQAPTPQTIFSYPDTLKA
WPATWKVSRELCHRFVVKRRWLFNMETDGRIGSDIPPSNASWKPCKRNIYFHKFTSGLGVRTQWKNVTDGG
VGAIQRGALYMVIAPGNGLTFTAHGQTRLYFKSVGNQ"

SQ Sequence 992 BP; 259 A; 254 C; 277 G; 202 T; 0 other;

GAATTCAGGG CAATCCCGGG CCATTTGTTC CAGGCACGGG ATAAGCATTC AGCCATGTCC
ACGTCCAAGA GGAAGCGGGG AGATGATTCG AATTGGAGTA AGCGGGTGAC TAAGAAGAAG
CCCTCGTCAG CTGGGCTGAA GAGGGCTGGA AGCAAGGCCG ATAGGCCATC CCTCCAAATC
CAGACACTCC AGCATGCTGG GACCACCATG ATAACTGTCC CATCCGGAGG AGTATGTGAC
CTCATCAACA CCTATGCCCG AGGATCTGAC GAGGGCAACC GCCACACCAG CGAGACTCTT
ACGTACAAGA TCGCCATCGA CTACCACTTC GTTGCCGACG CGGCAGCCTG CCGCTACTCC
AACACCGGAA CCGGTGTAAT GTGGCTGGTG TATGACACCA CTCCCGGCGG ACAAGCTCCG
ACCCCGCAAA CTATATTTTC CTACCCTGAC ACGCTAAAAG CGTGGCCGGC CACATGGAAG
GTGAGCCGGG AGCTGTGTCA TCGCTTCGTG GTGAAACGGC GATGGTTGTT CAACATGGAG
ACCGACGGGC GGATTGGTTC GGACATTCCT CCCTCGAATG CAAGTTGGAA GCCTTGCAAG
CGCAACATCT ACTTCCACAA GTTCACGAGC GGGTTGGGAG TTAGAACGCA GTGGAAGAAT
GTAACGGACG GAGGAGTTGG TGCCATCCAG AGAGGAGCTT TGTACATGGT CATTGCCCCC
GGCAATGGTC TTACATTTAC TGCCCATGGG CAGACCCGTC TGTACTTTAA GAGTGTTGGC
AACCAGTAAT GAATAAAAAC TCCCGTTTAA TTATATCTGA TGAATGCTGA AAGCTTACAT
TAATATGTCG TGCGATGGCA CGAAAAAACA CACGCAATCA ATACAGGGGG GTAGTAGGCG
GGCGGCTAAG GGTGGTGCTC GGCGGGCAGA ACATCGAAAA ATCAAGATCT ATCTGAATGT
ACTGCCTCCG TAGGAGGCTG CTCAGTTCTA GA

Features of the genome
Features of the genome: promoters, stem loops and ORF information see Mullineaux et al. (1984).
Sub-genomic mRNA found in infected cells (Morris-Krsinich et al., 1985).

Features of proteins
Virion protein(s) one; Mr 26900; coat protein.
Virus-coded non-virion proteins identified by genomic sequence analysis (Mullineaux et al. (1984)); one protein found. Mr 10900.

Replication
Genome replicates in nuclei. Replication does not depend on a helper virus.

Cytopathology

Virions found in leaves, mesophyll, epidermis, meristem, vascular parenchyma, companion cells and all parts of the host plant; in cytoplasm, in nuclei, and in cell vacuoles. Inclusions present in infected cells; are crystals in the nucleus and viroplasms; they contain virions. Other cellular changes: changes in chloroplasts and other organelles destroyed.

Taxonomy and relationships

monogeminivirus: Geminiviridae (type species)

Virus(es) with serologically unrelated virions:

African cassava mosaic and chloris striate mosaic viruses.

Best tests for diagnosis:

Use *Cicadulina mbila* to transmit to *Zea mays* cv. Golden Bantam.

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`Plant Viruses Online: Descriptions and Lists from the VIDE Database. Version: 16th January 1997.' URL http://biology.anu.edu.au/Groups/MES/vide/

APPENDIX C

DEFINITION Wheat histone H4 gene.

ACCESSION X00043

SOURCE wheat.

ORGANISM *Triticum aestivum*

Eukaryotae; mitochondrial eukaryotes; Viridiplantae; Charophyta/Embryophyta group; mbryophyta;Magnoliophyta; Liliopsida; Poales; Poaceae; Triticum.

REFERENCE 1 (bases 1 to 1200)

AUTHORS Tabata,T., Sasaki,K. and Iwabuchi,M.

TITLE The structural organization and DNA sequence of a wheat histone H4 gene

JOURNAL Nucleic Acids Res. 11 (17), 5865-5875 (1983)

FEATURES Location/Qualifiers mRNA: 669..1200 messenger RNA 736..1047

translation=MSGRGKGGKGLGKGGAKRHRKVLRDNIQGITKPAIRRLARRGGVKRISGLIYEETRGVLKIFLEN VIRDAVTYTEHARRKTVTAMDVVYALKRQGRTLYGFGG

5' REGULATORY REGION:

...tgataagcacgagatgggctcggggatgtcaataagctcgttcagttacacaaacagtactgtacatcagtgctggaagtgctcgttcagttaagtttcta

gcaccaattacctgaccgccaagctattacatgtaattattgtaacgtgttatctgaatgcttgaatcctaaaaaagtgaactccagtaagcgatgaaaaatg

agtatagcagtcactgcattcgagcaagtttcctgtagattatcttcagatctccagaacagttaggatgaaggaataataatcagtcattggaggacatgca

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